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Antenatal and postpartum immunological markers levels in women with HIV infection and malnutrition in a low resource setting: A pilot study

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Abstract

Objectives: Both, Human Immunodeficiency Virus (HIV) infection and malnutrition are major challenges in pregnancy and postpartum in low-resource settings and the respective cytokine levels remain poorly described. The main objectives of this study were to find immune markers that are associated with HIV infection and malnutrition in pregnant women and to determine how these would change at 14 weeks postpartum.

Method: Pregnant women of at least 20 weeks gestational age were enrolled into this longitudinal observational single centre pilot study at 4 primary health clinics in high-density areas around Harare, Zimbabwe. Socio-demographic and clinical data including plasma samples were collected in pregnancy and 14 weeks postpartum (PP). Mid-upper-arm circumference (MUAC) ≤23 cm was used as an indicator for malnourishment. Fifty-six cytokines and chemokines were assayed in plasma using the Mesoscale multiplex assay. We determined cytokine/chemokine levels including markers for vascular injury in HIV-infection and malnutrition. Associations remaining significant after multiple test correction were confirmed in multivariable analyses after controlling for confounders.

Results: Ninety-seven pregnant women were recruited for this study and from these, 44 were randomly selected for cytokine assaying of which 20 HIV infected, 15 malnourished, and 9 well-nourished HIV uninfected participants. HIV infection was associated with significantly higher interleukin (IL)-4 ($q < 0.05$) and IL-10 ($q < 0.001$) in pregnancy. Longitudinally, IL-4 ($q < 0.01$) and IL-10 ($q < 0.001$) significantly increased in HIV uninfected women whilst in the HIV-infected both were non-significantly decreased. IL-8 ($q < 0.05$) levels significantly increased in HIV-infected women from pregnancy to 14 weeks PP. Vascular Cell Adhesion Molecule 1 (VCAM-1) ($q < 0.05$) and interleukin-1 receptor antagonist (IL-1RA) ($q < 0.05$) were significantly lower in malnourished women in pregnancy and 14 weeks PP, respectively.

Conclusions: IL-4, IL-8, IL-10, and VCAM-1 are potential biomarkers for monitoring immune functioning in HIV-infected pregnant women and malnutrition. However, studies with larger sample size are warranted to confirm these findings.

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Introduction

Human Immunodeficiency Virus-1 (HIV) infection is one of the leading causes of death globally.[1] Sub-Saharan Africa (SSA) is the hardest hit region in the world with about 54% (20.6 million people) of HIV infected people in world.[2–4] The prevalence of HIV infection in women of child bearing age in Zimbabwe is estimated to be at 14.4%, indicating a high burden of HIV in this population.[5] In Zimbabwe, annual births stand at 300,000 per year of which almost 50,000 (16.7%) pregnant women are HIV infected.[6] The majority of the HIV infected women receive life-long combined antiretroviral therapy (cART).[6]

HIV infection leads to chronic inflammation with increased numbers of activated innate immune cells and T cells [7] as well as abnormal production of cytokines by infected cells and cells of the immune system.[8,9] The infection also results in dysregulation of the cytokine profiles with increased production of T helper type 2 (Th2) cytokines, interleukin (IL)-4 and IL-10, and a decreased secretion of T-helper type 1 (Th1) cytokines.[8,10,11]

Malnutrition is a global public health problem caused by an imbalance of nutrients required by the body and the intake of energy and nutrients.[12] Globally, almost 462 million people are underweight and malnutrition is also prevalent in developing countries like Zimbabwe.[12,13] The nutritional status of the mother at the time point of conception is critical with respect to embryonic development and development of the fetus.[14] Malnutrition remains a challenge in resource limited settings and can be associated with nutritionally acquired immunodeficiency [15] and/or systemic inflammation.

The immune system status in pregnancy has been suggested to have a Th2 bias to promote tolerance to the foreign fetus.[14,16,17] During induction of tolerance to a new fetus there is a decrease in Th1 cytokines such as IL-2 and interferon-gamma (IFN-γ) and an increase in Th2 cytokines including IL-4 and IL-10.[18,19] In pregnancy, disorders such as preterm birth,[20] fetal growth restriction, and preeclampsia are often associated with infections during pregnancy.[21] Infections due to bacteria, viruses, and parasites, which normally induce a Th1 immune response can have an impact on placental development and compromise fetal survival.[22]

At what time point the immune system returns to baseline functionality after pregnancy is not entirely clear. Some studies suggest that after delivery, the immune system tends to return to a non-pregnant state, shifting back to Th1 dominance, accompanied by a decrease in Th2 cells.[23–27] For some inflammatory chemokines like IL-8, more complex regulation patterns with an U-shaped curve with increasing levels at postpartum have been shown.[28]

HIV and malnutrition can disturb the delicate immune balance during pregnancy,[29,30] potentially exposing the fetus to an immune dysfunction which may cause adverse pregnancy/birth outcomes. Immune markers associated with HIV infection and malnutrition, both prevalent in resource limited settings have not been adequately described, more so in pregnancy. This pilot study aims to examine cytokines and chemokines levels during pregnancy and after birth depending on HIV and nutrition status to assess altered immune function.

The main objectives of this study were to find immune markers that are associated with HIV infection and malnutrition in pregnant women and determine how these would change at 14 weeks PP.

Methods and materials

Study population

This study is a longitudinal observational single centre pilot study nested in the University of Zimbabwe Birth Cohort Study (UZBCS).[31] Potential participants for the cohort were identified during routine antenatal care visits at four City of Harare Polyclinics namely, Kuwadzana, Rujeko, Budiriro and Glenview, serving populations with relatively poor socio-economic status, as previously described.[31] The inclusion criteria were: (1) pregnancy with at least 20 weeks' gestational age with plans to deliver at one of the four study sites and (2) informed consent for study participation for themselves, and parental consent for their infants to participate in the study after delivery. The exclusion criteria were: (1) maternal age below 15 years and (2) severe mental health disorders, compromising the ability to provide informed consent. Ethical approval was obtained from the Joint Research Ethics Committee (JREC/228/19) and the Medical Research Council of Zimbabwe (MRCZ/B/1824). Written informed consent was obtained from all subjects and parental consent for their infants to participate in the study. A total of 97 pregnant women were enrolled from February 2019 to September 2019. Plasma samples for 78 mothers were available at both enrolment and 14 weeks postpartum (PP) and these mothers were considered for further analysis.

Since our study is a pilot study and expected variations of most cytokines according to HIV and malnutrition according to HIV infection and malnutrition are unknown, a power analysis has not been feasible. However, findings of this pilot study will be used to inform planning of an independent study with a larger sample size.

In a pragmatic approach, out of 26 eligible HIV positive mothers, 20 mothers were randomly selected. Further, all

(n = 15) malnourished mothers (defined by a mid-upper arm circumference, MUAC ≤ 23 cm[32–35] were included. Moreover, nine mothers with a normal MUAC (MUAC > 23 cm – 28 cm) were randomly chosen. For the definition of malnutrition, we used MUAC because it has been reported to be a stable predictor of the nutritional status in pregnancy, not affected by metabolic changes in pregnancy, whereas BMI has been reported to be dependent on a woman`s gestational age.[32–35] Altogether, for our cytokine analysis a total of 44 mothers were selected.

For our pilot study we were interested in three outcomes: (1) we wanted to determine which immune markers are associated with HIV infection, or (2) malnutrition in pregnant women and (3) assess how the concentration of these immune markers would change from pregnancy to 14 weeks PP.

Data collection

At enrolment a comprehensive set of clinical, socio-demographic and nutritional data was collected using a validated questionnaire.[31,36] A physical examination was carried out which included measurements of height, pregnancy weight, MUAC, pulse and body mass index (BMI). The women were then followed up at 14 weeks PP for bio-sampling and reassessment of clinical data.

Sample collection, preparation, and assays

At enrolment and 14 weeks PP, 4 mL maternal venous blood was collected. Maternal HIV diagnosis was done using a qualitative rapid immune-chromatographic assay (Abbott Determine HIV-1/2). $CD4^+$ T-lymphocyte counts in EDTA blood samples were enumerated within a maximum of 6 h after sample acquisition for all HIV-infected mothers using a Partec Cyflow counter (Cyflow, Partec, Munster, Germany). Full blood counts (FBC) were determined from whole blood samples using a Mindray Haematology 3-part differential, BC3600 Analyser (Shenzhen, China). For HIV infected women, viral nucleic acids (viral load) were extracted from 1 mL maternal baseline plasma and the viral load was quantified using an automated Nuclisens EasyQ® (bioMérieux Clinical Diagnostics, Marcy-l'Étoile, France), according to the manufacturer's instructions. The detection limit for the assay is 100 cps/ml. The plasma samples at enrolment and at 14 weeks PP visit were stored at −80°C for the cytokine/chemokine assay.

Measurement of plasma cytokines

Quantitative determination of Th1, Th2, Th17, Treg, chemokines, acute phase proteins, and angiogenesis factors (Supplementary Table 1), was done by Meso Scale V-plex assays[65], a multi spot assay system (Meso Scale Diagnostics (MSD), Rockville, USA). The assay was done in plasma samples according to manufacturer instructions.

Statistical analysis

First, we measured cytokine plasma levels for different subgroups (i.e. HIV infected vs. uninfected) and time points (i.e. enrolment vs. 14 weeks PP). For this, we standardised each cytokines measured plasma level so that the new sample mean was 0 and the new sample standard deviation was 1 ($z = \frac{x-\bar{x}}{S}$, where z is the standardised value, $\bar{x}$ is the sample's mean and S the samples standard deviation). We then visualised these standardised cytokine plasma levels as a heat map and performed unsupervised hierarchical clustering using Euclidean distance and the complete linkage method. Aside from hierarchical clustering, cytokines were beforehand assigned to one of 7 groups (Th1, Th2, Th17, Treg, chemokines, acute phase proteins, and angiogenesis factors) based shared characteristics described in literature. A cytokine`s group was indicated on the heat map as well and could be compared with observed clusters.

Thereafter, we screened for significant differences in cytokine levels between (1) HIV, (2) malnourishment and (3) between time points of measurements (i.e. enrolment vs. 14 weeks PP). Change in cytokine levels were calculated by subtracting the enrolment value of a cytokine's measurement from its value at 14 weeks PP: $\Delta_{cytokine} = cytokine_{14\text{-weeks-PP}} - cytokine_{enrolment}$. Nourishment status was defined based on MUAC measurements at enrolment: pregnant mothers with a MUAC ≤23 cm were considered malnourished.[32,33]

Screening was done separately for the three associations of interest (Figure 1). (1) To detect differences in cytokine levels between HIV infected and uninfected women, we performed unpaired Wilcoxon tests (Wilcoxon-Mann-Whitney-Test) on data from enrolment and 14 weeks PP separately. (2) We similarly screened for the differences in cytokines in well-versus malnourished women. (3) To detect differences in cytokine levels between measurement time points (i.e. enrolment vs. 14 weeks PP) we performed paired Wilcoxon tests (Wilcoxon signed-rank test) on data of the different status groups (i.e. HIV negative vs. positive, well-vs. malnourished) separately. This approach resulted in 448 tests (56 cytokines * 2 types of tests [unpaired status groups and paired time points] * 2 types of status [HIV and nourishment status] * 2 status groups [HIV uninfected/infected and well-/malnourished]). Consequently, p-values were corrected for 448 tests by applying the Bonferroni-correction method to account for multiple testing problems. A corrected q-value <0.05 was considered significant.

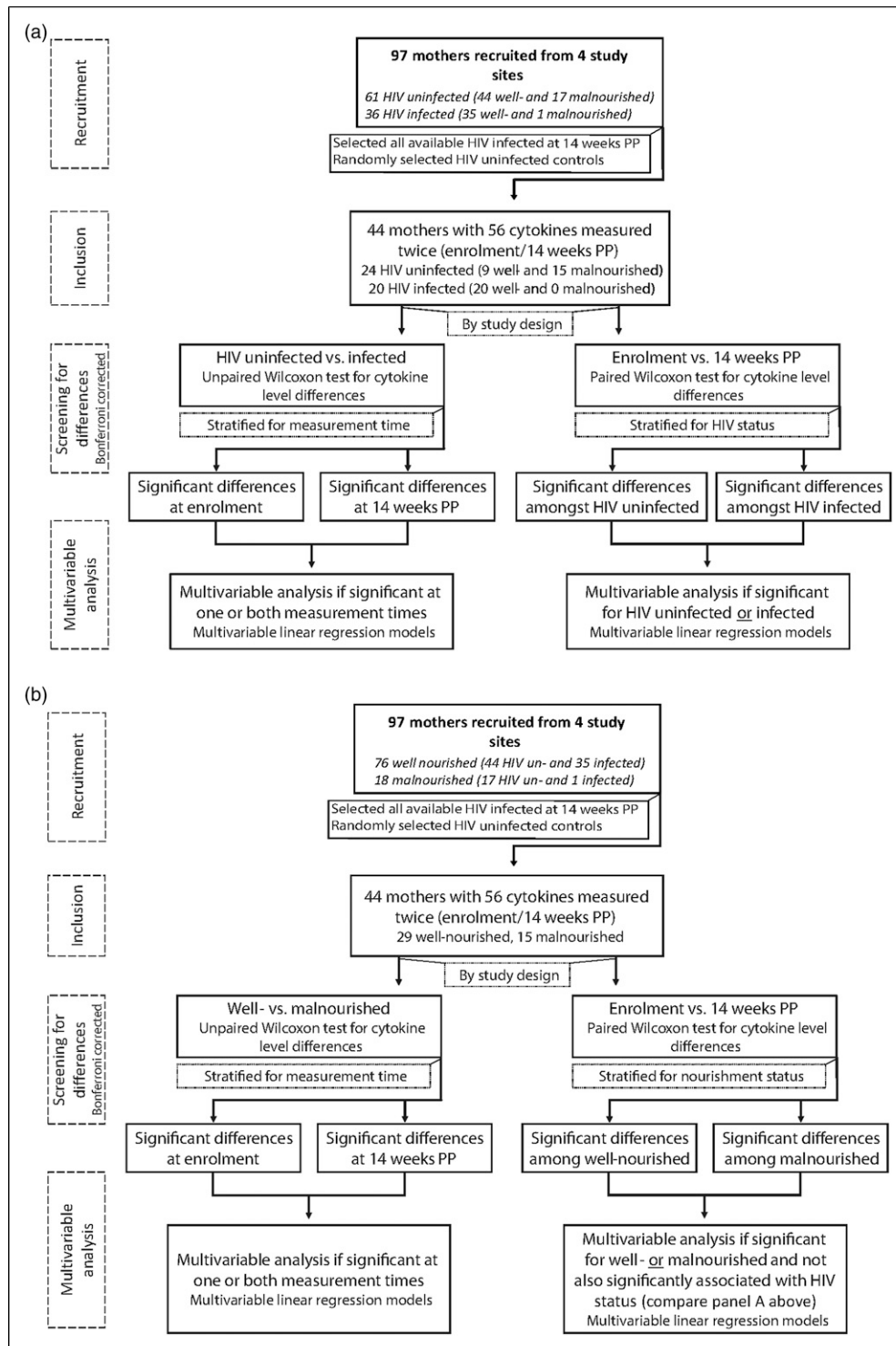For further analyses, we chose all cytokines with significant results in any screening test regarding HIV or

Figure 1. Flow charts of the study method. Panels A and B illustrate the main analysis steps for the HIV and the nourishment analysis, respectively. Abbreviations: PP: postpartum 339 × 492 mm (120 × 120 DPI).

nourishment status. For the longitudinal comparisons between enrolment and PP we included all cytokines with differential changes in cytokine plasma levels in the HIV or nourishment groups. These cytokines were considered

to show HIV or nourishment-dependent changes before and after pregnancy. Conversely, if significant differences were either observed for both HIV negative and positive mothers or if comparable differences were also observed

independent of HIV or nourishment status, we assumed that these changes might be associated with pregnancy status (pregnant vs. not pregnant) and/or the result of an unknown confounding variable associated with HIV and nourishment status and these cytokines were not further considered.

We then included the selected cytokines in multivariable linear regression models. The dependent variable was either the measured cytokine level or the change of cytokine level over time, depending on results of the screening test. The cytokines were modelled individually, and the models included either HIV or nourishment status along with independent variables (see below). We did not include nourishment and HIV status into the same model due to the observed high correlation between HIV and nourishment status which would violate the model's assumption of non-collinearity between independent variables.[37] Apart from HIV or nourishment status, the multivariable linear regression models included the independent variables maternal age, gestational age, and the number of previous gravidities. These independent variables were selected after being significant in univariate analysis in line with the assumption that these parameters represent relevant confounders. Models analysing measurement values at 14 weeks PP did not include gestational age. All data analyses were performed using R version 4.0.2 (2020-06-22).[38]

Results

To study the impact of HIV-infection and malnutrition on cytokine levels, we took advantage of the collected stored samples in the biobank of the UZBCS. We focused on 97 mothers recruited from February to September 2019. A total of 44 participants were selected for cytokine assaying (Figure 1(a) and (b)) in this pilot study (see methods). All mothers reside in a low-resource setting in high-density areas in Harare, communities that face significant economic and public health challenges.

Demographic and clinical data are compared in Tables 1 and 2. HIV infected mothers were significantly older and showed better nourishment with higher MUAC, body weight and BMI. Further, HIV infection was associated with more previous pregnancies, higher platelet counts, lower education and higher income used for food, (Table 1). All HIV infected women were on cART of Tenofovir, Lamivudine and Efavirenz (TENOLAM-E) treatment with a median of 1113 days of cART use. All HIV infected women were virally suppressed with all having an undetected HIV viral load and the majority of women were reaching an immune competent status with a median CD4$^+$ count of 347.5 cells/µl. On the other hand, malnourished women were significantly younger, with lower weight and lower BMI, smaller households and less monthly funds available to buy food (Table 2). There was no statistical difference on birth weight for both exposures that is HIV mothers and malnutrition mothers. Additionally, when compared the 44 participants included in cytokine assaying with the 53 excluded, there were no significant differences between both groups (Supplementary Tables 2a, b).

Changes in cytokine/chemokine levels between pregnancy and 14 weeks PP regardless of HIV infection

We then measured the levels of 56 cytokines/chemokines in the 44 participants at two time points: at enrolment (in pregnancy) and at 14 weeks postpartum. Overall, the cytokine and chemokine pattern changed according to pregnancy and HIV infection (Figure 2). For instance, acute phase proteins (C-reactive protein (CRP) and human serum amyloid A (SAA)) and transmembrane proteins (VCAM-1 and intercellular adhesion molecule-1 (ICAM-1)) showed a higher concentration in the HIV infected women both in pregnancy and at 14 weeks PP.

Unsupervised clustering revealed that differences between cytokines/chemokines according to time points (during pregnancy and at 14 weeks PP) were stronger than differences according to HIV status (Figure 2). Further, clustering revealed main three groups of cytokines (i) with strong increases between enrolment and 14 weeks PP, (ii) with only mild changes between enrolment and 14 weeks PP and (iii) with pronounced decreases between both time points. We grouped cytokines according to their proposed function (groups 1–7 in Figure 2), but no correlation between cytokine/chemokine group and the identified cluster was apparent.

HIV infection is associated with differences in IL-4, IL-8, and IL-10 levels

We screened for differences in the levels of 56 cytokines between HIV infected and uninfected mothers at enrolment and at 14 weeks PP (Supplementary Figure 1). IL-4 and IL-10 levels were higher in the HIV infected mothers ($q <$ 0.005) with significance remaining after multiple test correction (Table 3). However, no significant cytokine differences remained at 14 weeks PP after correcting for multiple testing.

Screening for effects of HIV status on longitudinal cytokine measurements between enrolment (pregnancy) and 14 weeks PP (Supplementary Figure 2), we identified three cytokines, IL-4, IL-8, and IL-10, for which HIV infection was associated with changes in plasma levels which remained significant after multiple test correction ($q <$ 0.005) (Table 3). For IL-4 and IL-10 concentrations decreased in HIV infected mothers but increased in HIV uninfected participants. In contrast, concentrations of IL-8 increased in the HIV infected group from pregnancy to 14

Table 1. Comparison of socio-demographic and clinical data stratified by HIV status at enrolment (in pregnancy).

Variable	HIV uninfected (n = 24)	HIV infected (n = 20)	p value
Age in years [median (Q1-Q3)]	24 (19.5–28.5)	32 (28.5–35)	<0.001
Monthly income in USD [median (Q1-Q3)]	275 (200–400)	350 (256.5–550)	0.331
Money used for food USD [median (Q1-Q3)]	80 (60–150)	150 (100–155)	0.008
Education: Completed secondary n (%)	20 (83.3%)	11 (55.0%)	0.007
Completed primary	4	9	
Maternal employment: Employed n (%)	5 (20.8%)	6 (30.0%)	0.407
Unemployed	19	14	
Spouse employment: Employed n (%)	23 (95.8)	19 (95.0%)	0.361
Unemployed	1	1	
Household size [median (Q1-Q3)]	3.5 (2–4.5)	4 (3-5)	0.106
Number of rooms used [median (Q1-Q3)]	1 (1-2)	2 (1–2)	0.132
Days with running water [median (Q1-Q3)]	7 (4.5–7)	5 (0–7)	0.0343
Meals normally eaten per day [median (Q1-Q3)]	3 (2.5–3)	3 (2-3)	0.487
Meals eaten per day during pregnancy [median (Q1-Q3)]	3 (3–4.5)	4 (3-5)	0.487
Fewer meals per days because family can't afford: YES n (%)	6 (25.0%)	3 (15.0%)	0.804
NEVER	18	17	
Number of total pregnancies [median (Q1-Q3)]	2 (1–2.5)	3 (3–3.5)	<0.001
MUAC in cm [median (Q1-Q3)]	22.5 (22.0–24.75)	31.75 (5.48)	0.006
Weight in kg [median (Q1-Q3)]	60 (53-65)	65 (61–74)	0.004
BMI [median (Q1-Q3)]	21.9 (20.6–24.9)	26.3 (23.6–27.5)	<0.001
White blood count 10⁹/L [median (Q1-Q3)]	7.4 (6.65–7.9)	7.7 (5.8–8.9)	0.232
Haemoglobin g/dL [median (Q1-Q3)]	11.8 (10.8–12.4)	11.7 (10.2–12.4)	0.2753
MCV fL [median (Q1-Q3)]	88.75 (83.5–90.25)	89.1 (82.7–96.6)	0.3243
Platelets 10⁹/L [median (Q1-Q3)]	199.5 (169.5–228.5)	211.8 (186–236)	0.049
CD4 cells/μl [median (Q1-Q3)]	n/a	347.5 (269–426)	
Viral load copies/ml [median (Q1-Q3)]	n/a	0 (0–0)	
		0 = undetected	
cART treatment: NO n (%)	N/A	0	
YES		20 (100%)	
cART duration in days		1113 (329–2191)	
Pregnancy outcomes			
Mode of delivery: Normal n (%)	24 (100%)	17 (85.0%)	0.164
Caesarean	0	3	
Baby birth weight in g [median (Q1-Q3)]	3000 (2768–3288)	3098 (2940–3350)	0.4594

weeks PP but did not significantly changed in women without HIV infection.

IL-4, IL-8 and IL-10 were further analysed in multivariable linear regression models which included HIV status, age, gestational age, and gravida (Supplementary Table 3a). For IL-4 and IL-10, differences between HIV infected and uninfected mothers at enrolment remained robust also after correction for confounders (Figure 3, Table 3). For longitudinal change of cytokine levels between enrolment and 14 weeks PP, only changes in IL-4 remained significant after multivariable correction (Figure 3, Supplementary Table 3b and Supplementary Figure 3, Supplementary Figure 4).

Malnutrition impacts on VCAM-1 and IL-1RA levels

In a similar approach, we also screened for differences between well- and malnourished mothers, at both enrolment

(in pregnancy) and 14 weeks PP (Supplementary Figure 5). VCAM-1 levels at enrolment were lower in malnourished women and significance (q < 0.005) remained after multiple test correction (Table 3). At 14 weeks PP, IL-1RA levels were lower in malnourished women with significance robust to correcting for multiple testing (q < 0.005) (Figure 4, Table 3). Multivariable linear regression models were implemented with both cytokines as dependent variables and statistical significance remained in both cases after correction for age, gestational age, and gravida as confounders (Supplementary Table 4a).

When testing for effects of malnourishment on cytokine level changes over time, we observed three cytokines (IL-7, IL-16, macrophage inflammatory protein-3 alpha (MIP-3α)) with significant (q < 0.005) nourishment dependent changes (Supplementary Figure 6) that fulfilled criteria for further analysis. However, no significant results remained

Table 2. Comparison of socio-demographic and clinical data, stratified by nourishment at enrolment (in pregnancy).

Variable	Well nourished (n = 29)	Malnourished (n = 15)	p value
Age in years [median (Q1–Q3)]	30 (26–33)	21 (18–28)	<0.001
Monthly income in USD [median (Q1–Q3)]	320 (250–500)	250 (150–250)	0.012
Money used for food USD [median (Q1–Q3)]	150 (100–200)	75 (50–100)	<0.001
Education: Completed secondary n (%)	19 (65.5%)	12 (80.0%)	0.467
Completed primary	10	3	
Maternal employment: Employed n (%)	9 (31.0%)	2 (13.3%)	0.247
Unemployed	20	13	
Spouse employment: Employed n (%)	28 (96.6%)	14 (93.3%)	0.267
Unemployed	1	1	
Household size [median (Q1–Q3)]	4 (3–5)	3 (2–5)	0.042
Number of rooms used [median (Q1–Q3)]	2 (1–2)	1 (1–2)	0.202
Days with running water [median (Q1–Q3)]	5 (4–7)	7 (1–7)	0.495
Meals normally eaten per day [median (Q1–Q3)]	3 (2–3)	3 (2–3)	0.581
Meals eaten per day during pregnancy [median (Q1–Q3)]	3 (3–5)	4 (3–4)	0.487
Fewer meals per days because family can't afford: YES n (%)	3 (10.3%)	6 (40.0%)	0.103
NEVER	26	9	
Number of total pregnancies [median (Q1-Q3)]	3 (2-3)	2 (1-2)	<0.001
HIV status: Negative n (%)	9 (31.0%)	15 (100%)	0.003
Positive	20	0	
Weight in kg [median (Q1–Q3)]	65 (62–75)	53 (49-60)	<0.001
BMI [median (Q1-Q3)]	26.2 (23.3–28.0)	20.9 (20.3–22.0)	<0.001
White blood count 10⁹/L [median (Q1-Q3)]	7.5 (6.4–8.9)	7.2 (6.7–7.7)	0.060
Haemoglobin g/dL [median (Q1-Q3)]	11.8 (10.6–12.1)	11.4 (10.2–12.8)	1
MCV fL [median (Q1-Q3)]	88.3 (83.1–91.8)	89.2 (83–91.1)	0.4478
Platelets 10⁹/L [median (Q1-Q3)]	203 (179–235)	192 (173–246)	0.1433
Pregnancy outcomes			
Mode of delivery: Normal n (%)	26 (89.7%)	15 (100%)	0.157
Caesarean	3	0	
Baby birth weight g [median (Q1-Q3)]	3125 (2900–3370)	2975 (2695–3260)	0.1751

in multivariable linear regression models (Figure 4, Supplementary Table 4b and Supplementary Figure 7, Supplementary Figure 8).

Discussion

Multiple challenges exist for the immune system during pregnancy in a low-resource setting including infectious diseases such as HIV and socio-economic problems associated with malnutrition. By testing over 50 immunological biomarkers in this pilot study, we found significantly higher concentrations of IL-4 and IL-10 in HIV infected women in late pregnancy compared to uninfected pregnant women. Further, concentrations of IL-4, IL-8 and IL-10 evolved differently between late pregnancy and 14 weeks PP, dependent on HIV status. Malnutrition was associated with lower VCAM-1 and IL-1RA levels.

Our finding of an elevated IL-4 concentration in HIV infection supports the idea that HIV infection skews the cytokine response to a predominant Th2 state.[39–41] IL-4 is

recognised as a definer of Th2 cells and has been identified as a critical cytokine promoting HIV immunopathology.[8,42] IL-4 contributes to HIV pathogenesis by impairing cell-mediated immunity.[8] Elevated IL-4 levels in HIV infected pregnant women were also reported by two studies from South Africa and Brazil even though differences did not yield statistical significance.[43,44] In contrast, another study from Zimbabwe reported that there was significant difference in IL-17A levels between HIV infected and uninfected while IL-4 was not affected.[45] These discrepancies are likely due to the different study populations since population investigated by Mlambo et al., consisted of cART naïve and not pregnant women.[45]

IL-4 production is increased throughout normal pregnancy, induced by progesterone.[46] IL-4 is produced by immune cells of the placenta and the maternal decidua, amniochorionic membranes, cytotrophoblasts, and maternal and fetal endothelial cells.[47] IL-4 reduces inflammation during pregnancy in a complex process that if disrupted can lead to persistent inflammation and pregnancy complications.[48]
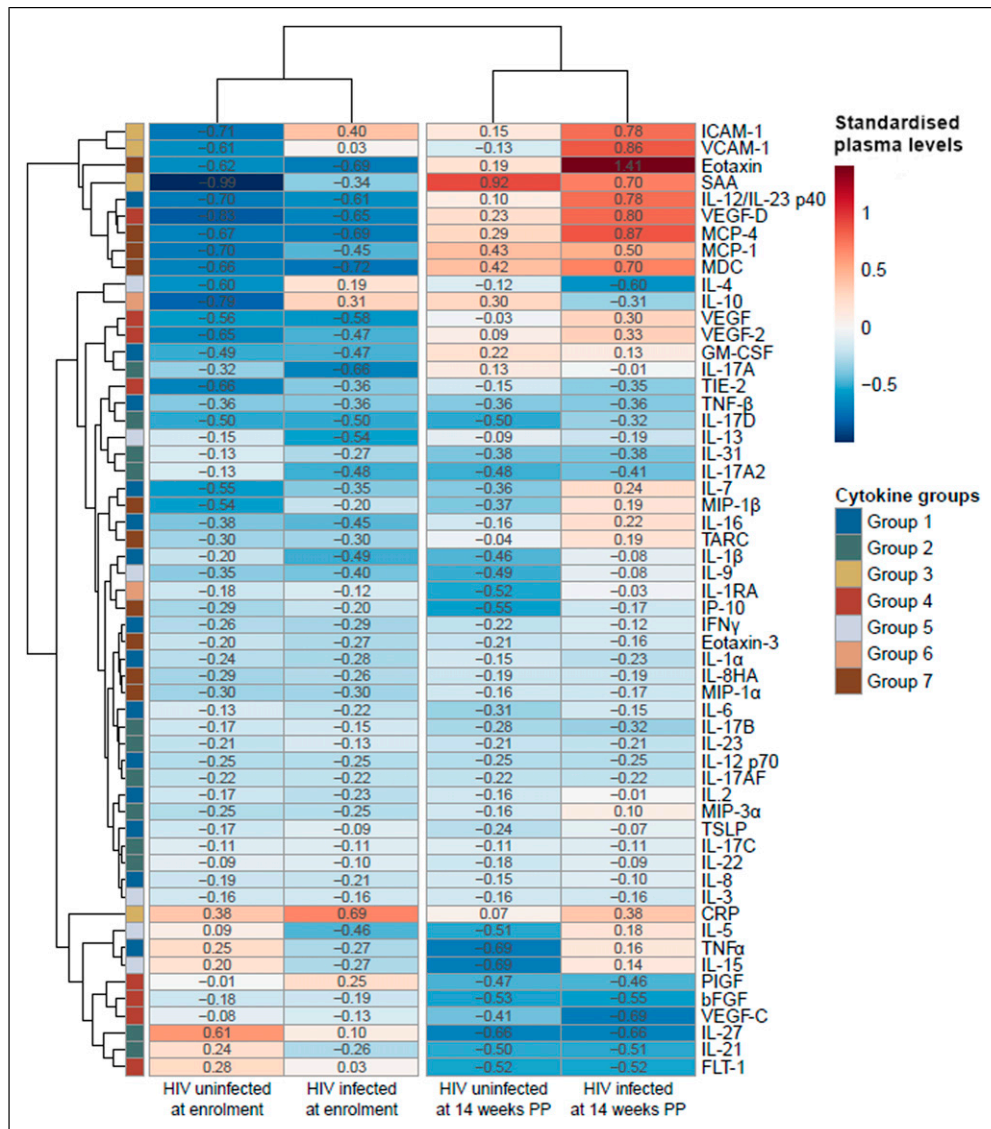
Figure 2. Heatmap showing cytokine/chemokine clusters and patterns of cytokine serum level changes. Heatmap of median standardised cytokine levels. Median standardised cytokine levels are additionally written as number in each corresponding cell. Membership of beforehand defined cytokine groups (group 1 Th1, group 2-Th17, group 3-acute phase proteins, group 4-angiogenesis factors, group 5-Th2, group 6-Treg, group 7-chemokine) are displayed on the left side as coloured squares. Dendrograms show hierarchical clusters of cytokine and patient groups. Abbreviations: PP: postpartum. 184 × 209 mm (96 × 96 DPI).

IL-10 and IL-4 have partially overlapping anti-inflammatory effects but IL-10 differs in its effects on macrophage gene expression and its inhibition of antigen-presenting cells. Persistent high circulating IL-10 levels are a characteristic feature of HIV infection and are of pathogenic importance in the progression of the disease.[49,50] The present study demonstrates that HIV infected pregnant women have significantly higher IL-10 levels than uninfected controls. These results are in line with recent findings from a South African study that observed significantly higher IL-10 levels in HIV infected pregnant women in the third trimester.[43] In contrast, a Brazilian

study reported lower concentrations of IL-10 in HIV infected pregnant women.[44]

High concentrations of IL-10 levels have been reported in pregnancy.[15,51] and anti-inflammatory characteristics of IL-10 supports successful completion of pregnancy.[51] IL-10 mediates the crosstalk between the placental decidua and the invading fetal trophoblasts and curtails maternal inflammation by decreasing the production of Th1 cytokines.[48] IL-10 shows a protective effect on the fetal-placental unit as it inhibits the secretion of inflammatory cytokines, such as IL-6, TNF-α, and IFN-γ,[52] all of which did not show any significant differences in this study. IL-10's

Table 3. Screening for differences in cytokine/chemokine levels according to HIV infection, nourishment and time points.

		Screening for differences in cytokine/chemokine levels			Multivariable analysis	
Cytokine name	Sample (stratification group)	Compared groups	Cytokine level pg/mL; median (IQR)	*p* value/q value	Estimate	*p* value
Comparison between cytokine levels of HIV uninfected and infected mothers at either enrolment and 14 weeks PP (unpaired Wilcoxon test)						
IL-4	Enrolment	HIV uninfected	0 (0–0)	<0.001/*	0.027	0.027
		HIV infected	0.02 (0–0.06)			
	14 weeks PP	HIV uninfected	0.01 (0–0.03)	0.37/ns		
		HIV infected	0 (0–0.01)			
IL-10	Enrolment	HIV uninfected	0 (0–0)	<0.001/***	0.163	<0.001
		HIV infected	0.18 (0.03–0.24)			
	14 weeks PP	HIV uninfected	0.18 (0.08–0.31)	0.09/ns		
		HIV infected	0.08 (0–0.26)			
Comparison between cytokine/chemokine levels at enrolment and 14 weeks PP amongst either HIV uninfected or infected mothers (paired wilcoxon test)						
IL-8	HIV uninfected	Enrolment	0.53 (0.33–0.88)	0.12/ns		
		14 weeks PP	0.85 (0.48–1.31)			
	HIV infected	Enrolment	0.33 (0.21–0.49)	<0.001/*	7.256	0.173
		14 weeks PP	1.27 (1.07–4.15)			
IL-4	HIV uninfected	Enrolment	0 (0–0)	<0.001/**		
		14 weeks PP	0.01 (0–0.03)			
	HIV infected	Enrolment	0.02 (0–0.06)	0.014/ns	−0.033	0.014
		14 weeks PP	0 (0–0.01)			
IL-10	HIV uninfected	Enrolment	0 (0–0)	<0.001/***		
		14 weeks PP	0.18 (0.08–0.31)			
	HIV infected	Enrolment	0.18 (0.03–0.24)	0.61/ns	−0.069	0.402
		14 weeks PP	0.08 (0–0.26)			
Comparison between cytokine/chemokine levels of well- and malnourished mothers at either enrolment or 14 weeks PP (unpaired wilcoxon test)						
VCAM 1	Enrolment	Well nourished	485424 (37,6116–6178,03)	<0.001/*	−182,557	0.01
		Malnourished	290214 (24,7945–3763,44)			
	14 weeks PP-	Well nourished	627914 (44,9955–7124,18)	0.039/ns		
		Malnourished	421628 (27,0441–5796,39)			
IL-1RA	Enrolment	Well nourished	130.96 (107.53–168.68)	0.088/ns		
		Malnourished	110.77 (93.14–136.54)			
	14 weeks PP-	Well nourished	149.9 (107.4–166.8)	<0.001/*	−83.999	0.003
		Malnourished	67.61 (56.22–72.57)			
Comparison between cytokine/chemokine levels at enrolment and 14 weeks PP amongst either well- or malnourished mothers (paired wilcoxon test)						
IL-16	Well nourished	Enrolment	63.20 (54.31–84.16)	<0.001/*		
		14 weeks PP	100.76 (81.13–128.6)			
	Malnourished	Enrolment	59.87 (40.14–65.96)	0.03/ns	−38.881	0.302
		14 weeks PP	71.57 (63.20–83.55)			
IL-7	Well nourished	Enrolment	0.94 (0.63–1.71)	<0.001/**		
		14 weeks PP	2.82 (1.68–4.76)			
	Malnourished	Enrolment	1.03 (0.67–2.57)	0.49/ns	−2.31	0.104
		14 weeks PP	1.03 (0.61–1.72)			
MIP-3α	Well nourished	Enrolment	14.90 (11.12–20.47)	<0.001/*		
		14 weeks PP	25.65 (16.93–56.56)			
	Malnourished	Enrolment	10.59 (6.61–15.88)	0.048/ns	−44.663	0.954
		14 weeks PP	24.05 (12.11–25.40)			

Only cytokines/chemokine with significant differences after Bonferroni correction are presented. The complete analysis can be found in the Supplementary figures 1-4. Multivariable linear regression models showing the association of HIV status and nourishment status with different cytokine levels are shown in the left two columns. *N* = 44 for all analysis. Abbreviations: PP: postpartum, q-value: Bonferroni-corrected *p*-value (correcting for 448 different tests) ns *q* > 0.05 **q* < 0.05, ***q* < 0.01 and ****q* < 0.001.
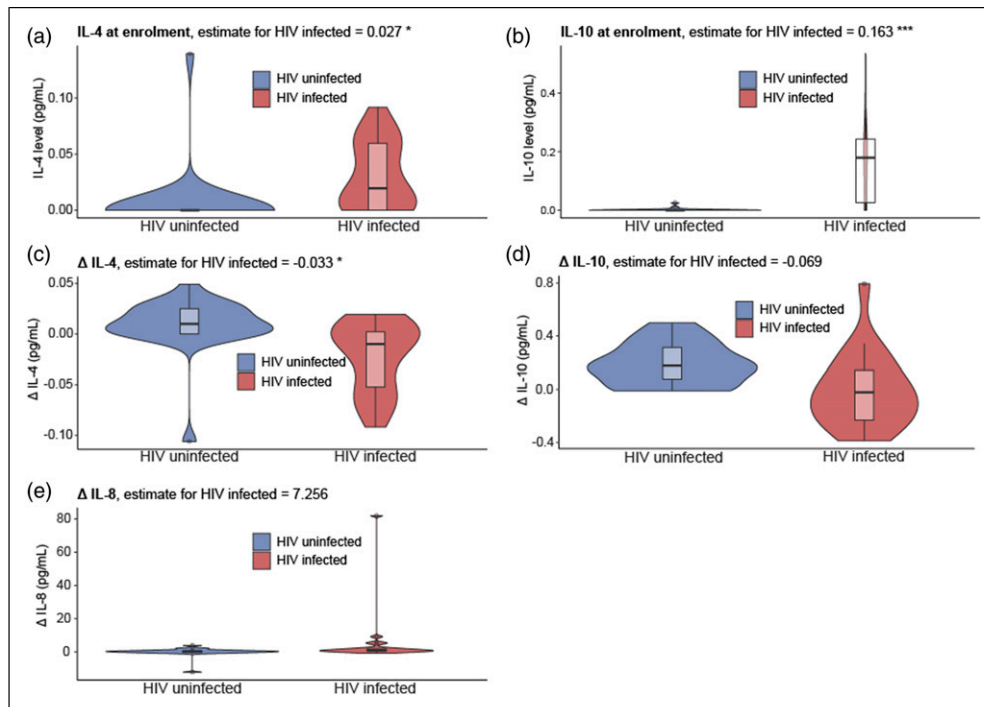
Figure 3. Violin plots of the distribution of cytokine/chemokine levels and cytokine/chemokine changes for HIV uninfected and infected mothers. Panels A and B show cytokine levels measured at enrolment whereas panels C-E show changes in cytokine/chemokine levels. The Δ of cytokines/chemokines was calculated by subtracting enrolment measurements of a cytokine/chemokine from its value at 14 weeks PP. P-values report the statistical differences between HIV uninfected and infected mothers and were obtained from multivariable linear regression models (compare with Table 1). Abbreviations: PP: postpartum, RC: regression coefficient. 333 × 243 mm (72 × 72 DPI).

immunosuppressive action in pregnancy contributes to the balance of pro- and anti-inflammatory signals that enable successful completion of pregnancy.[51]

In our study, levels of both, IL-4 and IL-10 significantly increased from pregnancy to 14 weeks PP in the HIV uninfected group. These results are in line with previous data reporting an increase of IL-4 and IL-10 from pregnancy to 2 months after delivery.[53] In contrast, for the HIV infected group, we observed a trend for a decrease in cytokine levels for both IL-4 and IL-10 from late pregnancy to 14 weeks PP. These results for the HIV infected group are in line with the notion that after pregnancy, the immune system shifts from the Th2 dominant state to the baseline Th1 state explaining the observed decrease in the Th2 related cytokines IL-4 and IL-10.[54,55]

We noticed that levels of IL-14 and IL-10 were undetectable or extremely low compared to reference levels for IL-10 and IL-4 (which are 0.298–23 pg/mL and 0.218–158 pg/mL, respectively). However, these reference levels are not necessarily relevant for black pregnant women.

There was a significant increase in concentrations of IL-8 between late pregnancy and 14 weeks PP in both HIV infected and HIV uninfected participants. For IL-8 concentrations, a U-shaped curve from early pregnancy to post

pregnancy has previously been reported with concentrations decreasing as pregnancy progresses, followed by an increase postpartum.[28] IL-8 is a pro-inflammatory related chemokine responsible for the recruitment and activation of immune cells to inflamed tissues.[56] It has been hypothesised that IL-8 may participate in the induction of a physiological delivery during pregnancy. This can partially explain the observed U-shaped curve and also the IL-8 increase from pregnancy to postpartum. In HIV, elevated IL-8 levels represent a hallmark of chronic inflammation and HIV disease progression.[56]

We also found non-significantly higher concentrations of acute phase proteins (CRP and SAA) and vascular signalling proteins (ICAM1 and VCAM1) in HIV infected women in pregnancy and 14 weeks PP. These proteins are important markers and assessors of vascular injury in humans. CRP, a prototype of acute phase proteins, increase in HIV infected population over time regardless of disease progression to AIDS.[57]

VCAM-1 concentration was lower in malnourished pregnant women than in the well-nourished women. In a study investigating disease related malnutrition, they observed that malnutrition was positively correlated with VCAM-1 levels,[58] and the concentration of VCAM-1 in high inflammation related diseases was higher.[59] Also IL-1RA, an
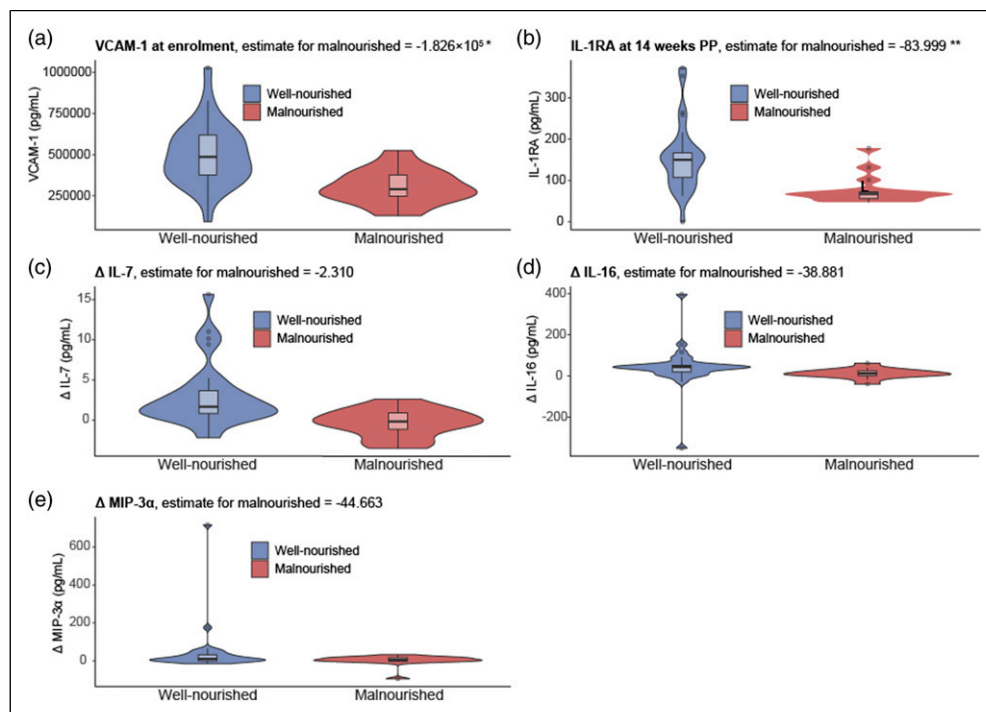
Figure 4. Violin plots of the distribution of cytokine/chemokine levels and cytokine/chemokine changes for well and malnourished mothers. Panels A and B show cytokine levels measured at enrolment and 14 weeks PP, respectively whereas panels C-E show changes in cytokine/chemokine levels. The Δ of cytokines/chemokines was calculated by subtracting enrolment measurements of a cytokine/chemokine from its value at 14 weeks PP. P-values report the statistical differences between well- and malnourished mothers and were obtained from multivariable linear regression models (compare with Table 1). Abbreviations: PP: postpartum, RC: regression coefficient. 333 × 243 mm (72 × 72 DPI).

anti-inflammatory cytokine, was lower in the malnourished women at 14 weeks PP. There are studies that have assessed IL-1RA that have seen the same pattern, with one Japanese study reporting lower IL-1Ra level in elderly malnourished patients[60] and was closely correlated with low body mass index.[61] These results may be a result of defective immune-inflammatory adaptation system.

In this study we did not confirm an increase in Th2 cytokines in malnourished individuals described in previous studies.[62,63] In fact, no significant difference between malnourished and well-nourished pregnant women regarding Th1 and Th2 cytokine levels could be found in this study. However, such a shift to a Th2 dominant cytokine profile might be due to deficiencies of micronutrients such as vitamin A rather than caloric deficiencies.[62,64] Vitamin A supplementation has been associated with an increased ratio of the Th1 cytokine IFN-γ to the Th2 cytokine IL-10 during pregnancy and postpartum.[63] Micronutrients and trace elements were not assessed in our analyses, which remains a limitation of our study.

Strengths of our study include the comprehensive approach with a large number of cytokines screened. Also the study assessed HIV and malnutrition as comorbidities in the same population. To correct for multiple testing problems,

Bonferroni-correction was applied. This correction makes our statistical analysis and models robust, though we lost some significance in other biomarkers, raising the possibility of type II errors. The main limitation of our study is the small sample size and we were underpowered to perform any sensitivity analyses. Therefore, we were unable to completely disaggregate the effects of HIV, malnutrition and pregnancy on cytokine levels. Therefore, our study should be considered a pilot study and future analyses with a larger sample size are warranted to provide an overview of effects HIV and malnutrition in pregnancy.

Conclusion

IL-4 and IL-10 were significantly higher in HIV infected women in late pregnancy compared to uninfected pregnant women and there were significant longitudinal differences in IL-4, IL-8, and IL-10 concentrations from pregnancy to 14 weeks PP. Significant differences in VCAM-1 and IL-1RA were associated with malnutrition. These results showed that HIV infection in pregnancy affects the cytokines by skewing the immune responses to the anti-inflammatory Th2 cytokines IL-4 and IL-10. These cytokines are both important for pregnancy

completion and also in the pathogenesis of HIV. Reductions of VCAM-1 and IL-1RA observed in pregnant women with malnutrition illustrate the broad systemic impact of malnutrition. The findings in this pilot study therefore warranty studies with a larger sample size to improve understanding of cytokine patterns associated with HIV infection and malnutrition. These might in the future enable for development of alternative novel biomarkers for diagnosis, monitoring and therapies for pregnant women to improve maternal and infant outcomes.

Appendix

List of abbreviations

Body mass index (BMI)
C-reactive protein (CRP)
Cluster of differentiation 4 (CD4$^+$)
Combined antiretroviral therapy (cART)
Full blood count (FBC)
Human serum amyloid A (SAA))
Human Immunodeficiency Virus-1 (HIV)
Intercellular adhesion molecule-1 (ICAM-1)
Interferon (IFN-γ)
Interleukin (IL)
Interleukin-1 receptor antagonist (IL-1RA)
Macrophage inflammatory protein-3 alpha (MIP-3α)
Mean corpuscular volume (MCV)
Mid upper arm circumference (MUAC)
Postpartum (PP)
Sub-Saharan African (SSA)
T helper type 1 (Th1)
T helper type 2 (Th2)
Regulatory T cells (Treg)
University of Zimbabwe Birth Cohort Study (UZBCS)

Authors' contributions

The study was conceived by KD and PC, and designed by PC, BY, BM and KD. PC, PTM and AJM were responsible for data collection, entry and validation. Recruitment and follow-up were done by PC and PTM overseen by KD and LRM. PC and BY performed the cytokine assays. SBUJ and PC performed the statistical analysis. The first draft of the manuscript was written by PC and all authors were involved in manuscript revisions and approved the final draft.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics approval

Ethical approval was obtained from the joint research ethics committee (JREC/228/19) of the University of Zimbabwe and the Medical Research Council of Zimbabwe (MRCZ/B/1824).

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Supplemental Material

Supplementary material for this article is available on the online.

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