

# **HIV, ANTIRETROVIRAL THERAPY, PREGNANCY, LACTATION AND BONE HEALTH IN UGANDA**

**Florence Nabwire**

MRC Elsie Widdowson Laboratory & Queens' College  
University of Cambridge

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# **Title: HIV, Antiretroviral Therapy, Pregnancy, Lactation and Bone Health in Uganda**

**Author: Florence Nabwire**

## **SUMMARY**

Globally, ~17 million women and ~2.1 million children are living with HIV. Sub-Saharan Africa accounts for 70% of HIV-infected (HIV<sup>+</sup>) persons. Mother-To-Child Transmission of HIV (MTCT) during pregnancy, delivery and breastfeeding, is the main route of HIV infection in children. The World Health Organisation recommends lifelong antiretroviral therapy (ART) for all HIV<sup>+</sup> pregnant and breastfeeding mothers to prevent MTCT, and breastfeeding for ≥24 months for optimal child health in resource limited settings (Option B<sup>+</sup> strategy).

Initiation of ART in HIV<sup>+</sup> adults is associated with a 2-6% decrease in areal bone mineral density (aBMD) regardless of ART regimen, but data are limited in pregnant and lactating women. Tenofovir, a preferred first-line drug in Option B<sup>+</sup> ART regimen, is associated with 1-2% greater decreases in aBMD. Pregnancy and lactation are associated with physiological changes in maternal bone mineral density, but most evidence shows that this is recovered after cessation of breastfeeding. The hypothesis of this thesis is that ART may accentuate the normal process of bone mobilisation during pregnancy and lactation, leading to bone loss that is not recovered in the mother and/or compromised infant growth and bone mineral accretion. The primary objective of this research was to investigate if HIV<sup>+</sup> women experience greater reductions in bone mineral compared to HIV-uninfected (HIV<sup>-</sup>) counterparts.

Two groups of pregnant women, 95 HIV<sup>+</sup> on ART (Tenofovir-Lamivudine-Efavirenz, previously ART naïve) and 96 HIV<sup>-</sup> were followed prospectively in Kampala, Uganda. Data were collected at 36 wks gestation (PG36), 2 (PP2) and 14 wks postpartum (PP14). Dual-energy x-ray absorptiometry was used to measure bone phenotype (aBMD, bone mineral content (BMC), bone area (BA), and size-adjusted BMC (SA-BMC, adjusted for height or length, weight and BA) of the whole body (WB) and lumbar spine (LS) in mother-baby pairs, and total hip (TH) in mothers. The primary outcome was the difference between groups in % change ( $\pm$  SE) in maternal LS aBMD between PP2 and PP14. Secondary outcomes included changes in maternal markers of bone formation (P1NP and BAP) and resorption (CTX), serum 25-hydroxy vitamin D (25(OH)D), parathyroid hormone (PTH), plasma and urine concentrations of creatinine (Cr), calcium (Ca), phosphate (PO<sub>4</sub>) and magnesium (Mg), urine mineral:creatinine ratios, TmCa/GFR and TMP/GFR, respectively), breastmilk mineral composition (Ca, P, Na, K and Na/K ratio); and infant growth Z-scores and bone mineral. Statistical models were adjusted for potential confounders.

Median maternal age was 24.5 (IQR 21.1, 26.9) yrs. Mean gestation was 40.9 $\pm$ 1.8 wks and not significantly different between groups. All women were breastfeeding at PP2 and PP14. More HIV<sup>+</sup> women reported exclusive breastfeeding (PP2: 82.9% v 58.7%, p=0.0008; PP14: 86.7% v 66.2%, p=0.002). Body weight was 4-5% lower in HIV<sup>+</sup> women. By PP14, mean duration of ART was 29.3 $\pm$ 5.1 wks, adherence was >95%, and the median CD<sub>4</sub> count was 403 (IQR 290-528) cells/mm<sup>3</sup>.

Maternal aBMD decreased between PP2 and PP14 at all skeletal sites in both groups as expected in lactation. Reductions in LS aBMD were not significantly different between groups (-1.8 $\pm$ 0.4% vs -

2.5±0.4%, p=0.3). However, HIV<sup>+</sup> women had a significantly greater reduction in TH aBMD which persisted after adjustment for body size (-3.7±0.3% vs -2.7±0.3%, p=0.04). Median serum 25(OH)D was 67.4 nmol/L (IQR 54.8, 83.7) at PG36 and 57.6 nmol/L (48.7, 70.1) at PP14 with no significant difference between groups. Changes in 25(OH)D and PTH from PG36 to PP14 were not significantly different between groups (25(OH)D: -13.9±4.1% vs -11.1±3.1%; PTH: +60.0±6.4% vs +57.6±6.4%; both p>0.05). However, HIV<sup>+</sup> women had 33-35% greater plasma PTH concentrations at both PG36 and PP14.

Bone formation and resorption markers increased in both groups between PG36 and PP14. HIV<sup>+</sup> women had greater increases (CTX: +74.6±5.9% vs +56.2±5.9%; P1NP: +100.3±5.0% vs +72.6±5.0%; BAP: +67.2±3.6% vs +57.1±3.6%, all p<0.05). They also had a greater decrease in plasma Ca (-6.6±0.5% vs -3.8±0.5%, p≤0.0001) and greater increase in plasma phosphate (+14.4±2.0% vs +7.7±2.0%, p=0.02). Changes in plasma Cr and Mg, TmP/GFR and urine mineral:creatinine ratios were not significantly different between the groups. However, at both PG36 and PP14, HIV<sup>+</sup> had significantly lower mean plasma Ca (PG36: -1.0±0.5%; PP14: -4.1±0.6%) and TmP/GFR (PG36: -11.4±3.1%; PP14: -7.2±3.0%) but higher PTH (PG36: +33.0±7.0%; PP14: +35.3±7.6%) compared to HIV<sup>-</sup> women (all p<0.05).

Mean breastmilk Ca decreased between PP2 and PP14, and the changes were not different between the groups (-19.9±3.0% vs -24.2±3.1%, p=0.3). There were no significant changes in breastmilk phosphorus (P) in both groups, but HIV<sup>+</sup> women had significantly higher concentrations (PP2: +9.7±3.8%, p=0.01; PP14: +9.6±3.5 %, p=0.007). Breastmilk P was significantly correlated with maternal plasma [CTX] in a separate ANCOVA model ( $\beta$  = +0.13±0.04% per 1% increase in CTX, p=0.0003). Mean breastmilk Na, K concentrations and Na/K decreased between PP2 and PP14 in both groups. However, HIV<sup>+</sup> women had a smaller decrease in breastmilk Na (-44.3±8.9% vs -72.6±9.0%, p=0.03). They also had a trend towards smaller reduction in Na/K ratio (-22.2±9.3% vs -46.6±9.5%, p=0.07).

Babies born to HIV<sup>+</sup> mothers (HIV-exposed infants, HEI) had significantly lower gains in weight (+53.0±1.4% vs +57.5±1.4%, p=0.02) compared to HIV-unexposed infants (HUI), and also lower weight-for-age (-0.47±0.16, p=0.003) and length-for-age (-0.53±0.18, p=0.005) Z-scores at PP14. HEI had a slower gain in WB BMC (+51.2±1.9% vs +57.3±1.9%, p=0.02), but the difference was not significant after adjustment for body size (-6.0±3.5% vs -7.6±3.8%, p=0.2); showing that the bone mineral accretion was appropriate for achieved infant size. In contrast, HEI had a greater increase in LS BMC (+29.5±1.7% vs +24.4±1.7%, p=0.03), a difference which remained after size-adjustment (+9.4±5.8% vs +4.3±6.2%, p=0.02).

This is the first study to compare changes in maternal bone health between HIV<sup>+</sup> mothers on Option B<sup>+</sup> ART and HIV<sup>-</sup> counterparts. The results show a greater reduction in TH aBMD in Ugandan HIV<sup>+</sup> women on Option-B<sup>+</sup> ART compared to HIV<sup>-</sup> in the first three months of lactation, consistent with their greater increases in bone turnover markers, lower TmP/GFR and plasma phosphate, and higher breastmilk phosphorus concentration. Also, HEI have slower growth and whole body bone mineral accretion compared to HUI. It is important to determine if these changes are temporary or have long-term consequences for the bone health of the mother and child.

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## ABBREVIATIONS AND GLOSSARY

**1,25(OH)<sub>2</sub>D:** 1,25 dihydroxy vitamin D

**25(OH)D:** 25-hydroxyvitamin D

**3TC:** Lamivudine [drug]

**ABC:** Abacavir [drug]

**aBMD: Areal Bone Mineral Density** (as measured by DXA) “represents the average mass of mineral (BMC) per unit scanned area (BA) and is expressed as g/cm<sup>2</sup>. It is not a true density as there is no measurement of bone depth available from DXA, and also cannot differentiate between anatomical regions within the bone with different material properties (i.e. It is an average across areas of cortical bone, trabecular bone, medullary cavity and intraosseous soft tissue)” (Prentice, Personal communication June 2018).

**AIDS:** Acquired Immune Deficiency Syndrome

**ALP:** Alkaline phosphatase

**ANC:** Antenatal Clinic

**ART:** Antiretroviral therapy

**ARV:** Antiretroviral [drug]

**BA: Bone area** (as measured by DXA) “represents the total scanned area (cm<sup>2</sup>) within the bone envelope in the scanned portion of the skeleton under examination. Bone edge detection is obtained by algorithms within the instrument that define the passage of the scanning beam from soft tissue (which absorbs little of the X-ray beam) to bone (which absorbs more of the X-ray beam)” (Prentice, Personal communication June 2018).

**BAP:** Bone alkaline phosphatase

**Baylor-Uganda:** Baylor College of Medicine Children’s Foundation Uganda

**BIPAI:** Baylor International Paediatric HIV/AIDS Initiative

**BM Ca/P:** Breastmilk calcium-phosphorus ratio

**BM Na/K:** Breastmilk sodium-potassium ratio

**BM:** Breastmilk



**BMC: Bone Mineral Content** (as measured by DXA) “represents the total mass of mineral (g) averaged over the scanned portion of the skeleton under examination. It is obtained by measuring the absorption of X-rays during the scan relative to phantom materials calibrated against hydroxyapatite (a calcium phosphate salt that closely resembles the mineral phase of bone)” (Prentice, Personal communication June 2018).

**BM-Ca:** Breastmilk calcium concentration

**BMD:** Bone Mineral Density

**BMI:** Body mass index

**BM-K:** Breastmilk potassium concentration

**BM-Na:** Breastmilk sodium concentration

**BM-P:** Breastmilk phosphorus concentration

**Bone health** “in the context of public health, is defined as the quality of being at low risk of three common skeletal disorders, stunting, rickets and osteomalacia, and osteoporosis and fragility fractures” (Prentice *et al.*, 2006b)

**Bone phenotype** in this thesis refers to all bone indices measured by DXA (aBMD, BMC, BA, SA-BMC).

**BP-3:** Binding protein-3 [IGF]

**BTM:** Bone turnover markers

**Ca:** Calcium

**CAP:** College of American Pathologists

**CD<sub>4</sub>:** Cluster of differentiation 4

**CKD-EPI:** The Chronic Kidney Disease Epidemiology Collaboration

**COE:** Center of Clinical Excellence

**Cr:** Creatinine

**CTX:** cross-linked C-terminal telopeptide

**DART:** The Development of AntiRetroviral Therapy in Africa

**DNA -PCR:** Deoxyribonucleic Acid Polymerase Chain Reaction

**DPD:** Deoxypyridinoline

**DTG:** Dolutegravir [drug]

**DTTM:** Dose-To-The-Mother method

**DXA:** Dual-energy X-ray absorptiometry

**EDTA:** Ethylenediaminetetraacetic acid

**EFV:** Efavirenz [drug]

**eGFR:** Estimated glomerular filtration rate

**ELISA:** Enzyme-linked immunosorbent assay

**eMTCT:** Elimination of mother-to-child transmission of HIV

**EWL:** MRC Elsie Widdowson Laboratory

**FGF-23:** Fibroblast growth factor -23

**FTC:** Emtricitabine [drug]

**GFR:** Glomerular Filtration Rate

**HAZ:** Height-for-Age Z-score

**HCAZ:** Head circumference-for-age Z-score

**HEI:** HIV Exposed Infants

**HIV:** Human Immune Virus

**HNR:** MRC Human Nutrition Research

**HUI:** HIV Unexposed Infants

**IAEA:** International Atomic Energy Agency

**ID:** Identification

**IGF-1:** Insulin-like growth factor 1

**IMPAACT:** International Maternal Pediatric Adolescent AIDS Clinical Trials Network

**IRB:** Institutional Review Board

**JCRC:** The Joint Clinical Research Centre

**K:** Potassium

**LAZ:** Length-for-Age-Z score

**LS:** Lumbar spine refers to the L1-L4 regions on a lumbar spine DXA scan.

**MDRD:** Modified Diet in Renal Disease

**Mg:** Magnesium

**MOH:** Ministry of Health

**MRC:** Medical Research Council

**mSv:** milliSievert

**MTCT:** Mother-To-Child Transmission

**MUJHU:** Makerere University Johns Hopkins University Research Collaboration

**Na:** Sodium

**NBH:** Nutrition and Bone Health

**NNRTI:** Non-Nucleoside Reverse Transcriptase Inhibitor

**NPNL:** Non-Pregnant Non-Lactating

**NRTI:** Nucleoside Reverse Transcriptase Inhibitor

**NTX:** Cross-linked N-terminal telopeptide

**NVP:** Nevirapine [drug]

**Osteomalacia** “is a failure of mineralisation on the trabecular and cortical surfaces of all bones, and can occur in both children and adults. Generally, children who present with rickets also have histological features of osteomalacia” (Prentice et al., 2006b).

**Osteopenia** or low bone density is a “condition where bone mineral density is lower than normal however not yet as low as osteoporosis and falls in the “T score range of -1.1 to -2 (WHO Study Group, 1994).

**Osteoporosis** is a “progressive systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture” (Kanis *et al.*, 1994). It is clinically defined as a BMD T-score less than or equal to -2.5 SD or more below the young female adult mean (WHO Study Group, 1994, Compston *et al.*, 2014).

**P:** Phosphorus

**P1CP:** Procollagen type-1 C-Terminal Propeptide

**P1NP:** Procollagen type 1 N-Terminal Propeptide

**P-Ca:** Plasma calcium concentration

**PCP:** Pneumocystis pneumonia

**P-Cr:** Plasma creatinine concentration

**PHACS:** USA-based Paediatric HIV/AIDS Cohort Study

**PI:** Protease Inhibitor

**P-Mg:** Plasma magnesium concentration

**PMTCT:** Prevention of Mother-To-Child Transmission

**PO<sub>4</sub>**: Phosphate

**P-PO<sub>4</sub>**: Plasma phosphate concentration

**pQCT**: Peripheral quantitative computed tomography

**PROMISE**: Promoting Maternal and Infant Survival Everywhere

**PTH**: Parathyroid hormone

**PTHrP**: Parathyroid hormone-related protein

**PYD**: Pyridinoline

**QUS**: Quantitative Ultrasound Scan

**Rickets** “by definition is a disease of growing bones and is characterized by a delay in or failure of mineralization at the unfused growth plates. This is associated with a failure of the normal apoptosis of the hypertrophied chondrocytes in the growth plate resulting in widening of the growth plate and a delay in appearance and irregularity of the provisional zone of calcification at the metaphyseal end of the growth plate. A clinical diagnosis of nutritional rickets is based on history and physical examination, supported by biochemical testing, and confirmed radiographically” (Pettifor and Prentice, 2011).

**RTG**: Raltegravir [drug]

**SA-BMC**: Size Adjusted Bone Mineral Content

**SACN (UK)**: Scientific Advisory Committee on Nutrition, UK

**SD**: Standard Deviation

**sdNVP**: Single Dose Nevirapine [drug]

**SE**: Standard Error

**Size adjusted BMC**: BMC adjusted for body size (weight and length).

**SMART**: Strategies for Management of Antiretroviral Therapy

**SSA**: Sub Saharan Africa

**Stunting** or poor linear growth is defined by low length or height-for-age (LAZ or HAZ) below -2SD of the reference population. WHO defines stunting as “the impaired growth and development that children experience from poor nutrition, repeated infection, and inadequate psychosocial stimulation” (Prentice et al., 2006b, WHO, 2018b).

**TAP**: Total Alkaline Phosphatase

**TB**: Tuberculosis

**TDF**: Tenofovir Disoproxil Fumarate

**TmCa/GFR:** Renal tubular maximum for reabsorption of calcium per unit of glomerular filtrate

**TmP/GFR:** Renal tubular maximum for reabsorption of phosphate per unit of glomerular filtrate

**TmP:** Tubular maximum reabsorption rate of phosphate

**TH:** Total hip refers to the combined phenotypes of the femoral neck, trochanter, and intertrochanteric regions on a hip DXA scan.

**TRP:** Tubular reabsorption for phosphate

**T-score** is “the number of standard deviations that a BMD measurement lies above or below the average value for young healthy women” (Compston et al., 2014).

**U-PO<sub>4</sub>/Cr:** Urine phosphate-creatinine ratio

**U-Ca/Cr:** Urine calcium-creatinine ratio

**U-Ca:** Urine calcium concentration

**U-Cr:** Urine creatinine concentration

**UDHS:** Uganda Demographic and Health Survey

**UMg/Cr:** Urine magnesium-creatinine ratio

**U-Mg:** Urine magnesium concentration

**UNAIDS:** Joint United Nations Programme on HIV/AIDS

**Underweight** is defined by low weight-for-age (WAZ) below -2SD of the reference population. A child who is underweight may be stunted, wasted, or both (Onyango and de Onis, 2008, WHO, 2018b).

**U-PO<sub>4</sub>:** Urine phosphate concentration

**UVB:** Ultraviolet B

**vBMD:** Volumetric Bone Mineral Density

**VOICE:** Vaginal and Oral Interventions to Control the Epidemic

**Wasting** or thinness is defined by low weight-for-height (WHZ) below -2SD of the reference population. In most cases, it indicates a recent and severe process of weight loss, which is often associated with acute starvation and/or severe disease. However, wasting may also be the result of a chronic unfavourable condition (Onyango and de Onis, 2008, WHO, 2018b).

**WAZ:** Weight-for-Age Z-score

**WB:** Whole Body

**WBLH:** Whole Body Less Head

**WBS:** The Women's Bone Study

**WHO:** The World Health Organisation

**WHZ:** Weight-for Height Z-score

**WLZ:** Weight-for Length Z-score

**Z-score** (also called a standard score) is the number of standard deviations above or below the mean for the population.

## CHAPTER 1: BACKGROUND AND CONTEXT OF PhD

### 1.1. Overview of HIV, ART and Bone

#### 1.1.1. HIV and global prevalence

The Human Immunodeficiency Virus (HIV) causes Acquired Immune Deficiency Syndrome (AIDS). The first cases of HIV-infection were reported in the USA in 1981. HIV belongs to the family of retroviruses, and two subtypes (HIV 1 and HIV 2) have been identified. HIV-1 accounts for the majority of HIV infections in Uganda and sub-Saharan Africa (Jaffar *et al.*, 2004); so in this thesis, the term HIV will refer to the HIV-1 subtype unless specified.

About 36.7 million people were globally living with HIV in 2016 of which 17.8 million were women above 15 years of age, and 2.1 million were children aged  $\leq 15$  years (UNAIDS, 2016). The highest burden of HIV infection is in Sub-Saharan Africa (25.5 million people) where nearly 1 in 25 (4.4%) adults were living with HIV, accounting for about 70% of all HIV-infected persons in the world (Figure 1). Within sub-Saharan Africa, the burden of HIV-infection was greatest in the Eastern and Southern Africa region. About 19.4 million people in the region were living with HIV of which 59% were women and girls aged  $>15$  years.

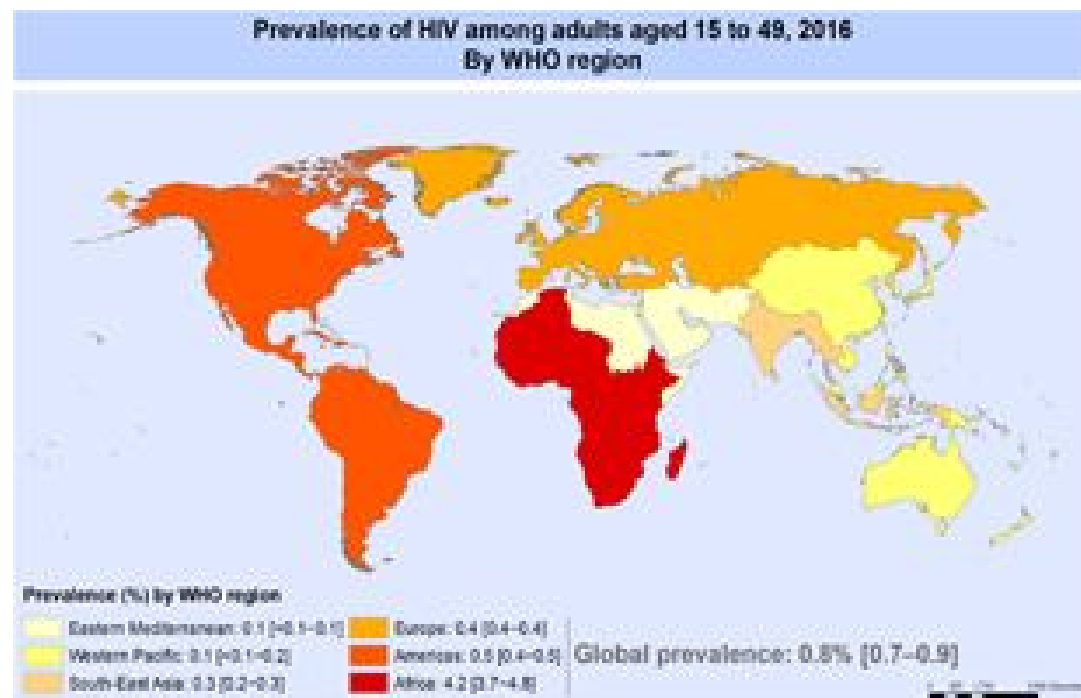


Figure 1: Global prevalence of HIV in adults and children in 2016

(WHO, 2018a) [Open access for reuse in academic thesis <http://www.who.int/about/licensing/extracts/en/> ]

### 1.1.2. Transmission routes of HIV

HIV is transmitted through contact with blood or genital fluids of infected persons and can also be transmitted from mother to the baby during pregnancy, labour/delivery or breastfeeding. About 90% of HIV infections in children are acquired through Mother-To-Child Transmission of HIV (MTCT) (UNICEF, 2014). Without interventions, the overall transmission rates range from 15-45% depending on the timing of infection and availability of services for Prevention of MTCT (PMTCT) as shown in Figure 2 (WHO, 2014).

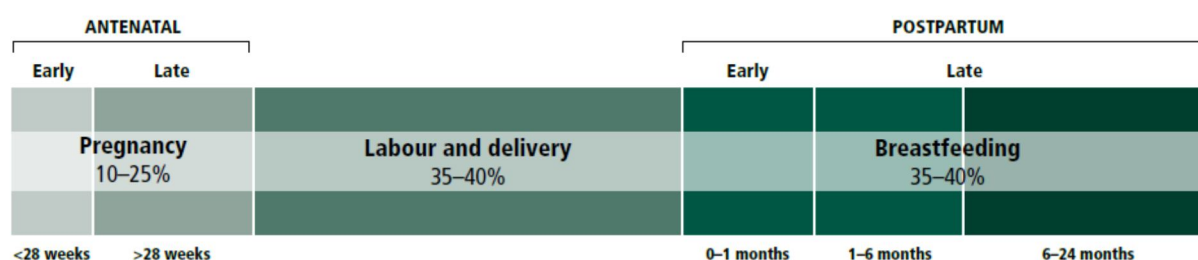


Figure 2: Rates of mother to child transition of HIV

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Once in the body, the HIV attacks the immune system by destroying CD<sub>4</sub> cells T cells (CD<sub>4</sub><sup>+</sup>). These are a type of white blood cell that are vital to fighting off infection. In general, CD<sub>4</sub> count decreases gradually as HIV-disease advances. The normal CD<sub>4</sub> count for a healthy adult ranges from 500-1500 cells/mm<sup>3</sup> of blood. The natural history of HIV has four distinct stages leading to AIDS or death. The primary HIV infection (stage 1, asymptomatic phase) lasts for a few weeks and is most often accompanied by a short viruslike illness which develops after 2-4 weeks post exposure and lasts for 1-3 weeks (Levy, 1993). Individuals in stage 1 of HIV-infection have a preserved CD<sub>4</sub> count ( $\geq 500$  cells/mm<sup>3</sup>). Over time, the immune system becomes severely damaged by HIV-infection, eventually fails and symptoms develop. The person initially develops mid symptoms (stage 2) and progresses to severe/advanced symptoms (stage 3) (Tindall and Cooper, 1991, Levy, 1993, Weiss, 1993). HIV-infected individuals with a CD<sub>4</sub> count below 200 cells/mm<sup>3</sup> are more likely to develop symptoms (WHO, 2007a). The common symptoms of HIV-infection (stage 2 onwards) include weight loss, chronic fever, chronic diarrhoea, persistent cough and night sweats, mouth and skin problems, regular infections and other serious illness or diseases. The symptoms are initially mild but worsen over time as HIV-infection progresses to AIDS (stage 4). At this stage, the individual develops increasingly severe opportunistic infections and cancers; ultimately a diagnosis of AIDS (Morgan *et al.*, 1997, WHO, 2007a).



### 1.1.3. Antiretroviral Therapy

To date, there is no cure for HIV. However, antiretroviral drugs (ARVs) delay progression of HIV-infection to AIDS disease by suppressing multiplication of the virus in the body (Sethi *et al.*, 2003, De Cock *et al.*, 2009, WHO, 2017b). Also, ARVs are effective in the prevention of HIV transmission through both sexual and MTCT (Cohen *et al.*, 2012, WHO, 2016a). The commonly available ARVs must be swallowed daily for life to maintain adequate drug levels for viral suppression. Thus adherence is critical to prevent drug resistance (Friedland and Williams, 1999, Sethi *et al.*, 2003). The four classes of common ARVs and their mode of action are presented in Table 1.

Table 1: Classes of antiretroviral drugs, their modes of action and common brand names

Class of ARV	Mode of action <sup>a</sup>	Common licenced drugs
<b>Nucleoside Reverse Transcriptase Inhibitors (NRTI)</b>	Interfere with the reverse transcription process (conversion of viral RNA into DNA). They are incorporated into HIV DNA resulting in incomplete DNA chain formation	Tenofovir Disoproxil Fumarate (TDF) Lamivudine (3TC) Emtricitabine (FTC) Zidovudine (AZT), Abacavir (ABC) Stavudine (d4T)
<b>Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI)</b>	Interfere with the reverse transcription process by binding to the reverse transcriptase enzyme hence interfering with the process of converting viral RNA into the required DNA	Efavirenz (EFV) Nevirapine (NVP)
<b>Protease Inhibitors (PIs)</b>	Inhibit the viral protease enzyme that is central to forming infectious viral particles	Ritonavir (RTV) Lopinavir (LPV) Atazanavir (ATV)
<b>Integrase Inhibitors (IIs)</b>	Block the HIV enzyme integrase, which the virus uses to integrate its genetic material into the DNA of the cell it has infected	Raltegravir (RTG) Dolutegravir (DTG)

<sup>a</sup>Source: National Institute of Allergy and Infectious Diseases website (NIH, 2013).

Currently, a combination of three ARV drugs from at least two classes is recommended for patients for the first time (first line regimen). When ARVs are given in a combination, they are referred to as antiretroviral therapy (ART). Several ARV combinations are available depending on the age and clinical presentation of the patient. The current preferred first-line ART combination for adults comprises of Efavirenz (EFV), Tenofovir Disoproxil Fumarate (TDF) and either Lamivudine (3TC) or Emtricitabine (FTC) (WHO, 2016a).

Effective ART was introduced over 20 years ago in developed countries and more recently has been introduced in Sub-Saharan Africa (Friedland and Williams, 1999, Sethi et al., 2003, Brown, 2013). ART is initiated based on criteria set-out in national clinical guidelines normally adapted from the WHO guidelines which provide “a public health approach” to the management of HIV. The WHO ART guidelines are mainly adopted in resource limited settings which need a pragmatic approach, and this lead to a single (or limited options) for ART regimen (Parkes-Ratanshi, Personal Communication May 2018). Previously, all HIV-infected persons aged >15 years were initiated on ART at a CD<sub>4</sub> cell count below 200 cells/mm<sup>3</sup> and/or WHO HIV clinical stage 3 or 4. The CD<sub>4</sub> cut-off was later increased to < 350 in 2010 (WHO, 2010). In 2013, WHO offered further guidance recommending ART for all children ≤ 15 years and pregnant and lactating women regardless of their CD<sub>4</sub> count or stage of HIV disease. ART was also recommended for all HIV-infected adults >15years with CD<sub>4</sub> counts ≤500 cells/mm<sup>3</sup> or advanced HIV disease (WHO, 2013). The current guidelines release in 2016 recommend that all persons (both adults and children) diagnosed with HIV should be initiated on ART as soon as possible - an approach referred to as test and treat. The 2016 guidelines are based on evidence that early initiation of ART is associated with better HIV treatment outcomes and reduced transmission rates among patients (Cohen et al., 2012, WHO, 2016a).

ART has been a game changer in the fight against AIDS and mother-to-child transmission of HIV. Following the success of ART, HIV-infection is now a manageable chronic disease and no longer a universally fatal infection (Friedland and Williams, 1999). Globally, HIV related deaths have significantly reduced, and life expectancy of HIV-infected persons has drastically increased over the years (van Sighem *et al.*, 2010, Bor *et al.*, 2013). ART has also dramatically reduced HIV-infection among children born to HIV-infected women. Effective ART can reduce the overall rate of MTCT to <5% in breastfeeding populations and to below 2% in non-breastfeeding populations when maintained throughout both pregnancy and breastfeeding (UNAIDS, 2011). The current global target is to achieve virtual elimination of mother-to-child transmission of HIV (eMTCT) (UNAIDS, 2011, UNAIDS, 2013a).

Currently, there is accelerated scale-up of ART to lower MTCT rates in Sub-Saharan Africa where women of reproductive age are disproportionately affected by the HIV epidemic (UNAIDS, 2013). The current WHO recommendation is that all pregnant and lactating women should initiate lifelong ART as soon as possible after diagnosis, and to breastfeed their babies for up to 24 months for optimal infant survival in resource-limited settings, strategy called PMTCT Option B-plus (WHO, 2013, WHO, 2016a). Consequently, more HIV-infected pregnant women, especially in Eastern and Southern Africa, face longer lifetime exposure to ART given the younger maternal age.

As the HIV population ages, a higher prevalence of age-related comorbidities are being observed especially in developed countries, and now these pose the most significant threat to the health and quality of life for HIV-infected persons (The Antiretroviral Therapy Cohort, 2010, Hasse *et al.*, 2011, Schouten *et al.*, 2014). The same trend is likely to be observed in developing countries especially in sub-Saharan Africa due to increasing longevity. The higher incidence of age-related co-morbidities has been described as 'accelerated ageing' in the HIV population, and is attributed to ART toxicities resulting from long-term exposure (Justice and Falutz, 2014).

#### 1.1.4. Evolution of WHO PMTCT guidelines

MTCT is the primary route of HIV transmission in children (AVERT, 2017). The WHO regularly provides guidance on PMTCT for countries in resource limited settings to adapt to their local circumstances. The first WHO PMTCT guidelines were launched in the year 2000. These guidelines recommended single dose Nevirapine (sdNVP) during labour and avoidance of breastfeeding for all HIV-infected women, where replacement feeding was AFASS (Acceptable, Feasible, Affordable, Sustainable and Safe), to reduce the risk of MTCT (WHO, 2003). Although MTCT rate during labour/delivery was slightly reduced, mortality was high in non-breastfed children, especially in resource-limited settings. Furthermore, avoidance of breastfeeding was associated with stigma in predominantly breastfeeding populations as non-breastfeeding women were assumed to be HIV-positive (Engebretsen *et al.*, 2010, Odeny *et al.*, 2016).

Later research showed that additional ARVs given from 28 weeks of pregnancy and maintained until delivery further reduced MTCT rates (Lallemant *et al.*, 2004, Shapiro *et al.*, 2006). This discovery guided the revision of WHO guidelines in 2006 recommending provision of AZT from 28 weeks, then 3TC from 32 weeks of gestation until delivery. sdNVP was still given during labour, but women with a CD<sub>4</sub> count  $\leq 350$  cells/mm<sup>3</sup> or advanced HIV infection (WHO stage III or IV) were initiated on lifelong ART (WHO, 2006). These guidelines still recommended avoidance of breastfeeding where it was Affordable, Feasible, Acceptable, Sustainable and Safe (AFASS). Women who did not meet the AFASS criteria were recommended to breastfeed exclusively for at least 3-6 months followed by abrupt cessation to avoid mixed feeding (WHO, 2006). Exclusive breastfeeding (EBF) was associated with lower postnatal risk of MTCT compared to mixed feeding (Coutsoudis *et al.*, 1999, Iliff *et al.*, 2005, Coovadia *et al.*, 2007, Becquet *et al.*, 2008). The mechanisms by which EBF reduces the risk of MTCT remain elusive (Filteau, 2010, Filteau, 2011) (see section 2.4.3). Under the 2006 guidelines, the MTCT rates during both pregnancy, and labour/delivery reduced. However, the survival of non-breastfed HIV-exposed uninfected infants was still poor due to the high incidence of malnutrition and acute

infections like pneumonia (Kuhn *et al.*, 2008, Creek *et al.*, 2010, Homsy *et al.*, 2010, Humphrey, 2010, Kafulafula *et al.*, 2010) . Thus, balancing the risks and benefits of breastfeeding on child survival was a challenge in resource limited settings where breastfeeding is the preferred and affordable infant feeding option (WHO, 2010b).

By 2010, clinical trials had provided substantial evidence that ARV prophylaxis maintained during pregnancy, delivery and breastfeeding improved HIV-free survival of infants (Kumwenda *et al.*, 2008, Chasela *et al.*, 2010, Shapiro *et al.*, 2010, Thomas *et al.*, 2011). Consequently, WHO released a third set of guidelines, primarily for low and middle income settings. The revised guidelines strongly recommended HIV-infected mothers to breastfeed for at least 12 months as long as either the mother or infant received ARV prophylaxis. Exclusive breastfeeding was recommended for the first six months followed by complementary feeding up to 12 months of life (WHO, 2010b). Concurrently, separate revised guidelines on use ART in adults and adolescents (WHO, 2010) and children (WHO, 2010c) were released.

The 2010 PMTCT guidelines recommended two options (A and B) for the provision of ARV prophylaxis for women with  $CD_4 \leq 350$  cells/mm<sup>3</sup>. Option A provided AZT from 14 weeks of gestation, maternal sdNVP + AZT/3TC at delivery, while Option B provided ART through pregnancy and breastfeeding. The preferred ART regimens for pregnant women were TDF+3TC+EFV (or NVP) or AZT+3TC+EFV (or NVP) (WHO, 2010a). In both options, the intervention (infant prophylaxis or maternal ART) was stopped four weeks after cessation of breastfeeding in women with a  $CD_4$  count  $> 350$  cells/mm<sup>3</sup>. Pregnant and lactating women with  $CD_4 \leq 350$  cells/mm<sup>3</sup> were maintained on lifelong ART. These guidelines had the potential to reduce overall MTCT rates to  $\leq 5\%$  in breastfeeding populations and significantly improve HIV-free survival of exposed infants. However, programmatic challenges were encountered in the implementation of those elaborate guidelines; and stopping ART after cessation of breastfeeding exposed women to the risk of developing resistance to preferred first-line ART (WHO, 2013).

Thus for programmatic purposes, WHO released the first combined ART guidelines in 2013 which consolidated all ART guidelines for adults, adolescents, children, pregnant and lactating women (WHO, 2013). The guidelines recommended a single ART regimen of TDF+EFV with either 3TC or FTC for all pregnant and breastfeeding women and that ART is maintained at least for the duration of MTCT risk. The cut-off for initiation of lifelong ART was raised from 350 to 500 cells/mm<sup>3</sup>. However, countries with generalised HIV epidemics were recommended to initiate all pregnant and breastfeeding women on lifelong ART (WHO, 2013). The 2010 infant feeding guidelines were retained, so HIV-infected women were advised to breastfeed for at least 12 months while on ART.

In 2014, WHO issued revised consolidated ART guidelines recommending initiation of lifelong ART in all HIV-infected pregnant and lactating women for the elimination of mother-to-child transmission of HIV (eMTCT), a strategy referred to as Option B-plus (B<sup>+</sup>) (WHO, 2014). Further guidance was provided in 2016, recommending that HIV-infected women in resource limited settings should breastfeed for at least 12 months and may continue breastfeeding up to 24 months while on Option B-plus ART (WHO, 2016a, WHO, 2016b). Overall, the PMTCT guidelines have increased uptake of HIV testing among pregnant and lactating women and reduced MTCT rates, but retention of mother-baby pairs along the eMTCT cascade on Option B+ remain a challenge (Gumede-Moyo *et al.*, 2017).

## 1.2. The situation in Uganda

### 1.2.1. History and prevalence of HIV in Uganda

The Republic of Uganda is located in East Africa and lies on the equator. The capital city is Kampala. According to the last census conducted in 2014, the total population was 34.6 million people. The median age of the population is 15.4 years, and the annual population growth rate is 3.0%. Kampala has a population of 1.5 million people. It is the second densely populated district after the surrounding Wakiso district which has 2.0 million people (Uganda Bureau of Statistics, 2016).



Map data © 2018 Google, INEGI United Kingdom (Reproduced with permission)



Dr Goldberg (PhD supervisor) and I at the equator crossing in Kayabwe, Mpigi District, about 72km from Kampala, Uganda.

Figure 3: Location of Uganda on the map of Africa and equator crossing landmark at Kayabwe

The first clinical cases of AIDS in Uganda were reported in 1982. A local name 'slim' was coined to refer to the disease (based on the characteristic symptoms of severe weight loss) that was later called AIDS by clinicians and scientists worldwide (Serwadda *et al.*, 1985). Since then Uganda has been leading in the fight against HIV infection, in both adults and children, making tremendous achievements between 1995 and 2006 when HIV prevalence reduced from 21% to 6.4%. This achievement made Uganda a model country in the implementation of HIV prevention programs (Slutkin *et al.*, 2006).

However, these impressive gains are slowly being reversed as adult HIV prevalence is gradually rising. The last national AIDS indicator survey (NAIS) of adults aged 15-49 years conducted in 2011 reported a national HIV prevalence of 7.3% among adults (MOH-Uganda, 2012b). Also, Uganda was among the three countries in the world where the number of new HIV infections in adults had increased (UNAIDS, 2013b). In 2015, the number of people living with HIV had increased from 1.4 million (in 2013) to 1.5 million, confirming a continued rise in the burden of HIV in Uganda since the last NAIS survey (Uganda AIDS Commission, 2015). However, preliminary results of a recent nationwide survey of 12,483 households indicated a lower HIV prevalence (6.0%) in adults aged 15-49 years, and a 0.5% prevalence among children aged <15 years (MOH-Uganda, 2017).

At a regional level, there is significant variation in the distribution of HIV-prevalence in Uganda. HIV prevalence rates range from 2.8% in the West-Nile to 7.7% in South-West Region (Figure 4). Kampala had a prevalence of 6.6%, which was lower than the national average (6.0%) and the surrounding Central one (7.4%) and two regions (7.6%). Overall, the burden of HIV was higher in urban areas compared to rural areas (7.1% vs 5.5%) (MOH-Uganda, 2017).

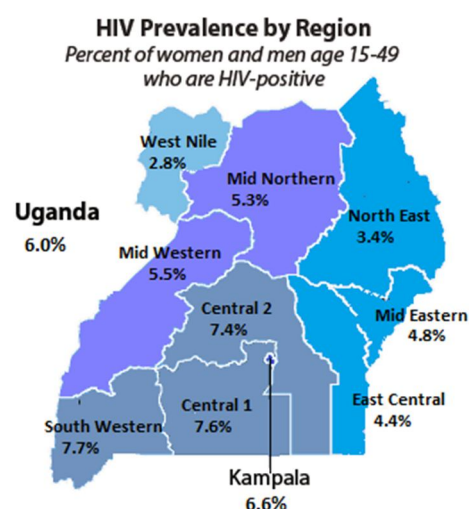


Figure 4: Map of Uganda showing HIV prevalence by region

(Updated map based on data by MOH-Uganda 2017)

With respect to gender, women are disproportionately affected by HIV throughout the country. In 2016, the prevalence of HIV infection in women was 7.5% compared to 4.3% in men (MOH-Uganda, 2017). Prevalence rates ranging from 13.5% to 4.8% have been reported in ANC surveillance surveys at different sentinel sites, and the figures are comparable to those observed in the MRC General Population cohort (Uganda AIDS Commission, 2015).

### *1.2.2. HIV and ART programmes in Uganda*

Effective ART was introduced in Uganda in 2006, and since then free ART programs have been scaling-up nationwide. The national HIV-care guidelines have also changed rapidly within the past five years based on global guidance from WHO (see section 1.1.3). In 2016, the country adopted the WHO test and treat strategy which recommends initiation of ART “at the earliest opportunity in all people with confirmed HIV infection, regardless of clinical stage or CD<sub>4</sub> cell count” (MOH-Uganda, 2016). The recommended first-line ART regimen for adults and adolescents aged >10 years is TDF-3TC-EFV, given as a daily fixed dose combination. The first line regimens for HIV-infected children aged <10 years are ABC-3TC-EFV (children aged 3-10 years) and ABC+3TC+LPV/r (for children below three years). These national guidelines also outline alternative regimens where the preferred first line is clinically contraindicated, as well as second line and salvage therapies for patients who fail on first and second line, respectively (MOH-Uganda, 2016).

HIV care programmes in the country are offered by various NGOs on behalf of the Ministry of Health and mainly funded by The US President’s Emergency Program for AIDS Relief (PEPFAR) through the American Centres for Disease control and Prevention (CDC) and the Global Fund (PEPFAR, 2017). The major NGOs and institutions providing HIV care countrywide include: The AIDS Support Organisation (TASO), Baylor College of Medicine Children’s Foundation-Uganda (Baylor-Uganda), Infectious Diseases Institute (IDI), Mildmay, Elizabeth Glazier Paediatric HIV/AIDS Foundation (EGPAF), Makerere University Johns Hopkins Research Collaboration (MUJHU) and Makerere University – Mbarara University Joint Aids Program (MJAP).

At a national level, delivery of HIV-care services has been rationalised by the Ministry of Health. Each partner organisation works with all health facilities within an allocated region (s) to avoid duplication of effort. The recommended implementation strategy is the Health Systems Strengthening (HSS) approach. WHO defines HSS as “improving the health system building blocks and managing interactions in ways that achieve more equitable and sustained improvements across health systems and outcomes” (WHO, 2007). Prior, to starting my PhD at Cambridge, I was privileged to work with over 400 government health facilities through an HSS approach while I was employed at Baylor-Uganda.



### 1.2.3. PMTCT in Uganda

Uganda has always been at the forefront of research and implementation of PMTCT guidelines. In 1999, Musoke et al. published an initial study that showed single dose Nevirapine (sdNVP) during labour was effective in reducing the risk of MTCT (Musoke *et al.*, 1999). This landmark study led to the development of the first WHO PMTCT guidelines for resource limited settings, and Uganda released its first national PMTCT guidelines in the same year. These recommended sdNVP during labour and avoidance of breastfeeding for all HIV-infected women (MOH-Uganda, 2003, Esiru, 2013). National PMTCT guidelines have also evolved in Uganda over the past 16 years to match the WHO recommendations. The milestones of PMTCT implementation in Uganda are shown in Figure 5.

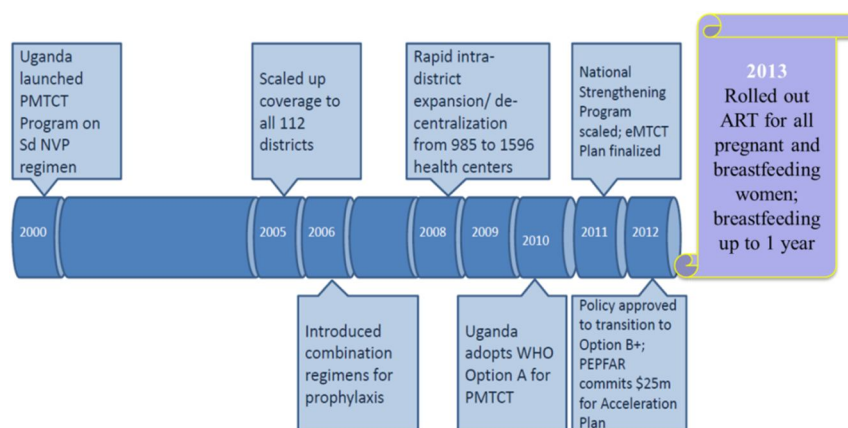


Figure 5: Flowchart illustrating evolution of PMTCT guidelines in Uganda

(Esiru, 2013) [Original figure without the scroll – reproduced and modified with permission]

In 2010, when WHO recommended two PMTCT Options (A and B), Uganda adopted Option A providing sdNVP and AZT/3TC because Option B which provided ART for the duration of MTCT was not affordable. Uganda transitioned to providing lifelong ART for all pregnant and breastfeeding women in 2012 following rapid advice from WHO; a strategy referred to as PMTCT Option B-plus (referred to as Option B-plus in this thesis). With financial commitment from PEPFAR, the country could then afford to maintain all pregnant and breastfeeding women on lifelong ART (MOH-Uganda, 2011).

The revised national PMTCT guidelines were released in early 2013. These recommended that all HIV infected pregnant and breastfeeding women should be initiated on lifelong ART as soon as possible regardless of their immune status, and strongly recommended HIV-exposed infant to be breastfed for at least 12 months of age. The recommended first-line regimen for pregnant and breastfeeding



women was TDF+3TC and EFV. Also, HIV-exposed infants were recommended NVP prophylaxis from birth to 6 weeks of age and thereafter cotrimoxazole prophylaxis until six weeks after cessation of breastfeeding. The first DNA-PCR HIV-test for the infants was done at six weeks of age and repeated at six weeks after cessation of breastfeeding. The infants were monitored until 18 months of age when a final confirmatory HIV-test is done and those confirmed HIV-negative at discharge from the PMTCT clinic. Infants who test HIV-positive at any point were referred to the ART clinic and initiated on ART as soon as possible (MOH-Uganda, 2013).

Uganda was the second country in Sub-Saharan Africa after Malawi to adopt Option B-plus guidelines at the time when I conceptualised the study described in this thesis and submitted my application for a Gates Cambridge Scholarship in December in 2012. The roll-out of Option B-plus in Uganda was implemented in phases. Mulago hospital, which runs the largest PMTCT clinic in the country, was among the first to implement. By 2014 when I wrote the full study protocol (first year of PhD) the Mulago PMTCT program had fully transitioned to Option B-plus and this presented a unique opportunity to investigate research questions on Option B-plus especially at that early stage of roll-out in sub-Saharan Africa.

Further revision of PMTCT guidelines has taken place after the current study commenced. In 2016 the national consolidated ART guidelines were revised to match the WHO recommendations, but the PMTCT Option B-plus ART guidelines were upheld. However, Uganda did not adopt the current WHO recommendation to extend the duration of breastfeeding to 24 months due to concerns about increased risk of MTCT during prolonged breastfeeding (MOH-Uganda, 2016, WHO, 2016b).

Implementation of the PMTCT programme in Uganda has rapidly scaled up since 2000 and dramatically reduced new infections among children. At the beginning of the program in 2000, the annual number of new HIV infections in children was about 25,000. In 2012 when the country started the transition to Option B+, new paediatric HIV-infections had reduced to about 15,000 per year. By 2015, after only three years of implementing Option B-plus, the number of new infections had drastically reduced to about 3,486 per year (Uganda AIDS Commission, 2016). Uganda is on course to meet the fast track target of less than 1,300 new infections in children by 2020 (Figure 6). In June 2016, 97% (117,784) of HIV-infected pregnant women received ART for eMTCT, an increase from 112,909 in 2014. (Uganda AIDS Commission, 2015, Uganda AIDS Commission, 2016). Given the high burden of HIV in women and the excellent coverage for eMTCT services, more HIV-infected mothers and their babies are having a longer duration of exposure to ART. Thus, there is a growing focus on prevention and management of ART side effects.

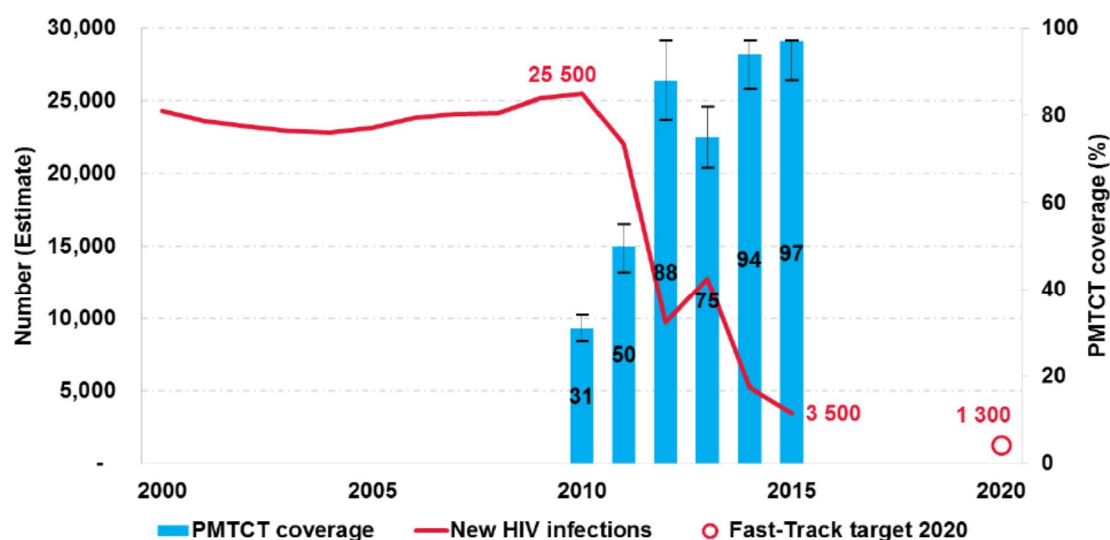


Figure 6: Uganda's progress towards 2020 fast track target of new HIV-infections in children

(Uganda AIDS Commission, 2016)[Open access for reuse in academic thesis]

### 1.3. HIV, ART and Bone

#### 1.3.1. Bone health

Bone health, in the context of public health, is defined as the quality of being at low risk of three common skeletal disorders, stunting, rickets and osteomalacia, and osteoporosis and fragility fractures (Prentice *et al.*, 2006a). Stunting is the impaired growth and development that children experience from poor nutrition, repeated infection, and inadequate psychosocial stimulation"; and is defined by low length or height-for-age below -2SD of the reference population and is commonly a result of suboptimal health and/or nutritional conditions (Prentice *et al.*, 2006b, WHO, 2018b). Rickets and osteomalacia are abnormalities of the skeleton caused by a combination of low bone mineral and collagen (Prentice *et al.*, 2006b). "Osteoporosis is a skeletal disease characterised by low bone mass, microarchitectural deterioration of bone tissue leading to enhanced bone fragility, and a consequent increase in fracture risk" (Consensus Development Conference, 1991).

Bone mineral content (BMC) and bone mineral density (BMD) are good predictors of fracture risk in both adults and children (Melton *et al.*, 1993, Marshall *et al.*, 1996, Melton *et al.*, 1998, Black *et al.*, 2018). Several non-invasive imaging techniques exist for *in vivo* assessment of bone BMC and BMD. These include dual-energy X-ray absorptiometry (DXA), quantitative ultrasound (QUS), quantitative computed tomography (QCT), and peripheral quantitative computed tomography (pQCT).

DXA, QCT and pQCT techniques use radiation while QUS uses ultrasound waves to estimate bone mineral. The study described in this thesis used DXA which is the current gold standard for measuring bone mineral and diagnosis of osteoporosis. Measurement of bone mineral using DXA is discussed in section 2.1.3.

### *1.3.2. Overview of bone health in the context of HIV and ART*

Greater incidence and prevalence of both osteoporosis and fractures have been reported among HIV-infected persons compared to HIV negative persons in Caucasian populations (Torti *et al.*, 2012, Yin *et al.*, 2012, Güerri-Fernandez *et al.*, 2013). There is some evidence that HIV infection *per se* may have a direct impact on bone through a variety of mechanisms that may increase resorption and decrease formation (Gibellini *et al.*, 2012). HIV-infected populations tend to have a higher prevalence of traditional risk factors for low bone mineral density (BMD) (Carr *et al.*, 2001, Dolan *et al.*, 2006, Prior *et al.*, 2007). Furthermore, the HIV-infected groups are likely to be confounded by exposure to ART.

Initiation of ART is associated with a 2-6% reduction in bone mineral density (BMD) at the spine and the hip within the first 96 weeks regardless of ART regimen used. Thereafter, BMD remains stable but does not return to baseline/pre-ART values (Brown *et al.*, 2009, Brown, 2013). The most significant declines in BMD (measured by DXA) occurs between 24-48 weeks following ART initiation. Lower baseline CD<sub>4</sub>, female gender, older age, lower BMI, higher HIV viral load are all associated with greater reductions in BMD (Grant *et al.*, 2013). Clinical trials have shown that TDF, a preferred drug in the current first-line ART regimen, causes greater reductions (1-2%) in BMD compared to other ARVs (Gallant *et al.*, 2004, Stellbrink *et al.*, 2010). HIV-infected persons on TDF based regimens are at increased risk of osteoporosis and fractures compared to persons on other ART regimens (Battalora *et al.*, 2014b).

A review of studies published on ART-related bone loss in HIV-infected women identified > 3% lower BMD at the femoral neck among women on PI-based ART compared to non-PI based ART, but there was no difference in BMD at the lumbar spine (Carvalho *et al.*, 2010). However, none of the studies included in that review was conducted in pregnant, lactating or African women. Recently, Hamill *et al.* reported a 2-3% mean decrease in BMD and size-adjusted bone mineral content (SA-BMC) at the total hip, femoral neck and lumbar spine at 12 months following initiation of ART in black South African women who were neither pregnant nor lactating (NPNL) (Hamill *et al.*, 2017). Overall, the majority of existing studies have excluded pregnant and breastfeeding women.

Pregnancy and lactation are associated with physiological changes in maternal bone mineral density to supply calcium for bone mineral accretion in the offspring in utero and through breastmilk, but most evidence shows that this is recovered after weaning or even higher than before (Olausson *et al.*, 2012). Therefore, ART may disrupt the normal process of bone mineral mobilisation in the mother, leading to bone loss in the mother that is not recovered and compromised growth/BMD accretion in the baby. Extensive reviews of studies that used DXA in HIV negative pregnant women identified a +0.5 to -2% mean change in the whole body BMD during pregnancy and a +1.8 to -4.5% mean change in BMD at the spine and/or hip (Olausson *et al.*, 2012, Kovacs, 2016). Recovery of BMD has been observed after cessation of breastfeeding in apparently healthy women, including even in African women on a very low calcium intake after long durations of breastfeeding (Sawo *et al.*, 2013). However, data is limited on the changes in BMD in HIV-infected breastfeeding women in either Caucasian or African populations.

At the time of designing the Gumba study, there was only one publication on the effect of ART on maternal bone mineral. Mora *et al.* studied bone mineral in 33 HIV-positive (on antepartum ART) and 116 HIV-negative Italian mother-baby pairs. The median exposure to ART during pregnancy was 33 weeks, and HIV-infected women did not breastfeed. However, only 17 HIV-positive and 57-HIV-negative mother-baby pairs were studied at 4 and 12 months. Mid-tibia cortical bone mineral was measured at baseline (within 4 days after delivery), 4 and 12 months postpartum using QUS. The investigators did not find significant differences in maternal cortical bone mineral at baseline or within the first year postpartum (Mora *et al.*, 2013). They also reported no significant differences in infant cortical bone mineral within 4 days of birth, 4 and 12 months of age; and bone mineral accrual was not significantly different between the groups. HIV-exposed infants had lower weight and length at baseline and 4 months of age, but there was no significant difference at 12 months. Adjusting for gestational age and weight did not have a significant effect on the results. The investigators also reported no significant differences in cord blood BAP and CTX between HIV-exposed and unexposed infants (Mora *et al.*, 2012).

The two papers by Mora *et al.* contribute to our understanding of postpartum changes in cortical bone mineral in Caucasian HIV-infected women and their infants following initiation of ART during pregnancy. However, the studies had a relatively small sample size of HIV-infected women especially in the longitudinal follow-up (17 women), so they may not have had adequate power to detect differences between the groups. Also, cortical bone was assessed although most evidence shows that mobilisation of bone mineral during pregnancy and lactation is more evident in the trabecular-rich bone sites (Kolthoff *et al.*, 1998). The bone imaging technique used (Qualitative ultrasound,

QUS, discussed in section 2.1.3) measures the speed of sound through bone, hence it is not a direct quantification of bone mineral. Furthermore, maternal ART and infant feeding practice mirrors the current recommendations in the western countries (i.e. HIV-infected women received ART during pregnancy and did not breastfeed), but not in SSA. Therefore, the findings by Mora et al. may not apply to HIV-positive women on lifelong ART under the current Option B+ especially in sub-Saharan Africa where breastfeeding is recommended. It is therefore important to describe bone mineral in breastfeeding HIV-infected African women on lifelong ART and bone mineral accretion in their babies. The Gumba study described in this thesis studied bone mineral using DXA, the gold standard for bone mineral assessment, in breastfeeding HIV-infected African mothers on Option B+ and their infants within the first 3 months of life.

Also at the time of designing the Gumba study, a multicentre clinical trial, PROMISE (Promoting Maternal and Infant Survival Everywhere) P1084s was ongoing. PROMISE P1084s compared bone and renal outcomes of mother-baby pairs on PMTCT Option A vs Option B in Uganda, Zimbabwe, Malawi and South Africa. The parent PROMISE study was a cross-over trial that randomised pregnant mothers into two PMTCT arms: Triple ART (ZDV/3TC/LPV/r or TDF/3TC/LPV/r) or ZDV in pregnancy + SdNVP + TDF/FTC at birth. Postpartum mothers (n=2431) and their infants (n=2444, including 13 sets of twins) were randomised within 14 days after delivery to either maternal ART or infant nevirapine prophylaxis. PROMISE P1084s recruited a subset of 400 mother-baby pairs (167 in the postpartum ART arm and 170 in the infant prophylaxis arm) who were not exposed to ART during pregnancy and did not have a control group of HIV-negative women.

Towards the end of my PhD, preliminary findings from PROMISE P1084s were presented at the 2016 HIV paediatrics workshop in Durban SA. Mothers in the maternal triple ART arm experienced greater reductions in bone mineral between 1 and 74 weeks postpartum compared to the infant prophylaxis arm (LS: -2.06% vs +1.09%,  $p<0.001$ ; THip: -5.37% vs -3.05%,  $p<0.001$ ) (Stranix Chibanda and PROMISE-team, 2016). However, potential confounders (especially changes in body weight, breastfeeding practices, duration of amenorrhoea and use of hormonal contraception/depo-provera) were not adjusted for. Hopefully, these limitations will be addressed when the full paper is published. The PROMISE study did not have a control group of HIV-negative women for comparison of either maternal or infant outcomes. Therefore, the study reported in this thesis was designed to overcome that limitation.

### 1.3.3. Overview of infant growth and bone health in the context of maternal HIV/ART

Maternal Tenofovir (TDF) is transferred to the offspring in utero across the placenta and postpartum through breast milk (Benaboud *et al.*, 2011, Flynn *et al.*, 2011). Animal studies have reported adverse effects of TDF exposure during gestation on fetal bone mineralisation in monkeys although this was at higher doses of TDF compared to those currently used in humans, and the effects of TDF on bone were dose-dependent (Tarantal *et al.*, 1999, Tarantal *et al.*, 2002). Pharmacokinetic studies have reported greater transfer of TDF to the infant in-utero compared to postpartum through breastmilk (Benaboud *et al.*, 2011). There is also evidence that 3TC and EFV (other preferred first line drugs in the current Option B+ regimen, see section 2.3.3 for their effect on bone) also penetrate into breastmilk in small quantities (Palombi *et al.*, 2016). To date, human studies have reported conflicting evidence on maternal TDF use during pregnancy on bone and growth outcomes in the offspring who are not HIV-infected (HIV exposed uninfected infants).

In PHACS (USA-based Paediatric HIV/AIDS Cohort) study, significantly lower mean length-for-age Z-scores ( $-0.17 \pm 1.00$  vs  $-0.02 \pm 1.14$ ;  $p=0.04$ ) and head circumference-for-age Z-scores ( $-0.23 \pm 1.10$  vs  $-0.41 \pm 1.25$ ;  $p=0.02$ ) were observed at age one year of age in TDF-exposed infants compared to TDF-unexposed infants born to HIV-infected American women; but WAZ was not significantly different between the groups. More data from PHACS showed lower mean whole body BMC in neonates with in-utero exposure to maternal TDF compared to TDF unexposed infants ( $-7.8\text{g}$  [95% CI  $-12.6\text{g}$ ,  $-3.1\text{g}$ ];  $p=0.01$ ). The difference between the groups remained significant after adjusting for clinical site in addition to age, smoking, CD<sub>4</sub> count, viral load, sex, gestational age, age at dual-energy X-ray absorptiometry, race, and body length ( $-5.3\text{g}$  (95% CI  $-9.5\text{g}$ ,  $-1.2\text{g}$ ;  $p=0.013$ ) (Siberry *et al.*, 2015). However, infant body weight was not initially adjusted for, and that was queried by Koo and colleagues (Koo *et al.*, 2016). After adjustment for both infant length and body mass, the difference between the groups reduced to  $-3.2\text{g}$  (95% CI  $-5.7\text{g}$ ,  $-0.7\text{g}$ ) and remained significant ( $p=0.014$ ) (Siberry and Jacobson, 2016). In the same study, there were no significant differences between the groups in mean length-for-age ( $-0.41$  vs  $-0.18$ ) or weight-for-age ( $-0.71$  vs  $-0.48$ ) Z-scores.

PROMISE P1084s study (described in section 1.3.2) also observed lower whole body bone mineral among maternal HIV and ART-exposed neonates compared to HIV-exposed but ART unexposed infants; but there were no significant differences in growth (Siberry *et al.*, 2016). These data suggest compromised bone mineral accretion in neonates exposed to maternal TDF during pregnancy. However, the longer term implications and clinical significance for infant bone health are not yet clear. This thesis provides data on bone mineral accretion in maternal HIV/ART exposed infants within the first three months of life.

In contrast to studies on maternal and infant bone mineral, there are many published studies on growth of HIV-exposed infants. The majority have reported poorer growth in HIV-exposed infants compared to HIV-unexposed (Evans *et al.*, 2016) (reviewed in section 2.4). Muhangi *et al.* reported two times greater odds of underweight in HIV-exposed Ugandan infants compared to unexposed infants at one year of age (Muhangi *et al.*, 2013a). The Development of AntiRetroviral Therapy in Africa (DART) trial, conducted between 2003-2009, did not observe significant differences in anthropometric Z-scores between in-utero TDF-exposed and unexposed (Ugandan and Zimbabwean) children within the first 3 years of life. However, Ugandan children had significantly lower Z-scores compared to Zimbabwean children.

To date, DART has the longest follow-up data published on growth of HIV-exposed infants following in-utero exposure to maternal ART. In Uganda, the DART trial was conducted in Kampala, and it did not have a control arm of HIV-unexposed children. The investigators argued that the mean Z-scores of Ugandan children were similar to a reference population in South-West Uganda (Gibb *et al.*, 2012). However, the latter were collected in 1988 when the prevalence of stunting and underweight was twice as high as Kampala (stunting 27.9% vs 14.2%; underweight 21.2% vs 10.5%)(MOH-Uganda, 1989). Since 1988, the region has consistently had a greater burden of malnutrition in the national demographic and health surveys. Therefore, the South –West reference data are not representative.

Pooled data from the parent PROMISE study did not reveal significant differences in growth between HIV-exposed infants in the maternal ART arm compared to the infant prophylaxis arm (Stranix-Chibanda and PROMISE-team, 2016). Contrary, a sub-analysis of the parent PROMISE data (only Uganda and Malawi) revealed significantly lower anthropometric mean Z-scores (weight, length and head circumference) in HIV and ART-exposed Ugandan infants compared to unexposed infants at 12 and 24 months of age, but the differences were not observed in Malawian infants at the same timepoints (Aizire *et al.*, 2016). Contrary, other studies have not observed adverse effects of maternal TDF use on infant outcomes (Nurutdinova *et al.*, 2008, Vigino *et al.*, 2011, Gibb *et al.*, 2012). However, all the three PROMISE publications/presentations do not present data on breastfeeding and infant feeding practices which are major determinants of infant growth. It is therefore important to investigate the disparities in growth in Ugandan HIV-exposed children in studies with a control group of HIV-unexposed children in the same environment.

Overall, the majority of the studies have concentrated on in-utero exposure to TDF although maternal EFV and 3TC may affect bone and lack control groups of HIV-unexposed uninfected infants for comparison of outcomes. Although, there is growing re-assurance that exposure to maternal TDF-based ART has minimal adverse effects on the offspring (Mofenson *et al.*, 2017), data are

limited on infant bone mineral accretion beyond the neonatal period following both in-utero and extended exposure to maternal ART/TDF through breastfeeding (12-24months) as per the current Option B+ guidelines. It is therefore important to describe bone mineral accretion in HIV-exposed African children in longitudinal studies.

#### *1.3.4. Bone health in Uganda*

The prevalence of osteoporosis in Uganda is currently unknown, and there is no national screening program. However, there are a few studies which have looked at disability among the elderly and asked questions on bone health. In the 2014 Uganda national household survey, 2,628 persons aged over 50 years were asked the question “Do you have difficulty walking or climbing steps?” Overall 19.6% and 8.8% respectively reported: “some difficulty” and “a lot of difficulty” walking or climbing the stairs. More women (33.6%) compared to men reported difficulty walking/climbing stairs (22.7%). Also, the proportion of respondents who reported a lot of difficulty walking/climbing stairs was greater among women (10.8%) compared to men (6.6%) (Wandera *et al.*, 2014b). A local non-governmental organisation, The Aged Family Uganda (TAFU) conducted a survey in 2006 among elderly persons aged 65-80 years in 7 districts of Kampala, in which they asked the question “Are you sick now”. Out of the 382 people surveyed, the highest proportion (81%) reported back and bone problems (Cumming, 2006).

Uganda has two DXA scanners located at MUJHU (within Mulago hospital) which are only used for research. These have been used in IMPAACT (International Maternal Pediatric Adolescent AIDS Clinical Trials Network) studies including PROMISE1048s (discussed in sections 1.3.2 and 1.3.3). Studies outside IMPAACT have also used DXA equipment at MUJHU. A study conducted among 181 HIV-infected adults aged 31-41 years who were failing on first-line ART reported 50.1% and 25% prevalence of low BMD at the spine and the hip, respectively (Wandera *et al.*, 2014a). In that study, the prevalence of osteoporosis at the lumbar spine and hip was 8% and 1.1%, respectively; and the prevalence of osteopenia was 46.6% and 27.1% at the lumbar spine and hip, respectively. BMI was associated with BMD at both the lumbar spine and hip while the use of TDF in first-line ART regimen was associated with low BMD at the spine. The investigators defined low BMD as T-score < -1, osteoporosis as T-score less than -2.5 and osteopenia as T-score between -2.5 and -1, respectively (Wandera *et al.* 2014).



National data on the prevalence of stunting among children aged less than five years is reported every five years in the Uganda Demographic and Health Surveys (UDHS). According to the last UDHS results released in 2016, the national prevalence of stunting was 29% (Uganda Bureau of Statistics and International, 2017). Studies conducted in Uganda have reported a higher burden of stunting among HIV-exposed uninfected children compared to HIV-unexposed uninfected children (Fadnes *et al.*, 2010, Muhangi *et al.*, 2013b, Engebretsen *et al.*, 2014) although others have not (Nalwoga *et al.*, 2010).

The majority of these studies, conducted in the pre-Option B-plus era, observed that stunting was associated with sub-optimal infant feeding practices and lower socio-economic status (Muhangi *et al.*, 2013a, Odongkara *et al.*, 2013). At Baylor Uganda, I conducted a retrospective study based on chart reviews for 4000 HIV-exposed infants aged <1 year at Mulago PMTCT clinic, following the change in WHO PMTCT and infant feeding guidelines. At the time WHO 2010 guidelines recommended breastfeeding for at least 12 months and maternal ART (either PMTCT Option A or B). The study found that a greater proportion of exposed infants on WHO 2010 guidelines were breastfed, but they were two times more likely to be stunted compared to infants on WHO 2006 guidelines (30% vs 16%,  $p \leq 0.0001$  based on WHO 2006 growth references (Nabwire *et al.* 2012). However, that study did not have a control group of HIV-unexposed uninfected infants for comparison of growth outcomes.

#### **1.4. Research problem**

Sub-Saharan Africa (SSA) already has twice the number of older adults (aged 60+) than northern Europe, and this figure is expected to grow faster than anywhere else in the world, increasing from 46 million in 2015 to 157 million by 2050 (International Osteoporosis Foundation, 2011). A demographic explosion is predicted for older people in Africa and the Middle East, such that those aged 50+ will account for 25% of the population by 2020 and 40% by 2050, conveying the highest proportional increase in hip fracture rates than any other region word (Cooper *et al.*, 2011). Hip fractures result in the loss of independence, lost wages, economic instability and increased mortality among the elderly. Women are at increased risk of fractures in old age compared to men. When aged 60, further life expectancy in SSA is 16 years for women and 14 for men, suggesting that for those who survive early life challenges, a long old age is already a reality (Aboderin and Beard, 2015).

Women of reproductive age are disproportionately affected by HIV in SSA. Scale-up of lifelong ART for pregnant and lactating women (Option B+ strategy) has dramatically improved maternal health and reduced MTCT over the past five years. Initiation of ART is associated with a 2-6% decrease in BMD regardless of the ART regimen used. Tenofovir, a preferred drug in the current first-line ART regimen, is associated with 1-2% greater decrease in BMD and increased risk of fractures compared to other ARVs (Stellbrink *et al.*, 2010). Pregnancy and lactation are associated with physiological changes in BMD (up to -4.5%), but recovery occurs after cessation of breastfeeding. However, the majority of published studies on HIV associated bone loss and fractures have been conducted in either men or non-pregnant and non-lactating women. Consequently, there is limited evidence on whether initiation of ART, pregnancy and lactation have an additive effect on bone loss among HIV-infected women on TDF- based ART, and if recovery of bone mineral occurs after cessation of breastfeeding. Therefore, research on bone health in these populations will inform interventions to mitigate the projected increase in fractures.

#### **1.5. Hypothesis**

The hypothesis of this thesis is that ART may accentuate the normal process of maternal bone mineral mobilisation, leading to bone loss in the mother that is not recovered, and compromised growth and/or bone mineral accretion in the baby.

## 1.6. Research plan

### *1.6.1. Overview of the study design and rationale*

This is a longitudinal observational cohort study with two groups of women and their infants namely: (1) HIV-infected pregnant women initiated on first-line ART under Option B-plus during the current pregnancy (HIV-positive), and (2) HIV-negative pregnant women who had never been on ART (HIV-negative). This longitudinal design was selected because it presented an opportunity for observation of both cross-sectional and longitudinal changes in outcomes between the study groups.

The study population was HIV-infected pregnant women antepartum and breastfeeding mother-baby pairs enrolled on the PMTCT Option B-plus programme at Mulago Hospital in Kampala, Uganda. HIV-infected pregnant women initiating ART under Option B-plus were selected because most of the existing studies on ART and bone excluded pregnant and breastfeeding women (discussed in section 1.3.2). Children born to both HIV-infected and HIV-negative women were followed for comparison of bone and growth outcomes following maternal ART exposure (discussed in section 1.3.3).

### *1.6.2. Study objectives and outcomes*

The primary objective was to determine if HIV-positive women initiated on lifelong ART during pregnancy experience a greater reduction in bone mineral during lactation compared to HIV-negative women. The primary outcome was the difference between the groups in % change ( $\pm$  SE) in maternal lumbar spine (LS) areal bone mineral (aBMD) between PP2 and PP14.

Secondary outcomes were the cross-sectional and longitudinal differences between the groups in the maternal total hip (TH) and whole body (WB) bone mineral, and body composition measured by DXA, anthropometry, breast milk composition, plasma and urine biochemistry, calciotropic hormones and bone turnover markers; and infant anthropometry, growth Z-scores, bone mineral content and body composition.

### *1.6.3. Approach taken to address objectives and test hypothesis*

Both HIV-negative and HIV-positive pregnant women who were ART naïve before the index pregnancy, were recruited from Mulago Hospital in Kampala, Uganda. Pregnant women were followed at 36 weeks of gestation (PG36). Thereafter, mother-baby pairs were followed postpartum at 2 and 14 weeks postpartum (PP2 and PP14, respectively). ART was provided under routine clinical care as per the prevailing clinical guidelines. Detailed descriptions of the conduct of the study visits are presented in chapter 3.

DXA scans were performed to measure bone phenotype (areal BMD (aBMD), bone area (BA), bone mineral content (BMC), and size-adjusted BMC (SA-BMC) in mother-baby pairs at 2 and 14 weeks postpartum. Physiological and biochemical data (blood and urine biochemistry), calciotropic hormones [25OHD and PTH], markers of bone turnover (procollagen type 1 N-terminal propeptide [P1NP], C-terminal telopeptide, [CTX], bone-specific alkaline phosphatase [BAP]) and breast milk mineral composition (Ca, P, Na and K) were collected to investigate potential disruptions in calcium-vitamin D and bone metabolism. Data were also collected on infant growth, and potential confounders (maternal anthropometry, medical history, maternal dietary intake of calcium and vitamin D rich foods, breastfeeding practices, and physical activity. Detailed description of the study methods and the rationale is presented in chapter 4 section 4.1.

## 1.7. Structure of thesis

This thesis is organised into the following chapters:

**Chapter 2:** Literature review. Presents an overview of HIV prevalence, ART, biology physiology and effects of pregnancy/ lactation on the bone, then builds up to a critical appraisal of studies on HIV/ART and pregnancy/lactation. The chapter also critiques studies on maternal HIV/ART and infant growth and maternal HIV/ART and lactation.

**Chapter 3:** Study design. Outlines the study objectives study design setting, participants, recruitment and a summary of the protocol.

**Chapter 4:** Study methods. Describes the methods/techniques used in data collection, laboratory, data handling and statistical analysis.

**Chapter 5:** Results at baseline and PG36. Presents the cross-sectional differences between the groups for maternal outcomes at 36 weeks of gestation, with and without adjustment for potential confounders where appropriate.

**Chapter 6:** Results at two weeks postpartum. Presents the cross-sectional differences between the groups for maternal and infant outcomes at two weeks postpartum, with and without adjustment for potential confounders where appropriate.

**Chapter 7:** Results at 14 weeks postpartum. Presents the cross-sectional differences between the groups for maternal and infant outcomes at 14 weeks postpartum, with and without adjustment for potential confounders where appropriate.

**Chapter 8:** Longitudinal results. Presents change in maternal and infant outcomes between 36 weeks of gestation and 14 weeks postpartum. The chapter compares the differences between the groups in mean percent changes presented with and without adjustment for potential confounders.

**Chapter 9:** Discussion. The chapter discusses the main findings in the context of the existing literature and gives recommendations for future research.

## CHAPTER 2: LITERATURE REVIEW

### 2.1. Overview of bone biology and metabolism

#### 2.1.1. Bone Biology

Bone is a tissue composed of an extracellular matrix and cells. The matrix contains both organic (35%) and inorganic (65%) molecules. The organic matrix is made predominantly of type I collagen (90%) with small amounts of non-collagenous proteins such as osteocalcin, glycoproteins and proteoglycans, and is mineralised by hydroxyapatite crystals which are formed by inorganic calcium and phosphate (Hlaing and Compston, 2014). The primary functions of bone are to provide mechanical support to the body, act as a reservoir for calcium and phosphate (discussed later in section 2.2.3) and a site for production of blood cells in the bone marrow (Grabowski, 2009).

Bone modelling and accretion starts during fetal growth and continues up to about 30 years of age when peak bone mass is achieved, and after that bone mineral remains relatively stable over the next 15-30 years. The adult skeleton contains about 1-1.5kg of calcium after peak bone mass is attained. The calcium required for bone mineralisation is acquired from the diet. There are two types of bone: cortical and trabecular, and the distribution differs at various sites of the skeleton. Cortical bone is more compact compared to trabecular bone and forms a bigger proportion of the long bones, giving structure and strength to bone. The shafts of long bones are predominantly made-up of cortical bone (Grabowski, 2009). Trabecular bone is more porous and metabolically active than cortical bone, and a greater proportion is found in the vertebrae and the ends of long bones.

Throughout life, bone continuously undergoes remodelling - a process by which old bone is removed and replaced by new bone under tightly controlled processes of bone formation and bone resorption. The three main types of bone cells are osteoblasts (bone forming cells), osteoclasts and osteocytes (Hlaing and Compston, 2014). Bone remodelling is mainly driven by osteoblasts and osteoclasts, which form a bone remodelling unit. The osteoclasts remove old bone (resorption), and osteoblasts lay down and mineralise new bone (formation) (Grabowski, 2009, Hlaing and Compston, 2014).

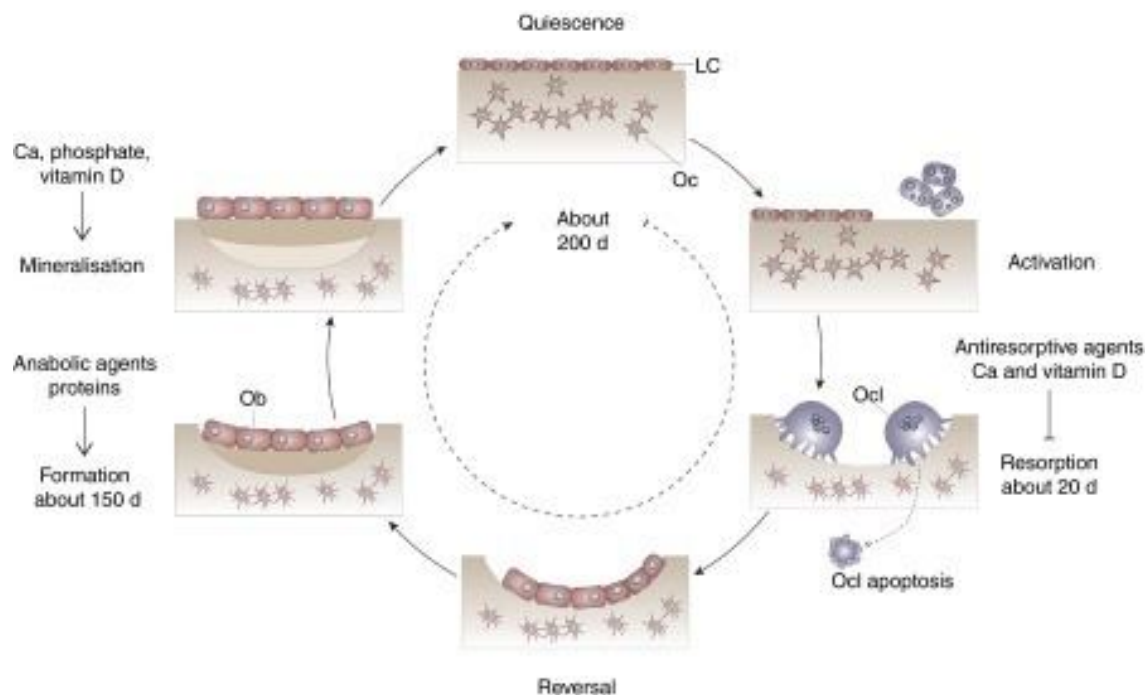


Figure 7: Bone remodelling cycle.

Bone remodelling cycle. Bone turnover follows a sequence of events that includes activation, recruitment of osteoclasts (Ocl) to begin resorption, degradation and removal of bone, reversal, and formation of new bone by osteoblasts (Ob). After this phase, a quiescent or resting period occurs. LC, lining cell; Oc, osteocyte (Bonjour *et al.*, 2014) [Reproduced with permission].

The bone remodelling cycle has four main phases that proceed in a sequence (Figure 7). The cycle starts with the release of cytokines from osteoblast precursors, leading to the formation of osteoclasts. The osteoclasts attach to the bone surface and remove old bone. Growth factors especially IGF-1 and transforming growth factor- $\beta$  are released during bone resorption, and these activate both bone lining cells and osteoblasts to lay down new bone (Bonjour *et al.*, 2014).

The bone remodelling cycle takes about 200 days in a healthy individual. Bone mineral accretion occurs when the rate of bone formation exceeds the rate of bone resorption, and bone loss occurs when the rate of resorption exceeds formation. The rate of bone remodelling is higher during growth in childhood and adolescence. Calcium, vitamin D, phosphate and other bone-forming minerals are required for adequate bone mineralisation (Bonjour *et al.*, 2014). Also, protein is required for bone formation – to form the collagen matrix. In women, hormonal changes associated with resumption of menses after lactational amenorrhea, hormonal contraception and menopause may also impact on the bone remodelling process (Olausson *et al.*, 2012). Other factors that influence bone resorption include mechanical stress, systemic hormone diseases, chronic infections, body weight, drug and substance use and some therapeutic drugs. Disruptions in the bone modelling unit which favour bone resorption may lead to accelerated loss of bone mineral weakening the bone microstructure (as in osteoporosis), hence increased risk of fracture.

The rate of bone remodelling is reflected in bone turnover markers which can be measured either in blood or urine. Markers of bone formation include osteocalcin, bone-specific alkaline phosphatase and propeptide type 1 extension peptides [procollagen type 1 N-terminal propeptide (P1NP) and procollagen type 1 C-terminal propeptide (P1CP)]. Resorption markers include: Hydroxyproline (OHP); collagen cross-link molecules [pyridinoline (PYD) and deoxypyridinoline (DPD)]; cross-linked telopeptides of collagen [N-terminal telopeptide (NTX) and C-terminal telopeptide (CTX) (Hlaing and Compston, 2014)].

Bone modelling and remodelling are dynamic processes. Hence a combination of assessments of bone mineral, bone turnover markers and anthropometric measurements provide a more comprehensive picture of bone health. The International Osteoporosis Foundation - International Federation of Clinical Chemistry (IFCC) and the Laboratory Medicine Bone Marker Standards Working Group recommend one bone formation marker (P1NP) and one bone resorption marker (CTX) for fracture risk prediction and monitoring for osteoporosis treatment (Vasikaran *et al.*, 2011).

### *2.1.2. Calcium, Vitamin D, Phosphate and Bone*

#### 2.1.2.1. Calcium-Phosphate-vitamin D homeostasis

About 99% of total calcium and 85% of total phosphate in the human body are stored in the skeleton embedded within hydroxyapatite crystals (formula  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ). Therefore, bone provides an adequate supply of both ionised calcium and phosphate to extracellular fluid, and also buffers against excess supply from dietary sources (Grabowski, 2009).

Serum calcium concentration is very tightly regulated (between 1.17 and 1.33 mmol/l) by the parathyroid hormone (PTH) and 1, 25 hydroxy vitamin D [ $1, 25(\text{OH})_2\text{D}$ ] as illustrated in Figure 8. In brief, when plasma calcium (P-Ca) levels fall below the normal range, there is increased production of PTH by the parathyroid gland. This acts on the kidneys to decrease urinary calcium loss. It also increases the formation of  $1, 25(\text{OH})_2\text{D}$ , which in turn, causes both increased intestinal absorption of calcium and mobilisation of calcium from bone to raise serum calcium concentrations back to the normal range. However, mobilisation of calcium from bone is accompanied by phosphate, and this elevates plasma phosphate concentration. Reduced production of PTH and increased production of fibroblast growth factor-23 (FGF-23) in bone increases renal phosphate excretion resulting in a fall in plasma phosphate (Prentice *et al.*, 2008).



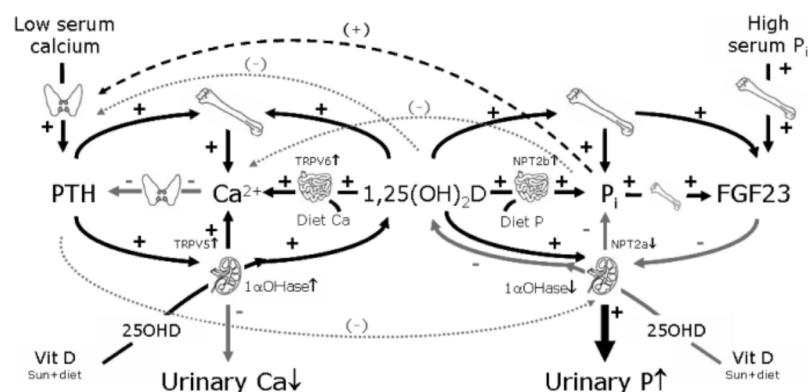


Figure 8: A schematic representation of the integrated Ca-P-vitamin D homeostatic system

(Prentice et al., 2008) [Reproduced with permission]

The renal system is central to vitamin D- calcium-phosphate homeostasis. Therefore, disease conditions and factors that compromise renal function may also affect bone health. For example, renal phosphate wasting (due to renal toxicities) is one of the mechanisms of bone loss in HIV-infected persons on TDF based ART (discussed later in section 2.5.3).

#### 2.2.3.2. Assessment of renal phosphate handling.

Renal phosphate handling is assessed by calculating the rate of phosphate clearance from blood by comparing phosphate concentrations in both blood and urine over a timed duration in the fasting state. Normally, more phosphate (95%) ions are reabsorbed by the kidneys during fasting and losses through urine are reduced. Postprandial (in approximately within 2 hours after eating) reabsorption of phosphate ions is reduced and there is increased excretion through urine (Anderson et al., 2012). Therefore, 2-hour fasted blood and urine collections are usually used in the assessment of renal phosphate clearance rate (Nordin and Fraser, 1960).

However, renal phosphate clearance is not a good measure of renal phosphate handling as it is affected by factors that influence the concentration of phosphate in the blood such as glomerular filtration rate, and rate of phosphate release from the gut, bones and cells into extracellular space. Consequently, tubular maximum reabsorption rate of phosphate (TmP) to glomerular filtration rate (GFR) is recommended. The normal TmP is >80% and the corresponding TmP/GFR is 2.6-4.4 mg/dl (0.80-1.35 mmol/l) (Payne, 1998).

### 2.1.3. Measurement of bone mineral using DXA

DXA imaging uses a low radiation dose similar to daily natural background radiation. This technique provides information on BMC, areal BMD (aBMD), bone area (BA) and body composition. aBMD is the most widely reported because it is measured with the greatest precision with reproducibility of 1-2% (Prentice *et al.*, 2006a). DXA measurements can be performed on the whole body and axial sites (commonly the hip and spine) and also the forearm. However, whole body and axial sites (hip and spine) of pregnant women are not scanned using QCT or DXA techniques to avoid unnecessary exposure of the fetus to radiation because the scans cannot distinguish maternal and fetal tissues (Olausson *et al.*, 2012). The international foundation for osteoporosis recommends the hip for diagnosis of osteoporosis in adults and the spine for monitoring osteoporosis treatment. In growing children, there is variability in skeletal development, especially at the hip. Hence, whole-body less head (WBLH) and spine measurements are recommended reference sites (Crabtree *et al.*, 2014, Kalkwarf *et al.*, 2014).

The international standard for clinical diagnosis of osteoporosis in postmenopausal women and men aged >50 years is based on T-scores from a comparison of the individual's aBMD measurement to the reference population data. A T-score lower than -2.5 is classified as osteoporotic, between -1 and -2.5 is classified as osteopenia or low BMD; and a T-score above -2.5 is classified as normal (WHO Study Group, 1994). However, the T-scores are based on data from postmenopausal Caucasian women, hence not applicable in either non-Caucasian women or premenopausal regardless of ancestry. To date, the majority of the reference data for DXA has been generated from developed/western countries, and there are no representative reference data for Africans. Therefore, Z-scores based on appropriate reference population are recommended for other age group and non-Caucasian populations (WHO, 2007b).

Caution should be exercised when comparing low aBMD or BMC and fracture risk across populations. This is because both BMC and aBMD are strongly influenced by body size. Populations of shorter stature (such as Africa and the Far East) have lower bone mineral compared to Western populations but they do not have higher rates of osteoporotic fractures. Also, aBMD is measured as  $\text{g}/\text{cm}^2$  which is equivalent to  $\text{BMC}/\text{BA}$ ; hence it is not a true volumetric density. To address these challenges, it is recommended that aBMD is adjusted for bone size in linear regression models, and all DXA readings (BMC, BA, aBMD and size-adjusted aBMD [SA-aBMD]) should be reported for proper interpretation of the data (Prentice *et al.*, 1994).

Low aBMD is an important predictor of fracture risk in adults, but other health and lifestyle factors also contribute to the risk of fractures (Kanis *et al.*, 2013). These factors include race, age, sex, weight, height, smoking, glucocorticoid use, alcohol intake of 3 or more standard units per day, previous fracture, family history of fractures, rheumatoid arthritis, other diseases associated with osteoporosis (including HIV). FRAX® is a free online risk assessment tool that was developed in 2008, by researchers at the University of Sheffield, to evaluate the risk of fractures in patients. The tool uses algorithms that integrate these risk factors with aBMD measurement at the femoral neck to give a 10-year probability of fracture (Kanis *et al.*, 2008, Kanis *et al.*, 2009).

## **2.2. Pregnancy, lactation and bone**

Maternal calcium is transferred across the placenta for fetal bone mineral deposition during pregnancy and later supplied to the mammary glands for secretion into breast milk during lactation. A full-term human fetus contains about 30g of calcium, 20g of phosphorus and 0.8g of magnesium, of which  $\approx 80\%$  is deposited during the third trimester. Demand for calcium and other bone forming minerals is high during pregnancy and lactation. Mean accretion rate for calcium in the fetus ranges from 50mg/day at 20 weeks to about 300-350mg/day at 35 weeks of gestation (Givens and Macy, 1933, Widdowson and Dickerson, 1964, Widdowson and McCance, 1965, Forbes, 1976, Ziegler *et al.*, 1976, Prentice and Bates, 1994). Depending on the individual, about 200 – 400 mg per day of calcium is secreted into breast milk at peak lactation, but varies for individual women (Prentice, 2003). Phosphorus accretion increases from 40mg/day at 24 weeks to 200mg/day from 35 weeks of gestation and of magnesium increases from 1.8 to 7.5mg/day in the last five weeks of pregnancy (Widdowson, 1962, Prentice, 2003).

Extensive literature reviews implicate three physiological adaptations of maternal calcium economy (1) up-regulation of intestinal absorption of calcium (2) altered renal calcium handling (3) altered skeletal turnover and mineral metabolism (Olausson *et al.*, 2012, Kovacs, 2016). Some of these adaptations start in the first trimester of pregnancy way before calcium requirements peak in the third trimester. Intestinal calcium absorption doubles and renal calcium excretion is increased in anticipation of peak accretion rates in the last trimester of pregnancy. Intestinal absorption is also up-regulated, and renal calcium is conserved in lactation. However, skeletal resorption is the dominant mechanism for meeting increased maternal calcium requirements during lactation.

A review of studies using DXA has shown a +1.8 to -4.5% mean change in maternal aBMD at the lumbar spine and/or hip during lactation (Olausson *et al.*, 2012). The magnitude of the changes in aBMD during lactation varies between individuals and is also dependent on other factors including resumption of menses, and the intensity and duration of breastfeeding. There is also some evidence that women who breastfeed for longer tend to mobilise more bone mineral over time compared to

those who breastfeed for shorter durations. The magnitude and duration of skeletal response to lactation are also related to resumption of menses (Laskey and Prentice, 1999). In British women, maternal height and volume of breast milk consumed by the infant have been associated with maternal lumbar spine bone mineral after adjustment for the bone area (Laskey *et al.*, 1998). Increasing calcium intakes does not attenuate the observed changes in the maternal bone mineral (Prentice, 2000, Jarjou *et al.*, 2010).

Pregnancy and lactation are also accompanied by significant increases in markers of bone turnover. Early increases in bone resorption markers (NTX, CTX and DPD) have been observed in the first trimester of pregnancy, without changes in bone formation markers (P1NP, P1CP and bone-specific ALP). Then, both resorption and formation markers reach their highest concentrations in the third trimester. Markers of bone formation and resorption increase within the first few weeks of lactation and are higher compared to late pregnancy. After that, the bone resorption markers decrease while formation markers either remain elevated or increase further before decreasing in late lactation (Prentice *et al.*, 1998, Holmberg-Marttila *et al.*, 2003, More *et al.*, 2003, Vargas Zapata *et al.*, 2004). The increased bone turnover during pregnancy and lactation is consistent with the observed changes in maternal bone mineral.

Changes in maternal aBMD during lactation are physiological. Most evidence shows that bone mineral density is recovered (or even higher) after stopping breastfeeding and resumption of menses both in women on calcium intakes close to international recommendations and very low calcium intakes (Kent *et al.*, 1990, Kalkwarf and Specker, 1995, Laskey and Prentice, 1999, Moller *et al.*, 2012). Recovery of bone mineral at the lumbar spine has been observed from six months of lactation regardless of breastfeeding status (Cooke-Hubley *et al.*, 2017), and also in Gambian women after long durations of breastfeeding (Sawo *et al.*, 2013). However, recovery at the total hip and the femoral neck take longer compared to the spine. Laskey *et al.* observed partial recovery of bone mineral at the femoral neck at 3 months post weaning, and residual effects on the maternal skeleton that were not related to the duration of lactation which were attributed to having been pregnant (Laskey and Prentice, 1999). It is thought that these changes in bone turnover markers account for the mobilisation of bone mineral in early lactation when resorption exceeds formation and replenishment of bone mineral in late lactation and after stopping breastfeeding - when formation exceeds resorption (Olausson *et al.*, 2012).

Recent studies using high resolution pQCT have reported micro-architectural changes in the maternal skeleton during lactation (Brembeck *et al.*, 2015, Bjørnerem *et al.*, 2017), but bone mineral content and strength is restored. Overall, most evidence shows that lactation is not associated with increased risk of low BMD and fragility fractures in later life (Paton *et al.*, 2003, Specker and Binkley, 2005, Wiklund *et al.*, 2012, Kovacs, 2017).

## 2.3. HIV, ART and Bone Health

### 2.3.1. HIV and bone health

There is evidence that HIV infection *per se* may have a direct impact on bone through several mechanisms that may increase bone resorption and decrease bone formation (Brown and McComsey, 2006a, Walker-Bone *et al.*, 2017). Studies conducted in predominantly Caucasian populations have reported an increased risk of fractures among HIV-infected compared to HIV negative persons (Torti *et al.*, 2012, Yin *et al.*, 2012, Güerri-Fernandez *et al.*, 2013). However, the majority of these studies have been conducted in HIV-infected Caucasian men who are also likely to have a higher prevalence of traditional risk factors for osteoporosis and fractures including drug use alcohol and smoking.

Contrary, other studies have not observed an association between HIV status and bone mineral density in either Caucasian or African cohorts (Hamill *et al.*, 2013, Tinago *et al.*, 2017). In the first study of HIV and bone in African women, Hamill *et al.* did not observe a significant difference in baseline aBMD between HIV-infected (>80% of the participants were on TDF-3TC-EFV) and HIV-negative premenopausal black South African women. The investigators concluded that the lack of difference between the groups could have been a result of true lack of effect of HIV infection on bone or a reflection of important differences in bone response to HIV between black South Africans and Caucasian women (Hamill *et al.*, 2013).

### 2.3.2. ART and Bone

Initiation of ART is associated with a 2-6% reduction in aBMD within the first two years regardless of ART regimen used (Brown and Qaqish, 2006b). Rapid decreases in aBMD occur between 24-48 weeks following ART initiation. After that, aBMD does not decrease further if the same ART regimen is maintained, but it remains lower compared to baseline (Brown *et al.*, 2009, Brown, 2013). Lower baseline CD<sub>4</sub>, female gender, old age, lower BMI and higher HIV viral load are associated with greater reductions in aBMD (Grant *et al.*, 2013). Calcium and vitamin D supplementation has been reported to attenuate ART-related bone loss by 50% in HIV-infected American adults (90% men) with median baseline serum 25(OH)D of 57 (Q1-Q3: 45-77) nmol/L (Overton *et al.*, 2015). However, some studies have not observed reductions in BMD within the after 1-2 years of initiating ART (Dolan *et al.*, 2006, Bolland *et al.*, 2012).

It would be plausible to hypothesise that initiation of ART would be beneficial for bone health as markers of systemic infections reduce (especially TNF- $\alpha$  and interleukin-6) and body mass increase, both which are associated with decreased risk of osteoporosis (Brown, 2013). Therefore, the general decrease in aBMD following initiation of ART is paradoxical. There is a growing school of thought that aBMD loss observed following initiation of ART could be mediated by immune reconstitution that occurs in persons with advanced HIV infection (Ofotokun *et al.*, 2012, McGinty *et al.*, 2016). Studies of patients on established long-term ART have reported stable or increasing aBMD (Nolan *et al.*, 2001, Bolland *et al.*, 2011, Bolland *et al.*, 2012), probably due to improvement in general health and body weight (Compston, 2016).

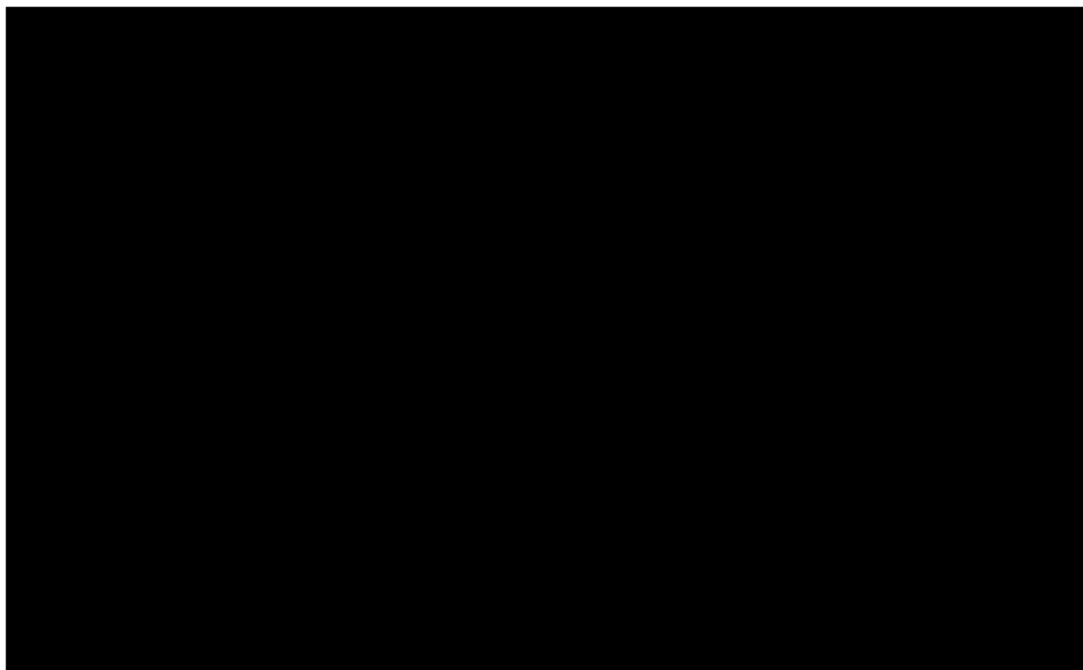
There is evidence that the decrease in aBMD following ART initiation is driven by increased bone remodelling rate resulting in changes in bone microstructure (Compston, 2015). Also, there is a clear inverse relationship between BMD and body fat in HIV- infected persons (Cotter and Mallon, 2012). Thus, reduction in lean: fat ratio could be one of the mechanisms underlying bone loss following initiation of ART. In, Hamill *et al.* observed a 10% gain in fat mass accompanied by a 2-3% decrease in aBMD after 12 months in premenopausal black South African women initiated on ART at low CD<sub>4</sub> (Hamill, 2013). This finding is alarming as overweight and obesity have been reported to be associated with increased risk of lower limb fractures in Caucasian populations (Compston, 2013).

Studies with fracture endpoints have been conducted among HIV-infected persons. In a five year longitudinal study of young HIV-positive individuals (83% men), higher rates of fractures were observed within the first two years after initiation of ART (0.53/100 person-years) compared to subsequent years (0.30/100 person-years) (Yin *et al.*, 2012). Recent reviews of the literature have concluded that HIV-infected individuals are at increased risk of fractures compared to HIV-uninfected persons (Battalora *et al.*, 2014c, Hoy and Young, 2016).

There is good evidence that ART itself might have a direct impact on bone. One study randomised patients to either intermittent ART (ART was stopped or deferred at study entry, and restarted when the CD<sub>4</sub> T-cell count declined below 250 cells/mL and stopped again at CD<sub>4</sub> T-cell counts above 350 cells/mL) or continuous ART (patients maintained on ART throughout the follow-up duration). Intermittent ART was associated with stabilisation or increase in aBMD accompanied by a reduction in markers of bone turnover compared to continuous ART but had adverse effects on the clinical outcomes of the patients (Hoy *et al.*, 2013). Also, the same investigators recently reported that immediate ART (initiation of ART at CD<sub>4</sub> >500 cells/mm<sup>3</sup> as per the current test and treat approach) is associated with accelerated bone loss compared to initiation at CD<sub>4</sub> <350 cells/mm<sup>3</sup> (Hoy *et al.*, 2017). However, the clinical significance of these findings is unknown because once started, ART

needs to be used continuously to suppress opportunistic infections; and early initiation of ART is associated with reduced risk of serious clinical outcomes (Hoy et al., 2013, Hoy et al., 2017).

The common limitation of studies on HIV and ART-associated bone loss arises from the selection of participants, especially in control groups (Figure 9). The majority of the studies have been conducted in Caucasian men – a group which tends to have a higher prevalence of traditional risk factors for low BMD compared to HIV-negative controls. Also, the HIV-positive groups are likely to be on ART which has a potential effect on aBMD. In longitudinal studies comparing ART-exposed and ART unexposed HIV-positive persons, the ART-exposed group is more likely to have had advanced HIV disease at baseline and/or ill health (due to ART side effects and immune reconstitution syndrome within the first six months of ART). These conditions are independently associated with lower aBMD, reduced physical activity, sunshine exposure and dietary intake. Thus, even with carefully designed studies, unravelling the effects of HIV infection *per se* from the effects of ART on bone may not be possible under the current test and treat approach that recommends early initiation of ART.



*Figure 9: Causes of low bone mass in patients with HIV*

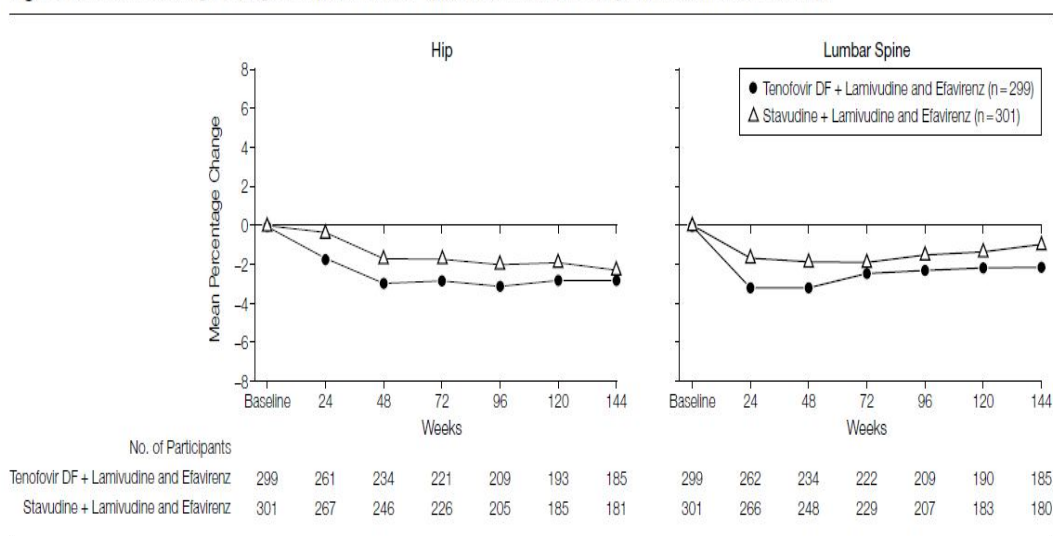
Osteoporosis in patients with HIV results from a combination of traditional, secondary and HIV-related risk factors. Abbreviations: NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor. (Walker-Bone et al., 2017).

### 2.3.3. Effects of specific ARVs on bone

There is a consensus that some commonly used ARVs have specific adverse effects on bone through a variety of mechanisms. AZT and 3TC are associated with enhanced formation of osteoclasts, while both TDF and EFV (preferred first-line ARVs for PMTCT) are associated with kidney toxicities which may interfere with the conversion of 25 (OH)D to 1,25 (OH)<sub>2</sub>D (Gibellini et al., 2012).

TDF is the most studied ART with regard to potential side effects on bone metabolism. Clinical trials have shown that TDF causes 1-2% greater reductions in BMD compared to other ARVs (Gallant et al., 2004, Stellbrink et al., 2010), as shown in Figure 10. The adverse effects of TDF on BMD have also been observed in a double-blind randomised trial in HIV-negative men initiated on TDF for Pre Exposure Prophylaxis (PrEP) in the Tenofovir PrEP study in San Francisco USA (Liu et al., 2011). A higher proportion of HIV-negative men in the TDF group experienced >5% reduction in aBMD at the femoral neck by 24 months compared to the placebo group (13% vs 6%) in the San Francisco study. These data provides evidence that TDF has an adverse effect on bone independent of HIV-infection.

**Figure 3.** Mean Percentage Change in Hip and Lumbar Spine Bone Mineral Density From Baseline to Week 144



DF indicates disoproxil fumarate. The range of variability (SD) of percentage change in lumbar spine and hip bone mineral density was from 2.5% to 5.2%.

**Figure 10:** Mean percentage change in hip and lumbar spine BMD from baseline to 144 weeks

(Gallant et al., 2004) [Reproduced with permission]

TDF-based ART has also been associated with subclinical kidney injury, a syndrome that has been shown to enhance bone loss. Subclinical kidney injury is characterised by isolated abnormalities in some of the markers of kidney function but without generalised clinical disease manifestation. Kidney toxicity can result in phosphate wasting with hypophosphataemia and secondary



hyperparathyroidism, leading to dysregulation of calcium-phosphate homeostasis and bone demineralisation (Holick, 1996, Grigsby *et al.*, 2010, Havens *et al.*, 2012).

TDF-based PrEP is associated with a small decline in estimated GFR (eGFR) in HIV-negative African men and women (Martin *et al.*, 2014, Solomon *et al.*, 2014, Mugwanya *et al.*, 2015). It is not known whether this low level of kidney injury is exacerbated in pregnant and lactating women with the prolonged use of TDF-based ART for PMTCT and for their own health.

Higher plasma concentration of TDF have been associated with higher vitamin D binding protein, lower 1,25-OH(2)D, lower estimated free 25- OHD, and increased parathyroid hormone (PTH) in HIV infected persons (Gibellini *et al.*, 2012). Although controversies remain regarding which vitamin D assay is the most accurate, and appropriate in different populations (Havens *et al.*, 2013, Aloia *et al.*, 2015, Denburg *et al.*, 2016, Henderson *et al.*, 2016, Nielson *et al.*, 2016a, Nielson *et al.*, 2016b) these associations suggest that TDF exposure can cause functional vitamin D deficiency (Havens *et al.*, 2012, Havens *et al.*, 2013).

Based on the consensus that TDF is associated with accelerated bone loss, several studies are exploring alternative first-line drugs including Abacavir (ABC), Raltegravir (RTG) and Dolutegravir (DTG). Switch studies have reported that RTG and DTG may have minimal adverse effects on renal function and bone metabolism compared to TDF (Battalora *et al.*, 2014a, Brown *et al.*, 2014, Yazdanpanah *et al.*, 2014, Tebas *et al.*, 2015, McComsey *et al.*, 2017). Also, phase two and three clinical trials of an investigational prodrug of Tenofovir, known as Tenofovir Alafenamide Fumarate (TAF) have reported improved renal and bone loss compared to TDF (Mills *et al.*, Sax *et al.*, 2014, Post *et al.*, 2017). These studies hold promise for maintaining bone health among people on ART. However, these new drugs are expensive and generic versions are not yet available. Except for Botswana which has rolled out DTG for all pregnant and breastfeeding women, the newer drugs are not yet used as first-line ART in sub-Saharan Africa. For now, TDF/3TC/EFV is still the affordable and preferred first-line regimen recommended by WHO for adults, including pregnant and lactating women.

#### 2.3.4. HIV, ART and bone in women

Overall, women are under-represented in HIV and bone studies although they have a higher lifetime risk of fractures compared to men. The few studies conducted in HIV-infected ART naïve women excluded pregnant and breastfeeding women. Therefore, changes in aBMD were not studied in HIV-infected ART naïve pregnant and breastfeeding in either Caucasian or African populations. Such studies have now been overtaken by advancements in ART for PMTCT making it unethical to conduct longitudinal studies in ART naïve HIV-infected pregnant/or breastfeeding women. Thus, it is more difficult to tease out effects of HIV and ART under the current guidelines.

Dolan et al. reported lower lumbar spine and total hip aBMD in premenopausal HIV-infected women compared HIV negative controls (54% vs 30%,  $p=0.004$ ) in a cross-sectional study (Dolan *et al.*, 2004). In a longitudinal study, BMD was lower in HIV-infected premenopausal women before ART compared to HIV negative counterparts (Dolan et al., 2006). The rates of change in aBMD were not significantly different between HIV-infected and negative women. However, BMD remained lower in HIV-infected women over two years of follow-up and was associated with lower weight, lean mass, duration of HIV, smoking, reduced androgen levels, abnormal menstrual function and increased bone turnover (Dolan *et al.*, 2007).

A review of studies published on ART-related bone loss among women (between 1987 and 2009) identified a >3% difference in BMD at the femoral neck among women on ART/PI based regimens (Carvalho et al., 2010). Other studies have also reported an association between HIV status and BMD, suggesting a possible effect of HIV-infection on aBMD in both postmenopausal (Yin *et al.*, 2005, Sharma *et al.*, 2011) and premenopausal women (Arnsten *et al.*, 2006). However, none of these studies was conducted in African women. Since, a few studies have been published on HIV/ART-associated bone loss in premenopausal, non-pregnant and non-lactating women in Africa. These are reviewed briefly below.

In a longitudinal study using DXA in urban black South African women of pre-menopausal age, women initiated on ART at low CD<sub>4</sub> had 2-3% significant decreases in aBMD at the lumbar spine and the femoral neck consistent with increased bone turnover. No significant changes aBMD were observed in HIV-infected ART naïve women with preserved CD<sub>4</sub>. Both HIV-status and ART exposure were not associated with vitamin D status, serum phosphate concentrations or renal phosphate handling (Hamill et al., 2017). To date, this is the only published study that has used DXA to investigate changes in aBMD following initiation of ART in premenopausal HIV-infected African women, who were neither pregnant nor lactating.

Greater loss in BMD has also been reported among young HIV-negative African women receiving oral TDF for HIV pre-exposure prophylaxis (PrEP) in Zimbabwe and Uganda, compared to the placebo group. Women TDF/FTC had a 1.4% lower mean percent change in lumbar spine BMD after 48 weeks compared to the placebo group. Changes in aBMD from the end of active treatment to 48 weeks were also significantly greater in the PrEP arm compared to the placebo with a net difference of approximately +0.9% at the LS ( $P = 0.007$ ) and +0.7% ( $P = 0.003$ ) at the TH (Mirembe *et al.*, 2016).

A cross-sectional study conducted in Senegal (66% women) using QUS reported low bone mineral among HIV-infected persons compared to negative controls but higher bone mineral in HIV-infected women compared to HIV-infected men (Cournil *et al.*, 2012). BMI accounted for the observed differences between men and women, and also between infected and negative persons. Although the study by Cournil *et al.* contributes to the literature on bone health in HIV-positive African women, it had limitations in its cross-sectional design and lack of DXA data for direct measurements of aBMD.

Only two studies have been published to date on postpartum changes in bone mineral in HIV-infected women on ART. Mora *et al.* reported no adverse effects of maternal ARVs during pregnancy on cortical bone measured by ultrasound at four days and 12 months postpartum in Italian women, and concluded that ARVs during pregnancy do not affect cortical bone (Mora *et al.*, 2013). This finding could be explained partly by existing evidence that most changes in bone mineral during pregnancy and lactation occur in areas rich in trabecular bone commonly the femoral neck and lumbar spine compared to sites rich in cortical bone (Olausson *et al.*, 2012). The main limitation of Mora *et al.*'s study was that it used ultrasound and so did not have actual measures of BMD or BMC. Also, HIV-infected women were underrepresented in the study (33 vs 116 HIV-negative controls), had a shorter duration of exposure to ART (only 14 weeks during pregnancy), and never breastfed. Thus, ART practice is different to that in sub-saharan Africa.

Preliminary results of the PROMISE study (P1084s) were presented at the 2016 AIDS conference in Durban, South Africa. Stanix-Chibanda *et al.* reported -3.14% (-4.44, -1.84,  $p \leq 0.001$ ) and -2.66% (-3.24, -1.42,  $p \leq 0.001$ ) mean difference in LS aBMD at 74 weeks of lactation among HIV infected women initiated on ART within the first 2 weeks postpartum (Option B) compared to HIV-infected women without exposure to ART (infant prophylaxis arm - Option A). HIV-infected women had 2.1% and 5.3% decreases in LS and total hip aBMD, respectively at 74 weeks of lactation (Stranix-Chibanda and PROMISE-team, 2016). This study did not have a control group of HIV-negative women, and the preliminary analysis did not investigate the recovery of bone mineral. A follow-up study (PROMOTE) is ongoing to investigate the longer term implications of these findings (Stranix-Chibanda, personal communication).

## 2.4. Maternal HIV/ART exposure, infant bone health and growth

### 2.4.1. Effect of HIV-infection/ ART on infant growth

Studies conducted in both developed and developing countries have reported poor growth outcomes among infants born to HIV-infected mothers (exposed non-infected infants) compared to infants of HIV-negative mothers (Makasa *et al.*, 2007, Fadnes *et al.*, 2009, Nielsen-Saines *et al.*, 2012, Lartey *et al.*, 2014, Ramokolo *et al.*, 2014). Birth weight, maternal viral load and early infant HIV infection have all been associated with the growth of exposed infants in Uganda and South Africa (Venkatesh *et al.*, 2010, Muhangi *et al.*, 2013a, Ramokolo *et al.*, 2014). Also, HIV-exposed infants are more likely to be born premature, small-for-gestation age compared to HIV-unexposed infants (Marinda *et al.*, 2007).

However, most of these studies are cross-sectional hence cannot prove causality. They also lack control groups of HIV-negative women making it difficult to determine if the high risk of poor growth results from HIV or from other aspects of poverty (Filteau, 2009). Maternal HIV infection may have a direct impact on child growth especially in women with advanced HIV disease (high HIV viral load). However, this could be confounded by a higher prevalence of other factors which directly impacts on child growth such infant feeding practices, maternal education, illness and access to medical care, low socioeconomic status among others. In Uganda, a higher prevalence of poor infant feeding practices, lower maternal education and socio-economic status have also been associated with poor infant growth (Fadnes *et al.*, 2009, Engebretsen *et al.*, 2010, Evans *et al.*, 2016).

In a case-control study among Zimbabwean children in the pre-maternal ART era, Prendergast *et al.* observed lower maternal IGF-1 among HIV-positive compared to HIV-negative women, and these were associated with stunting in the offspring. Also, markers of inflammation were higher among HIV-exposed infants compared to unexposed infants and were associated with lower IGF-I throughout infancy (Prendergast *et al.*, 2014). Human cytomegalovirus (CMV) infection is also associated with poor growth among HIV-exposed infants (Gompels *et al.*, 2012). Altered maternal microbiome and differences in breast milk oligosaccharides have also been observed in HIV-infected compared to uninfected mothers, and these may contribute to compromised growth among HIV-exposed infants (Van Niekerk *et al.*, 2014, Bender *et al.*, 2016, Monaco *et al.*, 2016). The question of whether maternal HIV infection *per se* has a direct impact on infant growth is not likely to be addressed in longitudinal studies because it is now unethical to study HIV-infected pregnant or breastfeeding women who are not on ART.

Besides the traditional determinants of growth in children, there are growing concerns that maternal ART exposure during pregnancy and breastfeeding may negatively affect infant growth. This is because ARVs are transferred to the offspring through the placenta and breast milk, and there is also increasing literature on the possible adverse effect of ART/TDF on the bone as discussed in section 2.5.3. Furthermore, animal studies have reported adverse effects of TDF exposure during gestation on fetal bone mineralisation in gravid monkeys, although at higher doses of TDF compared to those currently used in humans; and the effects of TDF on bone were dose-dependent (Tarantal et al., 1999, Tarantal et al., 2002).

To date, human studies have reported conflicting evidence on TDF exposure during pregnancy on infant growth outcomes. Sibbery et al. reported small but significantly lower height (HAZ) and head circumference (HCAZ) at 12 months of age which were not detected at birth, following in-utero TDF exposure in the USA-based Paediatric HIV/AIDS Cohort Study (PHACS) (Siberry *et al.*, 2012). This was the first study to report a possible adverse effect of maternal TDF exposure on infant growth and has been widely cited. However, the study had high rates of attrition so less than a third of the study participants were included in the 12 months analysis which may have introduced selection bias and measurement of potential confounders was limited (Kuhn and Bulterys, 2012).

On the contrary, Gibb et al. reported no long-term effect of in-utero maternal TDF exposure on growth within the first 5 years of life in African children within the multicentre DART (The Development of AntiRetroviral Therapy in Africa) trial conducted in Uganda and Zimbabwe. Ugandan children with >90% in-utero exposure to TDF had higher HAZ within the first 12 months compared to infants without TDF exposure, but WAZ was not significantly different between the groups. The authors also observed that Ugandan children with and without exposure to TDF were significantly lower HAZ at 1.5 and three years of age compared to WHO standards. The authors reported that attained Z-scores were similar to those predicted from a population of healthy children in South-West Uganda (Gibb et al., 2012). The limitations of this study are discussed in section 9.1. The difference in growth between Ugandan and Zimbabwean children observed by Gibb et al. demonstrates difficulties in assessing 'normal' growth in children across populations and underscores the need for control groups recruited from the same setting.

Overall, the majority of published studies on growth of HIV-exposed infants lacked a reference population of HIV/ART-unexposed children (Filteau, 2009), so evidence is limited on whether the observed deviations in linear growth are comparable to those of HIV/ART unexposed children in the same setting. Recently, Aizire et al. reported that Ugandan HEI in the maternal ART arm in the PROMISE study had significantly lower anthropometric mean Z-scores (weight, length and head circumference) compared to unexposed infants at 12 and 24 months. However, these differences

were not observed in Malawian infants (Aizire et al., 2016). Differences in breastfeeding practices and other environmental factors might account for the differences in growth trajectories of Ugandan HIV-exposed infants compared to Zimbabwean and Malawian infants. However, this needs further investigation. Overall, data are inconsistent on the potential effects of both antepartum and postnatal exposure to maternal ART on infant growth.

#### *2.4.2. Effect of maternal ART on infant bone mineral accrual*

A cross-sectional analysis within the Pediatric HIV/AIDS Cohort Study (PHACS) reported lower new-born BMC among infants with in-utero maternal TDF exposure, and the low BMC persisted after adjusting for infant body weight and length weight (Siberry et al., 2015, Siberry and Jacobson, 2016). More recently, Siberry et al. reported lower bone mineral at two weeks of age among African HIV-exposed infants on PMTCT Option B compared to Option A. These are initial data from the PROMISE P1084s study which followed mother-infant pairs up to 78 weeks of lactation. To date, these are the first studies which have reported differences in new-born BMC measured by DXA following maternal TDF exposure. However, these studies are cross-sectional. There are no published data on bone mineral among maternal HIV/ART-exposed infants beyond the neonatal period.

Overall, data are limited on the effects of maternal ART on bone mineral among HIV-exposed children with long-term exposure through breastfeeding. Consequently, more evidence collected prospectively is needed using DXA ideally with HIV/ART unexposed control groups and adjusted for infant feeding practices.

#### *2.4.3. Effect of maternal HIV/ART exposure on lactation*

Mastitis is the most common breast condition in breastfeeding mothers and is associated with poor infant growth. Mastitis is an inflammatory condition of the breast, which may or may not be accompanied by infection (WHO 2000). The condition is usually associated with lactation, causing a woman's breast tissue to become tender, painful and inflamed, sometimes the condition may progress to a local abscess. Mastitis is commonly caused by milk stasis when milk is not removed from the breast efficiently, but infection may also be the primary cause (WHO, 2000). Milk stasis may occur soon after delivery when the breasts are engorged, or at any time when the infant does not remove the milk that is produced from part or the entire breast (WHO, 2000). Mastitis is a major cause of the reduction in milk production and, according to WHO, it is cited by approximately a quarter of mothers as the main reason for stopping breastfeeding (WHO, 2000). Subclinical mastitis (SCM) is defined by raised milk sodium/potassium (Na/K) ratio ( $>1.0$ ) in one or both breasts, due to increased leakage of blood components into breast milk (McGregor *et al.*, 1985). In SCM there is no breast tenderness, but there is a reduction in milk output.

Mastitis can affect either one (unilateral) or both breasts (bilateral), although unilateral mastitis is more common due to breast preference. A greater burden of mastitis and sub-clinical mastitis has been reported in breastfeeding HIV-infected women in the pre-ART era, and both conditions are associated with poor infant growth (Kasonka *et al.*, 2006, Makasa *et al.*, 2007, Aryeetey *et al.*, 2008). Also, breast milk HIV viral load is increased in SCM, hence associated with increased risk of mother to child transmission of HIV (Semba *et al.*, 1999a, Willumsen *et al.*, 2003). The majority of studies on mastitis in the context of HIV have been conducted in Southern Africa (Semba *et al.*, 1999a, Semba *et al.*, 1999b, Willumsen *et al.*, 2003, Kasonka *et al.*, 2006). In some of these studies, a higher prevalence of both bilateral and unilateral SCM within 16 weeks postpartum was reported in HIV-infected women compared to their negative peers. Poor maternal and infant health was associated with SCM and also with earlier cessation of breastfeeding within the first six months (Kasonka *et al.*, 2006).

In another study, breast milk sodium concentrations fell after 12 weeks of treatment of subclinical mastitis using antibiotics but remained higher compared to the uninfected breast in HIV-infected women. In the same study, women were advised not to breastfeed but to express and discard the breast milk periodically. Antibiotics did not have a significant effect on the breastmilk HIV-viral load, so the study recommended evaluation of clinical monitoring protocols and advice on breastfeeding given to HIV-infected women with mastitis (Nussenblatt *et al.*, 2006). This study was conducted before the roll-out of ART for pregnant and breastfeeding women. Hence, the evidence is limited on SCM and risk of MTCT among HIV-infected women on lifelong ART under the current PMTCT Option B-plus.

A study conducted in Tanzania investigated the impact of nevirapine and subclinical mastitis HIV viral load in maternal plasma and breast milk following a single-dose of nevirapine (NVP) given during labour (combined with either 1- week tail of Combivir (zidovudine/lamivudine) or single-dose Truvada (tenofovir/emtricitabine). Subclinical mastitis was detected in 67% of the women at anytime within the first 6 weeks postpartum, and in 38% of the breastmilk samples. Breastmilk with sub-clinical mastitis had higher HIV viral load (Salado-Rasmussen *et al.*, 2015). These data are consistent with studies conducted in Zambia that have reported a high prevalence of sub-clinical mastitis in early lactation. However, it lacked a control group of HIV-negative women for comparison and ARVs were offered for a short duration (PMTCT Option A). Hence, it is not known whether maternal triple ART reduces the prevalence of mastitis in HIV-infected women on the current PMTCT Option B+.

WHO recommends skin –to-skin contact and early initiation of breastfeeding (within the first one hour after delivery) because it stimulates breastmilk production (lactogenesis II), increases the likelihood of exclusive breastfeeding for the first four months of life and also the overall duration of breastfeeding (WHO, 2017a). In a study of 425 Ghanaian women, HIV-negative status was associated with early onset of lactogenesis II compared to unknown or HIV-positive status, based on self-reports, suggesting a possible effect of HIV status on the onset of lactation. In turn, delayed lactogenesis II was associated with infant weight changes within the first two weeks (Otoo *et al.*, 2010). Contrary, Kasonka *et al.* did not observe delayed lactation in HIV-infected Zambian women: only one woman reported delayed lactation following delivery by C-section (Kasonka *et al.*, 2006). The study by Otoo *et al.* did not provide information on postnatal practices like skin-to skin contact which play a critical role in stimulating breastmilk production. Also, infant breastmilk intake was measured by test weighing technique which might disrupt breastfeeding. Overall, early studies published on breast milk output in the context of HIV were conducted in the pre-ART era (Kasonka *et al.*, 2006, Aryeetey *et al.*, 2008).

Drugs ingested by the mother can also affect initiation of lactation, milk secretion, impair milk ejection, diminish milk volume, and alter milk composition (Neville *et al.*, 2012). Thus, recent studies using Deuterium dilution methods in African women have investigated breastmilk output in HIV-positive and ART compared to HIV-negative women. A cross-sectional study conducted in Kenya reported higher rates of exclusive breastfeeding in HIV infected women compared to HIV-negative women, but there were no significant differences in breastmilk output. However, HIV-exposed infants had significantly lower fat mass compared to HIV-unexposed infants (Oiye *et al.*, 2017). Longitudinal data from South Africa shows that HIV-infected women on ART are able to produce adequate breastmilk throughout the first year of lactation without compromising their fat stores. In the same study, infants who were exclusively breastfed for the first 6 months had higher fat-free mass 12 months of age compared to non-exclusively breastfed infants (Mulol and Coutsoodis, 2017). Taken together, these data support the current WHO breastfeeding recommendations for HIV-infected women (WHO, 2016b).



## 2.5. Summary of literature

Pregnancy, lactation HIV-infection and ART are independently associated with reductions in bone mineral, but data are limited on whether these decreases are additive among HIV-infected women. The majority of the studies on HIV/ART-associated bone loss have been conducted in men and pregnant, and breastfeeding women were excluded from the few studies conducted in women.

There is also limited and conflicting evidence on both long and short-term effects of maternal HIV/ART especially TDF on infant outcomes. The majority of the studies on infant outcomes following ART/TDF exposure have only looked at growth indices and lacked measurements of bone mineral. Siberry et al. have reported lower infant bone mineral in both American and African children following in-utero exposure to TDF, but the mechanisms and clinical relevance are still unclear. Thus more longitudinal studies with DXA measurements are needed.

Delayed onset of lactation and mastitis are associated with reduced maternal breast milk output and poor infant growth. Further evidence from cross-sectional studies suggests that HIV- infected women experience delayed onset of lactation and/or are at increased susceptibility to sub-clinical mastitis. However, the evidence is not sufficient to conclude that HIV-infection compromises lactation capability as lactation performance is affected by various factors which need to be controlled for. In addition, it is unknown whether the current ART drugs affect lactation. Consequently, more longitudinal studies involving assessment of infant breast milk intake using stable isotope technique, infant growth assessment, breastfeeding practices and subclinical mastitis will significantly contribute to the knowledge base and inform the design of interventions.

In conclusion, "Additional research is needed on the safety and acceptability of lifelong ART for pregnant and breastfeeding women, and their infants, especially in low resource settings, where malnutrition and comorbidities are more common than in resource-rich countries and monitoring capacity is limited.....better data are needed on mothers' health outcomes, pregnancy outcomes (such as stillbirth, low birth weight and prematurity) birth defects and health outcomes for infants and young children" (WHO, 2013).

Therefore, more longitudinal studies using DXA are needed to understand better whether HIV-infected women on ART experience greater and/or longer-term postpartum reductions in BMD, especially in sub-Saharan-Africa where extended breastfeeding is recommended for HIV-infected women. In such studies, adjustments for breastfeeding (duration and infant breastmilk intake measured by deuterium dilution method) and dietary intake will provide better evidence when comparing HIV-infected and HIV-negative mothers in resource limited settings.

## CHAPTER 3: STUDY DESIGN AND SETTING

### 3.1. Study conceptual framework

- ❑ **Background:** Pregnancy (PG) and lactation (L) are associated with physiological decreases in bone mineral density (BMD), but most evidence shows that BMD is recovered after cessation of breastfeeding. Initiation of antiretroviral therapy (ART) in HIV-positive adults is associated with a 2-6% decrease in BMD, but data are limited in pregnant and lactating mothers.
  - ❑ **Hypothesis:** HIV/ART/TDF may accentuate the normal process of bone mobilisation during pregnancy and lactation, leading to bone loss that is not recovered in the mother and/or compromised infant growth and bone mineral accretion.
  - ❑ **Objectives:** To investigate the effect of HIV/ART/TDF on maternal BMD in the context of pregnancy and lactation.
  - ❑ **Primary outcome:** Changes in maternal bone mineral density during lactation in HIV-positive vs. HIV-negative Ugandan mothers
  - ❑ **Secondary outcomes:** Maternal bone turnover markers, calcitropic hormones, markers of Ca and phosphate metabolism in plasma and urine, markers of renal function and mineral handling, breastmilk mineral composition, and infant growth and bone mineral
- The figure below presents the conceptual framework for the Gumba study

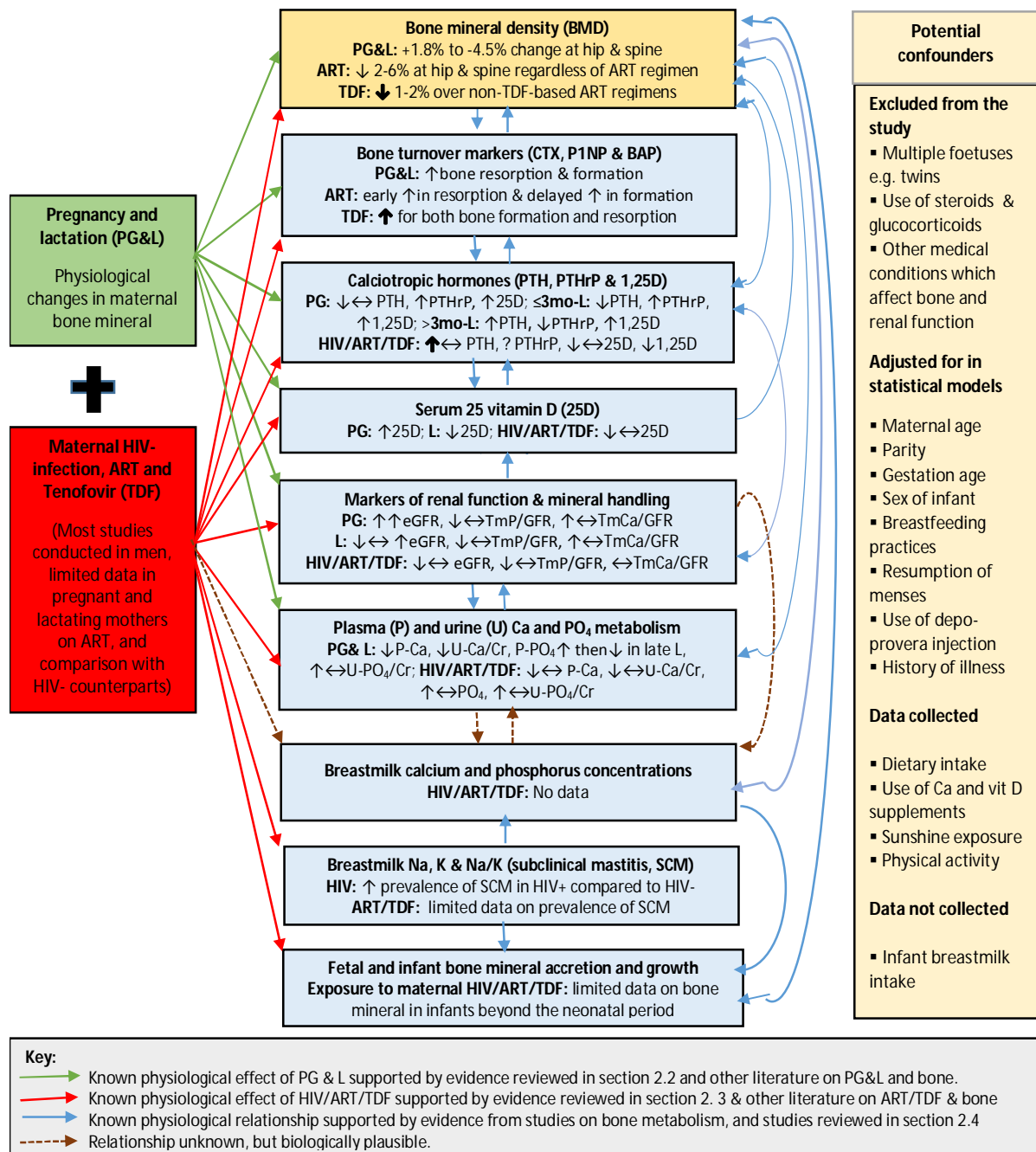


Figure 11: Study conceptual framework

### 3.2. Study design and protocol

Gumba is a longitudinal observational cohort study with two groups of women and their infants namely: (1) HIV-positive pregnant women initiated on first-line ART under Option B-plus during the current pregnancy, previously ART naïve (HIV-positive), and (2) HIV-negative pregnant women who had never been on ART (HIV-negative). The study objectives are presented in section 1.4.3. The conceptual framework is presented in figure 11.

Pregnant women were recruited at  $\leq 34$  weeks of gestation so that HIV-infected women had been on ART for 24-48 weeks by 14 weeks postpartum (assumed EDD at 40 weeks). This recruitment window was informed by literature that indicated (1) rapid declines in BMD are observed between 24 and 48 weeks after initiation of ART (2) there is minimal mobilisation of maternal bone mineral during the first and second trimesters of pregnancy.

Pregnant women were followed antepartum. Thereafter, mother-baby pairs were followed postpartum. The overall follow-up period for each participant was about 6 months depending on gestation age at enrollment. The first study visit was scheduled at around 36 weeks of gestation based on a routine obstetric ultrasound scan. Mother-baby pairs were followed at 2 and 14 weeks postpartum. The primary endpoint for the PhD work was 14 weeks postpartum (Figure 12 presents the study flow chart). Investigations for mother-baby pairs at each study visit are summarised in Tables 2 and 3, respectively. The scientific rationale and a comprehensive risk assessment for the procedures were detailed in the study protocol that was approved by the relevant research ethics and governing institutions in Uganda.

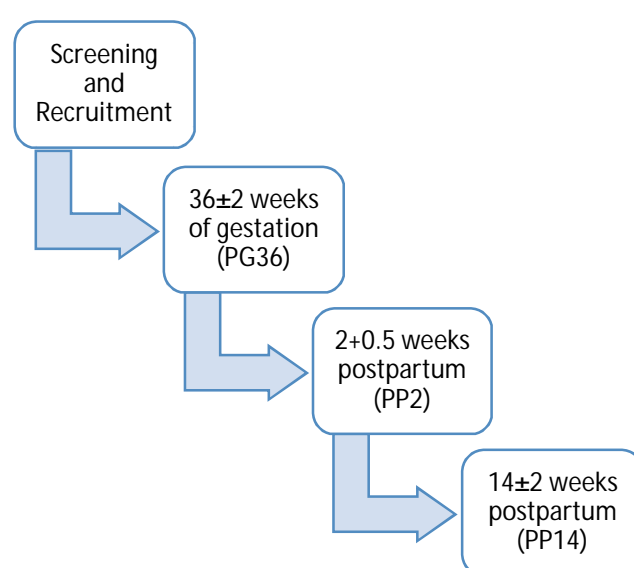


Figure 12: Study flow chart

Table 2: Summary of investigations for mothers at each study visit

Study visit Investigation	PG36	PP2	PP14
Biological samples <ul style="list-style-type: none"> <li>Overnight fasted blood (20 ml)</li> <li>Red blood cell pellets (1ml)</li> <li>2hr-fasted urine (20 ml)</li> <li>Breast milk (10 ml)</li> </ul>	√ √ √ X	X X √ √	√ √ √ √
DXA <ul style="list-style-type: none"> <li>Lumbar spine</li> <li>Hip</li> <li>Whole body</li> </ul>	X X X	√ √ √	√ √ √
Questionnaires <ul style="list-style-type: none"> <li>Socioeconomic status</li> <li>Medical history</li> <li>Dietary intake,</li> <li>Sunshine exposure</li> <li>Physical activity</li> </ul>	√ √ √ √ √	√ √ √ √ √	√ √ √ √ √
Anthropometry <ul style="list-style-type: none"> <li>Weight</li> <li>Height</li> <li>Mid-upper-arm circumference (MUAC)</li> </ul>	√ √ √	√ √ √	√ √ √

√ = scheduled; X =Not scheduled

Table 3: Summary of investigations for infants at each study visit

Study visit Investigation	Birth <sup>1</sup>	PP2	PP14
Biological samples <ul style="list-style-type: none"> <li>Blood (2 ml-spot sample)</li> </ul>	X	X	√
DXA <ul style="list-style-type: none"> <li>Whole body</li> <li>Lumbar Spine</li> </ul>	X X	√ √	√ √
Questionnaires <ul style="list-style-type: none"> <li>Medical history</li> <li>Breastfeeding</li> <li>Sunshine exposure</li> </ul>	X X X	√ √ √	√ √ √
Anthropometry <sup>2</sup> <ul style="list-style-type: none"> <li>Weight</li> <li>Length</li> <li>Head circumference</li> </ul>	√ <sup>3</sup> X X	√ √ √	√ √ √

√ = scheduled; X =Not scheduled

<sup>1</sup> Information on birth weight was obtained retrospectively at PP2 from maternity discharge forms and/or self-reports from mothers.

### 3.3. Sample size

The estimated sample size for this study was 200 pregnant women (100 HIV-infected ART-naive and 100 HIV-negative pregnant women (controls). This was calculated as detailed below in consultation with Dr Jianhua Wu (a statistician at HNR) and Dr Kate Ward (Senior Investigator Scientist, Nutrition and Bone Health (NBH) research group at MRC HNR/EWL).

*Assumptions primary outcome 1 (Minimum sample size 128 women, 64 per group)*

- i. Considering the independent effects of lactation and ART on bone, we anticipated at least a 2% difference between the groups in mean % change in maternal BMD at the lumbar spine between 2 and 14 weeks postpartum. The 2% difference was based on studies reviewed (sections 2.5.2 and 2.5.4)
- ii. The response within each group is normally distributed with a standard deviation (SD) of 4% based on evidence from previous pregnancy and lactation studies conducted by the NBH group (Olausson *et al.*, 2008, Jarjou *et al.*, 2010). We did not anticipate significant differences between the groups due to independent effects of pregnancy, lactation, and ageing.
- iii. Thus with a 2% mean difference between the groups in change in aBMD between PP2 and PP14, and a standard deviation of 4%, we needed to study at least 63 ART-exposed women and 63 HIV-negative women to be able to reject the null hypothesis that the population means of ART-exposed and ART groups are equal with power of 80%. The Type I error assumed was 0.05 (two-tailed). Calculation of sample size was done in OpenEpi software version 3.0 updated 06-April-2013 (Output presented in Figure 13; [http://www.openepi.com/Menu/OE\\_Menu.htm](http://www.openepi.com/Menu/OE_Menu.htm))

Sample Size For Comparing Two Means			
Input Data			
Confidence Interval (2-sided)	95%		
Power	80%		
Ratio of sample size (Group 2/Group 1)	1		
	Group 1	Group 2	Difference*
Mean			2
Standard deviation	4	4	
Variance	16	16	
Sample size of Group 1	63		
Sample size of Group 2	63		
Total sample size	126		
*Difference between the means			
Results from OpenEpi, Version 3, open source calculator--SSMean			

Figure 13: OpenEpi output for sample size calculation

Assumptions for final sample size (200 pregnant women, 100 per group)

- i. The following statistics from UDHS 2011 data for Kampala were used to make further assumptions regarding drop-outs from the study: maternal mortality (438/100,000 live births), infant mortality (47/1,000 live births); fertility rate (212/1,000 women aged 15-49 years)
- ii. A 3% rate of congenital abnormalities and 10% attrition rate have been reported in a study previously conducted in Kampala (Gibb et al., 2012), which translates into 17 mother-baby pairs.
- iii. We further anticipated infant deaths, successive pregnancies, the likelihood of missed visits, and challenges in acquiring DXA scans in infants.
- iv. Thus, we concluded that to answer the main objectives with adequate power at 14 weeks; we needed to recruit 200 pregnant women for at least 126 mother-baby pairs to complete all study procedures at 14 weeks postpartum (PP14).

### **3.4. Study setting**

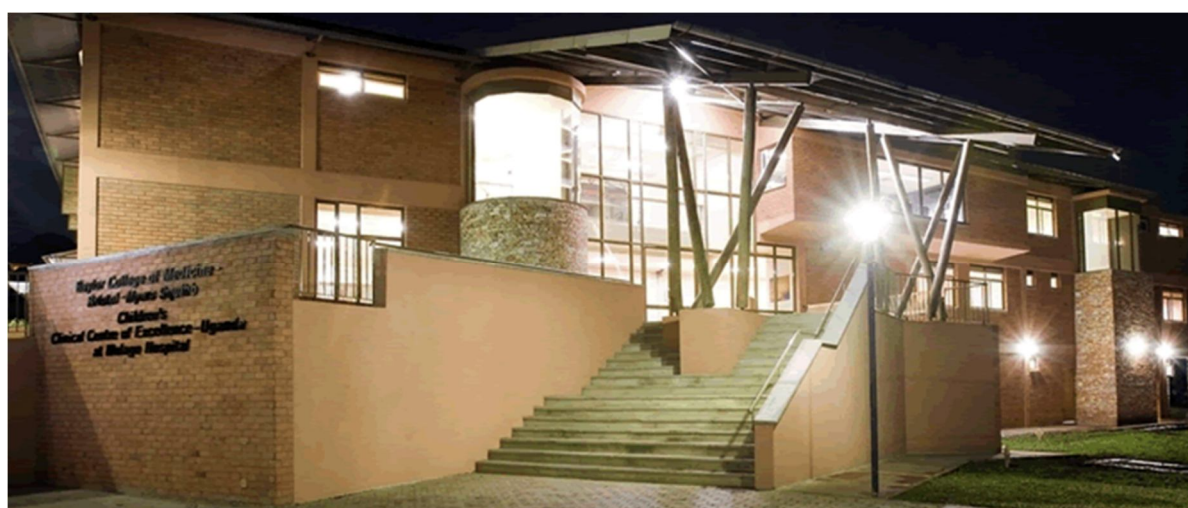
#### *3.4.1. Study Collaborators in Uganda*

This study was undertaken under a new research collaboration between MRC Elsie Widdowson Laboratory (EWL), (formerly known as MRC Human Nutrition Research, HNR), Baylor College of Medicine Children's Foundation Uganda (Baylor-Uganda) and Makerere University Johns Hopkins University Research Collaboration (MUJHU). A collaborative agreement was drawn up by the MRC contracts team in Cambridge and signed by all the collaborating institutions, and MRC was the overall legal sponsor for the study. Gumba study was funded by the NBH group at MRC EWL/HNR. All the collaborating institutions in Uganda operate on internationally acceptable policies that encourage the ethical conduct of research to ensure adequate protection of human subjects. They also have both technical and infrastructural capacity to carry out observational and intervention research with both local and international research collaborations (including MRC UK).

The fieldwork for this study was carried out in Kampala, Uganda within the Mulago National Teaching and Referral Hospital complex in collaboration with the Department of Obstetrics and Gynaecology. The hospital has a bed capacity of 1500 and has a directorate of obstetrics and gynaecology that runs two antenatal clinics and a maternity ward that conducts about 80 deliveries per day. The hospital complex also has several clinics and institutions offering specialised HIV care

services by the hospital itself, Makerere University and other organisations, including Baylor-Uganda and MUJHU.

Baylor Uganda operates the Children's HIV Clinical Center of Excellence (COE) and also doubles as the hospital's Pediatric Infectious Diseases Clinic. The COE offers comprehensive HIV services using a family-centred approach to over 6,000 children and their parents/caretakers, and is affiliated to Baylor International Pediatric AIDS Initiative (BIPAI) - a global partnership working to expand access to pediatric HIV/AIDS services in 11 countries within Africa, North America and Eastern Europe. Baylor Uganda COE has been involved in international multicentre clinical trials on paediatric ART and currently has among the largest paediatric HIV cohort studies. Prior to my PhD, I worked as a nutrition officer and later managed the nutrition programme at Baylor-Uganda. My position involved providing technical assistance to 23 districts and about 400 government health facilities on the integration of nutrition into HIV-care services. More information about Baylor-Uganda is available at <http://www.baylor-uganda.org/>



*Figure 14: Baylor-Uganda COE at Mulago hospital, courtesy of Baylor-Uganda*

*Photo credit: Baylor-Uganda [used with permission]*

MUJHU is a research-focused institution located less than 500 metres from Baylor-Uganda COE. Their research is focused primarily on reducing mother-to-child transmission (MTCT) of HIV, and over 7000 families have participated in their research studies and programs over the years. MUJHU is part of various international research networks including Improving Maternal Pediatric Adolescent AIDS Clinical Trials Network (IMPAACT) and Microbicides Trials Network (MTN).

MUJHU has participated in landmark multi-centre clinical trials that have contributed to the evolution of WHO PMTCT guidelines. The centre has two Dual Energy X-ray Absorptiometry (DXA) machines (Hologic models) and recently participated in the PROMISE (Promoting Maternal and Infant Survival Everywhere) and VOICE (Vaginal and Oral Interventions to Control the Epidemic) studies. PROMISE



1048s investigated the effects of maternal TDF exposure on maternal and infant bone while VOICE investigated the effect of TDF contain PreP on bone mineral among women of reproductive age. These studies are discussed later in sections 9.1, 9.2.1 and 9.2.6). More information about MUJHU is available at <http://www.mujhu.org/>



Figure 15: The main MUJHU building at Mulago Hospital. Courtesy of MUJHU

Photo credit: MUJHU Care Ltd [used with permission]

The laboratories at MUJHU and Baylor-Uganda are fully accredited by the College of American Pathologists (CAP), can process samples for routine investigations and HIV clinical monitoring, have experience with processing and shipping research samples for analysis at international laboratories and are equipped with Thermo Scientific -80°C freezers. However, they did not have the capacity to conduct specialist assays for bone metabolism and breast milk composition. Therefore, blood and urine samples for Gumba study were shipped for analysis at EWL in Cambridge, UK.



**Front row, left to Right:** Dr Dorothy Sebikari (chairperson MUJHU radiation committee), Dr Adeodata Kekitiinwa (Executive Director Baylor-Uganda, consultant study co-PI), Prof Ann Prentice (Director MRC HNR/EWL, study co-PI).

**Back row, left to right:** Prof Josaphat Byamugisha (Director Mulago hospital and Makerere University Dept. of Obs. & Gynae., study co-PI), Dr Vincent Tukei (Manager Research, Baylor-Uganda), Dr Jacqueline Balungi (Manager, Baylor COE clinic), Prof Mary Glenn Fowler (Executive Director MUJHU, study consultant co-PI), Dr Gail Goldberg (Senior Investigator Scientist at MRC HNR/EWL, Study co-PI and PhD supervisor), Florence Nabwire (PhD student MRC HNR/EWL and The University of Cambridge, study co-PI).

Figure 16: Gumba study collaborators at Baylor COE, Kampala on 14th August 2014.

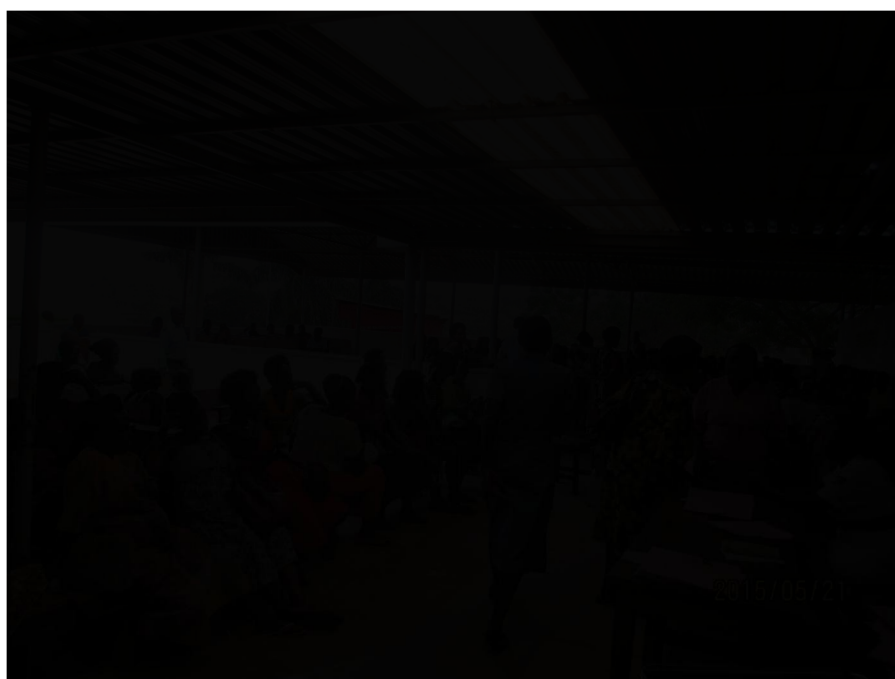
Photo credit: Baylor-Uganda communications team [used with permission]



### *3.4.2. Overview of Mulago Hospital antenatal and PMTCT programmes*

#### *3.4.2.1. Antenatal programme*

At the time of conducting fieldwork for the Gumba study, Mulago Hospital Department of Obstetrics and Gynaecology managed two antenatal clinics (ANCs): Upper Mulago and new Mulago antenatal clinics (ANC). The Upper Mulago was the main outpatient ANC for the hospital. It was managed by midwives and mainly served women with uncomplicated pregnancies. New Mulago ANC was managed by medical doctors and specialists. It served pregnant women with medical complications and non-pregnant women with gynaecological problems. The two clinics were merged during the study due to renovations in the new Mulago wing. Together, the ANC, gynaecology and obstetrics clinics had an attendance of about 500 women per day. The maternity conducted about 80 deliveries per day. The ANC clinics and maternity were located to Kawempe health centre level four in 2016 (after I completed data collection) to pave the way for renovations at the hospital.



*Figure 17: Upper Mulago ANC waiting area*

*Photo credit: Florence Nabwire*

The midwives conducted routine procedures including anthropometry, BP measurements and palpation in all pregnant women. The national ANC schedule recommended a minimum of four antenatal visits scheduled at 10-20, 20-28, 28-36 and > 36 weeks of gestation. According to the prevailing national standard of care, routine laboratory investigations which include syphilis (TPHA), Haemoglobin levels (Hb) and urinalysis were recommended at the first ANC visit (MOH-Uganda,

2012c, MOH-Uganda, 2012a). However, supplies were inadequate and often out of stock hence routine laboratory investigations were not offered to all women – priority was given to those who presented with complications when supplies were available. Referrals to private laboratories were made when the supplies were out of stock. At least one routine ultrasound scan was indicated during pregnancy. The hospital had an obstetric ultrasound facilities at new Mulago ward 5B and a subsidised fee of UGX 10,000/= was charged. However, the majority of women could not afford the scans, so referrals were done for women with complications.

#### *3.4.2.2. PMTCT programme*

PMTCT services were integrated with the routine ANC services. HIV testing and treatment services were offered at Upper Mulago ANC under the PMTCT program managed by MUJHU. HIV testing was mandatory for all pregnant women and their partners at the first ANC visit, in line with the national guidelines. Women who were not accompanied by their partners at first were encouraged to bring them for an HIV-test at the next visit. HIV-testing was provided free of charge to all women and supplies were always available.

In compliance with the national guidelines, women who tested HIV-positive were registered in the PMTCT program and initiated on ART, preferably on the same day. A single triple ART regimen, TDF/3TC/EFV, was initiated for all women unless contraindicated. A baseline blood sample for CD<sub>4</sub> test was sent to MUJHU laboratory, and the results were available in two weeks. Professional counsellors, nurses and doctors ran the PMTCT program. Experienced peer counsellors were also available to support newly diagnosed HIV+ women and guide them through the various service points within the hospital. The peers emphasised the benefits of adherence to ART, disclosure of HIV status to partners, delivering in a health facility and exclusive breastfeeding to reduce the risk of passing HIV to the baby.

The women attended the PMTCT clinic monthly for ART drug refills and clinical monitoring. HIV viral load tests were performed at 6 months after initiation of ART then annually to monitor ART treatment outcomes. Postpartum, HIV-infected women continued to receive PMTCT services from the PMTCT follow-up clinic run by MUJHU which was located about 100m from the antenatal clinic. All medical services were provided for HIV-infected women free of charge.

### *3.4.2.3. Early Infant Diagnosis (EID) programme*

All infants born to HIV infected women (HIV-exposed infants) were enrolled in the postnatal PMTCT follow-up clinic (PPNC) managed by Baylor-Uganda. The infants were tested for HIV-starting at 6 weeks of age and also received routine medical care free of charge. The first DNA PCR HIV-test for infants was done at 6 weeks of age according to the national Early Infant Diagnosis (EID) schedule (MOH-Uganda, 2012a).

The prevailing national guidelines recommended HIV-infected mothers to breastfeed their infants for at least 12 months while the mothers were on ART. ARV prophylaxis (nevirapine syrup) was provided to the infants from birth until 6 weeks of age when the first DNA polymerase chain reaction (PCR) HIV-test was done. Cotrimoxazole prophylaxis was provided (from 6 weeks until the results of the second DNA PCR test were known) to protect against *Pneumocystis pneumonia* (PCP), other bacterial infections and malaria. The second DNA PCR test was done at least 6 weeks after the infant stopped breastfeeding. HIV-exposed infants were followed in the EID clinic every three months until a confirmatory HIV-serology test was done at 18 months of age. Children who tested HIV positive at any point in the EID cascade were immediately started on paediatric ART as per the prevailing national guidelines (MOH-Uganda, 2013).

### *3.4.3. Study approvals*

A series of approvals were obtained between March and December 2014 before the study commenced in January 2015. The study protocol version 1.0 received initial approval from the MRC Human Nutrition Research, Research Review Board on 20 March 2014 (Appendix 1). Next, I obtained local ethics approvals in Uganda. The designated local Institutional Review and Ethics Boards (IRB) for Baylor Uganda, the main host institution in Uganda, was the Joint Clinical Research Centre (JCRC). According to the prevailing JCRC IRB requirements, all the study questionnaires and informed consent forms had to be translated into the local language “Luganda” that is widely spoken in Kampala. I thus obtained leave to work away (LTWA) from the University of Cambridge and travelled to Uganda in March 2014 to apply for ethics approvals early in my PhD because of anticipated bureaucracies in Uganda. Local supervision was provided by Profs Mary Glenn Fowler, Adeodata Kekitiinwa and Josaphat Byamugisha.

All the study documents were translated into Luganda, back-translated into English, and I reviewed for consistency before submission to the JCRC IRB on 25 March 2014. I received minor comments from JCRC IRB on 08 May 2014 regarding my application and responded on 24 June 2014. The revised study protocol version 2.0 was approved by JCRC IRB on 27 June 2014 initially for one year and continued annual renewals were subject to submission of satisfactory study progress reports. The JCRC IRB approval letter did not allocate the study a code number (Appendix 1). The next step was to obtain approval from the Uganda National Council for Science and Technology (UNCST), the overall body overseeing research activities in Uganda.

After my first year upgrade in July 2014, I was again granted LTWA by the University of Cambridge to obtain subsequent study approvals and conduct fieldwork in Uganda. I convened collaborators meeting in August 2014 to review progress and obtain guidance on the next approval steps. At the meeting, I was informed that Mulago National hospital had established their own ethics/institutional review board, so, I needed to get institutional clearance before applying to UNCST. I received institutional clearance from Mulago IRB on 24 August 2014 subject to annual renewals (Appendix 1).

I submitted the application to UNCST on 15 September 2014. UNCST clearance and study registration process involved both scientific approval and vetting of foreign study investigators by the Office of the President. By the end of October 2014, no feedback had been received from UNCST, so I appealed for expedited review on the basis that I was a PhD student with limited time for fieldwork. A provisional approval letter was issued on 24 November 2014 for implementation over 3 years (16 September 2014 to 16 September, 2017) under the study registration number HS 1680 (Appendix 1). Dr Gail Goldberg and I received clearance from the Office of the President on 25 March 2015.

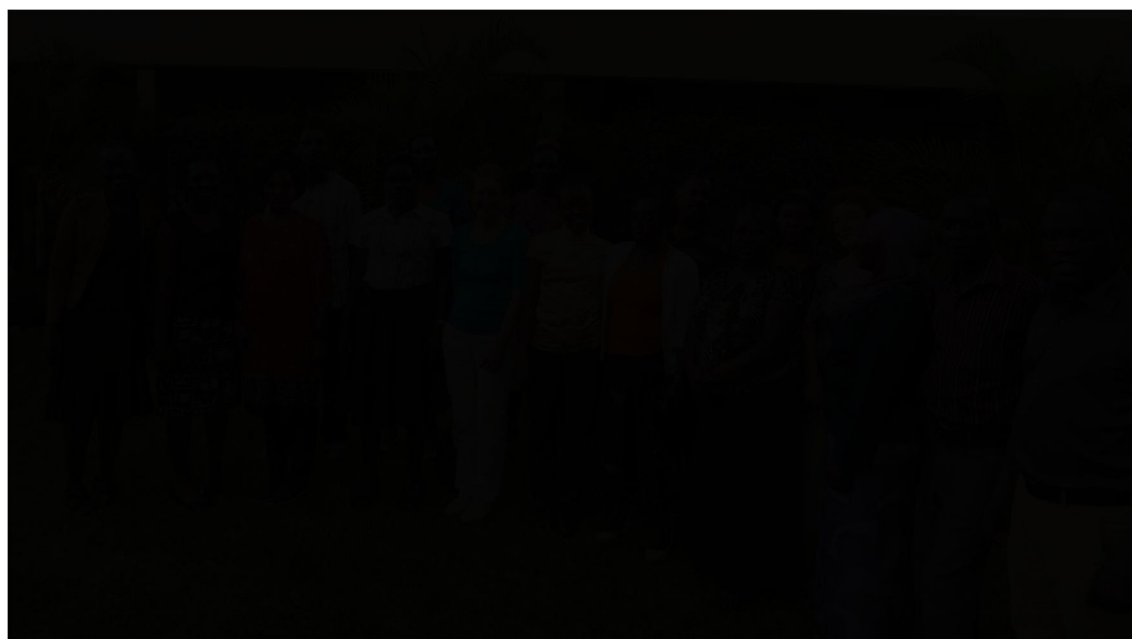
The next requirement was authorisation from UNCST for transfer of biological samples to HNR for analysis. This application could be made at any point during the study before samples were transferred and required a signed Materials Transfer Agreement (MTA) between HNR and Baylor-Uganda. However, at this time, drafting of collaborative agreements and MTA was still in progress. I, therefore, deferred the application until 27 August 2015. The approval was granted to on 30 November 2015 (Appendix 1).

### 3.5. Recruitment and training of staff

#### 3.5.1. Recruitment of staff

Between July and October 2014, I identified the staffing needs for the study and developed job descriptions. According to the prevailing research standards at both MUJHU and Baylor, only professional counsellors and nurses/midwives (to a lesser extent) can obtain informed consent from potential participants. As a minimum, data collection within the clinic environment is done by professional nurses, preferably with degree level training. Blood collection is done by qualified phlebotomists or trained nurses/midwives with prior experience in phlebotomy. Trained chaperones are required at Mulago ANC to guide potential study participants through various service points and provide additional counselling for HIV-infected women. A trained and experienced home visitor is also needed to map residences of participants and conduct home visits for missed visits. Only trained DXA technologists with a background in radiology were allowed by the MUHJU radiation safety committee to perform DXA.

Human Resource (HR) departments at Baylor Uganda and MUJHU hired staff (two nurses, one midwife, one counsellor, one home visitor and one laboratory technician) on my behalf. I was involved in all the recruitment procedures including advertising, shortlisting, interviewing and selection of candidates. The study also shared some of the existing staff who were allocated to support various aspects of the study (see Table 4 for a full list of staff and their roles).



*Figure 18: Prof Ann Prentice and Dr Gail Goldberg with the Gumba study team at Humura Hotel in Kampala  
Photo provided by Prof Ann Prentice*

Table 4: List of staff involved in the Gumba study both in Uganda

Title, Qualification (Employer)	Responsibility
<b>Principal Investigator, BSc, MSc (HNR)</b>	Overall coordination of all study activities including preparation and amendment of study protocols, questionnaires, SOPs and ethics documents, recruitment and training of study staff on protocols and SOPs, data QC2** and QA review for all study documents and reporting adverse events to IRBs, finance, and HR management for the team.
<b>Study Nurses*, BSc Nursing (Baylor)</b>	Pretesting questionnaires in local language, screening and enrollment of participants, data and sample collection (urine and breast milk), data QC1** reviewer
<b>Study Counsellors, BSc Counselling (MUJHU)</b>	Obtained informed consent from potential participants, data, and sample collection, conducted home visits for problematic cases; data QC1 reviewer.
<b>Study midwife, Dip. Midwifery &amp; Dip. Nursing ( MUJHU)</b>	Screening of potential study participants, enrollment of participants, data and sample collection (urine and breast milk), and data QC1 reviewer.
<b>Chaperones, O' level (MUJHU)</b>	Helped in identification of potential participants from the antenatal clinic, and chaperoned participants at ANC and within the Hospital.
<b>Home visitor, O level (Baylor)</b>	Conducted home visits for mapping, follow-up and missed appointments.
<b>Laboratory Technician (Baylor)</b>	Phlebotomy, sample processing, and storage.
<b>Laboratory Technologists, BMLT (Baylor)</b>	Phlebotomy, sample processing and storage, management of sample tracking system (Freezer works) and shipping samples.
<b>Senior Laboratory Technologists, BMLT (Baylor)</b>	Data QC1 reviewer for study laboratory documents.
<b>DXA Technologists, BSc Radiology, (MUJHU)</b>	Performed DXA scans, extracted scans from database and sent to HNR.
<b>Data Clerk, BSc (Baylor)</b>	In-charge of storage, access control for study records and data entry.
<b>Medical Officer (Baylor)</b>	Offered clinical care for sick participants.
<b>Paediatricians (Baylor)</b>	Offered routine clinical care for sick participants (infants) with complications.
<b>Physician (Baylor)</b>	Offered routine clinical care for sick participants (mothers) with complications.

\* One of the nurses took on the coordination of the day to day study activities from Jan-May 2015 after I returned to Cambridge. However, I still provided remote oversight for the study by conducting weekly study meetings via Skype, tracking of study appointment schedule/missed visits and reporting of adverse events to IRB. I also conducted monthly QA reviews on 10% of study visits conducted in months to monitor compliance to study protocol and SOPs.

QC1 = Quality Control level 1; QC2 = Quality Control level 2; QA=Quality Assurance. These procedures are described in section 4.6.1.

### *3.5.2. Training of staff*

Between July and October 2014, I developed study standard operating procedures (SOPs) for the study and associated forms. I trained all the study staff on the approved study protocols, SOPs, consent forms, questionnaires (both English and Luganda versions), and associated forms in compliance with regulatory requirements at both Baylor and MUJHU. I also worked with the staff to pre-test all the questionnaires, study SOPs and made simplified flow-charts for study procedures at every visit for quick reference.

In addition, all study staff and I completed mandatory online courses on Good Clinical Practices (GCP) and Human Subject's Protection (provided by the US National Institutes of Health). Additional training on general research protocols and technical aspects, e.g. phlebotomy, obtaining informed consent were provided by the relevant technical supervisors at Baylor and MUJHU. A training log was maintained and signed by both the trainer and trainee for each session.

## **3.6. Recruitment of participants**

### *3.6.1. Inclusion and exclusion criteria*

The study inclusion and exclusion criteria were:

- i. Known HIV status, with recent HIV test results (preferably tested at Mulago hospital).
  - a. If HIV-infected women, initiated ART during the current pregnancy
  - b. If HIV-negative women, never had exposure to antiretroviral drugs (ART naïve)
- ii. Aged 18.0-39.9 years. The lower cut-off of 18.0 years was set to match the legal age of informed consent in Uganda. The upper cut-off of 40 years was to allow adequate time to follow-up of participants before the onset of menopause, which is independently associated with 1-2% annual decreases in BMD. In Uganda, about 9% of women aged 30-49 years enter menopause, and the proportion of increases with advancing age to about 40% among women aged 48-49 years olds (UDHS, 2012).
- iii. Gestation age <34 weeks at enrollment to ensure HIV-infected women were on ART for 20-48 weeks at 14 weeks postpartum (assuming the EDD at 40 weeks). This cut-off provided adequate time for potential participants to complete the consent process and attend their first study visit at 36 weeks of gestation.
- iv. Planned to breastfeed their infant for at least six months. This would allow for observation of changes in maternal BMD during breastfeeding, which was central to the study rationale

- v. Without a known diagnosis of bone disease, diabetes mellitus, glycosuria, gestational diabetes, hypertension, preeclampsia/eclampsia, TB infection or TB suspected, hepatitis B or C co-infection, renal disease, proteinuria or any kidney-related medical condition. These conditions can also affect bone metabolism hence are potential confounders.
- vi. Lived within a 20km radius of Mulago Hospital and planned to stay within the study area for at least two years and consented to home visits. This area was covered by community volunteers at Baylor-Uganda COE enabling home visits for collection of urine samples and tracing of participants for follow-up visits.
- vii. Willing to undergo all study procedures as outlined in the approved study protocol and informed consent forms.
- viii. Consented on behalf of their unborn baby to participate in the study after delivery as per the study protocol.

Mothers who did not meet the above criteria were not enrolled in the study. Further exclusions after enrollment included:

- i. Pregnancies classified as high risk during the study.
- ii. Confirmed multiple fetuses based on ultrasound scan results.
- iii. Pre-term deliveries ( $\leq 37$  weeks of gestation based on ultrasound scan).
- iv. Fetal deaths, abortions, miscarriages, stillbirths and neonatal deaths.
- v. Stopped breastfeeding before 14 weeks postpartum.

These participant selection criteria were designed with a focus on the safety and wellbeing of participants to minimise potential confounders and attrition from the study.

### *3.6.2. Awareness and referral of potential participants*

Recruitment procedures commenced at Upper Mulago ANC clinic in January 2015. Pregnant women attending upper Mulago antenatal clinic were approached to participate in the study. While in the waiting area, the study staff (counsellor/nurse/midwife) gave 10-minute talks in English and Luganda (the main local language in Kampala) to raise awareness on the study objectives, eligibility criteria, and recruitment process. In these talks, the counsellor presented a 5 minute lay summary of the study and took questions from the audience. She then concluded by inviting interested women to talk to the midwife to obtain a referral or to come directly to the study office. Trained midwives approached potentially eligible women at their service stations in the clinic and completed referrals for interested women (see appendix 2). Referred women were escorted to the study office by the chaperones.



### *3.5.3. Screening and informed consent (IC) session 1*

The study nurses/midwife received pregnant women referred by the midwives and allocated a referral number. The referral forms were reviewed for completeness and eligibility of the potential participant. Women who were not eligible were informed and thanked for their interest in the study. Those deemed eligible based on the referral were given a verbal overview of the study procedures and asked if they wanted to initiate the study recruitment process on that day. The study nurse/midwife also obtained verbal informed consent to review medical records of interested women in order to complete part one of the screening questionnaire (appendix 3). A screening number was allocated for those who consented. Women with known medical complications were discontinued at this point, and those who passed initiated the informed consent (IC) process (session 1) with the study counsellor.

The counsellor introduced the informed consent form (ICF) to the participant in their preferred language (English or Luganda) and assessed comprehension of the participant (see appendix 4). Women who did not have a good understanding of either English or Luganda were discontinued. The counsellor discussed the key sections of the informed consent form, explained the procedures at the next visit (enrollment visit) and issued a return date for a full consent session. A copy of the informed consent form was given to the potential participants to take home, and they were encouraged to come with their partners at the next session 3.5.4. Screening and Informed consent session 2.

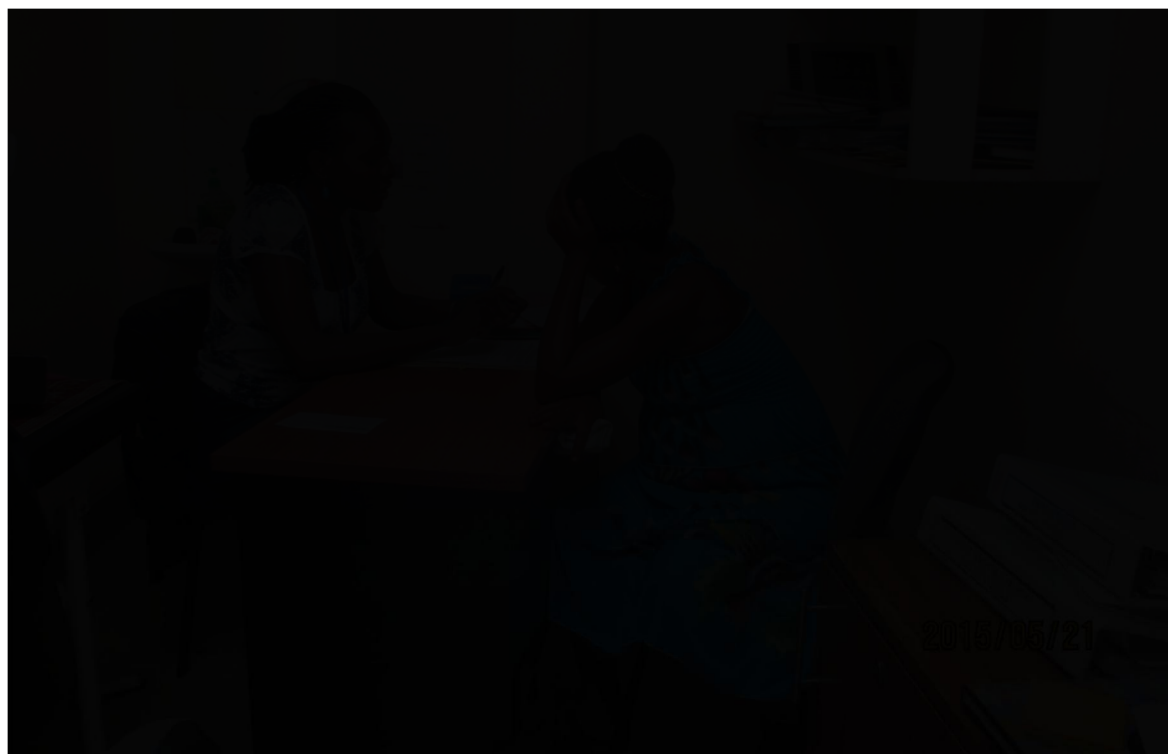
The counsellor had an in-depth discussion of study procedures with the potential participant and addressed their questions/concerns. It was emphasised to the mothers that participation in the study was entirely voluntary; and that if they decided not to join the study, their access to routine medical services at the hospital would not be affected. All sessions lasted at least an hour and were also witnessed by a trained chaperone. Husbands and personal witness (es) for illiterate women attended the session where available. Women who decided not to join the study were thanked for their time, and transport costs were reimbursed.

Women who decided to join the study signed the allocated section of the consent form. The counsellor, husband and witness (s) who attended the session also signed the appropriate sections of the form. For those unable to write, an inked thumbprint of their right hand was obtained as a signature. I reviewed all fully signed consent forms to ensure they were properly signed and issued a photocopy to the potential participant for future reference. The original was retained for study records and filled in a secure cabinet.

Screening level 2 procedures were conducted to confirm eligibility of consented women before enrollment into the study. Confirmation of full eligibility was done including a review of routine obstetric ultrasound and urinalysis results. Women with glycosuria, proteinuria +++, gestation age >37 weeks or multiple pregnancies were discontinued.

#### *3.6.4. Enrollment of participants*

Women who passed screening level 2 were enrolled into the study. The participants were allocated a unique study number and issued with a study identification card containing their study number and phone contact numbers for the study. The participant received reimbursement for transport costs. Enrolment of participants was conducted on a rolling basis from Monday-Friday until the target number of participants was achieved. These recruitment procedures were in line with those commonly used at Mulago clinics. Recruitment of study participants closed in February 2016.



*Figure 19: Study counsellor (left) obtaining informed consent from a potential participant*

*Photo credit: Florence Nabwire*

### 3.7. Study visits

#### 3.7.1. *Thirty-six weeks of gestation (PG36)*

The first study visit was scheduled at  $36 \pm 1$  weeks of gestation (PG36 visit). The timing of the visit was based on literature that late pregnancy is characterised by rapid bone turnover in the maternal skeleton to mobilise calcium for fetal bone mineral accretion (Olausson et al., 2012). This visit also matched with previous studies on pregnancy, lactation and bone health conducted by the NBH group in The Gambia and Cambridge. This presented an opportunity for comparison of the findings across studies.

At PG36, pregnant women came for the study visit in the morning after overnight fasting for at least 8 hours (participants were requested not to eat after 11pm). A two-hour timed fasting protocol was followed to collect blood and urine samples (described in section 4.3.1). While undergoing the 2-hour fasting procedure, anthropometric measurements were taken and questionnaires administered. At the end of the 2-hour fast, participants were offered breakfast, transport reimbursement and routine health services (PMTCT and/or antenatal care) as per prevailing practice. The study team checked the questionnaires for completeness and provided transport refund before the women left the clinic.

#### 3.7.2. *Delivery*

Participants were encouraged to deliver at Mulago National Hospital and to inform the study team via a toll-free line, at the onset of labour. An appointment for the first postpartum visit for the mother-baby pair was issued based on the date of delivery.

#### 3.7.3. *Two-week postpartum (PP2)*

The first postpartum visit was scheduled at  $2.0 \pm 0.5$  weeks postpartum. This was to obtain baseline maternal bone mineral by DXA to reflect pregnancy before lactation was fully established. It also enabled acquisition of DXA scans in infants to assess potential effects of in-utero exposure to maternal ART. The timing of the baseline postpartum DXA scan considered the duration required for recovery of women following delivery by Caesarean section as well as cultural norms regarding the movement of newborn infants outside the home. Rapid changes in body composition and water during the early puerperium period which could affect the estimation of bone mineral by DXA were

also considered. These considerations were to ensure valid DXA scans were obtained while minimising participant burden and risk of missed visits.

Previous studies on lactation and bone health in the Gambia and Cambridge and PROMISE studies (Laskey et al., 1998, Jarjou *et al.*, 2006, Olausson et al., 2008) . Therefore, scheduling baseline DXA scans at  $2 \pm 0.5$  weeks postpartum (PP2 visit) was consistent with both previous and ongoing studies on pregnancy, lactation, ART and bone health for comparison.

At PP2, mother-baby pairs came in the morning for study procedures. Participants were informed not to fast at this visit and to breastfeed their infants normally. On arrival at Baylor COE, anthropometric measurements were taken, breast milk samples collected and questionnaires administered as per the study protocol (appendix 9). The women were offered breakfast while the questionnaires were reviewed for completeness. Afterwards, infants with incomplete immunisation records were immunised at Baylor-COE clinic then mother-baby pairs were escorted to MUJHU for DXA scans. Participants were given a return appointment for the PP14 visit, and their transport costs were reimbursed.

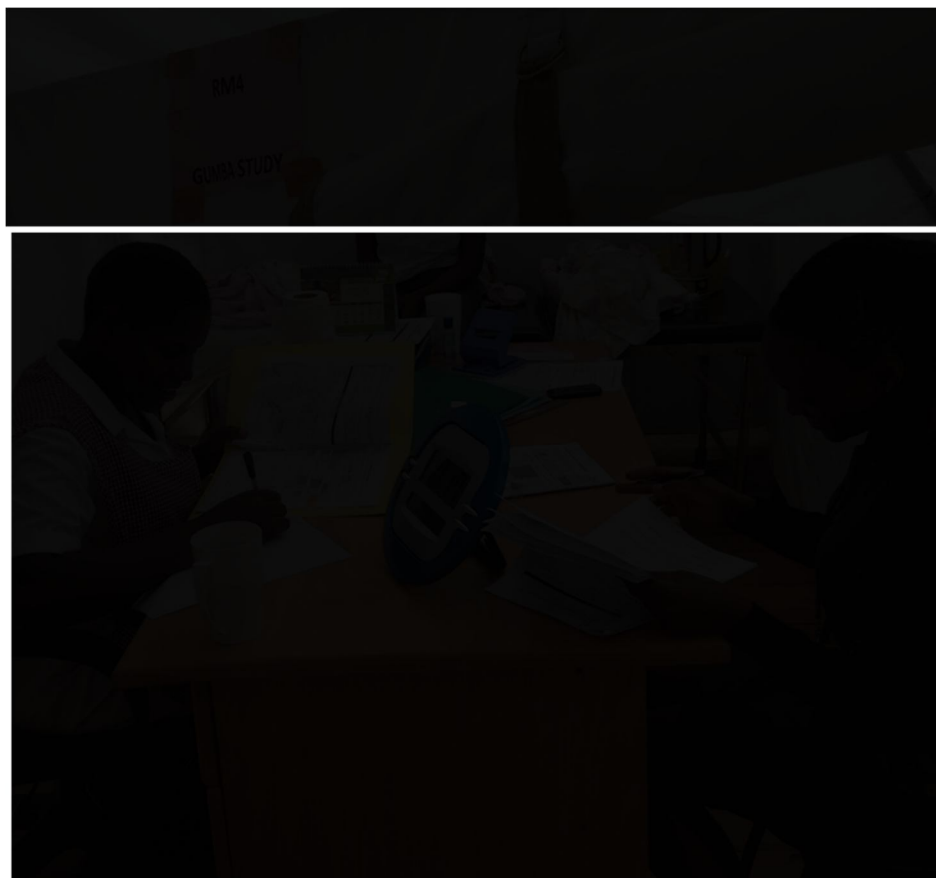


Figure 20: Study nurse (Lilian) and I reviewing questionnaires on a working day at the Baylor-COE

Photo credit: Dr Gail Goldberg

### 3.7.4. Fourteen weeks postpartum (PP14)

PP14 was scheduled at 14±2 weeks postpartum and was the primary endpoint of the PhD study. The timing of the visit was targeted around peak lactation which occurs at about 10-12 weeks postpartum. It was also desirable to schedule the visit when most women were still breastfeeding and when HIV-infected women had completed at least 24 weeks on ART to allow adequate time for observation of the combined effects of lactation and ART on the maternal skeleton. Previous lactation studies by NBH group have scheduled the visit at 13 weeks postpartum (Jarjou et al., 2010). Considering information that the median duration of predominant breastfeeding in Kampala was 3.2 months (UDHS, 2012) and there was a routine immunisation visit at 14 weeks postpartum, 14 weeks was selected as the end point of the study.

The PP14 visit had the same procedures as PG36 and PP2. In addition, a non-fasted blood sample was collected from the infants. Breastfeeding/feeding was not restricted during the 2hr-timed fasting procedure for mothers. Questionnaires were also completed (appendix 10). After study procedures and receipt of scheduled routine MCH services at Baylor COE, mother-infant pairs were escorted for DXA scans at MU-JHU. Data collection for PP14 visits was completed in May 2016.

### 3.7.5 Study coordination

The study had three sites for data collection within Mulago hospital. All study procedures during pregnancy (recruitment and PG36) took place at Upper Mulago, most of the postpartum activities were done at Baylor-Uganda (Baylor-COE laboratory, Baylor-COE study office) and DXA scans were performed at MUJHU. The laboratory at Baylor-Uganda was in charge of collection, processing and storage of biological samples. Cool boxes with ice packs were used to transport samples between study sites. Chaperones escorted participants between study sites for various procedures. A closed user group mobile phone service was used to facilitate voice calls between staff at various study sites. The overall flow of study procedures at each visit is summarised in Figure 21.

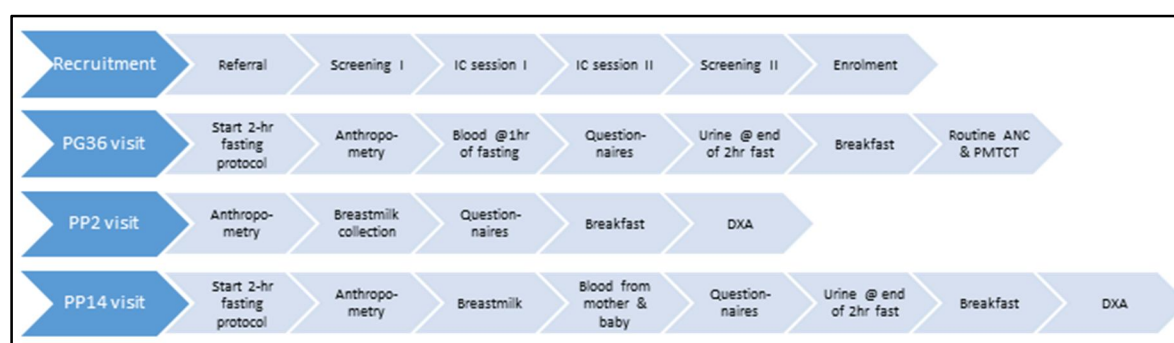


Figure 21: Flow of study activities at each visit

I provided overall supervision of staff and coordination of the study activities across the three sites, and I was involved in periodic staff appraisals. I identified supplies for all laboratory supplies especially sample collection and storage tubes. Prior to procurement, sample blank tubes (filled with demineralised water) were run on the analysis platforms in the NBH lab at EWL, and confirmed to be free of contamination for calcium and phosphorus. I then worked closely with the procurement team at Baylor to ensure all laboratory supplies were procured on time (both locally and from the UK) for smooth running of study activities. To keep all staff updated on progress, I conducted weekly study meetings and this offered opportunities to address challenges encountered by staff. In addition, I managed the study budget and approved all study transactions in Uganda. Regular meetings were also held with community volunteers to get updates from the community. I held meetings with all collaborators to keep them updated on the study progress and to seek guidance where necessary. I continued to remotely coordinate the study after I returned to Cambridge until May 2017 when the participants completed follow-up at 18 months postpartum.

During fieldwork in Uganda, I maintained regular contact with my PhD supervisor (Dr Gail Goldberg) and other staff at HNR via email and Skype. I also regularly attended the weekly Science club at EWL via Skype. Dr Goldberg and Prof Ann Prentice came to Uganda in October 2014 to meet the collaborators and provide guidance on setting up the study. I also had a second supervision visit by Dr Goldberg in May 2015.

### **3.8. Retention of participants in the study**

#### *3.8.1. Mapping of residences and home visits*

A home visitor accompanied participants to their homes after enrollment to map their residence. Participant tracing form F003 (appendix 5) and map to their residence was drawn for future use. The home visitor also introduced a trained field worker (community volunteer) to the participant. Home visits were only conducted for participants who were sick or not reached via phone contacts provided. When necessary, the community volunteers were contacted via phone and requested to conduct home visits to their catchment area. At every visit, participants were asked if they had changed their residence and/phone contacts and the information was updated on form F003 (Appendix 6). The mapping exercise was repeated for participants who changed residence, and a new map was drawn. There were two incidents of HIV-positive participants who took the study home visitor to a wrong address for mapping. Incidences of intentionally giving wrong phone numbers and address to hospital staff were common among newly diagnosed HIV-positive women in the PMTCT program. This is due to fear of stigma and home visits, which are associated with being HIV-positive in some communities.



*Figure 22: Community volunteers, Gumba staff and I celebrate end of data collection.*

*Photo provided by Florence Nabwire*

### *3.8.2. Phone calls and text messages*

The study maintained two mobile phone numbers and a toll-free line for communication with participants. The majority of the participants owned a mobile phone, and there was reliable network coverage. Alternative phone contacts given by the participants were phoned if the participant's primary phone number was off. Participants were reminded of their scheduled appointments at least one week in advance through mobile phone calls, text messages or home visits depending on the participant's contact details and preferred method of communication. In addition, the study nurse/midwife made weekly phone calls to all women who had completed PG36 to find out if they had delivered and to also check on their health. This is was implemented after a few participants did not phone in time and missed the PP2 visit.

Participants were also advised to contact the study via phone regarding study protocol, illness and inability to attend scheduled study visits. The study mobile phone numbers were kept open for 24hrs by the study nurse. A toll-free line was operated by Baylor-Uganda on weekdays between 8am and 5pm, and participants also used this service to contact the study team.



### *3.8.3. Transport arrangements and reimbursement*

Participants were paid transport refund of UGX. 15,000 per mother-infant pair to facilitate their movement to and from Mulago Hospital at every study visit. This amount was similar to that paid by ongoing studies at Baylor-Uganda and within limits recommended by UNCST to safeguard against coercion of participants.

At PP2, special transport arrangements were made for mother-baby pairs who had complications during/after delivery and for those who had travelled out of Kampala for postnatal care to maximise attendance. Women who lived in villages outside Kampala were sent adequate fare via mobile money to enable them to travel to Kampala by public transport for study procedures. The study was allocated vehicles at Baylor-Uganda to conduct home visits within a 20km radius and also pick and drop mother-baby pairs with complications following birth.

### *3.8.4. Integration of study visits with routine MCH visits*

Study visits were synchronised with the participants' antenatal and immunisation appointments to reduce participant burden. ANC services were integrated into study visits during pregnancy (enrolment and PG36). Postpartum, routine maternal and child health services like immunisation and growth monitoring services were offered at Baylor-Uganda at PP2 and PP14. Only the PP2 visit was outside the immunisation schedule. The integration of routine medical services and study appointments not only reduced participant burden but also improved their retention in the study.

## **3.9. Research governance**

### *3.9.1. Clinical care for participants*

The study did not directly provide routine medical services for participants but liaised with relevant service providers for participants to ensure participants received routine clinical services. In scenarios where the study identified gaps in care such as poor adherence to ART or clinical events, the study informed the relevant clinics for management. The study paid for routine ANC laboratory investigations for all women (syphilis screening, Hb, blood grouping, urinalysis and one ultrasound scan). The participants maintained the original results for their clinical care. The study only maintained a photocopy on the participant's file for financial accountability and reference. This was implemented based on guidance from the Director of the Department of Obstetrics and Gynaecology at the hospital. HIV-infected women and their exposed infants received PMTCT services



from Mulago Hospital PMTCT clinics run by MUJHU and Baylor-Uganda (see the description of these clinics in section 3.3.2).

Antepartum, sick participants were managed by midwives and specialists at Mulago ANC clinic. Sick participants who presented postpartum were managed at the Baylor COE clinic which had medical doctors and specialists including paediatricians and one physician (see section 3.4.1). The medical officers reviewed all sick patients and where necessary referred to specialists for further management. HIV-positive women and their babies received free medical services from the PMTCT clinic and Baylor-Uganda, respectively as part of the PMTCT-EID package. Sick HIV-negative participants received a free consultation with a doctor at Baylor-Uganda, and the study paid for prescribed medication up to a maximum of UGX 20,000/=.

### *3.9.2. Adverse events and abnormal laboratory results*

Although Gumba was not a clinical trial, all cases of illness and other adverse events were documented in a log and general notes added in the participant's file. I reviewed and countersigned all general notes then prepared a final report for submission to JCRC ethics committee, where necessary. The adverse events that were reportable to ethics committee included preterm deliveries, stillbirths, neonatal deaths, fracture caused by trauma (domestic violence) and hospital admissions. Serious adverse events were reported within 7 working days according to JCRC guidelines. Other reportable events were aggregated in the annual progress reports that accompanied the applications for renewal of study approvals.

### *3.9.3. Study audits*

The research regulatory office at Baylor-Uganda conducted internal audits to monitor compliance with approved study protocols, clinical and research standards. A pre-study audit was conducted in January 2015 before recruitment of participants commenced, and then spot audits were conducted routinely. I shared the audit reports with staff, and all the queries were addressed according to the prevailing requirements at Baylor-Uganda.

## CHAPTER 4: METHODS

### 4.1. Selection of study methods

Table 5: Selection of study methods

Study Outcome	Standard methods/ analytes/assays	Description, clinical utility and limitations	Method (s) used in the Gumba study and rationale
<b>Bone phenotype (BMD, BMC, BA and SA-BMC)</b>	Dual Energy X-ray Absorptiometry (DXA)	<ul style="list-style-type: none"> <li>The reference site for diagnosis of osteoporosis (by DXA) is the femoral neck.</li> <li>T-scores used for diagnosis of osteoporosis in postmenopausal women and men aged &gt;50 years.</li> <li>Interpretation of T-scores:  <b>Normal BMD:</b> above -1 SD:  <b>Osteopenia:</b> between -1.0 and -2.5 SD  <b>Osteoporosis:</b> -2.5 SD or lower</li> <li>Z-scores are recommended for premenopausal women, men aged &lt;50 years and children; and the cut-offs are similar T-scores.</li> <li>No reference standards for black Africans living in Africa, children, adolescents and/or pregnant and lactating women.</li> </ul>	<ul style="list-style-type: none"> <li>Measured bone mineral by DXA (the gold standard for assessment of bone mineral) in mothers and babies postpartum.</li> <li>Peripheral quantitative ultrasound (pQCT) equipment was not available in Uganda to assess maternal vBMD.</li> <li>The primary outcome was powered on maternal LS aBMD because both lactation and HIV/ART studies have reported greater losses in BMD at LS vs. other skeletal sites</li> <li>LS has a greater proportion of trabecular bone hence very sensitive to changes in aBMD compared to other skeletal sites.</li> <li>Infant bone mineral (secondary outcome) assessed to investigate potential effects of maternal HIV/ART on bone mineral accretion.</li> </ul> <p>In depth description of the DXA procedures presented in section 4.3</p>
<b>Bone turnover markers</b>	Markers of bone resorption: CTX, NTX, DPD/PYD, OPG, RANKL, hydroxyproline, hydroxylysine, cathepsin K, bone sialoprotein, TRACP5b	<ul style="list-style-type: none"> <li>Produced during bone resorption.</li> <li>The majority have high biologic and analytical variability.</li> <li>CTX and NTX are the most studied and used markers of bone resorption.</li> <li>Many studies on HIV/ART and bone have measured CTX.</li> </ul>	<p>Measured CTX, P1NP and BAP in mothers because:</p> <ul style="list-style-type: none"> <li>IOF/IFCC recommend P1NP and CTX for clinical monitoring of osteoporosis</li> <li>Greater increases in CTX, P1NP and BAP have been reported in HIV-infected persons after initiation of ART.</li> <li>Early increases in CTX and delayed increase in P1NP/BAP reported following ART - proposed as a potential mechanism for bone loss in the first 1-2 years of ART.</li> <li>Increases in bone turnover markers are greater among patients on Tenofovir (TDF)</li> </ul> <p>sample collection and laboratory procedures presented in sections 4.3.1, 4.4.1.2 and 4.4.1.3</p>
	Markers of bone formation: P1NP, P1CP, BAP, Osteocalcin	<ul style="list-style-type: none"> <li>Produced during bone formation.</li> <li>Assay methods for P1NP extensively described in the literature compared to P1CP.</li> <li>BAP is positively correlated with bone formation rate as measured by histomorphometry</li> <li>Osteocalcin is a sensitive marker of bone formation but its use in clinical settings is limited by sample instability, high biological and assay variability.</li> </ul>	

Study Outcome	Standard methods/ analytes/assays	Description, clinical utility and limitations	Method (s) used in the Gumba study and rationale
<b>Vitamin D and calciotropic hormones</b>	25hydroxyvitamin D (25(OH)D) and 1,25 hydroxyvitamin D (1,25(OH) <sub>2</sub> D)	<ul style="list-style-type: none"> <li>▪ Vitamin D is hydroxylated in the liver to form 25(OH)D, and further hydroxylated in the kidneys to form 1,25(OH)<sub>2</sub>D - the active metabolite involved in calcium homeostasis.</li> <li>▪ 25(OH)D is the commonly used indicator of vitamin D status because it has a longer half-life than 1,25(OH)<sub>2</sub>D.</li> <li>▪ 25(OH)D reflects dietary, endogenous sources and circulating supply of Vit D.</li> <li>▪ The Institute of Medicine (IOM) and the UK Scientific Advisory Committee on Nutrition (SACN) recommend serum 25(OH)D above 25 nmol/L to maintain bone health.</li> <li>▪ 25(OH)D measurements differ by assay type and across laboratories, hence implementation of DEQAS (the international vitamin D Quality Assessment Scheme and VDSP (international Vitamin D Standardization Program).</li> <li>▪ EWL participates in both DEQAS and VDSP.</li> </ul>	<p>Measured 25(OH)D in mothers because HIV/ART studies have reported:</p> <ul style="list-style-type: none"> <li>▪ Greater prevalence of serum 25(OH)D &lt;25 nmol/L) in HIV-positive vs negative persons in Northern latitudes.</li> <li>▪ Efavirenz, induces cytochrome P450 enzymes resulting in the accelerated breakdown of 25(OH)D to 1,25(OH)<sub>2</sub>D.</li> <li>▪ TDF is associated with ↓ free 1,25(OH)<sub>2</sub>D causing functional vitamin D deficiency, thought to explain the observed greater loss in aBMD with TDF use</li> </ul> <p>Sample collection and laboratory procedures presented in sections 4.3.1 and 4.4.2.1</p>
	Parathyroid hormone (PTH), Parathyroid hormone related protein (PTHrP) and calcitonin	<ul style="list-style-type: none"> <li>▪ PTH is produced in response to low serum calcium and acts on both bone and the kidneys to restore serum calcium levels to normal.</li> <li>▪ High circulating plasma concentration of PTH, hyperparathyroidism, is associated with increased bone loss.</li> <li>▪ Measurement of PTH is important in clinical diagnosis of disorders in calcium metabolism (hypercalcemia and hypocalcemia).</li> <li>▪ Renal impairment, vitamin D deficiency, low calcium intake and medication are among the causes of hyperparathyroidism.</li> <li>▪ PTH activity is suppressed in pregnancy and early lactation, its role in calcium homeostasis taken over by PTHrP.</li> <li>▪ PTHrP is produced by the mammary glands and released into the maternal blood and breastmilk. It can activate both the PTH/PTHrP receptors and renal 1,25(OH)<sub>2</sub>D production.</li> <li>▪ The role of PTHrP in human pregnancy and lactation is not fully understood because its biology is complex and the laboratory assays pose several methodological challenges.</li> <li>▪ Calcitonin is released in response to high plasma calcium and acts on both bone and kidneys to restore serum calcium levels to normal - acts opposite to the effects of PTH.</li> </ul>	<p>Measured PTH in maternal plasma because:</p> <ul style="list-style-type: none"> <li>▪ Higher PTH concentrations have been reported among HIV-positive persons.</li> <li>▪ Greater increases in PTH following initiation of ART in longitudinal studies of HIV-infected persons, increases are greater with TDF-based ART and thought to explain greater bone loss.</li> <li>▪ Both EFV and TDF are preferred drugs in the current Option B-plus ART regimen for pregnant and lactating women.</li> <li>▪ Calcitonin was not measured because its importance in humans has not been well established.</li> <li>▪ Sample collection and laboratory procedures presented in sections 4.3.1 and 4.4.2.2</li> </ul>

Study Outcome	Standard methods/ analytes/assays	Description, clinical utility and limitations	Method (s) used in the Gumba study and rationale
<b>Plasma and urine clinical biochemistry</b>	Plasma albumin, Total alkaline phosphatase (ATP); plasma and urine creatinine, calcium, phosphate and magnesium	<ul style="list-style-type: none"> <li>Measurement of plasma and urine mineral concentrations of Ca, PO<sub>4</sub> and Mg are important in diagnosis of disorders of bone mineral metabolism.</li> <li>A change in plasma concentrations outside the normal range affects both bone mineral mobilisation and renal reabsorption for the particular mineral.</li> <li>Plasma concentrations of plasma and mineral urine concentrations form the basis for calculation of indices assessing renal function and mineral handling.</li> </ul>	<p>Measured in the Gumba study to:</p> <ul style="list-style-type: none"> <li>Investigate HIV/ART related disturbances in plasma mineral concentrations and urinary excretion.</li> </ul> <p>Sample collection and laboratory procedures presented in sections 4.3.1 and 4.4.3</p>
<b>Markers of renal function, tubular damage and mineral handling</b>	Glomerular Filtration Rate (GFR), proteinuria and markers of renal tubular damage (RTD)	<ul style="list-style-type: none"> <li>Assessment of GFR is important in diagnosis of chronic kidney disease (CKD) - end stage CKD is renal failure.</li> <li>Renal tubular damage (RTD) is characterised by proteinuria (increased urinary excretion of proteins).</li> <li>Clinical diagnosis of CKD is based on serum creatinine (or Cystatin C) - based estimated GFR (eGFR) and/or presence of RTD</li> <li>eGFR &lt;60 ml/min/1.73 m<sup>2</sup> for ≥3 months classified as CKD, with/without RTD.</li> <li>HIV-positive patients may have renal disease diagnosed by RTD in absence of altered eGFR.</li> </ul>	<p>Measured eGFR and proteinuria because:</p> <ul style="list-style-type: none"> <li>Lower eGFR has been reported among HIV-infected adults.</li> <li>Faster decline in eGFR and progression to end stage CKD in HIV-positive adults.</li> <li>TDF-based ART associated with greater decline in renal function and renal tubular damage</li> <li>Specific proteins markers of RTD, were not measured because they are not routinely used in clinical practice.</li> <li>Calculation of eGFR presented in section 4.4.4.1</li> </ul>
	Markers of renal mineral handling: TmP/GFR and TmCa/GFR	<ul style="list-style-type: none"> <li>Renal tubular maxima for reabsorption of PO<sub>4</sub> (TmP) and calcium (TmCa) per unit volume of glomerular filtrate (GFR), measure the maximum rate of renal PO<sub>4</sub> and Ca (respectively) reabsorption.</li> <li>TmP/GFR and TmCa/GFR are used in diagnosis of disturbances in PO<sub>4</sub> and Ca homeostasis</li> <li>These indices are not affected by the release of PO<sub>4</sub>/Ca from bones, gut and other cells into the extracellular space.</li> <li>TmP/GFR is the important index of renal PO<sub>4</sub> handling and has replaced indices described in earlier literature.</li> </ul>	<p>Both TmP/GFR and TmCa/GFR were measured because:</p> <ul style="list-style-type: none"> <li>HIV/ART, especially TDF is associated with decline in renal function which might compromise renal phosphate and calcium handling.</li> <li>TDF is associated with renal phosphate wasting – one of the proposed mechanisms for greater bone loss with TDF use.</li> </ul> <p>More information and equations presented in sections 4.4.4.2 and 4.4.4.3</p>

Study Outcome	Standard methods/ analytes/assays	Description, clinical utility and limitations	Method (s) used in the Gumba study and rationale
<b>Breastmilk mineral content</b>	Breastmilk Ca and PO <sub>4</sub>	<ul style="list-style-type: none"> <li>Breastmilk (BM) Ca and PO<sub>4</sub> are important for infant skeletal growth.</li> <li>Maternal bone resorption occurs during lactation to supply Ca and PO<sub>4</sub> to the infant through BM.</li> <li>Predominantly breastfed infants depend on maternal supply of Ca and PO<sub>4</sub> through BM.</li> </ul>	<p>Measured BM Ca, phosphorus (P), Na and K because:</p> <ul style="list-style-type: none"> <li>There are no data on effects of maternal HIV/ART on BM Ca and PO<sub>4</sub> – but biologically plausible to speculate that disturbances in maternal Ca/PO<sub>4</sub> homeostasis following initiation of ART might alter BM composition, affecting infant growth.</li> <li>Measured breastmilk Na and K to identify SCM – which can cause leakage of plasma Ca, PO<sub>4</sub> into BM.</li> </ul>
	Breastmilk sodium (Na) and potassium (K).	<ul style="list-style-type: none"> <li>Raised BM Na/K ratio (&gt;1.0) is an indicator of subclinical mastitis (SCM).</li> <li>SCM causes opening of the paracellular pathways within the breast causing leakage of blood components into BM.</li> <li>SCM is associated with poor infant growth.</li> </ul>	<p>Laboratory methods for BM are described in section 4.4.5</p>
<b>Anthropometry</b>	Weight, height, mid-upper-arm and head circumferences, waist-hip ratio, skinfold thickness	<ul style="list-style-type: none"> <li>Anthropometry is the science of measuring human body size, weight and proportions.</li> <li>Important for clinical diagnosis and management for several disease conditions.</li> <li>Weight and height or length (the most common and basic anthropometry) measure body size.</li> <li>Hip and waist circumferences, BMI and skin fold thicknesses are used as proxy measures of body composition in adults.</li> <li>In children, weight, length, head circumference are used to assess growth for a given age and sex.</li> <li>Mid upper-arm-circumference (MUAC) is used to assess acute malnutrition (wasting) in both adults and children. Low MUAC associated with increased risk of mortality.</li> </ul>	<ul style="list-style-type: none"> <li>Measured weight and height/length in mothers and babies to adjust DXA measurements for body size.</li> <li>This is because BMD and BMC are highly dependent on body size, and changes in weight</li> <li>Infant anthropometry measurements were used to generate infant growth indices.</li> <li>Skinfold thickness and waist hip ratio) were not measured because body composition was estimated by DXA.</li> </ul> <p>Anthropometric procedures presented in section 4.2</p>
<b>Infant somatic growth</b>	Z-scores based on child growth reference standards: WHO	<ul style="list-style-type: none"> <li>Several international reference standards are available for monitoring fetal and postpartum growth in children – WHO, intergrowth and Fenton charts.</li> <li>WHO standards are derived from a multicentre study of breastfed infants and are the most widely used.</li> <li>Common growth indices are: weight-for-age, length/height-for age, weight-for height, BMI-for age, and growth velocity Z-scores.</li> <li>Uganda does not have its own growth standards. WHO standards are routinely used in the national growth monitoring program and clinical care.</li> </ul>	<p>Growth Z-scores based on WHO 2006 reference standards were used to investigate the potential effect of maternal ART on somatic growth of babies because:</p> <ul style="list-style-type: none"> <li>Many studies on maternal HIV/ART have reported poor growth among HIV-exposed infants compared to HIV-unexposed.</li> <li>To my best knowledge, there are no published papers containing both data on skeletal and somatic growth beyond the neonatal period in the context of maternal HIV/ART.</li> <li>Procedures for growth assessment are presented in section 4.1.2</li> </ul>

Potential confounder	Standard methods/ analytes/assays	Description, clinical utility and limitations	Method (s) used in the Gumba study and rationale
Dietary intake	Directly observed/real time weighed food diaries	<ul style="list-style-type: none"> <li>Observed weighed food diaries provides more accurate estimate of dietary intake, but difficult to implement in large studies.</li> </ul>	Not used in the Gumba study. Not feasible to implement for 200 participants scattered around Kampala city.
	Self-reports: weighed food records (WFR), food diaries, 24-hour recall, Food frequency questionnaire (FFQ), Dietary Diversity questionnaires (DDQ).	<ul style="list-style-type: none"> <li>Methods based on self-reports are commonly used because they are easier to implement, but are subject to recall bias and literacy level (WFR).</li> <li>FFQ provides an estimate of usual dietary intake (qualitative or quantitative) and is the most commonly used method in large epidemiological studies.</li> <li>Very important to use culturally appropriate methods and regional food composition tables</li> <li>FFQ and food diaries are available to assess calcium intakes.</li> <li>DDQ developed by FAO to assess household or individual food security and micronutrient intake (Fe &amp; Vit A) in resource limited settings.</li> <li>Newer methods, using web-based and smart phone applications, have been developed but these may not be applicable in resource limited settings.</li> <li>More sophisticated approaches (e.g. dietary patterns) and computer software are also available to analyse dietary data.</li> </ul>	<p>Calcium FFQ as used in Gambian studies was used to collect data on consumption of Ca and Vit D rich foods in mothers, to account for potential confounding when comparing BMD measurements between the groups.</p> <p>More information presented in section 4.5 and appendices (6, 7 and 8).</p>
Breastmilk volume and breastfeeding practices	Test-weighing and deuterium oxide dose-to-the-mother method (DTTM)	<ul style="list-style-type: none"> <li>Exclusively breastfed infants rely on breastmilk to meet all their nutritional needs.</li> <li>Infant size is positively correlated with volume of breastmilk consumed.</li> <li>Breastmilk volume is a major determinant of the lactation bone loss at the spine.</li> <li>Test-weighing method quantifies infant breastmilk intake by weighing the baby before and after each feed, but the method interferes with the breastfeeding routine.</li> <li>DTTM measures integrated infant BM intake over 14 days after administration of deuterium oxide to the mother and daily collections of urine or saliva from mother and baby.</li> <li>DTTM is safe and does not interfere with lifestyle or breastfeeding.</li> </ul>	<ul style="list-style-type: none"> <li>DDTM was planned but a decision was made not to implement as it would have overburdened participants and overstretched study staff, compromising the main study.</li> <li>Data on breastfeeding practices was incorporated into the dietary questionnaire (section 4.5 and appendices (6, 7 and 8).</li> </ul>
Other confounders	Demographic and socio-economic characteristics	<ul style="list-style-type: none"> <li>Potential confounders of both the primary and secondary study outcomes.</li> <li>For example, primiparity is associated with greater bone loss compared to multiparity.</li> </ul>	<p>Data were collected using questionnaires.</p> <p>More information presented in sections 4.5 and appendices (6, 7 and 8).</p>
	Medical history and physical activity	<ul style="list-style-type: none"> <li>History of illness (especially being bedridden) may affect bone loss and vitamin D status.</li> <li>Some disease conditions (e.g. diabetes), drugs and contraception may affect bone.</li> </ul>	
	Sunshine exposure	<ul style="list-style-type: none"> <li>Sunshine exposure (UVB) affects dermal synthesis of vitamin D, and vitamin D status.</li> </ul>	

## 4.2. Anthropometry

Anthropometry is the science of measuring human body size, weight and proportions. In the Gumba study, anthropometric measurements were done in mothers at PG36 and in mother-baby pairs at PP2 and PP14. All measurements were done by trained research assistants using standard clinical equipment according to SOPs in place.

### 4.2.1. Mothers

Height (cm) and weight (kg) were measured at every study visit using a digital weighing scale and stadiometer set (SECA 360; manufacturer SECA GmbH, Hamburg Germany). The equipment was calibrated daily using standard procedures at Baylor-Uganda.

Participants were measured in light clothing, without footwear and standing upright on the scale (Figure 23). Weight was measured to the nearest 0.1kg. Height was measured to the nearest 0.1 cm with the participant standing upright and without footwear. The mothers were positioned with the heels and shoulders against the back of the stadiometer keeping the back and knees straight with the Frankfort plane in the horizontal position. Irremovable hair extensions were gently compressed as close to the skull as possible, and note was made on the questionnaire alongside the measurement. Mean height based on measurements at PP2 and PP14, was used in all statistical models. This is because significant increases in height were not anticipated during the study duration. Body mass index (BMI) was calculated using the equation:  $BMI (kg/cm^2) = \text{Body weight}/(\text{height}^2)$ .

UNICEF approved MUAC tapes were used for pregnant and breastfeeding women to assess for malnutrition. As per the national guidelines, pregnancy and lactating (< 6 months postpartum) are classified as malnourished if they have MUAC < 22cm.

### 4.2.2. Infants

Weight, recumbent length and head circumference were obtained for all infants at every visit to compare growth outcomes between HIV-exposed and HIV-unexposed infants. Infants were weighed without clothes (or in light clothing during the rainy/cold season) using a digital infant weighing scale (SECA 374, manufacturer SECA GmbH, Hamburg Germany). The scale was calibrated daily using standard procedures at Baylor-Uganda. Recumbent length was measured using a standard wooden length board supplied by UNICEF. Standard tapes (SECA 212; manufacturer SECA GmbH, Hamburg Germany) were used to measure the head circumference.



Z-scores for growth indices (height-for-age, weight-for-age, weight-for-height and head circumference-for-age, Z-scores) were generated based on WHO 2010 growth standards in WHO Anthro software plugin for STATA. Six implausible head circumference measurements were flagged by the Anthro software and were excluded from data analysis.



*Figure 23: Volunteers undergoing anthropometric measurements.*

From top clockwise: study nurse taking maternal height and weight, head circumference of an infant, maternal MUAC and weight of an infant.

Photo credit: Lilian Birungi



### **4.3. Assessment of Bone mineral and body composition by DXA**

#### *4.3.1. Scan acquisition*

A Dual Energy X-ray Absorptiometer (DXA) scanner at MUJHU was used to assess bone mineral and body composition of mothers and infants (Model: Discovery W by Hologic, Inc., Waltham, MA, USA). Hologic QDR 4500A is a fan beam scanner which uses a broad X-ray beam and multiple detectors to measure the attenuation in a row of pixels in a 2D image. The DXA scanner automatically detects and separates the bone edges from soft tissue within the image, using the generated attenuation profiles based on inbuilt assumptions, and measures the total bone area (BA) in cm<sup>2</sup>. The DXA software then calculates BMC in grams and aBMD in g/cm<sup>2</sup> with the defined area (BA). Whole body DXA scans also measured total body fat and lean mass (g).

The study described in this thesis measured bone area (BA), bone mineral content (BMC), and areal bone mineral density (aBMD) of the whole body, total hip (TH) and lumbar spine (LS, L1-L4). All scans were performed by experienced and certified DXA operators engaged by MUJHU trained by the Hologic team in South Africa. The operators had previously performed scans for international clinical trials (VOICE and PROMISE) and for other studies. At the beginning of the study, I also trained the two DXA operators on the study protocol.

All women were offered a pregnancy test before referral for DXA scans after 2 weeks postpartum in line with the prevailing DXA standards of operation at MUJHU. A study nurse stationed at Baylor-Uganda completed a DXA referral form for all infants and non-pregnant women before they were escorted to MUJHU (referral forms attached in appendix 8). At the MUJHU DXA facility, chaperones helped the participants to remove any metallic accessories, including bras and jewellery and change into light cotton gowns. Prior to scanning the subject's details were checked by asking the participant to verbally confirm some of the details on the referral form (name, date of birth and residence); this was to ensure the correct individual was scanned. The participant's details (study number, initials, visit, weight and height) were then entered into the DXA software as per the referral form.

The participants were positioned on the DXA scanner according to manufacturer's instructions and SOPs in place. A set of three scans (whole body, hip and lumbar spine) were acquired in mothers, and two scans (whole body and lumbar spine) were acquired in infants at PP2 and PP14. Scans were performed in the default fast array mode for the majority of the participants. The selected scanning mode was maintained for each participant at all visits. The MUJHU Radiation Safety Committee

provided overall supervision for the conduct of DXA scans. The amount of radiation exposure from DXA is very low and much less compared to normal background radiation. The radiation delivered to participants in the whole study was about 0.0335mSv for mothers and 0.03mSv for infants. These radiation doses (although the study doses do not include natural background radiation received by individuals from natural sources like the sun, air, food, soil and from outer space, were much lower than the annual limit of 1mSv set for the public. The global annual background radiation is approximately 2.4mSv (Damilakis *et al.*, 2010). Uganda's average is unknown but, it is likely to be close to the global average. Therefore, the radiation doses for a set of scans in both mothers and infants were equivalent to approximately one day of exposure to natural background radiation in Uganda and much less than the annual radiation exposure.

#### *4.3.1.1. Maternal whole body scan*

The whole body scan was obtained with the participant lying on her back and looking at the ceiling while remaining still. The body was positioned straight within the scan field (outer lines on the DXA table, see photo) using the centre lines at the head and feet ends of the table. The arms were separated from the sides of the body with the hands placed palm down, within the scan field. Where the hands did not fit in the scan field, they were tucked under the thighs. The DXA operator verified that the participant was properly positioned before starting the scanner. If during scanning it was apparent that part of the participant's body was outside the scan field, the participant was repositioned, and the scan restarted.



*Figure 24: A volunteer undergoing a whole body DXA scan*  
*Photo credit: Florence Nabwire*

#### *4.3.1.2. Maternal lumbar spine scan*

The lumbar spine scan was acquired with the participant lying straight on her back with the spine centred on the table and shoulders at the upper scan limit line. The shoulders and pelvis were also aligned in a straight line based on centre line on the scan table. The knee positioning block was then placed under the participant's legs and moved inwards until the femurs were parallel with the edge of the block creating a 45 - 90° angle. The participant was requested to place her arms by her sides, remain still and to breathe normally during scanning.



*Figure 25: A volunteer undergoing a lumbar spine DXA scan  
Photo credit: Florence Nabwire*

#### *4.3.1.3. Maternal total hip scan*

The left hip scan was acquired with the participant lying on her back aligned on the centre line midline and with the foot positioner between their ankles. The hip was internally rotated 25° and the inner part of the foot placed against the triangle on the foot positioner. The feet were held in backward flexion and secured on the foot brace with a strap. The femur was aligned parallel to the edge of scan table for correct positioning of the neck box for analysis. The leg was then moved away from the midline in order to straighten the femur. The laser light guide from the scanner was positioned over the middle of the left thigh below the level of the greater trochanter, and the machine carried out the measurement.



*Figure 26: A volunteer undergoing a total hip DXA scan  
Photo credit: Florence Nabwire*

#### *4.3.1.4. Infant whole body and lumbar spine scans*

Infant whole body and lumbar spine DXA scans were performed without sedation while the babies were sleeping. The babies were breastfed and sometimes bathed to help them sleep before scanning. Both lumbar spine and whole body scans were acquired with the infant in a supine position. Manufacturer's guidelines and MUJHU SOPs on safety and positioning of infants on the scanner were observed. DXA scans were acquired using the automated infant whole-body and lumbar spine scanning modes (Apex software version 12.3.3; Hologic Inc.). Babies who woke up partway through the scanning were taken off the table, breastfed and soothed to sleep before a second attempt. If the second attempt failed, the participants were rescheduled for a repeat scan within 1 week. A maximum of three tries was attempted to acquire scans at each site.





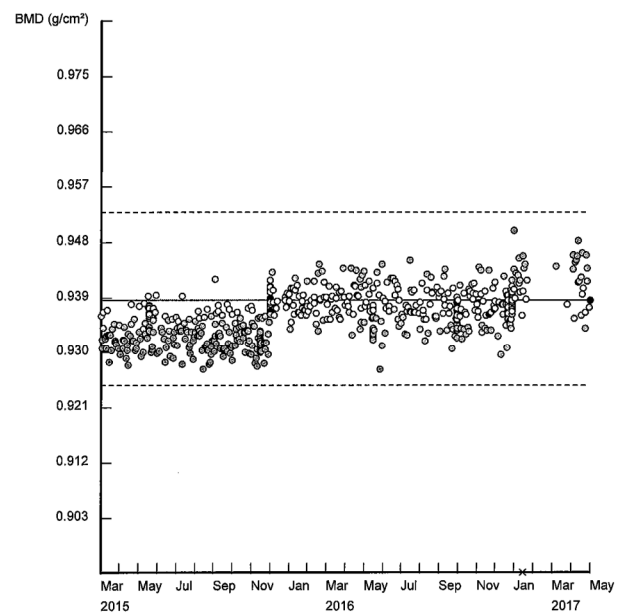
*Figure 27: A baby being soothed to sleep before a DXA scan  
Photo credit: Florence Nabwire*



*Figure 28: An infant undergoing whole body DXA scan in the supine position.  
Photo credit: Florence Nabwire*

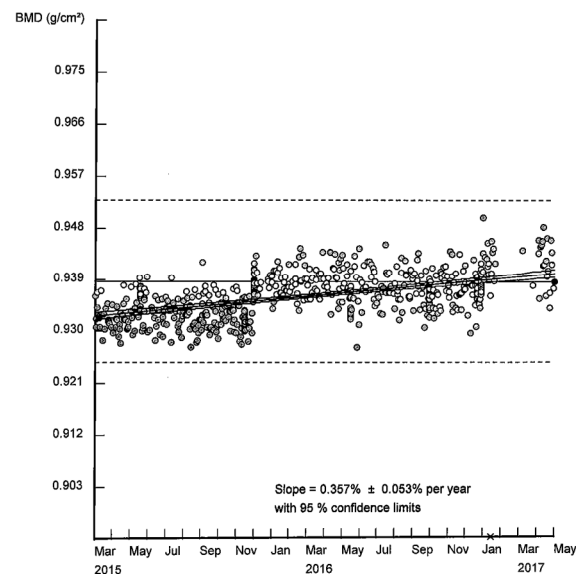
#### *4.3.2. Quality control for DXA scans*

Daily phantom calibration procedures were performed throughout the study before participants were scanned. Periodic maintenance of the machines was done by the Hologic team based in South Africa. The long term DXA stability for BMD measurements during the study was  $\pm 0.5\%$  (Figure 29). The allowed range for long term stability on Hologic densitometers is  $\pm 1.5\%$  (Lenchik *et al.*, 2002, Wilson and Smith, 2009).



Setup	Reference Values	Plot Statistics
a Lumbar Spine phantom #22315 System S/N: 84650	Limits: $\pm 1.5\%$ of mean Mean: 0.939 (g/cm <sup>3</sup> ) SD: 0.002 (g/cm <sup>3</sup> )	Number of Points: 610 Mean: 0.936 (g/cm <sup>3</sup> ) SD: 0.004 (g/cm <sup>3</sup> ) CV: 0.466 %

HOLOGIC®



Setup	Reference Values	Plot Statistics
a Lumbar Spine phantom #22315 System S/N: 84650	Limits: $\pm 1.5\%$ of mean Mean: 0.939 (g/cm <sup>3</sup> ) SD: 0.002 (g/cm <sup>3</sup> )	Number of Points: 610 Mean: 0.936 (g/cm <sup>3</sup> ) SD: 0.004 (g/cm <sup>3</sup> ) CV: 0.466 %

HOLOGIC®

Figure 29: Hologic DXA long term aBMD stability chart (Mar 2015-May 2017)

#### *4.3.3. Grading and evaluation of scans*

All the DXA images (~2000 images) were transferred to EWL. Hologic Apex software was purchased to enable grading and analysis of DXA images. One research assistant (L.O., experienced in scanning, grading and analysis of Lunar DXA scans for the Nutrition and Bone Health [NBH] group), one placement student (C.F) and I were trained on grading and analysis of Hologic scans by Dr Kate Ward (senior research scientist in NBH group at MRC EWL experienced in bone imaging). According to the standard procedures in NBH group, each scan is scored on a scale of 1-3 for both acquisition (quality of the image) and scan analysis. At the end of the training, each of us independently graded and analysed 20 scans according to an agreed-upon scoring system. When the results were compared, there was no significant difference. See sections 4.2.3.1 and 4.3.2.2. for descriptions of grading systems used.

For consistency, the placement student graded all maternal whole body scans for mothers and infants. The research assistant graded all maternal LS, TH and infant LS scans, and also provided day to day supervision for the placement student. Dr Ward provided overall technical guidance, and I supervised the whole exercise. After all scans were graded, I randomly checked 10 scans for each skeletal site for quality assurance. I then exported the data into access, organised into visits, cleaned the data prior to statistical analysis.

##### 4.3.3.1. Grading the quality of scans

The standard practice in NBH group at EWL is to grade each scan on a scale of 1-3 based on ability to extract valid data from the scan. The score takes into account the positioning of the subject and artefacts in the scan caused by movement, clothing and jewellery or the presence of metallic joint material. The grading scale for scan acquisition is presented below:

Grade 1: perfect scans, unaffected by the above considerations.

Grade 2: scans affected by one or more of the considerations above but the effect was judged less likely to affect the resultant data. Other reasons to grade a scan as a grade 2 included arms tucked into the fat tissue around the hips for bigger women, lumbar spine not straight, toes cut off from the whole body scan or for infant scans overlap of the hind and forearm in whole body scan.

Grade 3: scans in which one or more of the above considerations were deemed to have affected the scan quality and the ability to generate valid data.

All grade 3 scans were excluded from the reported DXA data. One maternal LS scan (PP14) and three WBLH scans (1 at PP2, and 2 at PP14) were grade 3. Two women with short femoral necks were not excluded because the study was interested in total hip bone mineral and not the femoral neck. For infants, one LS scan (PP2) and 24 WB scans (14 at PP2 and 10 at PP14) were grade 3.

#### 4.3.3.2. Scan analysis

Each scan was assigned an analysis grade from 1-3 as per standard practice in NBH group. Analysis grade was based on clarity of the scan image, ability to position lines for the region (s) of interest, movement and detection of bone edges in the scan. Where necessary, scans were reanalysed before grading, and this was noted on the grading sheet. Below is the grading scale for scan analysis.

Grade 1: scans had little or no movement.

Grade 2: Scans in which movement was discernible but not considered extensive enough to affect data validity.

Grade 3: Scans in which movement by the subject was deemed to have substantially affected the ability to extract valid data.

Hairstyles containing artificial hair extensions (braids and weaves) were common among women and also changed from visit to visit. The hair extensions could not be removed before scanning. The density of artificial hair is similar to soft tissue and thus anticipated to affect whole body DXA measurements in the head region. Therefore, all maternal whole body DXA measurements were analysed “less head” and are reported as whole body-less-head (WBLH). Hamill et al. used a similar approach in urban South African women (Hamill, 2013, Hamill et al., 2017).



## **4.4. Collection and handling of biological samples**

### *4.4.1. Two-hour fasted blood and urine collection in mothers*

The two-hour fasting blood and urine collection protocol were followed for mothers to assess renal phosphate handling (Nordin and Smith, 1965), one of the mechanisms implicated in bone loss among HIV-infected persons on TDF-based ART. The samples were also used for assays of other markers of bone metabolism. This protocol has been routinely used in NBH's studies in The Gambia, Bangladesh, China and UK. Sample collection form attached in Appendix 9.

Women came for the study visit in the morning after overnight fasting for at least eight hours. On arrival at the clinic, the mother was asked to void their bladder at the start of the two hours and then given 500ml of clean potable water. The participant was instructed to drink all the water within the two-hours of fasting to facilitate adequate urine production. At one hour into the fasting protocol, a trained phlebotomist collected 20ml of venous blood from the arm into appropriate blood tubes using standard procedures. Blood collection tubes (manufacturer BD) were used, and the following recommended order of draw was observed: serum separating tube with the yellow top (SST, 4ml), lithium heparin with green top (LH, 12ml), EDTA with the purple top (EDTA, 4ml).

At the end of the two hours, the participant was asked to completely empty their bladder into a disposable 500ml specimen bucket (Polypropylene + Lid White). The specimen buckets were supplied by Scientific Laboratory Supplies (SLS) the UK and were disposable/single use to avoid cross-contamination of samples, and also then avoided the need to acid wash the containers to remove contamination by calcium and phosphate residues. Participants were discouraged from emptying their bladder partway through the protocol unless it was absolutely necessary to ensure they had a urine sample at the end of two hours. Those who needed to empty their bladders partway during the two hours were asked to collect the urine and also asked to empty their bladder at the end of two hours. The total volume of urine produced over the two hours was thoroughly mixed and measured. The participant was offered breakfast after completing the two hours-fasting protocol.

#### *4.4.2. Collection of blood from infants*

Non-fasted venous blood samples were collected in infants to analyse markers of growth and bone development. A trained phlebotomist collected 2 ml of venous blood from the arm into a 2ml BD Plain Serum Tube (PST, red top) as per the study protocol. Approved phlebotomy procedures for infants were followed as per the Baylor-Uganda laboratory SOPs in place. The mothers were encouraged to breastfeed their infant during phlebotomy.

#### *4.4.3. Handling and processing of blood samples*

The urine and blood samples (EDTA and lithium Heparin) were kept in a cool box with ice packs and transferred to the laboratory where they were held in the fridge at 8-12°C while awaiting processing. Blood samples collected in SST and PST tubes were kept at room temperature for at least one hour after collection for maximum clotting before processing. All blood samples were centrifuged within two-hours of the collection at 4°C/3000rpm. Disposable pipette tips were used to prepare aliquots into free-standing screw cap microtubes (manufactured by Axygen and supplied by SLS, UK). Washed red blood cell pellets were harvested from the EDTA blood sample at PG36 and PP14.

The following aliquots were prepared: Serum (1.0ml aliquots, two aliquots from the maternal sample and one aliquot from infant sample), lithium heparin plasma (four aliquots of 1.5ml), EDTA plasma (two aliquots of 1.0ml), and washed blood cells (one aliquot of 1.0ml). The plasma and serum aliquots were stored at -80°C and washed red cells at -20 °C. All the samples were stored at these temperatures until the time of shipping on dry ice to EWL for analysis (sample processing and storage form attached in appendix 11).

#### *4.4.4. Handling and processing of 2-hour urine samples*

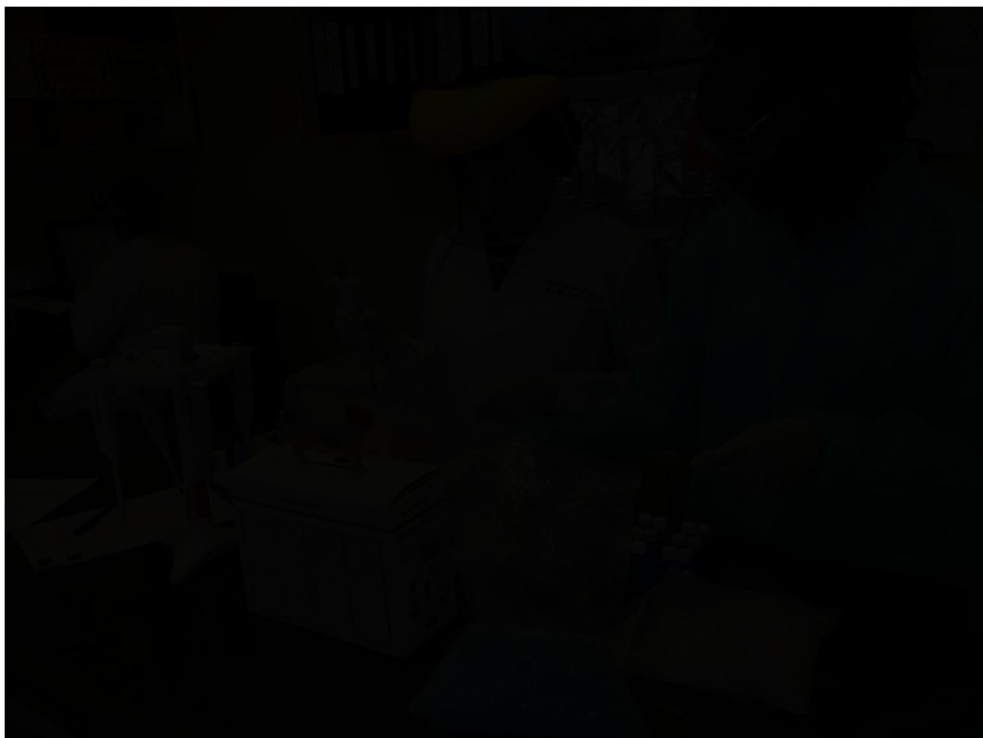
The 2-hour fasting urine samples were kept in a cool box with ice packs at the collection site. They were later transferred to the laboratory and held at 8-12°C in the fridge while awaiting processing. All urine samples were processed within 3 hours after collection. The urine in 500ml specimen buckets was swirled to mix before opening the container. In instances where the participant collected more than one bucket of urine, the urines were mixed before preparing aliquots.

Four 5ml aliquots were prepared into plain blood tubes (5ml Sterillin Blood Tube PP with Label, supplied by SLS, CAT No. TUB0032 and TUB0012). Disposable pipette tips were used to prepare the aliquots. Two of the aliquots were immediately capped and stored un-acidified. The other two aliquots were acidified with 1 drop (50µl) each of 37% concentrated hydrochloric acid (SIGMA,

Specific gravity 1.18; 12 Molar) and capped. All the urine aliquots were stored at -80C until shipping to EWL for analysis.

Standard urinalysis tests were performed using dipsticks on the leftover urine samples in the specimen bucket after preparing aliquots. This was to screen for urinary tract infections. Diagnosis of a UTI was based on the presence of both leucocytes and greater than five pus cells per high power field (hpf). All participants with abnormal urinalysis results were referred to antenatal medical staff for appropriate treatment and further investigations. Women diagnosed with UTIs received appropriate treatment, and the study paid for the medication prescribed by the antenatal staff. The 8 women who had protein in the urine, in the absence of a UTI, had their BP checked and hypertension/pre-eclampsia was ruled out. One woman whose urine contained glucose had a glucose tolerance test, and diabetes was ruled out.

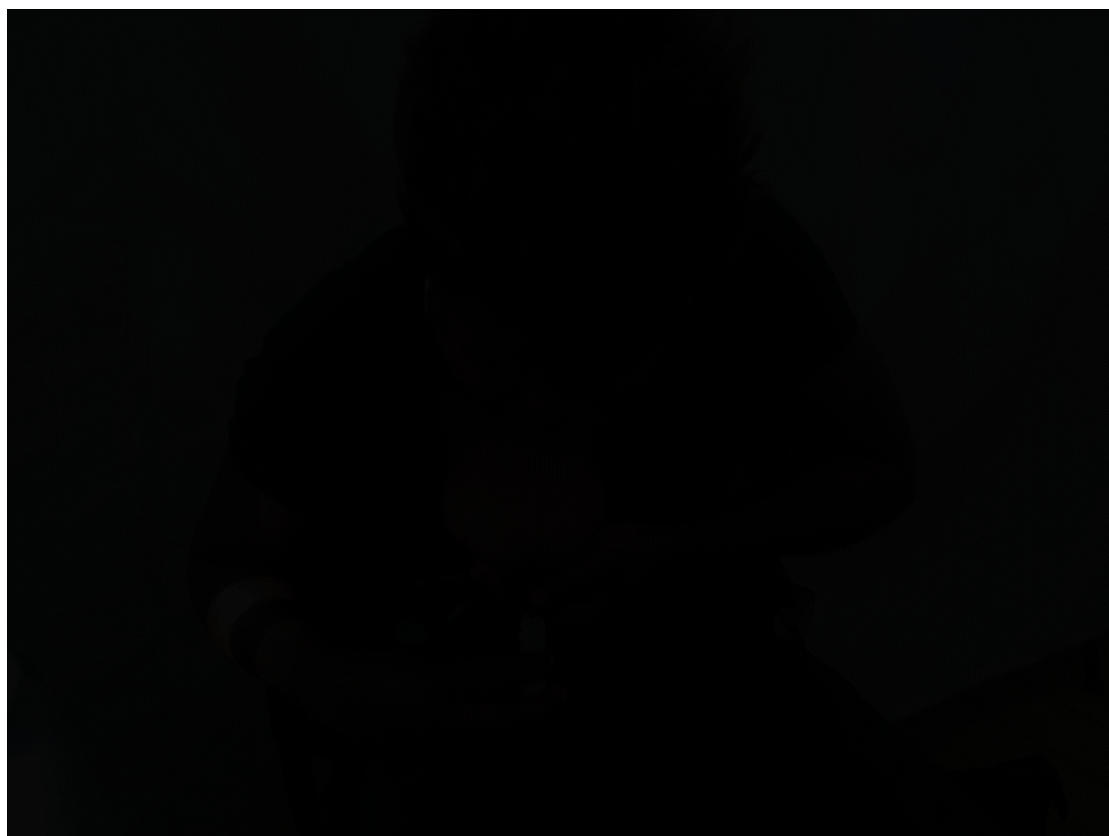
The total volume of urine left in the specimen bucket was then measured using a 500ml measuring cylinder and recorded. Then, 20ml (for aliquots) was added to the volume to obtain the final total volume of urine that was recorded for the participant. The urine in the specimen bucket was discarded and the container disposed of as per the laboratory standards of operation. The measuring cylinders were cleaned according to laboratory infection control procedures.



*Figure 30: Laboratory staff and I (in green lab coat) processing biological samples  
Photo credit: Dr Gail Goldberg*

#### *4.4.5. Collection and handling of breastmilk*

Breastfeeding mothers were requested to hand express 10ml (5ml from each breast) into plain blood tubes (5ml Sterillin Blood Tube PP with Label, supplied by SLS, CAT No. TUB0032 and TUB0012) (appendix 10). Prior to the procedure, the women were taught how to hand express breast milk and supported to do so where necessary. The breast milk samples were kept in a cool box with ice packs while awaiting transfer to the laboratory at Baylor-COE. The samples were stored at -80C while awaiting shipping for analysis at MRC EWL in Cambridge, UK.



*Figure 31: A volunteer hand expressing breastmilk into a tube*

*Photo credit: Lilian Birungi*

#### 4.5. Laboratory methods at EWL in Cambridge

All biological samples were airfreighted to the UK on dry ice and delivered to MRC EWL in Cambridge. All samples were received in good condition and stored at the same temperature as in Uganda. Laboratory assays were carried by trained staff in the NBH and Nutritional BioAssays (NBA) laboratories at EWL (previously HNR).

EWL places a strong emphasis on assuring the quality of its research activities. Quality assurance is governed by local management systems and external audit of specific research activities where required. There is a Quality Management System, certified to ISO 9001:2008 (since January 2006), which is maintained to control and monitor performance and to continually improve effectiveness. HNR is externally audited twice annually by Lloyd's Register Quality Assurance to ensure compliance with the requirements of ISO 9001:2008. The laboratories at EWL are accredited by both UK National External Quality Assessment Scheme (NEQAS) and Vitamin D External Quality Assessment Scheme (DEQAS).

All laboratory assays were measured using standard methods, and relevant quality control samples/materials were used. All the assays were within the specified range, and the mean intra-assay variation was 4% for all assays (a sample QC plot is presented in Figure 32).

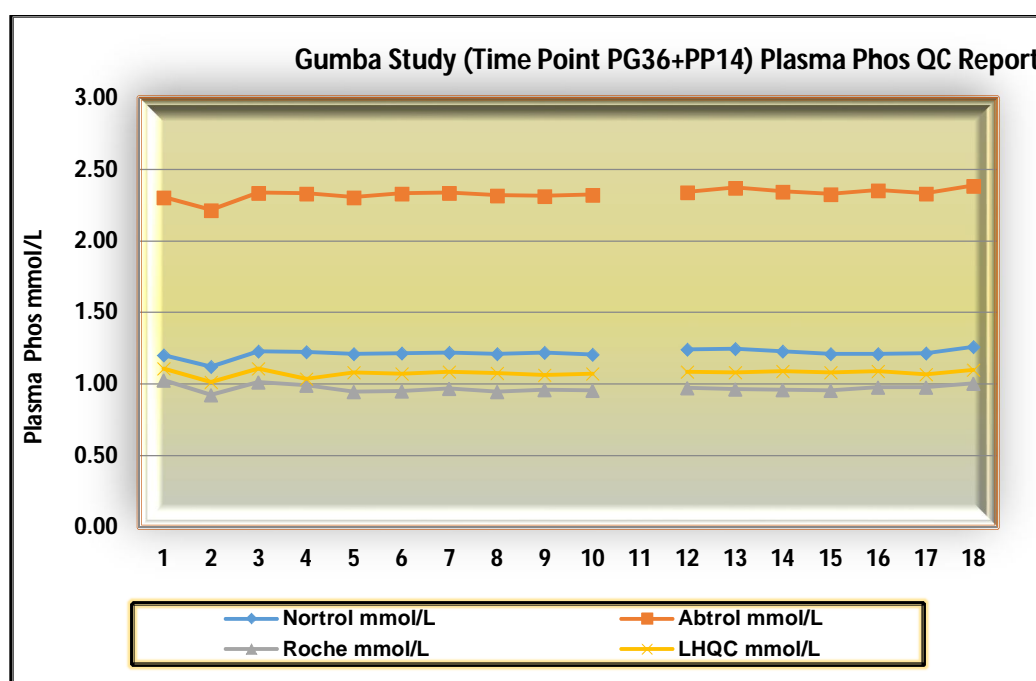


Figure 32: Laboratory QC data for plasma phosphate  
The plots represent commercial controls (Nortrol, Abtrol and Roche) and NBH lab internal control sample (LHQC).

#### 4.5.1. Bone turnover markers (BTM)

##### 4.5.1.1 CTX

Type I collagen accounts for >90% of the organic bone matrix and is synthesised primarily in bone. C-terminal cross-linked telopeptide of collagen I (CTX) is a peptide fragment generated by collagen degradation during bone renewal and thus an important marker of bone resorption. CTX is highly affected by circadian rhythm, but the variation is reduced when samples are collected in a fasting state (Ju *et al.*, 1997, Christgau, 2000). It can be measured in serum or 24hr urine. However, measurement in serum is preferred because it avoids the variation introduced by correction for creatinine excretion giving better reproducibility of the results (Hlaing and Compston, 2014).

In the Gumba study, CTX was assayed using the IDS iSyS platform (Immunodiagnosics Systems Ltd, Tyne and Wear, UK). The samples were analysed as singletons. The assay is based on chemiluminescence technology utilising two highly specific monoclonal antibodies. The antibodies are against the amino acid sequence of EKAHD- $\beta$ -GGR where the aspartic acid residue is (D)  $\beta$ -isomerised. Two chains of EKAHD- $\beta$ -GGR must be cross-linked in order to obtain a specific signal in the assay.

##### 4.5.1.2. P1NP

Procollagen type 1 N-terminal propeptide (P1NP) is the amino terminal of type I collagen formed outside the cell by proteolytic cleavage. Type 1 collagen is initially synthesised as type 1 procollagen and constitutes 90% of bone proteins. P1NP is the most specific and sensitive marker of bone formation. It circulates in blood as the intact original trimeric P1NP monomer and dimer or as one of several fragments. Intact P1NP has a half-life of 10 hours and is very unstable at body temperature hence rapidly degrades into the stable monomeric and dimeric forms (Samoszuk *et al.*, 2008). The release of P1NP is unaffected by food intake or diurnal variation and is stable at room temperature for up to 5 days (Garnero *et al.*, 2007).

In the Gumba study, P1NP was assayed using the IDS iSyS platform (Immunodiagnosics Systems Ltd, Tyne and Wear, UK). The samples were analysed as singletons. The assay is based on chemiluminescence technology which uses a biotinylated anti P1NP monoclonal antibody; an acridinium labelled antibody, streptavidin labelled magnetic particles and an assay buffer. After a series of steps, the acridinium label emits light which is proportional to the intact P1NP concentration in the sample.

#### 4.5.1.3. Total alkaline phosphatase (ALP)

Alkaline phosphatase (ALP) is an enzyme that catalyses the alkaline hydrolysis of a large variety of substrates. ALP is present in practically all tissues of the body (especially cell membranes) and occurs at particularly high levels in the intestinal epithelium, kidney tubules, bone (osteoblasts), liver and placenta. The enzyme is closely associated with the calcification process in bone. Physiological bone growth elevates total ALP (TAP) in serum, and this increase accounts for the fact that ALP enzyme activity is 1.5-2.5 times greater in sera of growing children compared to normal adult serum. TALP concentrations are also elevated during pregnancy due to increased placental synthesis, thus may not necessarily reflect the bone calcification process. Among bone diseases, the highest levels of ALP are observed in Paget's disease (10-25 times the upper limit of normal are not unusual). Very high levels are also present in patients with oestrogenic bone cancer. TAP levels are generally low in osteoporosis (Burtis and Ashwood, 2001).

In the Gumba study, plasma TAP was analysed on a Konelab analyser (Thermo Fischer Scientific, Vantaa, Finland) using Thermo Scientific kit based on IFCC plus method at 37 °C . The principle of the assay is that ALP catalyses the formation of p-nitrophenyl phosphate in alkaline solution and the formation of p-nitrophenol is measured at 405nm. The samples were analysed in duplicate as part of the clinical biochemistry described in section 4.3.1. The measurement range of the assay is 20-1000 U/l, and the detection limit is 1.0 U/l (Burtis and Ashwood, 2001). The expected normal values in adults are 35-105 U/l at 37°C. The reference range for non-pregnant women is 33-96 U/l and 38-229U/l during the third trimester of pregnancy (perinatology.com, 2010).

#### 4.5.1.4. BALP

Bone-specific alkaline phosphatase is produced by osteoblasts and is a marker of bone formation. Serum levels of BAP are believed to reflect the metabolic status of osteoblasts (Immunodiagnostic Systems Limited, 2015). Serum BALP was quantified using the Microvue BAP Assay which has low cross-reactivity with the liver enzyme (Quidel Corporation, Athens, USA). The samples were analysed in singleton.

#### 4.5.2. Vitamin D and calciotropic hormones

##### 4.5.2.1. 25 hydroxy-vitamin D

Vitamin D is a fat-soluble steroid hormone which plays a major role in calcium metabolism. Two forms of vitamin D have been identified. Vitamin D<sub>2</sub> (ergocalciferol) is the plant source synthesised from ergosterol. Vitamin D<sub>3</sub> (cholecalciferol) is synthesised in the skin from 7-dehydrocholesterol (7-DHC) under the influence of UVB sunlight and is the main source in animals. The rate of synthesis of vitamin D<sub>3</sub> in the skin is determined by the intensity of UVB and skin pigmentation. Melanin, sunscreen and clothing block UVB. UVB is available in sunlight all year round along the equator and the tropics, but the intensity decreases in temperate zones; such that, UVB is minimal during winter in the northern and southern latitudes (Müller *et al.*, 2011).

Vitamin D (both D<sub>2</sub> and D<sub>3</sub>) is hydroxylated by several hydroxylase enzymes, of which 25 hydroxylase is the most important, to form 25 hydroxyvitamin D (25(OH)D) in the liver. 25(OH)D is further hydroxylated in the kidneys by 1- $\alpha$  hydroxylase to form 1, 25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D), which is the active metabolite. 25(OH)D has a longer half-life compared to 1,25(OH)<sub>2</sub>D, and thus commonly used as an indicator of vitamin D status because it includes dietary and endogenous sources, and the circulating supply (Müller *et al.*, 2011). The Institute of Medicine (IOM) and the UK Scientific Advisory Committee on Nutrition (SACN) recommend serum 25(OH)D above 25 nmol/L to maintain bone health. However, there is lack of consensus on adequate serum 25(OH)D concentrations for non-skeletal functions (SACN, 2016).

Assays for 25(OH)D have evolved over time from manual competitive protein binding assays to now fully automated assays. The newest methods are based on Liquid Chromatography-Mass Spectrometry (LC-MS). The debate is ongoing on the metabolite that best represents functional vitamin D status. These methods give different results, and even the same method in different labs gives different results, hence need for standardisation through the DEQAS (the vitamin D Quality Assessment Scheme and VDSP (Vitamin D Standardization Program). Carter *et al.* present a detailed review of vitamin D assays from a historical perspective of DEQAS and VDSP (Carter *et al.*, 2017). EWL participates in both DEQAS and VSDP and the assay methods used are validated and standardised. It also has the capacity to quantify 25(OH)D using several assays including LC-MS method.



In the Gumba study, 25(OH)D was analysed using DiaSorin chemiluminescent immunoassay (Liaison; DiaSorin Inc., Stillwater, MN, USA) in duplicate. The Vitamin D Assay Kit is an ELISA method based on the principle of competitive binding. This method has been validated to be accurate and precise (Wagner *et al.*, 2009, Enko *et al.*, 2015).

#### 4.5.2.2. PTH

Parathyroid hormone is a polypeptide hormone of 84 amino acids secreted by the chief cells in the parathyroid glands (Keutmann *et al.*, 1978). The hormone is produced in response to low serum calcium and acts on both bone and the kidneys to restore serum calcium levels to normal. Thus PTH plays a major role in skeletal and mineral metabolism especially bone remodelling, renal 1,25(OH)D production and renal calcium and phosphate handling (Potts, 2005). PTH has two terminals, C and N. Biologically active PTH is found in the N-terminal region. C-terminal is bound to C-PTH receptor, and it exerts biological action opposite to classical action of PTH on the bone and kidneys. PTH has a half-life of 2-4 minutes and is rapidly degraded into C-terminal fragments which accounts for 80% of circulating PTH (Komaba *et al.*, 2009).

High circulating plasma concentration of PTH, hyperparathyroidism, is associated with increased bone loss. Renal impairment, vitamin D deficiency, low calcium intake and medication are among the causes of hyperparathyroidism (Stein *et al.*, 1996). Thus, measurement of PTH is important for the diagnosis of disorders in calcium metabolism (hypercalcemia and hypocalcemia). PTH assays have evolved from measurement of C-terminal (amino acids 39–84) and N terminal regions (amino acids 13–34) to the measurement of full length (intact or whole) PTH which is of clinical relevance. Komaba *et al.* have extensively discussed the generations of PTH assays over time (Komaba *et al.*, 2009).

In the Gumba study, plasma intact PTH was assayed using the IDS iSyS platform (Immunodiagnosics Systems Ltd, Tyne and Wear, UK). The samples were analysed as singletons. The method is based on chemiluminescence technology in which two polyclonal antibodies against human PTH are utilised. An antibody recognising the C-terminal region is used as the capture antibody, and an acridinium conjugated recognising the N-terminal is used for detection. In addition, the full-length PTH (amino acids 1-84) are also detected. The expected normal values are 8-51pg/mL in non-pregnant women and 9-26 pg/mL during the third trimester of pregnancy (Abbassi-Ghanavati *et al.*, 2009, perinatology.com, 2010).

#### 4.5.3. Blood and urine clinical biochemistry

All plasma and urine clinical biochemistry assays were measured in the NBH laboratory by standard methods on a Konelab analyser (Thermo Fischer Scientific, Vantaa, Finland). Low, medium and high control samples supplied by the kit manufacturers [Roche serum control (Roche Diagnostic Corporation, Indianapolis, USA), Lyphochek (Bio-Rad Laboratories, Herts, UK), NEQAS Clin Chem (Birmingham, UK)] and an internal plasma drift controls were used for quality assurance. The samples were analysed in duplicate with controls at the beginning and the end of each run.

##### 4.5.3.1. Plasma albumin

Albumin is the most abundant protein in human plasma accounting for approximately one half of plasma protein mass. It has no carbohydrate side chains, but it is highly soluble in water due to its high net negative charge at physiological value. Albumin is synthesised by the hepatic parenchymal cells. Its rate of synthesis is under primary control by the colloidal osmotic pressure (COP) and secondary control by protein intake. The normal plasma half-life of albumin is about 15-19 days. The primary function of albumin is the maintenance of COP in both vascular and extravascular spaces. It also binds and transports a large number of compounds (Burtis and Ashwood, 2001). Increased levels of albumin are present in acute dehydration but have no clinical significance. Decreased levels are seen in many clinical conditions including, inflammation, and hepatic disease, urinary excretion, protein-energy malnutrition, e.g. associated with wasting in HIV, oedema and ascites (Thermo Fisher Scientific Inc, 2009). In HIV-infected persons, serum albumin concentrations have been associated with progression of HIV- disease and mortality (Feldman *et al.*, 2000, Mehta *et al.*, 2006).

In the Gumba study, plasma albumin (P-Alb) was measured using a Thermo Scientific kit based on the bromocresol purple (BCP) method. The principle of this assay is that albumin reacts with BCP dye to form a coloured product. The formed colour intensity is measured at 600 nm and is proportional to the concentration of albumin in the sample (Pinnell and Northam, 1978). The measurement range for the assay is 4-6 g/L, and the minimum level of detection is 2 g/L (Thermo Fisher Scientific Inc, 2009). The expected normal values for serum/plasma in adults is 35-52 g/L. The normal ranges during the third trimester of pregnancy and in non-pregnant women are 2.3-4.2 g/L and 4.1-5.3g/L, respectively (perinatology.com, 2010).

#### *4.5.3.2. Plasma and urine creatinine*

Creatinine is a product of the breakdown of creatine and creatine phosphate. Creatine is synthesised in the kidneys, liver and pancreas; and transported in the blood to other organs like the brain and muscles. About 1-2% of muscle creatine is converted to creatinine daily and the amount produced is proportional to muscle mass and also varies with ethnicity, age and sex. Given the same glomerular filtration rate, men have higher creatinine concentrations compared to women, muscular individuals have higher levels than less muscular individuals, and younger persons have higher levels than the elderly. Depending on the individual's meat intake, diet may influence the value by about 10%, but this causes only a minor variation in daily creatinine excretion in an individual. Creatinine excretion is fairly constant hence used as a proxy for renal function (estimated glomerular filtration rate, eGFR) and also to correct for urine concentration. High creatinine levels are found in acute and chronic kidney insufficiency and dehydration (Burtis and Ashwood, 2001).

In the Gumba study, plasma and urine creatinine concentrations (P-Cr and U-Cr respectively) were measured by the compensated Jaffe' method where creatinine forms a red colour with an alkaline picrate solution (Jaffe, 1886). The method measures the rate at which creatinine forms the red complex. The intensity of the formed complex is measured at 510nm. The measuring ranges for the assay for plasma/serum and urine are 10-800  $\mu\text{mol/l}$  and 0.5-40.8mmol/l, respectively. The detection limit for the assay is 5  $\mu\text{mol/l}$ . The expected normal values are about 45-90  $\mu\text{mol/L}$  for women and 60-110  $\mu\text{mol/L}$  for men (Thermo Fisher Scientific Inc, 2012).

#### *4.5.3.3 Plasma and urine phosphate*

Phosphate in serum exists as both monovalent and divalent anions. Approximately, 10% of phosphate in serum is bound to protein, 35% is complexed with Na, Ca and Mg and the remainder are free. Inorganic phosphate is a major component of hydroxyapatite in bone. Phosphate also has a critical role in high-energy phosphate bonds. In soft tissues, most phosphate is organic and incorporated into cellular macromolecules. Phosphate is an essential element in phospholipid cell membranes, nucleic acids and phosphoproteins, gene transcription and growth.

Hypophosphataemia is common in hospitalised individuals often caused by carbohydrate-induced stimulation of insulin secretion, which enhances transport of phosphate into cells. Respiratory alkalosis and renal phosphate wasting may also cause hypophosphataemia. Hyperphosphataemia is usually related to renal failure. A decrease in glomerular filtration limits the renal excretion of phosphate, and hyperphosphataemia develops (Burtis and Ashwood, 2001).

In the Gumba study, plasma and urine phosphate concentrations (P-PO<sub>4</sub> and U-PO<sub>4</sub> respectively) were measured by a Thermo Scientific kit based on the ammonium molybdate method. This is based on the principle that phosphate forms a phosphomolybdate complex with ammonium molybdate in acidic medium. The absorbance at 340nm is directly proportional to the concentration of inorganic phosphate in the sample (Daly and Ertingshausen, 1972). The measurement ranges for the assay for plasma/serum and urine are 0.08-4.00 mmol/l and 2.5-84 mmol/l, respectively. The expected normal values are 0.87-1.45 mmol/l in plasma/urine and 12.9-42.0mmol/24hr in urine (for individuals on non-restricted diets). The detection limit for the assay is 0.03 mmol/ (Thermo Fisher Scientific Inc, 2013). Urine phosphate-creatinine ratio (UPO<sub>4</sub>/Cr) was calculated using the equation:

$$\text{U-PO}_4/\text{Cr} = [\text{U- PO}_4] / [\text{U-Cr}]$$

Where U-PO<sub>4</sub> = urine phosphate and U-Cr = urine creatinine each in mmol/L.

#### 4.5.3.4. Plasma and urine calcium

Calcium is found mainly in the skeleton with small amounts in the soft tissue and extracellular fluid. In blood 50% of the plasma calcium (P-Ca) is free, 40% is protein-bound, and 10% is complexed. About 80% of protein-bound calcium is associated with albumin, and the remaining 20% is associated with globulins. Extracellular calcium provides calcium ions for maintenance of intracellular calcium, bone mineralisation, blood coagulation and maintenance of plasma membrane potential. Calcium is also important in muscle contraction and second messenger affecting enzyme activity and hormone secretion.

A decrease in the free plasma calcium concentration causes increased neuromuscular excitability. Low total serum calcium (hypocalcemia) may be due to either a reduction in the albumin-bound or free fraction of calcium. Hypoalbuminemia is the most common cause of hypocalcemia. Serum calcium levels may also be low due to magnesium or vitamin D deficiency, hypoparathyroidism or acute pancreatitis. High serum calcium values (hypercalcemia) are commonly due to an increased influx of calcium into the extracellular fluid compartment from the skeleton, intestine, or kidney (Burtis and Ashwood, 2001) .

In the Gumba study, plasma and urine calcium concentrations (P-Ca and U-Ca respectively) were measured using the Thermo Scientific kit based on the Arsenazo III method. The principle of this assay is that calcium ions form a highly coloured complex with Arsenazo III at a neutral pH. The amount of the complex is measured at 660nm. The measuring range for the assay is 0.70-4.00 mmol/l. An extended measurement ranges of 0.70-12.00 mmol/l can be achieved after secondary dilution of the sample. P-Ca was normalised for albumin using the Payne equation:

**Adjusted P-Ca= Measured P-Ca+ ([40-plasma albumin]\*0.02).**

The expected normal range in serum/plasma in adults is 2.15-2.57 mmol/L, and the detection limit is 0.02 mmol/l (Thermo Fisher Scientific Inc, 2007).

Four participants had implausible P-Ca concentrations and their data were excluded from analysis (2 HIV positive -6.27 and 3.25 at PP14) and 2 HIV-negative (3.35 at PG36, 7.53 at PP14). The four samples could have been contaminated during processing. Their inclusion in the analysis increased the standard error of the means but did not change the interpretation of results.

Calcium excretion in urine can be measured by the ratio in a random sample or 24hr urine. Calcium excretion increases with increases in plasma calcium and PTH enhances tubular reabsorption of calcium. Urinary calcium-creatinine excretion ratio is usually less than 0.05, and 24hr urine gives a more accurate estimation than a random urine sample (Nordin and Smith, 1965). In clinical settings, hypercalcemia is diagnosed by the ratio of calcium to creatinine concentrations (UCa/Cr) in a spot sample (preferably fasted).

In the Gumba study, UCa/Cr was calculated based on 2-hour fasted urine sample using the equation:

$$\text{U-Ca/Cr} = [\text{U-Ca}] / [\text{U-Cr}]$$

Where U-Ca = urine calcium and U-Cr = urine creatinine each in mmol/L.

A total of 41 urine samples (7 PG36 and 34 PP14) had calcium concentrations below the detection limit and were assigned half a detection (0.01 mmol/l).

#### 4.5.5.8. Plasma and urine magnesium

Approximately, 55% of the total body magnesium is in the skeleton, and the remainder is intracellular, where it is the second most prevalent cation. Within the cells, magnesium is bound primarily to proteins and negatively charged molecules. In serum, about 55% of magnesium is free, 30% is associated with proteins, and 15% is complexed with phosphate, citrate and other anions. Extracellular magnesium provides magnesium for the maintenance of intracellular concentration (Burtis and Ashwood, 2001).

Magnesium is a co-factor for over 300 enzymes. It is essential for vitamin D synthesis, transport and activation, and for bone mineralisation by acting directly on bone cells and indirectly via PTH. A decrease in serum magnesium concentration results in muscular excitability. Magnesium deficiency may arise from intestinal caused by diarrhoea or malabsorption, so HIV-infected persons with advanced disease are at risk of deficiency. Urinary magnesium loss occurs in alcoholism and diabetes mellitus, with the use of certain drugs and with increased sodium and calcium excretion (Burtis and Ashwood, 2001).

In the Gumba study, plasma and urine magnesium (P-Mg and U-Mg respectively) was measured by a Thermo Scientific kit based on the xylidyl blue I method. The principle of the assay is that magnesium reacts with the sodium salt of xylidyl blue I (4-hydroxy-3-[2-hydroxy-3-(2, 4 dimethyl phenyl aminocarbonyl)-1-naphthylazo] benzene sulfonic acid) to form a red complex in alkaline solution. The absorbance at 510nm of the red complex is proportional to the concentration of magnesium in the sample. Interference by calcium is prevented by EGTA (ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid), also known as egtazic acid (Baginski et al. 1982).

The measurement range for the assay is 0.05-1.5 mmol/l. An extended measuring range of 0.05-10.9 mmol/l can be achieved after secondary dilution of the sample. The detection limit for the assay is 0.03 mmol/l. The expected normal values in serum/plasma are 0.66-1.07 mmol/L and normally higher in women during menses (Thermo Fisher Scientific Inc, 2014). Urine magnesium-creatinine ratio (UMg/Cr) was estimated using the equation:

$$\text{U-Mg/Cr} = [\text{U-Mg}] / [\text{U-Cr}]$$

Where U-Mg = urine magnesium and U-Cr = urine creatinine each in mmol/L.

#### 4.5.4. Renal function and mineral handling

##### 4.5.4.1. Estimated glomerular filtration rate (eGFR)

Estimated glomerular filtration rate (eGFR) was calculated based on plasma creatinine concentrations using the Chronic Kidney Disease –Epidemiology Collaboration (CKD-EPI) equation for females without the correction for black ethnicity (Levey *et al.*, 2009). The overall CKD equation for eGFR is:

$$\text{eGFR (mL/min/1.73m}^2\text{)} = 141 \times \min(P_{\text{Cr}}/\kappa, 1)^{\alpha} \times \max(P_{\text{Cr}}/\kappa, 1)^{-1.209} \times 0.993^{\text{Age}} \times 1.018 \text{ [if female]}$$

Where  $P_{\text{Cr}}$  =plasma creatinine in  $\mu\text{mol/L}$ ;  $\kappa=0.7$  and is a constant for females; min indicates the minimum of  $P_{\text{Cr}}/\kappa$  or 1, and max indicates the maximum of  $P_{\text{Cr}}/\kappa$  or 1.

However, for ease of calculation, the following simplified CKD-EPI equations for white women (not black ethnicity) were used in the Gumba study:

$$\text{For } P_{\text{Cr}} \leq 62 \mu\text{mol/L, eGFR (mL/min/1.73m}^2\text{)} = 144 \times (P_{\text{Cr}}/0.7)^{-0.329} \times (0.993)^{\text{Age}}$$

$$\text{For } P_{\text{Cr}} \geq 62 \mu\text{mol/L, eGFR (mL/min/1.73m}^2\text{)} = 144 \times (P_{\text{Cr}}/0.7)^{-1.209} \times (0.993)^{\text{Age}}$$

The CKD-EPI equation performs better than the Modified Diet in Renal Disease (MDRD) equation in healthy individuals. It has also been validated in African populations (Kenya and South Africa) and reported to perform better than MDRD and Cockcroft-Gault equations when the correction for black ethnicity is omitted (van Deventer *et al.*, 2011, Wyatt *et al.*, 2013).

##### 4.5.4.2. Renal Tubular Maximum for Reabsorption of Phosphate (TmP)

In 1969, Bijvoet introduced the use of renal tubular maximum for reabsorption of phosphate (TmP) divided by GFR to help distinguish hypercalcaemia due to hyperparathyroidism from other causes of hypercalcaemia (Bijvoet, 1969). TmP/GFR measures the maximum renal tubular phosphate reabsorption in mass per unit volume of glomerular filtrate (Payne, 1998). Over the years, assays for intact parathyroid hormone (PTH) have superseded the use of TmP/GFR in diagnosis of disturbances in calcium homeostasis, although the index is still clinically useful in diagnosis of disturbances in phosphate homeostasis. Unlike earlier indices of renal phosphate handling such as phosphate excretion index (PEI) and the index of phosphate excretion (IPE), only TmP/GFR has a physiological basis. TmP/GFR It is not influenced by factors which affect plasma phosphate concentrations, i.e. GFR and rate of release of phosphate from bones, gut and other cells into the extracellular space. Therefore, TmP/GFR is the most important index of renal phosphate excretion for diagnosing disorders in renal phosphate handling and has replaced both PEI and IPE (Payne, 1998).

Calculation of TmP/GFR is based on phosphate concentrations in blood and urine and follows several steps. The first step is calculation of the ratio of phosphate clearance to creatinine clearance ( $PO_4Cl/CrCl$ ).  $PO_4Cl/CrCl$  is a measure of the proportion of filtered phosphate that is not reabsorbed by renal tubules. This is based on the principle that creatinine clearance is approximately the same as glomerular filtration rate (GFR). Renal reabsorption of filtered phosphate occurs primarily in the proximal tubules, and the process is regulated by PTH. Low  $PO_4Cl/CrCl$  is associated with hypoparathyroidism and high values with hyperparathyroidism.

$PO_4Cl/CrCl$  is measured in two hours fasted urine or spot urine as the formula does not require the total volume of urine (Nordin and Smith, 1965).

$$P-PO_4Cl/CrCl = (U_P \times P_{Cr}) / (P_P \times U_{Cr})$$

Where  $P_P$  = plasma phosphate,  $U_P$  = urine phosphate,  $P_{Cr}$  = plasma creatinine and  $U_{Cr}$  = urine creatinine each in mmol/L.  $UP/Cr$  varies with plasma phosphate concentration but most values fall between 0.05 and 0.15. The corresponding normal tubular reabsorption for phosphate (TRP) is 85-95%. When  $C_P/C_{Cr}$  is subtracted from 1.0, it gives TRP.

$$\text{Thus, TRP} = 1 - [(U_P \times P_{Cr}) / (P_P \times U_{Cr})] \times 100\%$$

The maximum reabsorption of phosphate (TmP) is reached when the concentrations of phosphate in plasma and the glomerular filtrate are equal. Thus TRP multiplied by plasma phosphate gives the maximum reabsorption of phosphate per unit volume of glomerular filtrate (TmP/GFR) (Nordin and Fraser, 1960, Barth *et al.*, 2000).

**If TRP is 0.86 or less, then TmP/GFR = TRP X plasma phosphate ( $P_P$ )**

**If TRP greater than 0.86, then TmP/GFR =  $0.3 \times TRP / [(1-0.8 \times TRP)] \times$  plasma phosphate ( $P_P$ )**

The Gumba study used the above procedures to calculate TmP/GFR and the results are presented in mmol/mmol.



#### 4.5.4.3. Renal Tubular Maximum for Reabsorption of Calcium (TmCa)

Renal tubular maximum for reabsorption of calcium (TmCa) measures the maximum rate of renal calcium reabsorption which cannot be exceeded i.e when the concentration of calcium in plasma and glomerular filtrate are equal (Nordin *et al.*, 1976, Need *et al.*, 1985). TmCa is commonly reported per unit volume of glomerular filtrate (GFR). TmCa/GFR is not affected by factors that affect plasma calcium concentrations i.e the rate of flow of calcium into the extracellular space from gut or bone, filtration of calcium through the glomeruli and tubular reabsorption of calcium. It is therefore useful in defining the mechanism of hypercalcaemia or hypocalcaemia in any given individual (Nordin *et al.*, 1976).

In the Gumba study TmCa/GFR is calculated using the equation below derived by (Nordin *et al.*, 1976):

$$\text{TmCa/GFR} = [ (0.56 \text{ P-Ca}) - \text{CaE} ] / [ 1 - 0.08 \log e(0.56 \text{ P-Ca/CaE}) ] \text{ mmol/l GF}$$

Where: TmCa, tubular maximum for calcium reabsorption (mmol/l of glomerular filtrate (GF)); P-Ca, plasma total calcium concentration (mmol/l) (uncorrected for albumin); P-Cr, plasma creatinine concentration (mmol/l); (UCa/UCr), fasting urinary molar calcium creatinine ratio; CaE [(UCa/UCr) X PCr], calcium excretion (mmol/l of GF). The term (0.56 P-Ca) is an approximation to the plasma ultrafiltrable calcium concentration.

#### 4.5.5. Breastmilk mineral content

##### 4.5.5.1. Breast milk calcium and phosphorus

Calcium and phosphorus occur in various compartments in breast milk. The fat layer contains a significant proportion of calcium. Phosphorus is mainly in the aqueous layer, but recovery in clarified breast milk samples is only 30-40%. Thus, quantification of total calcium and phosphorus requires whole milk (Laskey *et al.*, 1991). Dorea reviewed studies conducted on breast milk calcium and phosphorus between the years 1949-1999. The median breastmilk concentrations were 252 mg/L (range: 84 -462 mg/L) for calcium (median) and 143 mg/L (range: 17- 278 mg/L for phosphate. The median calcium/phosphate ratio (Ca/P) was 1.7 (range: 0.8 to 6). The variation in breast milk calcium concentrations was found to be higher compared to phosphorus concentrations (Dorea, 1999).

In the Gumba study, breast milk calcium and phosphorus were analysed in samples from the left breast using the semi-automated micro method developed by Laskey *et al.* (Laskey *et al.*, 1991). Whole breast milk samples were warmed to 40°C, and then aliquots of 20µL prepared into acid washed tubes. The tubes were then frozen, freeze-dried and ashed in a muffle furnace at 400-500°C.

Next, the samples were cooled and digested with 0.5ml of 0.2 mol/L hydrochloric acid (Laskey *et al.*, 1991). Prepared samples were analysed in duplicate on a Konelab analyser (Thermo Fischer Scientific, Vantaa, Finland) using Thermo Scientific kits for P-Ca (Arsenazo III method) and phosphate (ammonium molybdate method) as discussed in sections 4.2.5.5 and 4.2.5.6, respectively.

#### 4.5.5.2. Sodium (Na) and potassium (K)

Breast milk contains about 5-6 mmol/L of sodium (Aperia *et al.*, 1979) and this concentration is tightly regulated. Breast milk sodium concentration is high within the first 3 days postpartum and progressively decreases with the transition to mature milk (about 2 weeks postpartum). The reduction in breast milk sodium concentration is due to the closing of the tight junctions between the mammary alveolar cells which separate milk from plasma and intercellular fluid. Afterwards, there is minimal diurnal variation in breast milk sodium and also no significant difference between the breasts or beginning and end of a feed in healthy women (Koo and Gupta, 1982). Maternal sodium intake does not affect breast milk concentration (Ereman *et al.*, 1987).

During mastitis, there is inflammation of breast tissue and opening of the tight junctions allowing plasma and intercellular fluid to enter breast milk through the paracellular pathways between alveoli cells (Prentice *et al.*, 1985). Thus, elevated breast milk sodium is a sensitive indicator of mastitis and/or subclinical mastitis. Due to complexities in the collection of breast milk and potential variations due to collection protocols used, breast milk sodium: potassium ratio is commonly used to permit the use of spot milk samples without consideration of time of sampling, use of fore or hindmilk, or time since the infant was last fed. Elevated sodium: potassium ratio ( $>1.0$ ) is an indicator of mammary epithelial permeability and/mastitis/subclinical mastitis (Filteau *et al.*, 1999, Kasonka *et al.*, 2006).

Breast milk sodium and potassium were analysed by the NBA laboratory at EWL using flame photometry (IL 945 flame photometer). The instrument was calibrated using a standard urine control containing 73.5-81.8 mmol/L of sodium and 27.8-34.2 mmol/L of potassium. Twenty four samples were analysed with and without centrifugation, and the results were not significantly different (correlation coefficients were 0.98 and 0.99 for Na and potassium, respectively when the origin = 0). Therefore, all breast milk samples were analysed as whole milk without centrifugation. The mean CVs for breast milk sodium and potassium were 4.0% and 3.6%, respectively.

## **4.6. Questionnaires**

Questionnaire were administered at all study visits. Each visit had a combination of several questionnaires (see appendices 6, 7 and 8). In summary, information was collected on the following:

### *4.6.1. Demographic and socio-economic characteristics*

Information on socio-economic characteristics was collected from participants using a semi-structured questionnaire based on the Uganda national household survey questionnaire. Several social and economic characteristics which could potentially impact on health and nutrition outcomes were compared between the groups.

### *4.6.2. Medical history*

Participants were asked brief questions about their health status. A review of medical records of HIV-infected women and exposed infants was done to capture additional information on their health status and PMTCT-EID care.

### *4.6.3. Sunshine exposure and physical activity*

A sunshine exposure questionnaire was used to collect information on clothing habits, outdoors activities, skin pigmentation and other practices which affect dermal synthesis of Vitamin D. The questionnaire was modified from those used in NBH studies in The Gambia, and UK.

### *4.6.4. Dietary intake of calcium and vitamin D*

A food frequency questionnaire was used to collect information about participants' habitual consumption of selected calcium and vitamin D rich foods. The questionnaire was combined with questions on maternal dietary intake, preferences and infant feeding practices. The questionnaire was modified from that used by NBH studies in The Gambia.

## **4.7. Data handling and statistical analysis**

### *4.7.1 Quality control and assurance checks*

Data Quality control (QC) reviews for study questionnaires, forms and hardcopy DXA scans were performed on a daily basis during fieldwork in Uganda. I developed data QC checklists which were implemented at two levels (level one [QC1] and two [QC2]) in compliance with the regulatory requirements at Baylor and MUJHU. QC1 reviews were done for all study documents by trained staff at the site of data collection before the participant left the premises (see Table 4). This was mainly aimed at checking accuracy and clarity of the information collected. Identified issues were addressed on the spot before the participant went home. The documents were reviewed and all correctly filled documents were signed on every page by the reviewer.

I conducted QC2 reviews for all documents after QC1 checking for accuracy, consistency in data collection and compliance to study protocols. For informed consent forms, the QC2 review was done before the participant was enrolled into the study. QC2 for study visits was done within 48 hours, and identified issues addressed within 3 days. QC2 reviews were conducted by the study coordinator at Baylor-Uganda after I had returned to Cambridge in February 2016.

Between February and May 2016, I conducted monthly Quality Assurance (QA) reviews on 10% of the study visits to monitor implementation of the study. I also conducted weekly study meetings to review progress and address any challenges.

### *4.7.2. Data handling and transformations*

All questionnaires and forms were scanned and sent to EWL via a secure online system (Filerun). The data were entered in an Ms Access database. Queries were run routinely to check for wrong entries and missing data and these were corrected. Anthropometry data were entered in duplicate. DXA data was exported into Ms Access and laboratory data compiled in Ms Excel. All datasets were compiled in Ms Access then imported into DataDesk 6.3.1 (Data Description Inc., Ithaca, NY, USA) for statistical analysis. Outliers were identified and investigated against the scanned forms to ascertain if they were correct entries. Implausible measurements were excluded from statistical analyses. Where relevant, the number of measurements or entries that were excluded have been presented in the footnotes for the tables of results in chapters 5, 6, 7 and 8.

With the exception of infant growth Z-scores, all continuous variables were transformed into natural logarithms before manipulation and analysis for differences between the groups. Multiplication of the natural log by 100 enabled presentation of mean percent differences between the groups and time points as sympercents.

The formula for calculation of Sympercents is: **Percentage difference=100× (difference/mean)**. A detailed explanation of sympercents and their application in reporting mean differences between the groups are described elsewhere (Cole, 2000, Cole and Altman, 2017b, Cole and Altman, 2017a). From this point onwards, the term percent refers to sympercents unless specified. Transformation into natural logarithms also normalised the distributions for positively skewed data. Outliers in transformed data were investigated using box plots for the variable of interest in DataDesk software (Figure 33). Extreme values are automatically flagged in data desk and plotted as stars. High values are indicated by a hollow dot. Statistical analysis was done with and without outliers, and the results are reported for both approaches where relevant.

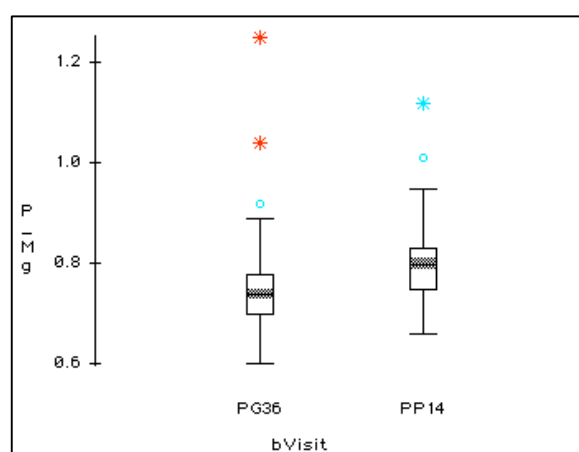


Figure 33: Box plot of plasma Mg by visit showing outliers by the visit.

Red colour= HIV-positive and Blue =HIV-negative. Analysis with and without the 3 outliers has no material effect on the results. Thus, exclusion of outliers is not necessary.

#### 4.7.3. Calculation of SA-BMC

BMC was adjusted for BA, weight and height (in mothers) or length (in infants) in linear regression models including a group term. All continuous variables are presented in natural logarithms, and the differences between the groups are reported as %±SE.

SA-BMC values for individuals were calculated by setting-up the model as described above but without the group term. The residuals for the model were calculated, added to mean In SA-BMC to give In SA-BMC values for individuals and these were antilogged to give SA-BMC values. Summary statistics for the entire group were then calculated.

#### *4.7.4. Summary statistics*

Descriptive statistics for continuous variables are presented as mean $\pm$ standard deviation (mean $\pm$ SD) for normally distributed variables and median (interquartile range (IQR), 25<sup>th</sup> and 75<sup>th</sup> quartiles) for skewed distributions. For all discrete variables, descriptive statistics are presented as proportions (%).

#### *4.7.5. Cross-sectional analysis*

Contingency tables and Chi-square tests were used for discrete variables to test if the proportions were significantly different between the groups. For continuous variables, mean cross-sectional differences between the groups were analysed in a general linear model (GLM). GLM brings together multiple linear regression, correlation, analysis of variance (ANOVA) and analysis of covariance (ANCOVA) into one platform, thus, providing a single consistent conceptual framework for data analysis (Vallenman, 1997). A *p*-value of  $\leq 0.05$  was considered significant for all tests.

Statistical analysis of continuous variables was done in two stages. First, ANOVA was used in GLM to compare means between the groups for each variable of interest. In this case, ANOVA is equivalent to a simple *t*-test since the study has only two groups. The mean differences between the groups are presented in the summary tables as sympercents  $\pm$  standard error of the mean (% $\pm$ SE) for HIV-positive vs HIV-negative group (Figure 34).

In the second stage of analysis, each outcome variable of interest was adjusted for potential confounders. This was achieved by adding all the identified confounders (both discrete and continuous variables) into the model developed above as co-factors to form a full model. Hence, the analysis automatically changed into ANCOVA (Figure 35). Non-significant variables were removed by backwards elimination to produce parsimonious models (Figure 36). The size effects for significant variables are presented in the text alongside the final mean % difference between the groups in the parsimonious model (see Figures 34-38 for sample models). Lists of potential confounders at each visit are presented at the beginning of each results chapter (chapters 5, 6, 7 and 8).

DESIGN

Dependent variables

Name	Code
mLnWT2/14	m24

Type of analysis: OLS ANOVA

Factors

Name	Code	Nested in	F/R	Kind
HIVstatus	HVs	()	Fix	Disc

Partial (Type 3) Sums of Squares

Interactions up to 1 - way

Source	F/R	max df	EMS	F-Denom
Const	-	1	Const	Error
HVs	F	1	HVs	Error
Error	R	398		
Total		399		

Modifications

cases selected according to cPP2

No variance variable

RESULTS

General Results

400 total cases of which 239 are missing

ANOVA

Analysis of Variance For  
cases selected according to  
400 total cases of which 239 are missing

mLnWT2/14  
cPP2

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	27.1293e6	27.1293e6	111.27e3	≤ 0.0001
HVs	1	1071.94	1071.94	4.3966	0.0376
Error	159	38765.8	243.81		
Total	160	39837.7			

Results for factor HVs

Coefficients

Coefficients of: mLnWT2/14 on HVs

Level of HVs	Coefficient	std. err.	t Ratio	prob
HIV-Neg	2.583	1.232	2.097	0.0376
HIV-Pos	-2.583	1.232	-2.097	0.0376

Expected Cell Means

Expected Cell Means of: mLnWT2/14 on HVs

Level of HVs	Expected Cell Mean	Cell Count
HIV-Neg	413.2	77
HIV-Pos	408	84

Scheffe Post Hoc Tests

	Difference	std. err.	Prob
HIV-Pos - HIV-Neg	-5.16551	2.464	0.037593

Figure 34: Sample ANOVA model for maternal weight at PP2

Interpretation: HIV-positive women (HIV-pos) have  $5.2 \pm 2.5\%$  lower body weight ( $p=0.04$ ) compared to HIV-negative (HIV-neg) women before adjustment for potential confounders.

DESIGN

Dependent variables

Name	Code
mLnWT2/14	m24

Type of analysis: OLS ANOVA

Factors

Name	Code	Nested in	F/R	Kind
HIVstatus	HVs	( )	Fix	Disc
mLnAge2	mL2	( )	Fix	Cont
PriGravida	P	( )	Fix	Disc
xLnAgeinfant_days	xL_	( )	Fix	Cont
priorDepo	pDo	( )	Fix	Disc
ExclBF2	EB2	( )	Fix	Disc

Partial (Type 3) Sums of Squares

Interactions up to 1 - way

Source	F/R	max df	EMS	F-Denom
Const	-	1	Const	Error
HVs	F	1	HVs	Error
mL2	F	1	mL2	Error
P	F	1	P	Error
xL_	F	1	xL_	Error
pDo	F	1	pDo	Error
EB2	F	1	EB2	Error
Error	R	393		
Total		399		

Modifications

cases selected according to cPP2

No variance variable

RESULTS

General Results

400 total cases of which 242 are missing

ANOVA

Analysis of Variance For

cases selected according to

400 total cases of which 242 are missing

mLnWT2/14

cPP2

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	26.6111e6	26.6111e6	129.49e3	≤ 0.0001
HVs	1	2127.91	2127.91	10.355	0.0016
mL2	1	234.543	234.543	1.1413	0.2871
P	1	2101.5	2101.5	10.226	0.0017
xL_	1	165.962	165.962	0.80759	0.3703
pDo	1	1892.01	1892.01	9.2067	0.0028
EB2	1	658.364	658.364	3.2037	0.0755
Error	151	31031	205.503		
Total	157	39624.8			

Results for factor HVs

Coefficients

Coefficients of: mLnWT2/14 on HVs

Level of HVs	Coefficient	std. err.	t Ratio	prob
HIV-Neg	3.966	1.233	3.218	0.0016
HIV-Pos	-3.966	1.233	-3.218	0.0016

Expected Cell Means

Expected Cell Means of: mLnWT2/14 on HVs

Level of HVs	Expected Cell Mean	Cell Count
HIV-Neg	416.5	77
HIV-Pos	408.6	81

Scheffe Post Hoc Tests

	Difference	std. err.	Prob
HIV-Pos - HIV-Neg	-7.93213	2.465	0.00158067

Figure 35: Sample fully adjusted ANCOVA model for maternal weight at PP2

Interpretation: HIV-positive group has 7.9±2.4% lower body weight (p=0.002) in the ANCOVA model including HIV-status, maternal age, primigravidity (yes=1 or no=0), infant age, previous use of depo-provera and exclusive breastfeeding (yes=1 or no=0). Variable names are listed in the order they appear in the model.



**DESIGN****Dependent variables**

Name	Code
mLnWT2/14	m24

Type of analysis: OLS ANOVA

**Factors**

Name	Code	Nested in	F/R	Kind
HIVstatus	HVs	()	Fix	Disc
PriGravida	P	()	Fix	Disc
priorDepo	pDo	()	Fix	Disc

Partial (Type 3) Sums of Squares

Interactions up to 1 - way

Source	F/R	max df	EMS	F-Denom
Const	-	1	Const	Error
HVs	F	1	HVs	Error
P	F	1	P	Error
pDo	F	1	pDo	Error
Error	R	396		
Total		399		

**Modifications**

cases selected according to cPP2

No variance variable

**RESULTS****General Results**

400 total cases of which 241 are missing

ANOVA

Analysis of Variance For	mLnWT2/14
cases selected according to	cPP2
400 total cases of which 241 are missing	

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	26.7805e6	26.7805e6	129.56e3	≤ 0.0001
HVs	1	2727.36	2727.36	13.195	0.0004
P	1	2086.78	2086.78	10.096	0.0018
pDo	1	2278.98	2278.98	11.026	0.0011
Error	155	32037.9	206.696		
Total	158	39626.2			

**Results for factor HVs**

Coefficients

Coefficients of: mLnWT2/14 on HVs

Level of HVs	Coefficient	std. err.	t Ratio	prob
HIV-Neg	4.28	1.178	3.632	0.0004
HIV-Pos	-4.28	1.178	-3.632	0.0004

Expected Cell Means

Expected Cell Means of: mLnWT2/14 on HVs

Level of HVs	Expected Cell Mean	Cell Count
HIV-Neg	416.1	77
HIV-Pos	407.5	82

Scheffe Post Hoc Tests

	Difference	std. err.	Prob
HIV-Pos - HIV-Neg	-8.55958	2.356	381.067e-6

Figure 36: Analysis output of parsimonious model for maternal weight at PP2

Interpretation: HIV-positive group has  $8.6 \pm 2.4\%$  lower body weight ( $p \leq 0.0001$ ) compared to HIV-negative women after adjustment for significant variables (primigravidity (yes/no) and previous exposure to depo-provera).

**DESIGN****Dependent variables**

Name	Code
mLnWT2/14	m24

Type of analysis: OLS ANOVA

**Factors**

Name	Code	Nested in	F/R	Kind
HIVstatus	HVs	()	Fix	Disc
PriGravida	P	()	Fix	Disc
priorDepo	pDo	()	Fix	Disc

Partial (Type 3) Sums of Squares

Interactions up to 1 - way

Source	F/R	max df	EMS	F-Denom
Const	-	1	Const	Error
HVs	F	1	HVs	Error
P	F	1	P	Error
pDo	F	1	pDo	Error
Error	R	396		
Total		399		

**Modifications**

cases selected according to cPP2

No variance variable

**RESULTS****General Results**

400 total cases of which 241 are missing

**ANOVA**

Analysis of Variance For **mLnWT2/14**  
cases selected according to **cPP2**  
400 total cases of which 241 are missing

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	26.7805e6	26.7805e6	129.56e3	≤ 0.0001
HVs	1	2727.36	2727.36	13.195	0.0004
P	1	2086.78	2086.78	10.096	0.0018
pDo	1	2278.98	2278.98	11.026	0.0011
Error	155	32037.9	206.696		
Total	158	39626.2			

**Results for factor P****Coefficients**

Coefficients of: mLnWT2/14 on P

Level of P	Coefficient	std. err.	t Ratio	prob
0	3.9	1.227	3.177	0.0018
1	-3.9	1.227	-3.177	0.0018

**Expected Cell Means**

Expected Cell Means of: mLnWT2/14 on P

Level of P	Expected Cell Mean	Cell Count
0	415.7	89
1	407.9	70

**Scheffe Post Hoc Tests**

	Difference	std. err.	Prob
1 - 0	-7.79984	2.455	0.00179386

Figure 37: Analysis output for size effect of parity on maternal weight at PP2

Interpretation: primigravidae women (coded as 1) have  $7.8 \pm 2.4\%$  lower body weight ( $p=0.002$ ) compared to multigravidae women taking into account HIV status and previous exposure to depo-provera.

**DESIGN****Dependent variables**

**Name**      **Code**  
 mLnWT2/14    m24

Type of analysis: OLS ANOVA

**Factors**

Name	Code	Nested in	F/R	Kind
HIVstatus	HVs	( )	Fix	Disc
PriGravida	P	( )	Fix	Disc
priorDepo	pDo	( )	Fix	Disc

Partial (Type 3) Sums of Squares

Interactions up to 1 - way

Source	F/R	max df	EMS	F-Denom
Const	-	1	Const	Error
HVs	F	1	HVs	Error
P	F	1	P	Error
pDo	F	1	pDo	Error
Error	R	396		
Total		399		

**Modifications**

cases selected according to cPP2

No variance variable

**RESULTS****General Results**

400 total cases of which 241 are missing

**ANOVA**

Analysis of Variance For **mLnWT2/14**  
 cases selected according to **cPP2**  
 400 total cases of which 241 are missing

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	26.7805e6	26.7805e6	129.56e3	≤ 0.0001
HVs	1	2727.36	2727.36	13.195	0.0004
P	1	2086.78	2086.78	10.096	0.0018
pDo	1	2278.98	2278.98	11.026	0.0011
Error	155	32037.9	206.696		
Total	158	39626.2			

**Results for factor pDo****Coefficients**

Coefficients of: mLnWT2/14 on pDo

Level of pDo	Coefficient	std. err.	t Ratio	prob
0	-4.457	1.342	-3.321	0.0011
1	4.457	1.342	3.321	0.0011

**Expected Cell Means**

Expected Cell Means of: mLnWT2/14 on pDo

Level of pDo	Expected Cell Mean	Cell Count
0	407.3	110
1	416.2	49

**Scheffe Post Hoc Tests**

	Difference	std. err.	Prob
1 - 0	8.91384	2.684	0.00112044

Figure 38: Analysis output for size effect of depo-provera on maternal weight at PP2

Interpretation: women with previous exposure to depo-provera (coded as 1) have  $8.9 \pm 2.7\%$  higher body weight ( $p=0.001$ ) compared to women without exposure taking into account HIV status and primigravidity.

#### *4.7.6. Longitudinal analysis*

Longitudinal analysis was performed in the hierarchical GLM models constructed for each variable with an individual identifier (nested by group), time point, group, and a group-by-time point interaction term (Vallenman, 1997). Scheffé posthoc tests in hierarchical repeated measures ANOVA and ANCOVA models were used to examine the differences between the predicted values of the dependent variable at different values of the specified factor (groups and time points). The Scheffé method has the property that the Type 1 error is at most  $\alpha$  for any of the possible comparisons between levels of the factor. The default  $\alpha$  (0.05) was used for each analysis. See Figure 39 for a sample model.

Hierarchical (or nested) GLM models evaluated the magnitude of within-group changes in the outcome of interest (presented as mean % change $\pm$ SE, p-value) and tested whether the changes differed significantly between the groups (gives a p-value for the group-by-time point interaction). The models also evaluated both the size and significance of between-group differences for each outcome variable for each time point (mean % difference between the groups $\pm$ SE, p-value). The mean % difference between the groups has less variation within an individual in the nested hierarchical models compared to analysis in the cross-sectional model. Therefore, the size effects for some outcomes that were not significantly different between the groups in the cross-sectional analysis become significant in the longitudinal analysis.

Adjustment for potential confounders was achieved by adding the variables into the model as co-factors to produce parsimonious models as discussed in section 4.6.5. Variables which were not anticipated to change over time (for example, parity and height) were not included in the models.

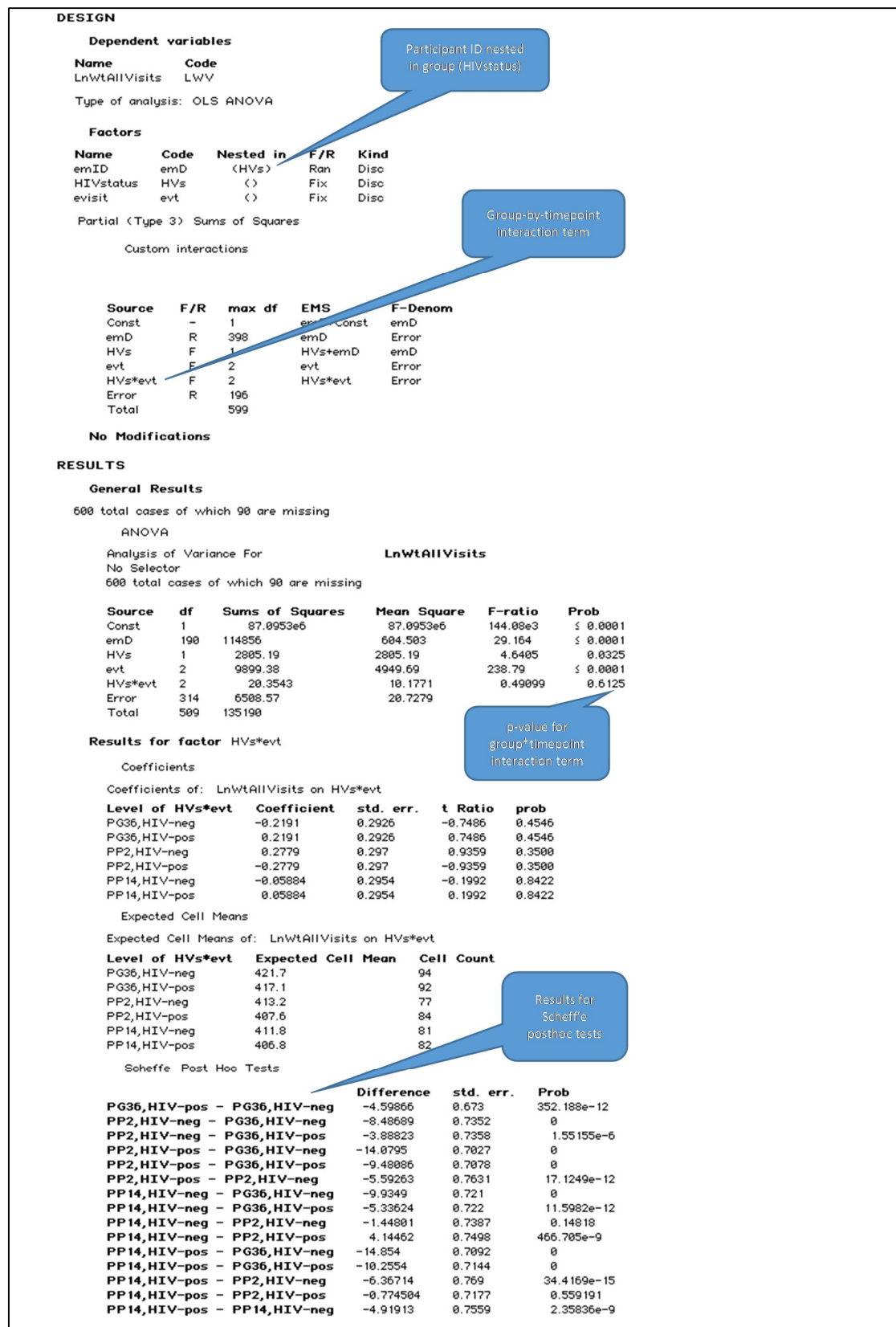


Figure 39: Sample Hierarchical model for maternal weight

DataDesk output showing nesting, group-by-time point term and associated p-value, and posthoc tests

## **CHAPTER 5: RESULTS DURING PREGNANCY**

This chapter presents participant characteristics at enrolment and outcomes at 36 weeks of gestation (PG36). For all comparisons, the reference group for maternal outcomes is the HIV-negative group. Therefore, the sign of all differences between the groups is for the comparison of HIV-positive vs HIV-negative.

All continuous outcomes at PG36 (sections 5.7 to 5.9) were adjusted for maternal age, gravidity (primigravidae vs multigravidae), gestation age at PG36 and previous use of depo-provera (yes vs no). Unadjusted mean percent differences between the groups are presented in the summary tables, and the adjusted results are discussed in the text. Unless explicitly mentioned, co-factors which are not mentioned in the text were not significant in the ANCOVA models.

### **5.1. Participant accrual**

Recruitment of study participants was carried out between January 2015 and February 2016 on a rolling basis. A total of 455 pregnant women were referred to the study by antenatal clinic staff, but 29 were no longer interested after a brief overview of the study. Four hundred and twenty-six women (210 HIV-positive and 216 HIV-negative) were assigned screening numbers and went through the study recruitment procedures as illustrated in Figure 40.

Only 399 women passed screening level 1 and were scheduled for informed consent session with the study counsellor. The scheduled women provided their phone contacts and consented for the study team to call them regarding their visit. However, 81 women did not attend their informed consent appointment and could not be traced via phone. It was discovered that these women had given wrong mobile phone numbers to both the study team and the ANC clinic. Further, 74 women did not attend informed consent appointment, and gave various reasons when contacted by the study team via phone (part 1 of Table 6).

The study successfully enrolled the target of 200 participants, 100 HIV-positive and 100 HIV-negative pregnant women, by mid-February 2016. Four women booked for enrolment in late February 2016 were informed that they were no longer needed because the target number had been reached and their appointments were cancelled. The distribution of excluded women (after screening level 2) is presented in part 2 of Table 6.

The number of women who were not enrolled into the study was not significantly different between the groups (HIV positive 52.4%, HIV-negative 53.7%). Their mean age was 24.7±4.0 years, and the median parity was one (IQR 0, 2). There was no significant difference in age and parity between HIV-positive and HIV-negative women participants and those who were eligible but were not enrolled in the study. Demographic characteristics of study participants are presented in section 5.3.

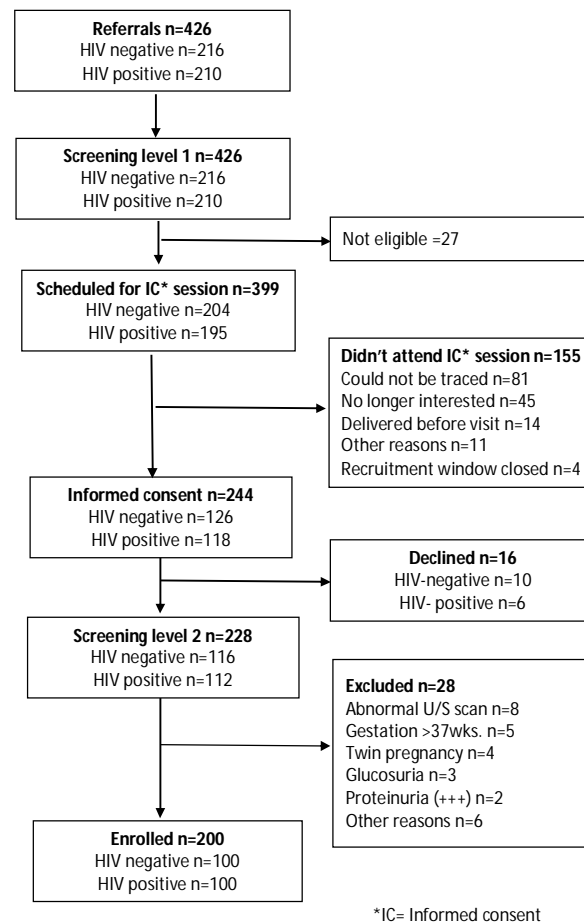


Figure 40: Flow-through of participant recruitment procedures

Table 6: Reasons why potential participants were not enrolled in the study

REASON	Total (n=226)	HIV-negative (n=116)	HIV-positive (n=110)
<b>Part 1: Did not attend informed consent (IC) session</b>	<b>182 (80.5)</b>	<b>90 (77.6)</b>	<b>92 (83.6)</b>
1.1 Not scheduled, did not meet eligibility criteria (n, %)	27 (14.8)	12 (13.3)	15 (16.3)
1.2 Missed visit and could not be traced via phone (n, %)	81 (44.5)	42 (46.7)	39 (42.4)
1.3 Not interested/busy/husband refused (n, %)	45 (24.7)	25 (27.8)	20 (21.7)
1.4 Delivered before visit (n, %)	14 (7.7)	6 (6.7)	8 (8.7)
1.5 Other social/medical reasons (n, %)	11 (6.0)	5 (5.6)	6 (6.5)
1.6 Recruitment window closed-no longer needed (n, %)	4 (2.2)	0	4 (4.3)
<b>Part 2: Attended IC session but were not enrolled</b>	<b>44 (19.5)</b>	<b>26 (22.4)</b>	<b>18 (16.4)</b>
2.1 Did not consent - (n, %)	16 (36.4)	10 (38.5)	6 (33.3)
2.2 Abnormal ultrasound scan results (n, %)	8 (18.2)	6 (23.1)	2 (11.1)
2.3 Gestation age out of range, >37 weeks (n, %)	5 (11.4)	3 (11.5)	2 (11.1)
2.4 Expecting twins (n, %)	4 (9.1)	2 (7.7)	2 (11.1)
2.5 Glycosuria (n, %)	3 (6.8)	2 (7.7)	1 (5.6)
2.6 Proteinuria +++ (n, %)	2 (4.5)	2 (7.7)	0
2.7 Other social/medical reasons (n, %)	6 (13.6)	1 (3.8)	5 (27.8)

## 5.2. Retention of participants

A total of 154 mother-baby pairs attended all study visits up to 14 weeks postpartum (PG36, PP2 and PP14). The overall attendance at each visit is presented Figure 41. A total of 154 mother-baby pairs attended PG36, PP2 and PP14 (Figure 41). The number of missed visits was highest at PP2 (16/24) because some women travelled to their parent's homes (in the village) for postnatal care, a common cultural practice in Uganda. Some women and their babies were unable to attend due to medical reasons including caesarean birth, neonatal sepsis, and prolonged hospital admission. Overall, more HIV-negative women missed their visits (15 mother-baby pairs) compared to 9 among HIV-positive women.

A total of 10 (8 HIV-positive, 2 HIV-negative) participants withdrew themselves from the study. Five HIV-positive women withdrew before their first study visit. The participants in this study were newly diagnosed with HIV hence the higher drop-out rate in the HIV-positive group could have been due to fear of home visits and potential stigmatisation in their community.

Five participants could not be traced after several attempts by the study team; so, they were declared lost to follow-up. One HIV-infected participant was excluded from the study because she had stopped breastfeeding before PP14 and had a mental health problem.



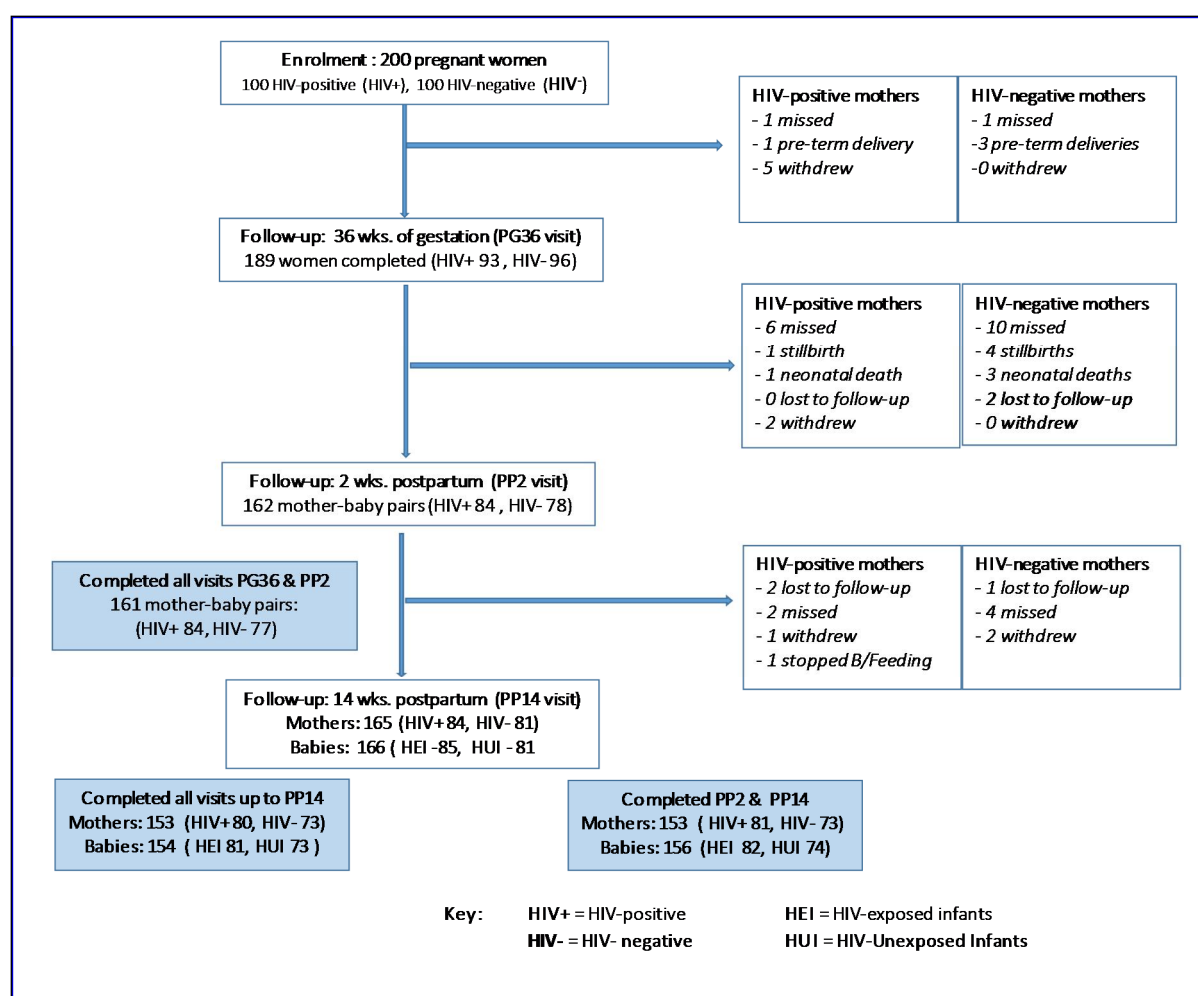


Figure 41: Study consort diagram

### 5.3. Demographic and social-economic characteristics

The median age of participants at enrolment was 24.5 (IQR 21.1, 26.9) years. There was a trend towards younger age among HIV-positive women compared to HIV-negative women, but the difference between the groups was not statistically significant ( $p=0.06$ , Table 7).

At enrolment, 46.5% of participants were primigravidae. Overall, there was a trend towards higher median gravidity among HIV-positive women compared to HIV-negative women ( $p=0.07$ ).

Significantly, fewer HIV-positive women were primigravidae compared to HIV-negative women (38.0%, vs 55.0%  $p=0.02$ ). Christianity was the most common religion practised by 71% of the participants. The distribution of participants across all religions was not significantly different between the groups. The majority (90%) of participants were married, but the proportion of married women was smaller among HIV-positive women compared to HIV-negative (85.3% vs 94.8%,  $p=0.03$ ). About half of the participants (53.6%) in both groups were housewives. There was a significant difference between groups in the distribution of the participants across maternal education ( $p=0.001$ ) and husband's education ( $p=0.004$ ).

Table 7: Baseline demographic and SES characteristics by group

Variable	HIV-negative (n=96) <sup>1</sup>	HIV-positive (n=95) <sup>1</sup>	p-value
<b>Age, yrs. (Median, IQR)</b>	24.1(22.1-27.6)	23.1 (20.6, 26.3)	0.06
<b>Parity, (median, IQR)</b>	0 (0, 1)	1 (0, 2)	0.07
0 (%)	55.6	37.5	
1 (%)	24.2	26.0	
2 (%)	8.1	18.8	
3 (%)	7.1	9.4	
4 (%)	5.1	8.3	
<b>Primigravidae (%)</b>	55.6	37.5	<b>0.02</b>
<b>Religion (%)</b>			0.6
Christian	73.2	69.5	
Muslim	26.8	30.5	
<b>Highest education attained by participant (%)</b>			<b>0.001</b>
None	2.1	3.2	
Primary	16.5	32.6	
Secondary school -O or A level	59.8	60.0	
Post-secondary	21.6	4.2	
<b>Occupation of the participant (%)</b>			0.8
Housewife	52.6	54.7	
Business	20.6	22.1	
Salaried employment	18.6	13.7	
<b>Marital status (%)</b>			<b>0.03</b>
Married	94.8	85.3	
Not married	5.21	14.7	
<b>Highest education attained by husband (%)</b>			<b>0.004</b>
None	0.0	1.2	
Primary	10.9	16.0	
Secondary school- O or A level	59.8	74.1	
Post-secondary	29.3	7.41	
<b>Occupation of husband (%)</b>			0.6
Business	46.3	48.8	
Salaried Employment	35.8	29.8	
Casual labour	17.9	19.0	

<sup>1</sup> Data includes 3 participants who missed PG36 visit (1 HIV-negative and 2 HIV-positive), information was collected retrospectively at PP2.

At the household level, the majority of the participants came from households with access to safe drinking water (80%) and electricity for lighting (91%). However, a significantly smaller proportion of HIV-positive women had access to safe drinking water compared to HIV-negative women (72.6%, vs 87.6%,  $p=0.0009$ ). Husbands were the main breadwinners for 83.9% of the households. The proportion of households provided for by husbands was smaller in the HIV-positive compared to HIV-negative group (77.9% vs 89.7%,  $p=0.03$ ), probably because fewer HIV-positive women were married. Other characteristics were not significantly different between the groups (Table 8). Three participants missed PG36, so their baseline data was collected at PP2.

Table 8: Summary of household characteristics

Variable	HIV-negative (n=97) <sup>1</sup>	HIV-positive (n=95) <sup>1</sup>	p-value
<b>Type of house (%)</b>			0.2
Detached	19.6	14.7	
Tenement (Muzigo)	78.4	85.3	
Shared house/flat/apartment	2.0	0.0	
<b>House tenure status (%)</b>			0.1
Owned	18.6	18.9	
Rented	79.4	78.9	
Supplied free by employer/someone else	2.0	2.1	
<b>Household size (median, IQR)</b>	3(2,4)	3(2,4)	0.1
<b>Number of under-fives in the household</b>	0(0,1)	0(0,1)	0.9
<b>Breadwinner for the household (%)</b>			<b>0.03</b>
Husband	89.7	77.9	
Self/Relative/other	10.3	22.1	
<b>Main source of drinking water</b>			<b>0.009</b>
Piped water/borehole	87.6	72.6	
Protected spring/well/others	12.4	27.4	
<b>Main source of lighting in the household</b>			0.4
Electricity (n, %)	92.7	89.5	
Paraffin lantern/tinlamp"taddoba"/candles	7.3	10.5	

<sup>1</sup> Data includes 3 participants who missed PG36 visit (1 HIV-negative and 2 HIV-positive), and information was collected retrospectively at PP2.

#### 5.4. Maternal health status during pregnancy

Mean gestation age at enrolment was 33.3±3.0 weeks and not significantly different between the groups (p=0.2, Table 9). All HIV-positive women were on first-line ART regimen comprising of TDF-3TC-EFV. The mean duration of ART at PG36 was 10.8±5.2 weeks. Median CD<sub>4</sub> count at initiation of ART was 394 (IQR 283, 519) cells/m<sup>3</sup> with a range of 44-970 cells/m<sup>3</sup>. Thus the majority of women had preserved immunity. Median adherence to ART was 100% (IQR 99%, 100%) based on the pill count method that is routinely used in clinical care. Viral load results were not available for participants at enrolment or at PG36. The prevailing clinical guidelines recommended the first viral load test at 6 months after initiation of ART, and thereafter annually for patients with suppressed viral load defined as <1000 copies/ml (UCG, 2016).

Complete blood counts were done women at enrolment. Mean white blood cell count was lower among HIV-positive women compared to HIV-negative women (-14.8%, p=0.003) as expected in chronically ill persons. There was a trend towards lower red blood cell count among HIV-positive women compared to HIV-negative women (-2.9%, p=0.06). Mean cell volume (MCV) was not significantly different between the groups (p=0.4).

Mean haemoglobin concentration at enrolment was  $11.6 \pm 1.1$  g/dL, and significantly lower in HIV-positive women compared to HIV-negative women ( $p=0.03$ ). Similarly, mean % haematocrit was 6.9% lower among HIV positive women compared to HIV-negative women ( $p=0.001$ ). Overall, 26.6% of the participants had a haemoglobin concentration  $<11$  g/dl in venous blood, the WHO cut-off for anaemia in pregnancy. Prevalence of anaemia was greater among HIV-positive women compared to HIV-negative women (33.0% vs 17.5%,  $p=0.03$ ) as expected in chronically ill persons.

Overall coverage for routine iron and folic acid supplementation (FEFOL) was 76.3% and significantly higher among HIV-positive women compared to HIV-negative women (83.9% vs 68.8%,  $p=0.02$ ).

Overall, 18.0% of the participants reported the use of multivitamin supplements and one HIV-positive participant was taking calcium and vitamin D supplements by 36 weeks of gestation.

More than half (57%) of the participants reported the use of at least one family planning method prior to the index pregnancy. A greater proportion of HIV-positive women had prior use of contraception compared to HIV-negative women (53.8% vs 32.3%,  $p=0.003$ ), probably because of fewer primigravidae among HIV-positive women. The common contraception methods were depo-provera injection (71.3%) and oral pills (18.8%). Again, a higher proportion of HIV-positive women reported previous use of depo-provera prior to the index pregnancy compared to HIV-negative women (44.1% vs 17.1%,  $p \leq 0.0001$ ).

None of the participants had pre-existing renal disease or diabetes mellitus at enrolment. Use of glucocorticoids was uncommon, although two HIV-positive participants were prescribed short course hydrocortisone and prednisolone to treat severe skin infections. None of the participants reported smoking or drinking alcohol during the index pregnancy.

Urinalysis was performed on the two-hour urine collection at PG36. The prevalence of urinary tract infection (UTIs) was 38.6% and not significantly different between the groups. Twenty-six women (19.4%) had protein in the urine (trace or + or ++) in the presence of a UTI but 8 women who did not have a concurrent diagnosis of a UTI. However, none of the 8 participants had protein in urine at +++ level, which is associated with a compromised renal function in the absence of a UTI. One HIV-positive participant had glucose (+) in urine.

Table 9: Maternal health status at PG36

Variable	HIV-negative (n=96)	HIV-positive (n=93)	Mean % difference±SE <sup>1</sup> (HIV+ vs. HIV-)	p-value
Weeks on ART (Mean±SD)	-	10.8±5.2	-	-
>95% adherence to ART & CTX (% , range)	-	100% (90-100)	-	-
Recent CD <sub>4</sub> , cells/mm <sup>3</sup> (Median, IQR)	-	394 (283, 519)	-	-
Gestation age at enrolment, wks. (Mean±SD)	33.6±2.7	33.0±3.4	-1.9±1.4	0.2
Gestation age at PG36 visit, wks. (Mean±SD)	36.5±0.9	36.4±0.7	-0.1±0.1	0.4
WBC (median, IQR) <sup>3</sup>	6.7 (5.7, 7.6)	5.6 (4.5, 7.2)	-14.8±4.9	<b>0.003</b>
Red Blood Cell count (Mean±SD) <sup>2</sup>	4.14±0.46	4.0±0.33	-2.9±1.6	0.06
Haemoglobin, (Mean±SD) <sup>2</sup>	12.0±1.15	11.3±1.04	-5.7±2.5	<b>0.03</b>
Hb<11 g/dL ,WHO cut-off, (%) <sup>2</sup>	17.5	33.0	-	<b>0.03</b>
Haematocrit, HCT% (Mean±SD) <sup>2</sup>	35.4±3.4	33.9±2.9	-6.9±2.1	<b>0.001</b>
MCV, (Mean±SD) <sup>2</sup>	85.8±7.0	84.7±7.0	-1.2±1.4	0.4
Any mineral/multivitamin supplement, (%)	77.4	84.9	-	0.2
FEFOL, (%)	68.8	83.9	-	<b>0.02</b>
Multivitamins, (%)	21.5	15.1	-	0.3
Vitamin D supplements, (%)	0	1	-	-
Calcium supplements, (%)	0	1	-	-
Consumed clay & clay products/herbs, (%)	50.8	21.7	-	<b>0.0003</b>
Previous use of any contraception (%)	32.3	53.8	-	<b>0.003</b>
Previous use of depo-provera injection (%)	17.1	44.1	-	<b>≤0.0001</b>
History of Smoking (%)	0.0	0.0	-	-
History of Alcohol (%)	0.0	0.0	-	-
Use of glucocorticoids, (%)	0.0	2.0 <sup>3</sup>	-	-
UTI - pus cells<5/hpf (%)	32.1	43.4	-	0.2
Proteins in urine, trace/+ /++ (%)	14.3	23.1	-	0.2
Proteins in urine in absence of UTI (%)	5.4	6.6	-	0.8

<sup>1</sup> Mean % differences between the groups presented as sympercents±SD. All variables were transformed into natural logarithms\*100 before statistical analysis using ANOVA function in Datadesk software.

<sup>2</sup> HIV-negative n= 63; HIV-positive n=91. CBC test was introduced after the study had begun

<sup>3</sup> Cream containing prednisolone prescribed for severe skin disease

## 5.5. Physical activity at PG36

The majority (>85%) of women were engaged in domestic chores and/or walking for at least 30 minutes on a daily basis. Other activities including hand washing clothes and ironing clothes were done by about 70% of women weekly. The proportions of women engaged in various physical activities were not significantly different between the groups as summarised in Table 10.

Table 10: Physical activity at PG36

Variable	HIV-negative (n=96)	HIV-positive (n=93)	p-value
<b>Cleaning the house (%)</b>			0.08
Never	3.2	10.8	
1-2 times a week	16.8	10.8	
Everyday	80.0	78.5	
<b>Hand washing utensils and pans (%)</b>			0.9
Never	7.3	6.5	
1-2 times a week	15.6	15.1	
Everyday	77.1	78.5	
<b>Hand washing clothes (%)</b>			0.9
Never	12.5	12.9	
1-2 times a week	74.0	72.0	
Everyday	13.5	15.1	
<b>Ironing clothes (%)</b>			0.2
Never	18.8	22.6	
1-2 times a week	62.5	67.7	
Everyday	18.8	9.7	
<b>Walking for at least 30 minutes (%)</b>			0.5
Never	2.1	5.3	
1-2 times a week	9.6	9.7	
Everyday	88.3	84.9	
<b>Carrying/Lifting heavy things inside or outside the house (%)</b>			0.9
Never	63.5	61.3	
1-2 times a week	27.1	28.0	
Everyday	9.4	10.8	

## 5.6. Consumption of selected calcium and vitamin D rich foods at PG36

Overall consumption of the selected food sources of calcium and vitamin D, based on food frequency questionnaire, was not significantly different between the groups (Figure 42).

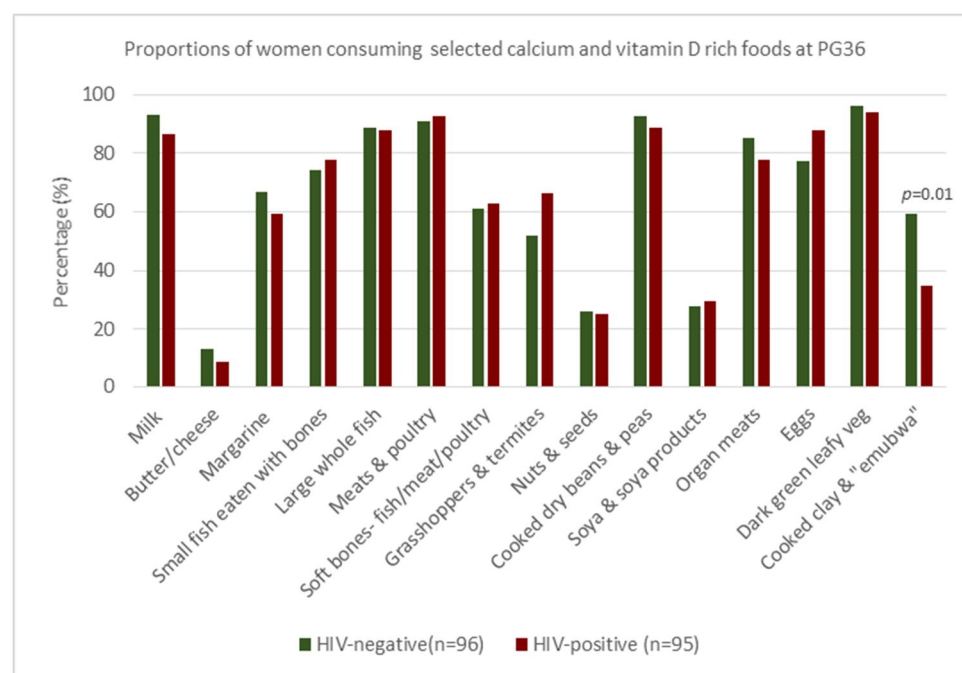


Figure 42: Consumption of selected calcium and vitamin D rich foods at PG36

The most commonly consumed calcium-rich foods were milk and milk products and cooked dry beans and peas. Small amounts of milk were regularly consumed in tea by about 90% of the participants. Over three-quarters of the participants (76.3%) reported consumption of small fish eaten with bones and there was no significant difference between the groups. Also, 62% of the participants reported regular ingestion of soft parts of bones of deep fried large fish, meat and poultry, generally not recorded as a source of calcium in this population. The proportion of women who reported eating soft parts of bones during pregnancy was not significantly different between the groups ( $p=0.1$ ).

Consumption of white cooked clay and clay products during pregnancy was common among participants. Overall, 44.4% of participants reported ingestion of clay either in the form of white cooked clay or mixed in a special herbal drink locally called *emubwa*. Significantly fewer HIV-positive women reported ingestion of white cooked clay or *emubwa*, compared to HIV-negative women (34.6% vs 59.3%,  $p=0.01$ ).

Eggs and organ meats were the most consumed vitamin D rich foods (Figure 42). Overall, consumption of vitamin D food sources was not significantly different between the groups.

## 5.7. Maternal anthropometry at PG36

Table 11: Summary of maternal anthropometry at PG36

Variable	HIV-negative (n=95) <sup>1</sup> Mean±SD	HIV-positive (n=92) <sup>1</sup> Mean±SD	Mean % Diff between the groups ±SE (HIV+ vs HIV-) <sup>2</sup>	p-value
Weight, kg	68.7±11.0	65.5±9.6	-4.6±2.2	0.03
MUAC, cm	27.2±3.4	26.7±3.0	-1.7±1.6	0.3

<sup>1</sup> Excludes two participants with missing anthropometric data at PG36 (1 HIV-positive and 1 HIV-negative).

<sup>2</sup> Mean % differences between the groups presented as sympercents±SD. All variables were transformed into natural logarithms\*100 before statistical analysis using ANOVA function in Datadesk software.

Overall, the mean height of participants was 157.4±5.7cm and not significantly different between the groups (157.0±5.0cm vs 157.8±6.3cm, p=0.3). Mean body weight at PG36 was 67.1±10.4 kg. HIV-positive women were 3.2±1.6kg (4.6%) significantly lighter compared to HIV-negative women (p=0.03, Table 11). Weight was significantly associated with both gravidity (multigravidae vs primigravidae: +5.1±2.2%, p=0.02) and previous use of depo-provera (yes vs no +6.5±2.5%, p=0.01) in the ANCOVA model. After adjusting the body weight for gravidity and previous exposure to depo-provera, the mean difference between the groups increased to -7.4±2.2% and was highly significant (p=≤0.0001).

Mean mid-upper arm circumference (MUAC) was 27.0±3.2cm, and not significantly different between the groups (-1.7%, p=0.3; Table 11). MUAC was significantly associated with gravidity in the ANCOVA model (multigravidae vs primigravidae: +3.9±1.7%, p=0.02). The difference between the groups increased to -2.4±1.7% after adjustment for gravidity but remained non-significant (p=0.1).

## 5.8. Maternal plasma and urine biochemistry at PG36

### 5.8.1. Plasma biochemistry at PG36

Overall mean plasma albumin concentration [P-Alb] was 26.5±2.4 g/L with no significant difference between the groups (-0.6±1.3, p=0.7, Table 12). However, mean [P-Alb] was below the normal range of 35-52g/L, probably due to haemodilution during pregnancy and poor nutrition status (protein deficiency). Mean [P-Alb] was negatively associated with gravidity (multigravidae vs primigravidae: -4.3±1.3%, p=0.001) and maternal age (β= -0.31±0.14% per year, p=0.04) in the ANCOVA model. Adjusting plasma albumin concentration for gravidity and maternal age reduced the difference between the groups (-0.1±1.3%, p=0.9).



Mean plasma calcium concentration [P-Ca], uncorrected for albumin, was  $2.19 \pm 0.08$  mmol/l and significantly lower among HIV-positive women compared to HIV-negative women (-1.2%,  $p=0.03$ , Table 12). Similarly, mean corrected [P-Ca] (corrected for albumin by Payne equation) was  $2.46 \pm 0.08$  mmol/l and significantly lower among HIV-positive women compared to HIV-negative women (-1.0%  $p=0.02$ ). Gravidity was significantly associated with uncorrected [P-Ca] (multigravidae vs primigravidae:  $-1.7 \pm 0.5\%$ ,  $p=0.002$ ) in the ANCOVA model. The mean percent difference between the groups in uncorrected P-Ca reduced after adjustment for gravidity and was no longer significant ( $-0.9 \pm 0.5\%$ ,  $p=0.09$ ).

Mean plasma phosphate concentration [PO<sub>4</sub>] was  $1.20 \pm 0.19$  mmol/L and significantly lower among HIV-positive women compared to HIV-negative women (-8.7%,  $p \leq 0.0001$ ). Mean plasma concentrations of creatinine [P-Cr] and magnesium [P-Mg] were  $63.3 \pm 7.71$   $\mu$ mol/l and  $0.74 \pm 0.06$  mmol/L, respectively, with no significant differences between the groups. Maternal age, gestation age at PG36, gravidity and previous use of depo-provera did not have significant effects on plasma phosphate, creatinine or magnesium concentrations in the models.

One HIV-positive participant had an extremely high [P-Mg] (1.25mmol/L); conducting the analysis with or without this subject did not have a material effect on the results (the difference between the groups reduced to  $+0.8 \pm 0.9$ ,  $p=0.4$ ). ARVs contain magnesium stearate as a filler, so it is possible the participant had swallowed her drugs in the morning close to the time of blood sample collection.

### *5.8.2. Urine biochemistry at PG36*

Overall median urine calcium/creatinine ratio (UCa/Cr) was 0.034 (IQR 0.014, 0.067) mmol/mmol and not significantly different between the groups ( $p=0.4$ , Table 12). UCa/Cr was not associated with maternal age, gravidity, gestation age or previous use of depo-provera in the ANCOVA model.

Overall median urine phosphate: creatinine ratio (UP/Cr) was 1.25 (IQR 0.950, 1.61) mmol/mmol, and not significantly different between the groups ( $p=0.7$ ). UP/Cr was significantly associated with gravidity (multigravidae vs primigravidae:  $-17.6 \pm 6.7\%$ ,  $p=0.009$ ) in the ANCOVA model. Adjusting (UP/Cr) for gravidity did not have a material effect on the mean difference between the groups (decreased to  $+0.8 \pm 6.9\%$ ,  $p=0.9$ ).

Overall median urine magnesium: creatinine ratio (UMg/Cr) was 0.189 (IQR 0.144, 0.254) mmol/mmol and not significantly different between the groups ( $p=0.3$ ). Maternal age, gestation age at PG36, gravidity and previous use of depo-provera did not have significant effects on UMg/Cr in the ANCOVA model.

Table 12: Summary of plasma and urine biochemistry at PG36

<i>Variables</i>	<b>HIV Negative (n=96)</b>	<b>HIV Positive (n=93)</b>	<b>Mean % Diff between the groups <math>\pm</math>SE (HIV+ vs HIV-)<sup>1</sup></b>	<b>Group P-value</b>
<b><i>Plasma</i></b>				
Albumin, g/L (Mean $\pm$ SD)	26.54 $\pm$ 1.97	26.47 $\pm$ 2.81	-0.6 $\pm$ 1.3	0.7
Calcium <sub>uncorrected</sub> , mmol/L (Mean $\pm$ SD) <sup>2</sup>	2.20 $\pm$ 0.08	2.17 $\pm$ 0.09	<b>-1.2<math>\pm</math>0.6</b>	<b>0.03</b>
Calcium <sub>Corrected</sub> , mmol/L (Mean $\pm$ SD) <sup>3</sup>	2.47 $\pm$ 0.07	2.44 $\pm$ 0.09	<b>-1.0<math>\pm</math>0.5</b>	<b>0.02</b>
Phosphate, mmol/L (Mean $\pm$ SD)	1.22 $\pm$ 0.16	1.12 $\pm$ 0.18	<b>-8.6<math>\pm</math>2.1</b>	<b><math>\leq</math>0.0001</b>
Magnesium, mmol/L (Mean $\pm$ SD)	0.736 $\pm$ 0.062	0.748 $\pm$ 0.082	+1.4 $\pm$ 1.3	0.3
Creatinine, $\mu$ mol/L (Mean $\pm$ SD)	62.9 $\pm$ 8.0	63.8 $\pm$ 7.43	+1.5 $\pm$ 1.8	0.4
<b><i>Urine</i><sup>4,5,7</sup></b>				
UCa/Cr mmol/mmol (Median, IQR) <sup>8</sup>	0.034 (0.017, 0.069)	0.032 (0.014, 0.066)	-15.0 $\pm$ 17.2	0.4
UP/Cr, mmol/mmol (Median, IQR) <sup>8</sup>	1.27 (0.966, 1.60)	1.21 (0.870, 1.66)	-2.2 $\pm$ 6.7	0.7
UMg/Cr, mmol/mmol (Median, IQR) <sup>8</sup>	0.197 (0.145, 0.259)	0.182 (0.143, 0.254)	-8.1 $\pm$ 7.1	0.3
<b><i>Renal parameters</i></b>				
eGFR, ml/min/1.73 m <sup>2</sup> (Median, IQR) <sup>9</sup>	126 (121, 131)	127 (122, 130)	+0.1 $\pm$ 1.0	0.9
TmP/GFR, mmol/L GFR (Mean $\pm$ SD)	1.35 $\pm$ 0.25	1.22 $\pm$ 0.27	<b>-11.4<math>\pm</math>3.1</b>	<b><math>\leq</math>0.0001</b>
TmCa/GFR, mmol/L GFR (Mean $\pm$ SD) <sup>10</sup>	1.24 $\pm$ 0.05	1.23 $\pm$ 0.05	<b>-1.4<math>\pm</math>0.6</b>	<b>0.02</b>

<sup>1</sup> Mean % differences between the groups presented as sympercents $\pm$ SD. All variables were transformed into natural logarithms\*100 before statistical analysis using ANOVA function in Datadesk software.

<sup>2</sup> Excludes data for one HIV-positive participant with an extreme value (7.53 mmol/L) which influences results.

<sup>3</sup> Plasma calcium normalised for albumin using Payne equation: Adjusted Serum Ca= Measured Ca+ ([40-serum albumin]\*0.02)

<sup>4</sup> Excludes data for one HIV-positive participant who did not collect a urine sample at the end of two-hour fasting protocol.

<sup>5</sup> Seven measurements below the detection limit (0.02 mmol/L) were assigned half a detection (0.01mmol/L) before analysis. These results are not significantly different to analysis with the original/unadjusted concentrations.

<sup>7</sup> IQR = inter quartile range presented as 25<sup>th</sup> and 75<sup>th</sup> quartiles.

<sup>8</sup> Urine UCa/Cr calcium/creatinine ratio, UP/Cr phosphate/creatinine ratio and UMg/Cr magnesium/creatinine ratio.

<sup>9</sup> Calculated using CKD-EPI equation: eGFR (ml/min/1.73m<sup>2</sup>) = 144 x min (PCr/0.7)-0.329 x 0.993Age if [PCr] is  $\leq$ 0.7; and eGFR (ml/min/1.73m<sup>2</sup>) = 144 x min (PCr/0.7)<sup>-1.209</sup> x 0.993Age if [PCr] is  $\geq$ 0.7. Data excludes two extreme values which influence the group\*visit interaction

<sup>10</sup> Excludes 3 extreme values (two in HIV-positive group; one in HIV-negative group)

### 5.8.3. Renal bone mineral handling at PG36

Overall median estimated glomerular filtration rate (eGFR) was 125 (IQR 121, 130) mL/min/1.73 m<sup>2</sup>, based on CKD-EPI equation, and was not significantly different between the groups ( $p=0.9$ , Table 12). eGFR was negatively associated with maternal age ( $\beta= -0.8\pm0.1\%$  per year,  $p\leq0.0001$ ) in the ANCOVA model. Adjusting eGFR for maternal age did not have a material effect on the mean difference between the groups ( $-0.8\pm0.9\%$ ,  $p=0.4$ ).

The overall mean renal tubular maxima for phosphate (TmP/GFR) and calcium (TmCa/GFR) were  $1.37\pm0.29$  mmol/L and  $1.24\pm0.06$  mmol/L, respectively. HIV-positive women had significantly lower TmP/GFR ( $-11.4\pm3.1\%$ ,  $p\leq0.0001$ ) and TmCa/GFR ( $-1.4\pm0.6\%$ ,  $p=0.02$ ) compared to HIV-negative women (Table 12). Maternal age, gravidity, gestation age at PG36, and previous use of depo-provera did not have significant effects on both TmP/GFR and TmCa/GFR in the models.

## 5.9. Maternal 25(OH)D, PTH and bone turnover markers

Table 13: Concentrations of calciotropic hormones and bone turnover markers at PG36

Variable	HIV-negative (n=96)	HIV-positive (n=93)	Mean % Diff between the groups $\pm$ SE (HIV+ vs HIV-) <sup>1</sup>	p-value
Serum 25(OH)D, nmol/L (Median, IQR)	65.9 (55.0, 76.9)	71.8 (52.3, 89.4)	+3.9 $\pm$ 5.2	0.5
Serum 25(OH)D <50nmol/L (n, %) <sup>2</sup>	13 (13.5)	20 (21.5)	-	0.2
Plasma PTH, pg/mL (Median, IQR)	23.1 (15.8, 35.1)	33.6 (23.6, 44.9)	<b>+33.0<math>\pm</math>7.0</b>	<b><math>\leq0.0001</math></b>
Plasma CTX, ng/mL (Median, IQR)	0.549 (0.433, 0.659)	0.507 (0.367, 0.723)	-3.0 $\pm$ 6.5	0.6
Plasma P1NP, ng/mL (Median, IQR)	72.4 (52.4, 103)	53.7 (38.5, 80.2)	<b>-25.7<math>\pm</math>6.8</b>	<b><math>\leq0.0001</math></b>
Plasma BAP, $\mu$ g/L (Mean $\pm$ SD)	20.5 $\pm$ 7.6	21.6 $\pm$ 9.6	+3.3 $\pm$ 5.5	0.5
Plasma TAP, U/L (Median, IQR)	147 (118, 196)	149 (110, 182)	-6.3 $\pm$ 5.3	0.2
Plasma BAP/TAP (Mean $\pm$ SD)	13.4 $\pm$ 0.1	14.8 $\pm$ 0.1	9.6 $\pm$ 5.5	0.08

<sup>1</sup> Mean % differences between the groups presented as sympercents $\pm$ SD. All variables were transformed into natural logarithms\*100 before statistical analysis using ANOVA function in Datadesk software.

<sup>2</sup> Proportion (n, %)

#### 5.9.1. Serum 25(OH)D at PG36

Overall median serum 25 hydroxyvitamin D concentration [25(OH)D] was 67.4 (54.8, 83.7) nmol/L and there was no significant difference between the groups ( $p=0.5$ , Table 13). Thirty-three participants (17.5 %) had serum [25(OH)D] below 50nmol/L, and their distribution was not significantly different across the groups. None of the participants had a serum [25(OH)D] below 25nmol/L. Serum [25(OH)D] was negatively associated with gestation age ( $\beta -2.0\pm0.9\%$  per week,  $p=0.02$ ) in the ANCOVA model. Adjusting [25(OH)D] for gestation age did not have a material effect on the mean percent difference between the groups ( $+2.2\pm5.1\%$ ,  $p=0.7$ ).

#### 5.9.2. PTH at PG36

Overall median plasma parathyroid hormone concentration [PTH] was 28.1 (IQR 19.6-40.9) pg/mL and significantly higher among HIV-positive compared to HIV-negative women ( $+26.4\%$ ,  $p\leq0.0001$ , Table 13). Maternal age, gravidity, and prior use of depo-provera did not have significant effects on plasma [PTH] in the ANCOVA model.

#### 5.9.3. CTX at PG36

Overall median plasma C-terminal cross-linked telopeptides of collagen I concentration [ $\beta$ -CTX] was 0.528 ng/mL (IQR 0.391-0.686) and not significantly different between the groups ( $p=0.6$ , Table 13). [ $\beta$ -CTX] was negatively associated with both gravidity (multigravidae vs primigravidae:  $-23.0\pm6.5\%$ ,  $p\leq0.0001$ ) and previous use of depo-provera (yes vs no:  $+17.9\pm7.3\%$ ,  $p=0.02$ ) in the ANCOVA model. The mean difference between the groups was greater after adjusting [ $\beta$ -CTX] for gravidity and previous use of depo-provera, but remained non-significant ( $+5.7\pm6.4\%$ ,  $p=0.4$ ).

#### 5.9.4. P1NP at PG36

Overall median procollagen type 1 N-terminal propeptide) concentration [P1NP] was 63.7 ng/mL (IQR 43.8-91.0) and significantly lower in HIV-positive compared to HIV-negative women ( $-26.4\%$ ,  $p\leq0.0001$ , Table 13). Plasma [P1NP] was negatively associated with gravidity (multigravidae vs primigravidae:  $-22.6\pm6.7\%$ ,  $p\leq0.0001$ ) in the ANCOVA model. The mean difference between the groups reduced after adjusting plasma [P1NP] for gravidity but remained significant ( $-1.6\pm6.7\%$ ,  $p=0.002$ ).

#### 5.9.5. TAP and BAP at PG36

Overall median total alkaline phosphatase concentration [TAP] was 148 (IQR 115, 191) U/L and not significantly different between the groups ( $p=0.2$ , see Table 13). Plasma [TAP] was significantly associated with maternal age ( $\beta +1.2\pm0.6\%$  per year,  $p=0.05$ ) in the ANCOVA model. The mean difference between the groups increased after adjusting [TAP] for maternal age but remained non-significant ( $+5.0\pm5.3\%$ ,  $p=0.3$ ).

Overall mean bone-specific alkaline phosphatase concentration [BAP] was  $21.0\pm8.6$  pg/mL, and there was no significant difference between the groups ( $p=0.5$ , see Table 13). Plasma [BAP] was negatively associated with gravidity (multigravidae vs primigravidae:  $-17.7\pm5.4\%$ ,  $p=0.001$ ) in the ANCOVA model. The mean difference between the groups increased after adjusting [BAP] for gravidity but remained non-significant ( $+6.6\pm5.7\%$ ,  $p=0.2$ ).

Overall mean percentage of [BAP] in [TAP] was  $14.1\pm4.9\%$ . There was a trend towards a higher BAP/TAP % among HIV-positive compared to HIV-negative women although the difference between the groups was not statistically significant ( $+9.6\pm5.5\%$ ,  $p=0.08$ ; see Table 13). Gravidity, maternal age, gestation age at PG36, and previous use of depo-provera did not have a significant effect on plasma BAP/TAP % in the ANCOVA model.

### 5.10. Summary of results at PG36

Median maternal age was 24.5 (IQR 21.1, 26.9) years, and not significantly different between the groups. Overall 47% of the participants were primigravidae. A significantly smaller proportion of HIV-positive women were primigravidae compared to HIV-negative women (38% vs 55%,  $p=0.002$ ). Overall, 71% of the women reported previous use of contraception and depo-provera injection was the most preferred method. Previous exposure to depo-provera was significantly higher among HIV-positive compared to HIV-negative women (44% vs 17%,  $p\leq 0.0001$ ). Consumption of calcium and vitamin D rich foods was comparable between the groups. However, a smaller proportion of HIV-positive women reported consumption of clay and clay products/herbs "*emubwa*" compared to HIV-negative women (35% vs 59%,  $p=0.01$ ).

Maternal height was not significantly different between the groups. However, body weight was 5% (~3.4 kg) significantly lower in the HIV-positive group ( $p=0.03$ ). Primigravidae women had 5.1% significantly lower weight compared to multigravidae, and exposure to depo-provera was associated with 6.5% more weight compared to no-exposure. After adjusting for parity and previous use of depo-provera, HIV-positive women had 7% lower weight, and the difference between the groups was highly significant ( $p\leq 0.0001$ ).

HIV-positive women had 1% significantly lower corrected [P-Ca], but the difference between the groups was not significant after adjustment for gravidity (-0.9%,  $p=0.09$ ). Plasma  $PO_4$ , TmP/GFR and TmCa/GFR were significantly lower in HIV-positive women (-9% and -11%, respectively). However, eGFR and other plasma and urine biomarkers were not significantly different between the groups. Mean plasma PTH concentration was 33% higher but mean [P1NP] was 26% lower among HIV-positive women. Mean plasma  $\beta$ -CTX, BAP, TAP and serum 25(OH)D concentrations were not significantly different between the groups. None of the participants had serum 25(OH)D below 25nmol/L. With the exception of [P-Ca], the differences between the groups in plasma and urine biomarkers were not explained by maternal age, gravidity, gestation age or previous use of depo-provera.

## **CHAPTER 6: RESULTS AT TWO WEEKS POSTPARTUM (PP2)**

This chapter presents both maternal (sections 6.6 to 6.8) and infant outcomes (sections 6.9 to 6.10) at 2 weeks postpartum (PP2). For all comparisons, the reference group for maternal outcomes is the HIV-negative group and the reference group for infant outcomes was the HUI. Therefore, the sign of all differences between the groups is for the comparison of HIV-positive vs HIV-negative (sections 6.6 to 6.8) or HEI vs HUI (sections 6.9 to 6.10).

All continuous maternal outcomes were adjusted for infant's age (days) and sex (boys/girls), exclusive breastfeeding (yes/no), maternal age (yrs.), parity (multiparae/primiparae), gestation age (weeks, based on obstetric ultrasound scan at enrolment), previous use of depo-provera (yes vs. no). All infant outcomes were adjusted for gestation age at birth (weeks), sex (male vs. female) birth order (firstborn yes vs no) maternal age (years), exclusive breastfeeding (yes/no) and having been ill within 2 weeks prior to study visit (yes/no).

Unadjusted mean percent differences between the groups are presented in the summary tables, and the adjusted results from parsimonious models are discussed in the text. All models contained a group term. Unless explicitly mentioned, co-factors which are not mentioned in the text were not significant in the fully adjusted models, and so they were excluded from the reported parsimonious models.

### **6.1. Birth outcomes**

A total of 191 women (97 HIV-positive, 94 HIV-negative) were active in the study at the end of PG36 and carried their pregnancy to term. Five women had stillbirths (1 HIV-positive women and 4 HIV-negative) and were no longer involved in the study as per the protocol. A total of 186 women had live births at term (96 HIV-positive and 90 HIV-negative women) and 5.5% of the deliveries were by caesarean section.

### **6.2. Attendance at PP2**

One HIV-exposed infant (HEI) and 3 HIV-unexposed infants (HUI) died within their first week of life, so these mother-baby pairs were no longer involved in the study. The remaining 182 mother-baby pairs were booked for a study visit at 2 weeks postpartum (PP2). Of these, 16 mother-baby pairs did not attend the visit due to various reasons. In addition, 2 pairs could not be traced by either phone or home visit, and 2 pairs withdrew themselves from the study. Eventually, 162 mother-baby pairs attended the PP2 visit (84 HIV-positive and 78 HIV-negative).

### 6.3. Infant health status and breastfeeding

Overall mean age of infants at the PP2 visit was  $2.1 \pm 0.4$  weeks, and there was a trend towards a younger mean age among HUI ( $p=0.07$ , Table 14). Mean gestation age and birth weight were  $40.9 \pm 1.8$  weeks and  $3.3 \pm 0.5$ kg, respectively, and not significantly different between the groups. Overall, 49.5% of the infants were male, and their sex distribution was also not significantly different between the groups (Table 14).

All infants were breastfeeding at PP2 visit. Overall, 71.9% of the infants had initiated breastfeeding within the first one hour of birth. However, only 71.3% of the infants were exclusively breastfed within the first 2 weeks of life. A greater proportion of HEI was exclusively breastfed compared to HUI (82.9% vs 58.7%,  $p=0.0008$ ). The most common drink offered to mixed-fed babies was plain water (26.1%), although 3 HUI were also offered glucose water and formula milk.

Overall, 15.5% of the infants were reported to have been ill within the first 2 weeks of life. Significantly fewer HEI were reported to have been sick compared to HUI (9.5% vs 22.1%,  $p=0.03$ ). Three percent of the infants were given over the counter multivitamin syrups. Overall, 90% of the infants had received the relevant immunisations (polio 0 and BCG) as per the national immunisation schedule. All HEI were on Nevirapine (NVP) syrup from birth as per the national PMTCT Option B-plus guidelines.

Table 14 Infant health and breastfeeding at PP2

Variable	HIV-unexposed (n=78)	HIV-exposed (n=84)	p-value
Age at PP2 visit, wks. (Mean $\pm$ SD)	2.1 $\pm$ 0.4	2.2 $\pm$ 0.5	0.07
Gestation age at birth, wks. (Mean $\pm$ SD)	40.8 $\pm$ 1.7	40.9 $\pm$ 2.0	0.8
Birth weight, kg (Mean $\pm$ SD) <sup>1</sup>	3.3 $\pm$ 0.6	3.3 $\pm$ 0.5	0.6
Sex, Male (%)	50.6	48.3	0.8
Initiation of breastfeeding with 1hour (%)	65.8	77.4	0.1
Lactogenesis II within 72 hours (%)	93.2	96.4	0.4
Exclusive breastfeeding (%)	58.7	82.9	<b>0.0008</b>
Been ill since birth (%)	22.1	9.5	<b>0.03</b>
Had a complete immunisation record (%)	86.8	92.9	0.2
Multivitamins (n, %)	3 (3.8)	3(3.8)	0.9

<sup>1</sup> birth weight was obtained from maternity discharge form where available. n= 71 HIV-unexposed infants and 79 HIV-exposed infants



#### **6.4. Maternal health status at PP2**

The mean duration of ART was  $17.4 \pm 5.7$  (range 7.6 - 32.6) weeks. Median CD<sub>4</sub> count was 400 (IQR 301-515; range 51-970) cells/m<sup>3</sup>. Median adherence to ART at PP2 100% (IQR 100%, 100%) based on pill counts.

All mother-baby pairs were breastfeeding. Overall, 94.9% of the mothers reported copious milk secretion (lactogenesis II) within the first 72 hours after delivery, and their distribution was not significantly different between the groups (Table 14). However, only 23.8% of the women had received postpartum vitamin A supplementation as per the national recommendation. Sixty percent of the women were still taking iron and folate supplements, and there was no significant difference between the groups (22.9% vs 24.7%  $p=0.09$ ). None of the participants reported taking vitamin D or calcium supplements, drinking alcohol or smoking.

#### **6.5. Consumption of calcium and vitamin D rich foods at PP2**

Overall, the most commonly consumed calcium-rich foods were milk, small fish eaten with bones, and cooked dry beans and peas (Figure 43). Consumption of milk and small fish eaten with bones was not significantly different between the groups. However, a significantly greater proportion of HIV-positive women reported consumption of cooked beans and peas compared to HIV-negative women (82.9% vs 65.4%,  $p=0.02$ ).

Unlike PG36, only 6 women reported consumption of cooked clay/clay products (Figure 43). A smaller proportion of HIV-positive women reported consumption of nuts and seeds compared to HIV-negative women (9.5% vs 21.8%,  $p=0.03$ ). However, consumption of grasshoppers “nseenene” and termites was higher among HIV-positive women (16.7% vs 3.9%,  $p=0.008$ ).

Overall, consumption of food sources of vitamin D was not significantly different between the groups (Figure 43). The most commonly consumed food sources of vitamin D were organ meats and eggs. However, there was a trend towards lower consumption of vitamin D fortified margarine among HIV-positive women compared to HIV-negative women (25.0% vs 39.7%,  $p=0.05$ ).

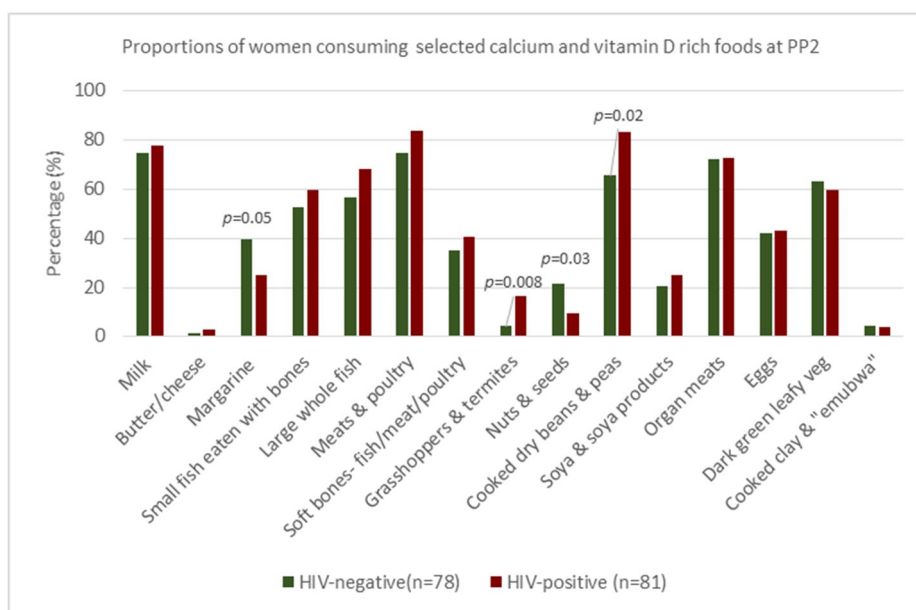


Figure 43: Consumption of selected calcium and vitamin D rich foods at PP2

## 6.6. Maternal anthropometry and body composition at PP2

### 6.6.1. Maternal anthropometry at PP2

Overall mean body weight was  $61.4 \pm 10.2$  kg, but HIV-positive women had significantly lower weight compared to HIV-negative women ( $-5.2\%$  ( $3.6 \pm 0.5$  kg)  $p=0.04$ , Table 15). Weight was significantly associated with parity (multiparae vs primiparae:  $4.6 \pm 1.6$  kg,  $p=0.004$ ), previous use of depo-provera (yes vs no:  $+5.7 \pm 1.7$  kg,  $p \leq 0.0001$ ) and gestation age at delivery ( $\beta = +0.8 \pm 0.3$  kg per week,  $p=0.05$ ) in the ANCOVA model. After adjusting body weight for parity and previous exposure to depo-provera, the mean difference between the groups increased to  $-8.6 \pm 2.3\%$  and was highly significant ( $p \leq 0.0001$ ).

Overall mean BMI was  $24.8 \pm 4.0$  kg/m<sup>2</sup> and not significantly different between the groups ( $p=0.2$ , Table 15). BMI was significantly associated with parity (multiparae vs primiparae:  $+2.0 \pm 0.6$  kg/m<sup>2</sup>,  $p \leq 0.0001$ ), previous use of depo-provera (yes vs no:  $+1.7 \pm 0.7$  kg/m<sup>2</sup>,  $p=0.01$ ) and gestation age at delivery ( $\beta = +0.3 \pm 0.2$  kg/m<sup>2</sup> per week,  $p=0.03$ ) in the ANCOVA model. After adjusting BMI for parity and previous exposure to depo-provera, the mean difference between the groups increased to  $-6.5 \pm 2.6\%$  and was highly significant ( $p=0.01$ ).

Overall mean MUAC was  $26.8 \pm 3.2$  cm and not significantly different between the groups ( $p=0.2$ , Table 15). MUAC was significantly associated with both parity (multiparae vs. primiparae:  $+7.1 \pm 1.8\%$ ,  $p \leq 0.0001$ ) and previous use of depo-provera (yes vs no:  $+4.6 \pm 2.0\%$ ,  $p=0.02$ ) in the ANCOVA model.

After adjusting MUAC for parity and previous exposure to depo-provera, the mean difference between the groups increased to  $-4.6 \pm 2.0\%$  and became significant ( $p=0.01$ ).

Table 15 Summary of maternal anthropometry and body composition at PP2

Variable	HIV-negative (n=78) Mean $\pm$ SD	HIV-positive (n=84) Mean $\pm$ SD	Mean % Diff between the groups $\pm$ SE (HIV+ vs HIV-) <sup>1</sup>	p-value
<b>Anthropometry</b>				
Weight, kg	63.2 $\pm$ 11.1	59.8 $\pm$ 9.0	<b>-5.2<math>\pm</math>2.5</b>	<b>0.04</b>
BMI, kg/m <sup>2</sup>	25.2 $\pm$ 4.2	24.4 $\pm$ 3.8	-3.3 $\pm$ 2.3	0.2
MUAC, cm	27.1 $\pm$ 3.4	26.5 $\pm$ 2.9	-2.2 $\pm$ 1.8	0.2
<b>WBLH Composition by DXA</b>				
Lean Mass, kg	36.9 $\pm$ 5.4	35.4 $\pm$ 5.1	-4.0 $\pm$ 2.3	0.08
Fat Mass, kg	20.8 $\pm$ 8.0	19.2 $\pm$ 7.0	-7.0 $\pm$ 6.2	0.3
% Fat	35.2 $\pm$ 8.7	34.5 $\pm$ 8.2	+2.8 $\pm$ 5.8	0.6
Fat:Lean <sup>2</sup> ratio (10 <sup>-3</sup> x kg/kg <sup>2</sup> )	15.9 $\pm$ 6.5	16.1 $\pm$ 6.7	+1.0 $\pm$ 7.4	0.9

<sup>1</sup> Mean % differences between the groups presented as sympercents $\pm$ SE. All variables were transformed into natural logarithms\*100 before statistical analysis using the ANOVA function in Datadesk software.

#### 6.6.2. Maternal whole body composition measured by DXA at PP2

Overall total whole body-less-head (WBLH) lean mass was 36.1 $\pm$ 5.3kg. There was a trend towards lower lean mass among HIV-positive women compared to HIV negative women, although the difference between the groups was not statistically significant ( $-4.0 \pm 2.3\%$ ,  $p=0.08$ ; Table 15). Lean mass was significantly associated with both parity (multipara vs primiparae:  $+5.8 \pm 2.3\%$ ,  $p=0.02$ ) and previous use of depo-provera (yes vs no:  $+6.1 \pm 2.6\%$ ,  $p=0.02$ ) in the ANCOVA model. After adjusting for parity and previous exposure to depo-provera, the difference in lean mass increased to  $6.3 \pm 2.3\%$ , and the trend towards lower lean mass in HIV-positive women persisted ( $p=0.05$ ).

Overall mean total WBLH fat mass was 20.0 $\pm$ 7.5kg, and not significantly different between the groups ( $p=0.3$ ). Fat mass was significantly associated with previous use of depo-provera in the ANCOVA model (yes vs no:  $+20.5 \pm 6.9\%$ ,  $p=0.003$ ). After adjustment for previous exposure to depo-provera, HIV-positive women had significantly lower fat mass compared to HIV-negative women ( $-11.7 \pm 6.3\%$ ,  $p=0.006$ ).

Overall mean fat:lean<sup>2</sup> ratio was 16.0 $\pm$ 6.7 (10<sup>-3</sup> x kg/kg<sup>2</sup>) and not significantly different between the groups ( $p=0.9$ ). Maternal age, parity, previous use of depo-provera, exclusive breastfeeding, gestation age at delivery, and sex of the infant did not have significant effects on fat:lean<sup>2</sup> ratio in the ANCOVA model.

## 6.7. Breast milk mineral content at PP2

Table 16: Breast milk mineral content at PP2

Variable	HIV-negative (n=78)	HIV-positive (n=84)	Mean % Diff between the groups $\pm$ SE (HIV+ vs HIV-) <sup>1</sup>	p-value
Calcium, mmol/L (Mean $\pm$ SD)	5.61 $\pm$ 1.03	5.74 $\pm$ 1.12	+2.0 $\pm$ 3.3	0.5
Phosphorus, mmol/L (Mean $\pm$ SD)	4.41 $\pm$ 1.00	4.86 $\pm$ 1.15	<b>+9.7<math>\pm</math>3.8</b>	<b>0.01</b>
Calcium/phosphate ratio (Mean $\pm$ SD)	1.32 $\pm$ 0.29	1.21 $\pm$ 0.23	<b>-7.7<math>\pm</math>3.2</b>	<b>0.02</b>
Sodium, mmol/L (Median, IQR) <sup>2</sup>	8.4 (6.4, 10.3)	7.25 (5.25, 10.1)	-15.2 $\pm$ 8.9	0.09
Potassium, mmol/L (Mean $\pm$ SD)	16.6 $\pm$ 2.9	16.7 $\pm$ 3.2	+0.7 $\pm$ 3.2	0.8
Sodium/Potassium ratio (Median, IQR)	0.447 (0.375, 0.647)	0.348 (0.297, 0.641)	-16.0 $\pm$ 10.9	0.1
Na/K ratio > 1.0 (n, %)	11 (14.5)	10 (11.9)	-	0.6

<sup>1</sup> Mean % differences between the groups presented as sympercents $\pm$ SE. All variables were transformed into natural logarithms\*100 before statistical analysis using the ANOVA function in Datadesk software.

### 6.7.1. Breast milk calcium and phosphorus at PP2

Overall mean breast milk phosphorus concentration [BM-P] was 4.64 $\pm$ 1.10 mmol/L and significantly higher among HIV-positive women compared to HIV-negative women (+9.7%, p=0.01). Mean breast milk calcium concentration [BM-Ca] was 5.68 $\pm$ 1.08 mmol/L and not significantly different between the groups (p=0.5, Table 16). Mean breast milk calcium-phosphorus ratio (BM Ca/P) was 1.26 $\pm$ 0.26 and lower among HIV-positive women compared to HIV-negative (-7.7%, p=0.02).

Maternal age, parity, previous use of depo-provera, exclusive breastfeeding, gestation age at delivery, and sex of the infant did not have significant effects on [BM-P], [BM-Ca] or Ca/P in the ANCOVA models.

### 6.7.2. Breast sodium and potassium at PP2

Overall median breast milk sodium concentration [BM-Na] was 7.60 mmol/L (IQR 5.60-10.2 mmol/L). There was a trend towards lower mean [BM-Na] among HIV-positive women compared to HIV-negative women, but the difference between the groups was not significant (-15.2 $\pm$ 8.9%, p=0.09; Table 16). Mean breast milk potassium concentration [BM-K] was 16.6 $\pm$ 3.0 mmol/L and not significantly different between the groups (p=0.8).

Median sodium-potassium ratio (BM Na/K) was 0.454 (IQR 0.338,-0.658 mmol/L). There was a -16.0 $\pm$ 10.9% difference in BM Na/K between the groups, but it was not significant (p=0.1). The overall prevalence of BM Na/K above 1.0, a diagnostic criterion for sub-clinical mastitis, was 13% and there was no significant difference between the groups (p=0.6).

Overall, maternal age, parity, previous use of depo-provera, gestation age at delivery, exclusive breastfeeding, and sex of the infant did not have significant effects on [BM-Na], [BM-K] or Na/K in the respective ANCOVA models.

## 6.8. Maternal bone mineral at PP2

Table 17: Maternal bone parameters at PP2

Variable	HIV-Negative (n=77) Mean±SD	HIV-Positive (n=84) Mean±SD	Mean % Diff between the groups ±SE (HIV+ vs HIV-) <sup>1</sup>	P-value
<b>Whole Body Less Head (WBLH)<sup>2</sup></b>				
Bone Mineral Content, BMC (g)	1470±193	1473±173	+0.3±2.0	1.0
Bone Area, BA (cm <sup>2</sup> )	1593±137	1578±114	-0.8±1.3	0.5
Bone Mineral Density, BMD (g/ cm <sup>2</sup> )	0.921±0.063	0.931±0.065	+1.1±1.1	0.3
Size adjusted BMC, SA-BMC (g) <sup>3</sup>	1477±92	1499±97	+1.6±1.1	0.1
<b>Total Hip (TH)<sup>4</sup></b>				
Bone Mineral Content, BMC (g)	26.7±4.1	27.1±4.23	+1.6±2.5	0.5
Bone Area, BA (cm <sup>2</sup> )	28.9±3.0	28.7±2.3	-0.5±1.5	0.8
Bone Mineral Density, BMD (g/ cm <sup>2</sup> )	0.921±0.092	0.943±0.121	+2.1±1.8	0.3
Size adjusted BMC, SA-BMC (g) <sup>3</sup>	26.2±2.6	27.1±3.3	+2.9±1.8	0.1
<b>Lumbar Spine (LS)</b>				
Bone Mineral Content, BMC (g)	49.7±9.2	49.3±7.4	-0.2±2.7	1.0
Bone Area, BA (cm <sup>2</sup> )	54.4±5.3	54.3±4.6	-0.1±1.4	1.0
Bone Mineral Density, BMD (g/ cm <sup>2</sup> )	0.909±0.110	0.906±0.094	-0.1±1.8	1.0
Size adjusted BMC, SA-BMC (g) <sup>3</sup>	48.7±5.1	49.1±4.8	+0.8±1.6	0.6

<sup>1</sup> Mean % differences between the groups presented as sympercents±SE. All variables were transformed into natural logarithms\*100 before statistical analysis using the ANOVA and ANCOVA functions in Datadesk software.

<sup>2</sup> Excludes one poor quality scan in the HIV-positive group.

<sup>3</sup> Adjusted for BA, weight and height

<sup>4</sup> Excludes two poor quality scans (one HIV-positive and one HIV-negative)

#### 6.8.1. Whole body-less-head (WBLH) at PP2

Overall mean WBLH bone mineral content (BMC) was  $1470 \pm 182$ g and not significantly different between the groups ( $p=1.0$ , Table 17). WBLH BMC was significantly associated with both maternal age ( $\beta = -0.4 \pm 0.2\%$  per year,  $p=0.05$ ) and previous use of depo-provera (yes vs no:  $+5.5 \pm 2.2\%$ ,  $p=0.01$ ) in the ANCOVA model. When WBLH BMC was adjusted for maternal age and previous use of depo-provera, the mean difference between the groups increased to  $-1.6 \pm 2.0\%$  but remained non-significant ( $p=0.4$ ).

Overall mean WBLH bone area (BA) was  $1585 \pm 125$ cm<sup>2</sup> and not significantly different between the groups ( $p=0.5$ , Table 17). WBLH BA was significantly associated with previous use of depo-provera (yes vs no:  $+3.5 \pm 1.4\%$ ,  $p=0.01$ ) in the ANCOVA model. When WBLH BA was adjusted for previous use of depo-provera using ANCOVA model, the difference between the groups increased to  $-1.7 \pm 1.3\%$  but remained non-significant ( $p=0.2$ ).

Overall mean WBLH areal bone mineral density (aBMD) was  $0.926 \pm 0.064$  g/cm<sup>2</sup> and not significantly different between the groups ( $p=0.3$ ). WBLH aBMD was significantly associated with previous use of depo-provera (yes vs no:  $+2.9 \pm 1.2\%$ ,  $p=0.02$ ) in the ANCOVA model. When WBLH aBMD was adjusted for previous use of depo-provera, the difference between the groups decreased to  $+0.4 \pm 1.1\%$  and remained non-significant ( $p=0.7$ ).

There was a non-significant mean difference between the groups in WBLH size-adjusted BMC, adjusted for BA, weight and height ( $+1.6\%$ ,  $p=0.1$ ). When maternal age, parity, previous use of depo-provera, sex of the infant and breastfeeding practice at PP2 were added into the ANCOVA model, only BA ( $\beta = +1.3 \pm 0.1\%$  per 1% increase in BA,  $p \leq 0.0001$ ) and weight ( $\beta = +0.40 \pm 0.05\%$  per 1% increase in weight,  $p \leq 0.0001$ ) remained significant. The difference between the groups increased to  $+2.5 \pm 1.5\%$  but remained non-significant ( $p=0.2$ ).

### 6.8.2. Total hip (TH) at PP2

Overall mean total hip (TH) BMC was  $25.9 \pm 4.1\text{g}$ , and there was a non-significant mean difference between groups ( $+1.6\%$ ,  $p=0.5$ , Table 17). TH BMC was significantly associated with previous use of depo-provera (yes vs no:  $+5.9 \pm 2.7\%$ ,  $p=0.03$ ) in the ANCOVA model. When TH BMC was adjusted for previous use of depo-provera using ANCOVA model, the difference between the groups decreased to  $+0.2 \pm 2.5\%$  ( $p=0.9$ ).

Overall mean TH aBMD was  $0.933 \pm 0.108 \text{ g/cm}^2$ . There was a non-significant  $+2.1\%$  mean difference in TH BMD between the groups but it was not statistically significant ( $p=0.3$ ). Mean TH BA was  $28.8 \pm 2.6 \text{ cm}^2$  and also not significantly different between the groups ( $p=0.8$ ). Maternal age, parity, previous use of depo-provera, gestation age at delivery, exclusive breastfeeding, and sex of the infant did not have significant effects on either TH aBMD or BA in the respective ANCOVA models.

There was a non-significant mean difference between the groups in TH SA-BMC, adjusted for BA, weight and height ( $+2.9\%$ ,  $p=0.1$ ). When maternal age, parity, previous use of depo-provera, sex of the infant and breastfeeding practice at PP2 were added into the ANCOVA model, only BA ( $\beta = +1.1 \pm 0.1\%$  per 1% increase in BA,  $p \leq 0.0001$ ) and weight ( $\beta = +0.2 \pm 0.1\%$  per 1% increase in weight,  $p=0.001$ ) were significant. There was a trend towards higher SA-BMC, adjusted for BA and weight, in HIV-positive women but the mean difference between the groups was not statistically significant ( $+3.1 \pm 1.8\%$ ,  $p=0.09$ ).

### 6.8.3. Lumbar spine (LS) at PP2

Overall mean lumbar spine (LS) BMC was  $49.4 \pm 8.3\text{g}$  and not significantly different between the groups ( $p=1.0$ , Table 17). Similarly, mean LS BA and aBMD were  $54.3 \pm 4.9 \text{ cm}^2$  and  $0.901 \pm 0.102 \text{ g/cm}^2$  respectively; and both were not significantly different between the groups. Maternal age, parity, previous use of depo-provera, gestation age at delivery, exclusive breastfeeding, and sex of the infant did not have significant effects on LS BMC, BA or aBMD in the respective ANCOVA models.

There was a non-significant mean difference between the groups in LS SA-BMC, adjusted for BA, weight and height ( $1.6 \pm 0.8\%$ ,  $p=0.6$ ). When maternal age, parity, previous use of depo-provera, sex of the infant and breastfeeding practice at PP2 were added into the ANCOVA model, only BA ( $\beta = +1.4 \pm 0.1\%$  per 1% increase in BA,  $p \leq 0.0001$ ) and weight ( $\beta = +0.20 \pm 0.05\%$  per 1% increase in weight,  $p=0.0002$ ) were significant and the difference between the groups remained non-significant ( $+1.1 \pm 1.6\%$ ,  $p=0.5$ ).

## 6.9. Infant anthropometry, growth and body composition at PP2

Table 18 Infant anthropometry, growth and body composition at PP2

Variable	HIV-unexposed (n=78)	HIV-exposed (n=84)	Mean % Difference between groups $\pm$ SE (HIV+ vs HIV-)	p-value
Age at PP2 visit, weeks (Mean $\pm$ SD)	2.09 $\pm$ 0.38	2.19 $\pm$ 0.47	+4.5 $\pm$ 3.1 <sup>1</sup>	0.1
Weight, kg (Mean $\pm$ SD)	3.62 $\pm$ 0.51	3.58 $\pm$ 0.55	-1.3 $\pm$ 2.4 <sup>1</sup>	0.6
Length, cm (Mean $\pm$ SD)	51.5 $\pm$ 2.2	51.3 $\pm$ 2.4	-0.5 $\pm$ 0.7 <sup>1</sup>	0.5
Head Circumference, cm (Mean $\pm$ SD) <sup>2</sup>	36.4 $\pm$ 1.2	36.3 $\pm$ 1.5	-0.4 $\pm$ 0.6 <sup>1</sup>	0.5
<b>Growth indices<sup>3</sup></b>				
Weight-for-Age Z-scores (Mean $\pm$ SD)	-0.15 $\pm$ 1.04	-0.32 $\pm$ 1.02	-0.16 $\pm$ 0.16 <sup>4</sup>	0.3
Length-for-Age Z-scores (Mean $\pm$ SD)	-0.24 $\pm$ 1.15	-0.43 $\pm$ 1.18	-0.19 $\pm$ 0.18 <sup>4</sup>	0.3
Head Circ-for-Age Z-scores (Mean $\pm$ SD) <sup>2</sup>	0.72 $\pm$ 1.04	0.53 $\pm$ 1.18	-0.23 $\pm$ 0.17 <sup>4</sup>	0.2
Weight-for-length Z-scores (Mean $\pm$ SD)	-0.26 $\pm$ 1.21	-0.23 $\pm$ 1.27	-0.03 $\pm$ 0.19 <sup>4</sup>	0.9
<b>Whole Body Composition<sup>5</sup></b>				
Lean mass, g (Median, IQR)	2883 (2644, 3196)	2913 (2619, 3255)	-0.2 $\pm$ 2.4	0.9
Fat mass, g (Median, IQR)	1065 (830, 1344)	1032 (842, 1235)	+2.5 $\pm$ 6.8	0.7
% Fat (Mean $\pm$ SD)	26.4 $\pm$ 8.5	26.5 $\pm$ 7.1	+2.8 $\pm$ 5.8	0.6

<sup>1</sup> Mean % differences between the groups presented as sympercents $\pm$ SE. All variables were transformed into natural logarithms\*100 before statistical analysis using the ANOVA and ANCOVA functions in Datadesk software.

<sup>2</sup> HIV-unexposed n 75, HIV-exposed n=81. Excludes 6 implausible measurements (4 due to documentation errors and 2 had Z scores > 4)

<sup>3</sup> age and sex standardised indices calculated in STATA using WHO Anthro plug-in based on WHO 2006 reference standards

<sup>4</sup> Mean difference between the groups presented as Z –scores  $\pm$ SE using the HIV-unexposed group as the reference.

<sup>5</sup> Excludes 13 poor quality WB DXA scans (10 HIV-exposed; 3 HIV-unexposed)

### 6.9.1. Infant anthropometry and growth at PP2

Overall mean infant weight was 3.6 $\pm$ 0.5 kg and not significantly different between the groups (-1.3%, p=0.6, Table 18). Weight was significantly associated with gestation age ( $\beta$  = +0.16 $\pm$ 0.02kg per week, p $\leq$ 0.0001), infant sex (boys vs. girls: +5.2 $\pm$ 2.0%, p=0.01) and age ( $\beta$ = +0.03 $\pm$ 0.01kg per day, p=0.02) in the ANCOVA model. The mean difference between the groups in weight increased to -2.4 $\pm$ 2.0% after adjusting for sex, age and gestation age, but remained non-significant (p=0.2).

Overall infant length was 51.4 $\pm$ 2.3 cm and not significantly different between the groups (p=0.5, Table 18). Infant length was significantly associated with gestation age ( $\beta$  = +0.6 $\pm$ 0.1 cm per week, p $\leq$ 0.0001) and sex (boys vs girls +2.2 $\pm$ 0.6%, p $\leq$ 0.0001) in the ANCOVA model. Adjusting length for sex and gestation age did not have a material effect on the mean difference between the groups (-0.7 $\pm$ 0.6%, p=0.3).



Overall mean infant head circumference was  $36.4 \pm 1.4$  cm and not significantly different between the groups ( $p=0.5$ , Table 18). Head circumference was significantly associated with gestation age ( $\beta = +1.2 \pm 0.1$  cm per week,  $p \leq 0.0001$ ), infant sex (boys vs. girls:  $+1.7 \pm 0.5\%$ ,  $p=0.001$ ) and age ( $\beta = +0.2 \pm 0.1$  cm per day,  $p=0.003$ ) in the ANCOVA model. The mean difference between the groups increased to  $-0.8 \pm 0.5\%$  after adjusting for sex, age and gestation age, but remained non-significant ( $p=0.08$ ).

All age and sex standardised growth indices, based on WHO 2006 growth standards, were also not significantly different between the groups (Table 18). Overall mean weight-for-age Z-scores (WAZ), length-for-age Z-scores (LAZ), head circumference-for-age Z-scores (HCAZ) and weight-for-length Z-scores (WFLZ) were  $-0.24 \pm 1.03$ ,  $-0.34 \pm 1.16$ ,  $-0.60 \pm 1.08$  and  $-0.24 \pm 1.24$ , respectively. There were no significant sex differences for all Z-scores. Also, there were no significant differences between the groups in all Z-scores when data were analysed separately for boys and girls.

#### *6.9.2. Infant body composition at PP2*

Overall median infant WB lean mass was 2885g (IQR 2641, 3240g) and not significantly different between the groups ( $p=0.7$ , Table 18). Lean mass was significantly associated with gestation age ( $\beta = +100.7 \pm 19.8$  g per week,  $p \leq 0.0001$ ), birth order (first born yes vs. no:  $\beta = -185.6 \pm 76.0$  g,  $p=0.02$ ) and sex (boys vs. girls  $+347.4 \pm 74.5$  g,  $p \leq 0.0001$ ) in the ANCOVA model. Adjusting lean mass for sex, birth order and gestation age did not have a material effect on the mean difference between the groups ( $-1.2 \pm 2.0\%$ ,  $p=0.5$ ).

Overall median infant whole body (WB) fat mass was 1051g (IQR 841, 1283g) and not significantly different between the groups ( $p=0.6$ , Table 18). Fat mass was significantly associated with gestation age ( $\beta = +3.5 \pm 0.7\%$ ,  $p \leq 0.0001$ ) and sex (boys vs girls:  $-14.7 \pm 6.3\%$ ,  $p=0.02$ ) in the ANCOVA model. The mean difference between the groups increased to  $+1.5 \pm 6.2\%$ , after adjusting fat mass for sex and gestation age, but remained non-significant ( $p=0.8$ ).

Overall, mean percent fat was  $26.5 \pm 7.8\%$  and not significantly different between the groups ( $p=0.6$ , Table 18). Percent fat was significantly associated with gestation age at birth ( $\beta = +82.6 \pm 15.7$ g per week,  $p \leq 0.0001$ ) and infant sex (boys vs girls:  $-133.1 \pm 55.3$ g,  $p=0.02$ ) in the ANCOVA model. The mean difference between the groups in % fat increased to  $+1.3 \pm 6.2\%$  in the final model (adjusted for gestation age at birth and sex) but remained non-significant ( $p=0.8$ ).

## 6.10. Infant bone mineral at PP2

Table 19 Infant bone mineral at PP2

Variable	HIV-unexposed (n=78) Mean±SD	HIV-exposed (n=83) Mean±SD	% Difference between the groups ±SE (HIV+ vs HIV-) <sup>1</sup>	p-value
<b>Whole body<sup>2</sup></b>				
Bone Mineral Content (g)	78.6±17.6	79.2±13.8	+1.9±3.5	0.6
Bone Area (cm <sup>2</sup> )	416±52	420±43	+1.1±1.9	0.6
Bone Mineral Density (g/cm <sup>2</sup> )	0.187±0.023	0.188±0.019	+0.8±1.9	0.7
Size adjusted BMC, SA-BMC (g) <sup>3</sup>	77.2±6.1	77.7±8.9	+0.3±1.4	0.8
<b>Lumbar Spine<sup>4</sup></b>				
Bone Mineral Content (g)	1.89±0.28	1.72±0.30	<b>-9.5±2.6</b>	<b>≤0.0001</b>
Bone Area (cm <sup>2</sup> )	9.19±0.76	8.74±0.9	<b>-5.2±1.6</b>	<b>0.001</b>
Bone Mineral Density (g/cm <sup>2</sup> )	0.205±0.024	0.197±0.028	<b>-4.3±2.1</b>	<b>0.04</b>
Size adjusted BMC, SA-BMC (g) <sup>3</sup>	1.83±0.21	1.76±0.24	<b>-4.9±2.2</b>	<b>0.02</b>

<sup>1</sup> Mean % differences between the groups presented as sympercents±SE. All variables were transformed into natural logarithms\*100 before statistical analysis using the ANOVA and ANCOVA functions in Datadesk software.

<sup>2</sup> Excludes 13 poor quality scans (10 HIV-exposed; 3 HIV-unexposed)

<sup>3</sup> Adjusted for BA, weight and length

<sup>4</sup> Excludes 15 poor quality scans (7 HIV-exposed; 8 HIV-unexposed)

### 6.10.1. Infant whole body (WB)

Mean infant whole body (WB) BMC was 78.9 ±15.8g and not significantly different between the groups (p=0.6, Table 19). WB BMC was significantly associated with gestation age ( $\beta$  = +7.0±0.7% per week, p≤0.0001) in the ANCOVA model, and when adjusted for, the mean difference between the groups reduced to +1.3±2.7% (p=0.6).

Overall mean infant WB BA was 418±47cm<sup>2</sup> and not significantly different between the groups (p=0.6). Infant WB BA was significantly associated with gestation age ( $\beta$  = +3.6±0.4% per week, p≤0.0001) and exclusive breastfeeding (yes vs no: +4.2±1.8%, p=0.02) in the ANCOVA model, and when adjusted for, the difference between the groups in WB BA remained non-significant (-0.3±1.6%, p=0.9).

Overall mean infant WB aBMD was 0.185±0.02g/cm<sup>2</sup> and not significantly different between the groups (p=0.7). Infant WB aBMD was significantly associated with gestation age ( $\beta$  = +3.5±0.4% per week, p≤0.0001) in the ANCOVA model, and when adjusted for, the difference between the groups reduced to +0.5±1.5% and remained non-significant (p=0.7).

SA-BMC (adjusted for WB BA, weight and length) was not significantly different between the groups ( $p=0.8$ ). When age, sex of infant, gestation age at birth, maternal age, birth order, breastfeeding status and history of illness within the past 2 weeks were added into the ANCOVA model, the significant variables were BA ( $\beta = +1.3 \pm 0.1\%$  per 1% increases in BA,  $p \leq 0.0001$ ), weight ( $\beta = +0.2 \pm 0.1\%$  per 1% increase in weight,  $p=0.02$ ), gestation age ( $\beta = +1.5 \pm 0.4\%$  per week,  $p=0.0004$ ) and sex (boys vs. girls:  $+2.9 \pm 1.3\%$ ,  $p=0.03$ ). Adjusting SA-BMC for gestation age and sex, did not have a material effect on the results ( $+0.4 \pm 1.3\%$ ,  $p=0.8$ ).

#### *6.10.2. Infant lumbar spine (infant LS) at PP2*

Overall mean infant LS BMC was  $1.80 \pm 0.30\text{g}$  and significantly lower in HEI compared to HUI ( $-9.5\%$ ,  $p \leq 0.0001$ , Table 19). Infant LS-BMC was significantly associated with gestation age ( $\beta = +2.4 \pm 0.7\%$  per week,  $p=0.006$ ) in the ANCOVA model. Adjusting infant LS BMC for gestation age did not have a material effect on the results ( $-9.7 \pm 2.6\%$ ,  $p \leq 0.0001$ ).

Overall mean infant LS BA was  $8.95 \pm 0.87\text{cm}^2$  and  $5.3\%$  lower in HEI compared to HUI after ( $p=0.001$ ). Infant LS-BA was significantly associated with gestation age ( $\beta = +1.4 \pm 0.4\%$  per week,  $p=0.001$ ) in the ANCOVA model. When infant LS BA was further adjusted for gestation, the difference between the groups increased to  $-5.3 \pm 1.5\%$  ( $p \leq 0.0001$ ).

Overall infant LS aBMD was lower among HIV-exposed infants compared to HIV-unexposed infants ( $-3.4\%$ ,  $p=0.04$ ). Gestation age at birth, age and sex, breastfeeding status, history of illness within the past 2 weeks, birth order and maternal age did not have significant effects on aBMD in the ANCOVA model.

Infant LS SA-BMC was significantly lower among HIV-exposed infants compared to HIV-unexposed infants ( $-4.9\%$ ,  $p=0.02$ , Table 19). When infant age, sex, gestation age at birth, maternal age, birth order, breastfeeding status and history of illness within the past 2 weeks were added into the ANCOVA model, only BA ( $\beta = +0.8 \pm 0.1\%$  per 1% increase in BA,  $p \leq 0.0001$ ) and length ( $\beta = +0.9 \pm 0.3\%$  per 1% increase in length,  $p=0.002$ ) were significant, and the mean difference between the groups remained significant ( $-4.9 \pm 2.3\%$ ,  $p=0.03$ ).

### 6.11. Summary of results at PP2

All women were breastfeeding. However, a greater proportion of HIV-positive women reported exclusive breastfeeding compared to HIV-negative women (82.9% vs 58.7%,  $p=0.0008$ ).

HIV-positive women had significantly lower body weight compared to HIV-negative women ( $-5.2\%$  ( $3.6\pm0.5\text{kg}$ ),  $p=0.04$ ). MUAC, BMI and body composition parameters were not significantly different between the groups. The mean difference in weight between the groups increased to  $-8.6\pm2.3\%$  ( $p\leq0.0001$ ) after adjusting for parity and previous exposure to depo-provera. Also, HIV-positive women had significantly lower BMI, MUAC, lean mass and fat mass after adjustment for parity and previous use of depo-provera.

Breast milk phosphorus concentration was significantly higher among HIV-positive women compared to HIV-negative women ( $-9.7\pm3.8\%$ ). Thus, breast milk Ca/P ratio was lower among HIV-positive compared to HIV-negative women ( $-7.7\pm2.6\%$ ). Breast milk calcium, sodium and potassium concentrations were not significantly different between the groups.

All maternal bone mineral parameters were not significantly different between the groups at all skeletal sites. Adjusting for maternal age, weight, height, parity, previous use of depo-provera, gestation age at delivery, sex of the infant or breastfeeding practice did not have material effects on the results.

Infant birth weight and anthropometric measurements at PP2 were not significantly different between the groups. Likewise, age and sex standardised growth indices and body composition were not significantly different between the groups. Gestation age at birth, age, sex, breastfeeding status, history of illness within the past 2 weeks, birth order and maternal age did not have a significant effect on weight, length and body composition parameters.

Infant whole body bone parameters were not significantly different between the groups. However, all LS bone parameters were significantly lower in HEI compared to HUI (BMC  $-9.5\%$ ,  $p\leq0.0001$ ; BA  $-5.3\%$ ,  $p=0.001$ ; aBMD  $-3.4\%$ ,  $p=0.04$ ; and SA-BMC  $-4.6\%$ ,  $p=0.02$ ). The differences between the groups were not explained by infant weight, length, age, sex, gestation age at birth, breastfeeding status, birth order or maternal age.

## **CHAPTER 7: RESULTS AT 14 WEEKS POSTPARTUM (PP14)**

This chapter presents both maternal (sections 7.6 to 7.9) and infant outcomes (sections 7.10 to 7.11) at 14 weeks postpartum (PP14). For all comparisons, the reference group for maternal outcomes is the HIV-negative group and the reference group for infant outcomes is HUI. Therefore, the sign of all differences between the groups is for the comparison of HIV-positive vs HIV-negative (sections 7.6 to 7.9) or HEI vs HUI (sections 7.10 to 7.11).

All continuous maternal outcomes were adjusted for sex of the infant, exclusive breastfeeding (yes vs no), maternal age, parity (multiparae vs primiparae), gestation age at delivery, both previous and current use of depo-provera (yes vs no), and resumption of menses. All infant outcomes were adjusted for gestation age at birth (weeks), sex, birth order, maternal age, exclusive breastfeeding (yes vs.no) and having been ill within 2 weeks prior to study visit. Unadjusted mean percent differences between the groups are presented in the summary tables and the adjusted results are discussed in the text. Unless explicitly mentioned, co-factors which are not mentioned in the text were not significant in the ANCOVA models.

### **7.1. Attendance at PP14**

A total of 178 mother-baby pairs (90 HIV-positive, 88 HIV-negative) were active in the study at the end of the PP2 visit (Figure 41). Six mother-baby pairs missed the visit, 3 were lost to follow-up, and 3 withdrew at PP14. In addition, one HIV-positive mother stopped breastfeeding before 14 weeks postpartum (because she was admitted to a mental health facility), hence, she was no longer involved in the study. A total of 165 mother-baby pairs attended PP14 visit (84 HIV-positive, 81 HIV-negative). One HIV-positive mother was confirmed pregnant on arrival for the visit. Therefore study measurements were done only for the baby.

### **7.2. Infant health status and breastfeeding**

Overall mean age of the infants at the PP14 visit was  $14.2 \pm 0.5$  weeks and not significantly different between the groups ( $p=0.8$ , Table 20). All the infants were breastfeeding, but only 76.9% were exclusively breastfed. The proportion of exclusively breastfed infants was significantly greater among HEI compared to HUI (86.7% vs 66.2%,  $p=0.002$ ). Cow's milk was the most common food given to mixed-fed infants.

Overall, 156 infants (98.1%) had a complete immunisation record, thus better coverage compared to PP2. Sixty-five infants (41.1%) were reported to have been ill since the PP2 visit. The burden of illness was significantly lower among HEI compared to HUI (32.5%, vs 50.7%,  $p=0.02$ ).

Seventy-five infants (45.5%) were on multivitamin syrups, commonly Grovit syrup, and the distribution was not significantly different between the groups. All HIV-exposed infants received nevirapine syrup from birth until 6 weeks of age and then switched to cotrimoxazole prophylaxis as per the prevailing clinical guidance.

Table 20: Infant health and breastfeeding at PP14

Variable	HIV-negative (n=81)	HIV-positive (n=83)	p-value
Age at visit, weeks (mean±SD)	14.1±0.5	14.3±0.5	0.8
Exclusive breastfeeding, (n, %)	51 (66.2)	72 (86.7)	<b>0.002</b>
Problem with breastfeeding (n, %)	11 (14.7)	4 (5.3)	0.06
Complete Immunisation record (n, %)	75 (98.7)	81 (97.6)	0.6
Been ill since PP2 visit (n, %)	38 (50.7)	27 (32.5)	<b>0.02</b>
Multivitamins (n, %)	41 (50.6)	34 (40.5)	0.2
Nevirapine ARV prophylaxis <sup>1</sup>	-	83 (100)	-
Cotrimoxazole prophylaxis <sup>1</sup>	-	83 (100)	-

<sup>1</sup> only for HIV-exposed infants.

### 7.3. Maternal health status at PP14

All participants were breastfeeding at the PP14 visit. Fifteen women (10%) reported challenges with breastfeeding (4 HIV-positive, 11 HIV-negative) and their distribution was not significantly different between the groups ( $p=0.06$ ). All HIV-positive women were still on first line PMTCT ART regimen at PP14, and the median adherence to ART based on pill count was 100% (IQR 100%, 100%). The mean duration of ART was  $29.3\pm 5.1$  weeks (range 19.9 - 44.3 wks.). Median CD<sub>4</sub> count was 403 (IQR 290-528; range 51-984) cells/cm<sup>3</sup>. Viral load results were available for only 3 participants, and all were undetectable (<50 copies/ml of venous blood).

Overall, 36.4% of the women had resumed menses, and their distribution was not significantly different between the groups (HIV-positive 37.8% vs HIV negative 35%,  $p=0.7$ ). Thus, 36.6 % of participants had taken up a contraception method by PP14. Uptake of contraception was significantly higher among HIV-positive compared to HIV-negative women (49.4% vs 23.5%,  $p=0.0006$ ). Depo-provera was the most preferred contraception method, used by 61.4% of the women on contraception. Overall, when data were categorised by current use of depo-provera (yes or no) for all the participants, 21.1% of the participants were on depo-provera. A significantly greater proportion of HIV-positive women had postpartum exposure to depo-provera at PP14 compared to HIV-negative women (28.6% vs 13.6%,  $p=0.02$ ).

Overall, use of supplements at PP14 was low compared to previous visits. Only 9 mothers reported the use of multivitamin tablets (3 HIV positive and 6 HIV-negative). None of the participants reported the use of Ca or vitamin D supplements, corticosteroids, smoking or drinking alcohol.

Fifteen women (9.5%) were diagnosed with UTIs based on urinalysis results from 2-hour fasted urine - a smaller proportion compared to PG36 (see section 5.8.2). Trace protein was detected in the urine of 17 women (10.8%), and the distribution was not significantly different between the groups. None of the participants had glucose in urine.

#### 7.4. Maternal physical activity at PP14

Table 21 Physical activity at PP14

Variable	HIV-negative (n=81)	HIV-positive (n=83)	p-value
<b>Cleaning the house</b>			0.7
Never (n, %)	6 (7.4)	5 (6.0)	
1-2 times a week (n, %)	13 (16.)	10 (12)	
Everyday (n, %)	62 (76.5)	68 (81.9)	
<b>Hand washing utensils and scrubbing pans</b>			0.6
Never (n, %)	13 (16)	10 (12)	
1-2 times a week (n, %)	10 (12.3)	8 (9.6)	
Everyday (n, %)	58 (71.6)	65 (78.3)	
<b>Hand washing clothes</b>			0.4
Never (n, %)	2 (2.5)	5 (6)	
1-2 times a week (n, %)	15 (18.5)	12 (14.5)	
Everyday (n, %)	64 (79)	66 (79.5)	
<b>Ironing clothes</b>			0.2
Never (n, %)	8 (9.9)	12 (14.5)	
1-2 times a week (n, %)	45 (55.6)	52 9 (62.7)	
Everyday (n, %)	28 (34.6)	19 (22.9)	
<b>Walking for at least 30 minutes</b>			<b>0.003</b>
Never (n, %)	15 (18.5)	26 (31.3)	
1-2 times a week (n, %)	26 (32.1)	37 (44.2)	
Everyday (n, %)	40 (49.4)	20 (24.1)	
<b>Carrying/Lifting heavy things</b>			0.4
Never (n, %)	52 (64.2)	53 (64.6)	
1-2 times a week (n, %)	22 (27.2)	26 (31.7)	
Everyday (n, %)	7 (8.6)	3 (3.7)	

The majority of women (80%) were engaged in household chores like cleaning the house and washing utensils on a daily basis as presented Table 21. Unlike PG36, a greater proportion of women (79.3%) reported hand washing clothes every day, as expected of women with small infants in this population. However, fewer HIV-positive women reported walking for at least 30 minutes compared to HIV-negative women (68.3% vs 81.5% p=0.003).

## 7.5. Maternal diet of calcium and vitamin D rich foods at PP14

Milk and milk products, cooked dry beans and peas, and small fish eaten with bones (*mukene* and *enkeje*) were the most commonly consumed food sources of calcium (Figure 44). There was a trend towards a lower consumption of milk and milk products among HIV-positive vs HIV-negative women (81.9% vs 92.3%,  $p=0.05$ ). However, consumption of small fish eaten with bones was significantly higher among HIV-positive compared to HIV-negative women (81.7% vs.63.0%,  $p=0.007$ ).

Eggs, organ meats and margarine were the most commonly consumed dietary sources of vitamin D (Figure 44). Significantly smaller proportions of HIV-positive women compared to HIV-negative women reported consumption of organ meats (42.5% vs 59.3%,  $p=0.03$ ); and there was a trend towards a lower consumption of vitamin D fortified margarine among HIV-positive compared to negative women (22.9% vs 37.0%,  $p=0.05$ ).

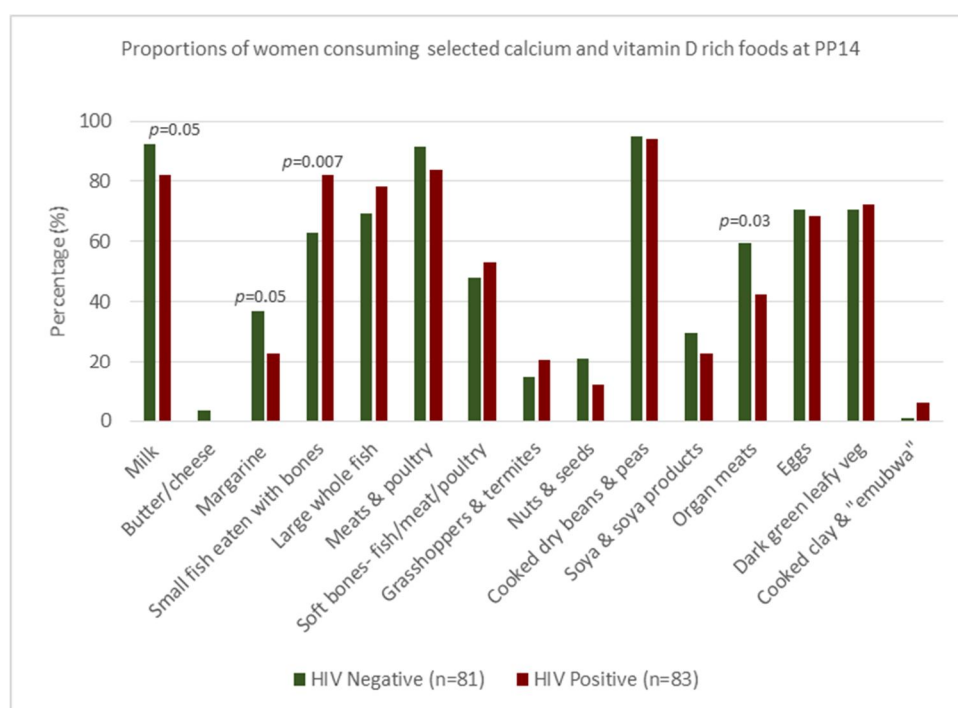


Figure 44: Consumption of selected vitamin D and calcium-rich foods at PP14



## 7.6. Maternal anthropometry and body composition at PP14

Table 22: Summary of maternal anthropometry and body composition at PP14

Variable	HIV-negative (n=81)	HIV-positive (n=83)	Mean % Diff between the groups $\pm$ SE (HIV+ vs HIV-) <sup>1</sup>	p-value
<b>Anthropometry</b>				
Weight, kg (Median, IQR)	59.9 (54.0, 67.8)	56.9 (52.9, 64.3)	-4.6 $\pm$ 2.6	0.07
BMI, kg/m <sup>2</sup> (Median, IQR)	23.5 (21.8, 30.0)	23.0 (21.6, 26.2)	-2.6 $\pm$ 2.5	0.3
MUAC	26.7 (24.5, 29.0)	26.0 (24.5, 28.9)	-1.7 $\pm$ 1.8	0.4
<b>WBLH Composition</b>				
Lean Mass, kg (Median, IQR)	35.3 (32.2, 39.1)	33.8 (31.4, 36.9)	<b>-5.1<math>\pm</math>2.1</b>	<b>0.01</b>
Fat Mass, kg (Median, IQR)	19.6 (15.2, 25.6)	19.0 (14.7, 22.9)	-2.6 $\pm$ 5.7	0.6
% Fat, Mean $\pm$ SD	35.5 $\pm$ 8.3	36.1 (7.0)	+2.7 $\pm$ 4.9	0.6
Fat:Lean <sup>2</sup> *10 <sup>-3</sup> kg/kg <sup>2</sup> , (Median, IQR) <sup>2</sup>	16.4 (11.5, 20.4)	17.4 (12.7, 21.9)	+7.6 $\pm$ 5.9	0.2

<sup>1</sup> Mean % differences between the groups presented as sympercents $\pm$ SE. All variables were transformed into natural logarithms\*100 before statistical analysis using the ANOVA function in Datadesk software.

### 7.6.1. Maternal anthropometry at PP14

Overall median weight was 57.6 (IQR 53.3, 66.0) kg. There was a trend towards lower body weight among HIV-positive compared to negative women, but the difference between the groups was not statistically significant (-4.6 $\pm$ 2.6%, p=0.07; Table 22). Weight was significantly associated with parity (multiparae vs primiparae: +8.9 $\pm$ 2.6%, p $\leq$ 0.0001) and previous use of depo-provera (yes vs no +8.7 $\pm$ 2.9%, p=0.003) in the ANCOVA model. After adjustment for parity and previous exposure to depo-provera, HIV-positive women had significantly lower weight compared to HIV-negative women (-8.0 $\pm$ 2.5%, p=0.002).

Overall median BMI was 23.6kg/m<sup>2</sup> (IQR 21.9, 26.9) and not significantly different between the groups (p=0.3). BMI was significantly associated with parity (multiparae vs primiparae: +10.0 $\pm$ 2.5%, p $\leq$ 0.0001) and previous use of depo-provera (yes vs no: +7.1 $\pm$ 2.8%, p=0.01) in the ANCOVA model. After adjustment for parity and previous exposure to depo-provera, HIV-positive women had significantly lower BMI compared to HIV-negative women (-5.6 $\pm$ 2.4%, p=0.02).

Overall median MUAC was 26.5cm (IQR 24.5, 29.0cm) and not significantly different between the groups (p=0.4). MUAC was significantly associated with parity (multiparae vs primiparae: +7.4 $\pm$ 1.8%, p $\leq$ 0.0001) and previous use of depo-provera (yes vs no +4.9 $\pm$ 2.0%, p=0.02) in the ANCOVA model. After adjustment for parity and previous exposure to depo-provera, HIV-positive women had significantly lower MUAC compared to HIV-negative women (-3.6 $\pm$ 1.7%, p=0.04).

### *7.6.2. Maternal body composition at PP14*

Overall lean mass was 35.6kg (IQR 32.2, 39.4), and lower in HIV-positive compared to negative women (-5.1%,  $p=0.01$ , Table 22). Lean mass was significantly associated with parity (multiparae vs primiparae:  $+6.0\pm2.1\%$ ,  $p=0.004$ ) in the ANCOVA model. After adjustment for parity, the difference between the groups increased to  $-6.1\pm2.0\%$  and was highly significant ( $p=0.003$ ).

Overall median WBLH fat mass was 19.1kg (IQR 15.0, 23.5) and not significantly different between the groups ( $p=0.6$ ). Fat mass was significantly associated with parity (multiparae vs primiparae:  $+14.6\pm5.7\%$ ,  $p=0.01$ ), previous use of depo-provera (yes vs no  $+24.9\pm6.5\%$ ,  $p\leq0.0001$ ) and age of the infant ( $\beta = -1.5\pm0.7\%$  per day,  $p=0.04$ ) in the ANCOVA model. After adjustment for age of the infant, parity, previous and use of depo-provera, the difference between the groups increased to  $-9.1\pm5.5\%$  but remained non-significant ( $p=0.1$ ).

Overall mean fat:lean<sup>2</sup> ratio was  $16.0\pm6.6\times10^{-3}$  kg/kg<sup>2</sup> and not significantly different between the groups ( $p=0.9$ ). Fat:lean<sup>2</sup> ratio was significantly associated with resumption of menses (yes vs no  $+20.7\pm5.6\%$ ,  $p\leq0.0001$ ), previous use of depo-provera (yes vs no  $+20.8\pm6.2\%$ ,  $p=0.001$ ) and age of the infant ( $\beta = -2.3\pm0.7\%$  per day,  $p=0.002$ ) in the ANCOVA model. Adjusting fat:lean<sup>2</sup> ratio for the resumption of menses, previous use of depo-provera and age of the infant did not have a material effect on the mean difference between the groups ( $-7.5\pm5.5\%$ ,  $p=0.2$ ).

## **7.7. Maternal plasma and urine biochemistry at PP14**

### *7.7.1 Plasma biochemistry at PP14*

Overall mean [P-Alb] was  $38.2\pm2.9$  g/L, and significantly lower in HIV-positive compared to negative women ( $-3.1\pm1.2\%$ ,  $p=0.008$ , Table 23). Maternal age, parity, resumption of menses, previous or current exposure to depo-provera, exclusive breastfeeding, and sex of the infant did not have significant effects on [P-Alb] in the ANCOVA model.

Overall mean uncorrected [P-Ca] was  $2.33\pm0.10$  mmol/L and lower in HIV-positive women compared to HIV-negative women ( $-5.3\pm0.7\%$ ,  $p\leq0.0001$ ). Mean corrected [P-Ca] was  $2.46\pm0.08$  mmol/L and also significantly lower in HIV-positive women ( $-4.1\pm0.6\%$ ,  $p\leq0.0001$ ). Parity was significantly associated with both uncorrected (multiparae vs. primiparae:  $-1.7\pm0.7\%$ ,  $p=0.03$ ) and corrected (multiparae vs. primiparae:  $-1.4\pm0.6\%$ ,  $p=0.03$ ) P-Ca concentrations in the ANCOVA model. Adjusting for parity did not have a material effect on the mean percent difference between the groups in both uncorrected ( $-5.0\pm0.7\%$ ,  $p\leq0.0001$ ) and corrected ( $-3.9\pm0.6\%$ ,  $p\leq0.0001$ ) plasma calcium.

Mean [P-PO<sub>4</sub>] across the groups was 1.31±0.19 mmol/L. Unlike PG36, mean [P-PO<sub>4</sub>] was not significantly different between the groups (-3.0±2.3%, p=0.2). Plasma phosphate concentration was significantly associated with previous use of depo-provera (yes vs no: -6.8±2.7%, p=0.01) in the ANCOVA model. Adjusting plasma [P-PO<sub>4</sub>] for previous use of depo-provera did not have a material effect on the mean difference between the groups (-2.1±2.4%, p=0.4).

Overall mean [P-Cr] was 63.3±7.7 µmol/L. There was a trend towards higher [P-Cr] in HIV-positive women, but the difference between the groups was not statistically significant (+3.4±1.8%, p=0.05). Mean [P-Mg] was 0.798±0.062 mmol/L, and not significantly different between the groups (p=0.5). Maternal age, parity, exclusive breastfeeding, resumption of menses, previous or current exposure to depo-provera, and sex of the infant did not have significant effects on either [P-Cr] or [P-Mg] in the respective ANCOVA models.

Table 23: Summary of maternal plasma and urine biochemistry at PP14

<b>Variables</b>	<b>HIV Negative (n=81)</b>	<b>HIV Positive (n=83)</b>	<b>Mean % Diff between the groups ±SE (HIV+ vs HIV-)<sup>1</sup></b>	<b>p-value</b>
<b>Plasma</b>				
Albumin, g/L (Mean±SD)	38.8 ±2.9	37.6±2.8	-3.1±1.2	<b>0.008</b>
Calcium <sub>uncorrected</sub> , mmol/L (Mean±SD)	2.36±0.09	2.24±0.12 <sup>2</sup>	-5.3±0.7	<b>≤0.0001</b>
Calcium <sub>corrected</sub> , mmol/L (Mean±SD) <sup>2</sup>	2.38±0.08	2.29±0.10	-4.1±0.6	<b>≤0.0001</b>
Phosphate, mmol/L (Mean±SD)	1.33±0.19	1.29±0.18	-3.0±2.3	0.2
Magnesium, mmol/L (Mean±SD)	0.802±0.071	0.795±0.051	-0.8±1.2	0.5
Creatinine, µmol/L (Mean±SD)	74.5±7.2	77.5±10.6	+3.4±1.8	<b>0.05</b>
<b>Urine <sup>3,4,5</sup></b>				
UCa/Cr, mmol/mmol (Median, IQR) <sup>6</sup>	0.015(0.006, 0.042)	0.009 (0.005, 0.026)	-36.9±19.8	0.06
UP/Cr, mmol/mmol (Median, IQR) <sup>6</sup>	1.02 (0.617, 1.48)	1.15 (0.786, 1.54)	+15.1±11.0	0.2
UMg/Cr, mmol/mmol (Median, IQR) <sup>6</sup>	0.181 (0.118, 0.260)	0.194 (0.137, 0.278)	-10.1±9.2	0.3
<b>Renal parameters</b>				
eGFR ml/min/1.73 m <sup>2</sup> (Median, IQR) <sup>7</sup>	113 (103, 122)	111 (98.6, 122)	-3.5±2.0	0.08
TmP/GFR, mmol/L GFR (Mean±SD)	1.52 ±0.28	1.42±0.26	-7.2±3.0	<b>0.02</b>
TmCa/GFR, mmol/L GFR (Mean±SD)	1.34±0.06	1.27±0.07	-5.8±0.8	<b>≤0.0001</b>

<sup>1</sup> Mean % differences between the groups presented as sympercents±SD. All variables were transformed into natural logarithms\*100 before statistical analysis using ANOVA function in Datadesk software.

<sup>2</sup> Plasma calcium normalised for albumin using Payne equation: Adjusted Serum Ca= Measured Ca+ ([40-serum albumin]\*0.02)

<sup>3</sup> The sample for one participant was not shipped from UK Uganda. Same participant without urine at PG36.

<sup>4</sup> Thirty-four measurements below the detection limit (0.02 mmol/L) were assigned half a detection (0.01mmol/L) before analysis. Data presented here is similar to analysis with the original data values.

<sup>5</sup> IQR = inter quartile range presented as 25th and 75th quartiles.

<sup>6</sup> Urine UCa/Cr calcium/creatinine clearance, UP/Cr phosphate/creatinine clearance and UMg/Cr magnesium/creatinine ratios.

<sup>7</sup> Calculated using CKD-EPI equation: eGFR (ml/min/1.73m<sup>2</sup>) = 144 x min (PCr/0.7)-0.329 x 0.993Age if [PCr] is ≤0.7; and eGFR (ml/min/1.73m<sup>2</sup>) = 144 x min (PCr/0.7)<sup>-1.209</sup> x 0.993Age<sup>0.718</sup> if [PCr] is >0.7.

### *7.7.2. Urine biochemistry at PP14*

Overall median UCa/Cr was 0.010 (IQR 0.005, 0.032) mmol/mmol and there was a trend towards a lower UCa/Cr in HIV-positive women ( $-36.9 \pm 19.8\%$ ,  $p=0.06$ ; Table 23). Median UMg/Cr was 0.189 (IQR 0.123, 0.278) mmol/mmol and also not significantly different between the groups ( $p=0.3$ ). Maternal age, parity, exclusive breastfeeding, resumption of menses, previous or current exposure to depo-provera, and sex of the infant did not have significant effects on either UCa/Cr or UMg/Cr in the respective ANCOVA models.

Overall median UP/Cr was 1.12 (IQR 0.699, 1.50) mmol/mmol and not significantly different between the groups ( $p=0.2$ ). UP/Cr was significantly associated with exclusive breastfeeding (yes vs no:  $+28.2 \pm 12.9\%$ ,  $p=0.03$ ) in the ANCOVA model, and when adjusted for, the difference between the groups reduced to  $+8.4 \pm 11.3$  ( $p=0.5$ ).

### *7.7.3. Renal parameters at PP14*

Overall mean eGFR was  $112 \pm 13$  mL/min/1.73 m<sup>2</sup>. There was a trend towards lower eGFR among HIV-positive compared to HIV-negative women, but the difference between the groups was not significant ( $-3.5 \pm 2.0\%$ ,  $p=0.08$ ; Table 23). Mean TmCa/GFR was  $1.31 \pm 0.08$  mmol/L was significantly lower in HIV-positive women ( $-5.8 \pm 0.8$ ,  $p \leq 0.0001$ ). Maternal age, parity, exclusive breastfeeding, resumption of menses, previous or current exposure to depo-provera, and sex of the infant did not have significant effects on either eGFR or TmCa/GFR in the respective ANCOVA models.

Mean TmP/GFR was  $1.47 \pm 0.28$  mmol/L and lower among HIV-positive women compared to HIV-negative women ( $-7.2\%$ ,  $p=0.02$ ). TmP/GFR was significantly associated with previous use of depo-provera (yes vs no:  $+30.2 \pm 11.9\%$ ,  $p=0.01$ ) in the ANCOVA model. Adjusting for previous use of depo-provera reduced the difference between the groups to  $-6.3 \pm 3.1\%$  but remained significant ( $p=0.04$ ).

## 7.8. Maternal 25(OH)D, PTH and bone turnover markers at PP14

Table 24: Concentrations of calciotropic hormones and bone turnover markers at PP14

Analyte	HIV Negative (n=81)	HIV Positive (n=80) <sup>1</sup>	Mean % Diff between the groups $\pm$ SE (HIV+ vs HIV-) <sup>2</sup>	P-value
Serum 25(OH)D (nmol/L), Median, IQR <sup>3</sup>	58.4 (49.7, 70.4)	57.6 (44.3, 71.3)	-2.1 $\pm$ 6.3	0.7
Plasma PTH (pg/mL), Median, IQR	41.4 (29.5, 58.8)	60.0 (44.7, 80.1)	<b>+35.3<math>\pm</math>7.6</b>	<b><math>\leq</math>0.0001</b>
Plasma CTX (ng/mL) (Mean $\pm$ SD)	1.01 $\pm$ 0.41	1.18 $\pm$ 0.49	<b>+15.6<math>\pm</math>7.5</b>	<b>0.04</b>
Plasma P1NP (ng/mL) (Mean $\pm$ SD)	158.3 $\pm$ 49.3	164.0 $\pm$ 65.6	+1.0 $\pm$ 5.5	0.9
Plasma BAP ( $\mu$ g/L) Median, IQR	33.9 (26.9, 44.3)	37.6 (31.3, 53.5)	<b>+16.3<math>\pm</math>5.5</b>	<b>0.003</b>
Plasma TAP (U/L) Median, IQR	121 (98.2, 138)	142 (113, 172) <sup>4</sup>	<b>+19.2<math>\pm</math>4.0</b>	<b><math>\leq</math>0.0001</b>
Plasma %BAP/TAP (Mean $\pm$ SD)	29.7 $\pm$ 4.9	29.0 $\pm$ 5.5	-0.7 $\pm$ 0.8	0.4

<sup>1</sup> Laboratory analysis was done for participants with both blood and urine samples. HIV-positive group n=80

<sup>2</sup> Mean % differences between the groups presented as sympercents $\pm$ SD. All variables were transformed into natural logarithms\*100 before statistical analysis using ANOVA function in Datadesk software.

<sup>3</sup> Not analysed for all participants n=75 in HIV-negative group, n=43 in HIV-positive group

<sup>4</sup> All samples analysed n=81 in HIV-positive group

### 7.8.1. Serum 25(OH)D at PP14

The median serum [25(OH)D] was 57.6 nmol/L (IQR 48.7-70.1) and not significantly different between the groups (p=0.7, Table 24). Overall, 27.1% of the participants had serum [25(OH)D] below 50nmol/L, and their distribution was not significantly different between the groups (32.6% vs 24.0%, p=0.3). None of the participants had [25(OH)D] below 25nmol/L.

Serum [25(OH)D] was significantly associated with maternal age ( $\beta$ = -1.7 $\pm$ 0.8% per year, p=0.02) and age of the infant ( $\beta$ = +2.0 $\pm$ 0.8% per day, p=0.01) in ANCOVA model. Adjusting [25(OH)D] for both maternal and infant age increased the mean difference between the groups to -7.4 $\pm$ 6.2% but remained non-significant p=0.2.

### 7.8.2. PTH at PP14

Overall median plasma [PTH] was 50.5 pg/mL (IQR 35.7-70.9 pg/mL). HIV-positive women had significantly higher [PTH] compared to HIV-negative women (+35.3%, p $\leq$ 0.0001, Table 24). Plasma [PTH] was significantly associated with maternal age ( $\beta$ = +1.8 $\pm$ 0.8% per year, p=0.03), parity (multiparae vs. primiparae: +18.7 $\pm$ 7.5%, p=0.01) and exclusive breastfeeding (yes vs. no: +19.3 $\pm$ 8.7%, p=0.03) in ANCOVA model. Adjusting [PTH] for maternal age, parity and exclusive breastfeeding reduced the mean difference between the groups to +30.1 $\pm$ 7.8% but remained highly significant (p $\leq$ 0.0001).

### 7.8.3. CTX at PP14

Overall mean [CTX] was  $1.09 \pm 0.46$  ng/mL. HIV-positive women had significantly higher [CTX] compared to HIV-negative women (+15.6%,  $p=0.04$ , Table 24). Plasma [CTX] was significantly associated with maternal age ( $\beta = +2.0 \pm 0.8\%$  per year,  $p=0.01$ ), parity (multiparae vs. primiparae: -16.7 $\pm$ 7.5%,  $p=0.03$ ), resumption of menses (yes vs. no: -21.0 $\pm$ 7.3%,  $p=0.004$ ) and previous use of depo-provera (yes vs. no: -22.5 $\pm$ 8.6%,  $p=0.01$ ) in ANCOVA model. Adjusting [CTX] for maternal age, parity, previous use of depo-provera and resumption of menses increased the mean difference between the groups to +25.0 $\pm$ 7.1% and was highly significant ( $p \leq 0.0001$ ).

### 7.8.4. P1NP at PP14

Overall median [P1NP] was 149 pg/mL (IQR 120-190 pg/mL) and not significantly different between the groups ( $p=0.9$ , Table 24). Plasma [P1NP] was significantly associated with maternal age ( $\beta = +1.2 \pm 0.6\%$  per year,  $p=0.05$ ) and previous use of depo (yes vs no: -20.5 $\pm$ 6.1%,  $p=0.001$ ) in ANCOVA model. Adjusting [P1NP] for maternal age and previous use of depo-provera did not have a material effect on the mean percent difference between the groups (+5.6 $\pm$ 5.4%,  $p=0.3$ ).

### 7.8.5. TAP and BAP at PP14

Overall median [TAP] was 128 (IQR 106, 152) U/L. HIV-positive women had significantly higher [TAP] compared to HIV-negative women (+19.2%,  $p \leq 0.0001$ , Table 24). Plasma [TAP] was negatively associated with resumption of menses (yes vs no: -8.6 $\pm$ 4.1%,  $p=0.04$ ) and sex of the baby (male vs female: -8.9 $\pm$ 4.0%,  $p=0.03$ ) in ANCOVA model. Adjusting [TAP] for the resumption of menses and sex of the baby did not have a material effect on the mean difference between the groups (+19.7 $\pm$ 4.0%,  $p \leq 0.0001$ ).

Overall median [BAP] was 35.6  $\mu$ g/L (IQR 28.8, 47.2). HIV positive women had significantly higher [BAP] compared to HIV-negative (+16.3%,  $p=0.003$ ). Maternal age, parity, resumption of menses, previous and current exposure to depo-provera, exclusive breastfeeding, and sex of the infant did not have significant effects on BAP in the ANCOVA model.

Overall mean percentage of BAP in TAP was 29.4 $\pm$ 0.1% and not significantly different between the groups ( $p=0.4$ ). Plasma BAP/TAP% was significantly associated with parity (multiparae vs primiparae: -6.4 $\pm$ 2.9%,  $p=0.03$ ) in ANCOVA model. Adjusting for parity did not have a material effect on the mean difference between the groups (-1.7 $\pm$ 2.9% ( $p=0.6$ )).

## 7.9. Breast milk mineral content at PP14

Table 25: Breast milk mineral content at PP14

Variable	HIV-Negative (n=77) Mean±SD	HIV-Positive (n=83) Mean±SD	Mean % Diff between the groups ±SE (HIV+ vs HIV-) <sup>1</sup>	P-value
Calcium, mmol/L, (Mean±SD) <sup>2</sup>	4.46 ±0.93	4.75 ±1.04	+6.1±3.7	0.1
Phosphorus, mmol/L (Mean±SD)	4.31 ±1.02	4.71 ±0.96	<b>+9.6±3.5</b>	<b>0.007</b>
Calcium/phosphorus ratio, Mean±SD	1.01±0.19	1.02±0.21	-3.5±3.1	0.3
Sodium, mmol/L Median IQR <sup>3</sup>	4.10 (3.30, 5.55)	4.40 (3.28, 5.70)	+11.1±10.5	0.1
Potassium, mmol/L (Mean±SD) <sup>4</sup>	13.0±2.09	13.3±2.39	+4.5±3.2	0.2
Sodium/potassium ratio, Median IQR <sup>5</sup>	0.326 (0.256, 0.432)	0.344 (0.254, 0.403)	+11.1±10.5	0.3

<sup>1</sup> Mean % differences between the groups presented as sympercents±SD. All variables were transformed into natural logarithms\*100 before statistical analysis using ANOVA function in Datadesk software.

<sup>2</sup> Includes 2 extreme values which don't affect results. Difference when included at PP2, +1.8±3.0, P=0.544; PP14 +4.7±3.0 p=0.113

<sup>3</sup> Includes outliers 8 extreme values (5 HIV-positive, 3 HIV-negative) 3 at PP2 (1 HIV-positive, 2 HIV-negative) PP14 (4 HIV-positive, 1 HIV-negative). When excluded, they did not affect the difference between the groups but the group\*visit interaction p-value became non-significant (changes from 0.026 to 0.275)

<sup>4</sup> Includes 2 HIV-negative women flagged as outliers at PP14. Exclusion does not affect results

<sup>5</sup> Includes 9 outliers overall (5 HIV-positive, 4 HIV-negative) 3 at PP2 (1 HIV-positive, 3 HIV-negative) PP14 (4 HIV-positive, 1 HIV-negative). When outliers are excluded there is no difference, although the almost significant group\* visit interaction p-values (p=0.0.68) becomes p=0.596

### 7.9.1. Breast milk calcium and phosphorus at PP14

Overall mean [BM-Ca] was 4.62±0.97 mmol/L and not significantly different between the groups (p=0.1, Table 25). Maternal age, parity, previous or current exposure to depo-provera, resumption of menses, exclusive breastfeeding, and sex of the infant and did not have significant effects on breast milk calcium in the ANCOVA model.

Overall mean [BM-P] was 4.51±1.01 mmol/L. Similar to PP2, HIV-positive women had significantly higher [BM-P] compared to HIV-negative women (+9.8%, p=0.002, Table 25). Resumption of menses was significantly associated with [BM-P] in ANCOVA model (yes vs no: -10.4±3.6%, p=0.004).

Adjustment [BM-P] for the resumption of menses did not have a material effect on the mean difference between the groups (+9.9±3.5%, p=0.005). Also, [BM-P] was significantly correlated with maternal plasma [CTX] in a separate ANCOVA model ( $\beta$  = +0.13±0.04% per 1% increase in CTX, p=0.0003).

Overall mean breast milk Ca/P ratio was 1.04±0.20 mmol/L and not significantly different between the groups (p=0.3, Table 25). Breast milk Ca/P ratio was significantly associated with sex of the infant (male vs female: +6.8±3.1%, p=0.03) in ANCOVA model. Adjusting breast milk phosphorus for infant sex did not have a material effect on the mean difference between the groups (-3.6±3.1%, p=0.3).



### 7.9.2. Breast milk sodium and potassium

Overall median [BM-Na] was 4.2 (IQR 3.3, 5.6) mmol/L and not significantly different between the groups ( $p=0.1$ , Table 25). Maternal age, parity, previous or current exposure to depo-provera, resumption of menses, breastfeeding practice, and sex of the infant did not have significant effects on [BM-Na] in the ANCOVA model.

Overall mean [BM-K] was  $13.2 \pm 2.2$  mmol/L and not significantly different between the groups ( $p=0.2$ , Table 25). BM-K was significantly associated with both maternal age ( $\beta = -0.6 \pm 0.3\%$  per year,  $p=0.05$ ) and age of the infant ( $\beta = +0.8 \pm 0.3\%$  per day,  $p=0.05$ ). Adjusting [BM-K] for maternal age and infant age did not have a material effect on the mean difference between the groups ( $+0.4 \pm 2.8\%$ ,  $p=0.9$ ).

Overall median breast milk Na/K ratio was 0.57 (IQR 0.48, 0.66) and was also not significantly different between the groups ( $p=0.3$ ). Eight women (2 HIV-negative and 6-HIV positive) had breast sodium/potassium ratio ratios  $> 1.0$ , a marker for sub-clinical mastitis. Maternal age, parity, previous or current exposure to depo-provera, resumption of menses, breastfeeding practice, and sex of the infant did not have significant effects on breast milk Na/K ratio in the ANCOVA model.

## 7.10. Maternal bone mineral at PP14

Table 26: Summary of maternal bone mineral at PP14

Variable	HIV-Negative (n=81) Mean $\pm$ SD	HIV-Positive (n=83) Mean $\pm$ SD	Mean % Diff between the groups $\pm$ SE (HIV+ vs HIV-) <sup>1</sup>	P-value
<b>Whole Body Less Head (WBLH)</b>				
Bone Mineral Content, BMC (g)	1451 $\pm$ 201	1449 $\pm$ 155	+0.2 $\pm$ 1.9	0.9
Bone Area, BA (cm <sup>2</sup> )	1583 $\pm$ 136	1573 $\pm$ 97	-0.5 $\pm$ 1.2	0.7
Bone Mineral Density, BMD (g/ cm <sup>2</sup> )	0.913 $\pm$ 0.069	0.920 $\pm$ 0.061	+0.6 $\pm$ 1.1	0.5
Size adjusted BMC, SA-BMC (g) <sup>2</sup>	1470 $\pm$ 98	1482 $\pm$ 89	+0.6 $\pm$ 1.1	0.6
<b>Total Hip (TH)</b>				
Bone Mineral Content, BMC (g)	25.8 $\pm$ 3.9	26.0 $\pm$ 4.4	+0.5 $\pm$ 2.5	0.8
Bone Area, BA (cm <sup>2</sup> )	28.8 $\pm$ 2.9	28.6 $\pm$ 2.3	-0.8 $\pm$ 1.4	0.6
Bone Mineral Density, BMD (g/ cm <sup>2</sup> )	0.894 $\pm$ 0.105	0.908 $\pm$ 0.121	+1.3 $\pm$ 2.0	0.5
Size adjusted BMC, SA-BMC (g) <sup>2</sup>	25.5 $\pm$ 2.9	26.1 $\pm$ 3.3	+2.3 $\pm$ 2.0	0.2
<b>Lumbar Spine (LS)<sup>a</sup></b>				
Bone Mineral Content, BMC (g)	48.4 $\pm$ 8.8	48.3 $\pm$ 7.1	+0.4 $\pm$ 2.6	0.9
Bone Area, BA (cm <sup>2</sup> )	54.3 $\pm$ 5.4	54.2 $\pm$ 4.9	+0.001 $\pm$ 1.5	1.0
Bone Mineral Density, BMD (g/ cm <sup>2</sup> )	0.887 $\pm$ 0.104	0.889 $\pm$ 0.089	+0.4 $\pm$ 1.7	0.8
Size adjusted BMC, SA-BMC (g) <sup>2</sup>	47.8 $\pm$ 4.8	48.3 $\pm$ 4.7	+1.0 $\pm$ 1.6	0.5

<sup>1</sup> Mean % differences between the groups presented as sympercents $\pm$ SE. All variables were transformed into natural logarithms\*100 before statistical analysis using the ANOVA and ANCOVA functions in Datadesk software.

<sup>2</sup> Adjusted for BA, body weight and height.



#### 7.10.1. Maternal WBLH bone mineral at PP14

Overall mean WBLH BMC was  $1450 \pm 178$  g and not significantly different between the groups ( $p=0.9$ , Table 26). WBLH BMC was significantly associated with both previous (yes vs no:  $+5.9 \pm 2.2\%$ ,  $p=0.007$ ) and current use of depo-provera (yes vs no:  $-6.2 \pm 2.4\%$ ,  $p=0.01$ ) in the ANCOVA model. Adjusting WBLH BMC for both previous and current use of depo-provera further diminished the mean difference between the groups ( $+0.02 \pm 2.0\%$ ,  $p=0.9$ ).

Overall mean WBLH BA was  $1578 \pm 118 \text{ cm}^2$  and not significantly different between the groups ( $p=0.7$ , Table 26). WBLH BA was significantly associated with both previous (yes vs no:  $+3.1 \pm 1.3\%$ ,  $p=0.02$ ) and current use of depo-provera (yes vs no:  $-3.5 \pm 1.5\%$ ,  $p=0.02$ ) in the ANCOVA model. Adjusting WBLH BA for both previous and current use of depo-provera did not change the mean difference between the groups ( $-0.5 \pm 1.2\%$ ,  $p=0.7$ ).

Overall mean WBLH aBMD was  $0.916 \pm 0.065 \text{ g/cm}^2$  and not significantly different between the groups ( $p=0.5$ , Table 26). WBLH aBMD was significantly associated with previous use of depo-provera (yes vs no:  $+2.9 \pm 1.3\%$ ,  $p=0.02$ ) in the ANCOVA model. Adjusting WBLH aBMD for previous use of depo-provera using ANCOVA model further diminished the difference between the groups ( $+0.1 \pm 1.1\%$ ,  $p=0.9$ ).

Mean WBLH SA-BMC (adjusted for BA, weight and height) was not significantly different between the groups ( $p=0.6$ , Table 26). When maternal age, parity, previous use of depo-provera, sex of the infant age and breastfeeding practice at PP14 were added into the ANCOVA model, only BA remained significant ( $\beta = +1.4 \pm 0.1\%$  per 1% increase in BA,  $p \leq 0.0001$ ); and the difference between the groups remained non-significant ( $+0.9 \pm 1.0\%$ ,  $p=0.4$ ).

### 7.10.2. TH bone mineral at PP14

Overall mean TH BMC was  $25.9 \pm 4.1$  g and not significantly different between the groups ( $p=0.8$ , Table 26). TH BMC was significantly associated with previous use of depo-provera (yes vs no:  $+9.9 \pm 2.8\%$ ,  $p \leq 0.0001$ ) in the ANCOVA model. Adjusting TH BMC for previous use of depo-provera using ANCOVA model increased the difference between the groups to  $+1.4 \pm 2.5\%$ , but remained non-significant ( $p=0.6$ ).

Overall mean TH BA was  $28.9 \pm 2.6$  cm<sup>2</sup>, and not significantly different between the groups ( $p=0.6$ , Table 26). Maternal age, parity, previous or current exposure to depo-provera, resumption of menses, breastfeeding practice, and sex of the infant did not have significant effects on TH BA in the ANCOVA model.

Overall mean TH aBMD was  $0.901 \pm 0.113$  g/cm<sup>2</sup>. There was a  $+1.3\%$  mean difference between the groups in TH aBMD but was not statistically significant ( $p=0.5$ , Table 26). TH aBMD was significantly associated with resumption of menses (yes vs no:  $+5.8 \pm 2.2\%$ ,  $p=0.008$ ), previous use of depo-provera (yes vs no:  $+6.4 \pm 2.3\%$ ,  $p=0.006$ ) and current use of depo-provera (yes vs no:  $-5.6 \pm 2.7\%$ ,  $p=0.04$ ) in the ANCOVA model. Adjusting TH aBMD for the resumption of menses, current and previous use of depo-provera decreased the difference between the groups to  $+0.7 \pm 2.1\%$  ( $p=0.7$ ).

There was a  $+2.3\%$  mean difference between the groups in TH SA-BMC (adjusted for BA, weight and height) but was not statistically significant ( $p=0.2$ , Table 26). When maternal age, parity, previous use of depo-provera, sex of the infant age and breastfeeding practice at PP14 were added to the ANCOVA model, only BA ( $\beta=+1.0 \pm 0.1\%$  per 1% increase in BA,  $p \leq 0.0001$ ), weight ( $\beta=+0.24 \pm 0.06\%$  per 1% increase in weight,  $p=0.002$ ) and resumption of menses (yes vs. no:  $+4.6 \pm 2.0\%$ ,  $p=0.02$ ) were significant; and the difference between the groups remained non-significant ( $+2.2 \pm 1.9\%$ ,  $p=0.3$ ).

### 7.10.3. Maternal LS bone mineral at PP14

Overall mean LS BMC was  $48.3 \pm 7.9$  g and not significantly different between the groups ( $p=0.9$ , Table 26). LS BMC was significantly associated with current use of depo-provera (yes vs no:  $-6.3 \pm 3.2\%$ ,  $p=0.05$ ) in the ANCOVA model. Adjusting LS BMC for current use of depo-provera increased the difference between the groups to  $+1.4 \pm 2.6\%$ , but remained non-significant ( $p=0.6$ ).

Overall mean LS BA was  $54.3 \pm 5.1$  cm<sup>2</sup> and not significantly different between the groups ( $p=1.0$ , Table 26). LS BA was significantly associated with current use of depo-provera (yes vs no:  $-4.0 \pm 1.8\%$ ,  $p=0.03$ ) in the ANCOVA model. Adjusting LS BA for current use of depo-provera increased the difference between the groups to  $+0.6 \pm 1.5\%$ , but remained non-significant ( $p=0.7$ ).

Overall mean LS aBMD was  $0.907 \pm 0.102 \text{ g/cm}^2$  and not significantly different between the groups ( $p=0.8$ , Table 26). LS aBMD was significantly associated with age of the infant ( $\beta = -0.6 \pm 0.2\%$  per day,  $p=0.01$ ) in the ANCOVA model. Adjusting LS aBMD for the age of infant increased the difference between the groups to  $+1.1 \pm 1.7\%$ , but remained non-significant ( $p=0.5$ ).

There was a non-significant  $+1.0\%$  mean difference between the groups in LS SA-BMC (adjusted for BA, weight and height) ( $p=0.5$ , Table 26). When maternal age, parity, previous use of depo-provera, sex of the infant and breastfeeding practice at PP14 were added into the model, only BA ( $\beta = +1.3 \pm 0.1\%$  per 1% increase in BA,  $p \leq 0.0001$ ), weight ( $\beta = +0.17 \pm 0.05\%$  per 1% increase in weight,  $p=0.005$ ) and age of the infant ( $\beta = -0.6 \pm 0.2\%$  per day,  $p=0.01$ ) were significant. Adjusting SA-BMC for BA, weight and age of infant increased the mean difference between the groups to  $+1.7 \pm 1.6\%$ , but remained non-significant ( $p=0.3$ ).

## 7.11. Infant anthropometry, growth and body composition at PP14

Table 27: Infant anthropometry, growth and body composition at PP14

Variable	HIV-Unexposed (n=81)	HIV-Exposed (n=85)	Mean % Diff between the groups $\pm$ SE (HIV+ vs HIV-) <sup>1</sup>	P-value
<b>Anthropometry</b>				
Weight, kg (Mean $\pm$ SD)	6.5 $\pm$ 0.8	6.1 $\pm$ 0.8	-5.9 $\pm$ 2.1 <sup>1</sup>	<b>0.004</b>
Length, cm (Mean $\pm$ SD)	61.5 $\pm$ 2.6	60.3 $\pm$ 2.5	-2.0 $\pm$ 0.7 <sup>1</sup>	<b>0.002</b>
Head circumference, cm (Mean $\pm$ SD) <sup>2</sup>	41.1 $\pm$ 1.5	40.9 $\pm$ 1.4	-0.7 $\pm$ 0.6 <sup>1</sup>	0.2
<b>Growth indices<sup>3</sup></b>				
Weight-for-Age Z-scores, WAZ (Mean $\pm$ SD)	0.21 $\pm$ 1.04	-0.27 $\pm$ 1.02	-0.47 $\pm$ 0.16 <sup>4</sup>	<b>0.003</b>
Length-for-Age Z-scores, LAZ (Mean $\pm$ SD)	0.03 $\pm$ 1.27	-0.49 $\pm$ 1.09	-0.53 $\pm$ 0.18 <sup>4</sup>	<b>0.005</b>
Head Circ-for-Age Z-scores, (Mean $\pm$ SD) <sup>2</sup>	0.66 $\pm$ 1.16	0.43 $\pm$ 1.08	-0.23 $\pm$ 0.18 <sup>4</sup>	0.2
Weight-for-length Z-scores, WFLZ (Mean $\pm$ SD)	0.29 $\pm$ 1.17	0.20 $\pm$ 1.19	-0.09 $\pm$ 0.18 <sup>4</sup>	0.6
<b>Whole body composition<sup>5</sup></b>				
Lean Mass (g) (Mean $\pm$ SD)	3789 $\pm$ 847	3556 $\pm$ 628	-5.2 $\pm$ 3.2	0.1
Fat Mass (g) (Mean $\pm$ SD)	3158 $\pm$ 993	2980 $\pm$ 869	-2.7 $\pm$ 6.1	0.7
% Fat, Mean $\pm$ SD	44.6 $\pm$ 12.1	44.9 $\pm$ 9.8	+2.7 $\pm$ 4.9	0.6

<sup>1</sup> Mean % differences between the groups presented as sympercents $\pm$ SE. All variables were transformed into natural logarithms\*100 before statistical analysis using the ANOVA function in Datadesk software

<sup>2</sup> excludes 4 extreme measurements (HIV-unexposed n 79, HIV-exposed n=83)

<sup>3</sup> age and sex standardised indices calculated in STATA using WHO Anthro plug-in based on WHO 2006 reference standards

<sup>4</sup> difference in Z-scores  $\pm$ SE. Data not log transformed

<sup>5</sup> adjusted for sex and age

### *7.11.1. Infant anthropometry and growth indices at PP14*

Overall mean infant weight was  $6.3 \pm 0.8$  kg. HEI had 5.9% significantly lower weight compared to HUI ( $p=0.004$ , Table 27). Weight was significantly associated with gestation age at birth ( $\beta = +0.14 \pm 0.03$  kg per week,  $p \leq 0.0001$ ), infant sex (boys vs. girls:  $+0.5 \pm 0.1$  kg,  $p \leq 0.0001$ ) and age at PP14 ( $\beta = +0.03 \pm 0.02$  kg per day,  $p=0.02$ ). The mean difference between the groups was highly significant after adjusting infant weight for age at PP14, gestation age and sex in the ANCOVA model ( $-6.1 \pm 1.9\%$ ,  $p=0.001$ ).

Overall mean infant length was  $60.9 \pm 2.3$  cm. HEI were significantly shorter compared to HUI (2.0%,  $p=0.002$ , Table 27). Length was significantly associated with gestation age at birth ( $\beta = +0.3 \pm 0.1$  cm per week,  $p=0.0004$ ), age at PP14 ( $\beta = +0.14 \pm 0.05$  cm per day,  $p=0.005$ ), infant sex (boys vs. girls:  $+2.0 \pm 0.3$  cm,  $p \leq 0.0001$ ) and maternal age ( $\beta = +0.10 \pm 0.04$  cm per year,  $p=0.01$ ). Adjusting infant length for gestation age, maternal age, infant age and sex did not have a material effect on the mean difference between the groups in the ANCOVA model ( $-1.8 \pm 0.6\%$ ,  $p=0.003$ ).

Mean head circumference was  $40.9 \pm 1.5$  cm and not significantly different between the groups ( $p=0.2$ ). Head circumference was significantly associated with gestation age at birth ( $\beta = +0.32 \pm 0.05$  cm per week,  $p \leq 0.0001$ ), infant sex (boys vs. girls:  $+1.0 \pm 0.1$  cm,  $p \leq 0.0001$ ) and age at PP14 ( $\beta = +1.0 \pm 0.3$  cm,  $p=0.0008$ ) in the ANCOVA model. There was a trend towards lower mean head circumference in HEI after adjusting for gestation age, age at PP14 and sex ( $-0.9 \pm 0.5\%$ ,  $p=0.05$ ).

Mean WAZ and LAZ were  $-0.03 \pm 1.05$  and  $-0.24 \pm 1.21$ , respectively. HEI had significantly lower WAZ and LAZ compared to HUI. Mean HCAZ and WFLZ were respectively  $+0.54 \pm 1.12$  and  $-0.24 \pm 1.18$  and not significantly different between the groups (Table 27). The differences between the groups were similar when WAZ was analysed by sex (boys:  $-0.5 \pm 0.2\%$ ,  $p=0.02$ ; girls:  $0.5 \pm 0.2\%$ ,  $p=0.03$ ). There was also a  $-0.5 \pm 0.2\%$  mean difference between the groups in HAZ for both boys and girls, but the difference in girls was not significant (boys  $p=0.04$ ; girls  $p=0.06$ ). There were no significant differences between boys and girls in both HCAZ and WFLZ.

### 7.11.2. Infant body composition at PP14

Median WB lean mass was 3534 g (IQR 3190, 4037g) and not significantly different between the groups ( $p=0.1$ , Table 27). Lean mass was significantly associated with sex (boys vs. girls:  $+410\pm118$  g,  $p\leq0.0001$ ), birth order (first born yes vs. no:  $-244\pm121$  g,  $p=0.05$ ) and maternal age ( $\beta = +32.1\pm13.3$  g per year  $p=0.02$ ) in the ANCOVA model. Adjusting infant lean mass for maternal age, birth order and infant sex did not have a material effect on the mean difference between the groups ( $-4.9\pm3.1\%$ ,  $p=0.1$ ).

Overall median WB fat mass was 3070g (IQR 2438, 3649g) and not significantly different between the groups ( $p=0.7$ , Table 27). Fat mass was significantly associated with gestation age at birth ( $\beta = +95\pm39$  g per week,  $p=0.01$ ) and exclusive breastfeeding (yes vs no:  $+392\pm169$ g,  $p=0.02$ ) in the ANCOVA model. Adjusting fat mass for gestation age and exclusive breastfeeding did not have a material effect on the results ( $-6.5\pm6.0\%$ ,  $p=0.2$ ).

The mean percent fat was  $44.8\pm11.0\%$  and not significantly different between the groups ( $p=0.6$ , Table 27). Infant age, sex, gestation age at birth, history of illness since the PP2 visit, exclusive breastfeeding, birth order and maternal age) did not have significant effects on the percent fat in ANCOVA model.

### 7.12. Infant bone mineral at PP14

Table 28: Infant bone mineral at PP14

Variable	HIV-Unexposed (n=76) Mean $\pm$ SD	HIV-Exposed (n=80) Mean $\pm$ SD	Mean % Diff between the groups $\pm$ SE (HIV+ vs HIV-) <sup>1</sup>	P-value
<b>Whole body</b>				
Bone Mineral Content, BMC (g)	140 $\pm$ 20	133 $\pm$ 19	-4.8 $\pm$ 2.6	0.06
Bone Area, BA (cm <sup>2</sup> )	634 $\pm$ 56	612 $\pm$ 55	-3.5 $\pm$ 1.5	<b>0.02</b>
Bone Mineral Density, BMD (g/cm <sup>2</sup> )	0.219 $\pm$ 0.017	0.216 $\pm$ 0.017	-1.3 $\pm$ 1.3	0.3
SA-BMC (g) <sup>2</sup>	134 $\pm$ 7	136 $\pm$ 9	+0.7 $\pm$ 1.0	0.5
<b>Lumbar Spine</b>				
Bone Mineral Content, BMC (g)	2.37 $\pm$ 0.37	2.29 $\pm$ 0.39	-3.3 $\pm$ 2.8	0.2
Bone Area, BA (cm <sup>2</sup> )	11.7 $\pm$ 0.9	11.2 $\pm$ 1.0	-4.3 $\pm$ 1.4	<b>0.002</b>
Bone Mineral Density, BMD (g/cm <sup>2</sup> )	0.201 $\pm$ 0.024	0.203 $\pm$ 0.024	+1.1 $\pm$ 2.0	0.6
SA-BMC (g) <sup>2</sup>	2.28 $\pm$ 0.26	2.35 $\pm$ 0.26	+3.6 $\pm$ 2.0	0.07

<sup>1</sup> Mean % differences between the groups presented as sympercents $\pm$ SE. All variables were transformed into natural logarithms\*100 before statistical analysis using the ANOVA function in Datadesk software.

<sup>2</sup> Adjusted for BA, body weight and length

### 7.12.1. Infant WB bone mineral at PP14

Mean infant WB BMC was  $136.2 \pm 19.8$  g. There was a trend towards lower WB BMC among HIV-exposed infants compared to HIV-unexposed infants, but the difference between the groups was not significant ( $-4.8\%$ ,  $p=0.06$ ; Table 28). Infant WB BMC was significantly associated with gestation age at birth ( $\beta = +2.9 \pm 0.6\%$  per week,  $p \leq 0.0001$ ) in the ANCOVA model. Adjusting WB BMC for gestation age reduced the difference between the groups to  $-3.1 \pm 2.6\%$  ( $p=0.2$ ).

Mean infant WB BA was  $623 \pm 56$  cm<sup>2</sup>. HEI had significantly lower WB BA compared to HUI ( $3.5\%$ ,  $p=0.02$ , Table 28). WB BA was significantly associated with gestation age at birth ( $\beta = +1.6 \pm 0.4\%$  per week,  $p \leq 0.0001$ ), sex (boys vs. girls:  $+6.0 \pm 1.4\%$ ,  $p \leq 0.0001$ ), history of illness since PP2 visit (yes vs. no:  $-4.9 \pm 2.0\%$ ,  $p=0.01$ ) and maternal age ( $\beta = +0.4 \pm 0.1\%$  per year,  $p=0.01$ ) in the ANCOVA model. Adjusting WB BA for gestation age, sex, history of illness visit and maternal age slightly reduced the mean difference between the groups but remained significant ( $-3.3 \pm 1.5\%$ ,  $p=0.03$ ).

Mean infant WB aBMD was  $0.219 \pm 0.017$  g/cm<sup>2</sup> and not significantly different between the groups ( $p=0.3$ , Table 28). WB aBMD was significantly associated with gestation age at birth ( $\beta = +1.5 \pm 0.3\%$  per week,  $p \leq 0.0001$ ) and age at PP14 ( $\beta = +0.4 \pm 0.2\%$  per day,  $p=0.02$ ) in the ANCOVA model. Adjusting WB aBMD for gestation age and age of infant increased the difference between the groups to  $+1.8 \pm 1.2\%$  but remained non-significant ( $p=0.1$ ).

Mean infant WB SA-BMC (adjusted for WB BA, weight and length) was not significantly different between the groups ( $p=0.5$ , Table 28). When infant age and sex, gestation age at birth, maternal age, birth order, breastfeeding status and history of illness within the past 2 weeks were added into the ANCOVA model, the significant variables were BA ( $\beta = +1.3 \pm 1.1\%$  per 1% increase in BA,  $p \leq 0.0001$ ), weight ( $\beta = +0.3 \pm 0.1\%$  per 1% increase in weight,  $p=0.001$ ), length ( $\beta = -0.5 \pm 0.2\%$  per 1% increase in length,  $p=0.001$ ), gestation age at birth ( $\beta = +0.7 \pm 0.3\%$  per week,  $p=0.01$ ), age at PP14 ( $\beta = +0.3 \pm 0.1\%$  per day,  $p=0.05$ ), and history of illness (yes vs. no:  $+3.4 \pm 1.4\%$ ,  $p=0.01$ ). Adjusting SA-BMC for gestation age, age at PP14 and history of illness did not have a material effect on the mean difference between the groups ( $+0.4 \pm 1.1\%$ ,  $p=0.7$ ).

### 7.12.2. Infant LS bone mineral at PP14

Overall mean infant LS BMC was  $2.33 \pm 0.38$  g and not significantly different between the groups ( $p=0.2$ , Table 28). Infant LS BMC was significantly associated with gestation age at birth ( $\beta = +2.9 \pm 0.7\%$  per week,  $p \leq 0.0001$ ) in the ANCOVA model. Adjusting infant LS BMC for gestation age did not have a material effect on the mean difference between the groups ( $-3.2 \pm 2.6\%$   $p=0.2$ ).

Mean infant LS BA was  $11.5 \pm 1.0$  cm<sup>2</sup>. HEI had significantly lower LS BA compared to HUI ( $-4.3\%$   $p=0.002$ , Table 28). LS BA was significantly associated with gestation age at birth ( $\beta = +1.1 \pm 0.3\%$  per week,  $p=0.003$ ), infant age ( $\beta = +0.4 \pm 0.2\%$  per day,  $p=0.03$ ) and sex (boys vs. girls:  $+4.0 \pm 1.3\%$ ,  $p=0.002$ ) in the ANCOVA model. Adjusting infant LS BA for gestation age, infant age and sex did not have a material effect on the results ( $-4.3 \pm 1.3\%$ ,  $p=0.001$ ).

Mean infant LS aBMD was  $0.204 \pm 0.024$  g/cm<sup>2</sup> and not significantly different between the groups ( $p=0.6$ , Table 28). LS aBMD was significantly associated with gestation age at birth ( $\beta = +1.9 \pm 0.5\%$  per week,  $p=0.0004$ ) in the ANCOVA model. Adjusting infant LS aBMD for gestation age did not have an effect on the difference between the groups ( $+1.1 \pm 1.9\%$ ,  $p=0.6$ ).

There was a trend towards higher mean LS SA-BMC among HIV-exposed infants compared to HIV exposed infants after LS BMC was adjusted for BA, weight and length in ANCOVA model ( $+3.6 \pm 2.0\%$ ,  $p=0.07$ , Table 28). When infant age and sex, gestation, maternal age, birth order, breastfeeding status and history of illness within the past 2 weeks were added into the ANCOVA model, only BA ( $\beta = +1.3 \pm 1.1\%$  per 1% increase in BA,  $p \leq 0.0001$ ) and gestation age at birth were significant ( $\beta = +1.5 \pm 0.5\%$  per week,  $p=0.005$ ). Adjusting infant LS SA-BMC for gestation age at birth reduced the mean difference between the groups to  $+3.0 \pm 2.0\%$  ( $p=0.1$ ).

### 7.13. Summary of results at PP14

All mother-baby pairs were breastfeeding, but a significantly greater proportion of HIV-positive women (86.7%) were exclusively breastfeeding compared to 66.2% among HIV-negative women ( $p=0.002$ ). Also, the proportion of women with postpartum exposure to depo-provera was greater in the HIV-positive group compared to the HIV-negative group.

Overall maternal consumption of dietary sources of calcium and vitamin D was not significantly different between the groups. However, a greater proportion of HIV-positive compared to negative women reported consumption of small fish with bones. The majority of participants routinely engaged in physical activities mostly household chores like cleaning utensils, hand washing clothes among others, but fewer HIV-positive women reported walking for at least 30 minutes a day.

HIV-positive women had lower lean mass compared to HIV-negative women (-5.1%). Mean fat mass, body weight, BMI and MUAC) were not significantly different between the groups although the mean values for all anthropometric measurements were lower in HIV-positive women.

There were no significant differences between the groups in mean maternal bone mineral parameters (BMC, BA, aBMD and SA-BMC) at all the skeletal sites. However, there was a trend towards higher TH SA-BMC (adjusted for weight, height and BA) compared to HIV-negative women although the difference between the groups was not significant ( $+2.3 \pm 2.0\%$ ,  $p=0.2$ ).

Overall mean albumin-corrected [P-Ca], estimated GFR (by CKD-EPI equation), TmP/GFR and TmCa/GFR were significantly lower but [P-Cr] was higher in HIV-positive compared to negative women. Other plasma biochemistry were not significantly different between the groups. Mean [PTH], [CTX] and [BAP] were significantly higher in HIV-positive women compared to HIV-negative women. However, mean [25(OH)D] and [P1NP] were not significantly different between the groups. Mean [BM-P] was higher among HIV-positive compared to negative women ( $+9.8\%$ ,  $p=0.002$ ), and significantly correlated with maternal plasma [CTX] in a separate ANCOVA model ( $\beta = +0.13 \pm 0.04\%$  per 1% increase in CTX,  $p=0.0003$ ). However, [BM-Ca], [BM-Na], BM Ca/P, [BM-Na] and [BM-K] were not significantly different between the groups.

HEI had significantly lower weight (-5.9%) and length (-2.0%) compared to HUI. Also, WAZ and LAZ were significantly lower in HEI compared to HUI although the group means were within the normal range. HEI also had 6.3% lower lean mass compared to HUI. Both head circumference and HCAZ were not significantly different between the groups.



HEI had significantly lower WB BMC compared to HIV-unexposed infants (-5.3%,  $p \leq 0.0001$ ). They also had a trend towards lower LS BMC although the difference between the groups was not significant (-3.7%,  $p = 0.06$ ). Both WB and LS BA were lower in HEI compared to HUI. However, WB and LS aBMD were not significantly different between the groups. Also, both WBLH and LS SA-BMC (adjusted for BA, body weight and length) was not significantly different between the groups, showing that HEI had adequate bone for attained body size.

## CHAPTER 8: LONGITUDINAL RESULTS

This chapter presents longitudinal changes in maternal (sections 8.1 to 8.5) and infant outcomes (sections 8.6 to 8.7). Results were obtained from Scheffé post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA and ANCOVA models that included subject (nested by group), group, visit, and group\*visit interaction. For all comparisons, the reference group for maternal outcomes is the HIV-negative group and the reference group for infant outcomes is HUI. Therefore, the sign of all differences between the groups is for the comparison of HIV-positive vs HIV-negative (sections 8.1 to 8.5) or HEI vs HUI (sections 8.6 to 8.7).

The variance for each group at each visit is smaller in the nested longitudinal models than in cross-sectional models because the subject is nested within the group and thereby between-individual variation is minimised. Therefore, some of the differences between the groups that were not significant in the cross-sectional analysis (presented in chapters 5, 6 and 7) became significant in the longitudinal models. The significant group differences in the longitudinal models are highlighted on the figures by an asterisk at the corresponding visit, presented as the difference (Diff, p=value)\* and discussed in the text.

All maternal outcomes were adjusted for exclusive breastfeeding (yes vs no), illness in the baby, and exposure to depo-provera (yes vs no). All infant outcomes were adjusted for breastfeeding status and illness within 2 weeks of the study visit. Other factors (maternal age, parity and infant sex) were not included in the longitudinal models because they were not anticipated to change. Resumption of menses was also not included because data were available only at PP14. Unadjusted results are presented in the summary tables, and the adjusted results are discussed in the text. Unless explicitly mentioned, co-factors which are not mentioned in the text were not significant in the nested GLM models.

## 8.1. Changes in maternal anthropometry and body composition

### 8.1.1. Changes in maternal anthropometry

Table 29: Mean percent changes in maternal anthropometry between visits

	HIV-Negative		HIV-Positive		Group*visit interaction p-value
	(%Δ±SE) <sup>1</sup>	p-value	(%Δ±SE) <sup>1</sup>	p-value	
<b>PG36-PP2</b>	<b>(n=77)</b>		<b>(n=84)</b>		
Weight (kg)	-8.7±0.7	<b>≤0.0001</b>	-9.5±0.7	<b>≤0.0001</b>	0.2
MUAC (cm)	-0.003±0.8	0.9	-0.8±0.7	0.6	0.5
<b>PP2-PP14</b>	<b>(n=73)</b>		<b>(n=81)</b>		
Weight (kg)	-1.4±0.7	0.1	-0.8±0.7	0.5	0.8
BMI (kg/m <sup>2</sup> )	-1.0±0.7	0.4	-0.7±0.7	0.6	0.7
MUAC (cm)	+0.3±0.8	0.9	+1.3±0.7	0.2	0.1
<b>PG36-PP14</b>	<b>(n=73)</b>		<b>(n=80)</b>		
Weight (kg)	-10.1±0.7	<b>≤0.0001</b>	-10.3±0.7	<b>≤0.0001</b>	0.7
MUAC (cm)	+0.3±0.8	0.9	+0.6±0.7	0.8	0.6

<sup>1</sup> Mean % changes presented as sympercents±SE. All variables were transformed into natural logarithms\*100 before statistical analysis in DataDesk software. Results were obtained from Scheffé's post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction.

As expected, both groups significantly lost weight between PG36 and PP2; but there were no significant changes in weight between PP2 and PP14 (Table 29). Also, there were no significant changes in MUAC between PG36 and PP14 in both groups. The magnitudes of change in both weight and MUAC were not significantly different between the groups (Figure 45). Exposure to depo-provera was negatively associated with weight in the nested ANCOVA model (yes vs no: -3.6±1.1%,  $p \leq 0.0001$ ). Adjusting for the use of depo-provera (either pre-or post-index pregnancy) did not have a material effect on the difference between the groups in changes in weight (-10.5% vs -10.1%,  $p=0.7$ ).

The mean difference in weight between the groups at each visit was significant in the unadjusted model, and the differences were similar to those observed in the cross-sectional analysis (e.g. at PP14 longitudinal model: -4.9±0.7%,  $p \leq 0.0001$  vs cross-sectional model: -4.6±2.6%,  $p=0.07$ ).

Figure 45(a)

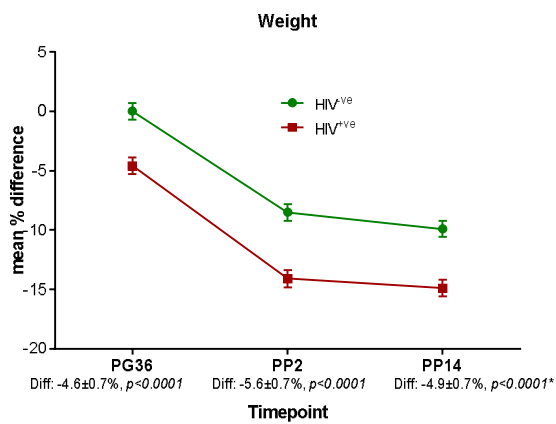


Figure 45(b)

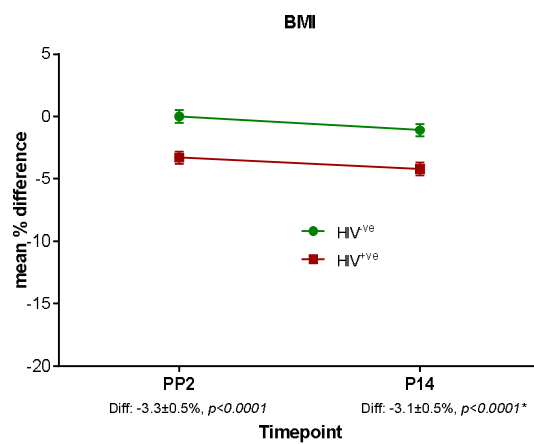


Figure 45(c)

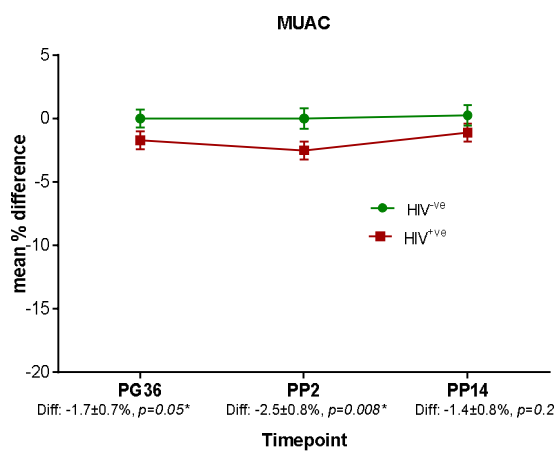


Figure 45 (d)

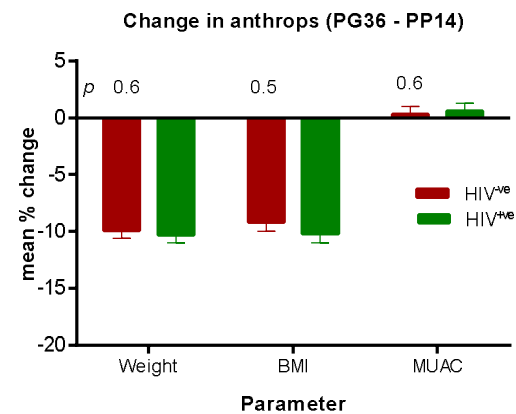


Figure 45: Change in maternal anthropometry (PG36-PP14)

BMI=Body mass index ( $\text{kg}/\text{m}^2$ ), MUAC= Mid-upper-arm circumference (cm); Diff = mean sympercent difference between the groups  $\pm$  SD. Results were obtained from Scheff'e post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction. The number of subjects included in the analysis was 96 HIV-negative and 93 HIV-positive mothers. Figure 45 (d) Bars and error bars represent the mean  $\pm$  SE sympercent changes within the groups between PG36 and PP14. The p- values shown above the bars are for between-group comparison of mean % changes between PG36 and PP 14 for the corresponding parameter.

### 8.1.2. Changes in maternal WBLH body composition

Table 30: Mean percent change in maternal body composition measured by DXA (PP2 - PP14)

	HIV-Negative (n=74)		HIV-Positive (n=81)		Group*visit interaction p-value
	(%Δ±SE)	p-value	(%Δ±SE)	p-value	
Fat Mass (g)	+2.2±3.2	0.5	+6.1±3.2	<b>0.05</b>	0.4
Lean Mass (g)	-2.0±1.3	0.1	-2.4±1.3	<b>0.05</b>	0.8
Fat:Lean <sup>2</sup> ratio (kg/kg <sup>2</sup> )	+6.1±5.5	0.3	+11.0±5.3	<b>0.04</b>	0.5

<sup>†</sup> Mean % changes presented as sympercents±SE. All variables were transformed into natural logarithms\*100 before statistical analysis. Results were obtained from Scheffe post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction.

Significant changes in fat, lean mass and fat:lean<sup>2</sup> were observed only in HIV-positive women (fat mass +6.1%, lean mass - 2.4% and fat:lean<sup>2</sup> + 11%), but the magnitudes of the changes were not significantly different between the groups (Table 30). Exposure to depo-provera was negatively associated with body composition (fat -6.7±3.8%, p=0.07; lean -2.7±1.5%, p=0.07 and fat:lean<sup>2</sup> - 12.1±6.3, p=0.06). Changes in body composition in HIV-positive women were no longer significant after adjusting for the use of depo-provera (fat +5.7±3.2%, p=0.07; lean -2.1±1.2%, p=0.08; fat:lean<sup>2</sup> +10.0±5.4%, p=0.06).

The mean difference between the groups in lean mass at PP2 was significant in the unadjusted hierarchical model (-4.5±1.3%, p≤0.0001, Figure 46), but the magnitude was similar the cross-sectional analysis (-4.0±2.3%, p=0.08). Both lean and fat:lean<sup>2</sup> were not significantly different between the groups at either PP2 or PP14 in both unadjusted and adjusted models.

Figure 46(a)

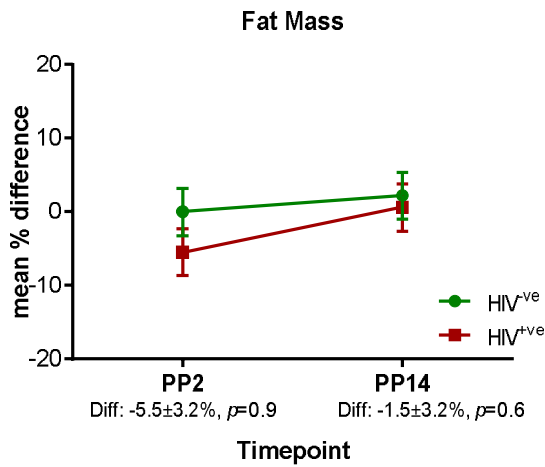


Figure 46(b)

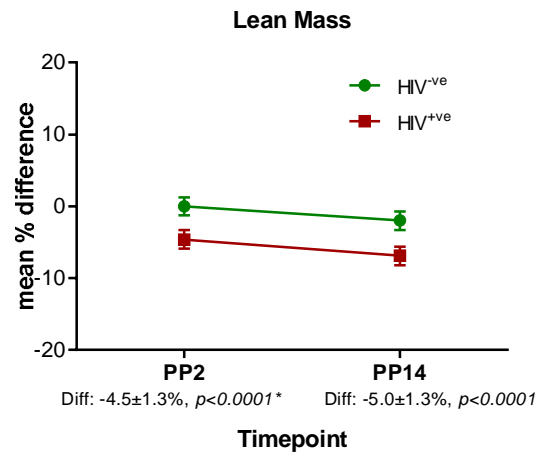


Figure 46(c)

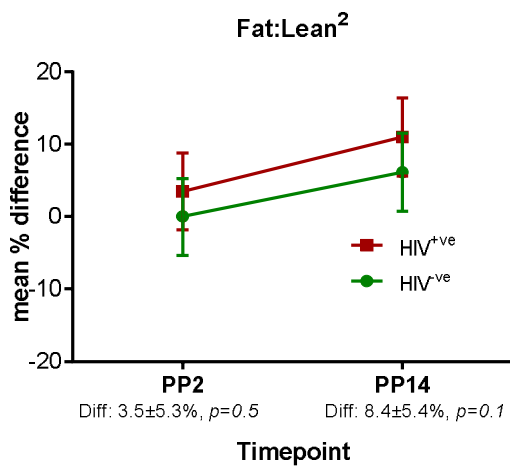


Figure 46(d)

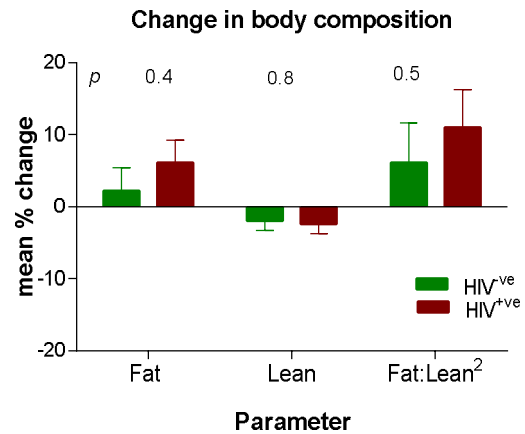


Figure 46: Changes in maternal body composition (PP2-PP14)

Units for lean and fat mass =g. Diff = mean sympercent difference between the groups±SD. Results were obtained from Scheff'e post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction. The number of subjects included in the analysis was 81 HIV-negative and 83 HIV-positive mothers. Figure 46 (d) Bars and error bars represent the mean±SE sympercent changes within groups between PP2 and PP14. The p-values shown above the bars are for comparison of mean % changes between the groups at 14 weeks for the corresponding parameter.

## 8.2. Change in maternal plasma and urine biochemistry

Table 31: Summary of changes in maternal biochemistry (PG36 - PP14)

Variable	HIV-Negative (n=74)		HIV-Positive (n=81)		Group*visit interaction p-value
	(%Δ±SE) <sup>1</sup>	p-value	(%Δ±SE) <sup>1</sup>	p-value	
<b>Plasma biochemistry</b>					
Plasma albumin (g/L)	+37.6±1.0	≤0.0001	+35.5±1.0	≤0.0001	0.1
Plasma calcium <sub>uncorrected</sub> (mmol/L) <sup>2</sup>	+6.6±0.5	≤0.0001	+3.0±0.5	≤0.0001	≤0.0001
Plasma calcium <sub>Corrected</sub> (mmol/L) <sup>2,4</sup>	-3.8±0.5	≤0.0001	-6.6±0.5	≤0.0001	≤0.0001
Plasma phosphate (mmol/L)	+7.7±2.0	≤0.0001	+14.4±2.0	≤0.0001	0.02
Plasma magnesium (mmol/L)	+8.6±1.1	≤0.0001	+6.6±1.1	≤0.0001	0.2
Plasma creatinine (μmol/L)	+16.6±1.3	≤0.0001	+18.7±1.3	≤0.0001	0.3
<b>Urine biochemistry</b> <sup>5,6</sup>					
UCa/Cr ratio (mmol/mmol)	-79.9±17.5	≤0.0001	-97.8±17.6	≤0.0001	0.5
UP/Cr ratio (mmol/mmol)	-32.1±8.7	≤0.0001	-22.6±8.7	0.01	0.4
UMg/Cr ratio (mmol/mmol)	-11.8±7.6	0.1	+5.6±7.6	0.4	0.1
<b>Renal parameters</b>					
eGFR, CKD-EP (ml/min/1.73m <sup>2</sup> BSA) <sup>7</sup>	-10.2±1.1	≤0.0001	-13.6±1.1	≤0.0001	0.04
TmP/GFR (mmol/L GFR)	+11.3±2.7	≤0.0001	+18.1±2.9	≤0.0001	0.1
TmCa/GFR (mmol/L GFR)	+7.6±0.7	≤0.0001	+3.2±0.7	≤0.0001	≤0.0001

<sup>1</sup> Mean % changes presented as sympercents±SE. All variables were transformed into natural logarithms\*100 before statistical analysis in DataDesk software. Results were obtained from Scheffé's post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction.

<sup>2</sup> Excludes 4 outliers. 2 HIV positive -6.27, 3.25 at PP14) and 2 HIV-negative (3.35 at PG36, 7.53 at PP14. group n=79, n=80

<sup>3</sup> Normalised for albumin using a linear model with albumin as a covariant

<sup>4</sup> Normalised for albumin using the Payne equation: Adjusted plasma Ca= Measured Ca+ ([40-serum albumin]\*0.02).

<sup>5</sup> Thirty-four measurements below the detection of limit (0.02 mmol/L) were assigned half a detection (0.01mmol/L) before analysis.

<sup>6</sup> Less one participant whose samples were not shipped from Uganda.

<sup>7</sup>CKD –EPI. Excludes two low values (outliers) at PG36 which influence group\*visit interaction term (p=0.04). One participant per group.

### 8.2.1. Change in plasma biochemistry

#### 8.2.1.1. Plasma albumin and calcium

Mean [P-Alb] and uncorrected [P-Ca] significantly increased in both groups between PG36 and PP14 (Table 31). Changes in [P-Alb] were not significantly different between the groups. However, HIV-positive women had a significantly smaller change in uncorrected [P-Ca] compared to HIV-negative women (+3.0±0.5 vs +6.6±0.5, p= ≤0.0001). Mean albumin-corrected [P-Ca] decreased in both groups, but HIV-positive women had a significantly greater decrease (-6.6% vs -3.8%, p= ≤0.0001).

Similar to cross-sectional analysis, HIV-positive women had lower [P-Alb], and both corrected and uncorrected [P-Ca] at PP14, but no significant difference between the groups at PG36 (Figure 47). Depo-provera was not significantly associated with either [P-Alb] or [P-Ca] in the respective nested models.

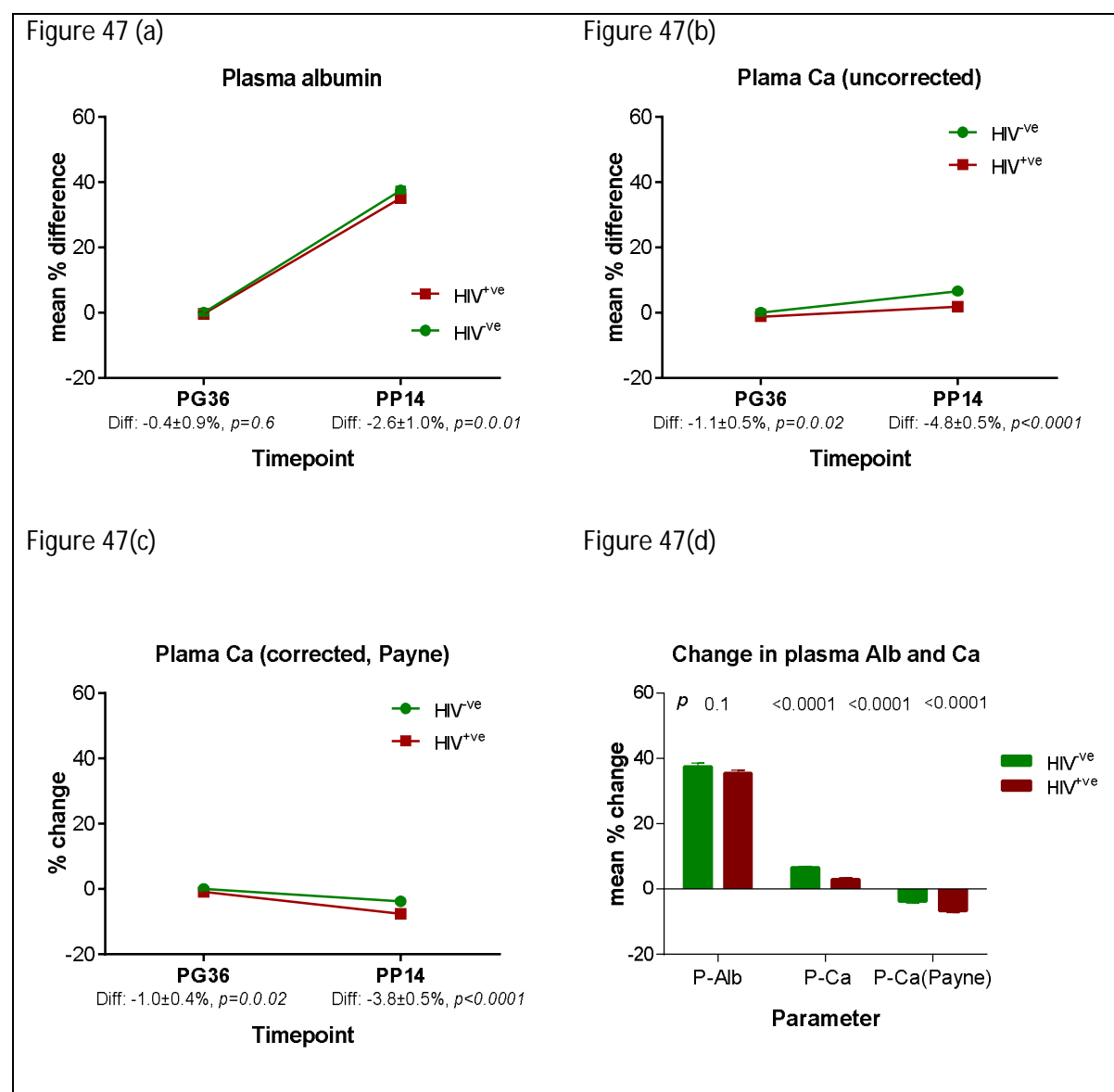


Figure 47: Change in maternal plasma albumin and calcium (PG36 and PP14)

Plasma albumin units = g/L, plasma calcium units = mmol/L; Diff = mean sympercent difference between the groups ± SD. Results were obtained from Scheffé post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction. The number of subjects included in the analysis was 96 HIV-negative and 93 HIV-positive mothers. Figure 47 (d): Bars and error bars represent the mean ± SE sympercent changes within the groups between PP2 and PP14. The p-values shown above the bars are for comparison of mean % changes between the groups at 14 weeks for the corresponding parameter



### 8.3.1.2. Plasma phosphate, magnesium and creatinine

Mean [P-PO<sub>4</sub>], [P-Mg] and [P-Cr] increased in both groups. However, HIV-positive women had a significantly greater increase in [P-PO<sub>4</sub>] compared to HIV-negative women (+14.4 vs 7.7%,  $p=0.02$ ; Table 31). Changes in [P-Mg] and [P-Cr] were not significantly different between the groups. Similar to cross-sectional analysis, HIV-positive women had significantly lower [P-PO<sub>4</sub>] at PG36, but there was no significant difference between the groups at PP14 ( $p=0.4$ , Figure 48a). Depo-provera was not significantly associated with [P-PO<sub>4</sub>], [P-Mg] or [P-Cr] in the respective nested models.

### 8.2.2. Change in urine biochemistry

Mean UCa/Cr and UP/Cr ratios significantly reduced in both groups (Figure 49a-b) and the changes were not significantly different between the groups. In contrast, there were no significant changes in UMg/Cr in both groups. Adjusting for the use of depo-provera did not have a material effect on UCa/Cr, UP/Cr and UMg/Cr ratios. Overall, the magnitudes of mean differences between the groups in UCa/Cr, UP/Cr and UMg/Cr at PG36 and PP14 were very similar to those observed in the respective cross-sectional models.

### 8.2.3. Change in renal parameters

eGFR significantly reduced in both groups (Figure 50 and Table 31). HIV-positive women had a significantly greater reduction in eGFR compared to HIV-negative women ( $-13.6 \pm 1.1\%$  vs.  $-10.2 \pm 1.1\%$ ;  $p=0.04$ ). TmP/GFR and TmCa/GFR significantly increased in both groups. However, HIV-positive women had a significantly smaller increase in TmCa/GFR ( $+7.6 \pm 0.7\%$  vs.  $+3.2 \pm 0.8\%$ ,  $p \leq 0.0001$ ; Figure 50). The changes in TmP/GFR were not significantly different between the groups ( $+18.1 \pm 2.9\%$  vs  $+11 \pm 2.7\%$ ,  $p=0.1$ ). Unlike in the cross-sectional model, mean TmP/GFR was not significantly different between the groups at PP14 (nested model:  $-4.7 \pm 3.1\%$ ,  $p=0.2$ ; cross-sectional model:  $-7.2 \pm 3.0\%$ ,  $p=0.02$ ). Overall, adjusting both TmP/GFR and TmCa/GFR for depo-provera did not have a material effect on the results in the nested models.

Figure 48(a)

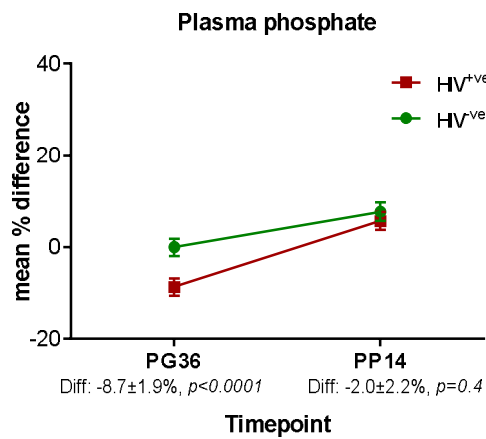


Figure 48(b)

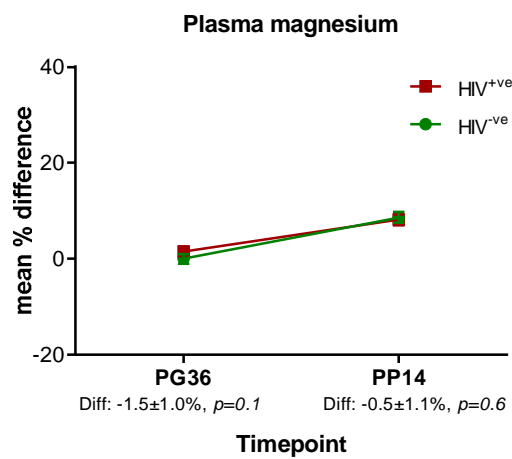


Figure 48(c)

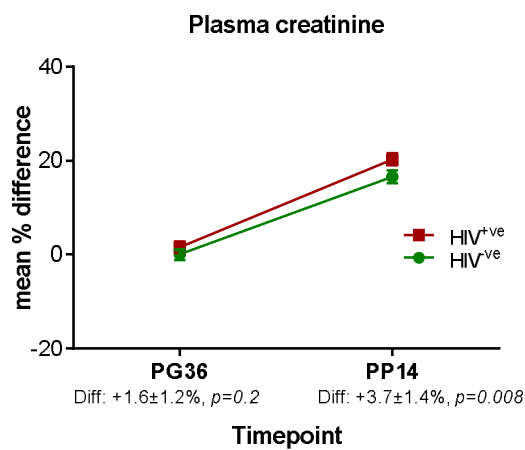


Figure 48(d)

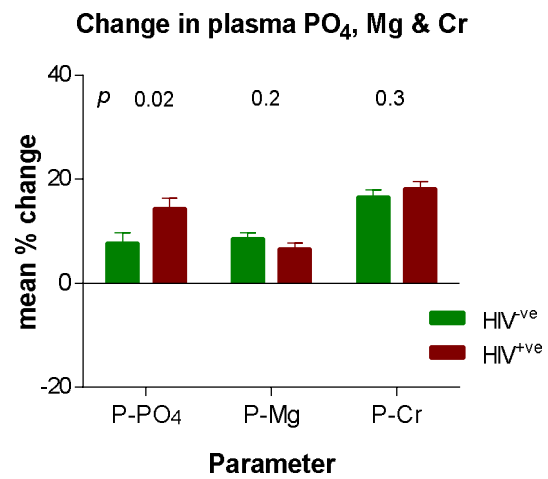


Figure 48: Change in maternal plasma phosphate, magnesium and creatinine (PG36-PP14)

Plasma phosphate and magnesium units = mmol/L, plasma creatinine units =  $\mu$ mol/L. Diff = mean sympercent difference between the groups  $\pm$  SD. Results were obtained from Scheffé's post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction. The number of subjects included in the analysis was 96 HIV-negative and 93 HIV-positive mothers. Figure 48(d): Bars and error bars represent the mean  $\pm$  SE sympercent changes within groups between PG36 and PP14. The p-values shown above the bars are for comparison of mean % changes between the groups at 14 weeks for the corresponding parameter.

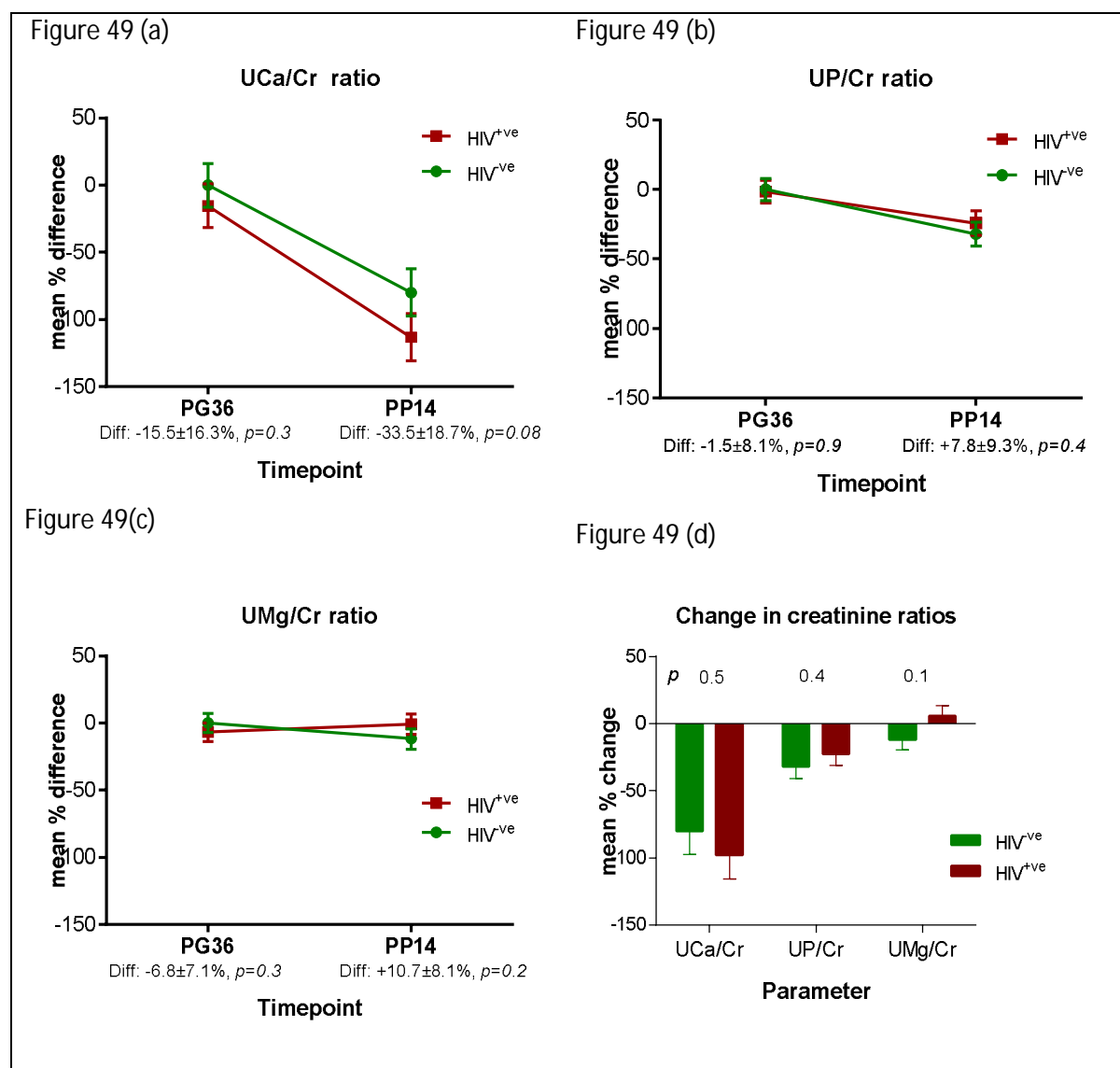


Figure 49: Change in maternal Ca/Cr, P/Cr and Mg/Cr ratios (PG36-PP14)

Units mmol/mmol. UCa/Cr = Urine calcium/creatinine ratio; UP/Cr = Urine phosphate/creatinine ratio; UMg/Cr = Urine magnesium/creatinine ratio; Diff = mean sympercent difference between the groups ± SD. Results were obtained from Scheffé's post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction. The number of subjects included in the analysis was 96 HIV-negative and 93 HIV-positive mothers. Figure 49(d) Bars and error bars represent the mean ± SE sympercent changes within groups at 14 weeks postpartum. The *p*-values shown above the bars are for comparison of mean % changes between the groups at 14 weeks for the corresponding parameter.

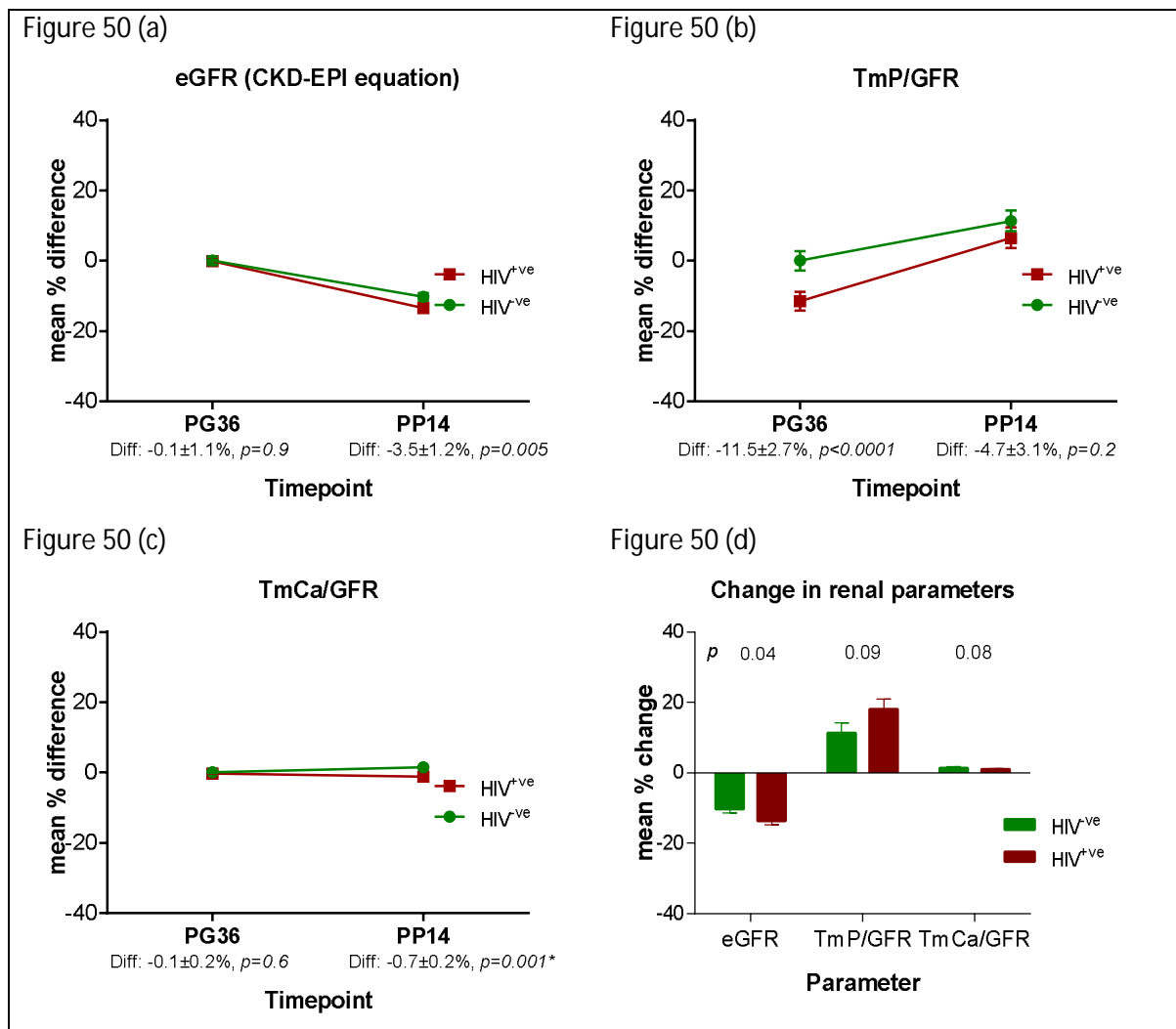


Figure 50: Change in maternal eGFR-MDRD, TmP/GFR and TmCa/GFR (PG36-PP14)

eGFR CKD-EPI= estimated glomerular filtration rate based on Chronic Kidney Disease Epidemiology Collaboration equation, TmP/GFR and TmCa/GFR =tubular maxima for phosphate and calcium, respectively/glomerular filtrate. Diff = mean sympercent difference between the groups±SD. Results were obtained from Scheffé's post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction. The number of subjects included in the analysis was 96 HIV-negative and 93 HIV-positive mothers. Figure 50(d) Bars and error bars represent the mean±SE sympercent changes within the groups between PG36 and PP14. The p- values shown above the bars are for comparison of mean % changes between the groups at 14 weeks for the corresponding parameter

### 8.3. Change in maternal PTH, 25OHD and bone turnover markers

Table 32: Summary of changes in bone turnover markers (PG36 to PP14)

Analyte	HIV-Negative (n=74)		HIV-Positive (n=81)		Group*visit interaction p-value
	(%Δ±SE) <sup>1</sup>	p-value	(%Δ±SE) <sup>1</sup>	p-value	
Serum 25(OH)D (nmol/L)	-11.1±3.1	≤0.0001	-13.9±4.1	0.001	0.6
Plasma PTH (pg/mL)	+57.6±6.4	≤0.0001	+60.0±6.4	≤0.0001	0.8
Plasma CTX (ng/mL)	+56.2±5.9	≤0.0001	+74.6±5.9	≤0.0001	0.04
Plasma P1NP (ng/mL)	+72.6±5.0	≤0.0001	+100.3±5.0	≤0.0001	0.0001
Plasma BAP (μg/mL)	+57.1±3.6	≤0.0001	+67.2±3.6	≤0.0001	0.05
Plasma TAP (U/L)	-26.1±4.2	≤0.0001	-4.6±4.2	≤0.0001	0.0003
Plasma TAP/BAP ratio	-83.2±3.9	0.009	-71.9±3.9	≤0.0001	0.04

<sup>1</sup> Mean % changes presented as sympercents±SE. All variables were transformed into natural logarithms\*100 before statistical analysis in DataDesk software. Results were obtained from Scheffé's post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction.

#### 8.3.1. Change in 25(OH)D, PTH and CTX

Mean serum [25(OH)D] significantly decreased; and mean plasma [PTH] increased in both groups as expected in lactation (Figure 51a-b). The changes in serum [25(OH)D] and PTH were not significantly different between the groups (Table 32). Also, there were no significant differences between the groups in mean [25(OH)D] at either PG36 or PP14 (Figure 51d). Mean plasma [CTX] increased in both groups (Figure 51c), but HIV-positive women had a significantly greater increase compared to HIV-negative women (74.6% vs 56.2%,  $p=0.03$ ). Adjusting for depo-provera did not have a material effect on both serum [25(OH)D] and [PTH].

Similar to cross-sectional analysis, HIV-positive women had higher [PTH] concentrations compared to HIV-negative women at both PG36 and PP14, respectively (+32% and +34%, both  $p\leq 0.0001$ ). They also had higher CTX at PP14 (+14.6%,  $p=0.02$ ). Mean 25(OH)D was not significantly different between the groups at either PG36 or PP14.

Figure 51(a)

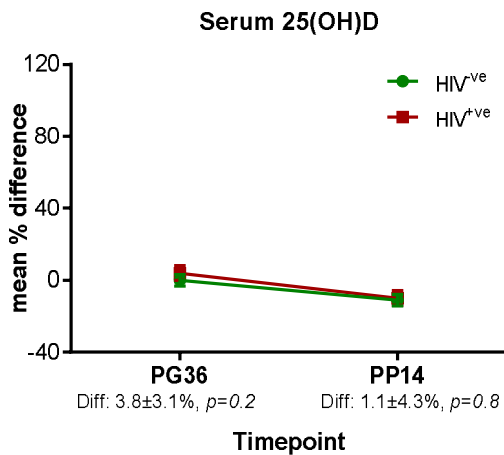


Figure 51(b)

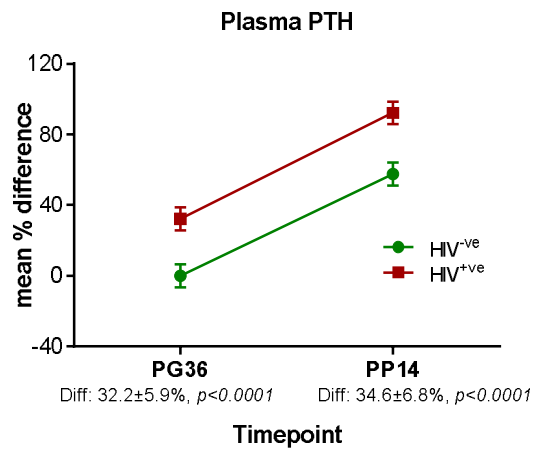


Figure 51(c)

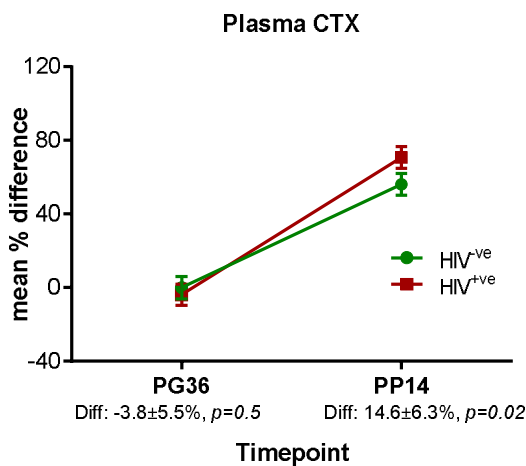


Figure 51(d)

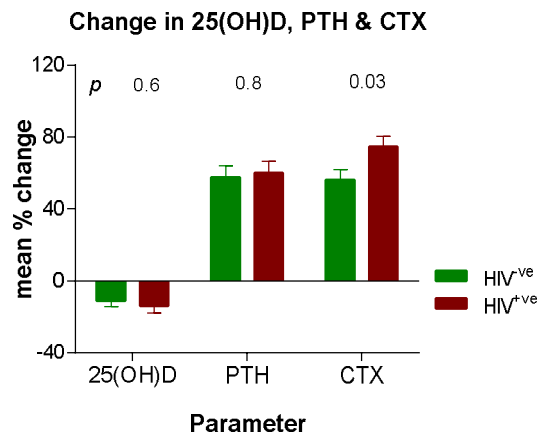


Figure 51: Change in 25OHD, PTH and CTX (PG36-PP14)

25(OH)D = 25 hydroxyvitamin D, PTH = parathyroid hormone and CTX = collagen type 1 cross-linked C-telopeptide. Diff = mean sympercent difference between the groups  $\pm$  SD. Results were obtained from Scheffé's post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction. The number of subjects included in the analysis was 96 HIV-negative and 93 HIV-positive mothers. Figure 51(d) Bars and error bars represent the mean  $\pm$  SE sympercent changes within groups between PG36 and PP14. The p-values shown above the bars are for comparison of mean % changes between the groups at 14 weeks for the corresponding parameter.

### 8.3.2. Change in P1NP, BAP and TAP

Mean plasma [P1NP] increased in both groups (Table 32 and Figure 52a). HIV-positive women had a significantly greater increase in [P1NP] compared to HIV-negative women ( $+100.3 \pm 5.0\%$  vs.  $+72.6 \pm 5.0\%$ ,  $p=0.0001$ ). Also, HIV-positive women had significantly lower [P1NP] at PG36 ( $-26.4\%$ ,  $\leq 0.0001$ ), which was also seen in the cross-sectional model, but there was no significant difference between the groups at PP14 ( $+1.3\%$ ,  $p=0.8$ ).

Mean plasma [TAP] decreased in both groups (Figure 52c). HIV-positive women experienced a smaller reduction in [TAP] compared to HIV-negative women ( $-4.6\%$  vs.  $-26.1\%$ ,  $p=0.0003$ ). However, mean [BAP] increased in both groups. There was a trend towards a greater increase in [BAP] among HIV-positive women compared to HIV-negative women ( $+67.2 \pm 3.6\%$  vs  $+57.1 \pm 3.6\%$ ,  $p=0.05$ ).

BAP/TAP ratio increased in both groups, but HIV-positive women had a significantly greater increase compared to HIV-negative counterparts ( $-83.2 \pm 3.9\%$  vs  $71.9 \pm 3.9\%$ ,  $p=0.04$ ).

The differences between the groups in the longitudinal analysis were comparable to the cross-sectional analysis. Adjusting for depo-provera did not have a material effect on plasma [P1NP], [TAP], [BAP] or BAP/TAP ratio.

Figure 52 (a)

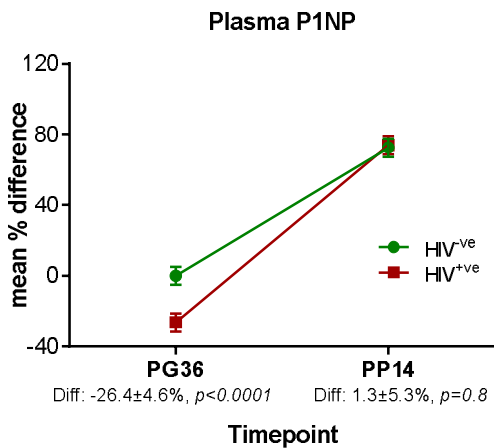


Figure 52 (b)

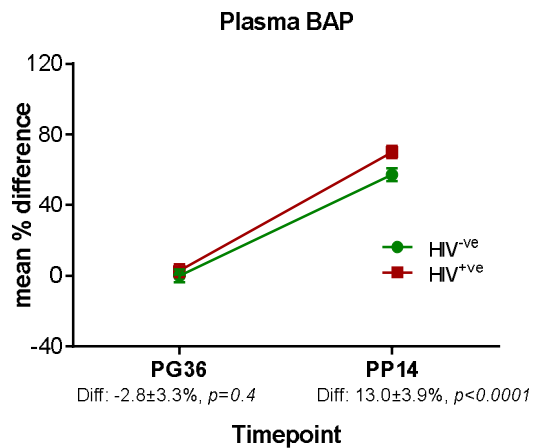


Figure 52(c)

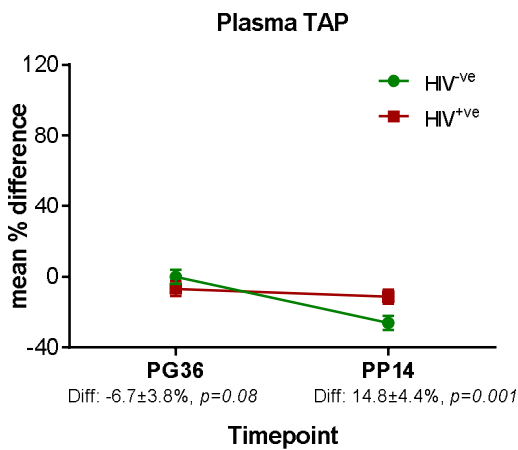


Figure 52(d)

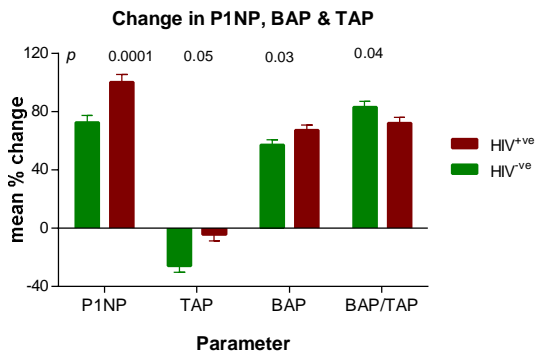


Figure 52: Change in maternal P1NP, TAP and BAP concentrations PG36- PP14)

P1NP = N-terminal propeptide of type 1 procollagen, BAP = bone-specific alkaline phosphatase and TAP= total alkaline phosphatase. Diff = mean sympercent difference between the groups $\pm$ SD. Results were obtained from Scheffés post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction. The number of subjects included in the analysis was 96 HIV-negative and 93 HIV-positive mothers. Figure 52(d) Bars and error bars represent the mean $\pm$ SE sympercent changes within groups at 14 weeks postpartum. The p- values shown above the bars are for comparison of mean % changes between the groups at 14 weeks for the corresponding parameter



## 8.4. Change in breast milk mineral content

Table 33: Summary of changes in breast milk mineral content (PP2- PP14)

Variable	HIV-Negative (n=74)		HIV-Positive (n=81)		Group*visit interaction p-value
	(%Δ±SE)	p-value	(%Δ±SE)	p-value	
Calcium (mmol/L) <sup>2</sup>	-24.2±3.1	≤0.0001	-19.9±3.0	≤0.0001	0.3
Phosphorus (mmol/L)	-2.73±3.2	0.4	-2.10±3.2	0.5	0.9
Calcium/Phosphorus ratio	-21.4±2.7	≤0.0001	-17.8±2.6	≤0.0001	0.3
Sodium (mmol/L) <sup>3</sup>	-72.6±9.0	≤0.0001	-44.3±8.9	≤0.0001	0.03
Potassium (mmol/L) <sup>4</sup>	-26.1±3.0	≤0.0001	-22.1±2.9	≤0.0001	0.4
Sodium/Potassium ratio <sup>5</sup>	-46.6±9.5	≤0.0001	-22.2±9.3	0.02	0.07

<sup>1</sup> Mean % changes presented as sympercents±SE. All variables were transformed into natural logarithms\*100 before statistical analysis in DataDesk software. Results were obtained from Scheffé's post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction.

<sup>2</sup> Includes 2 outliers who don't affect results. Difference when excluded PP2, +1.8±3.0, P=0.544; PP14 +4.7±3.0 p=0.113

<sup>3</sup> Includes 8 outliers overall (5 HIV-positive, 3 HIV-negative) 3 at PP2 (1 HIV-positive, 2 HIV-negative) and PP14 (4 HIV-positive, 1 HIV-negative). When outliers are excluded there is still no difference between groups (PP2 = -9.4±7.0%, P=0.309; PP14 = -3.8±7.0%, P=0.590) but, within groups changes reduce (HIV-ve -64.8±7.0%, p≤0.0001; HIV+ve -53.8±7.0%, P≤0.0001) and group\*visit interaction p-value become non-significant (0.275). Visit p-value unaffected.

<sup>4</sup> Includes 2 TR women flagged as outliers at PP14. Exclusion does not affect results

<sup>5</sup> Includes 9 outliers overall (5 HIV-positive, 4 HIV-negative) 3 at PP2 (1 HIV-positive, 3 HIV-negative) PP14 (4 HIV-positive, 1 HIV-negative). When outliers are excluded there is still no difference in group differences (PP2 = -7.2±7.0%, P=0.178; PP14 = -4.1±6.9%, P=0.552) but, within groups changes reduce (HIV-ve -37.4±7.0%, p≤0.0001; HIV+ve -32.1±7.0%, P≤0.0001) and group\*visit interaction p-value becomes non-significant (0.591).

### 8.4.1. Change in breast milk calcium and phosphorus

Mean [BM-Ca] significantly decreased in both groups between PP2 and PP14 (Figure 53a) and the changes were not significantly different between the groups (-24.4% vs -19.9%, p=0.3). There were no significant changes in mean [BM-P] in both groups. Mean breast milk Ca/P ratio decreased in both groups between PP2 and PP14 (Table 33), and the changes were not significantly different between the groups (p=0.3). Adjusting for infant age, breastfeeding status, having been ill within the past 2 weeks did not have a material effect on the results.

Similar to cross-sectional analysis, HIV-positive women had higher mean [BM-P] compared to HIV-negative women (PP2: +9.2±3.2%, p=0.005; PP14: +9.8±3.2%, p=0.002). There was a trend towards higher [BM-Ca] at PP14, although the difference between the groups was not significant (+5.7±3.0%, p=0.06). Mean breast milk Ca/P ratio was 7.7% lower in HIV-positive women at PP2, but the group means were not significantly different at PP14 (-4.1±2.6%, p=0.1; Figure 53c).

Figure 53(a)

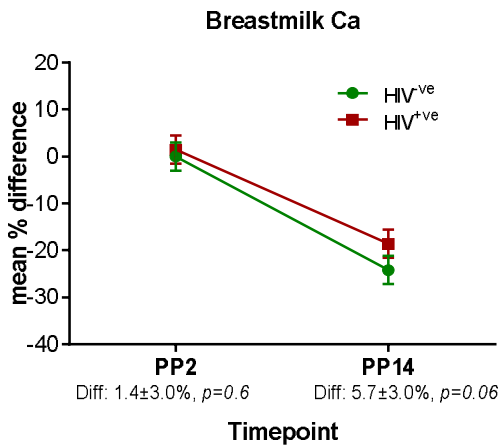


Figure 53(b)

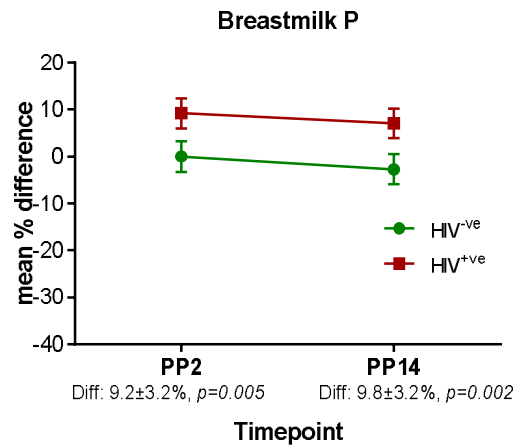


Figure 53(c)

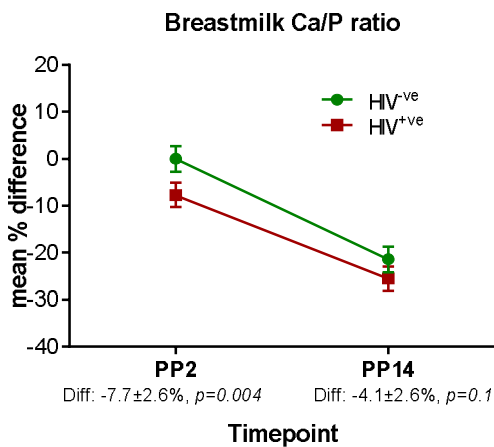


Figure 53(d)

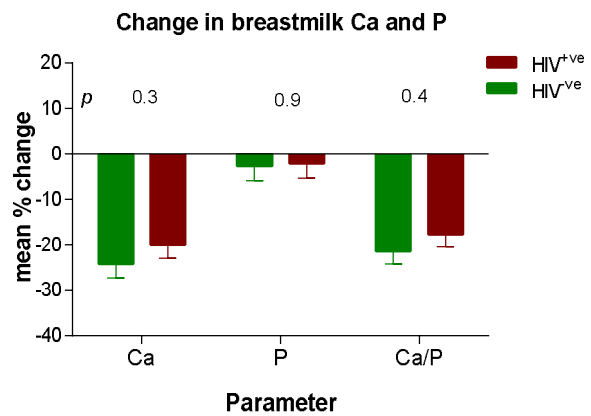


Figure 53: Change in breast milk calcium and phosphate (PP2-PP14)

Units for breast milk calcium and phosphate = mmol/L. Diff = mean sympercent difference between the groups  $\pm$  SD. Results were obtained from Scheffés post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction. The number of subjects included in the analysis was 81 HIV-negative and 83 HIV-positive mothers. Figure 53(d) Bars and error bars represent the mean  $\pm$  SE sympercent changes within groups at 14 weeks postpartum. The p-values shown above the bars are for comparison of mean % changes between the groups at 14 weeks for the corresponding parameter.

#### *8.4.2. Change in breast milk sodium and potassium*

Overall, mean [BM-Na] significantly decreased in both groups (Figure 54a). HIV-positive women had a significantly smaller decrease in [BM-Na] compared to HIV-negative ( $-44.3 \pm 8.9\%$  vs.  $-72.6 \pm 9.0\%$ ,  $p = 0.03$ ). They also had 14.5% lower [BM-Na] compared to HIV-negative women at PP2, but 13.8% higher concentration at PP14.

Mean [BM-K] and Na/K ratio decreased in both groups (Figure 54b), and the changes were not significantly different between the groups (Table 33). However, there was a trend towards a smaller decrease in breast milk Na/K ratio in HIV-positive compared to HIV negative women ( $-22.2 \pm 9.3\%$  vs  $-46.6 \pm 9.5\%$ ;  $p = 0.07$ ).

Eight measurements were flagged as outliers (5 HIV-positive, 3 HIV-negative); 3 at PP2 (1 HIV-positive, 2 HIV-negative) and 5 at PP14 (4 HIV-positive, 1 HIV-negative). When these were excluded from statistical analysis, both groups still had significant decreases in mean [BM-Na], but the changes were not significantly different between the groups ( $-64.8 \pm 7.0\%$  vs  $-53.8 \pm 7.0$ ,  $p = 0.3$ ). Breast milk Na/K ratios reduced in both groups, and there was a trend towards a lower Na/K in the HIV-positive group (HIV-positive  $-22.2 \pm 9.3\%$ ,  $p = 0.02$ ; HIV-negative  $-46.6 \pm 9.5\%$ ,  $p \leq 0.0001$ ; group\*visit  $p$ -value = 0.07).

These results were comparable to the cross-sectional analysis. Adjusting for infant age, breastfeeding status, having been ill within the past 2 weeks did not have a material effect on breast milk Na, K or Na/K ratio.

Figure 54(a)

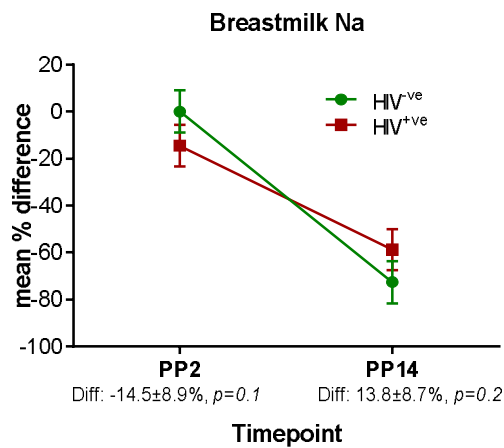


Figure 54(b)

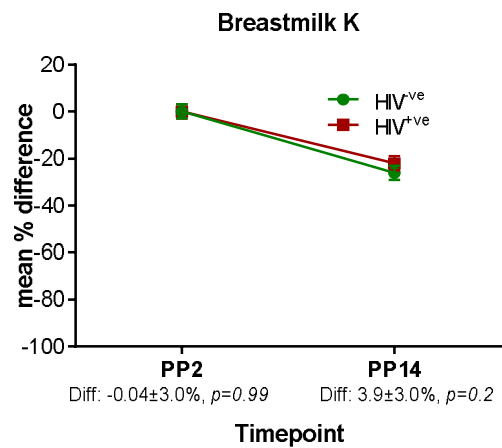


Figure 54(c)

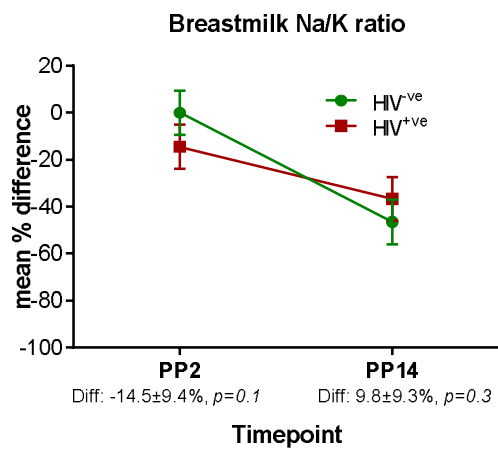


Figure 54(d)

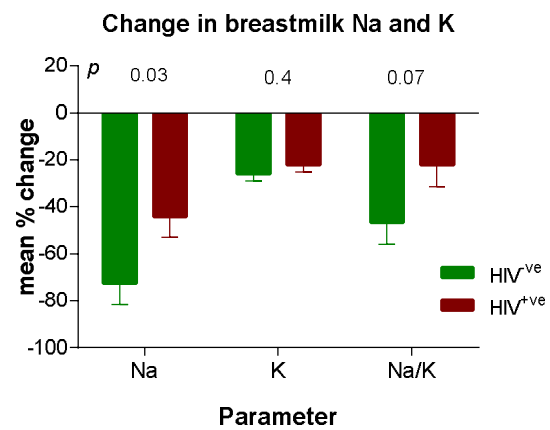


Figure 54: Change in breast milk sodium and potassium concentrations (PP2-PP14)

Units = mmol/L. Diff = mean sympercent difference between the groups±SD. Results were obtained from Scheffé's post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction. The number of subjects included in the analysis was 81 HIV-negative and 83 HIV-positive mothers. Figure 54(d) Bars and error bars represent the mean±SE sympercent changes within groups at 14 weeks postpartum. The p-values shown above the bars are for comparison of mean % changes between the groups at 14 weeks for the corresponding parameter.

## 8.5. Changes in maternal bone mineral

Table 34: Percent change in maternal bone mineral parameters (PP2- PP14)

	HIV-Negative (n=74)		HIV-Positive (n=81)		Group*visit interaction <i>p</i> -value
	(%Δ±SE) <sup>1</sup>	p-value	(%Δ±SE) <sup>1</sup>	p-value	
<b><i>Whole body Less Head (WBLH)<sup>a</sup></i></b>					
BMC (g)	-0.9±0.4	<b>0.02</b>	-1.0±0.4	<b>0.005</b>	0.8
BA (cm <sup>2</sup> )	-0.3±0.3	0.5	+0.2±0.3	0.5	0.3
aBMD (g/cm <sup>2</sup> )	-0.6±0.2	<b>0.004</b>	-1.2±0.2	<b>≤0.0001</b>	<b>0.05</b>
SA-BMC (g) <sup>2</sup>	-0.7±0.2	<b>0.003</b>	-1.1±0.2	<b>≤0.0001</b>	0.1
<b><i>Total Hip (TH)</i></b>					
BMC (g)	-2.5±0.9	<b>0.008</b>	-4.3±0.9	<b>≤0.0001</b>	0.2
BA (cm <sup>2</sup> )	-0.2±0.6	0.7	-0.3±0.6	0.2	0.6
aBMD (g/cm <sup>2</sup> )	-2.7±0.4	<b>≤0.0001</b>	-4.0±0.4	<b>≤0.0001</b>	<b>0.04</b>
SA-BMC (g) <sup>2</sup>	-2.7±0.3	<b>≤0.0001</b>	-3.7±0.3	<b>≤0.0001</b>	<b>0.04</b>
<b><i>Lumbar Spine (LS)</i></b>					
BMC (g)	-2.9±0.6	<b>≤0.0001</b>	-1.7±0.6	<b>0.002</b>	0.1
BA (cm <sup>2</sup> )	-0.4±0.3	<b>0.3</b>	+0.1±0.3	0.8	0.3
aBMD (g/cm <sup>2</sup> )	-2.5±0.4	<b>≤0.0001</b>	-1.8±0.4	<b>≤0.0001</b>	0.3
SA-BMC (g) <sup>2</sup>	-2.2±0.4	<b>≤0.0001</b>	-1.7±0.4	<b>≤0.0001</b>	0.4

<sup>1</sup> Mean % changes presented as sympercents±SE. All variables were transformed into natural logarithms\*100 before statistical analysis in DataDesk software. Results were obtained from Scheffé's post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction.

<sup>2</sup> Adjusted for BA and body weight. Height was not included in the models because it was not expected to change over time.

### 8.5.1. Change in maternal WBLH bone mineral

Overall, mean WBLH BMC, aBMD and SA-BMC significantly decreased in both groups as expected during lactation. Changes in BMC were not significantly different between the groups (-1.0±0.4% vs -0.9±0.4%, p=0.8), and there were no significant differences between the groups at either PP2 or PP14. There were no significant changes in BA in both groups (Figure 55a-d).

However, there was a trend towards a greater reduction in WBLH aBMD among HIV-positive compared to HIV-negative women (aBMD:-1.2% vs -0.6%, p=0.05). Changes in SA-BMC were not significantly different between the groups (-1.1% vs -0.7%, p=0.1). HIV-positive women had significantly higher aBMD and SA-BMC compared to HIV-negative at both PP2 (+1.2±0.2%, p≤0.0001 and +1.1±0.2%, p≤0.0001, respectively) and PP14 (+0.6±0.2%, p=0.006 and +0.7±0.2%, p=0.01, respectively). These differences between the groups were very similar to those observed in the cross-sectional analyses. Potential were not significantly associated with all maternal WBLH bone parameters.

Figure 55(a)

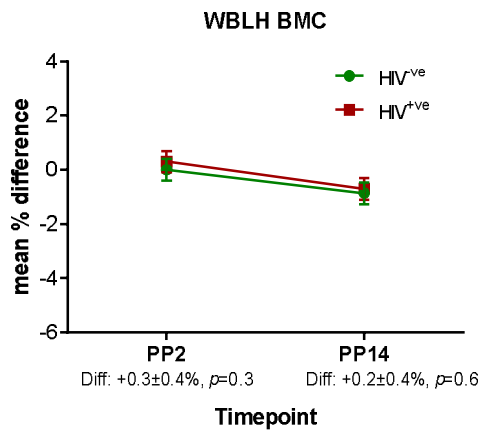


Figure 55(b)

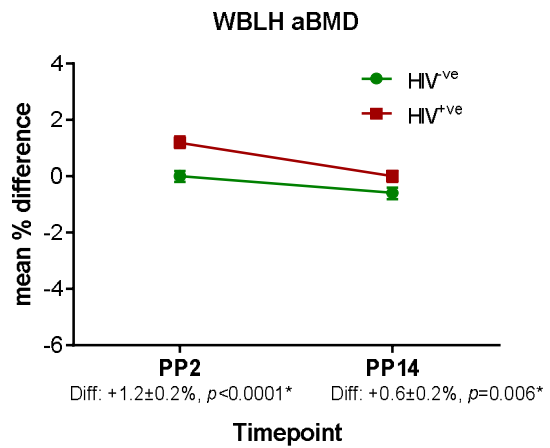


Figure 55(c)

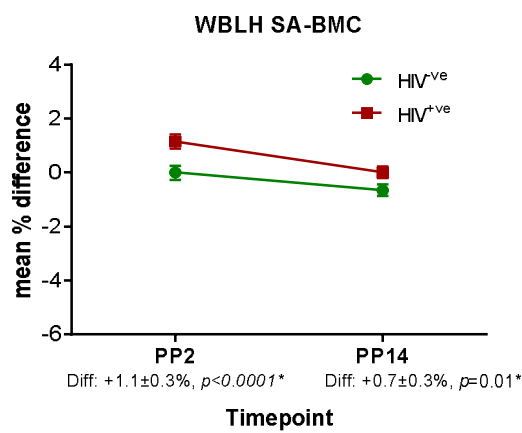


Figure 55(d)

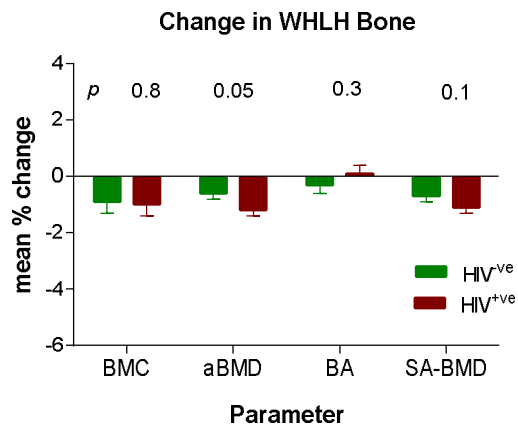


Figure 55: Percent change in the whole body (less head) maternal bone (PP2 - PP14)

WBLH= whole body less head, BMC=Bone mineral content (g), BA=Bone area (cm<sup>2</sup>), aBMD= areal Bone mineral density (g/cm<sup>2</sup>), SA-BMC =Size adjusted BMC – BMC adjusted for BA and body weight. Diff = mean sympercent difference between the groups±SD. Results were obtained from Scheffé's post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction. The number of subjects included in the analysis was 81 HIV-negative and 83 HIV-positive breastfeeding mothers. Figure 55(d) Bars and error bars represent the mean±SE sympercent changes within groups at 14 weeks postpartum. The P values shown above the bars are for comparison of mean % change between the groups at 14 weeks for the corresponding bone parameter.

### 8.5.2. Change in maternal TH bone mineral

Overall, mean TH BMC, aBMD and SA-BMC significantly reduced in both groups, as expected during lactation, and there were no significant changes in BA in both groups (Table 34 and Figure 56a-d). Changes in BMC were not significantly different between the groups ( $p=0.2$ ). There was a trend towards higher BMC among HIV-positive compared to HIV-negative women at PP2 ( $+1.7\%\pm0.9\%$ ), but the difference between the groups was not significant ( $p=0.07$ ), similar to cross-sectional analysis.

However, HIV-positive women had significantly greater reductions in TH aBMD ( $-4.0\%$  vs.  $-2.7\%$ ,  $p=0.04$ ) and SA-BMC ( $-3.7\%$  vs.  $-2.7\%$ ,  $p=0.04$ ). They also had significantly higher aBMD at PP2 ( $+2.0\pm0.4\%$ ,  $p\leq0.0001$ ) and higher SA-BMC at both PP2 ( $+3.2\pm0.4\%$ ,  $p\leq0.0001$ ) and PP14 ( $+2.2\pm0.4\%$ ,  $p\leq0.0001$ ). The size effects at PP2 and PP14 were very similar to the cross-sectional model.

Potential confounders (use of depo-provera, baby having been ill within two weeks to the visit and exclusive breastfeeding) were not significantly associated with all maternal TH bone parameters - BMC, BA, aBMD or SA-BMC.

Figure 56(a)

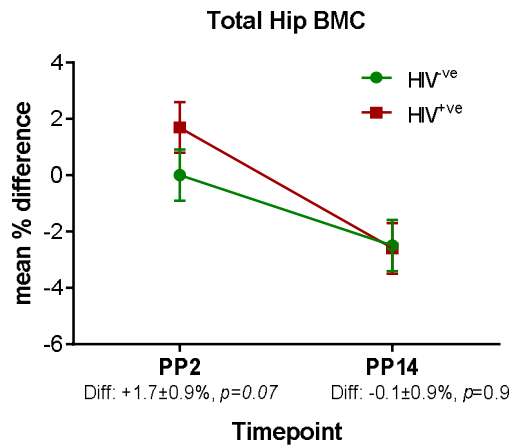


Figure 56(b)

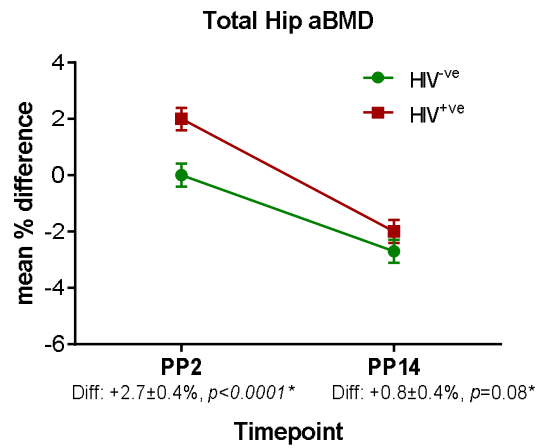


Figure 56(c)

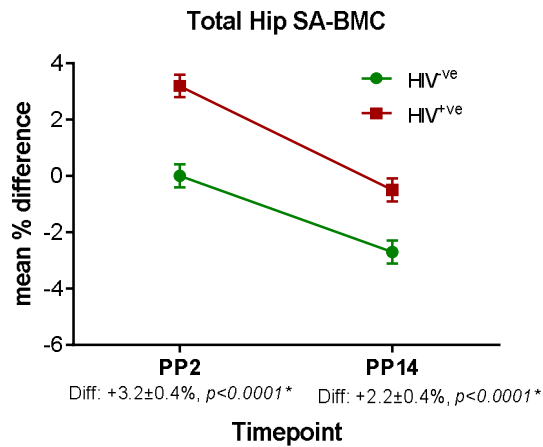


Figure 56(d)

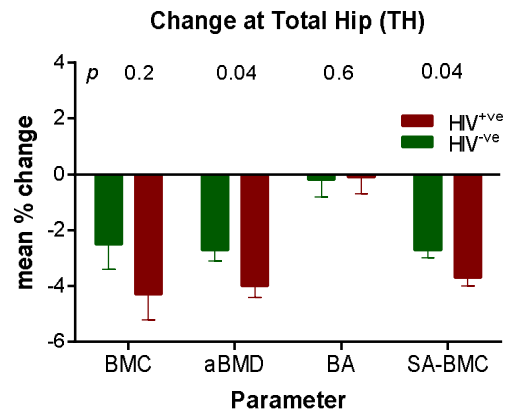


Figure 56: Changes in maternal bone parameters at the total hip (PP2-PP14)

BMC=Bone mineral content (g), BA=Bone area (cm<sup>2</sup>), aBMD= areal Bone mineral density (g/cm<sup>2</sup>), SA-BMC =Size adjusted BMC – BMC adjusted for BA and body weight. Diff = mean sympercent difference between the groups±SD. Results were obtained from Scheffés post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction. The number of subjects included in the analysis was 81 HIV-negative and 83 HIV-positive breastfeeding mothers. Figure 56(d) Bars and error bars represent the mean±SE sympercent changes within groups at 14 weeks postpartum. The P values shown above the bars are for comparison of mean % change between the groups at 14 weeks for the corresponding bone parameter.



### 8.5.3. Change in maternal LS bone mineral

Overall, mean maternal LS BMC, aBMD and SA-BMC significantly reduced in both groups, but there were no significant changes in LS BA (Table 34 and Figure 57 a-d). There was a trend towards a smaller reduction in mean LS BMC in HIV-positive women, but the changes were not significantly different between the groups ( $-1.7 \pm 0.5\%$  vs  $-2.9 \pm 0.6\%$ ,  $p=0.1$ ; Figure 57d). Changes in LS aBMD and SA-BMC were also not significantly different between the groups (aBMD:  $-1.8 \pm 0.4\%$  vs  $-2.5 \pm 0.4\%$ ,  $p=0.2$ ; SA-BMC  $-1.7 \pm 0.4\%$  vs  $-2.2 \pm 0.4\%$ ,  $p=0.4$ ).

HIV-positive women had a significantly higher SA-BMC compared to HIV-negative women at PP14 ( $+1.3 \pm 0.5\%$ ,  $p=0.02$ ), and the size effects were very similar to the cross-sectional analysis ( $+1.0 \pm 1.6\%$ ,  $p=0.5$ ). Mean LS BMC and aBMD were not significantly different between the groups at either PP2 or PP14, and the size effects were very similar to the cross-sectional models.

Potential confounders (use of depo-provera, baby having been ill within two weeks to the visit and exclusive breastfeeding) were not significantly associated with all maternal LS bone parameters - BMC, BA, aBMD or SA-BMC.

Figure 57(a)

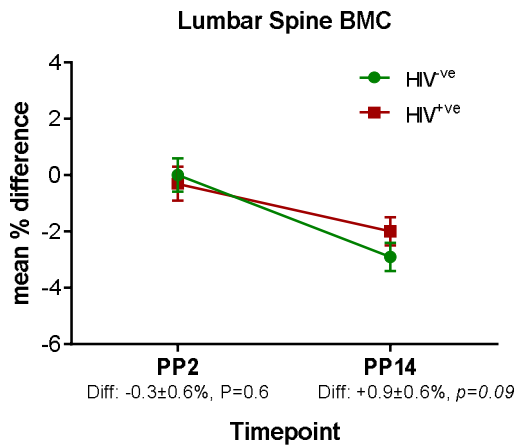


Figure 57 (b)

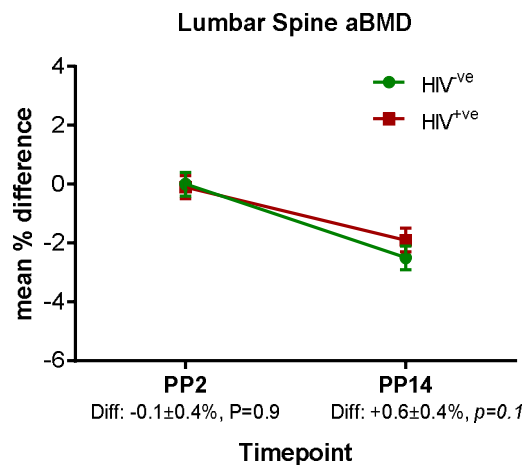


Figure 57(c)

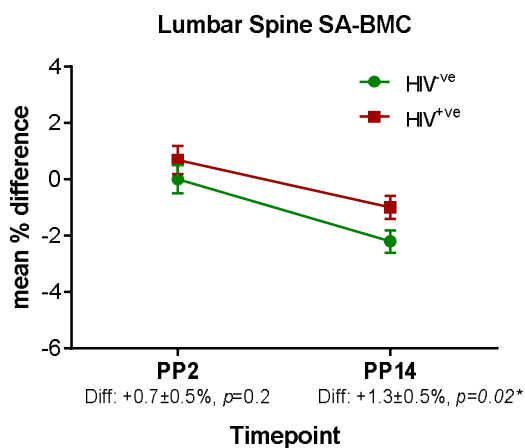


Figure 57(d)

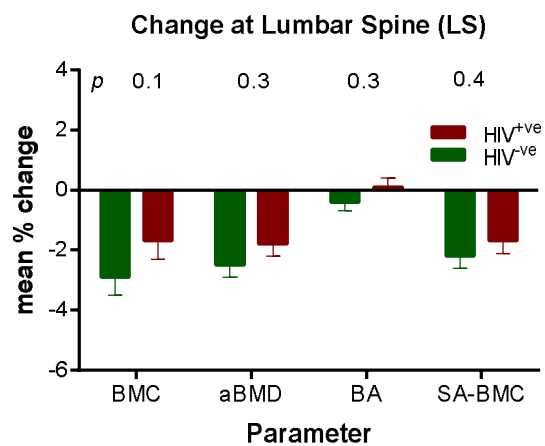


Figure 57: Change in maternal bone parameters at the lumbar spine (L1-L4) from PP2 to PP14

BMC=Bone mineral content (g), BA=Bone area (cm<sup>2</sup>), aBMD= areal Bone mineral density (g/cm<sup>2</sup>), SA-BMC =Size adjusted BMC – BMC adjusted for BA and body weight. Diff = mean sympercent difference between the groups±SD. Results were obtained from Scheffés post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction. The number of subjects included in the analysis was 81 HIV-negative and 83 HIV-positive breastfeeding mothers. Figure 57(d) Bars and error bars represent the mean±SE sympercent changes within groups at 14 weeks postpartum. The P values shown above the bars are for comparison of mean % change between the groups at 14 weeks for the corresponding bone parameter.

## 8.6. Change in infant anthropometry and body composition

Table 35: Longitudinal change in infant anthropometry (PP2-PP14)

	HIV-Unexposed (n=81)		HIV-Exposed (n=85)		Group*visit interaction p=value
	(%Δ±SE) <sup>1</sup>	p-value	(%Δ±SE) <sup>1</sup>	p-value	
<i><b>Anthropometry</b></i>					
Weight (kg)	+57.5±1.4	≤0.0001	+53.0±1.4	≤0.0001	0.02
Length (cm)	+17.3±0.5	≤0.0001	+16.1±0.4	≤0.0001	0.05
Head Circumference (cm)	+12.3±0.3 <sup>2</sup>	≤0.0001	+11.9±0.3	≤0.0001	0.4
<i><b>Whole Body Composition</b></i>					
Lean mass (g)	+23.7±2.4	≤0.0001	+17.9±2.4	≤0.0001	0.09
Fat mass (g)	+110.4±5.7	≤0.0001	+104.9±5.7	≤0.0001	0.5
% Fat	+55.8±5.1	≤0.0001	+56.3±5.1	≤0.0001	0.9

<sup>1</sup> Mean % changes presented as sympercents±SE. All variables were transformed into natural logarithms\*100 before statistical analysis in DataDesk software. Results were obtained from Scheffé's post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction.

### 8.6.1. Change in infant anthropometry

Overall, mean weight, length and head circumference increased in both groups as expected in growing infants (Table 35 and Figure 58a-d). HEI had a slower increase in weight compared to HUI (+53.9±1.4 vs +57.5±1.5 p=0.02). Similar to the cross-sectional analysis, HEI were significantly lighter at PP14 (-5.9±1.4%, p≤0.0001). There was no significant difference in weight between the groups at PP2 (-1.6±1.4%, p=0.3).

There was a trend towards a slower increase in length in HEI compared to HUI (+16.1±1.4% vs +17.3±0.5%, p=0.05). Similar to cross-sectional analysis, HEI were significantly shorter compared to HUI at PP14 (-1.8±0.4%, p≤0.0001), and there was no significant difference between the groups at PP2 (-0.6±0.5%, p=0.2).

Changes in head circumference were not significantly different between the groups (+11.9±0.3% vs 12.3±0.3%, p=0.4). HEI had significantly lower HC compared to HUI at PP14 (-0.8±0.3% p=0.01), and the size effects were very similar to the cross-sectional analysis (-0.7±0.6% p=0.2).

Breastfeeding practices and health status were not associated with any of the infant anthropometric measurements.

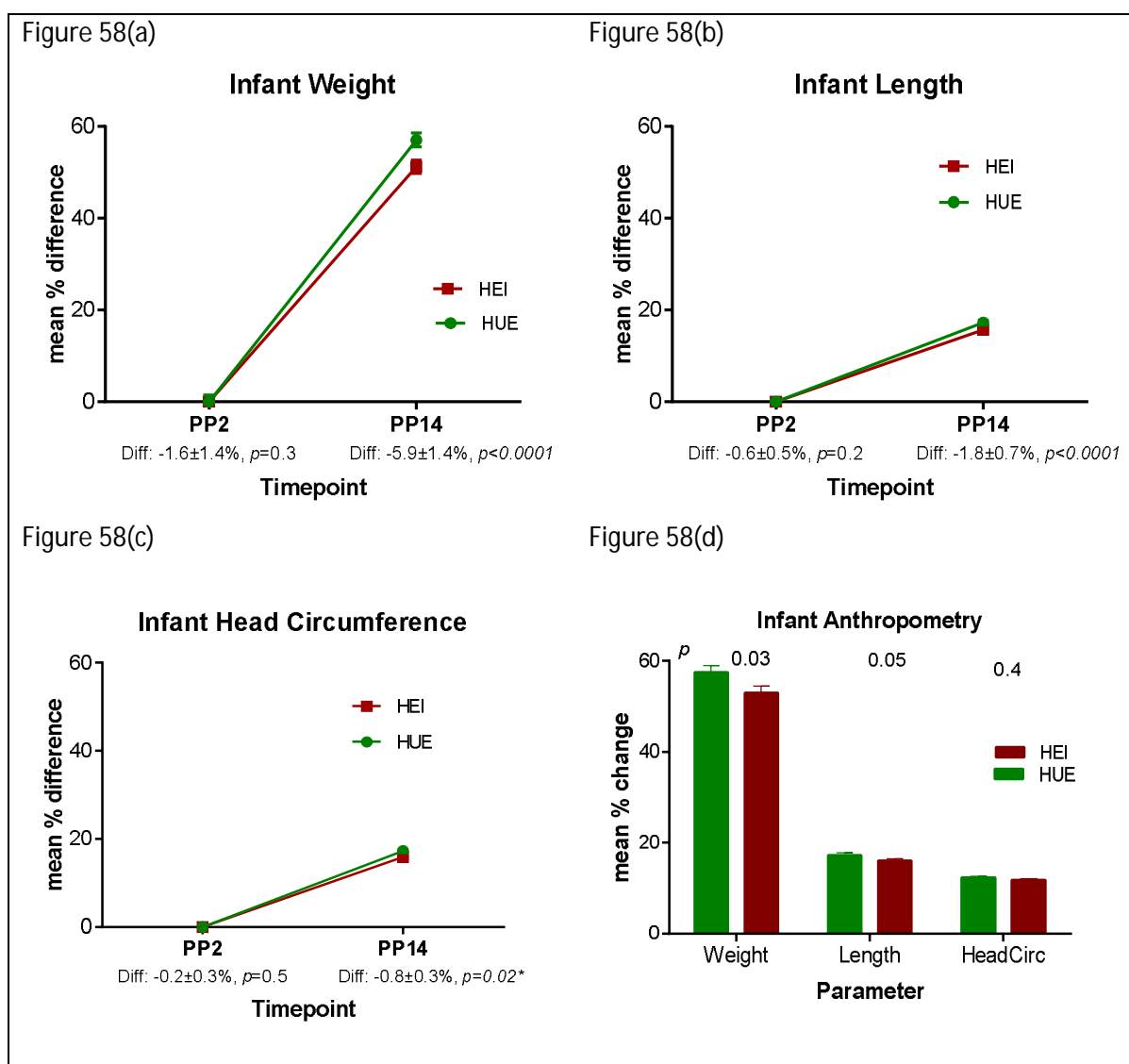


Figure 58: Change in infant anthropometry (PP2-PP14)

HeadCirc = head circumference; Diff = mean sympercent difference between the groups ± SD. Results were obtained from Scheffé's post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction. The number of subjects included in the analysis was 81 HIV-negative and 83 HIV-positive breastfeeding infants. Figure 58(d) Bars and error bars represent the mean ± SE sympercent changes within groups at 14 weeks postpartum. The P values shown above the bars are for comparison of mean % change between the groups at 14 weeks for the corresponding bone parameter.

### *8.6.2. Change in infant body composition*

Overall, mean lean mass, fat mass and % fat significantly increased in both groups as expected in growing infants (Table 35 and Figure 59a-c). There was a trend towards a slower increase in lean mass in HEI compared to HUI, but the changes were not significantly different between the groups (+17.9% vs +23.7%,  $p=0.09$ ). Similar to cross-sectional analysis, HEI had significantly lower lean mass compared to HUI at PP14 ( $-6.3 \pm 2.3\%$ ,  $p=0.007$ ); and there was no significant difference between the groups at PP2.

Fat mass doubled in both groups (HEI +104%,  $p \leq 0.0001$ ; HUI +110%,  $p \leq 0.0001$ ), hence the changes were comparable between the groups ( $p=0.5$ ). Changes in mean %fat were not significantly different between the groups (+56.3% vs +55.8%,  $p=0.9$ ). Similar to cross-sectional analysis, both fat mass and % fat were not significantly different between the groups at either PP2 or PP14.

Potential confounders (age of the infant, breastfeeding status and illness within two weeks to the visit) were not significantly associated with all infant body composition parameters (fat, lean and % fat).

Figure 59(a)

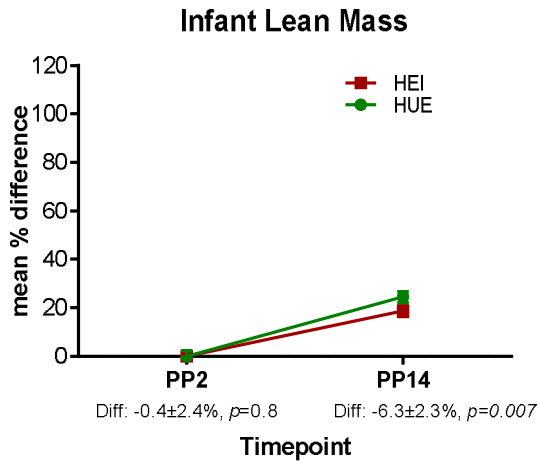


Figure 59(b)

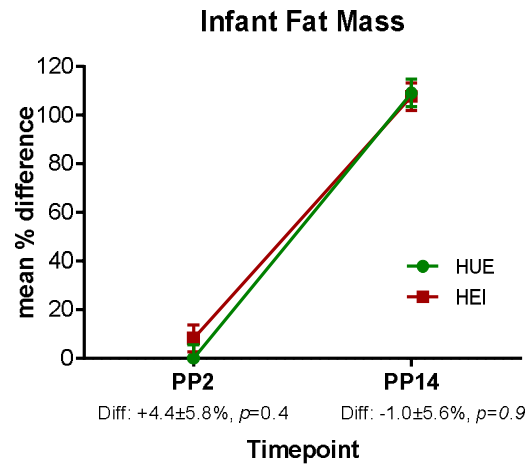


Figure 59(c)

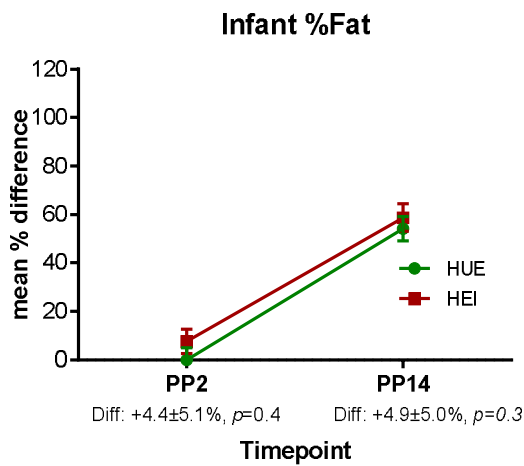


Figure 59(d)

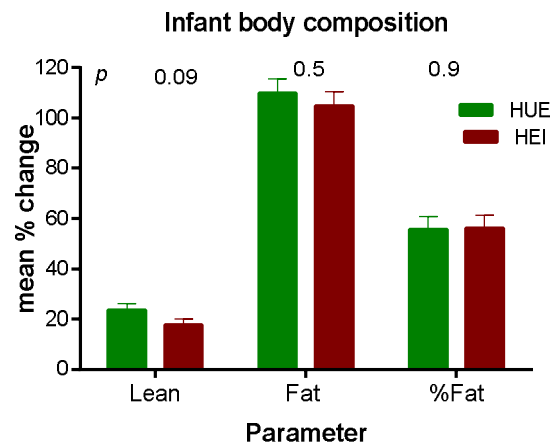


Figure 59: Change in infant body composition (PP2-PP14)

Diff = mean sympercent difference between the groups  $\pm$  SD. Results were obtained from Scheffé's post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction. The number of subjects included in the analysis was 81 HIV-negative and 83 HIV-positive breastfeeding infants. Figure 59(d) Bars and error bars represent the mean  $\pm$  SE sympercent changes within groups at 14 weeks postpartum. The P values shown above the bars are for comparison of mean % change between the groups at 14 weeks for the corresponding bone parameter.

## 8.7. Change in infant bone mineral at PP14

Table 36: Summary of longitudinal changes in infant bone mineral (PP2-PP14)

	HIV-Unexposed (n=81)		HIV-Exposed (n=85)		Group*visit interaction p-value
	(%Δ±SE) <sup>1</sup>	p-value	(%Δ±SE) <sup>1</sup>	p-value	
<i><b>Whole body</b></i>					
BMC (g)	+57.3±1.9	≤0.0001	+51.2±1.9	≤0.0001	0.02
BA (cm²)	+41.9±1.1	≤0.0001	+36.9±1.1	≤0.0001	0.001
aBMD (g/cm²)	+15.4±1.1	≤0.0001	+14.4±1.1	≤0.0001	0.5
SA-BMC (g) <sup>2</sup>	-7.6±3.8	0.05	-6.0±3.5	0.09	0.2
<i><b>Lumbar Spine</b></i>					
BMC (g)	+24.4±1.7	≤0.0001	+29.5±1.7	≤0.0001	0.03
BA (cm²)	+25.6±1.1	≤0.0001	+25.6±1.1	≤0.0001	0.9
aBMD (g/cm²)	-1.1±1.5	0.4	+3.0±1.4	0.008	0.02
SA-BMC (g) <sup>2</sup>	+4.3±6.2	0.5	+9.4±5.8	0.1	0.02

<sup>1</sup> Mean % changes presented as sympercents±SE. All variables were transformed into natural logarithms\*100 before statistical analysis in DataDesk software. Results were obtained from Scheffés post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction.

<sup>2</sup> Adjusted for BA, body weight and length

### 8.7.1. Change in infant WB bone mineral

Overall, mean WB BMC, BA and aBMD significantly increased in both groups as expected in growing infants, but there were no significant changes in SA-BMC (Table 36 and Figure 60a-d)

HEI had a significantly slower increase in both BMC and BA compared to HIV-unexposed infants (BMC +51.2% vs. +57.3%, p=0.02; BA +36.9% vs. +41.9%, p=0.001). Similar to cross-sectional analysis, HEI had significantly lower BMC and BA compared to HUI at PP14 (BMC -5.3±1.8%, p=0.005; BA -4.2±1.0%, p≤0.0001); and there was no significant difference between the groups at PP2.

Mean WB aBMD increased in both groups, and the changes were not significantly different between the groups (+14.4% vs +15.4%, p=0.5; Table 36). Similar to the cross-sectional analysis, there were no significant differences between the groups at either PP2 or PP14. WB aBMD was not significantly associated with having been ill within 2 weeks before the study visit and breastfeeding status in the nested model.

There were trends towards decreases in SA-BMC (adjusted for BA, weight and length) in both groups (HEI -6.0±3.5%, p=0.05; HUI -7.6±3.5%, p=0.09). Changes in SA-BMC were not significantly different between the groups (p=0.2), and also there were no significant differences between the groups at either PP2 or PP14. Also, SA-BMC was not significantly associated with infant age, having been ill within two weeks before the study visit and breastfeeding status in the nested model.

Figure 60(a)

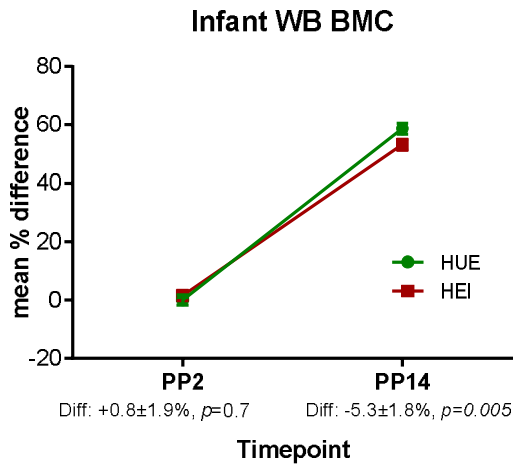


Figure 60(b)

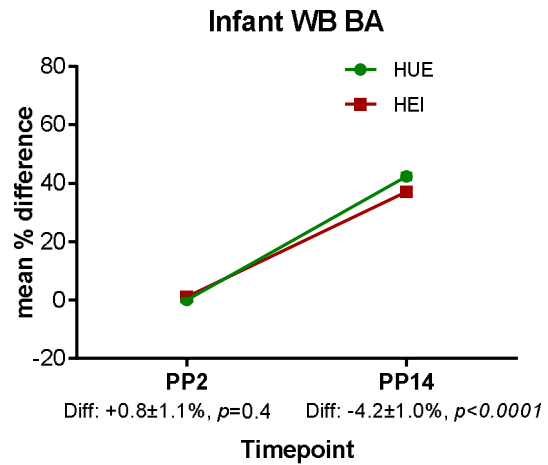


Figure 60(c)

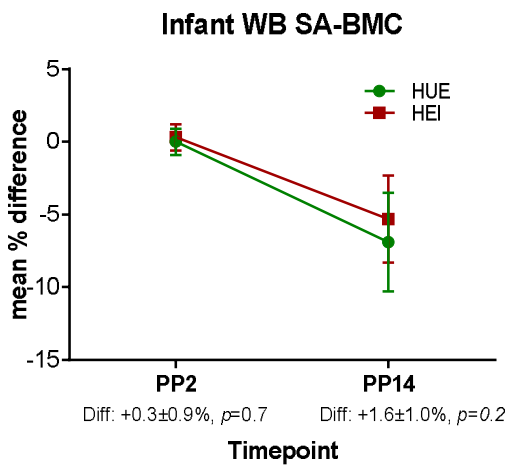


Figure 60(d)

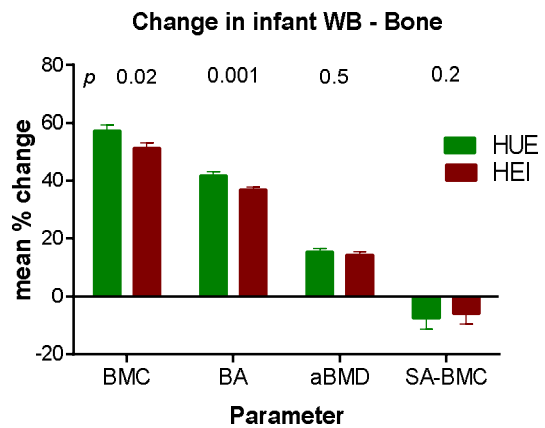


Figure 60: Change in infant whole body (with head) bone mineral (PP2-PP14)

WB=whole body with head, BMC=Bone mineral content (g), BA=Bone area (cm<sup>2</sup>), aBMD= areal Bone mineral density (g/cm<sup>2</sup>), SA-BMC =Size adjusted BMC – BMC adjusted for BA and body weight. Diff = mean sympercent difference between the groups $\pm$ SD. Results were obtained from Scheffé's post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction. The number of subjects included in the analysis was 81 HIV-negative and 83 HIV-positive breastfeeding infants. Figure 60(d) Bars and error bars represent the mean $\pm$ SE sympercent changes within groups at 14 weeks postpartum. The P values shown above the bars are for comparison of mean % change between the groups at 14 weeks for the corresponding bone parameter.



### 8.7.2. Change in infant LS bone mineral

Overall, mean infant LS BMC and BA increased in both groups as expected in growing infants. aBMD significantly increased only among HIV-exposed infants. There were no significant changes in SA-BMC (adjusted for BA, weight and length) in both groups (Table 36 and Figure 61a-d).

Contrary to changes in WB BMC, HEI had a significantly greater increase in LS BMC compared to HUI (+29.5% vs +24.4%,  $p=0.03$ ). HEI had significantly lower LS BMC at PP2 ( $-8.1\pm1.6\%$ ,  $p\leq0.0001$ ), and the size effect was similar (or even bigger) in the cross-sectional analysis. There was a trend towards a lower BMC in HEI vs HUI at PP14 ( $-3.3\pm1.6\%$ ), but the difference between the groups was not significant ( $p=0.06$ ).

Mean LS BA increased by the same magnitude in both groups ( $+25.6\pm1.1\%$ ,  $p\leq0.0001$  for both). Similar to the cross-sectional analysis, HEI had significantly lower LS BA at both PP2 ( $-4.7\pm1.0\%$ ,  $p\leq0.0001$ ) and PP14 ( $-4.7\pm1.1\%$ ,  $p\leq0.0001$ ).

Mean LS aBMD significantly increased only in HEI ( $+3.9\pm1.4\%$  vs  $-1.1\pm1.5\%$ ,  $p=0.4$ ). Consequently, HEI had a significantly greater increase in aBMD compared to HUI ( $p=0.02$ ). Similar to the cross-sectional analysis, HEI had significantly lower LS aBMD at PP2 ( $-3.4\pm1.5\%$ ,  $p=0.02$ ) and there was no significant difference between the groups at PP14 ( $+1.6\pm1.4\%$ ,  $p=0.3$ ).

Non-significant increases in SA-BMC were observed in both groups (HEI  $+9.4\pm5.8\%$ ,  $p=0.1$ ; HUI  $+4.3\pm6.2\%$ ,  $p=0.5$ ). Even so, the increase in SA-BMC was significantly greater in HEI compared to HUI ( $p=0.02$ ). Similar to the cross-sectional analysis, HEI had a significantly lower SA-BMC at PP2 ( $-4.6\pm1.6\%$ ,  $p=0.004$ ), and there was no significant difference between the groups at PP14 ( $+0.5\pm1.6\%$ ,  $p=0.8$ ). Potential confounders (age of the infant, breastfeeding status and illness within two weeks to the visit) were not significantly associated with all infant bone parameters at the lumbar spine.

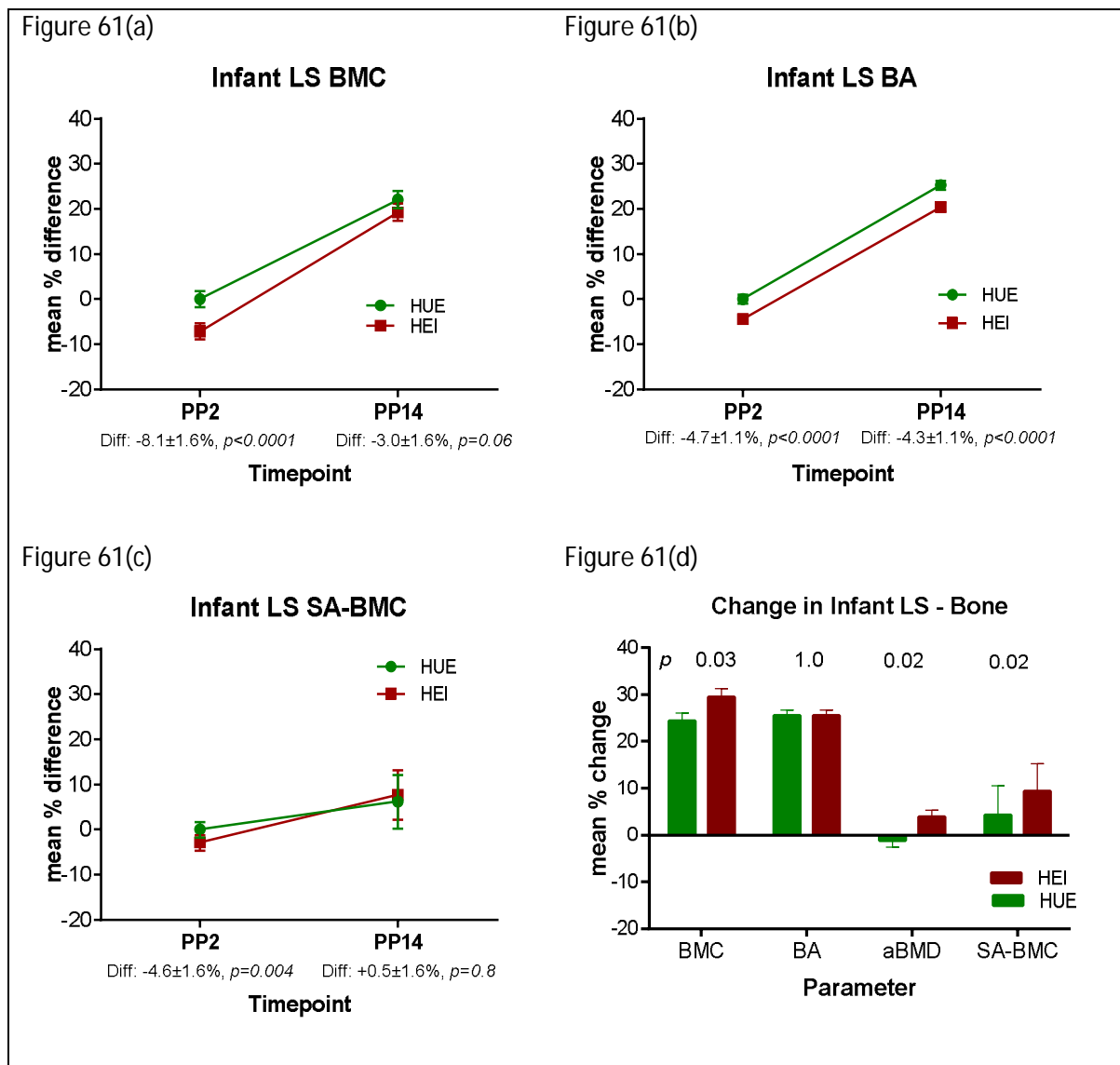


Figure 61: Change in infant lumbar spine bone mineral (PP2-PP14)

LS = lumbar spine, BMC=Bone mineral content (g), BA=Bone area (cm<sup>2</sup>), aBMD= areal Bone mineral density (g/cm<sup>2</sup>), SA-BMC =Size adjusted BMC – BMC adjusted for BA and body weight. Diff = mean sympercent difference between the groups±SD. Results were obtained from Scheffés post hoc tests for group\*visit (time point) interaction terms in hierarchical repeated measures ANOVA models that included subject (nested by group), group, visit, and group\*visit interaction. The number of subjects included in the analysis was 81 HIV-negative and 83 HIV-positive breastfeeding infants. Figure 61(d) Bars and error bars represent the mean±SE sympercent changes within groups at PP14. The P values shown above the bars are for comparison of mean % change between the groups at PP14 for the corresponding bone parameter

## 8.8. Summary of longitudinal results

Maternal body weight, lean mass and fat:lean<sup>2</sup> significantly reduced in both groups and the magnitudes of change were not significantly different.

Plasma calcium, PO<sub>4</sub>, Mg and Cr concentrations significantly increased in both groups between PG36 and PP14. However, the mean increase in corrected plasma calcium (uncorrected for albumin) was significantly smaller in HIV-positive women compared to HIV-negative women (+3.0±0.5% vs -6.6±0.5%, p≤0.0001). Similarly, albumin corrected plasma calcium decreased in both groups but HIV-positive women had a significantly greater decrease compared to HIV-negative women (-6.6±0.5% vs -3.8±0.5%, p≤0.0001).

HIV-positive women had a significantly greater mean increase in plasma phosphate concentration compared to HIV-negative women (+14.4±0.2% vs +7.7±2.0%, p=0.022), such that the significantly lower concentration of HIV-positive women at PG36 was no longer different to the HIV negative women at PP14. The magnitude of changes in plasma magnesium and creatinine were not significantly different between the groups.

Estimated GFR significantly reduced in both groups, but HIV-positive women had significantly greater reductions compared to HIV-negative women (-13.6±1.1% vs -10.2±1.1%, p=0.04). Changes in TmP/GFR were not significantly different between the groups. However, HIV-positive women had a significantly smaller increase in TmCa/GFR compared to HIV-negative (+3.4±0.5% vs +7.3±0.5, p≤0.0001). Urine Ca and phosphate creatinine ratios (UCa/Cr and UP/Cr, respectively) significantly reduced in both groups between PG36 and PP14, and the magnitudes of changes were not significantly different between the groups. There were no significant changes in UMg/Cr ratio in both groups.

There were no significant changes in breast milk phosphorus in both groups, but HIV-positive women had about 9% significantly higher concentrations compared to HIV-negative women at both PP2 and PP14. Breastmilk calcium and potassium concentrations significantly reduced in both groups between PP2 and PP14, and the mean changes were not significantly different between the groups. HIV-positive women had significantly lower breastmilk calcium concentration at PP2 but not at PP14. They also had a significantly smaller reduction in mean breast milk sodium concentration, and a trend towards a smaller reduction in Na/K compared to HIV-negative women.

Mean plasma PTH, and markers of bone resorption (CTX) and bone formation (P1NP and BAP) significantly increased between PG36 and PP14, and the increases were significantly greater in HIV-positive compared to HIV-negative women. As expected, plasma TAP and serum 25(OH)D concentrations significantly reduced in both groups between PG36 and PP14. The magnitude of change in serum 25OHD was not significantly different between the groups. However, HIV-positive women had a significantly smaller decrease in TAP compared to HIV-negative women.

Maternal bone mineral (BMC, aBMD and SA-BMC) reduced at all skeletal sites in both groups, as expected during lactation. However, HIV-positive women experienced a significantly greater reduction in total hip aBMD and SA-BMC compared to HIV-negative women (BMD  $-4.0 \pm 0.4\%$  vs.  $-2.7 \pm 0.4$ ,  $p=0.04$ ; SA-BMC  $-3.7 \pm 0.3\%$  vs.  $-2.7 \pm 0.3$ ,  $p=0.04$ ). The magnitudes of changes in both lumbar spine and WBLH bone mineral (BMD and SA-BMC) were not significantly different between the groups, although, there was a trend towards a greater reduction in WB aBMD among HIV-positive compared to negative women ( $-1.2 \pm 0.2\%$  vs  $-0.6 \pm 0.2$ ,  $p=0.05$ ).

All infant anthropometric measurements (weight, length and head circumference) increased between PP2 and PP14 as expected in infancy, but HEI had slower increases compared to HUI. Also, infant whole body lean and fat mass (measured by DXA) significantly increased in both groups, but the increases were not significantly different between the groups.

Infant BMC and BA significantly increased at the WB and LS in both groups as expected during infancy, but HEI had slower increases compared to HUI. Lumbar spine aBMD increased only among HIV-exposed infants. They also had significantly greater mean percent increases in LS aBMD and SA-BMC compared to HUI. However, the magnitudes of changes in WB aBMD and SA-BMC were not significantly different between the groups, showing that both HEI and HUI had adequate bone mineral for their attained bone size and body weight.

## CHAPTER 9: DISCUSSION

The study that forms this thesis (referred to as the Gumba study in this chapter) was a longitudinal observational study conducted in Kampala, Uganda. Two groups of pregnant women, 95 HIV+ (on Tenofovir-Lamivudine-Efavirenz, previously ART naïve) and 96 HIV- were followed prospectively. Data were collected at 36 wks gestation (PG36), 2 (PP2) and 14 wks postpartum (PP14). Dual-energy x-ray absorptiometry was used to measure bone phenotype (bone mineral content (BMC); bone area (BA); areal bone mineral density (aBMD), and size- adjusted BMC (SA-BMC, adjusted for height or length, weight and BA) of the whole body (WB) and lumbar spine (LS) in mother-baby pairs, and total hip (TH) in mothers.

Gumba study sought to compare bone health between HIV-positive women on Option B+ ART vs HIV-negative Ugandan women, and their babies in the context of pregnancy and lactation. The primary outcome was the difference between groups in % change ( $\pm$  SE) in maternal LS aBMD between PP2 and PP14. Secondary outcomes included changes in maternal bone turnover markers, calciotropic hormones, markers of bone mineral metabolism in urine and plasma, breastmilk mineral composition; and infant bone mineral, anthropometry and growth. Data were also collected on breastfeeding, reproductive history, health, and lifestyle characteristics in order to adjust statistical models for potential confounders.

The Gumba study was designed to test the hypothesis that maternal ART may accentuate the normal process of bone mineral mobilisation during pregnancy and lactation, leading to bone loss that is not recovered in the mother, and compromised infant growth and/or bone mineral accretion. Gumba study hypothesis was developed after a review of the literature on the independent effects of pregnancy, lactation and ART on bone health in adults, and also on infant outcomes following exposure to maternal ART.

## 9.1. Novel data presented in this thesis

The research presented in this thesis has several key findings which significantly contribute to the body of knowledge on bone metabolism in breastfeeding HIV-infected women on TDF-based ART and their babies in the following areas: maternal bone mineral and biochemistry, breastmilk mineral content, and infant growth and bone mineral accretion. These are discussed below in more detail.

### 9.1.1. Maternal bone mineral

#### 9.1.1.1. Lactation and bone

To the best of my knowledge, this is the first study to describe changes in bone mineral during lactation in HIV-positive African women on Option B+ ART in comparison to HIV-negative counterparts. A consistent pattern of decrease in aBMD was found between 2 and 14 weeks of lactation at all skeletal sites in both HIV-positive (LS:  $-1.8 \pm 0.4\%$ , TH:  $-4.0 \pm 0.4\%$ , WBLH:  $-1.2 \pm 0.2\%$ ;  $p < 0.05$  for all) and HIV-negative women (LS:  $-2.5 \pm 0.4\%$ , TH:  $-2.7 \pm 0.4\%$ , WBLH:  $-0.6 \pm 0.2\%$ ;  $p < 0.05$  for all). These findings are consistent with the existing evidence, in apparently healthy women and in African women, that lactation is associated with physiological decreases in maternal aBMD during lactation (Olausson et al., 2012).

Longitudinal studies conducted using DXA or DPA in Caucasian and Gambian women have reported decreases in maternal bone mineral from shortly after delivery to 3 months of lactation. The changes in aBMD range from -6.0 to 3.1% at the lumbar spine, -2.3% to -1.5% at the total hip and 0.8% to -1.5% in the whole body (Kalkwarf and Specker, 1995, Affinito *et al.*, 1996, Krebs *et al.*, 1997, Laskey et al., 1998, Hopkinson *et al.*, 2000, Åkesson *et al.*, 2004, Pearson *et al.*, 2004).

Only one study has investigated changes in maternal bone mineral during lactation in African women. A placebo controlled calcium supplementation study conducted in rural Gambian women on habitual low calcium diets observed decreases in maternal bone mineral by DXA at 2 and 13 weeks of lactation. The changes at the lumbar spine and whole body in the placebo group were very similar to those observed in HIV-negative women in the Gumba study (LS aBMD:  $-2.3 \pm 0.7\%$ , LS SA-BMC:  $-2.4 \pm 0.7\%$ ; WB aBMD:  $-0.7 \pm 0.3\%$ ; WB SA-BMC:  $-0.7 \pm 0.3\%$ ). However, the magnitude of the changes at the TH in HIV-negative women in the Gumba study was smaller than observed in Gambian women (TH aBMD  $4.0 \pm 0.8\%$ ; TH SA-BMC  $3.7 \pm 0.8\%$ ) (Jarjou et al., 2010).

Different parts of the skeleton may respond differently to increased calcium requirements during pregnancy and lactation (Laskey and Prentice, 1999). Lactation studies have reported greater mobilisation of bone mineral at the lumbar spine in Caucasian women, a trabecular rich site,

compared to total hip and whole body at 3 months of lactation (Affinito et al., 1996, Kolthoff et al., 1998, Laskey and Prentice, 1999, Jarjou et al., 2010). However, greater decreases in aBMD have been observed at the total hip compared to the lumbar spine in Gambian women (Jarjou et al., 2010).

The magnitude of changes in maternal bone mineral depends on the pattern of breastfeeding. Women who breastfeed for longer durations experience greater changes in bone mineral during the first 3-6 months postpartum compared to those who breastfeed for shorter durations (Sowers *et al.*, 1993, Laskey and Prentice, 1999, Hopkinson et al., 2000). This might be due to differences in the intensity and frequency of suckling, infant breastmilk intake and timing of introduction of complementary or supplementary foods (Laskey et al., 1998, Prentice *et al.*, 1999, Olausson et al., 2012). Also, infant breastmilk intake (quantified by the deuterium dilution technique) and maternal height have been identified to predict the magnitude of bone loss in Gambian women (Laskey et al., 1998). The Gumba study did not quantify infant breastmilk intake but used self-reports of breastfeeding practices as a proxy for intensity of breastfeeding and infant breastmilk intake. This is because DTTM procedures would have overburdened participants and overstretched study staff, compromising the main study.

In the Gumba study, more HIV-positive women reported exclusive breastfeeding compared to HIV-negative women (PP2: 82.9% vs. 58.7%,  $p=0.0008$ ; PP14: 86.7% vs 66.2%,  $p=0.002$ ). Nonetheless, LS aBMD was significantly associated with age of the infant ( $\beta = -0.6 \pm 0.2\%$  per day,  $p=0.01$ ) in the analysis of covariance, but adjusting for it did not have a significant effect on the results. There was no significant association between maternal aBMD (at any site) and height. Adjusting for breastfeeding practices did not attenuate the difference in changes in aBMD between the groups.

Other factors which might influence mobilisation of bone mineral during lactation include maternal age, body weight, parity, duration of breastfeeding, resumption of menses, maternal hormones (estradiol, prolactin and progesterone), use of hormonal contraception, infant weight and breastmilk intake (Olausson et al., 2012). Except for maternal hormones (not measured in the current study), the above factors did not account for the greater mobilisation of bone mineral in HIV-infected women. Overall, the effect of lactation was very similar between the groups.

### 9.1.1.2. HIV, ART, Lactation and Bone

In the Gumba study, HIV-positive vs HIV-negative women had significantly greater reductions in aBMD at both WBLH ( $p=0.05$ ) and TH ( $p=0.04$ ); and TH SA-BMC, adjusted for weight and bone area ( $-3.7\pm0.3\%$  vs.  $-2.7\pm0.3\%$ ,  $p=0.04$ ). There was a trend towards a smaller reduction in LS aBMD compared to HIV-negative women, although the changes were not significantly different between the groups ( $p=0.3$ ). The decreases in aBMD observed at both the LS and TH in HIV-positive women in the Gumba study are consistent with other studies following initiation of ART (Dolan et al., 2004, Gallant et al., 2004, Stellbrink et al., 2010). Therefore, the observed greater reduction in maternal aBMD at the TH in HIV-positive women is likely to be an effect of ART initiation during pregnancy.

In a multicentre ASSERT clinical trial, greater decreases in TH aBMD were observed in HIV-infected adults on TDF containing ART compared to HIV-positive adults on non-TDF-based ART at week 48 after initiation of ART (TH:  $-3.6\%$  vs  $-2.4\%$ ; LS:  $-1.9\%$  vs  $-1.6\%$ ). The participants were 80% Caucasian men with mean  $CD_4 < 250$  cells/ $ml^3$  (Stellbrink et al., 2010). The steepest decreases in aBMD have been observed within the first 24 weeks, with evidence of some recovery in lumbar spine aBMD between 24 and 48 weeks after initiating ART (Gallant et al., 2004, Stellbrink et al., 2010, Moyle et al., 2013). The mean duration of exposure to maternal ART at PP14 was  $29.3\pm5.1$  weeks, and the majority of the women had preserved  $CD_4$  at initiation of ART. Thus, the time points in the Gumba study are within the window when greatest reductions in aBMD are expected following initiation of TDF-based ART.

In the Women's Bone Study (WBS), a greater decrease in aBMD at the TH compared to LS was also observed after 12 months of ART in HIV-positive black South African women with preserved  $CD_4$  (TH  $-1.9\pm0.6\%$ ; LS  $-0.2\pm0.5\%$ ; WBLH  $+0.4\pm0.3\%$ ) (Hamill et al., 2017). The change in TH aBMD was no longer significant, in women with preserved  $CD_4$ , after adjusting for bone area and body weight ( $-0.3\pm0.6\%$ ). Size adjustment did not have a material effect on the changes at LS and WBLH. Conversely, women with advanced HIV disease (low  $CD_4$ ) in WBS had significant decreases in aBMD at the LS ( $-2.0\pm0.6\%$ ) and WBLH ( $-0.9\pm0.3\%$ ) but not at TH ( $+0.4\pm0.6\%$ ). They also had significant decreases in SA-BMC at both TH ( $-1.7\pm0.6\%$ ) and LS ( $-2.3\pm0.6\%$ ), but not WBLH SA-BMC ( $-0.7\pm0.3$ ) (Hamill et al., 2017).

Significant decreases in aBMD were also observed in HIV-negative African women after 48 weeks on oral TDF for prevention of HIV-infection in the Vaginal and Oral Interventions to Control the Epidemic (VOICE) study, but with greater losses at the LS ( $-1.4\%$ ) compared to TH ( $-0.9\%$ ) (Mirembe et al., 2016). Changes in aBMD were reversed after the study drug was stopped. However, the participants in the VOICE study had poor compliance to the study drug, and the reported



changes in aBMD were not adjusted for changes in body weight and bone area. Both the WBS and the VOICE studies significantly contribute to our understanding of changes in bone mineral following initiation of ART in African women who are neither pregnant nor lactating.

Only one study (PROMISE 1048s) has published data on changes in bone mineral during lactation in HIV-infected mothers initiated on ART postpartum. Greater reductions in aBMD have been reported at 74 weeks postpartum in ART-exposed HIV-infected African mothers compared to ART unexposed (Stranix-Chibanda and PROMISE-team, 2016). The change in LS aBMD in the TDF-based ART arm was comparable to that observed in HIV-positive women in the Gumba study. Similar to other studies on ART, the magnitude of changes in maternal aBMD in the PROMISE study was greater at the TH compared to the LS (TH: -5.37% vs -3.05%,  $p < 0.001$ ; LS: -2.06% vs +1.09%,  $p < 0.001$ ). Taken together, these studies suggest a greater vulnerability of the TH vs LS to the effects of ART on maternal bone mineral.

The median duration of breastfeeding in PROMISE P1084s was 61 weeks, and the measurements were done at 74 weeks (~3 months after cessation of breastfeeding for most of the participants (Stranix-Chibanda and PROMISE-team, 2016). Lactation studies have reported recovery of bone mineral at the LS from 6 months postpartum even in women who are still breastfeeding (Cooke-Hubley et al., 2017). Most evidence shows that there are no significant differences from baseline (2 weeks postpartum) in LS aBMD by 3 months after cessation of breastfeeding, but recovery at the total hip may take longer (Laskey et al., 1998, Cooke-Hubley et al., 2017). Recovery of bone mineral has also been observed in Gambian women on very low calcium intakes after long durations of breastfeeding and successive cycles of pregnancy and lactation (Sawo et al., 2013). Therefore, the data from PROMISE P1084s could suggest a slower recovery or even a permanent decrease in maternal bone mineral at the LS among HIV-positive women on postpartum ART, compared to those not on ART. The clinical significance of these findings is still unclear as maternal ART is very effective at preventing mother-to-child transmission of HIV during lactation. However, P1084s did not have a control group of HIV-negative mothers to give an insight on the normal pattern of changes in bone mineral during lactation in healthy Ugandan women; and the preliminary data were not adjusted for changes in weight or other potential confounders. It will be interesting to see the full paper once published and to compare to post-lactation data collected in the Gumba study.

In the Gumba study, HIV-positive women had significantly lower weight compared to HIV-negative women at all visits (PG36:  $-4.6 \pm 2.2\%$ ,  $p = 0.03$ ; PP2:  $-5.2 \pm 2.5\%$ ,  $p = 0.04$ ; PP14:  $-4.6 \pm 2.6\%$ ,  $p = 0.07$ ). The longitudinal changes in weight were not significantly different between the groups (PP14-PG36:  $-10.3 \pm 0.7\%$  vs  $-10.1 \pm 0.7\%$ ,  $p = 0.7$ ; PP14-PP2:  $-0.8 \pm 0.7\%$  vs  $-1.4 \pm 0.7\%$ ,  $p = 0.8$ ). Although HIV positive women had a greater mean decrease in TH aBMD (as discussed above), there was an overall trend

towards higher TH aBMD compared to HIV-negative women at both PP2 (TH:  $+2.1 \pm 1.8\%$ ,  $p=0.3$ ; WBLH:  $+1.1 \pm 1.1\%$ ,  $p=0.3$ ) and PP14 (TH:  $+1.3 \pm 2.0\%$ ,  $p=0.5$ ; WBLH:  $+0.6 \pm 1.1\%$ ,  $p=0.5$ ). The mean % differences between the groups were accentuated after adjusting for body weight and bone area at PP2 (TH:  $+2.9 \pm 1.8\%$ ,  $p=0.1$ ; WBLH:  $+1.6 \pm 1.1\%$ ,  $p=0.1$ ) and PP14 (TH:  $+2.1 \pm 1.8\%$ ,  $p=0.3$ ; WBLH:  $+0.6 \pm 1.1\%$ ,  $p=0.6$ ), because HIV-positive women had lower weight.

#### *9.1.1.3. Body size, weight and bone*

DXA estimates areal bone mineral density within a 2-dimension region of interest hence it is not a true volumetric density (vBMD). Thus, one should be cautious when comparing DXA measurements between groups or individuals as smaller bones will have lower aBMD (BMC/BA) than larger bones, even when the vBMD is the same. This is because BA does not fully adjust for differences in bone size and this is a major limitation of the areal bone density measured by DXA. To circumvent this limitation, Prentice et al. recommend size adjustments using multiple regression models using BMC as the dependent variable and BA, weight and height as independent variables to avoid size-related artefacts (Prentice et al. 1994), and this was performed in Gumba.

Lack of adjustments for bone and body size in studies using DXA is a common limitation in the majority of the studies investigating changes in bone mineral following initiation of ART. Bone mineral density measured by DXA is highly dependent on bone area. Therefore, differences in the positioning of the subject on the scanner affect bone edge detection and interpretation of BMD data, especially in longitudinal studies. Also, changes in weight are likely to occur which might confound interpretation of bone mineral density when comparing groups.

Weight gain during pregnancy causes changes in the pelvis and also the positioning of the femoral neck changes, but it gradually returns to the normal position after delivery (Laskey and Prentice, 1999). These changes in the pelvis are likely to affect the precision and comparability of DXA measurements at the femoral neck. Also, redistribution of bone mineral between the femoral neck and the greater trochanter occurs during lactation, such that the final measurement at the TH is similar to baseline. Hence this redistribution might mask the changes in the TH aBMD during lactation; and thus, relying on TH measurements alone might not give the full picture (Laskey and Prentice, 1999). The Gumba study had a contemporaneous control group of HIV-infected women. Therefore, the observed greater decrease in TH aBMD in HIV-positive vs HIV-negative women is not likely to be due to artefacts, but a result of maternal ART.

#### 9.1.2.4. Depo-provera and bone

Use of depo-provera contraception in healthy women is associated with weight gain and reductions in aBMD ranging from 2.7% at the LS and 4.1% at the total hip. The side effects of depo-provera are reversed after discontinuation, and similar to lactation, recovery of bone mineral is faster at the spine compared to other skeletal sites (Kaunitz *et al.*, 2008). In the Gumba study, a greater proportion of HIV-infected women had used depo-provera before their index pregnancy and also by 14 weeks postpartum. Use of depo-provera prior to the index pregnancy was associated with 6-9% significantly greater body weight and 3-6% greater aBMD at both PP2 and PP14. Current use of depo-provera at PP14 was not associated with body weight. However, women who reported current use of depo-provera at PP14 had significantly lower TH aBMD ( $-5.6 \pm 2.7$ ,  $p=0.04$ ), consistent with other studies (Kaunitz *et al.*, 2008). Adjusting BMC, aBMD and SA-BMC for either current or previous use of depo-provera did not have a material effect on the longitudinal results at all skeletal sites.

Overall, the results from the Gumba study suggest the greater mobilisation of bone mineral in HIV-infected women is independent of breastfeeding practices and other potential confounders, hence likely to be an effect of maternal ART. Continued follow-up of the participants is recommended to investigate if these changes are permanent.

#### 9.2.2. Maternal bone turnover markers (BTM)

To my best knowledge, Gumba is the first study to compare changes in bone turnover markers (BTM) during pregnancy and in lactation between HIV-positive and HIV-negative women. Overall, bone turnover markers increased in both HIV-positive and HIV-negative women consistent with previous studies that have reported increased bone turnover at 3-6 months of lactation (Prentice *et al.*, 1998, Holmberg-Marttila *et al.*, 2003, More *et al.*, 2003, Vargas Zapata *et al.*, 2004).

Greater increases in BTM were observed in HIV-positive compared to HIV-negative women in the Gumba study (P1NP:  $+100.3 \pm 5.0\%$  vs.  $+72.6 \pm 5.0\%$ ,  $p=0.0001$ ; CTX:  $+74.6 \pm 5.9\%$  vs.  $+56.2 \pm 5.9\%$ ,  $p=0.04$ ; BAP:  $+67.2 \pm 3.6\%$  vs.  $+57.1 \pm 3.6\%$ ,  $p=0.05$ ). These data are consistent with some of the previous studies which have reported greater changes in BTM markers in HIV-infected individuals, predominantly Caucasian men, regardless of ART regimen (Haskelberg *et al.*, 2011).

Early increases in bone resorption markers and delayed increases in bone formation markers have been observed at 4 months after initiation of ART in HIV-infected men, revealing a catabolic window for bone loss (van Vonderen *et al.*, 2011). The greatest increases in resorption markers were at 4 months after initiation of ART while greater increases in bone formation markers were at 12 months. Increases in BTM at 4 months predicted hip aBMD at 12 months (van Vonderen *et al.*, 2011). Other

studies have also observed increases in BTM (osteocalcin, P1NP, BAP, and CTX) in the first 24 weeks after initiation of ART, and then stabilise or decrease thereafter (Stellbrink et al., 2010, Haskelberg *et al.*, 2012). In the ASSERT study, greater increases in all BTM markers were observed in the TDF/FTC group compared to ABC/3TC group; and all markers except CTX remained significantly different between the groups at 48 weeks (Stellbrink et al., 2010). In the SMART trial, intermittent ART was associated with significant decreases in both formation (osteocalcin, BAP and P1NP) and resorption markers (NTX and CTX) compared to continuous ART, suggesting that ART has a direct effect on bone turnover/loss (Hoy et al., 2013).

Few studies have investigated bone metabolism in HIV-infected NPWL women following initiation of ART (Dolan et al., 2006, Hamill et al., 2017). Greater increases in osteocalcin and NTX were observed in HIV-infected NPWL women on various ART regimens compared to HIV-negative controls over 24 months. The change in NTX predicted change in BMD at the femoral neck in women on PI/NNRTI and at LS aBMD in women on NNRTI (Dolan et al., 2006). Only one study has investigated changes in bone turnover markers following initiation of ART in HIV-infected African women. Greater increases in BAP, P1NP and CTX, were observed at 12 months after initiation of ART in black NPWL South African women with low CD<sub>4</sub> at initiation of ART, and changes in BTM were associated with decreases in femoral neck and LS aBMD (Hamill et al., 2017). There are no published studies on BTM in pregnant and breastfeeding women on ART.

In the Gumba study, HIV-positive women had 26% significantly lower P1NP at PG36, but there was no significant difference in CTX. These data suggest slower/delayed bone formation compared to HIV-negative women. P1NP concentrations doubled in HIV-positive women and thus by PP14 there was no significant difference between the groups. However, CTX concentrations were 15.6% higher in HIV-positive women at PP14 suggesting greater mobilisation of bone mineral during lactation. Taken together, these changes in P1NP and CTX reveal a catabolic window for bone loss in HIV-positive women, consistent with previous studies in men and non-pregnant non-lactating women.

The data from the Gumba study suggest higher bone turnover in HIV-infected women compared to HIV-negative counterparts between late pregnancy (10.8±5.2 weeks on ART) and 3 months of lactation (29.3±5.1 weeks on ART) and support the observed greater decreases in bone mineral at the TH and WBLH in HIV-positive women.

By design, Gumba did not measure bone turnover markers before and during pregnancy or before initiation of ART. Thus, the magnitude of changes during pregnancy and following initiation of ART could not be assessed.

### 9.2.3. Maternal calciotropic hormones

#### 9.2.3.1. 25(OH)D

Mean serum 25(OH)D concentrations were not significantly different between the groups at either PG36 or PP14, and all the participants had concentrations above 50nm/L indicating good vitamin D status. These data are similar to those reported in pregnant and lactating women in The Gambia (13°N, all year sunshine and UVB exposure) (Prentice *et al.*, 1998). Mean [25(OH)D] decreased in both groups between PG36 and PP14 and the changes were not significantly different between the groups ( $-13.9 \pm 4.1$  % vs  $-11.1 \pm 3.1$ %,  $p=0.6$ ). The decrease in [25(OH)D] is consistent with other studies in pregnancy and lactation and is attributed to an increase in DBP during pregnancy (Jones *et al.*, 2016).

There is some evidence that TDF is associated with an increase in DBP and a decrease in free 1,25(OH)<sub>2</sub>D causing functional vitamin D deficiency, and this might explain the observed greater loss in aBMD with TDF use (Overton *et al.*, 2015). Efavirenz, another first-line drug for pregnant and lactating women in Uganda, has been reported to induce cytochrome P450 enzymes resulting in the accelerated breakdown of 25(OH)D to 1,25(OH)<sub>2</sub>D (Gyllenstein *et al.*, 2006, Avihingsanon *et al.*, 2015). Thus, both TDF and EFV, which are preferred drugs in the current first-line Option B+ ART regimen, can alter vitamin D metabolism resulting in a functional deficiency.

Studies conducted in HIV-positive individuals, predominantly men in the USA, have reported greater prevalence of vitamin D deficiency compared to HIV-negative persons (Hileman *et al.*, 2016). On the contrary, the Gumba study did not observe poor vitamin D status in HIV-infected women either during late pregnancy (PG36) or at 3 months postpartum (PP14). These findings are consistent with data reported by Hamill *et al.* in non-pregnant non-lactating black South African women at baseline and 12 months of ART in the WBS study (Hamill *et al.*, 2017).

Taken together, the data from both Gumba and WBS studies suggest that HIV-infection is not associated with poor vitamin D status and that initiation of TDF-containing ART does not adversely affect serum vitamin D status in African women with the ability to synthesize vitamin D all year. However, both studies did not measure 1,25(OH)<sub>2</sub>D - the active metabolite of 25(OH)D which plays a major role in calcium homeostasis, hence cannot rule out functional vitamin D deficiency following initiation of TDF and EFV. In the future, it will be interesting to measure 1,25(OH)<sub>2</sub>D in stored samples in the Gumba and WBS studies to investigate functional vitamin D deficiency.

### 9.1.3.2. PTH

Median PTH concentrations at 14 weeks of lactation were 60.0 (44.7, 80.1) pg/ml in HIV-positive and 41.4 (IQR 29.5, 58.8) pg/ml in HIV-negative women. These concentrations in Ugandan women, regardless of HIV-status, were comparable to the medians reported at 13 weeks of lactation in Gambian women on low calcium intakes with good vitamin D status (Prentice *et al.*, 1998). Gambian women have been reported to have higher PTH concentrations compared to British women at 13 weeks of lactation ( $47.2 \pm 3.0$  ng/L vs  $20.5 \pm 2.5$  ng/L;  $p \leq 0.001$ ) and after stopping breastfeeding ( $53.1 \pm 7.0$  ng/L vs  $34.3 \pm 7.2$  ng/L;  $p \leq 0.001$ ) (Prentice *et al.*, 1998). The higher PTH in lactating Gambian women is accompanied by higher  $1,25(\text{OH})_2\text{D}$  and bone formation markers. Higher PTH has also been observed in pre and postmenopausal Gambian compared to and British women (Aspray *et al.*, 2005).

Under normal physiological circumstances, PTH maintains plasma calcium concentrations, increases urine phosphate excretion and activates renal production of  $1,25(\text{OH})_2\text{D}$  (Blaine *et al.*, 2015). However, PTH gradually increases as renal function declines resulting in an increase in plasma phosphate and fibroblast growth factor-23 (FGF-23) concentrations, which decreases renal synthesis of  $1,25(\text{OH})_2\text{D}$  and consequently a decrease in serum calcium concentrations – hence secondary hyperparathyroidism (Goodman and Quarles, 2008). In secondary hyperparathyroidism, PTH is not suppressed by FGF-23, hence continued decline in renal function leads to further increases in PTH and worsening of secondary hyperthyroidism because of suppressed  $1,25(\text{OH})_2\text{D}$  production and resistance to FGF-23 (Ben-Dov *et al.*, 2007).

It is unclear whether the higher PTH concentrations in both Gambian and Ugandan women compared to British women are physiological adaptations to low dietary calcium intakes in an environment of good vitamin D status (due to year-round UVB exposure). However, Dibba *et al.* did not observe differences in calciotropic hormones between British Caucasians in England and Gambians raised in West Africa but resident in England, suggesting a lack of racial differences (Dibba, 1994). Data from a calcium supplementation study in Gambian women suggest that the habitually low calcium intakes may not account for the observed elevated PTH during lactation (Prentice *et al.*, 1998). However, the Gumba study did not quantify calcium intakes, hence unable to make comparisons of calcium intakes between the groups, and with both Gambian and British diets; and investigate associations between calcium intakes and PTH. To my best knowledge, Gumba is the first study to report PTH concentrations in pregnant and lactating women in Uganda.

HIV-positive women had 33-35% significantly higher mean PTH concentration at both PG36 and PP14. Elevated PTH concentrations have been observed in HIV-positive individuals especially those on TDF-containing ART. In a cross-sectional study of HIV-infected persons in central Europe, TDF use, low vitamin D status and low plasma calcium were significantly associated with higher PTH concentrations in Caucasian but not in African patients. In particular, elevated PTH was not associated with the use of TDF in first generation Africans living in central Europe, although there was an association in Caucasians suggesting that PTH mediated effects on bone might be less pronounced in persons of African ancestry living in Europe (Noe *et al.*, 2017). However, that study had an uneven distribution of patients by sex across the groups (87% male in the Caucasian group and 63% women in the African group) which was not adjusted for, and furthermore, data on reproductive history (i.e. breastfeeding and contraception) were not reported.

The median PTH values reported in NPNL women in the Gambia by Prentice et al. are very similar to those reported by Noe et al. in HIV-infected African patients in Europe [(52.8 (33.7-71.3) pg/ml. Recently, median PTH values between 12 and 38 pg/ml were reported in NPNL HIV-infected and HIV-uninfected black South African women, and women with low baseline CD<sub>4</sub> had significantly higher PTH after 12 months on ART compared to those with preserved baseline CD<sub>4</sub> (Hamill et al., 2017). The median PTH concentrations in the South African study are comparable to those reported in Caucasians in Europe and much lower compared to those observed in African women in The Gambia, and Uganda (in the Gumba study).

PTH is known to be suppressed in pregnancy and lactation. Data from a supplementation study in Gambian women suggest that PTH is not responsive to calcium intake during lactation, and only rises to concentrations at or higher than those of NPNL women during the time of weaning the child from the breast (Prentice et al., 1998). The Gumba study did not quantify calcium intakes, hence unable to determine whether there were differences in calcium intakes between the groups, but it is unlikely that the group differences in PTH reflect the observed modest differences in consumption of calcium rich foods (esp. small fish eaten with bones). In addition, the HIV+ mothers were mostly exclusively breastfeeding at PP14. It is therefore likely that the higher PTH concentrations in HIV+/ART mothers at P36 and PP14 reflect a response to the HIV-infection and/or ART than to differences in calcium intake or in the intensity of breastfeeding.

PTH increased in both groups between PG36 and PP14, but the changes were not significantly different between the groups ( $+60.0 \pm 6.4\%$  vs  $+57.6 \pm 6.4\%$ ,  $p=0.8$ ). Most evidence shows that PTH is either unchanged or decreased during pregnancy (Olausson et al., 2012). Postpartum, PTH is lower in breastfeeding compared to non-breastfeeding mothers at the same time point. A decrease in PTH has been observed within the first 3 months of lactation followed by an increase in the later stages of lactation and after cessation of breastfeeding (Affinito et al., 1996, Krebs et al., 1997, Prentice et al., 1998). The increases in both PTH and  $1,25(\text{OH})_2\text{D}$  in late lactation and after breastfeeding stops are thought to play a role in the recovery of bone mineral. However, early postpartum changes in the calciotropic hormones (PTH,  $1,25(\text{OH})_2\text{D}$  and calcitonin) are not associated with changes in maternal bone mineral, bone turnover markers or breast milk calcium; suggesting that these hormones do not play a major role in breastmilk calcium homeostasis in early lactation. It is thought that PTH related protein (PTHrP) takes over the role of PTH in calcium homeostasis during pregnancy and early lactation (Prentice et al., 1998, Kovacs, 2016).

PTHrP is produced by the mammary glands and released into the maternal blood and breastmilk. It can activate both the PTH/PTHrP receptors and renal  $1,25(\text{OH})_2\text{D}$  production. Greater postpartum decreases in maternal bone mineral at both the lumbar spine and the femoral neck in women with higher PTHrP concentrations have been observed in early lactation (Dobnig *et al.*, 1995) However, More et al. did not observe an association between PTHrP and maternal bone mineral in women with established lactation (More et al., 2003), probably because PTHrP concentration in plasma is higher at delivery, decreases gradually in lactation, and is undetectable by 6 months postpartum.

The role of PTHrP in human pregnancy and lactation is not fully understood because its biology is complex and the laboratory assays pose several methodological challenges. PTHrP is rapidly broken down and degraded in serum (Hutchesson *et al.*, 1994, Olausson et al., 2012). Therefore, the samples should be collected in chilled EDTA tubes containing a protease inhibitor then kept chilled on ice, centrifuged at low temperature and frozen within 15 minutes after collection. Even with such a robust protocol, some of the PTHrP is still degraded (Hutchesson et al., 1994). The Gumba study did not measure PTHrP and also did not collect suitable samples for the assay.

The observed higher PTH concentrations in HIV-positive women in the Gumba study is likely to be an effect of ART. However,  $1,25(\text{OH})_2\text{D}$ , FGF-23, and other hormones/cytokines involved in modifying bone turnover, including prolactin and other lactation hormones were not measured. It would be interesting to measure these in the future to better understand the mechanisms underlying bone loss in HIV-infected women following initiation of TDF-based ART during pregnancy.



#### 9.2.4. Maternal plasma and urine clinical biochemistry

Overall, mean albumin-corrected plasma calcium (P-Ca) reduced, and TmCa/GFR increased in both groups between PG36 and PP14. However, HIV-positive women had a significantly greater reduction in corrected P-Ca ( $-6.6 \pm 0.5\%$  vs  $-3.8 \pm 0.5\%$ ,  $p \leq 0.0001$ ) and a smaller increase in TmCa/GFR compared to HIV-negative women. Also, HIV positive women had significantly lower corrected P-Ca concentrations and TmCa/GFR compared to HIV-negative women at both 36 weeks of gestation and 14 weeks of lactation. This profile suggests greater renal excretion of calcium in HIV-positive women leading to a fall in plasma calcium. The low plasma calcium is likely the driver for the observed higher PTH and greater mobilisation of bone mineral in HIV-positive women to maintain plasma calcium concentrations within the normal range.

Plasma phosphate increased in both groups. HIV-positive women had greater mean increases compared to HIV-negative women ( $+14.4 \pm 2.0\%$  vs  $+7.7 \pm 2.0\%$ ,  $p = 0.02$ ) such that the significantly lower plasma phosphate concentration at PG36 ( $-8.6 \pm 2.1\%$ ,  $p \leq 0.0001$ ) was no longer different to the HIV negative women at PP14 ( $-3.0 \pm 2.3\%$ ,  $p = 0.2$ ). TmP/GFR increased in both groups but was significantly lower in HIV-positive women at both PG36 ( $-11.4 \pm 3.1\%$ ,  $p \leq 0.0001$ ) and PP14 ( $-7.2 \pm 30\%$ ,  $p = 0.02$ ). The mean urine phosphate/creatinine ratio (UP/Cr) decreased in both groups, and the decrease in HIV-positive women was smaller although it was not significantly different between the groups ( $-22.6 \pm 8.7\%$  vs  $-32.1 \pm 8.7\%$ ,  $p = 0.4$ ). Also, mean UP/Cr was higher in HIV-positive women at PP14, but not at PG36, although the difference between the groups was not significant ( $+15.1 \pm 11.0$ ,  $p = 0.2$ ). These data suggest renal phosphate wasting in HIV-positive women, probably a consequence of greater mobilisation of bone mineral to maintain plasma calcium concentrations within the normal range (further discussed in section 9.2.5).

Estimated glomerular filtration rate (eGFR) in healthy women increased in lactation from late pregnancy in both HIV-positive and HIV-negative women in the Gumba study, consistent with existing literature (Sims and Krantz, 1958). However, HIV-positive women had a significantly greater decline in eGFR (estimated by CKD-EPI equation) among HIV-positive women compared to HIV-negative women ( $-13.6 \pm 1.1\%$  vs  $-10.2 \pm 1.1\%$ ,  $p = 0.04$ ). These data suggest early onset and gradual decline in renal function accompanied by greater renal excretion of calcium in HIV-infected women, probably a consequence of TDF-based ART. TDF is associated with renal tubular toxicities leading to increased renal phosphate wasting (Kinai and Hanabusa, 2005, Moyle et al., 2013, Hamzah et al., 2015). A cross-sectional study conducted in Uganda did not observe differences in renal function between TDF-exposed and TDF-unexposed HIV-positive patients (mean duration on ART was 9.3years) (Salome et al., 2016).

Baseline renal function was not formally assessed before initiation of TDF-based ART in the Gumba study. None of the participants had proteinuria +++ at enrolment. A study conducted in Malawi reported a very low prevalence of pre-existing CKD in HIV-infected pregnant women at the initiation of TDF-based ART and recommended that baseline assessment of renal function was not necessary for the roll-out of Option B+ guidelines (initiating TDF-based ART in pregnant and lactating women) (Johnson *et al.*, 2012). Thus, the observed greater decline in renal function in HIV-positive vs HIV-negative mothers in the Gumba study could be a consequence of TDF-based ART.

#### 9.2.5. Breast milk mineral content

Mean breast milk Ca concentrations were not significantly different between the groups. However, HIV-positive Ugandan women had 9-10% significantly higher breast milk P at both PP2 and PP14 and lower calcium/phosphate ratio at PP2 (PP2:  $-7.7 \pm 3.2\%$ ,  $p=0.02$ ; PP14:  $-3.5 \pm 3.1\%$ ,  $p=0.3$ ). [BM-P] was significantly correlated with maternal plasma CTX concentration ( $\beta = +0.13 \pm 0.04\%$  per 1% increase in CTX,  $p=0.0003$ ). To my best knowledge, Gumba is the first study to compare breastmilk calcium and phosphorus concentrations between HIV-positive and HIV-negative women.

In the Gumba study, breastmilk calcium concentrations significantly decreased between PP2 and PP14 in both groups. These data are consistent with a previous study that reported a gradual decrease in breastmilk calcium within the first 6 months of lactation; and also lower mean breastmilk calcium concentrations in Gambian compared to British mothers (Laskey 1990). In the same study, a significant decrease in breastmilk phosphorus was observed in British women, but concentrations remained constant in Gambian women. The Gumba study did not observe significant changes in breastmilk phosphorus in either HIV-positive or HIV-negative Ugandan women. The changes in breastmilk phosphorus in Ugandans are consistent with Gambian data, and suggest ethnic differences in breastmilk phosphorus between African and Caucasian mothers. However, changes in breastmilk calcium/phosphorus ratio were not significantly different between HIV-positive and HIV-negative women in the Gumba study.

The finding of higher mean breastmilk phosphorus concentration in HIV-positive vs HIV-negative women in the Gumba study is intriguing. These data suggest that HIV-positive women transferred more phosphorus into breastmilk in addition to the greater urinary excretion, possibly to maintain plasma phosphate concentrations within the normal range. However, the transfer of phosphorus into breast milk does not seem to be accompanied by a matching increase in the transfer of calcium into breastmilk. In fact, breastmilk calcium concentrations were higher in HIV positive women at both PP2 and PP14, although the differences between the groups were not significant. The greater

influx of phosphorus into breastmilk suggests alterations in ionic breastmilk fractions and possibly different phosphate species in HIV-positive women, and is likely to be an effect of ART.

Infection of the mammary glands, especially sub-clinical mastitis defined by elevated breast milk sodium concentrations and a sodium/potassium ratio  $>1$ , can activate paracellular transfer of phosphate into breast milk which could also lead to elevated breastmilk phosphorus concentrations. Breastmilk sodium and potassium concentrations were measured to investigate subclinical mastitis. The prevalence of sodium/potassium ratio  $>1$  was 13% at PP2 and 0.5% at PP14; and not significantly different between the groups. Mean breast milk sodium and potassium concentrations significantly decreased in both groups consistent with previous studies (Neville *et al.*, 1991). Changes in mean breastmilk potassium and sodium/potassium ratio were not significantly different between the groups. However, HIV-positive women had a significantly smaller decrease in breast milk Na ( $-44.3 \pm 8.9\%$  vs  $-72.6 \pm 9.0\%$ ,  $p=0.03$ ). They also had a trend towards a smaller decrease in Na/K ratio ( $-22.2 \pm 9.3\%$ , vs  $-46.6 \pm 9.5\%$ ,  $p=0.07$ ) suggesting a greater (but mild) burden of sub-clinical mastitis in HIV-positive women at PP14 consistent with data from Zambia (Kasonka *et al.*, 2006). Even so, sub-clinical mastitis is unlikely to explain the higher breastmilk phosphorus in HIV-positive women as the mean concentrations were already elevated by 2 weeks postpartum (without significant differences in breastmilk sodium or sodium/potassium ratio).

PTHrP is present in breastmilk in large quantities and is thought to play a role in regulating mammary gland secretion of calcium into breast milk. Studies have reported significant associations between the C-terminal fragment of PTHrP and breastmilk calcium concentrations in both bovine and human milk (Law *et al.*, 1991, Seki *et al.*, 1997, Uemura *et al.*, 1997). Immunoreactive calcitonin, another calciotropic hormone found in human milk, has also been reported to be significantly associated with total calcium in breastmilk (Arver *et al.*, 1984). However, it is unknown if these hormones also play a role in the secretion of breastmilk phosphorus. Collection of breastmilk samples and laboratory assays for PTHrP in breastmilk are still complex, thus similar to blood samples, breastmilk samples collected in the Gumba study are not likely to be suitable for PTHrP assay.

Overall, literature is scanty on the regulation of phosphate secretion in breastmilk. Phosphate, citrate and bicarbonate are the major ions that bind a significant amount of calcium (Neville, 2005). The human casein molecule is not fully phosphorylated compared to bovine milk, and the calcium binding capacity of human milk is only about 14 mol/mol of casein (Neville, 2005, Kent *et al.*, 2009). Replacement of citrate by phosphate ions in the aqueous phase of breastmilk has been proposed as a possible mechanism to accommodate the greater influx of phosphate, without a matching increase in calcium concentration in breastmilk of HIV-infected women in the Gumba study (Profs Malcolm Peaker and Ann Prentice, personal communications).

Thus, analysis of citrate and possibly bicarbonate in stored breast milk samples from the Gumba study could be explored in the future to investigate this. At the moment, the mechanisms behind the elevated breastmilk phosphorus in HIV-infected Ugandan women and the implications on their breastfed infants are unknown.

#### *9.2.5. Infant anthropometry, growth and body composition*

HIV exposed infants (HEI) had significantly lower gains in weight ( $+53.0 \pm 1.4\%$  vs  $+57.5 \pm 1.4\%$ ,  $p=0.03$ ) and length ( $+16.1 \pm 0.4\%$  vs  $+17.3 \pm 0.5\%$ ,  $p=0.05$ ) between 2 and 14 weeks of age compared to HIV-unexposed infants (HUI). Thus, they had significantly lower weight ( $-5.9 \pm 2.1\%$ ,  $p=0.004$ ), length ( $-2.0 \pm 0.7\%$ ,  $p=0.002$ ), WAZ ( $-0.47 \pm 0.16$ ,  $p=0.003$ ) and LAZ ( $-0.53 \pm 0.18$ ,  $p=0.005$ ) at PP14. These results suggest differences in growth at 3 months of age in breastfeeding HIV-exposed infants born to mothers initiated on Option B+ ART during pregnancy.

These findings are consistent with other studies which have reported poorer growth in HIV-exposed infants in sub-Saharan Africa. Significantly lower birth LAZ, WAZ, and WLZ were observed among infants exposed to maternal ART compared to zidovudine monotherapy in Botswana and, LAZ were significantly lower during the first 6 months of life (Powis *et al.*, 2011, Powis *et al.*, 2016). In the Zimbabwe Vitamin A for Mothers and Babies (ZVITAMBO) trial, differences in growth (LAZ and WAZ) between HEI and HUI peaked at 6 weeks of age and remained lower throughout the first 2 years of life. Also, HEI had significantly lower HCAZ in the first year of life. In the same study, lower WAZ and WLZ were associated with lower maternal education (primary vs A level) despite high levels of education in the study population (Evans *et al.*, 2016).

Poorer growth has also been observed in Ugandan HEI exposed to maternal ART. Ugandan HEI in the maternal ART arm in the PROMISE study had significantly lower anthropometric mean Z-scores (weight, length and head circumference) at 12 and 24 months compared to a control group of HUI recruited from well-baby clinics, but the differences were not observed in Malawi (Aizire *et al.*, 2016). Another study observed a non-significant trend towards higher rates of stunting among HEI compared to HUI at 12 months of age [12-month adjusted odds ratio (OR) 1.55, 95% CI 0.92–2.61] (Muhangi *et al.*, 2013a). However, the DART trial did not observe significant differences in growth in Ugandan and Zimbabwean children as discussed in section 9.1.

Infant feeding practices greatly influence growth, hence these should be considered when comparing the growth of HEI vs HUI in all settings. The rate of growth in exclusively breastfed babies within the first 6 months of age is slower compared to formula fed babies (Agostoni *et al.*, 1999, Ziegler, 2006). Also, both non-breastfed and mixed-fed babies tend to have poorer growth compared to exclusively breastfed babies especially in resource limited settings.

A clinical trial conducted in Uganda observed a more than six fold lower mortality in HEI breastfed for longer than 6 months compared to those breastfed for less than 6 months (Homsy *et al.*, 2010). The current national guidelines in Uganda recommend exclusive breastfeeding for HEI in the first 6 months of life and continued breastfeeding (complementary feeding) up to 1 year of age. In the Gumba study, more HIV-positive women reported exclusive breastfeeding compared to negative women at both PP2 and PP14. Adjusting for differences in breastfeeding practices and history of illness did not attenuate the observed differences in growth at PP14.

Earlier studies conducted in the pre-Option B+ ART era reported a greater risk of MTCT in mixed feeding compared to exclusively breastfeeding infants. Subclinical mastitis was more prevalent in mixed feeding mothers and was associated with greater risk of MTCT and poor infant growth. However, the mechanisms by which exclusive breastfeeding protects against MTCT remain elusive (Filteau, 2010, Lunney *et al.*, 2010). Evidence shows that HIV-infected women are more likely to exclusively breastfeed compared to HIV-negative women (Oiye *et al.*, 2017), probably because they are motivated by the need to protect their babies from HIV-transmission. Thus, the observed differences in growth could be either a consequence of exposure to maternal HIV/ART or differences in breastmilk and dietary intakes.

Caution should be exercised when comparing growth between HIV-exposed infants and HIV-unexposed infants, especially in breastfeeding populations, in the absence of objective measurements of breastfeeding practices. Self-reports greatly over-estimate exclusive breastfeeding rates compared to deuterium dose-to-the-mother method (DTTM). In Kenya, a huge overestimation of exclusive breastfeeding rates was recently reported in both HIV-positive (94.1% vs 23.5% at 6 weeks postpartum and 75.0% vs 43.3% at 6 months postpartum) and HIV negative women (76.9% vs. 13.8% at 6 weeks postpartum and 59.7% vs. 24.2% at 6 months postpartum) (Oiye *et al.*, 2017). The majority of recent studies conducted on the effects of maternal ART on infant growth have not accounted for potential differences in infant feeding practices either by self-reports or DTTM.

Apart from feeding practices, several hypotheses have been proposed to explain the poorer growth in HEI compared to HUI. Congenital cytomegalovirus (CMV) infection is associated with poor fetal growth. In Zambian HEI and HUI, CMV seropositivity was associated with stunting at 18 months of age and smaller head circumference and lower psychomotor development in HEI, suggesting that the effects of CMV infection might be more pronounced in HEI (Gompels et al., 2012). There is some evidence that HIV-infection can distort the maternal microbiota leading to abnormal assembly of the gut microbiota in HEI, thus predisposing HEI to subclinical intestinal damage and inflammation, causing growth failure (Funkhouser and Bordenstein, 2013, Lozupone *et al.*, 2013, Monaco et al., 2016). Furthermore, higher levels of systemic inflammation in the mother might lead to reduced insulin-like growth factor 1 (IGF-1) and consequently growth failure in HEI (Prendergast et al., 2014). However, more data are needed to test these hypotheses in HEI.

Overall, data are inconsistent on the effects of maternal HIV/ART exposure on infant growth compared to HIV/ART unexposed infants; and this is probably due to differences in other environmental, health and cultural factors that may impact on infant growth but have not been adequately controlled for in existing studies. In the Gumba study, effort was made to control for breastfeeding practices and health status, but objective measurements of infant breastmilk intake were not done.

#### 9.2.6. Infant bone mineral

HEI had a significantly slower increase in WB BMC between PP2 and PP14 ( $+51.2 \pm 1.9\%$  vs  $+57.3 \pm 1.9\%$ ,  $p=0.02$ ) compared to HUI, but the difference was not significant after size-adjustment. Also, there was a trend towards lower WB BMC in HEI at PP14, but the difference between the groups was not significant. In contrast there was a greater increase in LS bone mineral in HEI than HUI (BMC:  $+29.5 \pm 1.7\%$  vs  $+24.4 \pm 1.7\%$ ,  $p=0.03$ ; aBMD:  $+3.0 \pm 1.4\%$  vs  $-1.1 \pm 1.5\%$ ,  $p=0.02$ ; and SA-BMC:  $+11.4 \pm 5.4\%$  vs  $+6.5 \pm 5.8\%$ ,  $p=0.02$ ). LS BMC and BA were significantly lower in HEI compared to HUI ( $-9.5 \pm 2.6\%$ ,  $p \leq 0.0001$ ) at PP2, but not at PP14 ( $-3.3 \pm 2.8\%$ ,  $p=0.2$ ). Overall, these data suggest slower WB bone mineral accretion consistent with a slower increase in bone area and body weight, although BMC was proportional to attained body weight and size.

To my best knowledge, Gumba is the first study to compare bone mineral accretion between HIV/ART-exposed uninfected and HIV/ART unexposed infants within the first 3 months of life. At the time of writing this thesis, there were only two studies using DXA (both published by Siberry et al.) that have reported infant bone outcomes within the neonatal period, (discussed in section 9.1). At CROI 2016, Siberry et al. reported lower infant WB BMC in African neonates exposed to maternal ART (Option B) compared to neonates on Option A, and there was no significant difference in BMC between TDF exposed and TDF-unexposed infants (Siberry et al., 2016).

Previously, the same investigators reported a 12% lower mean whole body BMC in American neonates with in-utero exposure to maternal TDF compared to TDF unexposed infants, and the lower BMC persisted after adjustment for body weight and length (Siberry *et al.*, 2015). To circumvent the limitations of using DXA in children, it is recommended to adjust BMC for bone area, body weight and length using multiple regression models as discussed in section 9.2.1.3. However, both the studies by Siberry *et al.* did not adjust for bone area. In the Gumba study, the differences in infant WB BMC between the groups was no longer significant after adjustment for bone area, body weight and length.

These data suggest altered bone mineral deposition within the first 3 months of life in infants exposed to maternal ART consistent with the observed slower growth, but the BMC is proportional to attained body weight and size. The mechanisms and clinical implications of these findings are unknown. Children with growth hormone deficiency have been reported to be stunted but have normal bone mineral for their muscle force (Högler *et al.*, 2003). Biochemical markers of bone metabolism and growth, dietary intake were not measured in the Gumba study. The stored biological samples in the Gumba study could be analysed in the future to investigate the mechanisms behind the observed lower bone mineral accretion in HEI vs. HUI. Therefore, it is important to continue following-up these Gumba infants to investigate longer-term implications for their bone health.

#### 9.2.7. *Pregnancy outcomes and infant health*

Gestation age at delivery was not significantly different between HIV-positive and HIV-negative women. However, fewer cases of stillbirths (1 vs 4), pre-term deliveries (1 vs 3) and neonatal deaths (1 vs 3) were observed in HIV-positive compared to HIV-negative women. Also, significantly fewer HEI had a history of previous illness at PP2 (9.5% vs. 22.1%,  $p=0.03$ ) and PP14 (32.5%, vs. 50.7%,  $p=0.021$ ). These results are contrary to studies that have reported poorer pregnancy outcomes among HIV-infected women on various ART regimens (Chen *et al.*, 2012, Short and Taylor, 2014). The observed better pregnancy and infant health outcomes in the HIV-positive group probably reflect differences in access to medical services between the groups.

As per the set-up at Mulago Hospital, group education sessions were delivered to all women at the beginning of the antenatal clinic. In addition to these routine ANC services, HIV-positive women attended a monthly PMTCT clinic where they received free specialised comprehensive clinical care and intensive one-one counselling sessions. Also, it is likely that fewer cases of poor pregnancy outcomes and neonatal deaths occurred in the HIV-positive group because the majority were multigravidae. Thus they might have been more aware of danger signs in pregnancy and the newborn compared to HIV-negative women.

From 6 weeks of age, HIV-exposed infants attended a specialised monthly EID clinic where they received free clinical monitoring by doctors, hence were likely to receive prompt diagnosis and treatment. In case of illness between visits, the babies were brought into the clinic and referred for free medical treatment. Also, HEI received cotrimoxazole prophylaxis from 6 weeks of age. There is evidence that cotrimoxazole prophylaxis combined with the use of insecticide-treated bed nets reduces the incidence of malaria, a major cause of infant morbidity and mortality in infants in Uganda (Kamya *et al.*, 2007)

Also, HIV-negative women and their babies attended the routine immunisation clinic managed by midwives, and in case of illness they received the standard free medical services offered by the hospital after waiting for long hours and in most instances they were expected to purchase their medication. Thus, the Gumba study PI negotiated with Baylor-Uganda for free medical consultation for sick HIV-negative mothers and their babies and the study provided prescribed medication to reduce these disparities in access to medical care.



## **9.2. Strengths of the Gumba study**

Gumba study is the first to compare changes in maternal bone mineral, infant growth and bone mineral accretion within the first 3 months postpartum, around peak lactation, when calcium requirements are highest. Unlike previous studies, the study has a contemporaneous group of HIV-negative mother-baby pairs for comparison of both cross-sectional and longitudinal outcomes. It also used DXA, which is the gold standard bone imaging technique.

Gumba is also the first study to report extensive data on bone turnover markers and calciotropic hormones during pregnancy and lactation in HIV-infected women on ART. The study followed mother-baby pairs hence is able to investigate the interrelationships between maternal and infant outcomes. Extensive data were also collected on health, diet, breastfeeding and lifestyle factors to adjust for potential confounding and to help explain the results. Unlike previous studies, the current study adjusted both maternal and infant outcomes for breastfeeding practices.

All HIV-infected women were on one ART regimen (TDF/3TC/EFV), the current recommended first-line ART for pregnant and lactation women and reported good adherence based on pill counts. ART was provided according to the prevailing national guidelines in Uganda, which mirrors the Option B+ strategy that is currently being scaled-up in Sub-Saharan Africa. Therefore, the findings presented in this thesis significantly contribute to our understanding of bone health in HIV-infected women in Uganda and their babies from a clinical perspective.

### 9.3. Limitations of the Gumba study

This was an observational study. Although effort was made to adjust for potential confounders, it can only be speculated that ART may be responsible for the observed differences between the groups. A randomised clinical trial design was not feasible because only HIV-infected pregnant women received ART, and also a single first-line triple ART regimen comprising of TDF-3TC-EFV was recommended for PMTCT.

It would have been desirable to study a control group of HIV-infected pregnant women not receiving ART to isolate the effects of maternal ART from the effects of HIV infection, but such a study would have been unethical under the prevailing Option B-plus guidelines in Uganda. Also, a case-control design was not feasible within the time allocated for fieldwork (controlled for age and parity) as it would have taken a longer duration to recruit participants. The preferred approach was to recruit both HIV-infected and HIV-negative pregnant women as they presented at the antenatal clinic based on a set inclusion and exclusion criteria (discussed in section 3.2.7) and later control for differences in age and parity during data analysis.

There were no baseline measurements either before pregnancy or initiation of ART. A longitudinal observational design with DXA scans acquired pre-pregnancy would have been ideal, but such a study was not feasible within the PhD timelines. It would also have been desirable to acquire baseline BMD scans at the time of ART initiation in HIV-infected women. However, DXA scans (spine, hip and whole body BMD measurements) are not currently recommended during pregnancy because they are unable to differentiate between maternal and fetal bone. Other bone imaging techniques which can be safely used in pregnancy and are not affected by inclusion of fetal tissues (e.g pQCT), were not available in Uganda. Therefore, DXA scans were acquired postpartum similar to the majority of previous studies on changes in bone mineral during lactation.

There were no measurements at delivery to compare growth outcomes. Consequently, data on birth weight was obtained from the labour ward discharge forms for some of the participants who delivered at Mulago hospital, and the measurements were not collected using a standardised procedure. The first study measurements were done at 2 weeks postpartum. In the initial study design that was presented in my first-year report, the plan was to collect cord blood (both maternal and infant side) and also take infant anthropometric measurements at delivery. These investigations were intended to provide an extensive comparison of maternal and infant outcomes between the groups. Conducting a study visit at delivery required several logistics especially transport facilitation for all women in labour to ensure they delivered at the health facility, 24-hour availability of staff on the labour ward to conduct deliveries and collect cord, and cooling facilities for storage of cord blood

in the maternity ward and at night. According to prevailing standards of care, this aspect of the study would have needed to hire at least two obstetricians and two midwives to work in shifts on the labour ward to attend to study participants. Thus, the visit was not implemented after careful consideration of study logistics, feasibility and funding.

Biological samples were not collected at 2 weeks postpartum to match the DXA data. Baseline biochemistry data were collected at 36 weeks of gestation, so these might not reflect the change between 2 and 14 weeks postpartum. The initial study protocol had planned to collect cord blood at birth so collecting more blood samples from the mothers at 2 weeks was not thought necessary. In hindsight, it would have been useful to collect biological samples at 2 weeks after the decision was made not to collect cord blood.

Information on breastfeeding practices was collected based on self-reports hence it might be subject to recall bias and over-estimation. Deuterium Dose-To-the-Mother (DDTM) procedures were also planned and approved at 2 and 10 weeks postpartum, to assess maternal breastmilk output and exclusivity of breastfeeding. Altered maternal breastmilk output in HIV-infected women could potentially affect growth in their children as discussed in section 9.2.5. Regrettably, it was finally decided not to include DDTM in Gumba as it would have overburdened participants and overstretched study staff, compromising the main study.

Results from the Gumba study suggest declining renal function in HIV-positive women. However, markers of proximal tubule damage (i.e. RBP, beta-2 microglobulin) were not measured at baseline to rule out pre-existing renal disease. Urine protein and glucose concentrations were used as proxy markers of renal function and participants with abnormal values at enrolment were excluded from the study. Also, viral load test results were not available to verify the reported good compliance to ART. Even so, it is plausible to speculate that the observed higher plasma creatinine concentrations and altered renal mineral handling in HIV-infected women at PP14 might be due to ART-induced renal tubular damage.

The calcium intakes during pregnancy and lactation were not quantified. The Gumba study observed higher consumption of small fish (eaten with bones) among HIV-positive compared to HIV-negative women based on a qualitative calcium food frequency questionnaire which has not been validated in Uganda. Also, there were differences between the groups in consumption of other local foods, non-food/herbs (especially cooked clay and emubwa) which might contain hidden sources of calcium. Other macro- and micronutrients also play a role in maintaining bone health, but they were not assessed due to logistical challenges, costs, and participant burden.

Overall, the statistical analysis focussed on groups so the results cannot be interpreted at the individual level. The data presented in these study were collected in Kampala Uganda hence it might not be generalizable to other regions of Uganda or other countries.

#### **9.4. Future directions**

Follow-up of participants is desirable to investigate if the observed greater decreases in bone mineral in HIV-infected women are permanent, and if the differences in infant bone mineral and growth persist. Data have been collected at 26, 52 and 78 weeks postpartum, and 14 weeks after cessation of breastfeeding (NPNL); but it is outside the scope of this thesis.

Further laboratory analysis of stored maternal blood and urine samples to investigate mechanisms behind the greater bone loss in HIV-infected women, especially  $1,25(\text{OH})_2\text{D}$ , lactation hormones and markers of renal proximal tubal damage are planned if funding allows. It would also be useful to analyse the stored infant serum samples to investigate markers of Ca-P-vitamin D, bone metabolism and growth (especially IGF-1 and BP-3) following exposure to maternal ART during pregnancy and lactation.

Subject to availability of adequate sample volumes and funding for laboratory work, screening both maternal and infant blood samples for CMV infection might also provide more insight into the mechanisms behind the observed slower growth and bone mineral accretion in maternal HIV/ART-exposed infants. As discussed earlier, altered infant microbiome might also affect growth. However, the Gumba study did not collect suitable samples; this could be considered in the future when designing studies involving primary data collection.

Quantitative estimates of dietary intakes using robust methodologies like the validated 24-hour or weighed food records will provide a better insight on calcium intakes. The weighed food records are labour intensive and might be difficult to implement in an urban setting where most women work outside their homes. Therefore, validation of the FAO standard 21 food group FFQ in estimating calcium intakes in Uganda would provide a simple and rapid tool for estimating calcium intakes with minimum participant burden especially in urban areas like Kampala.

Infant breastmilk intake is a major determinant of maternal bone mobilisation and infant growth and might potentially help explain the greater mobilisation of bone mineral in HIV-positive women and slower growth/bone mineral accretion in their babies. Therefore, it would be useful to compare infant breastmilk intake between HIV-positive and HIV-negative women within the study setting in Kampala. Deuterium oxide and supplies are available in Uganda. I am exploring funding opportunities to cover fieldwork and laboratory costs for a new study.

## 9.5. Summary

This thesis has reported several key findings which significantly enhance our understanding of bone health in HIV-positive mothers on TDF-based ART and their babies under the current WHO PMTCT Option B+ PMTCT strategy.

Firstly, HIV-infected Ugandan women initiated on TDF-based ART during pregnancy experienced greater reductions in total hip aBMD compared to HIV-negative women between 2 and 14 weeks postpartum ( $-4.0 \pm 0.4\%$  vs  $-2.7 \pm 0.4\%$ ,  $p = 0.04$ ), which persisted after size adjustment ( $-3.7 \pm 0.3\%$  vs  $-2.7 \pm 0.3\%$ ,  $p = 0.04$ ), suggesting a greater vulnerability of the total hip vs. the lumbar spine to the effects of HIV and ART on bone.

Second, HIV-positive women also had greater increases in plasma phosphate concentration, markers of bone formation (P1NP and BAP) and resorption (CTX) suggesting higher bone turnover, consistent with the increased mobilisation of bone mineral. They also had higher PTH, plasma calcium and phosphate concentrations, TmP/GFR and TmCa/GFR both at PG36 and PP14, and a greater reduction in eGFR compared to HIV-negative women. Also, HIV-infected women had significantly higher breastmilk phosphorus concentrations at both PP2 and PP14, but breastmilk calcium concentrations were not significantly different between the groups. These biochemistry and breastmilk mineral composition profiles suggest a disruption in calcium and phosphate metabolism in HIV-infected women on ART leading to a greater influx of phosphate from plasma into breastmilk.

Third, HIV-exposed infants had significantly lower somatic growth and whole body bone mineral accretion by 14 weeks of age compared to HIV-unexposed infants; but their bone mineral was adequate for attained body weight and size. All infants were breastfed in the first three months of life, but a greater proportion of HIV-exposed infants were exclusively breastfed compared to HUI at both PP2 and PP14. The observed slower growth of HIV/ART-exposed infants (HEI) compared to HUI might be a consequence of exposure to maternal HIV-infection/ART or simply reflects more rapid growth in HUI.

Overall, these data suggest early disruptions in maternal plasma and breastmilk Ca-P metabolism accompanied by higher bone turnover and bone loss in HIV-infected mothers, and slower growth in their babies by 3 months postpartum. It is likely that the observed greater bone loss and disruptions in bone mineral metabolism are consequences of TDF-based ART initiation during pregnancy under PMTCT Option B+. However, the study had a relatively short duration of follow-up and the clinical implications of these findings are unclear. Follow-up studies are needed to provide insights into the mechanisms and longer-term implications for bone health in both the mothers and their babies.

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## APPENDICES

### Appendix 1: Ethics approval letters



JCRC IRB/REC Chair,  
Plot 101 Upper Lubowa Estates  
P O Box 10005,  
Kampala  
Uganda

20 March 2014

Dear Sir

**Ms Florence Nabwire - Effects of antiretroviral therapy during pregnancy and lactation on the bone health of urban Ugandan women, and on infant growth**

The detailed proposals for this project have been peer reviewed by the HNR Research Review Board (RRB), of which I am chair, ahead of submission to JCRC. The RRB review is overseen by a lead senior scientist (Dr Jonathan Powell in this case) and constitutes two scientists, a biostatistician and Head of Operations and Governance who all provide written comments. All reviewers are outside the research group involved. In summary in relation to this project, the protocol was very well prepared and the review raised no substantive scientific issues. A number of suggestions to improve clarity and some logistical considerations were raised. I can confirm these have been addressed in the submitted final protocol version, and/or will be put in place at HNR.

I also confirm that HNR will fulfil its responsibilities as sponsor in accordance with the Research Governance Framework for the management and monitoring of this project to ensure that the research is of high quality and that any risks are effectively identified and managed. Research monitoring is overseen by the HNR Research Review Board, which reports to the Unit Director. Research is conducted according to MRC Good Research Practice, and in line with GCP where necessary and appropriate, and all necessary arrangements will be made to protect the safety and well-being of research participants in conjunction with Collaborators and the Collaborative Agreements being drafted.

HNR has an established Quality Management System (QMS) maintained to control and monitor performance and continually improve effectiveness. This is certified to ISO 9001:2008. As part of the QMS, HNR has an internal audit programme and is externally audited under the requirements of ISO every 6 months.

I hope this provides the information you need, however should you need anything further please don't hesitate to contact me.

Yours faithfully

A handwritten signature in black ink, appearing to read 'Polly Page', written over a horizontal line.

Polly Page  
HNR Research Review Board Chair  
Head of Operations and Governance  
direct line: 01223 437584  
direct fax: 01223 437515  
email: [Polly.page@mrc-hnr.cam.ac.uk](mailto:Polly.page@mrc-hnr.cam.ac.uk)



# Joint Clinical Research Centre

*Vision: A Vibrant Self Sustaining Centre of Excellence in Medical Research, Training and Healthcare Services*

Plot 101 Lubowa, off Entebbe Road  
P.O.Box 10005, Kampala Uganda  
Fax: 256-414- 342632  
Email: [jcrc@jcrc.org.ug](mailto:jcrc@jcrc.org.ug)  
Website: <http://www.jcrc.org.ug>



Telephone: 256-414 - 201148  
201147  
201146

Helpline: 256 - 414 - 273062

## IRB OFFICE

27<sup>th</sup> June 2014

Florence Nabwire  
MRC Human Nutrition Unit  
Elsie Widdowson Laboratory, UK

Dear Florence

RE: PROTOCOL APPROVAL

The protocol, "Effects of antiretroviral therapy during pregnancy and lactation on the bone health of urban Ugandan women, and on infant growth", Version 2 dated 24th June 2014 that you submitted to the JCRC-IRB/REC was reviewed on 26<sup>th</sup> June 2014.

This is to inform you that the Board has approved the study for implementation for a period of one (1) year, with effect from 26<sup>th</sup> June 2014 through 26<sup>th</sup> June 2015. The following documents have been herewith approved;

- a) HNR study no. 6522: GUMBA study protocol Version 2, Dated 24th June, 2014
- b) English Informed Consent Form Version 2, Dated 24th June, 2014

Please note that requests for annual renewal should be submitted to this office at least 4 weeks in advance of expiry, including a request for renewal, annual report and any related publications/abstracts. Note also that you are required to clear your study with the Uganda National Council for Science and Technology before the study can commence. We would like to congratulate you and wish you luck with this important study.

Yours sincerely

  
Jesse Kagimba, M.D., M.Sc.  
Chairman JCRC-IRB/REC  
c.c. Uganda National Council for Science and Technology

JCRC's Mission is to Conduct Quality Medical Research and Training, Provide Equitable and Sustainable HIV/AIDS Care and other Healthcare Services in Uganda and Internationally

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Website: <http://www.jcrc.org.ug>



Telephone: 256-414 - 201148  
201147  
201146

Helpline: 256 - 414 - 273062

## IRB OFFICE

18<sup>th</sup> June 2015

Florence Nabwire  
MRC Human Nutrition Unit  
Elsie Widdowson Laboratory, UK

Dear Florence

RE: RENEWAL APPROVAL

The protocol, "Effects of antiretroviral therapy during pregnancy and lactation on the bone health of urban Ugandan women, and on infant growth," that you submitted to the JCRC-IRB/REC was reviewed on 18<sup>th</sup> June 2015.

This is to inform you that the Board has approved the study for continuation for a period of one (1) year, with effect from 26<sup>th</sup> June 2015 through 25<sup>th</sup> June 2016. The following documents have been herewith approved;

- Progress report, dated 11<sup>th</sup> May 2015
- Study Protocol version 3.0 dated 11<sup>th</sup> May 2015
- English and Luganda Informed Consent Forms, version 3.1 dated 11<sup>th</sup> May 2015
- Study Tools and Questionnaires, version 2.0 dated 11<sup>th</sup> May 2015

Please note that requests for annual renewal should be submitted to this office at least 4 weeks in advance of expiry, including a request for renewal, annual report and any related publications/abstracts. Note also that you are required to clear your study with the Uganda National Council for Science and Technology before the study can commence. We would like to congratulate you and wish you luck with this important study.

Yours sincerely

  
Jesse Kagimba, M.D., M.Sc.  
Chairman JCRC-IRB/REC  
c.c. Uganda National Council for Science and Technology

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Plot 101 Lubowa, off Entebbe Road  
P.O. Box 10005, Kampala Uganda  
Fax: 256-414-342632  
Email: [jrcr@jrcr.org.ug](mailto:jrcr@jrcr.org.ug)  
Website: <http://www.jrcr.org.ug>



Telephone: 256-414 - 201148  
201147  
201146

Helpline: 256 - 414 - 273062

## IRB OFFICE

June 23 2016

Florence Nabwire  
MRC Human Nutrition Unit  
Elsie Widdowson Laboratory, UK

Dear Florence

RE: RENEWAL APPROVAL



The protocol, "Effects of antiretroviral therapy during pregnancy and lactation on the bone health of urban Ugandan women, and on infant growth," that you submitted to the JCRC-IRB/REC was reviewed on June 23 2016.

This is to inform you that the Board has approved the study for continuation for a period of one (1) year, with effect from June 23 2016 through June 25 2017. The following documents have been herewith approved;

- Progress report, dated May 24 2016
- Study Protocol version 4.0 dated May 24 2016

Please note that requests for annual renewal should be submitted to this office at least 4 weeks in advance of expiry, including a request for renewal, annual report and any related publications/abstracts. Note also that you are required to clear your study with the Uganda National Council for Science and Technology before the study can commence. We would like to congratulate you and wish you luck with this important study.

Yours sincerely

Jesse Kagimba, M.D., M.Sc.  
Chairman JCRC-IRB/REC

c.c. Uganda National Council for Science and Technology

# Joint Clinical Research Centre

Vision: A Vibrant Self-Sustaining Centre of Excellence in Medical Research, Training and Healthcare Services

Plot 101 Lubowa, off Entebbe Road  
P.O. Box 10005, Kampala Uganda  
Fax: 256-414-342632  
Email: [jrcr@jrcr.org.ug](mailto:jrcr@jrcr.org.ug)  
Website: <http://www.jrcr.org.ug>



Telephone: 256-414 - 201148  
201147  
201146

Helpline: 256 - 414 - 273062

## IRB OFFICE

June 22 2017

Florence Nabwire  
MRC Human Nutrition Unit  
Elsie Widdowson Laboratory, UK

Dear Florence

RE: RENEWAL APPROVAL



The protocol, "Effects of antiretroviral therapy during pregnancy and lactation on the bone health of urban Ugandan women, and on infant growth," that you submitted to the JCRC-IRB/REC was reviewed on June 22 2017.

This is to inform you that the Board has approved the study for continuation for a period of one (1) year, with effect from June 26 2017 through June 25 2018. The following documents have been herewith approved;

- Study Protocol version 5.0 dated June 15 2017

Please note that requests for annual renewal should be submitted to this office at least 4 weeks in advance of expiry, including a request for renewal, annual report and any related publications/abstracts. Note also that you are required to clear your study with the Uganda National Council for Science and Technology before the study can commence. We would like to congratulate you and wish you luck with this important study.

Yours sincerely

Jesse Kagimba, M.D., M.Sc.  
Chairman JCRC-IRB/REC

c.c. Uganda National Council for Science and Technology

TELEPHONE: +256-41554008/1  
HOSPITAL  
FAX: +256-414-5325591  
E-mail: [admin@mulago.or.ug](mailto:admin@mulago.or.ug)  
Website: [www.mulago.or.ug](http://www.mulago.or.ug)



MULAGO NATIONAL REFERRAL  
P.O. Box 7051  
KAMPALA, UGANDA

IN ANY CORRESPONDENCE ON THIS  
SUBJECT PLEASE QUOTE NO...

THE REPUBLIC OF UGANDA

25<sup>th</sup> Aug. 2014.

Ms. Florence Nabwire  
MRC Human Nutrition Unit  
Elsie Widdows Laboratory, UK.

Dear Nabwire,

RE: ADMINISTRATIVE CLEARANCE: MREC:630: EFFECTS OF  
ANTIRETROVIRAL THERAPY DURING PREGNANCY AND LACTATION ON THE  
BONE HEALTH OF URBAN UGANDAN WOMEN AND ON INFANT GROWTH,  
Version 2 dated 24<sup>th</sup> June 2014.

The Mulago Research and Ethics Committee consider your request and hereby grant  
you permission to carry out the above study up to 26<sup>th</sup> June, 2015.

- (i) The study is subject to monitoring by the Mulago research and ethics  
committee at any time.
- (ii) Remit 1,330,000 Uganda shillings as 10% institutional fee to Mulago hospital.

Attached is the Mulago hospital policy on institutional fees for research projects.

Yours sincerely;

DR. NAKWAGALA FREDERICK NELSON  
CHAIRMAN- MULAGO RESEARCH & ETHICS COMMITTEE

Vision: "To be the leading centre of Health Care Services"

TELEPHONE: +256-41554008/1  
FAX: +256-414-5325591  
E-mail: [admin@mulago.or.ug](mailto:admin@mulago.or.ug)  
Website: [www.mulago.or.ug](http://www.mulago.or.ug)



MULAGO NATIONAL REFERRAL HOSPITAL  
P.O. Box 7051  
KAMPALA, UGANDA

IN ANY CORRESPONDENCE ON THIS  
SUBJECT PLEASE QUOTE NO...

THE REPUBLIC OF UGANDA

10<sup>th</sup> July, 2015.

Ms Florence Nabwire  
Principal Investigator  
MRC Human Nutrition Unit  
Elsie Widdowson Laboratory, UK.

Dear Nabwire,

Re: Protocol Renewal; MREC: 630: "Effects of Antiretroviral Therapy during  
Pregnancy and Lactation on the Bone Health of Urban Ugandan Women and on  
Infant Growth, Version 3.0 11<sup>th</sup> May 2015".

Thank you for your early submission of the above subject. The Mulago Hospital  
Research and Ethics Committee have considered your request for the above and  
hereby grant a renewal approval for the conduct of this study for a period of one year  
from 26<sup>th</sup> June, 2015 to 25<sup>th</sup> June, 2016.

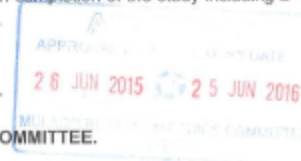
This approval is subject to the following conditions:

1. That the study site may be monitored by the Mulago research and ethics  
committee at any time.
2. That you will abide by the regulations governing research in the country as set by  
the Ugandan National Council for Science and Technology including abiding to  
all reporting requirements for serious adverse events, unanticipated events and  
protocol violations.
3. That no changes to the protocol and study documents will be implemented until  
they are reviewed and approved by the Mulago Research and Ethics Committee.
4. That you provide annual progressive reports and request for renewal of approval  
at least 60 days before expiry of the current approval.
5. That you provide an end of study report upon completion of the study including a  
summary of the results and any publications.

I wish you the best in this Endeavour.

DR. NAKWAGALA FREDERICK NELSON  
CHAIRMAN - MULAGO RESEARCH & ETHICS COMMITTEE.

Vision: "To be the leading centre of Health Care Services"





## Uganda National Council for Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

Our Ref: HS 1688

26<sup>th</sup> November 2014

Dr. Florence Nabwire  
Baylor College of Medicine Children's Foundation-Uganda  
Mulago  
Kampala

**Re: Research Approval:** Effects of antiretroviral therapy during pregnancy and lactation on the bone health of urban Ugandan women and on infant growth

I am pleased to inform you that on 16/09/2014, the Uganda National Council for Science and Technology (UNCST) approved the above referenced research project. The Approval of the research project is for the period of 16/09/2014 to 16/09/2017.

Your research registration number with the UNCST is HS 1688. Please, cite this number in all your future correspondences with UNCST in respect of the above research project.

As Principal Investigator of the research project, you are responsible for fulfilling the following requirements of approval:

1. All co-investigators must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the research protocol or the consent form (where applicable) must be submitted to the designated local Research Ethics Committee (REC) or Lead Agency for re-review and approval prior to the activation of the changes. UNCST must be notified of the approved changes within five working days.
3. For clinical trials, all serious adverse events must be reported promptly to the designated local REC for review with copies to the National Drug Authority.
4. Unanticipated problems involving risks to research subjects/participants or other must be reported promptly to the UNCST. New information that becomes available which could change the risk/benefit ratio must be submitted promptly for UNCST review.
5. Only approved study procedures are to be implemented. The UNCST may conduct impromptu audits of all study records.
6. A progress report must be submitted electronically to UNCST within four weeks after every 12 months. Failure to do so may result in termination of the research project.

Below is a list of documents approved with this application:

	Document Title	Language	Version	Version Date
1	Research proposal	English	2	4 <sup>th</sup> June 2014
2	Participant informed consent forms	English, Luganda	1.0	19 <sup>th</sup> March 2014
3	Study referral and screening form	English	1.0	13 <sup>th</sup> March 2014
4	Clinical chart review form	English	N/A	N/A
5	Questionnaires	English, Luganda	N/A	N/A

Yours sincerely,

  
Leah N Omongo  
for: Executive Secretary  
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

cc: Chair, Joint Clinical Research Center-Research Ethics Committee, Kampala  
cc: Chair, Mulago Hospital-Research Ethics Committee, Kampala

### LOCATION/CORRESPONDENCE

Plot 6 Kintere Road, Ntinda  
P. O. Box 6884  
KAMPALA, UGANDA

### COMMUNICATION

TEL: (256) 414 795580  
FAX: (256) 414-234579  
EMAIL: info@uncst.go.ug  
WEBSITE: <http://www.uncst.go.ug>



## Uganda National Council for Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

Our Ref: HS 1688

30<sup>th</sup> November 2015

Florence Nabwire  
Baylor College of Medicine  
Children's Foundation -Uganda  
Kampala

**RE: APPROVAL OF TRANSFER OF SAMPLES FROM BAYLOR COLLEGE OF MEDICINE CHILDREN'S FOUNDATION -UGANDA TO MEDICAL RESEARCH COUNCIL, UNITED KINGDOM**

We refer to the application dated 27/08/2015 requesting for permission to transfer samples obtained under the research project titled, 'Effects of Antiretroviral therapy during Pregnancy and Lactation on the bone Health of Urban Ugandan Women, and Infant growth: Locally known as GUMBA Study'.

The UNCST on 25/11/2015 approved your application to transfer samples listed below through Entebbe International Airport to Medical Research Council, United Kingdom.

Sample type and source	Number of participants	Number of study visits/ participant	Total volume of sample stored per participant per visit	Total No of unique samples/aliquots to be shipped for analysis
Maternal Blood-serum	200	5	2.0ml (2x1.0ml aliquots)	1000 samples (2000 aliquots)
Maternal blood - Lithium Heparin and EDTA plasma	200	5	8.0ml (LH 4x1.5ml aliquots and EDTA 2x1.0ml aliquots)	1000 samples (6000 aliquots)
Infant blood - Serum	200	4	1.0ml (1x1.0ml aliquots)	800 samples (800 aliquots)
Maternal 2-hour fasted urine	200	5	20.0ml (2X5ml acidified aliquots) (2X5ml non-acidified aliquots)	1000 samples (4000 aliquots)
Maternal blood - cellular materials	200	2	2.0ml (1x1.0ml)	400 (800 aliquots)
Maternal Breast milk	200	3	10.0ml (2x5ml aliquots)	600 (1200 aliquots)

### LOCATION/CORRESPONDENCE

Plot 6 Kintere Road, Ntinda  
P. O. Box 6884  
KAMPALA, UGANDA

### COMMUNICATION

TEL: (256) 414 795580  
FAX: (256) 414-234579  
EMAIL: info@uncst.go.ug  
WEBSITE: <http://www.uncst.go.ug>



## Appendix 2: Referral form



### REFERRAL FORM (GUMBA- F001)

(To be completed by referring health workers at various referral points within ANC)

Date of referral							Study Referral Number: R _ _   _ _   _ _
Mother's Name							ANC No.
Mother's DOB							Age (years)
							EXCL. <18.0 or >39.9y
Address (Exclude if >20km)	District		Parish		Zone/Village		
Mobile Phone Number (s)							
Primary contact/Owner of Phone						Best time to phone	
LMP (dd-mm-YYYY)							Gestation Age (wks)
Estimated EDD							EXCL. <24 or > 28
							G P A
Date of last HIV testing							PMTCT Code
						ART Code	
Received Post-test Counselling?						<input type="checkbox"/> YES <input type="checkbox"/> NO	
If TRR, is the mother initiating HAART during current pregnancy?				<input type="checkbox"/> YES <input type="checkbox"/> NO		If yes, HAART Regimen?	
						EXCL. If not 1 <sup>st</sup> line	
Date of HAART initiation							EXCL. If not initiated during current pregnancy
Latest CD4 count results						Date of results	
Received counselling on infant feeding options, and has chosen or intends to breastfeed for at least 6 months						<input type="checkbox"/> YES <input type="checkbox"/> NO	
Pregnancy currently classified as low risk (as per ANC protocol)						<input type="checkbox"/> YES <input type="checkbox"/> NO	
Explained to the mother baseline information about GUMBA study						<input type="checkbox"/> YES <input type="checkbox"/> NO	
Mother interested in learning more about the GUMBA study						<input type="checkbox"/> YES <input type="checkbox"/> NO	
Date of next ANC appointment							
Date of next PMTCT appointment (if applicable)							
Comments (if any)							
Name of ANC staff making referral:							
Signature and Date:							

## Appendix 3: Screening questionnaire



### SCREENING FORM (GUMBA - F002)

Study Screening ID: S\_\_|\_|\_|

Date: 

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(DD/MM/YYYY)

Study Referral Number: R\_\_|\_|\_|

#### SECTION A: ANTHROPOMETRY

A1. Mid-Upper Arm Circumference (MUAC) 

....	....	....	*	....
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 cm

#### SECTION B: PRIMARY INCLUSION CRITERIA

B1:	Is the potential participant willing to have a study staff review her hospital file?	<input type="checkbox"/> YES <input type="checkbox"/> NO*
B2:	Is the potential participant between 18.00 and 39.99 years old?	<input type="checkbox"/> YES <input type="checkbox"/> NO*
B3:	Is the gestation age < 28 weeks? (Based on fundal height)	<input type="checkbox"/> YES <input type="checkbox"/> NO*
B4:	Is it a singleton pregnancy? (If known)	<input type="checkbox"/> YES <input type="checkbox"/> NO*
B5:	Is the potential participant willing to deliver at Mulago Hospital?	<input type="checkbox"/> YES <input type="checkbox"/> NO*
B6:	Does the potential participant live within a reasonable distance (< 20km) of Mulago hospital?	<input type="checkbox"/> YES <input type="checkbox"/> NO*
B7:	Is the potential participant willing to be visited at home?	<input type="checkbox"/> YES <input type="checkbox"/> NO*
B8:	Does the potential participant plan to breastfeed for at least 6 months?	<input type="checkbox"/> YES <input type="checkbox"/> NO*
B9:	Does the potential participant have recent HIV test results (<3months)?	<input type="checkbox"/> YES <input type="checkbox"/> NO*
B10:	Is the potential participant ART naïve or if HIV* only initiated HAART during current pregnancy? <i>Please ask about use of PEP/PrEP</i>	<input type="checkbox"/> YES <input type="checkbox"/> NO*
B11:	If HIV positive, is the participant on or due to initiate first line HAART?	<input type="checkbox"/> YES <input type="checkbox"/> NO*
<b>*EXCL. If NO to any of statements B1-B11</b>		

B12: Is the participant currently enrolled in another study (s)?  
If Yes, which study ..... ☐ YES ☐ NO  
**(EXCL. IF YES)**

Screening questionnaire, Version 1.1. Dated 13<sup>th</sup> March 2014

Page 1 of 6

QC1: Initials..... Date .....

QC2: Initials..... Date .....



### SCREENING FORM (GUMBA - F002)

Study Screening ID: S\_\_|\_|\_|

#### SECTION C: MEDICAL HISTORY (CHART REVIEW)

B13: Does the potential participant have a known diagnosis of:	<input type="checkbox"/> YES <input type="checkbox"/> NO
a) Renal disease	<input type="checkbox"/> YES <input type="checkbox"/> NO
b) Type 1 or 2 or gestational Diabetes Mellitus	<input type="checkbox"/> YES <input type="checkbox"/> NO
c) Hepatitis B or C	<input type="checkbox"/> YES <input type="checkbox"/> NO
d) Hypertension	<input type="checkbox"/> YES <input type="checkbox"/> NO
e) TB/TB suspect	<input type="checkbox"/> YES <input type="checkbox"/> NO
f) Fractures/Bone disease	<input type="checkbox"/> YES <input type="checkbox"/> NO
g) Proteinuria	<input type="checkbox"/> YES <input type="checkbox"/> NO
h) Other pregnancy related complications	<input type="checkbox"/> YES <input type="checkbox"/> NO
<b>EXCL. If Yes to any</b>	
B14: Smoking	<input type="checkbox"/> YES <input type="checkbox"/> NO
a) History of smoking prior to current pregnancy	<input type="checkbox"/> YES <input type="checkbox"/> NO
b) Currently smoking	<input type="checkbox"/> YES <input type="checkbox"/> NO
c) If yes, how many cigarettes per day .....	
B15: Alcohol consumption	<input type="checkbox"/> YES <input type="checkbox"/> NO
a) History of alcohol consumption	<input type="checkbox"/> YES <input type="checkbox"/> NO
b) Alcohol consumption during current pregnancy	<input type="checkbox"/> YES <input type="checkbox"/> NO
c) If yes, how much per day .....	
B16: Calcium supplementation	<input type="checkbox"/> YES <input type="checkbox"/> NO
a) Currently on calcium supplements	<input type="checkbox"/> YES <input type="checkbox"/> NO
b) If yes, List preparations.....	<b>(EXCL. If &gt;.....)</b>
c) Dose/number of tablets.....	
B17: Vitamin D supplementation	<input type="checkbox"/> YES <input type="checkbox"/> NO
a) Currently on calcium supplements	<input type="checkbox"/> YES <input type="checkbox"/> NO
b) If yes, List preparations.....	<b>(EXCL. If &gt;.....)</b>
c) Dose/number of tablets.....	
B18: Other Vitamin/mineral supplements/nutrient supplements (e.g. multivitamins, if YES list)	<input type="checkbox"/> YES <input type="checkbox"/> NO
1. .... Dose .....	
2. .... Dose .....	
B19: Current medication(s) & dose – list (if YES). Other than ARV or nutrient supplements	<input type="checkbox"/> YES <input type="checkbox"/> NO
1. .... Dose .....	
2. .... Dose .....	

Thank you for your interest in this study. With your permission, I will introduce you to the study counsellor who will go through the informed consent form with you. Please feel free to ask questions and or seek clarifications where necessary.

**END OF SCREENING QUESTIONNAIRE – PART 1**

Screening questionnaire, Version 1.1. Dated 13<sup>th</sup> March 2014

Page 2 of 6

QC1: Initials..... Date .....

QC2: Initials..... Date .....

**SCREENING FORM (GUMBA - F002)**

Study Screening ID: S \_ \_ | \_ \_ | \_ \_

**SECTION D: OUTCOME OF INFORMED CONSENT PROCESS** (To be completed by counsellor)**PART 1:** To be completed at initiation of IC process

Mother potentially eligible to participate in the study (review screening Q above with focus on points for exclusion)	<input type="checkbox"/> YES	<input type="checkbox"/> NO*
Informed consent process initiated, mother introduced to informed consent (IC) form	<input type="checkbox"/> YES	<input type="checkbox"/> NO*
Given both English and Luganda copies of approved informed consent form to take home	<input type="checkbox"/> YES	<input type="checkbox"/> NO*
Scheduled for enrolment visit at 32 weeks of gestation	<input type="checkbox"/> YES	<input type="checkbox"/> NO*
Date of next appointment (dd/mm/yyyy)		
Name of person initiating consent process		
Signature:	Date:	Time:

**PART 2:** To be completed on day IC is obtained.

Study information discussed with participant and given a chance to ask questions	<input type="checkbox"/> YES	<input type="checkbox"/> NO*
Language in which informed consent was obtained	<input type="checkbox"/> English <input type="checkbox"/> Luganda	
Informed consent obtained	<input type="checkbox"/> YES	<input type="checkbox"/> NO*
Participant agreed to home escort on day of consent	<input type="checkbox"/> YES	<input type="checkbox"/> NO*
Home visitor assigned	<input type="checkbox"/> YES <input type="checkbox"/> NO	Name of home visitor
Name of person who obtained consent		
Signature:	Date:	Time:

**\*If No to any of above- document reasons here** (insert additional paper if necessary)

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Screening questionnaire, Version 1.1, Dated 13<sup>th</sup> March 2014

Page 3 of 6

QC1: Initials..... Date .....

QC2: Initials..... Date .....

**SCREENING FORM (F002)**

Study Screening ID: S \_ \_ | \_ \_ | \_ \_

**SECTION E: SCREENING Q - PART 2 [CHECKLIST]**

(To be completed by study nurse at enrolment visit after informed consent has been obtained)

Date 

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 (DD/MM/YYYY)

## CHECKLIST FOR BASIC INCLUSION CRITERIA

B2.1:	Is the potential participant between 18.00 and 39.99 years old?	<input type="checkbox"/> YES	<input type="checkbox"/> NO*
B3.1:	Is the gestation age $\leq$ 37 weeks? (Based on ultrasound scan)	<input type="checkbox"/> YES	<input type="checkbox"/> NO
B3.2:	Current gestation age in weeks -----		
B4.1:	Is it a singleton pregnancy? (based on ultrasound scan)	<input type="checkbox"/> YES	<input type="checkbox"/> NO*
B7.1:	Is the potential participant willing to be visited at home?	<input type="checkbox"/> YES	<input type="checkbox"/> NO*
B8.1:	Does the potential participant plan to breastfeed for at least 6 months?	<input type="checkbox"/> YES	<input type="checkbox"/> NO*
B10.1:	Is the potential participant ART naive or if HIV* only initiated HAART during current pregnancy? <i>Confirm about use of PEP/PrEP</i>	<input type="checkbox"/> YES	<input type="checkbox"/> NO*
B11.1:	If HIV positive, is the participant still on first line HAART (currently TDF/3TC/EFV)?	<input type="checkbox"/> YES	<input type="checkbox"/> NO*
<b>*EXCL. If NO to any of statements above</b>			

## CHECKLIST FOR MEDICAL HISTORY (review file, ANC lab results, ultrasound scan and ask participant where necessary)

B13.1:	Does the potential participant have a known diagnosis of:	<input type="checkbox"/> YES*	<input type="checkbox"/> NO
a)	Renal disease	<input type="checkbox"/> YES*	<input type="checkbox"/> NO
b)	Glycosuria (+)	<input type="checkbox"/> YES*	<input type="checkbox"/> NO
c)	Hepatitis B or C	<input type="checkbox"/> YES*	<input type="checkbox"/> NO
d)	Hypertension	<input type="checkbox"/> YES*	<input type="checkbox"/> NO
e)	TB/TB suspect	<input type="checkbox"/> YES*	<input type="checkbox"/> NO
f)	Fractures/Bone disease	<input type="checkbox"/> YES*	<input type="checkbox"/> NO
g)	Proteinuria (++) or (+++) or (++++)	<input type="checkbox"/> YES*	<input type="checkbox"/> NO
h)	Other pregnancy related complications	<input type="checkbox"/> YES*	<input type="checkbox"/> NO
<b>* EXCL. if Yes to any</b>			

Screening questionnaire, Version 1.1, Dated 13<sup>th</sup> March 2014

Page 4 of 6

QC1: Initials..... Date .....

QC2: Initials..... Date .....

**SCREENING FORM (F002)**

Study Screening ID: S\_ \_ \_ | \_ \_ | \_ \_

**SECTION F: STUDY ENROLLMENT DETAILS** (To be completed by study nurse)

Participant understands study protocol and is voluntarily interested to join the study		<input type="checkbox"/> YES	<input type="checkbox"/> NO*
Participant enrolled into the Study		<input type="checkbox"/> YES	<input type="checkbox"/> NO*
STUDY ID assigned	Attach PED label here for the mother	Attach PED label here for infant after delivery	
Name of study staff who enrolled the participant:			
Signature:	Date:	Time:	

Participant scheduled for study visit at 36 weeks of gestation		<input type="checkbox"/> YES	<input type="checkbox"/> NO*
If YES, Date (dd/mm/yyyy)		Time	

\*If No to any of above- document reasons here (insert additional paper if necessary)

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**SCREENING FORM (F002)**

Study Screening ID: S\_ \_ \_ | \_ \_ | \_ \_

**SECTION G: OUTCOME OF HOME ESCORT** (To be completed by home visitor)

Participant lives within location of interest		<input type="checkbox"/> YES	<input type="checkbox"/> NO*
Baseline social economic Questionnaire completed		<input type="checkbox"/> YES	<input type="checkbox"/> NO*
Home visit successfully done		<input type="checkbox"/> YES	<input type="checkbox"/> NO*
Contact Details form completed		<input type="checkbox"/> YES	<input type="checkbox"/> NO*
Participant linked to the area community volunteer on the study		<input type="checkbox"/> YES	<input type="checkbox"/> NO*
Name of CV attached to participant		Phone no.	
Home escorted done by (NAME)		Signature:	
Date (dd/mm/yyyy)		Time	

\*If No to any of above- document reasons here (insert additional paper if necessary)

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## Appendix 4: Informed consent form (English version)

HNR study no. 6522- GUMBA study  
Protocol Version 2, Dated 24<sup>th</sup> June, 2014

Place PID Label Here

### RESEARCH PARTICIPANT INFORMED CONSENT FORM

**Protocol Title:** Effects of exposure to antiretroviral therapy during pregnancy and breastfeeding, on maternal bone health and infant growth in urban Ugandans

**Short title:** Maternal ART and bone health in Ugandan mothers, and their infants (to be known locally to participants in Uganda as the GUMBA ['Bone'] study)

**Principal Investigator (s):** Ms. Florence Nabwire (PhD student), Dr Gail Goldberg and Dr Ann Prentice of MRC Human Nutrition Research Unit in Cambridge, UK

**Funder:** UK Medical Research Council, UK.

**Date/Revision:** 24<sup>th</sup> June, 2014

#### 1. Introduction:

- You are being invited to take part in a research study. This study is being organized by the Nutrition and Bone Health Group at the Human Nutrition Research Unit (HNR) of the Medical Research Council, in Cambridge United Kingdom (UK), in collaboration with Mulago National Referral Hospital (Mulago Hospital), Baylor-Uganda and MUJHU CARE Ltd (MUJHU).
- You have been approached to participate in this study because you are pregnant and seeking antenatal services here at Mulago Hospital. We are looking to recruit women aged 18-40 years at less than 28 weeks (about 5 months) of pregnancy. We plan to recruit about 200 pregnant women, that is 100 HIV-infected and 100 HIV- uninfected pregnant women.
- Before you decide if you want to join, we would like to explain the purpose, the procedures, the risks and benefits of participating, and what will be expected of you and your baby.
- Please read this consent form with care. You may also have this consent form read to you.
- Ask questions about things that are not clear to you now or when you think of them later.
- After the study has been fully explained to you, and all of your questions have been answered, you can decide freely if you want to join the study. Your participation is entirely voluntary. If you decide not to join the study, or decide later to leave the study, your and your baby's care will not be affected.
- At the end of the study, you will be told when the study results will be ready and how you can learn about them if you wish.
- You will be followed in the study for between 2 and 3 years (depending on your current stage of pregnancy and when you stop breastfeeding); and your baby will be followed up for about 1.5 years. We hope to be able to extend the study period in the future if funding is available. So will ask you if you consent to be invited to participate in future research

Page 1 of 10



HNR study no. 6522- GUMBA study  
Protocol Version 2, Dated 24<sup>th</sup> June, 2014

Place PID Label Here

#### 2. Why is the GUMBA study being done?

The main objective of this study is to find out if HIV-infected pregnant women started on ARVs lose more bone after delivery compared to HIV-uninfected women, and if HIV-infected women on antiretroviral drugs (ARV) regain their bone after they stop breastfeeding. We also want to find out if the ARVs taken by HIV-infected pregnant or breastfeeding women affect the bones and growth of their babies who are not born HIV-infected. Studying bone loss is important because when someone loses too much bone, their bones become weak and so they are more likely to get fractures in the future.

Bone mineral loss in women during pregnancy and breastfeeding is natural. It happens so that the baby's skeleton can grow and to have important nutrients such as calcium, in breast milk. The bone mineral is replaced again later and does not seem to cause more fractures than expected compared to women who have never been pregnant or breastfed. Some studies (but not done in pregnant or breastfeeding women) have reported bone loss in HIV-infected people within the first two years of starting ARVs. However, we do not know whether any losses in bone are greater in HIV-infected pregnant and breastfeeding women on ARV, and we also do not know if and when their bones return to normal after they stop breastfeeding. Furthermore, ARVs are passed to the babies during pregnancy and breastfeeding to protect them from HIV infection but we have little information on whether this has an effect on the bones and growth of babies born to HIV-infected women on ARVs. This study will help us better understand the effects of ARVs on the bones of mothers and their babies.

This study has been approved by Institutional Review Boards (IRBs) and Ethics Committees (EC) in the UK and in Uganda; and received a research permit from the Uganda National Council for Science and technology. IRBs are special groups of people that decide if a study is good science and ECs watch over the safety and rights of research participants.

#### 3. What will happen if you and your baby join the GUMBA study?

You will be asked to come for a total of eight study visits over the next 2-3 years. During pregnancy, your study visit will be scheduled at 36 weeks of gestation and you will be expected to deliver at Mulago Hospital. After delivery, you and your baby will be asked to come for study visits at 2 weeks then at 3, 6, and 12 months; thereafter you will also have a study visit at 3 months after you stop breastfeeding, (so the time cannot be known for this at the moment); and your baby will have a visit at 18 months of age. Apart from the 2 week visit, all the other visits will be scheduled to match with your postnatal, immunization and growth monitoring appointments. All study visits will be at Baylor-Uganda and MUJHU (located about 50 metres from this ANC clinic) and you will continue to receive routine health services for mothers during pregnancy and for mother-baby pairs after delivery as per the national guidelines. We will also access your health records for information of relevance to this study, this will include your antenatal, delivery, postnatal, immunization, family planning and HIV care records (where applicable) within the hospital. All the information will be treated in the strictest confidence. You will be given a study number and no names will be used with the information collected. At

Page 2 of 10



the end of the study you will continue to receive routine health services within Mulago Hospital or at another clinic of your choice.

#### 4. Tests and Procedures at the study visits

**Baseline (enrolment):** With your permission, we will keep any of your left over blood that was collected today in the laboratory for routine antenatal tests, for future information relating to this study.

**Visits during pregnancy:** You will be asked to come to Baylor-Uganda Clinic in Mulago at about 7:00 AM after fasting overnight (no eating or drinking except water since 11:00 PM the night before). We will ask you to empty your bladder when you arrive and give you some water to drink, then after 1 hour we will take a blood sample (about 2 tablespoonsful) and you will collect urine at the end of 2 hours. You will not be allowed to eat within this time but we will give you breakfast after the 2hrs. We will also measure your weight and height, and ask about your food intake, sunshine exposure and any changes in your socioeconomic status and/or residence since your last visit. You will also receive routine antenatal services before you go home.

**At delivery:** We will ask you to try and deliver at Mulago Hospital. Depending on where and when you deliver, we will collect cord blood and we will measure your baby's birth weight, length and head circumference. Cord blood is collected from the umbilical cord immediately after the birth of the baby; after the cord has been cut. This will not cause any pain to you or your baby.

#### Visits after delivery:

**At 2 weeks after delivery:** You will be asked to come to Baylor-Uganda clinic with your baby without fasting overnight. We will measure your weight and height, and then ask about your food intake, breastfeeding practices and collect urine samples from you and your baby. We will also ask you to collect about 1 tablespoonful of breast milk and then give you a dose of tracer water to drink. The tracer water is safe and is used in studies all over the world. It will enable us measure how much breast milk your baby takes. You and your baby will then have a bone scan before you go home. A home visitor will then come to your home for the next 13 days to collect urine samples from you and your baby at a time of your convenience. The urine samples will be used to estimate how much breast milk your baby takes and the breast milk sample will be used to measure the amount of nutrients in your breast milk.

**At 3, 6, 12 months after delivery, and 3 months after you stop breastfeeding:** You will be asked to come to Baylor-Uganda Clinic in Mulago with your baby at about 7:00 AM after fasting overnight (no eating or drinking except water since 11:00 PM the night before). Put in something about pregnancy test here. **YOUR BABY WILL NOT BE REQUIRED TO FAST; THEREFORE YOU CAN BREASTFEED YOUR BABY NORMALLY BOTH AT HOME AND IN THE CLINIC.** We will ask you to empty your bladder and then give you some water to drink to help with urine production. At this point you will be asked to collect a small amount of that urine for pregnancy testing and if found pregnant, you will not continue with the study procedures. If confirmed not to be pregnant, we will take a blood sample from you (about 2 table spoonful) after 1 hour and then ask you to



collect your urine at the end of 2 hours. A blood sample will also be taken from your baby (about 1/4 tablespoonful) anytime within the 2 hours. You will not be allowed to eat within this time but we will give you breakfast after the 2hrs. We will also measure your weight and height, and then ask about your food intake, breastfeeding practices, sunshine exposure and any changes in your socioeconomic status and/or residence since your last visit and ask you to collect a breast milk sample (if still breastfeeding). In addition, we will measure your baby's length, weight and head circumference, and ask about how she/he is feeding. Thereafter, you and your baby will receive routine services (postnatal and immunization) and then be escorted for bone scans at MUJHU. For the visit at 3 months, we will again give you a dose of tracer water and collect urine samples from your home for the next 13 days.

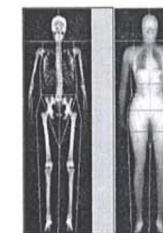
#### 5. What is a bone scan (DXA)?

The DXA is a special X-ray that measures how much mineral your skeleton contains in the whole body or at particular parts of the body like the hip and spine. It also measures how much of your body is fat, muscle and bone.

For the DXA scan, you will lie still on a bed for up to 15 minutes while a machine passes over your body and takes a picture of your bones. Each maternal DXA scan visit involves scans of the whole body, spine and hip. There will be up to three tries for each scan per visit. Before each DXA scan we will perform a pregnancy test on your urine sample to make sure you are not pregnant. The DXA scan will not be done if you are pregnant, because the information they will provide will not be useful to the study. Therefore, we will not unnecessarily expose your unborn child to X-rays.



Whole-body DXA position



Sample Whole body DXA scan

For your baby's scan, your baby may be held while the scan passes over him/her. We may wrap your baby to help him/her keep still during the DXA scan, and they may be asleep, which may take up to 15 minutes. The DXA will be taken at 2, 6 and 12 months of age but not at 18 months, because it's difficult to maintain a child in a motionless position. There will be about 3 trial times for every picture taken during your visit.





You or your baby will not feel anything while this DXA scan is being done. You will not be informed about the results of the DXA scan during the study as the DXA data will be sent away to be read by experts in Cambridge. However, your study doctor will be informed if there is any information that they would need for your care.

#### 6. What is Tracer water?

All water is made up of two parts called hydrogen and oxygen. The special water we are going to give you to drink contains some hydrogen parts that are heavier than ordinary hydrogen and is called deuterium. It tastes the same as ordinary water.

Deuterium has been in use in human behavior studies for over 50 years now. When it is absorbed in the stomach, the tracer water mixes well with body fluid that helps us locate the fluid's movement in and outside the body by measuring the amount of oxygen in the fluid (better than in urine or saliva). Tracer water leaves the body mainly through urine within 14 days. In this study, a small amount of tracer water will be given to the mother, and then we shall obtain urine or saliva from the mother and her child. Tracer water will go into the child through breast milk, so obtaining urine or saliva from the mother and the child in 14 days; we hope to estimate the amount of breast milk the child takes. Therefore, we will send a home visitor at your home every day over the 13 days, to collect urine samples at your convenient time.

#### 7. Summary of the tests that will be done in this study:

- Tests of blood and urine to see if you or your baby are losing or gaining bone.
- Tests of hormones, minerals and vitamins that affect bone metabolism through testing of blood, cord blood, breast milk and urine.
- Blood tests that measure how much the body is reacting to infections.
- Tests of tracer water in urine to estimate how much breast milk your baby suckles at 2 and 14 weeks of age.
- A special x-ray, called a DXA scan, to measure how much mineral there is in the bones.
- We also request to keep your blood cells for genetic tests related to bone metabolism

Some of the urine test results will be made available to you at the study visits (e.g pregnancy test and urinalysis). At the end of the study, we will send the results of the last DXA scan to your doctor so that your doctor can counsel you about your bone health and the bone health of your baby. Some of the other special test results on you and your baby will not be given to you because they will not be available during the study.

**Sample storage:** The blood, urine and breast milk samples that are collected during the study will be stored in freezers at Baylor-Uganda and HNR for tests mentioned above and for future studies relevant to bone health, breastfeeding, HIV and ARV in mothers and babies.



#### 8. Are you willing to have a study staff come visit you at home?

If you decide to participate in this study, a study staff called a health visitor will accompany you to your home today to know where you live. This is important so the health visitor can help remind you of clinic visits if you are not able to come to the clinic on your planned day or if a test result is not normal and you are required to be reviewed by the study clinician at the study clinic. If you do not come for a scheduled visit or if a test result comes back abnormal, the study health visitor will contact you. The home visitor will also help collect urine samples from your home after you have drunk the tracer water.

#### 9. What are the risks of participating in this study?

Taking part in this study may involve some risks or discomforts. There is a risk of minor bruising from the collection of the blood samples but this is minimized as only well-trained and experienced staff will take the samples. For your baby, we can apply a numbing cream before drawing blood to reduce the pain if you wish so. There are no risks in collection of urine, although sometimes it may be uncomfortable for some people. There are no risks in the collection of cord blood.

##### A. Risk of the DXA Scans

###### *Risk of the DXA Procedure:*

No complications are expected with the DXA procedures which measure the mineral content of your and your baby's bones. Care is taken during DXA procedure to use the lowest radiation dose possible while producing the best pictures for evaluation. National and international radiology protection councils continually go back and look and update the technique standards used in DXA procedure. DXA systems have tightly controlled X-ray beams with significant filtration and dose control methods to reduce stray or scatter radiation. This ensures those parts of a participant's body not being imaged receive very little radiation exposure. The radiation dose from DXA is very low so the staff operating the scanners do not have to wear special protective clothing and the room does not have to be shielded.

###### *Risk of Radiation:*

There is a risk from being near X-rays, also known as radiation. Radiation is energy in the form of waves. Every person is exposed to a small level of radiation from the sun, naturally (called background radiation). Exposure to X-rays, particularly at high doses, can lead to cancer. However, the level of X-rays used in a DXA scan is very low and is about the same as used for a chest X-ray. The study staff has been trained to do the DXA scan using the smallest amount of X-ray possible.

The radiation exposure in this study is for research purposes only and is not part of your medical care. X-rays can damage cells, but at low doses the body is usually able to repair these cells. The total amount of radiation that you and your baby will receive in the whole study will be comparable to a few days of natural radiation exposure in Kampala, although this will be in addition to the background radiation. This is much less than the radiation that the average person gets each year from natural sources like the sun, air, food, and soil. The risk from the radiation exposure in this research is very small.



The radiation exposure talked about here is what you or your baby will get from this research study only. It does not include any exposure you or your baby may have received or will receive from other medical tests outside of this study that are done as part of your regular medical care. If you have questions about the total amounts of radiation you or your baby will be receiving, you should ask the study doctor.

**Risks Related To Pregnancy:**

We will not do DXA when you are pregnant because the X-ray will not be able to differentiate your bone from your unborn baby's bones; such scans will not be useful in this study. Furthermore, the cells that multiply very fast like those of the unborn baby are more sensitive to radiation. Therefore pregnant women will not take part in the DXA part of the study. Before the DXA scans are done you will have a urine pregnancy test. The urine pregnancy test will be done on some of the urine that you will collect the beginning of the two hour fasted procedure. No pregnancy test is needed before DXA scans at 2 week after delivery of your baby, since that is too early after delivery for you to be pregnant again. If you are found to be pregnant you will not be able to continue with all the study procedures including DXA. You will be removed from the study at this point because the pregnancy will affect the measurements from your blood and urine samples and also the DXA scans will not provide accurate information about your bone health. However, we will ask you to continue bringing your baby (already enrolled in the study) for the study visits as scheduled.

**B. Risks of information about you becoming known by others**

Study staff will make every effort to protect your privacy while you are in this study. Your visits here will take place in private. Some of the study staff will know your HIV status. These workers are very serious about your privacy and will make a lot of effort to make sure that others do not learn about your HIV status.

**C. Risk of new clinical diagnosis:**

If we find any abnormalities in your bone scans and/or blood and/or urine samples which are considered to be clinically reportable, we will inform you and then refer you to a specialist within Mulago Hospital for further investigations. This might lead to a new diagnosis for which the study will not pay for subsequent examinations or treatment.

**D. Risks related to Overnight Fasting**

We will ask you to fast overnight and come early morning (7-8 am) to the clinic on some of the study visits. You will only be allowed to drink water during the first 2-hours in the clinic, so you may feel hungry and weak. However, you will be served breakfast at the end of the 2hrs (about 9-10am depending on when you come in), which should not be too different from your usual habits, and you will have access to a comfortable sitting area with entertainment suitable for both children and adults.

**E. Safety of Tracer Water**

The amount of deuterium oxide that will be used in preparation of tracer water dose is considered safe. The tracer water is excreted out of the body through urine in both adults and children. Scientist at HNR are experts in this technique and have been using this method for over 30 years all over the world and no adverse effects have been observed in the mothers or infants.

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**10. Are there benefits to you or your baby for taking part in this study?**

There may not be a direct benefit to you and your baby. However, the study will pay for your routine ultrasound scan recommended by the midwife, if you haven't had one already and cannot afford to pay for yourself.

Above all, your participation in this study will benefit the wider society, because it will enable us to gain valuable information on whether HIV-infected women on ART lose more bone compared to HIV negative women. This has the potential to improve care for HIV-infected women and also their children.

Having a DXA scan may provide important information for your doctor about your bone health and the bone health of your baby. You may get some satisfaction from knowing that you and your baby participated in this study.

**11. Do you have to take part in this study?**

Joining this study is entirely voluntary. It is up to you to decide whether or not to take part. You may decide not to take part now or take time to think through before you make a decision. If you do decide to take part now you will be asked to sign the page provided at the end of this consent form. However, you will be free to withdraw from the study at any time, if you wish. Your decision now or in the future will not influence the medical care you and your baby child will receive within the hospital. If you decide to withdraw from the study in the future, with your consent, samples and data obtained may be kept and used to contribute to study results or for future studies. However, should you request your samples and data to be destroyed along with any other information relating to you, we will ensure that this takes place.

**12. Why might you and your baby be taken off this study early?**

You and/or your baby may be taken off the study early if:

- If you are found to be expecting twins or more children
- If you experience a miscarriage or deliver before 37 weeks of gestation (preterm birth)
- If you stop breastfeeding before 14 weeks after delivery
- If you have a subsequent pregnancy during the postpartum period
- The study staff decide that staying in the study would be harmful to you or your baby
- If there is a major problem with equipment
- You and your baby are unable to follow study procedures as told;

**13. Will it cost you anything or will you be paid to be in this study?**

There is no cost to you for your study visits, exams, blood tests or DXA scans or those of your baby. You will receive 15,000 Uganda shillings as compensation for your time and travel for each scheduled study visits at Baylor-Uganda.

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Place PID Label Here

**14. What information is kept private and what information may be given out?**

The study staff will do all they can to keep information about you and your baby's part in this study as private as possible. On your and your baby's study records, a code will be used instead of your names. Only the study staff will know this code. The study staff may see the details about your and your baby's health. The study staff, and the study review boards, may see details about your and your baby's health in research records. The study staff will not give out any information that tells who you are to any other groups without your written consent. All the study forms used for the study visits and collection of samples will only have your study number and age for identification. All records and information will be kept secure in locked rooms. Any publication of this study will not use you or your baby's name or identify you or your baby personally

**15. What happens if you or your baby is injured in this study?**

It is extremely unlikely that there will be any harm caused to you or your baby as a result of participating in this study. If the study staff determines that you or your baby have/has been negligently harmed as a direct result of participating in this study, you will be given immediate treatment for those injuries at no cost to you. There are no other special compensation arrangements. By signing this consent form you will not be giving up any of your legal rights in such situations.

**16. Whom should you call if you have study questions, or a study problem?**

If you ever have questions about this study or if you have a study related medical problem or injury, you should contact Dr Vincent Tukei or Ms Florence Nabwire at Block 5 Mulago Hospital on the toll free line **0800 111011** on weekdays (**Monday to Friday**) from **08am to 5pm**; .If unable to reach them, you can contact Dr Dorothy Sebikari at the Makerere University-Johns Hopkins University (MU-JHU) Research Collaboration, Upper Mulago Hill Road on telephone number 0414-541-044 (not toll free ).If you have questions about your rights as a research participant, you may contact the Joint Clinical Research Center (JCRC), the Uganda IRB, through its chairman Dr. Jesse Kagimba on telephone number 0414 270 622 or 0414 270 283.

**17. What does your signature mean?**

Your signature or thumbprint on the next page means that you understand the information given to you about the study and in this consent form. If you sign or place your thumbprint on the form, it means that you agree to join the study. By signing this consent form, you have not waived any of the legal rights that you otherwise would have as a participant in a research study.

**WE WILL OFFER YOU A COPY OF THIS CONSENT FORM.**

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Place PID Label Here

**STATEMENT OF CONSENT:** I have read (or someone has read and explained to me) the information in this consent form. I understand the purpose of this Bone Health and Child Growth study also known as GUMBA study, the clinical and research methods to be followed and which ones are extra, and the risks and benefits as described in this written summary. I agree by my own choice to join and to allow my baby to take part in the GUMBA study.

Participant's Name (print)

Participant's Signature or  
Thumbprint

Date

**For all participants:** I have explained the purpose of this study to the participant and have answered all of her questions. To the best of my knowledge, she understands the purpose, methods, risks and benefits of taking part in the Bone Health and Child Growth study. She willingly agrees to her and her baby's taking part.

Name of person obtaining  
consent (Print)

Signature of person obtaining  
consent

Date

**For illiterate participants** I attest that the information contained in this written consent form has been read and explained to the volunteer. To the best of my knowledge, the information provided was complete and accurate. The volunteer appears to understand the purpose, methods, risks and benefits of taking part in the Bone Health and Child Growth study also known as GUMBA study. She willingly agrees to her and her baby's taking part.

**For those placing a thumbprint only:** I attest that the participant who states that her name is

\_\_\_\_\_ has placed her thumbprint on this consent form out

of her own free will on this day \_\_\_\_\_.





## Appendix 5: Home visit contact details form

GUMBA STUDY	CONTACT DETAILS FORM (F003)	Version 1: Dated 01 December, 2014
<div style="display: flex; justify-content: space-between;"> <div>DATE: ____/____/____ <small>(DD/MM/YYYY)</small></div> <div>MULAGO ANC NUMBER: _____</div> <div>STUDY SCREENING NUMBER: SN ____ ____ ____ </div> </div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"> <div>DOB: ____/____/____ <small>(DD/MM/YYYY)</small></div> <div>STUDY NUMBER: MAB ____ ____ ____  - ____ ____  <small>(If already enrolled into study)</small></div> </div>		
<p><b>This form should be completed for all mothers enrolled into Gumba study or as an update when the contact details of the mother or child change</b></p> <p>Type of entry: <input type="checkbox"/> Form completed at enrolment (Tick relevant box) <input type="checkbox"/> An update of new details</p>		
PRIMARY CONTACT DETAILS		
<p>(1a). FULL NAME OF MOTHER: _____ <small>(please use capital letters with good spacing)</small></p> <p>(1b). FULL NAME OF BABY: _____ <small>(If form updated postpartum) (please use capital letters with good spacing)</small></p> <p>(1c). ADDRESS/LOCALITY* _____ <small>(* name directions to residence &amp; draw map below)</small></p> <p>_____</p> <p>_____</p> <p>(1d). PHONE NUMBER (S): ____ ____ ____  - ____ ____  - ____ ____  - ____ ____  ____ ____ ____  - ____ ____  - ____ ____  - ____ ____ </p> <p>(1e). Is this a personal phone number: N/A <input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> <small>If No, specify _____</small></p> <p>(1f). If yes: Are you happy for us to phone you if necessary? Yes <input type="checkbox"/> No <input type="checkbox"/></p> <p>(1g). Are you happy for us to visit you at home if necessary? Yes <input type="checkbox"/> No <input type="checkbox"/></p> <p><small>*Draw map here</small></p> <div style="border: 1px solid black; height: 150px; width: 100%; margin-top: 10px;"></div>		
<p>QC1: _____ Page 1 of 2 QC2: _____</p>		

GUMBA STUDY	CONTACT DETAILS FORM (F003)	Version 1: Dated 01 December, 2014
ALTERNATIVE CONTACT DETAILS		
<p>(2a). Do you have an alternative phone number which we can call if your phone is not available? Yes <input type="checkbox"/> No <input type="checkbox"/></p> <p>(2b). PHONE NUMBER (S): ____ ____ ____  - ____ ____  - ____ ____  - ____ ____  ____ ____ ____  - ____ ____  - ____ ____  - ____ ____ </p> <p>DETAILS OF PHONE OWNER</p> <p>(2c). FULL NAME : _____</p> <p>(2d). RELATIONSHIP: _____</p> <p>(2e). Is this a personal phone number: N/A <input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> <small>If No, specify _____</small></p> <p>(2f). If yes: Are you happy for us to phone this person if necessary? Yes <input type="checkbox"/> No <input type="checkbox"/></p> <p>(2g). Do you stay with this person in the same house? Yes <input type="checkbox"/> No <input type="checkbox"/></p>		
HOUSEHOLD DETAILS		
<p>(3). Whom do you stay with? <input type="checkbox"/> Alone <input type="checkbox"/> Relatives <input type="checkbox"/> Friends <input type="checkbox"/> Husband <input type="checkbox"/> Parents</p> <p>(4a). How many adults (&gt;18 years) currently live in your household? <span style="border: 1px solid black; display: inline-block; width: 30px; height: 20px; vertical-align: middle;"></span></p> <p>(4b). How many children (&lt; 18 years) currently live in your household? <span style="border: 1px solid black; display: inline-block; width: 30px; height: 20px; vertical-align: middle;"></span></p> <p>(4c). How many children are aged &lt; 5 years? <span style="border: 1px solid black; display: inline-block; width: 30px; height: 20px; vertical-align: middle;"></span></p> <p>(5). Specify the type of house the mother lives in? (Tick all that apply)</p> <p>(5a). Roofing: <input type="checkbox"/> Grass thatched <input type="checkbox"/> Iron sheets <input type="checkbox"/> Tiles <input type="checkbox"/> Other, specify _____</p> <p>(5b). Walls: <input type="checkbox"/> Thatch/mud/clay <input type="checkbox"/> sun-dried bricks <input type="checkbox"/> burnt bricks <input type="checkbox"/> stone <input type="checkbox"/> Cement</p> <p>(5c). Floor: <input type="checkbox"/> Earth <input type="checkbox"/> <input type="checkbox"/> Cement <input type="checkbox"/> Tiles <input type="checkbox"/> wood/planks <input type="checkbox"/> broken bricks</p> <p>(5d). Tenure: <input type="checkbox"/> Earth <input type="checkbox"/> <input type="checkbox"/> Cement <input type="checkbox"/> Tiles <input type="checkbox"/> wood/planks <input type="checkbox"/> broken bricks</p> <p>(6). How many rooms does your household use for sleeping? <span style="border: 1px solid black; display: inline-block; width: 30px; height: 20px; vertical-align: middle;"></span></p> <p>(7). Do you work away from home? N/A <input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/></p> <p>(8a). When is the best day to visit you at home, if necessary?</p> <p>(8b). When is the best time to visit you on the preferred day? ____ ____  : ____ ____  <input type="checkbox"/> AM <input type="checkbox"/> PM <small>(In 12hour clock)</small></p>		
COMPLETED BY: (Print name in space below)		SIGNATURE: _____
<p>QC1: _____ Page 2 of 2 QC2: _____</p>		

## Appendix 6: PG36 questionnaire pack

<p style="font-size: small;">INR study no. 6522: Gambia study Protocol Version 3.0, Dated 11<sup>th</sup> May, 2015</p> <div style="text-align: center; margin-top: 20px;">  </div> <p style="text-align: center; font-weight: bold;">QUESTIONNAIRE PACK FOR PG36 VISIT – COVER PAGE</p> <p>DATE COMPLETED: <input type="text"/>/ <input type="text"/>/ <input type="text"/> (dd/mm/yy)</p> <p>MOTHER'S DETAILS:</p> <p>Mother's Study No: <input style="width: 100px;" type="text"/></p> <p>Mother's D.O.B: <input type="text"/>/ <input type="text"/>/ <input type="text"/> (dd/mm/yy)</p> <p>Residence: .....</p> <p>EDD (based on ultrasound scan): Date <input type="text"/>/ <input type="text"/>/ <input type="text"/></p> <p>NAME OF INTERVIEWER: ..... SIGNATURE .....</p> <p style="font-size: x-small; margin-top: 20px;">PG36 Questionnaire, Version 2.0, Dated 11<sup>th</sup> May 2015 <span style="float: right;">Page 1 of 16</span></p> <p style="font-size: x-small;">QC1: Initials..... Date ..... QC2: Initials..... Date .....</p>	<div style="text-align: center; margin-bottom: 20px;">  </div> <p style="text-align: center; font-weight: bold;">BASELINE DEMOGRAPHIC, SOCIO-ECONOMIC STATUS AND MEDICAL HISTORY QUESTIONNAIRE (MAB-Q1)</p> <p><b>SECTION A: ANTHROPOMETRY FOR MOTHER</b></p> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 15%;">Weight</td> <td style="width: 10%;"><input type="text"/></td> <td style="width: 10%;"><input type="text"/></td> <td style="width: 10%;"><input type="text"/></td> <td style="width: 10%;"><input type="text"/></td> <td style="width: 10%;"><input type="text"/></td> <td style="width: 10%;"><input type="text"/></td> <td style="width: 10%;"><input type="text"/></td> <td style="width: 10%;"><input type="text"/></td> <td style="width: 10%;"><input type="text"/></td> <td style="width: 10%; text-align: right;">kg</td> </tr> <tr> <td>Height</td> <td><input type="text"/></td> <td><input type="text"/></td> <td><input type="text"/></td> <td><input type="text"/></td> <td><input type="text"/></td> <td><input type="text"/></td> <td><input type="text"/></td> <td><input type="text"/></td> <td><input type="text"/></td> <td style="text-align: right;">cm</td> </tr> <tr> <td>Mid-Upper Arm Circumference (MUAC)</td> <td><input type="text"/></td> <td><input type="text"/></td> <td><input type="text"/></td> <td><input type="text"/></td> <td><input type="text"/></td> <td><input type="text"/></td> <td><input type="text"/></td> <td><input type="text"/></td> <td><input type="text"/></td> <td style="text-align: right;">cm</td> </tr> </table> <p><b>SECTION B: GENERAL INDIVIDUAL AND HOUSEHOLD CHARACTERISTICS</b></p> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 30%;">1. When is your date of Birth?</td> <td>...../...../..... (dd/mm/yyyy)</td> </tr> <tr> <td>2. What is your Religion?</td> <td> <input type="checkbox"/> [1] Protestant  <input type="checkbox"/> [2] Roman Catholic  <input type="checkbox"/> [3] Muslim  <input type="checkbox"/> [4] Pentecostal/ Born again Christian  <input type="checkbox"/> [5] None  <input type="checkbox"/> [6] Other (Specify)         </td> </tr> <tr> <td>3. What is your highest level of education?</td> <td> <input type="checkbox"/> [1] None  <input type="checkbox"/> [2] Primary  <input type="checkbox"/> [3] O-level  <input type="checkbox"/> [4] A' – level  <input type="checkbox"/> [5] Diploma  <input type="checkbox"/> [6] Certificate  <input type="checkbox"/> [7] Vocational  <input type="checkbox"/> [8] University         </td> </tr> <tr> <td>4. What do you do to earn a living (Occupation)?</td> <td> <input type="checkbox"/> [1] Housewife  <input type="checkbox"/> [2] Petty trader  <input type="checkbox"/> [3] Businessperson  <input type="checkbox"/> [4] Casual laborer  <input type="checkbox"/> [5] Salaried employment  <input type="checkbox"/> [6] Unemployed  <input type="checkbox"/> [7] Student  <input type="checkbox"/> [8] Other (Specify)         </td> </tr> </table> <p style="font-size: x-small; margin-top: 10px;">PG36 questionnaire, Version 2.0, Dated 11<sup>th</sup> May, 2015 <span style="float: right;">Page 2 of 16</span></p> <p style="font-size: x-small;">QC1: Initials..... Date ..... QC2: Initials..... Date .....</p>	Weight	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	kg	Height	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	cm	Mid-Upper Arm Circumference (MUAC)	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	cm	1. When is your date of Birth?	...../...../..... (dd/mm/yyyy)	2. What is your Religion?	<input type="checkbox"/> [1] Protestant <input type="checkbox"/> [2] Roman Catholic <input type="checkbox"/> [3] Muslim <input type="checkbox"/> [4] Pentecostal/ Born again Christian <input type="checkbox"/> [5] None <input type="checkbox"/> [6] Other (Specify)	3. 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Mid-Upper Arm Circumference (MUAC)	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	cm																																
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2. What is your Religion?	<input type="checkbox"/> [1] Protestant <input type="checkbox"/> [2] Roman Catholic <input type="checkbox"/> [3] Muslim <input type="checkbox"/> [4] Pentecostal/ Born again Christian <input type="checkbox"/> [5] None <input type="checkbox"/> [6] Other (Specify)																																									
3. What is your highest level of education?	<input type="checkbox"/> [1] None <input type="checkbox"/> [2] Primary <input type="checkbox"/> [3] O-level <input type="checkbox"/> [4] A' – level <input type="checkbox"/> [5] Diploma <input type="checkbox"/> [6] Certificate <input type="checkbox"/> [7] Vocational <input type="checkbox"/> [8] University																																									
4. What do you do to earn a living (Occupation)?	<input type="checkbox"/> [1] Housewife <input type="checkbox"/> [2] Petty trader <input type="checkbox"/> [3] Businessperson <input type="checkbox"/> [4] Casual laborer <input type="checkbox"/> [5] Salaried employment <input type="checkbox"/> [6] Unemployed <input type="checkbox"/> [7] Student <input type="checkbox"/> [8] Other (Specify)																																									



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5. How much do you earn in a month?	.....UGX
6. What is your Marital status?	[1] Married, monogamous [2] Married, polygamous [3] Cohabiting [4] Single, never married (skip to Qn 9) [5] Divorced (skip to Qn 9) [6] Separated (skip to Qn 9) [7] Widowed (skip to Qn 9)
6. If married, what is the highest education level attained by your husband?	[1] None [2] Primary [3] O-level [4] A' - level [5] Diploma [6] Certificate [7] Vocational [8] University
7. If married, what is the occupation of your husband?	[1] Petty trader [2] Businessperson [3] Casual laborer [4] Salaried employment [5] Unemployed [6] Student [7] Other (Specify)
8. If married, How much does your husband earn in a month?	.....UGX <input type="checkbox"/> Do not know
9. Who is the main bread winner of the household?	[1] Self [2] Husband [3] Parents [3] Relative [4] Friend [5] Other specify.....
10. What is the most important source of earning for your household?	[1] Salaried employment [2] Casual labour [3] Donations [4] Remittances [5] Sale of farm produce [6] Other specify.....
11. Do you have other sources of earning for your household ? If yes, list the sources (tick all that apply)	[1] None [2] Salaried employment [3] Casual labour [4] Donations [5] Remittances [6] Sale of farm produce [7] Other specify.....

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**SECTION C: HOUSING CONDITIONS<sup>1</sup>**

Now I would like to ask you about your housing conditions: all the rooms and all separate building used by your household members.

12. Whom do you stay with?	[1] Alone [2] Relatives [3] Friends [4] Husband [5] Parents
13. How many people currently live in your household?	13A. .... 13B Children..... 13C. < 5 years ..... 13D. Adults.....
14. What type of house are you staying in?	[1] Independent house [2] Tenement (Muzigo) [3] Independent flat/apartment [4] Sharing house/flat/apartment [5] Boys quarters [6] Garage [7] Hut [8] Uniport [9] Other (specify).....
15. What is the tenure status for your house?	[1] Owned [2] Rented (Normal) [3] Rented (subsidized) [4] Supplied free by employer [5] Supplied free or rent paid by other person [6] Other (specify)
16. How many rooms does your household use for sleeping?	.....
17. What is the major construction material of the roof?	[1] Thatch, Straw [2] Iron sheets [3] Tiles [4] Other (specify)
18. What is the major construction material of the external wall?	[1] Thatch, Straw [2] Mud and poles [3] Timber [4] Un-burnt bricks [5] Burnt bricks with mud [6] Burnt bricks with cement [7] Cement blocks [8] Stone [9] Other (specify)

<sup>1</sup> Questions from the Uganda National Household Survey report for 2009/2010. Published by the Uganda Bureau of statistics (enapt1-3)

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19. What is the major material of the floor?	[1] Earth [2] Earth and cow dung [3] Cement [4] Mosaic or tiles [5] Other (specify)
20. What is the main source of drinking water for your household?	[1] Private connection to pipeline [2] Public taps [3] Bore-hole [4] Protected well/spring [5] River, stream, lake, pond [6] Vendor/Tanker truck [7] Gravity flow scheme [8] Rain water [9] Other (specify)
21. What is the type of toilet that is mainly used in your household?	[1] Covered pit latrine private [2] Covered pit latrine shared [3] VIP latrine private [4] VIP latrine shared [5] Uncovered pit latrine [6] Flush toilet private [7] Flush toilet shared [8] Bush [9] Other (specify)
22. With how many other households do you share this toilet with?	.....
23. Do you have a hand washing facility at the toilet?	[1] No [2] Yes with water only [3] Yes with water and soap
24. What is the main source of lighting in your dwelling?	[1] Electricity-Grid [2] Electricity-Generator [3] Electricity-Solar [4] Paraffin lantern [5] Tadooba [6] Firewood [7] Other (specify)
25. What type of fuel do you use most often for cooking?	[1] Electricity-Grid [2] Electricity-Generator [3] Electricity-Solar [4] Firewood [5] Charcoal [6] Paraffin/kerosene [7] Gas [8] Other (specify)
26. What type of cooking technology do you use in your household?	[1] Traditional stove (Sigiri) [2] Traditional 3-stone open fire [3] Improved charcoal stove [4] Improved firewood stove

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	[5] Gas stove/cooker [6] Paraffin stove [7] Electric plate/cooker [8] Other (specify)
--	--

**SECTION D: HOUSEHOLD ASSETS<sup>2</sup>**

Now I would like to ask you about assets owned by your household.

Type of assets	Asset Code	Does any member of your household own [ASSET] at present? 1=Yes 2=No 3= Don't know	How many [...] does your household own at present?	
			Number	Total estimated value (in UGX)
House	001			
Other Buildings	002			
Land	003			
Computers/laptops	006			
Generators	007			
Solar panel/electric inverters	008			
Bicycle	009			
Motor cycle	010			
Motor vehicle	011			
Mobile phone	015			
Other specify	017			
Other specify	018			

<sup>2</sup> Questions from the Uganda National Household Survey report for 2009/2010. Published by the Uganda Bureau of StatisticsPG36 questionnaire, Version 2.0, Dated 11<sup>th</sup> May, 2015

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## SECTION C: BASELINE MEDICAL HISTORY

11. Repeat HIV test since last visit (if recent test > 6 months old)	NO <input type="checkbox"/> (skip 11.1) YES <input type="checkbox"/>										
11.1. HIV test Result (0=negative, 1=positive) ..... (If newly diagnosed positive inform PI or study coordinator)	NEGATIVE <input type="checkbox"/> POSITIVE <input type="checkbox"/>										
17. Were you on a family planning method before this pregnancy?	NO <input type="checkbox"/> (skip to 18) YES <input type="checkbox"/>										
17.1 If Y, which method? (tick all that apply):	[1] Pills [2] Depo provera injection [3] Condoms [4] Natural method (s) [5] Other specify.....										
17.2. When did you start using this method?	<table border="1"><tr><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr></table>										
17.3. When did you stop using this method?	<table border="1"><tr><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr></table>										
18. Current smoker	NO <input type="checkbox"/> (skip to 19) YES <input type="checkbox"/>										
18.1. If Yes, how many cigarettes do you smoke per day?	.....										
19. Have you ever had a bone fracture?	NO <input type="checkbox"/> (skip to Qn.20) YES <input type="checkbox"/>										
19.1. If YES, on which part of your body?	[1] Hip [2] Arm-radius (s) [3] Leg (s) [4] Back [5] Other, specify.....										
19.2. How was it caused?	[1] Car, taxi, motorbike or cycle crash [2] Fall down the stairs [3] Fall from object - ladder/box /stool [4] Standing up [5] Other, specify.....										

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20. Have you been on calcium supplements since last visit? If YES, 20.1. List preparations..... 20.2. Dose/number of tablets.....	NO <input type="checkbox"/> (skip to 21) YES <input type="checkbox"/> ..... .....
21. Have you been on vitamin D supplements since last visit? If YES, 21.1. List preparations..... 21.2. Dose/number of tablets.....	NO <input type="checkbox"/> (skip to 22) YES <input type="checkbox"/> ..... .....
22. Have you been on any multivitamins or a vitamin-mineral mix since last visit?, If Yes, 22.1. List preparations..... 22.3. Dose/number of tablets.....	NO <input type="checkbox"/> (skip to 23) YES <input type="checkbox"/> ..... .....
23. Have you taken other drugs since your last visit? (other than ARVs, and those already mentioned above) If Y: 23.1. List preparations..... 23.2. Dose/number of tablets..... 23.3. Number of months taken..... 23.4. Why did you take these drugs?	NO <input type="checkbox"/> (skip to 24) YES <input type="checkbox"/> ..... ..... ..... [1] As part of TB treatment [2] To treat cancer [3] To treat low blood platelets [4] Hepatitis B [5] Asthma [6] Serious skin condition [7] Other specify.....
24. Are you currently sick? If Y, list what 24.1. Illness 1.....	NO <input type="checkbox"/> (skip to 25) YES <input type="checkbox"/> .....

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24.2. For current diarrhea/vomiting, note the	no. of episodes per day .....
25. Do you have any metallic parts in your body inserted during operations?	NO <input type="checkbox"/> YES <input type="checkbox"/>
25.1 If yes, which type of operation?	.....
25.2 Which part of the body?	.....



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## CLINICAL CHART REVIEW FORM FOR TRR MOTHERS (MAB-F4-A)

Mother's PMTCT NO. .... Mother's Study NO. ....

Date: .../.../.....

Time point.....

71. Current (most recent) CD4 count and date	CD4 count: ..... Date: .....
72. Current (most recent) HIV Viral loads	Viral load: ..... Date: .....
73. Bactrim (PCP) prophylaxis (Cotrimoxazole or Dapsone)	NO <input type="checkbox"/> YES <input type="checkbox"/>
74. Has the participant changed ARV combination since last visit? 74.1 If yes, New combination ..... (If changed regimen inform PI) 74.2. Start date: ..... 74.3. Reason for change in ART regimen	NO <input type="checkbox"/> (skip to 75) YES <input type="checkbox"/>  [1] Substitution due to side effects [2] Switch to second line (failed 1 <sup>st</sup> line) [3] Other (specify).....
75. WHO clinical staging	.....
76. Drug Adherence at current visit (Pill count) 76.1. ART ..... 76.2. Cotrimoxazole/Dapsone .....	

END OF BASELINE SOCIO-ECONOMIC QUESTIONNAIRE



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**DIETARY ASSESSMENT QUESTIONNAIRE FOR MOTHERS (MAB-Q3-A)**

Date of interview:

--	--	--	--	--	--	--	--

**Study visit/Time point .....****Name of interviewer: .....**34<sup>2</sup>. Has your appetite changed since your last visit?

[0] NO (skip 34.1) [1] YES

34.1<sup>2</sup> If **Yes** has it increased or decreased?

[1] Increased [2] Decreased

35. Are there specific foods that you currently do not eat/avoid?

[0] NO (skip to Qn. 36 ) [1] YES

35.1. If yes, Please list the foods and reason why

Food	Reason for avoidance
35.1.1. Food 1:	35.1.2. Reason 1:
35.2.1. Food 2:	35.2.2. Reason 2:
35.3.1. Food 3:	35.3.2. Reason 3:

36. Are there specific foods you are eating which you weren't eating before?

[0] NO (skip to 37) [1] YES

36.1. If yes, Please list the foods and reason why

Food	Reason for inclusion
36.1.1. Food 1:	36.1.2. Reason 1:
36.2.1. Food 2:	36.2.2. Reason 2:
36.3.1. Food 3:	36.3.2. Reason 3:

<sup>2</sup> Adapted from the South African MRC Food Frequency Questionnaire (FFQ) used by Hamill, 2013

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37<sup>2</sup>. Are you on special diet/herbal products e.g. that have been prescribed for you by a doctor, or one that you have adopted from someone e.g. a TV star/magazine/friends?

[0] NO (skip to section B)

[1] YES

37.1<sup>2</sup>. If **YES**, Where did you get this advice to change your diet (tick all that apply)?

- [1] Clinic/Hospital  
 [2] Friend/Family  
 [3] Magazine/TV/radio/internet/newspaper  
 [4] My own decision  
 [5] Other – please write in:

37.2<sup>2</sup>. Describe what kind of diet you are on and list the food/drinks that are included or exclude from your diet:[1] food/drinks **ADDED** to the diet: .....[2] food/drinks **EXCLUDED** from the diet: .....37.3<sup>2</sup>. How long have you been on that diet?.....  
(weeks/months/years).

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**SECTION B: FOOD FREQUENCY QUESTIONNAIRE** (Please tick in appropriate column)

Q71. I would like to ask you about how you have been eating from the time you discovered that you are pregnant up to now. I have a list of some foods which I would like to know whether you eat them or not and how often you eat these foods

	Food group	Selected local foods	YES = 1 NO = 0	Eaten Everyday ...../day	Eaten every week ...../week	Eaten occasionally	Never eaten
1	Milk and Milk products	Yoghurt and fresh, dried, tinned cow, sheep, goat milk (milk from any mammal)					
2	Butter and Cheese	Butter, Cheese,					
3	Margarine from Vegetable Oil	Blue band					
4	Small fish eaten whole with bones	Mukene, Enkejje					
5	Large whole fish/dried fish/shellfish, eaten without bones	Fresh, dried or fried- Ngege/Tilapia, Nile perch (emputa) lungfish					
6	Meats and Poultry	Beef, pork, veal, lamb, goat, game meat; Chicken, duck, turkey, pigeon, guinea hen, game birds					
7	Fish/Meat/Poultry eaten with bones	Soft bones of chicken, beef or deep fried fish etc					
8	Grasshoppers and termites	Grasshopper (Nsenene), termites (nkuyege), white ants (enswa)					
9	Special cooked clay (emubwa)	Emubwa					
10	Nuts and seeds	Simsim, Amaranth seeds, sunflower seeds					
11	Cooked dry beans and peas	Black beans, pinto beans, white beans, mung beans, cranberry (kanyewa); cowpeas					
12	Soya and Soya products	Soya beans, soya flour, Corn soya blend					
13	Organ meat	livers, kidneys, intestines, heart, other organ meats or blood based foods					
14	Eggs	From any bird					
15	Dark green leafy vegetables *	Nakati, Ensugga, green dodo, cowpeas leaves (egobe), sukuma wiki, okra, spinach, pumpkin leaves, etc					

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**SUNLIGHT EXPOSURE AND PHYSICAL ACTIVITY QUESTIONNAIRE**  
(MAB-Q4)

Vitamin D is important for keeping the bones strong and our skin is able to make it when exposed to the sunlight. To help us understand you and your baby's vitamin D status measured in the blood sample you have provided, we would like to ask some questions about your and your infant's habitual sunlight exposure. Please consider times when you and your infant were out together, and when your infant may have been outside with another family member or babysitter. Also think about your daily lives particularly in the last two weeks.

Study ID: MAB..... Date SEQ completed.....

Name of Study staff completing the SEQ:.....

**A. General sunlight exposure**

53. How often were you outdoors during the last two weeks?

## 53.1 Mother

- ☐ Never  
☐ Less than once a week  
☐ 1-2 times a week  
☐ Half the week  
☐ Every day

54. At what time of the day were you outside during the last 2 weeks?

## 54.1 Mother

- ☐ Morning 07:00 to 10:00  
☐ Middle of the day 10:00-15:00  
☐ Late afternoon 15:00-17:00  
☐ Evening 17:00-20:00

55. How long did you stay outside during the last 2 weeks?

## 55.1 Mother

- ☐ Less than 30 minutes  
☐ 30 minutes to 1 hour  
☐ 1-2 hours  
☐ More than 2 hours

56. When you were outside during the last two weeks, did you: Please tick

- ☐ Try to avoid staying in direct sunshine (i.e. seeking shade)  
☐ Stay sometimes in the direct sunshine  
☐ Stay often in direct sunshine

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**2. Skin-type and Clothing**

58. Please rate the mother's skin colour

58.1 Mother

- ☐ Very dark  
☐ Dark  
☐ Light  
☐ Very light

59. Do you apply skin lightening agents?

59.1 Mother

- ☐ Yes  
☐ No

61. When outside during the last two weeks, what did you mostly wear:  
 (Please tick all that apply to make one complete attire commonly worn)

61.1 Mother

- ☐ Head scarf/Hijab/veil  
☐ Long sleeves blouses  
☐ Short sleeved blouses  
☐ Sleeveless blouses  
☐ Long Skirts or trousers  
☐ Long Tunics/religious wear  
☐ Stockings and/or gloves  
☐ Short/Knee height skirts

62. Which parts of your body were normally exposed to sunshine while outside within the past 2 weeks (tick all that apply)

62.1 Mother

- ☐ Arms  
☐ Neck  
☐ Legs  
☐ Face  
☐ Head



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**5. Physical activities**

Now I would like to ask your routine activities outside and inside the home to understand how active you have been over the past month. This will help us to better understand your bone, diet and nutrition outcomes observed in this study.

69. Have you been sick within the past one month?

- ☐ Yes  
☐ No (skip to 70)

69.1. Were you bedridden or unable to do your daily activities during the past one month?

- ☐ Yes  
☐ No (skip to 70)

69.2 How long were you/have you been bedridden?



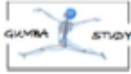
- ☐ Less than 3 days  
☐ 4- 7 days  
☐ 1-2 weeks  
☐ More than 2 weeks

70. Which of the following activities have you engaged in within the past one month?

Activity	During the last two weeks		
	Never	Once/ twice per week	Every day
Cleaning the house/compound, windows etc.			
Hand washing utensils and scrubbing pans			
Hand washing clothes			
Ironing Clothes			
Walking for at least 30 minutes			
Carrying the baby while walking outdoors			
Gardening/digging/ Pounding food			
Carrying/lifting heavy things inside or outside the house			
Playing Sports/games Specify .....			

**Thank for completing this questionnaire!**

## Appendix 7: PP2 questionnaire Pack

<div style="text-align: center;">  <div style="display: flex; justify-content: space-around; align-items: center;"> <div>OFFICIAL – SENSITIVE</div> <div style="border: 1px dashed black; padding: 2px 10px;">Place PID Label Here</div> </div>  </div> <p style="text-align: center; margin-top: 20px;"><b>QUESTIONNAIRE PACK FOR PP2 VISIT – COVER PAGE</b></p> <p>DATE COMPLETED: ____/____/____ (dd/mm/yy)</p> <p><b>MOTHER'S DETAILS:</b></p> <p>Mother's Study No: <span style="border: 1px dashed black; display: inline-block; width: 100px; height: 20px; vertical-align: middle;"></span></p> <p>Mother's D.O.B: ____/____/____ (dd/mm/yy)</p> <p>Residence: .....</p> <p><b>BABY'S DETAILS:</b></p> <p>Baby's Study Number: <span style="border: 1px dashed black; display: inline-block; width: 100px; height: 20px; vertical-align: middle;"></span></p> <p>Baby's D.O.B: ____/____/____ (dd/mm/yy)</p> <p>Sex:    <input type="checkbox"/> Male        <input type="checkbox"/> Female</p> <p>Birth Weight: ____ . ____ Kg</p> <p>Mode of delivery:    <input type="checkbox"/> Normal    <input type="checkbox"/> Caesarian section    <input type="checkbox"/> Other (use F010)</p> <p>NEXT STUDY APPOINTMENT (PP14): Date ____/____/____</p> <p>NAME OF INTERVIEWER: ..... SIGNATURE .....</p> <p style="font-size: small;">PP2 questionnaire. Version 2.0. Dated 11<sup>th</sup> May, 2015 <span style="float: right;">Page 1 of 11</span></p> <p style="font-size: x-small;">QC1: Initials..... Date .....      QC2: Initials..... Date .....</p>	<div style="text-align: center;">  <div style="display: flex; justify-content: space-around; align-items: center;"> <div>OFFICIAL – SENSITIVE</div> <div style="border: 1px dashed black; padding: 2px 10px;">Place PID Label Here</div> </div> </div> <p style="text-align: center; margin-top: 10px;"><b>FOLLOW-UP SOCIO-DEMOGRAPHIC AND QUESTIONNAIRE (MAB-Q2)</b></p> <p>Date of interview: <span style="border: 1px solid black; display: inline-block; width: 60px; height: 20px; vertical-align: middle;"></span></p> <p>Study Visit/ Time Point ..... Name of interviewer: .....</p> <p><b>SECTION A: ANTHROPOMETRY</b></p> <p><b>A1: MOTHER</b></p> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 30%;">A1. Weight</td> <td style="width: 10%; text-align: center;">****</td> <td style="width: 10%; text-align: center;">****</td> <td style="width: 10%; text-align: center;">****</td> <td style="width: 10%; text-align: center;">*</td> <td style="width: 10%; text-align: center;">****</td> <td style="width: 10%; text-align: right;">kg</td> </tr> <tr> <td>A2. Height</td> <td style="text-align: center;">****</td> <td style="text-align: center;">****</td> <td style="text-align: center;">****</td> <td style="text-align: center;">*</td> <td style="text-align: center;">****</td> <td style="text-align: right;">cm</td> </tr> <tr> <td>A3. Mid-Upper Arm Circumference (MUAC)</td> <td style="text-align: center;">****</td> <td style="text-align: center;">****</td> <td style="text-align: center;">****</td> <td style="text-align: center;">*</td> <td style="text-align: center;">****</td> <td style="text-align: right;">cm</td> </tr> </table> <p><b>A2: BABY</b></p> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 30%;">2.1 Weight</td> <td style="width: 10%; text-align: center;">****</td> <td style="width: 10%; text-align: center;">****</td> <td style="width: 10%; text-align: center;">*</td> <td style="width: 10%; text-align: center;">****</td> <td style="width: 10%; text-align: right;">kg</td> </tr> <tr> <td>2.2. Length</td> <td style="text-align: center;">****</td> <td style="text-align: center;">****</td> <td style="text-align: center;">*</td> <td style="text-align: center;">****</td> <td style="text-align: right;">cm</td> </tr> <tr> <td>2.4. Head Circumference</td> <td style="text-align: center;">****</td> <td style="text-align: center;">****</td> <td style="text-align: center;">*</td> <td style="text-align: center;">****</td> <td style="text-align: right;">Cm</td> </tr> </table> <div style="border: 1px solid black; padding: 5px; margin-top: 10px;"> <p>3. Have you changed residence since last visit?    <input type="checkbox"/> YES    <input type="checkbox"/> NO (skip Qn 3.1)</p> <p>3.1. If YES New residence.....</p> </div> <p style="font-size: small; margin-top: 10px;"><b>PLEASE GIVE THE MOTHER A HOT DRINK AT THIS POINT BEFORE YOU CONTINUE WITH THE QUESTIONNAIRE. THIS WILL HELP IN COLLECTION OF BREASTMILK SAMPLES</b></p> <p style="font-size: x-small;">PP2 questionnaire. Version 2.0. Dated 11<sup>th</sup> May, 2015 <span style="float: right;">Page 2 of 11</span></p> <p style="font-size: x-small;">QC1: Initials..... Date .....      QC2: Initials..... Date .....</p>	A1. Weight	****	****	****	*	****	kg	A2. Height	****	****	****	*	****	cm	A3. Mid-Upper Arm Circumference (MUAC)	****	****	****	*	****	cm	2.1 Weight	****	****	*	****	kg	2.2. Length	****	****	*	****	cm	2.4. Head Circumference	****	****	*	****	Cm
A1. Weight	****	****	****	*	****	kg																																		
A2. Height	****	****	****	*	****	cm																																		
A3. Mid-Upper Arm Circumference (MUAC)	****	****	****	*	****	cm																																		
2.1 Weight	****	****	*	****	kg																																			
2.2. Length	****	****	*	****	cm																																			
2.4. Head Circumference	****	****	*	****	Cm																																			

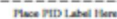
**SECTION C: MOTHER'S CLINICAL/MEDICAL HISTORY**

PP2 questionnaire, Version 2.0, Dated 11<sup>th</sup> May, 2015

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QC1: Initials \_\_\_\_\_ Date \_\_\_\_\_

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**SECTION D: CHILD'S CLINICAL/ MEDICAL HISTORY**

PP2 questionnaire. Version 2.0. Dated 11<sup>th</sup> May, 2015

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immunization card/observe)	
27. Has your baby been on multivitamins or a vitamin-mineral mix since the last visit? If Y: 27.1. List preparations: _____ 27.2. Dose/number of tablets: _____	NO <input type="checkbox"/> (skip to 28) YES <input type="checkbox"/>
28. Has your baby taken other drugs since your last visit? (other than ARVs, and multivites) If Y: 28.1. List preparations: _____ 28.2. Dose/number of tablets: _____ 28.3. Number of months taken: _____ 28.4. Why did your baby take these drugs? [1] ARV prophylaxis [2] Confirmed HIV positive [3] TB [4] Malaria [5] Other specify: _____	NO <input type="checkbox"/> (skip to 29) YES <input type="checkbox"/>
29. Did your baby have a major illness since last visit? If Y, list what & dates 29.1. Illness 1: _____ 29.1.1 Dates: _____ 29.2. Illness 2: _____ 29.2.1 Dates: _____	NO <input type="checkbox"/> (skip to 30) YES <input type="checkbox"/>

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QC1: Initials: \_\_\_\_\_ Date: \_\_\_\_\_

QC2: Initials: \_\_\_\_\_ Date: \_\_\_\_\_



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## CLINICAL CHART REVIEW FORM FOR TRR MOTHERS (MAB-F4-A)

PMTCT NO. .... Mother's Study NO. ....

Date: ...../...../.....

Time point: .....

71. Current (most recent) CD4 count and date	CD4 count: ..... Date: .....
72. Current (most recent) HIV Viral loads	Viral load: ..... Date: .....
73. Bactrim (PCP) prophylaxis (Cotrimoxazole or Dapsone)	NO <input type="checkbox"/> YES <input type="checkbox"/>
74. Has the participant changed ARV combination since last visit? 74.1 If yes, New combination: ..... (If changed regimen inform PI) 74.2. Start date: ..... 74.3. Reason for change in ART regimen	NO <input type="checkbox"/> (skip to 75) YES <input type="checkbox"/> [1] Substitution due to side effects [2] Switch to second line (failed 1 <sup>st</sup> line) [3] Other (specify): .....
75. WHO clinical staging	.....
76. Drug Adherence at current visit (Pill count) 76.1. ART: ..... 76.2. Cotrimoxazole/Dapsone: .....	

Other diagnoses and/or drugs of relevance to the study (Use General Notes form (F010)): .....

PP2 questionnaire. Version 2.0. Dated 11<sup>th</sup> May, 2015

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QC1: Initials: \_\_\_\_\_ Date: \_\_\_\_\_

QC2: Initials: \_\_\_\_\_ Date: \_\_\_\_\_



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## CLINICAL CHART REVIEW FORM FOR EXPOSED INFANTS (MAB-F4-B)

Infant's EID NO. ....

Infant's Study NO. ....

Date: ...../...../.....

Time point.....

77. Most recent HIV result (DBS/serology according testing algorithm)	Y <input type="checkbox"/> N <input type="checkbox"/>
Result (0=negative, 1=positive)	0 <input type="checkbox"/> 1 <input type="checkbox"/>
78. On ARV prophylaxis (NVP syrup)	Y <input type="checkbox"/> N <input type="checkbox"/>
If yes, Start only.....	
If yes Adherence.....	
If No, stop date.....	
79. Baby on PCP (Cotrimoxazole) prophylaxis	Y <input type="checkbox"/> N <input type="checkbox"/>
Adherence at current visit .....	
80. Baby on HAART	Y <input type="checkbox"/> N <input type="checkbox"/>
If yes,	
Date of initiation.....	

Other diagnoses and/or drugs of relevance to the study (Use general notes form (F010))

<b>Data extracted from file by:</b>	<b>Data Entered by:</b>
Name.....	Name.....
Signature.....	Signature.....
Date.....	Date.....
Time .....	Time .....



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## SECTION B: FOOD FREQUENCY QUESTIONNAIRE FOR MOTHERS (Please tick in appropriate column)

Q71. I would like to ask you about the foods and drinks that you have been eating in the past TWO weeks since you delivered. I have a list of some foods which I would like to know whether you eat them or not and how often you eat these foods

	Food group	Selected local foods	YES = 1 NO = 0	Eaten Everyday ...../day	Eaten every week ...../week	Eaten occasionally	Never eaten
1	Milk and Milk products	Yoghurt and fresh, dried, tinned cow, sheep, goat milk (milk from any mammal)					
2	Butter and Cheese	Butter, Cheese,					
3	Margarine from Vegetable Oil	Blue band					
4	Small fish eaten whole with bones	Mukene, Enkeje					
5	Large whole fish/dried fish/shellfish, eaten without bones	Fresh, dried or fried Ngege/Tilapia, Nile perch (emputa) lungfish					
6	Meats and Poultry	Beef, pork, veal, lamb, goat, game meat; Chicken, duck, turkey, pigeon, guinea hen, game birds					
7	Fish/Meat/Poultry eaten with bones	Soft bones of chicken, beef or deep fried fish etc.					
8	Grasshoppers and termites	Grasshopper (Nsenene), termites (nkuyege), white ants (enswa)					
9	Special cooked clay (emubwa)	Emubwa					
10	Nuts and seeds	Simsim, Amaranth seeds, sunflower seeds					
11	Cooked dry beans and peas	Black beans, pinto beans, white beans, mung beans, cranberry (kanyewa), cowpeas					
12	Soya and Soya products	Soya beans, soya flour, Corn soya blend					
13	Organ meat	livers, kidneys, intestines, heart, other organ meats or blood based foods					
14	Eggs	From any bird					
15	Dark green leafy vegetables *	Nakati, Ensugga, green dodo, cowpeas leaves (egobe), sukuma wiki, okra, spinach, pumpkin leaves, etc					



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**BREASTFEEDING QUESTIONNAIRE (MAB-Q3-B)**

[To be completed at 2 weeks postpartum on study visit PP2]

38. After you delivered, how long did you take before initiate breastfeeding for this baby?

- [1] Immediately after birth
- [2] Within first 1 hour
- [3] After one hour but within 1 day
- [4] After 1 day
- [5] Decided not to breastfeed

39. How many days after delivery did you get full breasts (did your breast milk 'come')  
..... days

40. Did you express out the first thick yellowish breast milk (colostrum) before you started breastfeeding?

- [0] NO (skip 40.1)
- [1] YES

40.1. Did you give the expressed thick yellowish breast milk (colostrum) to your baby?

- [0] NO
- [1] YES

41. Have you or anyone else given other feeds (not breast milk) to your baby since birth?

- [0] NO (skip to 42)
- [1] YES

41.1. If yes, what was your baby given?

- [1] Plain water
- [2] Glucose water
- [3] Cow's milk
- [4] Infant formula
- [5] Local herbs

41.2. Reason for giving other feeds

- [1] Breast milk delayed
- [2] Breast milk not enough
- [3] It is a tradition/custom
- [4] Mother sick
- [5] Other specify.....

PP2 questionnaire. Version 2.0. Dated 11<sup>th</sup> May, 2015

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QC1: Initials..... Date .....

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Now am going to ask you about how you fed your baby yesterday.

42. Did you breastfeed your baby or was your baby fed breast milk yesterday?  
[0] NO [1] YES (skip 42.1)

42.1. Why didn't you breastfeed your baby yesterday?

- [1] Stopped breastfeeding (skip to 46)
- [2] Baby sick
- [3] Mother was away
- [4] Mother has breast problems, specify.....

43. Do you have any problems breastfeeding your baby?

- [1] No
- [2] Breasts painful (cracked nipples, engorgement)
- [2] Breast infection in one breast (Request to observe)
- [3] Do not have enough breast milk

44. How many times did you breastfeed your baby yesterday (day and night)?

- [1] 1-2 times
- [2] 3-4 times
- [3] 5-6 times
- [4] 7-8 times
- [5] More than 8 times/on demand

45. Did your baby breastfeed normally, more or less yesterday?

- [1] Normally (Skip to 46)
- [2] More than usual (Go to 45.1)
- [3] Less than usual (Go to 45.2)

45.1. What is the main reason why your baby breastfed more yesterday?

- [1] Offered breast more times/ was at home
- [2] Recovering from illness
- [3] More hungry
- [4] Stayed at home with the baby

45.2. What is the main reason why your baby breastfed less yesterday?

- [1] Mother was busy
- [2] Baby was sick, specify illness.....

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QC1: Initials..... Date .....

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- [3] Mother was sick, specify illness.....  
[4] Mother was away  
[4] Baby refused to breastfeed  
[5] Other specify.....

46. Have you introduced your baby to food/drinks other than breast milk (including plain water, gripe water)?

[0] NO (end infant diet assessment here) [1] YES

47. If yes, when did you introduce these foods? .....DD/MM/YYYY

48. Please list for me the food and drinks that your baby consumed yesterday starting in the morning and throughout the night (24 hours)

Breakfast	Snack	Lunch	Snack	Supper	Snack

53. Please ask the mother to put the baby on the breast and you observe the following:

53.1. Positioning of baby at breast [1] Good [2] Poor (support the mother)




53.1. Attachment of the baby to the breast [1] Good [2] Poor (support the mother)

**N.B**

1. Please collect breast milk samples at this point into 5ml Z/S tubes (5ml per breast) and complete section B of form f005

2. Escort mother-infant pair for DXA

## Appendix 8: PP14 questionnaire Pack

<div style="text-align: center;">  <div style="display: flex; justify-content: space-around; align-items: center;"> <div>OFFICIAL - SENSITIVE</div> <div style="border: 1px dashed black; padding: 2px 10px;">Place PID Label Here</div> </div>  </div> <p style="text-align: center; margin-top: 20px;"><b>QUESTIONNAIRE PACK FOR PP14 VISIT – COVER PAGE</b></p> <p>DATE COMPLETED: <input type="text"/>/ <input type="text"/>/ <input type="text"/> (dd/mm/yy)</p> <p><b>MOTHER'S DETAILS:</b></p> <p>Mother's Study No: <input style="width: 100px;" type="text"/></p> <p>Mother's D.O.B: <input type="text"/>/ <input type="text"/>/ <input type="text"/> (dd/mm/yy)</p> <p>Residence: .....</p> <p><b>BABY'S DETAILS:</b></p> <p>Baby's Study Number: <input style="width: 100px;" type="text"/></p> <p>Baby's D.O.B: <input type="text"/>/ <input type="text"/>/ <input type="text"/> (dd/mm/yy)</p> <p>Sex: <input type="checkbox"/> Male <input type="checkbox"/> Female</p> <p>NEXT STUDY APPOINTMENT (PP16): Date <input type="text"/>/ <input type="text"/>/ <input type="text"/></p> <p>NAME OF INTERVIEWER: ..... SIGNATURE .....</p> <div style="display: flex; justify-content: space-between; font-size: small; margin-top: 20px;"> <span>PP14 questionnaire. Version 2.0. Dated 11<sup>th</sup> May, 2015</span> <span>Page 1 of 15</span> </div> <div style="display: flex; justify-content: space-between; font-size: x-small; margin-top: 10px;"> <span>QC1: Initials..... Date .....</span> <span>QC2: Initials..... Date .....</span> </div>	<div style="text-align: center;">  <div style="display: flex; justify-content: space-around; align-items: center;"> <div>OFFICIAL - SENSITIVE</div> <div style="border: 1px dashed black; padding: 2px 10px;">Place PID Label Here</div> </div> </div> <p style="text-align: center; margin-top: 10px;"><b>FOLLOW-UP SOCIO-DEMOGRAPHIC AND QUESTIONNAIRE (MAB-Q2)</b></p> <p>Date of interview: <input type="text"/>/ <input type="text"/>/ <input type="text"/></p> <p>Study Visit/ Time Point ..... Name of interviewer: .....</p> <p><b>SECTION A: ANTHROPOMETRY</b></p> <p><b>A1: MOTHER</b></p> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 30%;">A1. Weight</td> <td style="width: 10%; text-align: center;">****</td> <td style="width: 10%; text-align: center;">****</td> <td style="width: 10%; text-align: center;">****</td> <td style="width: 10%; text-align: center;">*</td> <td style="width: 10%; text-align: center;">****</td> <td style="width: 10%; text-align: right;">kg</td> </tr> <tr> <td>A2. Height</td> <td style="text-align: center;">****</td> <td style="text-align: center;">****</td> <td style="text-align: center;">****</td> <td style="text-align: center;">*</td> <td style="text-align: center;">****</td> <td style="text-align: right;">cm</td> </tr> <tr> <td>A3. Mid-Upper Arm Circumference (MUAC)</td> <td style="text-align: center;">****</td> <td style="text-align: center;">****</td> <td style="text-align: center;">****</td> <td style="text-align: center;">*</td> <td style="text-align: center;">****</td> <td style="text-align: right;">cm</td> </tr> </table> <p><b>A2: BABY</b></p> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 30%;">2.1 Weight</td> <td style="width: 10%; text-align: center;">****</td> <td style="width: 10%; text-align: center;">****</td> <td style="width: 10%; text-align: center;">*</td> <td style="width: 10%; text-align: center;">****</td> <td style="width: 10%; text-align: right;">kg</td> </tr> <tr> <td>2.2. Length</td> <td style="text-align: center;">****</td> <td style="text-align: center;">****</td> <td style="text-align: center;">*</td> <td style="text-align: center;">****</td> <td style="text-align: right;">cm</td> </tr> <tr> <td>2.4. Head Circumference</td> <td style="text-align: center;">****</td> <td style="text-align: center;">****</td> <td style="text-align: center;">*</td> <td style="text-align: center;">****</td> <td style="text-align: right;">Cm</td> </tr> </table> <div style="border: 1px solid black; padding: 5px; margin-top: 10px;"> <p>3. Have you changed residence since last visit? <input type="checkbox"/> YES <input type="checkbox"/> NO (skip Qn 3.1)</p> <p>3.1. If YES New residence.....</p> </div> <div style="display: flex; justify-content: space-between; font-size: small; margin-top: 20px;"> <span>PP14 questionnaire. Version 2.0. Dated 11<sup>th</sup> May, 2015</span> <span>Page 2 of 15</span> </div> <div style="display: flex; justify-content: space-between; font-size: x-small; margin-top: 10px;"> <span>QC1: Initials..... Date .....</span> <span>QC2: Initials..... Date .....</span> </div>	A1. Weight	****	****	****	*	****	kg	A2. Height	****	****	****	*	****	cm	A3. Mid-Upper Arm Circumference (MUAC)	****	****	****	*	****	cm	2.1 Weight	****	****	*	****	kg	2.2. Length	****	****	*	****	cm	2.4. Head Circumference	****	****	*	****	Cm
A1. Weight	****	****	****	*	****	kg																																		
A2. Height	****	****	****	*	****	cm																																		
A3. Mid-Upper Arm Circumference (MUAC)	****	****	****	*	****	cm																																		
2.1 Weight	****	****	*	****	kg																																			
2.2. Length	****	****	*	****	cm																																			
2.4. Head Circumference	****	****	*	****	Cm																																			





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## SECTION C: MOTHER'S CLINICAL/MEDICAL HISTORY

11. Repeat HIV test since last visit (if recent test > 6 months old)	NO <input type="checkbox"/> (skip 11.1) YES <input type="checkbox"/>							
11.1. HIV test Result (0=negative, 1=positive) ..... (If newly diagnosed positive inform PI or study coordinator)	NEGATIVE <input type="checkbox"/> POSITIVE <input type="checkbox"/>							
13. Pregnancy test done at current visit (to be done at all postpartum visits except at 2 weeks)	NO <input type="checkbox"/> (skip 13.1) YES <input type="checkbox"/>							
13.1. Pregnancy test Result (0=negative, 1=positive) (If positive inform PI - exclude from DXA measurements)	NEGATIVE <input type="checkbox"/> POSITIVE <input type="checkbox"/>							
15. Have you resumed your menses/periods?	NO <input type="checkbox"/> (skip 15.1) YES <input type="checkbox"/>							
15.1. If YES, when?	<table border="1"><tr><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr></table> (dd/mm/yyyy)							
17. Have you started using a family planning method since your last visit?	NO <input type="checkbox"/> (skip to 18) YES <input type="checkbox"/>							
17.1 If Y, which method? (tick all that apply):	[1] Pills, specify ..... [2] Depo provera injection [3] Condoms [4] Natural method (s) [5] Other specify.....							
17.2. When did you start using this method?	<table border="1"><tr><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr></table> (dd/mm/yyyy)							

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QC1: Initials..... Date .....

QC2: Initials..... Date .....



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18. Current smoker	NO <input type="checkbox"/> (skip to 19) YES <input type="checkbox"/>
18.1. If Yes, how many cigarettes do you smoke per day? .....	
19. Have you had a <b>New</b> fracture since last visit?	NO <input type="checkbox"/> (skip to Qn.20) YES <input type="checkbox"/>
19.1. If YES, on which part of your body?	[1] Hip [2] Arm-radius (s) [3] Leg (s) [4] Back [5] Other, specify
19.2. How was it caused?	[1] Car, taxi, motorbike or cycle crash [2] Fall down the stairs [3] Fall from object - ladder/box /stool [4] Standing up [5] Other, specify.....
20. Have you been on calcium supplements since last visit?	NO <input type="checkbox"/> (skip to 21) YES <input type="checkbox"/>
If YES	
20.1. List preparations.....	
20.2. Dose/number of tablets.....	
21. Have you been on vitamin D supplements since last visit?	NO <input type="checkbox"/> (skip to 22) YES <input type="checkbox"/>
If YES,	
21.1. List preparations.....	
21.2. Dose/number of tablets.....	
22. Have you been on any multivitamins or a vitamin-mineral mix since last visit?	NO <input type="checkbox"/> (skip to 23) YES <input type="checkbox"/>
If Y	
22.1. List preparations.....	

PP14 questionnaire. Version 2.0. Dated 11<sup>th</sup> May, 2015

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QC1: Initials..... Date .....

QC2: Initials..... Date .....



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<p>22.3. Dose/number of tablets_____</p>	
<p>23. Have you taken other drugs since your last visit? (other than ARVs, and those already mentioned above)</p> <p>If Y:</p> <p>23.1. List preparations_____</p> <p>23.2. Dose/number of tablets_____</p> <p>23.3. Number of months taken_____</p> <p>23.4. Why did you take these drugs?</p>	<p>NO <input type="checkbox"/> (skip to 24) YES <input type="checkbox"/></p> <p>[1] As part of TB treatment          [2] To treat cancer          [3] To treat low blood platelets          [4] Hepatitis B          [5] Asthma          [6] Serious skin condition          [7] Other specify_____</p>
<p>24. Have you had a major illness since last visit</p> <p>If Y, list what &amp; dates</p> <p>24.1. Illness 1. _____</p> <p>24.1.1 Dates: _____</p> <p>24.2. Illness 2: _____</p> <p>24.2.1. Dates: _____</p> <p>24.3. For current diarrhoea/vomiting, note the no. of episodes per day .....</p>	<p>NO <input type="checkbox"/> (skip to 25) YES <input type="checkbox"/></p>



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**SECTION D: CHILD'S CLINICAL/ MEDICAL HISTORY**

25. Immunization record up-to-date (check immunization card) – If Not up to date refer for immunization on the study visit day	NO <input type="checkbox"/> YES <input type="checkbox"/>
26. Attaining developmental milestones (check immunization card/observe)	NO <input type="checkbox"/> YES <input type="checkbox"/>
27. Has your baby been on multivitamins or a vitamin-mineral mix since the last visit? If Y 27.1. List preparations _____ 27.2 Dose/number of tablets _____	NO <input type="checkbox"/> (skip to 28) YES <input type="checkbox"/>
28. Has your baby taken other drugs since your last visit? (other than NVP, and multivites) If Y: 28.1. List preparations and dose _____ 28.4. Why did your baby take these drugs?	NO <input type="checkbox"/> (skip to 29) YES <input type="checkbox"/>  _____ [1] ARV prophylaxis [2] Confirmed HIV positive [3] TB [4] Malaria [5] Other specify _____
29. Did your baby have a major illness since last visit? If Y, list what & dates 29.1. Illness 1. _____ 29.1.1 Dates: _____ Illness 2: _____	NO <input type="checkbox"/> (skip to 30) YES <input type="checkbox"/>



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## CLINICAL CHART REVIEW FORM FOR TRR MOTHERS (MAB-F4-A)

PMTCT NO. .... Mother's Study NO. ....

Date: ...../...../.....

Time point.....

71. Current (most recent) CD4 count and date	CD4 count: ..... Date: .....
72. Current (most recent) HIV Viral loads	Viral load: ..... Date: .....
73. Bactrim (PCP) prophylaxis (Cotrimoxazole or Dapsone)	NO <input type="checkbox"/> YES <input type="checkbox"/>
74. Has the participant changed ARV combination since last visit? 74.1 If yes, New combination ..... (If changed regimen inform PI) 74.2. Start date: ..... 74.3. Reason for change in ART regimen	NO <input type="checkbox"/> (skip to 75) YES <input type="checkbox"/> [1] Substitution due to side effects [2] Switch to second line (failed 1 <sup>st</sup> line) [3] Other (specify).....
75. WHO clinical staging	.....
76. Drug Adherence at current visit (Pill count) 76.1. ART ..... 76.2. Cotrimoxazole/Dapsone .....	

Other diagnoses and/or drugs of relevance to the study (Use General Notes form (F010)).....

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QC1: Initials..... Date .....

QC2: Initials..... Date .....



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## CLINICAL CHART REVIEW FORM FOR EXPOSED INFANTS (MAB-F4-B)

Infant's EID NO. ....

Infant's Study NO. ....

Date: ...../...../.....

Time point.....

77. Most recent HIV result (DBS/serology according testing algorithm) Result (0=negative, 1=positive)	Y <input type="checkbox"/> N <input type="checkbox"/> 0 <input type="checkbox"/> 1 <input type="checkbox"/>
78. On ARV prophylaxis (NVP syrup) If yes, Start only..... If yes Adherence..... If No, stop date.....	Y <input type="checkbox"/> N <input type="checkbox"/>
79. Baby on PCP (Cotrimoxazole) prophylaxis Adherence at current visit .....	Y <input type="checkbox"/> N <input type="checkbox"/>
80. Baby on HAART If yes, Date of initiation.....	Y <input type="checkbox"/> N <input type="checkbox"/>

Other diagnoses and/or drugs of relevance to the study (Use general notes form (F010))

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**SECTION B: FOOD FREQUENCY QUESTIONNAIRE FOR MOTHERS** (Please tick in appropriate column)

Q71. I would like to ask you about the foods and drinks that you have been eating in the past TWO weeks since you delivered. I have a list of some foods which I would like to know whether you eat them or not and how often you eat these foods

	Food group	Selected local foods	YES = 1 NO = 0	Eaten Everyday ...../day	Eaten every week ...../week	Eaten occasionally	Never eaten
1	Milk and Milk products	Yoghurt and fresh, dried, tinned cow, sheep, goat milk (milk from any mammal)					
2	Butter and Cheese	Butter, Cheese,					
3	Margarine from Vegetable Oil	Blue band					
4	Small fish eaten whole with bones	Mukene, Enkejje					
5	Large whole fish/dried fish/shellfish, eaten without bones	Fresh, dried or fried Ngege/Tilapia, Nile perch (emputa) lungfish					
6	Meats and Poultry	Beef, pork, veal, lamb, goat, game meat; Chicken, duck, turkey, pigeon, guinea hen, game birds					
7	Fish/Meat/Poultry eaten with bones	Soft bones of chicken, beef or deep fried fish etc.					
8	Grasshoppers and termites	Grasshopper (Nsenene), termites (nkuyegge), white ants (enswa)					
9	Special cooked clay (emubwa)	Emubwa					
10	Nuts and seeds	Simsim, Amaranth seeds, sunflower seeds					
11	Cooked dry beans and peas	Black beans, pinto beans, white beans, mung beans, cranberry (kanyewa), cowpeas					
12	Soya and Soya products	Soya beans, soya flour, Corn soya blend					
13	Organ meat	Livers, kidneys, intestines, heart, other organ meats or blood based foods					
14	Eggs	From any bird					
15	Dark green leafy vegetables *	Nakati, Ensugga, green dodo, cowpeas leaves (egobe), sukuma wiki, okra, spinach, pumpkin leaves, etc.					



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34<sup>1</sup>. Has your appetite changed since your last visit?  
[0] NO (skip 34.1) [1] YES

34.1<sup>c</sup> If **Yes** has it increased or decreased?  
[1] Increased [2] Decreased

35. Are there specific foods that you currently do not eat/avoid?  
[0] NO (skip to Qn. 36) [1] YES

35.1. If yes, Please list the foods and reason why

Food	Reason for avoidance
35.1.1. Food 1:	35.1.2. Reason 1:
35.2.1. Food 2:	35.2.2. Reason 2:
35.3.1. Food 3:	35.3.2. Reason 3:

36. Are there specific foods you are eating which you weren't eating before?  
[0] NO (skip to 37) [1] YES

36.1. If yes, Please list the foods and reason why

Food	Reason for inclusion
36.1.1. Food 1:	36.1.2. Reason 1:
36.2.1. Food 2:	36.2.2. Reason 2:
36.3.1. Food 3:	36.3.2. Reason 3:

37<sup>c</sup>. Are you on special diet/herbal products e.g. that have been prescribed for you by a doctor, or one that you have adopted from someone e.g. a TV star/magazine/friends?

[0] NO (skip to section B) [1] YES

37.1<sup>c</sup>. If **YES**, Where did you get this advice to change your diet (tick all that apply)?

- [1] Clinic/Hospital  
[2] Friend/Family  
[3] Magazine/TV/radio/internet/newspaper  
[4] My own decision  
[5] Other – please write in:

37.3<sup>c</sup>. How long have you been on that diet? ..... (Months)

<sup>1</sup> Adapted from the South African MRC Food Frequency Questionnaire (FFQ) used by Hamill, 2013



Place PID Label Here

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**DIETARY INTAKE QUESTIONNAIRE FOR CHILD (MAB-Q3-B)**

Now am going to ask you about how you have been feeding your baby since last visit

42. Did you breastfeed your baby or was your baby fed breast milk every day since the last visit?

- [0] NO [1] YES (skip 42.1)

42.1. Why didn't you breastfeed your baby?

- [1] Stopped breastfeeding (skip to 46) (**Urgently inform P1**)
- [2] Baby sick
- [3] Mother was away
- [4] Mother has breast problems, specify.....

43. Do you have any problems breastfeeding your baby?

- [1] No
- [2] Breasts painful (cracked nipples, engorgement)
- [2] Breast infection in one breast (Request to observe)
- [3] Do not have enough breast milk

44. How many times did you breastfeed your baby yesterday (day and night)?

- [1] 1-2 times
- [2] 3-4 times
- [3] 5-6 times
- [4] 7-8 times
- [5] More than 8 times/on demand
- [6] Stopped breastfeeding (**Urgently inform P1**)

45. Did your baby breastfeed normally, more or less yesterday?

- [1] Normally (Skip to 46)
- [2] More than usual (Go to 45.1)
- [3] Less than usual (Go to 45.2)

45.1. What is the main reason why your baby breastfed more yesterday?

- [1] Offered breast more times/ was at home
- [2] Recovering from illness
- [3] More hungry
- [4] Stayed at home with the baby



Place PID Label Here

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45.2. What is the main reason why your baby breastfed less yesterday?

- [1] Mother was busy
- [2] Baby was sick, specify illness.....
- [3] Mother was sick, specify illness.....
- [4] Mother was away
- [4] Baby refused to breastfeed
- [5] Other specify.....

46. Have you introduced your baby to food/drinks other than breast milk (including plain water, gripe water)?

- [0] NO (end infant diet assessment here) [1] YES

47. If yes, when did you introduce these foods? .....DD/MM/YYYY

48. Please list for me the food and drinks that your baby consumed yesterday starting in the morning and throughout the night (24 hours)

Breakfast	Snack	Lunch	Snack	Supper	Snack

**N.B**

**1. Please collect breast milk samples at this point into 5ml Z/5 tubes (5ml per breast) and complete section of form f005**



Place PID Label Here

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## SUNSHINE EXPOSURE AND PHYSICAL ACTIVITY QUESTIONNAIRE (MAB-Q4)

To help us understand you and your baby's vitamin D status measured in the blood sample you have provided, we would like to ask some questions about your and your infant's habitual sunlight exposure. Please consider times when you and your infant were out together, and when your infant may have been outside with another family member or babysitter. Also think about your daily lives particularly in the last two weeks.

### A. General sunlight exposure

53. How often were you and your infant outdoors during the last two weeks?

53.1 Mother

- ☐ Never  
☐ Less than once a week  
☐ 1-2 times a week  
☐ Half the week  
☐ Every day

53.2 Infant

- ☐ Never  
☐ Less than once a week  
☐ 1-2 times a week  
☐ Half the week  
☐ Every day

54. At what time of the day were you and your infant usually outside during the last 2 weeks?

54.1 Mother

- ☐ Morning 07:00 to 10:00  
☐ Middle of the day 10:00-15:00  
☐ Late afternoon 15:00-17:00  
☐ Evening 17:00-20:00

54.2 Infant

- ☐ Morning 07:00 to 10:00  
☐ Middle of the day 10:00-15:00  
☐ Late afternoon 15:00-17:00  
☐ Evening 17:00-20:00

55. How long did you and/or your baby usually stay outside during the last 2 weeks?

55.1 Mother

- ☐ Less than 30 minutes  
☐ 30 minutes to 1 hour  
☐ 1-2 hours  
☐ More than 2 hours

55.2 Infant

- ☐ Less than 30 minutes  
☐ 30 minutes to 1 hour  
☐ 1-2 hours  
☐ More than 2 hours

56. When you were outside during the last two weeks, did you: Please tick

- ☐ Try to avoid staying in direct sunshine (i.e. seeking shade)  
☐ Stay sometimes in the direct sunshine  
☐ Stay often in direct sunshine

57. When outside in the last two weeks, was your infant: Please tick

- ☐ Kept in the shade  
☐ Sometimes in the direct sunshine  
☐ Often in the direct sunshine



Place PID Label Here

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## 2. Skin-type and Clothing

58. Please rate the mother and infant's skin colour

58.1 Mother

- ☐ Very dark  
☐ Dark  
☐ Light  
☐ Very light

58.2 Infant

- ☐ Very Dark  
☐ Dark  
☐ Light  
☐ Very Light

59. Do you apply skin lightening agents?

59.1 Mother

- ☐ Yes  
☐ No

59.2 Infant

- ☐ Yes  
☐ No

60. Do you or your infant use sunscreen/sunblock or creams with sunscreen?

60.1. Mother

- ☐ Yes (If yes, SPF.....)  
☐ No  
☐ Unknown

60.2. Infant

- ☐ Yes (If yes, SPF.....)  
☐ No  
☐ Unknown

61. When outside during the last two weeks, what did you mostly wear:  
(Please tick all that apply to make one complete attire commonly worn)

61.1 Mother

- ☐ Head scarf/Hijab/veil  
☐ Long sleeves blouses  
☐ Short sleeved blouses  
☐ Sleeveless blouses  
☐ Long Skirts or trousers  
☐ Long Tunics/religious wear  
☐ Stockings and/or gloves  
☐ Short/Knee height skirts

61.2. Infant

- ☐ Wrapped in shawl  
☐ Vest and shorts  
☐ Long sleeves and trousers  
☐ Short sleeved dresses  
☐ Socks and/or gloves  
☐ Hat

62. Which parts of your body or your baby were normally exposed to sunshine while outside within the past 2 weeks (tick all that apply)

62.1. Mother

- ☐ Arms  
☐ Neck  
☐ Legs  
☐ Face  
☐ Head

62.2. Infant

- ☐ Arms  
☐ Neck  
☐ Legs  
☐ Face  
☐ Head



Place PID Label Here

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63. When outside, is your infant kept shaded/covered from the direct sunshine (e.g. using an umbrella, baby shawl etc.)?

- ☐ Yes  
☐ No

**5. Physical activities** (*This section applies only to the mother*)

Now I would like to ask your routine activities outside and inside the home to understand how active you have been over the past month. This will help us to better understand your bone, diet and nutrition outcomes observed in this study.

69. Have you been sick within the past one month?

- ☐ Yes  
☐ No (skip to 70)

69.1. Were you bedridden or unable to do your daily activities during the past one month?

- ☐ Yes  
☐ No (skip to 70)

69.2 How long were you/have you been bedridden?

- ☐ Less than 3 days  
☐ 4- 7 days  
☐ 1-2 weeks  
☐ More than 2 weeks

70. Which of the following activities have you engaged in within the past one month?

Activity	During the last two weeks		
	Never	Once/ twice per week	Every day
Cleaning the house/compound, windows etc.			
Hand washing utensils and scrubbing pans			
Hand washing clothes			
Ironing Clothes			
Walking for at least 30 minutes			
Carrying the baby while walking outdoors			
Gardening/digging/ Pounding food			
Carrying/lifting heavy things inside or outside the house			
Playing Sports/games Specify .....			

**Thank for completing this questionnaire!**

PP14 questionnaire. Version 2.0. Dated 11<sup>th</sup> May, 2015

Page 15 of 15

QC1: Initials..... Date .....


QC2: Initials..... Date .....



## Appendix 9: Two-hour fasted blood and urine collection form

<p>TWO-HOUR FASTED BLOOD &amp; URINE COLLECTION / URINALYSIS FORM – F006</p> <div style="text-align: right; margin-bottom: 10px;"> </div> <p>Study Number: MAB [ ] [ ] [ ] [ ] - [ ]      Date of Birth: [ ] [ ] - [ ] [ ] - [ ] [ ] [ ] [ ]</p> <p>Date [ ] [ ] / [ ] [ ] / [ ] [ ] [ ] [ ] (dd/mm/yyyy)      Study Visit [ ] [ ] [ ] [ ]</p>	<p>TWO-HOUR FASTED BLOOD &amp; URINE COLLECTION / URINALYSIS FORM – F006</p> <div style="text-align: right; margin-bottom: 10px;"> </div> <p>SECTION C: Urinalysis and HCG (Please use this section to report urinalysis and HCG results for participant)</p>																					
<p><b>SECTION A: Urine collection</b></p> <p>1.1. Is participant overnight fasted (at least 8 hours)? <input type="checkbox"/> YES <input type="checkbox"/> NO* [consult with PI/study nurse]</p> <p>1.2. Time start urine collection: [ ] [ ] [ ] [ ] (use 24 hour clock)</p> <p>1.3. Time end of urine collection: [ ] [ ] [ ] [ ] (use 24 hour clock)</p> <p>1.4. Volume of water drunk: [ ] [ ] [ ] [ ] ml</p> <p>1.5. Time of urine collection: Bucket 1: [ ] [ ] [ ] [ ] (use 24 hour clock) Bucket 2: [ ] [ ] [ ] [ ] (use 24 hour clock)</p> <p>1.6. Complete 2-hour fasted urine sample collected <input type="checkbox"/> YES <input type="checkbox"/> NO* [give reason below and inform PI]</p> <p>1.6b * Reason _____</p> <p>1.7. Urine collection done by ((Name, signature, date): _____)</p>	<p><b>Urinalysis Test used:</b> <input type="checkbox"/> Urinary dipstick <input type="checkbox"/> Urine Microscopy</p> <p><b>Results:</b></p> <table style="width: 100%; border: none;"> <tr> <td>Colour: _____</td> <td>Proteins: _____</td> <td>Pus cells: _____/hpf</td> </tr> <tr> <td>Clarity: _____</td> <td>Glucose: _____</td> <td>RBCs: _____/hpf</td> </tr> <tr> <td>Sp. Gravity: _____</td> <td>Ketones: _____</td> <td>Epith: _____/hpf</td> </tr> <tr> <td>PH: _____</td> <td>Urobil: _____</td> <td>Casts: _____/hpf</td> </tr> <tr> <td>Leucocytes: _____</td> <td>Bilirubin: _____</td> <td>Crystals: _____/hpf</td> </tr> <tr> <td>Nitrites: _____</td> <td>Erythrocytes: _____</td> <td>Yeasts: _____/hpf</td> </tr> <tr> <td></td> <td></td> <td>Orgni: _____/hpf</td> </tr> </table> <p><b>HCG Results:</b> <input type="checkbox"/> NEGATIVE <input type="checkbox"/> POSITIVE</p> <p>Tested By: [ ] [ ]      Date: [ ] [ ] - [ ] [ ] - [ ] [ ] [ ] [ ]      Time: [ ] [ ] [ ] [ ] hrs (24hr clock)</p> <p>Reviewed By: [ ] [ ]      Date: [ ] [ ] - [ ] [ ] - [ ] [ ] [ ] [ ]      Time: [ ] [ ] [ ] [ ] hrs (24hr clock)</p>	Colour: _____	Proteins: _____	Pus cells: _____/hpf	Clarity: _____	Glucose: _____	RBCs: _____/hpf	Sp. Gravity: _____	Ketones: _____	Epith: _____/hpf	PH: _____	Urobil: _____	Casts: _____/hpf	Leucocytes: _____	Bilirubin: _____	Crystals: _____/hpf	Nitrites: _____	Erythrocytes: _____	Yeasts: _____/hpf			Orgni: _____/hpf
Colour: _____	Proteins: _____	Pus cells: _____/hpf																				
Clarity: _____	Glucose: _____	RBCs: _____/hpf																				
Sp. Gravity: _____	Ketones: _____	Epith: _____/hpf																				
PH: _____	Urobil: _____	Casts: _____/hpf																				
Leucocytes: _____	Bilirubin: _____	Crystals: _____/hpf																				
Nitrites: _____	Erythrocytes: _____	Yeasts: _____/hpf																				
		Orgni: _____/hpf																				
<p><b>SECTION B: Blood collection:</b></p> <p>2.1. Has a 20ml Blood sample been collected? <input type="checkbox"/> YES <input type="checkbox"/> NO* [give reason below and inform PI]</p> <p>2.1b. * Reason _____</p> <p>2.2. Time of blood collection: [ ] [ ] [ ] [ ] (is this 2 hour after 'time start urine collection?')</p> <p>2.3. Blood Volumes drawn: SST/PST (4ml) [ ] [ ] [ ] [ ] ml LH 12ml (2X6ml) [ ] [ ] [ ] [ ] ml EDTA (1X4ml) [ ] [ ] [ ] [ ] ml</p> <p>2.4. Phlebotomist (Name, signature, date): _____</p>	<p><b>SECTION D: Laboratory Notes</b></p> <p><i>Please note here additional information that might be relevant to use of stored samples (e.g observed abnormalities, breakdowns in processing equipment, and any deviations from the SOP etc.) and interpretation of urinalysis results</i></p>   <p>Name of laboratory personnel: _____ Signature: _____ Date: _____</p>																					
<p>2hr Fasted BBU collection form. Version 1.2. Dated 01<sup>st</sup> Sept, 2015      Page 1 of 2</p> <p>QC1: Initials _____ Date _____      QC2: Initials _____ Date _____</p>	<p><b>SECTION E: Quality Control Check</b> (To be completed by lab supervisor, QC/QA or designated laboratory staff)</p> <table border="1" style="width: 100%; border-collapse: collapse; text-align: center;"> <tr> <th style="width: 25%;">Laboratory personnel signature</th> <th style="width: 25%;">Print name</th> <th style="width: 25%;">Date</th> <th style="width: 25%;">Time</th> </tr> <tr> <td style="height: 40px;"></td> <td></td> <td></td> <td></td> </tr> </table> <p>2hr Fasted BBU collection form. Version 1.2. Dated 01<sup>st</sup> Sept, 2015      Page 2 of 2</p> <p>QC1: Initials _____ Date _____      QC2: Initials _____ Date _____</p>	Laboratory personnel signature	Print name	Date	Time																	
Laboratory personnel signature	Print name	Date	Time																			

## Appendix 10: Breastmilk collection form



**BREAST MILK COLLECTION FORM – GUMBA F007**

Study Number: MAB [ ] [ ] [ ] [ ] - [ ]      Date of Birth: [ ] [ ] - [ ] [ ] - [ ] [ ] [ ] [ ]

Date [ ] [ ] / [ ] [ ] / [ ] [ ] [ ] [ ] (dd/mm/yyyy)      Study Visit [ ] [ ] [ ] [ ]

	LEFT BREAST	RIGHT BREAST
Breast milk sample collected <small>[* If No, Inform PI/study nurse]</small>	<input type="checkbox"/> YES <input type="checkbox"/> NO*	<input type="checkbox"/> YES <input type="checkbox"/> NO*
Time of previous feed	[ ] [ ] : [ ] [ ]	[ ] [ ] : [ ] [ ]
Volume of breast milk collected (1x5ml per breast): <small>[collect at least 4.0ml from each breast]</small>	[ ] [ ] . [ ] [ ] ml	[ ] [ ] . [ ] [ ] ml
Time sample collected:	[ ] [ ] : [ ] [ ]	[ ] [ ] : [ ] [ ]

\* Reason

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

Urine collected by ((Name, signature, date)): \_\_\_\_\_

\_\_\_\_\_

Tracer water dosing/breast milk collection form  
Version Dated 04 Jan 2016


Page 1 of 1

QC1: Initials..... Date .....

QC2: Initials..... Date .....

## Appendix 11: Laboratory sample processing and sample storage forms

### LABORATORY REQUEST AND STORAGE FORM (MOTHER) – F004



Study No: \_\_\_\_\_

DOB: \_\_\_\_\_

Sample No: \_\_\_\_\_


Study Visit:	<input type="checkbox"/> PG36	<input type="checkbox"/> PP2	<input type="checkbox"/> PP10	<input type="checkbox"/> PP14	<input type="checkbox"/> PP26	<input type="checkbox"/> PP52	<input type="checkbox"/> NPPL
Deuterium Urine Day	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

**Section A: Specimens and Test Type (Tick all that apply for the study visit as per the protocol):**

Specimen type and volumes	Collected By	Delivery By	Received By	Processed By	Stored By
<input type="checkbox"/> LH Tube (2X6ml)	Date: _____	Date: _____	Date: _____	Date: _____	Date: _____
<input type="checkbox"/> SST Tube (1X4ml)	Time: _____ By: _____	Time: _____ By: _____	Time: _____ By: _____	Time: _____ By: _____	Time: _____ By: _____
<input type="checkbox"/> EDTA tube (1X4ml)	Date: _____	Date: _____	Date: _____	Date: _____	Date: _____
<input type="checkbox"/> Urine 2 hour fasted	Time: _____ By: _____	Time: _____ By: _____	Time: _____ By: _____	Time: _____ By: _____	Time: _____ By: _____
<input type="checkbox"/> Day 0 DTTM urine	Date: _____	Date: _____	Date: _____	Date: _____	Date: _____
<input type="checkbox"/> Day 1 DTTM urine	Time: _____ By: _____	Time: _____ By: _____	Time: _____ By: _____	Time: _____ By: _____	Time: _____ By: _____
<input type="checkbox"/> Day 2 DTTM urine	Date: _____	Date: _____	Date: _____	Date: _____	Date: _____
<input type="checkbox"/> Day 3 DTTM urine	Time: _____ By: _____	Time: _____ By: _____	Time: _____ By: _____	Time: _____ By: _____	Time: _____ By: _____
<input type="checkbox"/> Day 4 DTTM urine	Date: _____	Date: _____	Date: _____	Date: _____	Date: _____
<input type="checkbox"/> Day 13 DTTM urine	Time: _____ By: _____	Time: _____ By: _____	Time: _____ By: _____	Time: _____ By: _____	Time: _____ By: _____
<input type="checkbox"/> Day 14 DTTM urine	Date: _____	Date: _____	Date: _____	Date: _____	Date: _____

Dated 01 Sept 2015    QC1: Initials: \_\_\_\_\_ Date: \_\_\_\_\_    QC2: Initials: \_\_\_\_\_ Date: \_\_\_\_\_

### LABORATORY REQUEST AND STORAGE FORM (MOTHER) – F004



☐ Breast milk (Left)

Date: \_\_\_\_\_

Date: \_\_\_\_\_

Date: \_\_\_\_\_

Date: \_\_\_\_\_

Date: \_\_\_\_\_

☐ Breast milk (Right)

Date: \_\_\_\_\_

Date: \_\_\_\_\_

Date: \_\_\_\_\_

Date: \_\_\_\_\_

Date: \_\_\_\_\_

**Section B: Sample processing and storage information (To be completed by study laboratory staff)**

Aliquot Type	Volume stored	Rack	Tray/ box number	Position	Aliquot pellet ID number
LH plasma aliquot #1 - 1.5ml					
LH plasma aliquot #2 - 1.5ml					
LH plasma aliquot #3 - 1.5ml					
LH plasma aliquot #4 - 1.5ml					
Serum aliquot #1 - 1.0ml					
Serum aliquot #2 - 1.0ml					
EDTA plasma aliquot #1 - 1.0ml					
EDTA plasma aliquot #2 - 1.0ml					
RBC concentrates #1 - 1.0ml					
RBC concentrates #2 - 1.0ml					
Acidified urine #1 (5.0ml)					
Acidified urine #2 - 5.0ml					
Non Acidified urine #1 - 5.0ml					
Non Acidified urine #2 - 5.0ml					
Total volume 2-hour Urine (ml)					
Breast milk (Left) #1 - 5.0ml					
Breast milk (Right) #1 - 5.0ml					
Deuterium urine (Day 0) #1 - 5.0ml					
Deuterium urine (Day 1) #1 - 5.0ml					
Deuterium urine (Day 2) #1 - 5.0ml					
Deuterium urine (Day 3) #1 - 5.0ml					
Deuterium urine (Day 4) #1 - 5.0ml					
Deuterium urine (Day 13) #1 - 5.0ml					
Deuterium urine (Day 14) #1 - 5.0ml					

**Section C: Quality Control Check (To be completed by lab supervisor, QC/QA or designated laboratory staff)**

Laboratory personnel signature	Print name	Date	Time

Dated 01 Sept 2015    QC1: Initials: \_\_\_\_\_ Date: \_\_\_\_\_    QC2: Initials: \_\_\_\_\_ Date: \_\_\_\_\_

# LABORATORY REQUEST AND STORAGE FORM (BABY) – F005



Study No: \_\_\_\_\_ DOB: \_\_\_\_\_ Sample No: \_\_\_\_\_  
SEX: \_\_\_\_\_

Study Visit:	<input type="checkbox"/> PG36	<input type="checkbox"/> PP2	<input type="checkbox"/> PP10	<input type="checkbox"/> PP14	<input type="checkbox"/> PP26	<input type="checkbox"/> PP52	<input type="checkbox"/> PP78
Deuterium Urine Day	<input type="checkbox"/> 0	<input type="checkbox"/> 1	<input type="checkbox"/> 2	<input type="checkbox"/> 3	<input type="checkbox"/> 4	<input type="checkbox"/> 13	<input type="checkbox"/> 14

## Section A: Specimens and Test Type (Tick all that apply for the study visit as per the protocol):

Specimen type and volumes	Collected By	Delivery By	Received By	Processed By	Stored By
<input type="checkbox"/> Serum Tube (1x2ml)	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____
<input type="checkbox"/> Day 0 DTTM urine (1x5ml)	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____
<input type="checkbox"/> Day 1 DTTM urine (1x5ml)	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____
<input type="checkbox"/> Day 2 DTTM urine (1x5ml)	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____
<input type="checkbox"/> Day 3 DTTM urine (1x5ml)	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____
<input type="checkbox"/> Day 4 DTTM urine (1x5ml)	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____
<input type="checkbox"/> Day 13 DTTM urine (1x5ml)	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____
<input type="checkbox"/> Day 14 DTTM urine (1x5ml)	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____	Date: _____ Time: _____ By: _____

Dated 01<sup>st</sup> Sept, 2015 QC1: Initials: \_\_\_\_\_ Date: \_\_\_\_\_ QC2: Initials: \_\_\_\_\_ Date: \_\_\_\_\_

# LABORATORY REQUEST AND STORAGE FORM (BABY) – F005



## Section B: Sample storage information (To be completed by study laboratory staff):

Aliquot Type	Volume stored	Rack	Box number	Position	Aliquot pellet ID number
Serum aliquot # 1 - 1.0ml					
DTTM urine (Day 0) # 1 - 5.0ml					
DTTM urine (Day 1) # 1 - 5.0ml					
DTTM urine (Day 2) # 1 - 5.0ml					
DTTM urine (Day 3) # 1 - 5.0ml					
DTTM urine (Day 4) # 1 - 5.0ml					
DTTM urine (Day 13) # 1 - 1.0ml					
DTTM urine (Day 14) # 1 - 1.0ml					

## SECTION D: Laboratory Notes

Please note here additional information that might be relevant to use of stored samples (e.g. observed abnormalities, breakdowns in processing equipment, and any deviations from the SOP etc.) and challenges in drawing blood.

Name of laboratory personnel: \_\_\_\_\_ Signature: \_\_\_\_\_ Date: \_\_\_\_\_

## Section D: Quality Control Check (To be completed by lab supervisor, QC/QA or designated laboratory staff)

Laboratory personnel signature	Print name	Date	Time

Dated 01<sup>st</sup> Sept, 2015 QC1: Initials: \_\_\_\_\_ Date: \_\_\_\_\_ QC2: Initials: \_\_\_\_\_ Date: \_\_\_\_\_



# LABORATORY REQUEST AND STORAGE FORM (MOTHER) – F004



Study No: .....		DOB: .....		Sample No: .....	
Study Visit:	<input type="checkbox"/> PG36	<input type="checkbox"/> PP2	<input type="checkbox"/> PP10	<input type="checkbox"/> PP14	<input type="checkbox"/> PP26
	<input type="checkbox"/> PPS2	<input type="checkbox"/> NPNI			
Deuterium Urine Day	<input type="checkbox"/> 0	<input type="checkbox"/> 1	<input type="checkbox"/> 2	<input type="checkbox"/> 3	<input type="checkbox"/> 4
	<input type="checkbox"/> 13	<input type="checkbox"/> 14	<input type="checkbox"/> 0	<input type="checkbox"/> 1	<input type="checkbox"/> 2
	<input type="checkbox"/> 3	<input type="checkbox"/> 4	<input type="checkbox"/> 13	<input type="checkbox"/> 14	

Section A: Specimens and Test Type (Tick all that apply for the study visit as per the protocol):

Specimen type and volumes	Collected By	Delivery By	Received By	Processed By	Stored By
<input type="checkbox"/> LH Tube (2X6ml)	Date: .....	Date: .....	Date: .....	Date: .....	Date: .....
<input type="checkbox"/> SST Tube (1X4ml)	Time: .....	Time: .....	Time: .....	Time: .....	Time: .....
<input type="checkbox"/> EDTA tube 1X4ml	By: .....	By: .....	By: .....	By: .....	By: .....
<input type="checkbox"/> Urine 2 hour fasted	Date: .....	Date: .....	Date: .....	Date: .....	Date: .....
	Time: .....	Time: .....	Time: .....	Time: .....	Time: .....
	By: .....	By: .....	By: .....	By: .....	By: .....
<input type="checkbox"/> Day 0 DTTM urine	Date: .....	Date: .....	Date: .....	Date: .....	Date: .....
	Time: .....	Time: .....	Time: .....	Time: .....	Time: .....
	By: .....	By: .....	By: .....	By: .....	By: .....
<input type="checkbox"/> Day 1 DTTM urine	Date: .....	Date: .....	Date: .....	Date: .....	Date: .....
	Time: .....	Time: .....	Time: .....	Time: .....	Time: .....
	By: .....	By: .....	By: .....	By: .....	By: .....
<input type="checkbox"/> Day 2 DTTM urine	Date: .....	Date: .....	Date: .....	Date: .....	Date: .....
	Time: .....	Time: .....	Time: .....	Time: .....	Time: .....
	By: .....	By: .....	By: .....	By: .....	By: .....
<input type="checkbox"/> Day 3 DTTM urine	Date: .....	Date: .....	Date: .....	Date: .....	Date: .....
	Time: .....	Time: .....	Time: .....	Time: .....	Time: .....
	By: .....	By: .....	By: .....	By: .....	By: .....
<input type="checkbox"/> Day 4 DTTM urine	Date: .....	Date: .....	Date: .....	Date: .....	Date: .....
	Time: .....	Time: .....	Time: .....	Time: .....	Time: .....
	By: .....	By: .....	By: .....	By: .....	By: .....
<input type="checkbox"/> Day 13 DTTM urine	Date: .....	Date: .....	Date: .....	Date: .....	Date: .....
	Time: .....	Time: .....	Time: .....	Time: .....	Time: .....
	By: .....	By: .....	By: .....	By: .....	By: .....
<input type="checkbox"/> Day 14 DTTM urine	Date: .....	Date: .....	Date: .....	Date: .....	Date: .....
	Time: .....	Time: .....	Time: .....	Time: .....	Time: .....
	By: .....	By: .....	By: .....	By: .....	By: .....

Dated 01 Sept 2015 QC1: Initials: ..... Date: ..... QC2: Initials: ..... Date: .....

# LABORATORY REQUEST AND STORAGE FORM (MOTHER) – F004



<input type="checkbox"/> Breast milk (Left)	Date: .....	Date: .....	Date: .....	Date: .....	Date: .....
	Time: .....	Time: .....	Time: .....	Time: .....	Time: .....
	By: .....	By: .....	By: .....	By: .....	By: .....
<input type="checkbox"/> Breast milk (Right)	Date: .....	Date: .....	Date: .....	Date: .....	Date: .....
	Time: .....	Time: .....	Time: .....	Time: .....	Time: .....
	By: .....	By: .....	By: .....	By: .....	By: .....

Section B: Sample processing and storage information (To be completed by study laboratory staff)



Allotment Type	Volume stored	Rack	Tray/ box number	Position	Allotment pellet ID number
LH plasma aliquot #1 - 1.5ml					
LH plasma aliquot #2 - 1.5ml					
LH plasma aliquot #3 - 1.5ml					
LH plasma aliquot #4 - 1.5ml					
Serum aliquot #1 - 1.0ml					
Serum aliquot #2 - 1.0ml					
EDTA plasma aliquot #1 - 1.0ml					
EDTA plasma aliquot #1 - 1.0ml					
RBC concentrates #1 - 1.0ml					
RBC concentrates #2 - 1.0ml					
Acidified urine #1 (5.0ml)					
Acidified urine #2 - 5.0ml					
Non Acidified urine #1 - 5.0ml					
Non Acidified urine #2 - 5.0ml					
Total volume 2-hour Urine (ml)					
Breast milk (Left) #1 - 5.0ml					
Breast milk (Right) #1 - 5.0ml					
Deuterium urine (Day 0) #1 - 5.0ml					
Deuterium urine (Day 1) #1 - 5.0ml					
Deuterium urine (Day 2) #1 - 5.0ml					
Deuterium urine (Day 3) #1 - 5.0ml					
Deuterium urine (Day 4) #1 - 5.0ml					
Deuterium urine (Day 13) #1 - 5.0ml					
Deuterium urine (Day 14) #1 - 5.0ml					

Section C: Quality Control Check (To be completed by lab supervisor, QC/QA or designated laboratory staff)

Laboratory personnel signature	Print name	Date	Time

Dated 01 Sept 2015 QC1: Initials: ..... Date: ..... QC2: Initials: ..... Date: .....

## Appendix 12: DXA referral forms

 <b>GUMBA STUDY DXA REFERRAL FORM FOR MOTHER (GUMBA- F008)</b>	 <b>GUMBA STUDY DXA REFERRAL FORM FOR BABY (GUMBA- F009)</b>
DATE: ____/____/____ (DD/MM/YYYY)	DATE: ____/____/____ (DD/MM/YYYY)
<b>SECTION A: PARTICIPANT DETAILS</b>	
<b>A1. Study Number (Mother):</b> [MAB ____] - M <b>A2. Residence:</b> _____ <b>A3. DOB:</b> ____/____/____ (DD/MM/YYYY) <b>A4. Participant Initials</b> _____ <b>A5. Sex</b> <input type="checkbox"/> Female	<b>A4. Visit:</b> <input type="checkbox"/> PP2 <input type="checkbox"/> PP14 <input type="checkbox"/> PP26 <input type="checkbox"/> PP52 <input type="checkbox"/> NPPL <b>A3. Sex</b> <input type="checkbox"/> Male <input type="checkbox"/> Female <b>A4. Participant Initials</b> _____
<b>SECTION B: DXA REQUISITION DETAILS</b>	
<b>Anthropometry:</b> <b>B1. Weight:</b> ____ . ____ <b>B2. Height:</b> ____ . ____	
<b>B3. Pregnancy Test Results (HCG)</b> <input type="checkbox"/> Negative <input type="checkbox"/> Negative <input type="checkbox"/> N/A <b>B4. Date HCG test done:</b> ____/____/____ <b>B5. Presence of metallic parts in/on the body</b> <input type="checkbox"/> NO <input type="checkbox"/> YES* *Please provide further information _____ <b>B6. DXA Scans requested:</b> <input type="checkbox"/> Lumbar spine (Please request for the 3 scans) <input type="checkbox"/> Hip <input type="checkbox"/> Whole body <b>B7. DXA requested by (Name):</b> _____ <b>Signature:</b> _____ <b>Date:</b> _____	
<b>SECTION C: FEEDBACK FROM DXA TECHNOLOGIST</b>	
<b>C1. DXA scans done:</b> Lumbar spine <input type="checkbox"/> YES <input type="checkbox"/> NO* Hip <input type="checkbox"/> YES <input type="checkbox"/> NO* Whole body <input type="checkbox"/> YES <input type="checkbox"/> NO* <b>C2. * Reason why scan was not done (please attach further information where necessary):</b> _____ <b>C3. DXA Technologist (Name):</b> _____ <b>Signature:</b> _____ <b>Date:</b> _____	
DXA referral form, Version 1.1, Dated 30 <sup>th</sup> March 2015 QC1: Initials: _____ Date: _____ QC2: Initials: _____ Date: _____	DXA referral form, Version 1.1, Dated 30 <sup>th</sup> March 2015 QC1: Initials: _____ Date: _____ QC2: Initials: _____ Date: _____

