Association Between Immune Activation and Birth Outcomes Among HIV-Infected and Uninfected Pregnant Women in Zambia

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Abstract

Rates of neonatal mortality and morbidity are highest in Sub-Saharan Africa, with intrapartum complications and adverse birth outcomes being primary contributors to these disconcerting trends. Maternal human immunodeficiency virus (HIV) infection can increase the risk of these complications, either as a direct result of vertical HIV transmission or, possibly, through pro-inflammatory states and accompanying immune processes during pregnancy. Even in women without HIV heightened immune activity has been associated with neurodevelopmental and psychological abnormalities. This observational study examined the association between immune activation during early pregnancy and selected birth outcomes among HIV-infected and uninfected women in the Sub-Saharan country of Zambia.

Demographic, clinical, and birth outcome data were obtained from the ongoing prospective Zambian Infant Cohort Study (ZICS), which enrolls HIV infected and uninfected women in early pregnancy in a 1:1 ratio. Baseline immune activation was defined as the percentage of CD8 T cells expressing inflammatory surface markers during a woman's first or early second trimester.

Adjusted regression models were performed to examine the association between immune activation and (1) infant birthweight, (2) low birthweight (weight <2500g at birth), and (3) preterm birth. Exploratory analyses evaluated whether these associations differed based on HIV status. Among women with HIV, we also examined the associations between pre-conception combined antiretroviral therapy (cART) and the adverse birth outcomes listed above. 714 pregnant women (mean age = 27.9 ±5.8 years, n=396 (55.0%) with HIV) were included in the analyses. Among the women with HIV,

n=329 (83.1%) were on cART before conception, of which n=285 (86.6%) had cART duration of greater than 6 months preconception.

The mean level of immune activation in the total cohort was 32.9% (± 16.9%), with women in the HIV cohort showing higher levels of immune activation on average compared to women without HIV (38.2% vs. 26.3%, $p<0.001$). There was no statistically significant association between immune activation and low birthweight (adjusted OR = 1.01 95% CI 1.00-1.02) or preterm birth (adjusted OR = 1.00, 95% CI 0.98-1.01), and no evidence of effect modification of immune activation by HIV status in this cohort. Overall, there was also no significant association seen between immune activation and infant birthweight as a continuous outcome ($p=0.43$) in the total cohort. However, among the women with HIV, there was a statistically significant inverse relationship between immune activation and infant birthweight, such that a 10% increase in IA during pregnancy was associated with a mean decrease of 86g in infant birthweight. ($p=0.04$).

The association between immune activation and birthweight seen in women with HIV suggests that in utero exposure to heightened immune states may be indirectly linked to fetal growth and development, possibly through the presence of accompanying immunologic processes and pro-inflammatory signals. Future analyses should examine the long-term effects of heightened maternal immune activation in infants and the role of other immunologic components impacting the in-utero environment and fetal development, particularly in women with HIV.

Dedication

This work is dedicated to my family. To my parents, Malgorzata and Mariusz Pawlak, thank you for always supporting me and telling me to follow my heart. Mom – you especially have encouraged me to “defy gravity” and I would not be where I am today without you. To my grandmother, Alina Borkowska, and in loving memory of my grandfather, Roman Borkowski, who have reminded me over the years to be as proud of my accomplishments as they are of me. I love you all to the moon and back.

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To the CTSI faculty whom I've had the pleasure to learn from - especially Dr. Angie Mae Rodday and Dr. Robert Goldberg – you are such incredible teachers and have played an important role in making me the researcher I am today. Finally, I would like to thank our research team in Chawama, Zambia, for their hard work, effort, and dedication.

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List of Abbreviations

cART	Combined antiretroviral therapy
HEU	HIV exposed uninfected
HIV	Human immunodeficiency virus
IA	Immune activation
LBW	Low birthweight
LMIC	Low- and middle-income country
UN	United Nations

Chapter 1: Introduction

More than two-thirds of the world's population of people living with HIV are located in Sub-Saharan Africa.[1] Within Sub-Saharan Africa, four low- and middle-income countries (LMICs) in particular (Ethiopia, Nigeria, Zambia, and Zimbabwe) have the largest burden of HIV in this region. In Zambia, young women are often disproportionally affected by HIV compared with men and contribute a significant proportion to the 15 million women of reproductive age who are living with HIV worldwide.[1,2] Zambia was also identified by the United Nations (UN) as one of 22 countries with extremely high mother to child transmission of HIV that would need intensified support and targeted policies in order to achieve the UN goal of eliminating acquired immune deficiency syndrome by 2030.[3] To this end, the Zambian Ministry of Health approved the implementation of the Option B+ policy in 2013 to reduce mother to child transmission by making first-line combination antiretroviral therapy (cART) immediately available to all women testing positive for HIV.[4] Improved cART coverage of pregnant and breastfeeding women, combined with improved knowledge about the transmission of HIV, has significantly reduced the rate of mother-to-child transmission of HIV and the number of new HIV infections in young children during recent years.[4–6]

Despite these achievements, there is limited understanding how maternal HIV infection and its associated immunologic changes influence birth outcomes. Some studies report a higher risk of pre-term birth, low birthweight (LBW), and small for gestational age infants among mothers with HIV, while other studies have found no significant difference in the risk of these adverse outcomes among women with HIV.[7–10] Research

on the impact of cART on pregnancy outcomes is also conflicting, with several studies supporting a decreased risk of preterm birth and LBW infants in women who initiated cART preconception while other studies support an increased risk of these outcomes despite preconception cART and in the absence of plasma viremia .[11–14] However, these prior studies have several major limitations, including: suboptimal seronegative or cART-naïve control groups and low statistical power to detect clinically meaningful between group differences[10,11,13,15], shifts in ART guidelines and regimens available (especially those coinciding with the early adoption of Option B+)[4,14,16,17], inability to control for several potentially confounding factors[1–4], and lack of availability of ultrasound data to accurately date the time of gestation.[8,18,19] The latter can be attributed to challenges in implementing data collection with prenatal ultrasound and the availability of skilled ultrasound personnel on the research team, particularly in low-middle income countries. Moreover, few previously conducted studies have assessed neonatal morbidity and mortality as outcomes.

During pregnancy, shifts in the immune system occur to achieve a delicate balance between protecting the mother and fetus against pathogens and immune quiescence to accommodate the presence of paternal antigens associated with the fetus.[20,21] However, knowledge of how these processes affect outcomes in both HIV-infected and uninfected women is limited. Immune activation (IA) is defined as the signaling by cell surface markers that cell-mediated immune responses have been triggered. More specifically, IA is the process by which naïve T cells acquire an active phenotype, and this activation is expressed as the proportion of T cells expressing this activated phenotype over the total number of that population of T cells. In the context of

CD8+ T cells, which play an important role in sustained viral control and other adaptive immune system functioning, the cell-surface markers of IA are HLA-DR and CD38. These markers signify that the CD8+ cells are active in their effector cell functioning, which includes immune cell proliferation, cytotoxicity, and cytokine production.[22,23] In patients with HIV, immune activation of CD8+ is beneficial for sustained viral control and countering HIV's negative impact on immune function.[23] Acute activation of these cells is also beneficial to preventing harmful immune alterations and maintaining system functioning in seronegative individuals. Additionally, IA has been shown to occur in both HIV-positive and seronegative pregnant women.[20,21,24,25]

On the other hand, while helpful acutely, prolonged IA can have negative consequences, as maintenance of this state can result in subsequent loss of activated CD8+functional abilities, increased expression of inhibitory molecules related to immune exhaustion, and self-induced cell death from overactivation.[23] The negative consequences of prolonged IA has also been associated with an increased risk of adverse clinical outcomes. Perturbances in the levels of IA in seronegative women have been associated with disruptions in fetal brain development and psychological abnormalities, which emphasizes the importance of balancing immune cell populations during pregnancy.[21,26,27] Furthermore, among HIV-positive women, increased IA during pregnancy has been associated with adverse metabolic changes, poor immune reconstitution, and worsened disease progression and prognosis.[20,28] However, it is still unclear whether heightened maternal IA is a cause of these adverse outcomes or whether it acts as a marker of pathological processes and poor baseline maternal health predisposing to adverse outcomes. It is also important to note that although cART

reduces viral load in women with HIV, a considerable portion (15-20%) of individuals retain a heightened degree of IA even with undetectable viral loads.[29, 33]

The purpose of this nested retrospective analysis within a large prospective study is to examine the association between IA during the first or second trimester of pregnancy with the outcomes of birthweight and preterm birth in infants and to explore whether these associations change by HIV status and cART use.

Chapter 2: Association Between Immune Activation and Birth Outcomes Among HIV-Infected and Uninfected Pregnant Women in Zambia

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2.1 INTRODUCTION

Throughout the world, over 15 million reproductive aged women are living with human immunodeficiency virus (HIV).[2] Improved knowledge about HIV transmission and the use of combined antiretroviral therapy (cART) by pregnant and breastfeeding women has significantly reduced the rate of mother-to-child transmission of HIV, as well as the number of new HIV infections in children under age five during recent years.[4–6]

Despite these achievements, there is limited understanding as to how maternal HIV infection influences birth outcomes, such as preterm birth (birth at <37 weeks gestational age) and low birthweight (birthweight ≤2500g). Some studies report a higher risk of pre-term birth and low birthweight (LBW) infants among mothers with HIV, while other studies have found no significant difference in these outcomes maternal HIV infection and the risk of pre-term birth or LBW, even in the presence of sustained viral control and undetectable viral loads. [7,10,15,17,18]

A proposed mechanism contributing to the association between HIV infection and adverse birth outcomes is in-utero exposure to accompanying immune processes that may exist even in the absence of maternal plasma viremia. Previous studies have investigated immune activation (IA), defined as the percentage of T cells expressing an "active" phenotype, during pregnancy in seronegative women and its potential impact on disease progression in mothers with HIV as well.[20,24,25,31] In the context of the CD8+ cell population, IA is defined as the percentage of total CD8+ cells that signal activation through the expression of cell-surface markers CD38 and HLA-DR.[20,23,24] Among HIV-infected and uninfected women, pregnancy is associated with an increase in CD8+ cells compared to non-pregnant seronegative controls, as well as a persistently increased

expression of HLA-DR and CD38. Previous studies have also found that IA can be seen starting in the first trimester and remains persistent at a constant elevated level as gestation continues.[20,24]

Perturbances in the levels of IA (specifically, those associated with greater pro-inflammatory states) in seronegative women have been associated with disruptions in fetal brain development and psychological abnormalities.[31] Among HIV-positive women, increased IA during pregnancy has been associated with adverse metabolic changes, poor immune reconstitution, and poor prognosis.[20,22,28] However, it is still unclear whether heightened maternal IA is a cause of these adverse outcomes or whether it acts as a marker of pathological processes and poor baseline maternal health predisposing to adverse outcomes. It is also important to note that although cART reduces viral load in women with HIV, a considerable portion (15-20%) of individuals retain a heightened degree of IA even with undetectable viral loads.[29, 33] While the effect of IA during pregnancy on HIV disease outcomes and progression has been explored, its association with perinatal outcomes and infant health remains unknown. This is particularly relevant to LMICs in Sub-Saharan Africa, where the rates of neonatal morbidity and mortality are highest.[32]

2.2 METHODS

2.2.1 Study Design

This analysis uses data on HIV-positive and seronegative women and their infants who enrolled in the Zambia Infant Cohort Study (ZICS). Initiated in the Chawama township of the Zambian capital city, Lusaka in May 2019, the primary objective of ZICS

is to determine if infants born to HIV+ mothers have increased morbidity and mortality compared to those born to HIV- mothers.

2.2.2 Study Setting

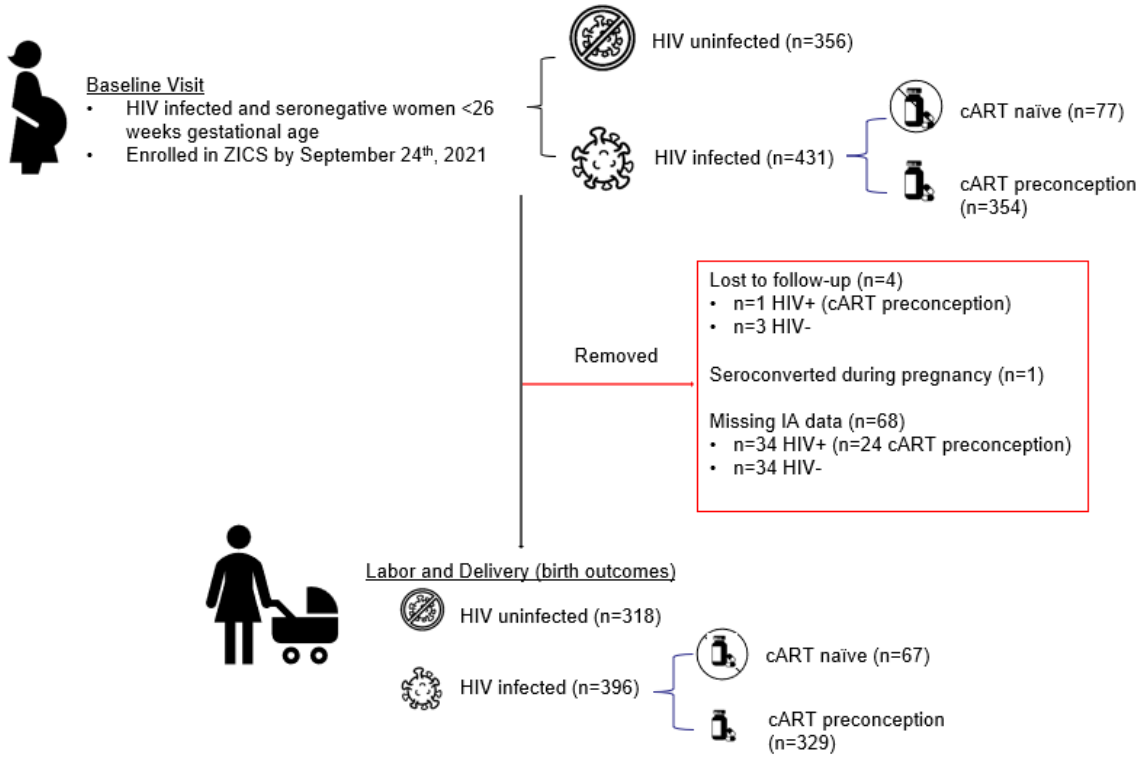
Chawama is one of the largest periurban townships (population =70, 181, 2010 census) located in Lusaka. Like many of Lusaka's townships, it is extremely impoverished and socio-demographically homogenous. All primary healthcare (including antenatal care and post-natal care) is provided free to the local community at the Chawama primary care facility where ZICS enrollment takes place. This primary care facility provides antenatal, postnatal, and well-baby care to > 95% of mothers in Chawama.

2.2.3 Participants

ZICS plans to enroll approximately 1,500 HIV-positive and seronegative pregnant women (in a 1:1 ratio) during routine antenatal care visits who are <26 weeks gestational age at the time of their baseline ultrasound. The reason for excluding women >26 weeks gestational age is because gestational dating methods by ultrasound are the least reliable in the third trimester, which would affect the accuracy with which the outcome of preterm birth and gestational age at birth are determined. Women were excluded from this analysis if they tested HIV-negative at baseline but seroconverted during pregnancy, if they were HIV-positive but refused cART during their pregnancy, and if there were missing information on immune activation at baseline visit or information on birth outcomes due to loss of follow up. Women who had not given birth to live infants would

also have been excluded; however, no women experienced stillbirth in this cohort. The study design and participant selection process are outlined in Figure 2.1.

Figure 2.1 Study Sample



2.2.4 Data Collection

Maternal demographic (e.g., age, BMI) and clinical variables (e.g., HIV status, CD4+ and CD8+ cell counts, obstetric history, cART use) were collected at the time of the baseline enrollment visit, where the self-reported date of the woman's last menstrual period and ultrasound data were used to estimate gestational age. Women with HIV were classified as being cART naïve if they had not been on cART prior to conception. Certain clinical variables, such as HIV seropositivity, cART use, maternal adverse events (e.g.

sexually transmitted diseases, hospitalizations, obstetric complications, or self-reported alcohol use during pregnancy) were also collected at baseline and subsequent antenatal care visits. Information on birth outcomes was collected during the Labor and Delivery visit.

T-cell immune activation was determined with flow cytometry (FC500 flow cytometer, Beckman Coulter) using freshly collected whole blood directly stained with antibody fluorochrome combinations in conjunction with absolute counting beads (LeukoSure Fluorospheres; Beckman Coulter Life Sciences). CD8+ lymphocytes are isolated and evaluated for co-expression of CD38 and HLA-DR. The degree of immune activation is calculated as a percentage of CD38+HLA-DR+ expressed on CD3+CD8+ T cells, though there is currently no standardized guideline to define the upper or lower limits of normal IA for pregnant or non-pregnant women.

The outcomes of interest in this study were birthweight and preterm birth. Birthweight was studied as both a continuous variable and as a dichotomous outcome of LBW (> 2500 g vs. ≤ 2500 g). Preterm birth was defined as <37 weeks gestational age.

2.2.5 Statistical Analysis

Data analysis was conducted using R studio version 1.3.1073 (R Core Team, Vienna, Austria). Point estimates and measures of association are reported with 95% confidence intervals.

Regarding a priori power analysis, no prior pilot studies had been done on the association between immune activation and peri-neonatal outcomes in HIV+ and seronegative women or in HIV+ women with preconception initiation of cART vs. post-

conception initiation. The sample size of this study was estimated to be n=800 women, with a 1:1 ratio of HIV positive vs. seronegative women. The prevalence of preterm birth in Zambia was estimated to be 13%, whereas the prevalence of low birth weight in the Lusaka province (where Chawama is located) is 11%. Using a logistic regression of the binary response variable of preterm vs. full-term birth, with the prevalence of preterm birth in the population = 13%, and an alpha of 0.05, we calculated that this study would have 69% power to detect an odds ratio of 1.3. The same approach for the binary study outcome variable of low birth weight, alpha = 0.05, and n=800 observations estimated that the study would achieve 63% power to detect an odds ratio of 1.3.

Baseline characteristics of the HIV positive and negative groups were compared using Student's t-tests for most continuous variables, Wilcoxon rank-sum test for maternal BMI, and Chi-square tests for categorical variables. Chi-square tests were also utilized to compare the proportion of women in each group who developed various adverse events (alcohol use, tuberculosis, pneumonia, hospitalization, or obstetric complications) during pregnancy. Among women with HIV, demographic and clinical characteristics (including HIV-relevant characteristics such as CD4 count and viral load) among those who received pre-conception cART were compared with those who were cART naïve, using Student's t-test or Chi-square test where appropriate. The duration of cART in women who were treated pre-conception was also examined

Unadjusted and adjusted logistic and linear regression models were used to examine the association between maternal immune activation and the birth outcomes of interest. The adjusted models included confounders identified a priori based on prior literature and expert consensus: HIV status, age, BMI, and parity. For the outcomes of

birthweight and LBW, gestational age at time of IA measurement was also adjusted for in the regression models. Given the interrelatedness between infants who are born preterm and having low birthweight, a sensitivity analysis was performed for the outcomes of birthweight as a continuous variable and low birthweight in which the unadjusted and adjusted regression analyses was repeated only among infants born full-term (87% of births in the cohort). Exploratory interaction terms were added to these models to evaluate possible effect modification by HIV status.

To explore the association between pre-conception initiation of cART and birth outcomes, regression models were performed among women with HIV. Because of the small sample sizes in this analysis, the only confounders included in the model were those that altered the unadjusted beta-coefficient by more than 15% (age, BMI, and baseline CD4 count).

2.3 Results

A total of 756 women enrolled in ZICS and who had given birth to live infants were identified. After removing those who had missing data on study outcomes due to data collection disruptions during the Covid-19 pandemic, n= 714 women were included in the analysis dataset. No women were excluded due to stillbirth or miscarriage. The mean maternal age was 27.9 ±5.8 years and mean BMI was 24.7 (±4.9 kg/ m^2. Most participants (89%) were married, 21% were nulliparous, and 72% were unemployed or not working in the formal sector. The majority of women (66.8%) in the cohort had completed secondary school. The mean IA in the total cohort was 32.9% ± 16.9%.

2.3.1 Characteristics of women with and without HIV

A total of 714 women were included in the study, of which n=396 (55.0%) were HIV-positive. The demographic and clinical characteristics of women with and without HIV are shown in Table 2.1. Compared to seronegative women, women with HIV were significantly older and had a significantly lower BMI. A higher percentage of women with HIV were married (91.7% vs. 86.5%, p=0.04) and had ≥4 previous pregnancies (24.0% vs. 9.4%) compared to women without HIV.

Over the course of their pregnancies, a greater proportion of women with HIV reported alcohol use (14.9% vs. 13.4%) and sexually transmitted disease (3.6% vs. 0%) compared to women without HIV. Women with HIV were more likely to report maternal sick visits requiring medical treatment (9.7% vs. 7.5%), with the most common indication being urinary tract infections/pyelonephritis (15/32, 47%), but this difference was not statistically significant. There were no statistically significant differences in rates of hospitalization or Cesarean section during delivery between the two groups. Mean IA was significantly higher in women with HIV compared to those in the seronegative cohort (38.2% vs. 26.3%, p<0.001).

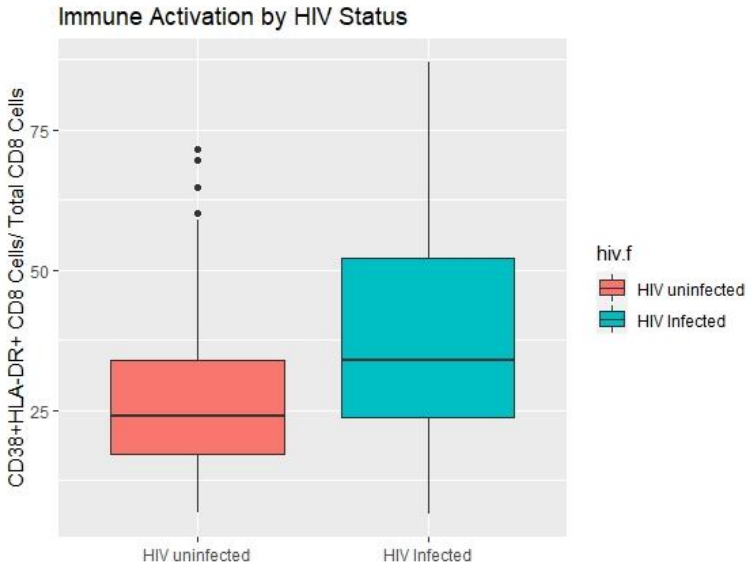
Table 2.1 Characteristics of women with and without HIV (n=714)

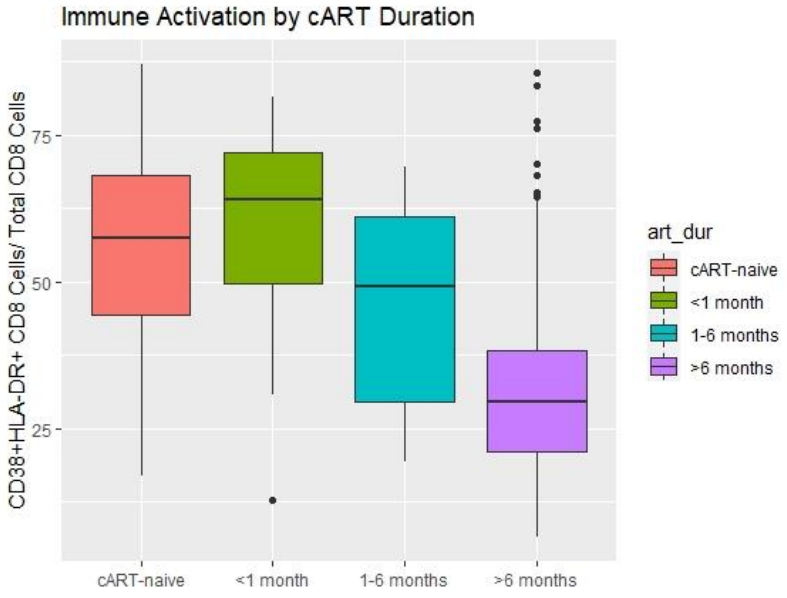
	HIV negative (n=318)	HIV positive (n=396)	P value
Baseline Demographic Characteristics			
Age (years), Mean (SD)	25.83 (5.66)	29.49 (5.49)	<0.001
BMI, Mean (SD)	25.49 (5.15)	24.30 (4.32)	0.046
Married, No. (%)	275 (86.5)	363 (91.7)	0.035
Employment, No. (%)			0.40
Unemployed	234 (73.6)	277 (69.9)	
Employed, Part-time	29 (9.1)	48 (12.1)	
Employed, Full-time	55 (17.3)	71 (17.9)	
Highest Level of Education Achieved, No. (%)			<0.001
No formal education	6 (1.9)	8 (2.0)	
Primary	52 (16.4)	115 (29.0)	
Secondary	225 (70.8)	252 (63.6)	
College and beyond	35 (11.0)	21 (5.3)	
Clinical and Pregnancy-related characteristics			
Number of Previous Pregnancies, No. (%)			<0.001
0	107 (33.6)	42 (10.6)	
1 - 3	181 (56.9)	259 (65.4)	
≥4	30 (9.4)	95 (24.0)	
Alcohol Use During Pregnancy, No. (%)	17 (5.3)	58 (14.6)	<0.001
STD during pregnancy, No. (%)	0 (0.0)	12 (3.6)	0.23
Any maternal sick visits for non-obstetric medical conditions, No. (%)	5 (7.5)	32 (9.7)	0.73
Hospitalization during pregnancy, No. (%)	1 (1.5)	11 (3.3)	0.68
Required C-section during delivery, No. (%)	7 (10.4)	28 (8.5)	0.79
Immune Activation*, Mean (SD)	26.3 (12.09)	38.2 (18.36)	<0.001

*Immune activation is defined as the percentage of CD8+ T cells expressing CD38 and HLA-DR.

Notably, even with prolonged cART use greater than 6 months, the levels of IA did not fully equalize to the same levels as HIV uninfected women (31.7 vs. 26.3, p<0.001), with the differences and variability in IA highlighted in Figure 2.2.

Figure 2.2 Immune Activation in Subgroups by HIV Status and cART Duration





For the total sample of 714 women, the mean infant birthweight was 2921g (±510g) and 162 (23%) of infants born were LBW. There was no statistically significant difference in mean birthweight among infants who were born to mothers with HIV compared to those born to seronegative women. However, a larger proportion of infants born to mothers with HIV were classified as LBW (27.0% vs. 17.3%, p=0.003) or were preterm (13.4% vs. 7.9%, p=0.026) compared to infants of seronegative mothers (Table 2.2).

Table 2.2 Birth Outcomes in Study Cohort

Outcome	**HIV negative (n=318)**	**HIV positive (n=396)**	**P value**
Birthweight of infant (g), Mean (SD)	2957.7 (498.8)	2891.7 (517.2)	0.085
Low birthweight infant (<2500g), No. (%)	55 (17.3)	107 (27.0)	0.003
Preterm birth (<37 weeks), No. (%)	25.0 (7.9)	53.0 (13.4)	0.026
Outcome	**cART-naïve (n=77)**	**cART preconception (n=354)**	**P value**
Birthweight of infant (g), Mean (SD)	2823.4 (604.3)	2905.5 (497.6)	0.24
Low birthweight infant (<2500g), No. (%)	21 (31.3)	86 (26.1)	0.47
Preterm birth (<37 weeks), No. (%)	10 (14.9)	43 (13.1)	0.83

2.3.2 Subgroup Characteristics: cART-preconception vs. cART-naïve

Among women with HIV, those who had been on cART prior to conception were older (29.5 years vs. 25.8 years, p<0.001) with a lower average BMI (24.3 vs. 25.5, p = 0.046) and a higher number of previous pregnancies (p<0.001) (Table 2.3).

Table 2.3 Characteristics of women on cART preconception and cART naïve

	cART-naïve (n=67)	cART pre-conception (n=329)	P value
Age (years), Mean (SD)	27.27 (4.65)	29.95 (5.55)	<0.001
BMI, Mean (SD)	25.49 (5.15)	24.30 (4.32)	0.046
Married, No. (%)	59 (88.1)	304 (92.4)	0.353
Employment, No. (%)			0.56
Unemployed	45 (67.2)	232 (70.5)	
Employed, Part-time	7 (10.4)	41 (12.5)	
Employed, Full-time	15 (22.4)	56 (17.0)	
Highest Level of Education Achieved, No. (%)			0.20
No formal education	0 (0.0)	8 (2.4)	
Primary	14 (20.9)	101 (30.7)	
Secondary	49 (73.1)	203 (61.7)	
College and beyond	4 (6.0)	17 (5.2)	
Baseline CD4, Mean (SD)	374.41 (223.85)	534.25 (247.76)	<0.001
Detectable Viral Load**, No. (%)	58 (86.6)	46 (14.5)	<0.001
Baseline viral load***, Mean (SD)	3.80 (1.60)	1.04 (1.43)	<0.001
Duration of ART Therapy, No. (%)			
>1 month		22 (6.7)	
1-6 months		22 (6.7)	
>6 months		285 (86.6)	
Parity, No. (%)			<0.001
0	15 (22.4)	27 (8.2)	
1 - 3	45 (67.2)	214 (65.0)	
≥4	7 (10.4)	88 (26.7)	
Immune Activation, Mean (SD)	56.22 (16.57)	34.50 (16.45)	<0.001

** >20 $\log_{10}$ HIV RNA copies/mL plasma, ***Log_{10} HIV RNA copies/mL plasma

There were no statistically significant differences in marital status, employment, or education level. Women who were on cART pre-conception had higher average CD4 counts (534 vs. 374) and lower log baseline viral loads (1.4 vs. 3.8 log10 HIV RNA copies/mL plasma), with a lower proportion of women (14.5% vs. 86.6%) showing detectable viral levels.

Among the women who were on pre-conception cART, the majority (86.6%) had been on cART for more than six months. Being on cART for more than 6 months was

associated with higher baseline CD4 counts and lower baseline viral loads compared to a treatment duration between one to six months (Table 2.4). The difference in baseline CD4 counts and viral load was even greater when comparing women who were on cART for more than six months to those who had been on cART for less than one month (Table 2.4).

Table 2.4 Descriptive Analysis by cART Duration Before Conception

	<1 month n=22	**1-6 months n=22**	**>6 months n=285**
Baseline CD4 count, Mean (SD)	346.4 (199.0)	455 (266.6)	555.3 (243.1)
Detectable Viral Load, No. (%)	13 (4.1)	3 (0.94)	30 (9.4)
Baseline Viral Load, Mean (SD)	3.0 (1.8)	1.4 (1.2)	0.87 (1.3)
Immune Activation, Mean (SD)	58.9 (17.7)	46.1 (17.4)	31.7 (14.2)

Women who were on cART prior to conception had lower levels of IA compared to women who were cART naïve (34.5% vs. 56.2%, $p<0.001$). An inverse relationship was observed between IA and cART-duration among women in the pre-conception cART subgroup (Table 2.4), and cART duration and IA were found to be correlated ($r = -0.45$, $p<0.001$). An unadjusted linear regression showed that the association between mean IA and cART duration (subdivided into the three categories specified above) was statistically significant ($p<0.001$). The average infant birthweight and proportion of infants who were LBW or born preterm did not significantly differ between cART-naïve women and women who were on pre-conception cART (Table 2.2).

2.3.3. Birthweight and Risk of Low Birthweight

In the unadjusted linear regression analysis, there was a non-significant inverse relationship between IA and birthweight (Table 2.5), such that a 10% increase in IA

corresponded with a mean decrease of 18 g in birthweight (95% CI: -40.0, 3.90; p=0.11). In the adjusted linear regression, which included HIV status, maternal age, maternal BMI, gestational age at IA measurement, and parity as confounders, the inverse relationship between IA and birthweight was further attenuated, with a mean decrease in birthweight of 6.7 g per 10% increase of immune activation (95% CI: -29.9, 16.6; p=0.57). The exploratory interaction term between IA and HIV status was not statistically significant and was therefore not retained in the adjusted model. In the unadjusted logistic regression for the risk of LBW, there was a statistically significant association between IA and the odds of having a LBW infant (Table 2.5) (unadjusted OR = 1.02, 95% CI: 1.01, 1.03; p=0.009). In the adjusted logistic regression model, the association between IA and the odds of LBW infants was no longer statistically significant after controlling for same confounders as above (adjusted OR = 1.01, 95% CI: 1.00, 1.02).

Table 2.5 Regression Analyses for the Association Between Immune Activation and Birth Outcomes

Continuous Outcomes	**Unadjusted Analysis**		**Adjusted Analysis***	
Variable	**Difference per 10% increase in IA**	**95% Confidence Interval**	**Difference (per 10% increase in IA)**	**95% Confidence Interval**
Birthweight (g)	-18g	-40.0, 3.9	-6.7g	-29.9, 16.6
Dichotomous Outcomes	**Unadjusted Analysis**		**Adjusted Analysis**	
Variable	**Odds Ratio**	**95% Confidence Interval**	**Odds Ratio**	**95% Confidence Interval**
Low birthweight (<2500g)	1.02	1.01, 1.03	1.01	1.00, 1.02
Preterm Birth (<37 weeks)	1.00	0.98, 1.02	1.00	0.98, 1.01

*Controlling for HIV status, age, BMI, and parity. Adjusted regression analysis for birthweight and LBW also controlled for gestational age at IA measurement.

Since preterm birth and LBW are highly correlated, a sensitivity analysis was performed in which the above analyses were repeated only among infants born full-term (about 89% of all deliveries in the cohort). The sensitivity analysis yielded similar findings seen in the total cohort. In the adjusted linear regression, there was an inverse relationship between IA and birthweight, such that a 10% increase in IA was associated with a mean decrease in birthweight of 10.0g, while holding the covariates specified above constant, and this association was not statistically significant (95% CI: -31.3, 11.5; p=0.36). The adjusted logistic regression found no statistically significant association between IA and LBW (OR = 1.01, 95% CI: 1.00, 1.02). When added to the adjusted models, neither of the regression models identified significant effect modification by HIV status (p=0.86 for birthweight and p=0.70 for LBW) and these interaction terms were therefore not retained.

2.3.4 Preterm Birth

In the unadjusted linear regression analysis, there was no association found between IA and preterm birth (OR=1.00, 95% CI: 0.98-1.02, p=0.79), which is shown in Table 2.5. As above, the adjusted linear regression model included HIV status, maternal age, maternal BMI, and parity as confounders, as well as an exploratory interaction term to assess effect modification by HIV infected or uninfected status. Just as in the unadjusted analysis, the adjusted model found no significant association between IA and preterm birth in the cohort (OR=1.00, 95% CI: 0.98, 1.01, p=0.42), as depicted in Table 2.5. There was also no significant effect modification by HIV status on the association between IA and preterm birth (p=0.37) and the term was dropped from the model.

2.3.5 Immune Activation and Birth Outcomes in HIV+ Mothers

Among women with HIV, the adjusted linear regression models included cART status, as well as age, maternal BMI, and baseline CD4 cell count, selected using the criteria of a change in the beta-coefficient ≥15% (Table 2.6). Gestational age at IA measurement did not meet this criterion and was therefore excluded from the adjusted regression model for birthweight and LBW infants. Although HIV viral load is an important disease characteristic that may affect infant birthweight, it was excluded due to its significant correlation with IA ($p<0.001$). The adjusted model found a significant association between IA and birthweight (Table 2.6). Specifically, a 10% increase in immune activation was associated with a mean decrease of 86 g in birthweight (95% CI: -171.1, -0.90, p=0.048) (Table 2.6).

Table 2.6 Association Between Immune Activation and Birth Outcomes in Women with HIV

Continuous Outcomes		
Variable	**Adjusted Difference per 10% increase in IA***	**95% Confidence Interval**
Birthweight (g)	-86.0 g	-171.1, -0.90
Dichotomous Outcomes		
Variable	**Adjusted Odds Ratio***	**95% Confidence Interval**
Low birthweight (<2500g)	1.01	0.99, 1.03
Preterm Birth (<37 weeks)	1.00	0.98, 1.02

*Controlling for age, BMI, cART status, and baseline CD4 cell count

The exploratory interaction term between IA and cART status did not reach statistical significance (p=0.06) and was therefore removed from the final adjusted regression model. There was no association between IA and having a LBW infant (adjusted OR = 1.01, 95% CI: 0.99, 1.03, p=0.21) or preterm birth (adjusted OR = 1.00,

95% CI: 0.98, 1.02, p=0.83) in the adjusted logistic regression models, controlling for the confounders mentioned above.

2.3.6 Immune Activation and Birth Outcomes in Seronegative Mothers

Among women without HIV, the adjusted linear regression model of birthweight included maternal age and BMI, selected using the criteria of a change in the beta-coefficient ≥15%. The adjusted linear model found an inverse relationship between IA and birthweight that was even smaller than that found in the total cohort, with a 10% increase in IA associated with a mean decrease of 4.2g in birthweight (CI: -49.6, 41.3). There was no association between IA and having a LBW infant (adjusted OR = 1.00, 95% CI: 0.98, 1.03; p=0.86) or preterm birth (adjusted OR = 0.98, 95% CI: 0.94, 1.02; p=0.29) in the adjusted logistic regression models, controlling for the confounders mentioned above.

2.4 Discussion

2.4.1 Immune Activation and Birth Outcomes in HIV Infected and Uninfected Women

The presence of IA among the sample of n=714 women suggests that pregnancy itself may act as a trigger for increased IA, or more generally, it serves to support that triggers for IA exist outside of viral processes that target CD8 cells. Increased IA is seen even among the cohort of n=318 women without HIV, whose mean IA was 26.3% – a level that is considerably higher than the IA ranging from 5-10% of CD8 cells previously described in a study of n=10 non-pregnant women, although these women were slightly older (mean age: 32.3) but with no HIV infection or other comorbidities.[34] These findings support the notion that pregnancy involves a balance of both IA and immune suppression

to protect the mother and fetus, rather than the traditionally held notion of pregnancy being a predominantly immunosuppressed state.

The findings of no statistically significant association between IA and study outcomes, after adjustment for confounding, in the total sample of n=714 women indicate that heightened IA alone does not significantly contribute to the risk of having a LBW infant or preterm birth. An alternative explanation is that a threshold level of IA exists (one beyond the acceptable upper limit of normal, which has not yet been defined) beyond which an association could be observed; it is possible that the women in our cohort, particularly those without HIV, did not have sufficient exposure to activating triggers and did not achieve the threshold of elevated IA needed to see this association. Interestingly, even though the adjusted regression models failed to show a statistically significant association between IA and birthweight in the total sample and in the cohort of seronegative women, the two were consistently inversely related to one another across the HIV infected and uninfected subgroups. This supports the hypothesis that IA may negatively impact intrauterine development, but the context in which the IA occurs, rather than the mechanism alone, determines the degree to which growth is affected. Factors leading to increased T cell activation may be the more important determinant, with immune activation serving as a proxy of the specific host response, infectious burden, or viral load (regarding the HIV infected women in our study cohort), as well as a measure of maternal immune signaling due to any illness during pregnancy. Alternatively, IA might influence individual organ development (which would be in line with the findings of disrupted neural development from Boulanger-Bertolus et al) rather than total body growth.[27] This relationship would still be reflected in the minor changes

in birthweight (as seen in infants with asymmetrical intrauterine growth retardation) that could be associated with changes in IA.

As expected, IA was considerably higher in women with HIV compared to seronegative women. Use of pre-conception cART among women with HIV lowered the IA closer to levels seen in HIV uninfected women, though this effect was notable only in the women who had been on pre-conception cART for more than six months. Thus, while it takes several months after initiating cART for patients with HIV to achieve an undetectable viral load, the duration of cART needed to observe a change in IA appears to be longer. Moreover, even with prolonged cART use, the levels of IA were not fully equalized to the same levels as HIV uninfected women. Taken together, these findings indicate that the provision of cART might not affect IA as quickly or fully as it does plasma viremia, nor does it normalize maternal IA in women with HIV compared to HIV-negative pregnant women. However, these findings should be interpreted with caution, as a heightened level of IA is expected during pregnancy regardless of HIV status, and non-pregnant controls would be needed to further explore the influence of cART on IA.

In terms of birthweight, on adjusted analysis a 10% increase in immune activation was associated with a mean decrease of 86g in birthweight in women with HIV – which is twenty-fold greater than the mean change calculated for HIV uninfected women. This further supports the idea that the driver and duration of IA, rather than the levels of IA alone, holds potential importance to influencing infant growth. In the setting of chronic HIV infection, IA might serve as a proxy for the viral load that the immune system is or previously was burdened with, or it may serve as a proxy for sustained viral replication

even in the setting of low plasma viremia (considering that the majority of pre-conception cART group was undetectable). The persistence of IA was previously described in a cross-sectional study with n=1,000 women with HIV who had sustained undetectable viral loads for 10 years. In either of these scenarios, the influence of immune activation on fetal growth arises due to the chronic viral infection that triggered the IA, and the magnitude of the association between IA and birthweight could look different had the underlying cause been another cause of chronic IA. In the exploratory analysis, there was a non- significant interaction (p=0.06) between IA and cART use on the outcome of birthweight, with cART use diminishing the inverse relationship between IA and birthweight; since this analysis was underpowered to detect these effects, it was excluded from the final model. Larger studies may uncover evidence of an association between IA or other proinflammatory processes and infant outcomes that is modified by cART use; this would be encouraging since cART has become widely accessible to pregnant women with HIV in Zambia under recent initiatives by the Zambian Ministry of Health.

2.4.2 Strengths and Limitations

The strengths of this study include the availability of a large sample of both seronegative women and women with HIV with longitudinal follow-up throughout their pregnancy and delivery. The sociodemographic homogeneity of this cohort can also be considered a strength, as it allows for the control of potential confounders of this type. Additionally, all of the women in this study had gestational dating performed by ultrasound in the first or second trimester, which is a more reliable method of determining gestational age at birth compared to the last menstrual period or symphysiofundal height that is often used in research settings in LMICs. Additionally,

among women with HIV, those who were on pre-conception cART were initiated on their regimens using the same guidelines under Option B+. Finally, our determination of maternal IA using freshly collected whole blood and flow cytometry is a more sensitive and accurate measurement of IA compared to the presence or absence of symptoms of underlying causes as an indication of IA used in previous studies.[27, 31]

Limitations of this study include the potential for the exploratory analyses by HIV subgroups to be underpowered. Additionally, due to the small number of women who had medical sick visits or hospitalizations in the HIV-infected and uninfected cohorts, it is not feasible to control for possible illnesses that might exacerbate IA, such as upper respiratory illness, especially considering that many patients might not have sought medical care for minor symptoms; the adjusted models could not control for viral loads among women with HIV due to the high correlation with IA and the fact that many women had undetectable viral levels. This study is also limited by the fact that we only looked at one cell line in determining IA and may therefore overlook the contributions of other important immune cell populations that play a role in immune tolerance or proinflammatory states. These accompanying immune processes might play a bigger role in influencing birth outcomes than IA alone. Finally, as mentioned, this study specifically looked at outcomes at the time of birth, and it is possible that the effect of in utero exposure to maternal IA manifests in outcomes later in development, which ZICS is designed to investigate.

2.4.3 Collaboration

A key study strength worth mentioning separately is the collaborative work involved in creating and executing this global health research project. This would not have been

possible without the bilateral and longitudinal partnership between the project mentors Drs. Christopher Gill and Donald Thea at Boston University and collaborators at the University of Zambia in Lusaka. Data collection was executed by Chawama clinic healthcare workers and research associates for the Zambian Infant Cohort Study, including but not limited to screening and enrollment, ultrasound gestational age dating, clinical and demographic variable collection, and maternal whole blood sampling. Ascertainment of seropositivity and immune activation was executed by laboratory personnel associated with the University of Zambia. The planning of this thesis project, including the creation of the study criteria, database, and analysis plan, as well as the execution of the study analysis, was led by the MSc student Natalie Pawlak.

2.5 Conclusion

Our study highlighted the variability in immune activation experienced during pregnancy, with a wide range of values seen in both HIV positive and negative women, though on average women with HIV still had significantly higher levels, as expected. Immune activation during pregnancy in women with HIV had a statistically significant inverse relationship with birthweight that was not seen in seronegative women. This suggests that in utero exposure to immune activation may be indirectly linked to dysregulated fetal growth and development, though the causal agent of immune activation and the presence of accompanying pro-inflammatory signals may be more important in determining this effect than the levels of immune activation alone. There was no statistically significant association found between immune activation and the outcomes of having a LBW infant or preterm birth, possibly due to the crudeness of binary outcomes, the alleviating effects of cART exposure in women with HIV, or the

existence of a threshold of elevated immune activation needed for observable outcomes that was not reached in our cohort. Finally, we observed that immune activation may be heightened in pregnant women with HIV even in the absence of detectable viral loads. The use of cART, particularly durations of more than six months, is associated with lower immune activation in women with HIV closer to levels seen in seronegative women. Future analyses should examine the long-term effects of heightened maternal immune activation in infants and the role of other immunologic components impacting the in-utero environment and fetal development, particularly in women with HIV.

Chapter 3: Discussion

This study aimed to address a key gap in the knowledge of mechanisms contributing to the high rates of neonatal morbidity and mortality in Zambia, particularly among women with HIV who make up ~14% of the country's population of reproductive age women.[35] Increased risk of adverse birth outcomes, such as preterm birth and low birthweight, among women with HIV has been found even in the setting of long-term cART use. In-utero exposure to pro-inflammatory and immune processes, such as maternal immune activation, that may exist even in the absence of plasma viremia was therefore proposed as a potential alternative mechanism contributing to these observed associations.

Results from this cohort of n=714 women showed evidence of immune activation during pregnancy. IA was seen even among the cohort of n=318 women without HIV, whose mean IA was 26.3% – a level that is considerably higher than the IA ranging from 5-10% of CD8 cells previously described in a study of n=10 non-pregnant HIV uninfected women.[34] Although this study did not include non-pregnant controls, the study findings suggest that IA may be triggered by processes (such as pregnancy) that do not involve direct viral targeting of T cells. This favors the increasingly accepted theory that pregnancy involves a balance of both IA and immune suppression to protect the mother and fetus, rather than the traditionally held notion of pregnancy being a predominantly immunosuppressed state.

The findings of no statistically significant association between IA and study outcomes, after adjustment for confounding indicate that heightened IA alone does not significantly contribute to the risk of having a LBW infant or preterm birth. An

alternative explanation is that a threshold level of IA exists (one beyond the acceptable upper limit of normal, which has not yet been defined) beyond which an association could be observed. It is possible that the women in our cohort, particularly those without HIV, did not have sufficient exposure to activating triggers and did not achieve the threshold of elevated IA needed to see this association.

However, even though adjusted regression models failed to show a statistically significant association between IA and birthweight in the total sample and in the cohort of seronegative women, the two were consistently inversely related to one another across the HIV infected and uninfected subgroups. This supports the hypothesis that IA may negatively impact intrauterine development, but the context in which the IA occurs, rather than the mechanism alone, determines the degree to which growth is affected. Alternatively, IA may be an accompanying process to proinflammatory mechanisms that more directly impact growth. Factors leading to increased activity in the adaptive immune system may be the more important determinant, with IA serving as a proxy of the specific host response or previous triggers (e.g., previous viral loads for the HIV infected women in our study cohort), as well as a measure of maternal immune signaling due to any illness during pregnancy. Alternatively, IA might influence individual organ development (which would be in line with the findings of disrupted neural development from Boulanger-Bertolus et al) rather than total body growth.[27] This relationship would still be reflected in the minor changes in birthweight, as seen in infants with asymmetrical intrauterine growth retardation.

As expected, IA was considerably higher in women with HIV compared to seronegative women. Use of pre-conception cART among women with HIV lowered the

IA closer to levels seen in HIV uninfected women, though this effect was notable only in the women who had been on pre-conception cART for more than six months. Thus, while it takes several months after initiating cART for patients with HIV to achieve an undetectable viral load, the duration of cART needed to observe a change in IA appears to be longer. Moreover, even with prolonged cART use, the levels of IA were not fully equalized to the same levels as HIV uninfected women. Taken together, these findings indicate that the provision of cART might not affect IA as quickly or fully as it does plasma viremia, nor does it normalize maternal IA in women with HIV compared to HIV-negative pregnant women. However, these findings should be interpreted with caution in the absence of non-pregnant controls to provide insight into the increase in IA attributable to pregnancy.

In women with HIV, adjusted regression analysis found no significant association between IA and LBW or preterm birth. However, on adjusted analysis, a 10% increase in IA was associated with a mean decrease of 86g in birthweight – which is twenty-fold greater than the mean change calculated for HIV uninfected women. This association was found to be statistically significant. The finding further supports the idea that the driver and duration of IA, rather than the levels of IA alone, hold potential importance to influencing infant growth. In the setting of chronic HIV infection, IA might serve as a proxy for the viral load that the immune system is or previously was burdened with, or it may serve as a proxy for sustained viral replication even in the setting of low plasma viremia (considering that the majority of pre-conception cART group was undetectable). The persistence of IA was previously described in a cross-sectional study with n=1,000 women with HIV who had sustained undetectable viral loads for 10 years. In either of

these scenarios, the influence of immune activation on fetal growth arises due to the chronic viral infection that triggered the IA, and the magnitude of the association between IA and birthweight could look different had the underlying cause been another cause of chronic IA. In the exploratory analysis, there was a non- significant interaction (p=0.06) between IA and cART use on the outcome of birthweight, with cART use diminishing the inverse relationship between IA and birthweight. However, since this analysis was underpowered to detect these effects, it was excluded from the final model. Larger studies may uncover evidence of an association between IA and infant outcomes that is modified by cART use; this would be encouraging since cART has become widely accessible to pregnant women with HIV in Zambia under recent initiatives by the Zambian Ministry of Health. Future studies may also uncover that cART regimens are more beneficial to controlling other proinflammatory processes that have a negative impact in utero.

The strengths of this study include the availability of a large sample of both seronegative women and women with HIV with longitudinal follow-up throughout their pregnancy and delivery. The sociodemographic homogeneity of this cohort can also be considered a strength, as it allows for the control of potential confounders of this type. Additionally, all of the women in this study had gestational dating performed by ultrasound in the first or second trimester, which is a more reliable method of determining gestational age at birth compared to the last menstrual period or symphysiofundal height that is often used in research settings in LMICs. Another strength is the use of freshly collected maternal whole blood and flow cytometry to provide the most sensitive and accurate measurement of IA compared to the presence or

absence of symptoms of underlying causes as an indication of IA used in previous studies.[27, 31] Finally, when considering the limitations and challenges to global health research, particularly in conducting studies in a setting different from where one typically lives and practices, a key strength of this study was the longitudinal bilateral partnership between study members. Stakeholder engagement included consultation and communication during the preparation phase with study clinicians and researchers affiliated with the University Teaching Hospital in Lusaka, Zambia, as well as the healthcare personnel and research staff at the Chawama clinic and Labor and Delivery unit. The conduction of research phase also included consultation and co-production with the above-mentioned stakeholders for choosing relevant outcomes and data collection.

Limitations of this study include the potential for the exploratory analyses by HIV subgroups to be underpowered. Additionally, due to the small number of women who had medical sick visits or hospitalizations in the HIV-infected and uninfected cohorts, it is not feasible to control for possible illnesses that might exacerbate IA, such as upper respiratory illness, especially considering that many patients might not have sought medical care for minor symptoms; the adjusted models could not control for viral loads among women with HIV due to the high correlation with IA and the fact that many women had undetectable viral levels. This study is also limited by the fact that we only looked at one cell line in determining IA and may therefore overlook the contributions of other important immune cell populations that play a role in immune tolerance or proinflammatory states. These accompanying immune processes might play a bigger role in influencing birth outcomes than IA alone. Finally, as mentioned, this study specifically looked at outcomes at the time of birth, and it is possible that the effect of in utero

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