The immunological basis of preterm birth in HIV-1 infected pregnant women

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Abstract

Hypotheses

HIV infection and combination antiretroviral therapy (cART) used to prevent mother-to-child-transmission of HIV in pregnancy increase the risk of preterm birth (PTB). We hypothesise that the use of cART, particularly protease inhibitor (PI) regimes, induce a pro-inflammatory environment at the fetal maternal unit, which triggers labour to occur at an earlier point in gestation than would occur without HIV-1 infection or cART. We also hypothesise that local cervicovaginal fluid (CVF) will be more informative regarding immune function than plasma.

Specific aims

-To understand the inflammatory mechanisms underlying PTB in HIV1infected and uninfected women

-To elucidate if PI-based cART regimes are associated with greater concentrations of inflammatory cytokines compared to non-PI-based cART regimes in pregnancy

-To characterise inflammatory and immune proteins in the CVF of HIV-1 infected pregnant women and compare by ART exposure and prematurity with a view to identifying potential PTB biomarkers.

Results

PTB in HIV-1 infected women was associated with greater circulating activated T cells and abundance of vaginal *Gardnerella* and *Prevotella* species. HIV-1 infected pregnant women have a high prevalence of *L. iners* and diverse anaerobic bacteria dominant vaginal community state types. Pre-conception cART was associated with greater abundance of *Gardnerella vaginalis* and a reduction in circulating activated T cells but not to the same level as observed in uninfected pregnant women. Length of time on cART was associated with an increase in plasma IL-12, the macrophage activating cytokine, indicating a pro-inflammatory

shift in cytokine environment. HIV-1 infected pregnant women had much higher inflammatory cytokines in their CVF. In HIV-1 infected pregnant women vaginal bacterial diversity was positively correlated with CVF pro-inflammatory cytokine IL-1β, known to be associated with PTB. Directed exploration of the CVF proteome in HIV-1 infected pregnant women revealed high abundance of immune proteins associated with macrophage and neutrophil activation, and Extracellular Matrix (ECM) modifiers such as matrix metalloproteinases (MMPs). These proteins were especially abundant in women receiving PI-based cART.

Significance

These data indicate that ART may modulate the local bacterial species; perhaps through selective pressure and that the vaginal bacterial communities observed in HIV-1 infected women are pro-inflammatory and associated with PTB. Local CVF inflammation and immune activation is exaggerated in these HIV-1 infected women and not fully reversed with cART. The presence of bacterial antigen in the context of enhanced local innate and adaptive inflammatory immune response is likely responsible for up-regulating downstream NFκB pathways such as induction of MMPs, known to be association with induction of labour.

Contribution to academic area

A greater understanding of the mechanisms behind this phenomenon enable us to greater define the toxicity of certain antiretroviral classes or specific drugs in pregnancy and assess newer drugs using the same techniques. Optimising the risk stratification in these women, perhaps through application of improved PTB biomarkers or regular vaginal bacterial screening, may enable improved targeted risk reduction strategies e.g. progesterone, antibiotics or probiotics.

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To all the women who took part in these studies, the Wellcome Trust and the March of Dimes, thank you, without you none of this work would be possible.

Testimony that own work

I declare that this original work undertaken and written by myself and that all else is appropriately referenced.

Copyright

Publications in press

- Short CE, Quinlan R, Brown R, et al. The vaginal microbiome of HIV – infected pregnant mothers: associations with local inflammation and gestational age at delivery. Rev Anti Therapy & Infect Dis. 2018; 8:82.
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- Short CE, Brown R, Quinlan R, Lee Y, Shattock R, Bennett P, Taylor GP and MacIntyre D. Dysbiotic vaginal microbiota in HIV-infected pregnant women is associated with local inflammation and preterm birth.- Lancet HIV
- Short CE, Skyes L, Bennett P and MacIntyre D. The reproductive tract microbiome and pregnancy outcomes: the vagina dialogues. Commissioned review on the FGT microbiome and immunological causes of preterm birth. Nature reviews Urology.

Abbreviations

1D SDS-PAGE One dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis
2D-LC multi-dimensional liquid chromatography
3TC Lamivudine
ABC Abacavir
AIDS Acquired immune deficiency syndrome
AMP Anti microbial protein
ATV/r Atazanavir &ritonavir
BCA Bicinchoninic acid
BHIVA British HIV Association
CAM Chorioamniontitis
CAPRISA Centre for the AIDS Programme of Research in South Africa
cART Combination antiretroviral therapy
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CCR5I C-C chemokine receptor type 5 inhibitor
CD4 Cluster of differentiation 4
CDC Center for Disease Control and Prevention
CHI3L1 Chitinase 3-like 1
COX-2 Cyclo-oxygenase-2
CRH Corticotrophin releasing hormone
CRP C-Reactive Protein
CST Community state type
CTLA Cytotoxic T-Lymphocyte Associated Protein
CVF Cervicovaginal fluid
CVL Cervicovaginal Fluid
CX3CL1 C-X3-C Motif Chemokine Ligand 1
d4T Stavudine
DKK-1 Dickkopf-1
DPPIV : Dipeptidyl-peptidase IV
DTG Dolutegravir
E coli Escherichia coli
ECM Extracellular matrix
ECS European Collaborative Study
EDTA Ethylenediaminetetraacetic acid
EFV Efavirenz
EMMPRIN Extracellular Matrix Metalloproteinase Inducer
FFN Fetal fibronectin
FGT Female Genital Tract
FOXP3 Forkhead box P3
FRESH Females Rising through Education, Support and Health
FTC Emtricitabine
G-CSF Granulocyte colony-stimulating factor
GDF-15 Growth/differentiation factor 15
GM-CSF Granulocyte-macrophage colony-stimulating factor
HC-HIV Human contraception and HIV acquisition study
HIV Human Immunodeficiency Virus
HLA-DR Human Leukocyte Antigen – DR isotype
ICAM Intercellular Adhesion Molecule-1

ICL Imperial College London
IFN Interferon
IL Interleukin
IMPAACT International Maternal Pediatric Adolescent AIDS Clinical Trial
INSTI Integrase strand transfer inhibitor
IP-10 Interferon gamma-induced protein-10
IQR Interquartile range
IRGF Insulin Related Growth Factor
IRGF BP IGRF Binding Protein
IUGR Intra uterine growth restriction
kDa Kiladalton
LEfSE Linear discriminant analysis with effect size
LLETZ Lower Loop Excision of the cervical Transformation Zone
LPS Lipopolysaccharide
LPV/r Lopinavir & ritonavir
MC menstrual cup
MCP Monocyte chemo attractant protein
MHC Major histocompatibility complex
MIF Macrophage migration inhibitory factor
MIP Macrophage inflammatory protein
MMP Matrix metalloproteinase
MPO Myeloperoxidase
mRNA messenger RNA
MSD Meso Scale Discovery
MTCT Mother to child transmission
NFKB nuclear factor-kappa B
NFV Nelfinavir
NIHR National Institute for Health Research
NK Natural Killer
NMA Network meta-analysis
NNRTI Non-nucleoside reverse transcriptase inhibitor
NRTI Nucleoside reverse transcriptase inhibitor
NSIG non-significant
NVP Nevirapine
OTU Operational taxonomic units
PBMCS Peripheral blood mononuclear cells
PI Protease Inhibitor
PMTCT Prevention of MTCT
PPROM Prelabour premature rupture of membraes
PREP Pre-exposure prophylaxis
PROMISE Promoting Maternal and Infant Survival Everywhere
PTB Preterm birth
PTL Preterm labour
PVA Polyvinyl acetyl
RA Receptor Antagonist
RANTES Regulated upon Activation, Normal T cell Expressed, and Secreted
RCT Randomised Control Trial
RDP Ribosomal Database Project
rRNA ribosomal RNA

sdNVP single dose Nevirapine
SELDI-TOF Mass Spectrometry
SERPINE1 Serine proteinase inhibitor E1
SHBG Steroid Hormone Binding Globulin
SLPI Secretory Leukocyte Peptidase Inhibitor
sPTB Spontaneous preterm birth
SPTL Spontaneous preterm labour
STAMP Statistical Analysis of Metagenomic Profiles
TCR T cell receptor
TDF Tenofovir Disoproxil Fumarate
TFF3 Trefoil factor-3
TGF-β Transforming growth factor beta
Th1 Type 1 T helper
Th17 Type 17 T helper
Th2 Type 2 T helper
TLR Toll Like Receptor
TNF-α Tumour necrosis factor alpha
TV USS: Transvaginal ultrasound scan
uPAR Urokinase receptor
VEGF Vascular Endothelial Growth Factor
VIT D-BP Vitamin D binding protein
VMB Vaginal microbiota
WHO World health organisation
WIHS Women's Interagency HIV Study
WITS Women and Infants Transmission study
ZDV zidovudine monotherapy
ZDV Zidovudine
ZDV mono Zidovudine monotherapy

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1. Chapter 1. Literature review

1.1.1 The paradoxical puzzle

Suppression of maternal plasma HIV RNA to less than 50 copies/mL with combination antiretroviral therapy (cART, three or more active drugs use in combination) can virtually eliminate mother to child transmission of HIV (MTCT) with rates of 0.1% achievable(Peters *et al.*, 2017). However rates of preterm birth (PTB) in HIV infected women are higher than in uninfected women (15-18% vs. 6-9%)(Thorne *et al.*, 2000; Boer *et al.*, 2007; Rudin *et al.*, 2011; Wedi *et al.*, 2016). Untreated HIV infected pregnant women have a PTB rate of approximately 15% whereas with cART this can increase to 20-40%(Lorenzi, V. M. Spicher, Laubereau, Hirschel, *et al.*, 1998; Thorne *et al.*, 2000; Short and Taylor, 2014). The rates of PTB have not decreased with increasing availability of antiretroviral therapy to HIV infected pregnant women.

1.1.2 Global importance of PTB

Worldwide the incidence of PTB varies from 6% of all births in Europe, 11% in the USA and 9-18% in African countries(Beck *et al.*, 2010). Globally approximately 15 million neonates are born preterm annually(Blencowe *et al.*, 2012). PTB is the leading cause of neonatal mortality accounting directly for approximately 1 million and up to 80% of neonatal deaths annually (Blencowe *et al.*, 2013; Global Burden of Disease Study, 2016).

The consequences of PTB are a spectrum that depends not only on gestational age at delivery but also the available neonatal support facilities. In resource rich settings very preterm infants, born before 32 weeks gestation, have increased rates of mortality and cerebral palsy (Tucker and Mcguire, 2004; Sukhov *et al.*, 2012). In the same setting moderate-late preterm infants, born between 32-36 weeks gestation, have comparable survival rates to infants born after 37 weeks gestation. However, these infants have increased risk of recurrent hospital admissions, respiratory problems, later behavioral problems and having an IQ <85(Talge *et al.*, 2011; Boyle *et al.*, 2012).

In low and middle income countries, where 85% of PTBs occur, and the majority of HIV infected pregnant women reside, neonatal mortality rates are higher for all preterm infants(Beck *et al.*, 2010; Smid, Stringer and Stringer, 2016; Brink *et al.*, 2019). Marchant et al.'s meta-analysis of East African data estimated rates of neonatal mortality for moderate preterm infants (<34 weeks) to be 47% (Marchant et al., 2012). This mortality rate, equivalent to the limit of viability, is found at lower gestations (around 24 weeks) in most high income countries(Blencowe *et al.*, 2012; Costeloe *et al.*, 2012). The rates of survival between high and low income countries are widening. This is largely due to lack of access to simple neonatal care, with up to half of women delivering at home in low income countries and poor health facility capacity for small and sick newborns(Lawn *et al.*, 2013; Narayanan *et al.*, 2019)

1.1.3 ART for all: expanding ART exposure

Guidelines for Prevention of MTCT (PMTCT) have rapidly advanced over recent years. At the time of the conception of this body of work HIV infected women were offered different prophylactic options in pregnancy dependent on their CD4 cell count and health. In women with CD4 counts > 350 cells/mcL, contemporaneous 2010 World Health Organisation (WHO) and 2012 British HIV Association (BHIVA) guidelines recommended pregnant women could either start cART or Zidovudine monotherapy (ZDV mono), if their plasma viral concentrations was <10000 HIV RNA copies/ml and were planning a caesarian section)(World Health Organization, 2010; Taylor *et al.*, 2012). A boosted Protease Inhibitor (PI) or triple Nucleoside –Reverse-Transcriptase-Inhibitor (NRTI) based cART were the recommended options for cART and the WHO guideline also recommended the Non-NRTI (NNRTI): Efavirenz (EFV) –based cART.

In 2013 the WHO updated HIV treatment guidelines recommending CD4 count treatment threshold for maternal health be increased to 500 cells/mcL(World Health Organization, 2013). The guidelines stated that ART use for PMTCT alone (CD4 > 500 cells/mcL) could be ZDV mono (Option A) or EFV-based cART (Option B), which could be continued after pregnancy irrespective of CD4 count

(Option B+) for maternal and infant feeding and health, health of future pregnancies, and ease of interpretation and programmatic implementation.

In 2015 the WHO recommended immediate roll out of life long cART for every person infected with HIV (World Health Organization, 2015b). This edict followed data from several important randomised control trials demonstrating both survival benefits for individuals with CD4 counts >500 cells/mcL and that cART provided an important public health measure for preventing onward HIV transmission, thereby offering an intervention to reduce HIV incidence (Cohen *et al.*, 2011; 'Initiation of Antiretroviral Therapy in Early Asymptomatic HIV Infection', 2015). In July 2019 the WHO changed its recommended first line EFV-based cART regime to Integrase Strand Inhibitor (INSTI): Dolutegravir (DTG) based cART(World Health Organization, 2018b).

Practically this expansion of cART access has also resulted in more women of reproductive age receiving and conceiving on cART (unaids.org, 2018). This up scaling of cART has resulted in increasing numbers of women receiving cART for PMTCT (82% worldwide) and effected important reduction in rates of MTCT. A side effect of this is increased antenatal exposure to these drugs and the potential for associated adverse obstetric outcomes e.g. PTB. Early studies of the effect of this expansion of cART access and exposure have not shown an increase in PTB rates (Rempis *et al.*, 2017) but many countries are yet to reach the UNAIDS goal of 90% ART coverage and 90% of individuals with fully suppressed plasma HIV (Joint United Nations Program on HIV/AIDS, 2014; World Health Organization, 2018a). It has been predicted with theoretical mathematical models that an expansion of PTB rates will increase (Correia and Williams, 2017).

1.1.4 HIV associated risk factors for PTB

HIV infection per se is a risk factor for PTB and appears to be linked to the degree of immunosuppression. Women with lower CD4+ cell counts and more advanced clinical disease (CDC stage C) are at greater risk of PTB(Thorne *et al.*, 2000; Schulte *et al.*, 2007; Townsend, Cortina-Borja, *et al.*, 2007; Wedi *et al.*,

2016; Favarato *et al.*, 2018) Detectable HIV-1 viraemia also correlates with increased risk(Traisathit *et al.*, 2009; Turner *et al.*, 2013; Kreitchmann *et al.*, 2014; Slyker *et al.*, 2014).

HIV infected women have a high prevalence of established risk factors for PTB. Within this population the following risk factors have been shown to increase risk of PTB: increasing maternal age(Aebi-Popp *et al.*, 2010; Brown *et al.*, 2012; Salihu *et al.*, 2012); black ethnicity (Tuomala *et al.*, 2005; Schulte *et al.*, 2007); single relationship status (Olusanya and Ofovwe, 2010); smoking (Haeri *et al.*, 2009; J. Y. J. Chen *et al.*, 2012; Aliyu *et al.*, 2013); alcohol use during pregnancy (Lambert *et al.*, 2000); illicit drugs e.g. cocaine, opiates and marijuana(Schulte *et al.*, 2007; Haeri *et al.*, 2009; Brown *et al.*, 2012; Thorne, Semenenko and Malyuta, 2012; Yu *et al.*, 2012); past history of PTB (Lambert *et al.*, 2000; Parekh *et al.*, 2011); hypertension and pre-eclampsia(Wimalasundera *et al.*, 2002; Tuomala *et al.*, 2005; Olusanya and Ofovwe, 2010; Parekh *et al.*, 2011; J. Y. J. Chen *et al.*, 2012); anemia(Traisathit *et al.*, 2009; J. Y. J. Chen *et al.*, 2012) and low maternal weight gain(Young *et al.*, 2012; Darak *et al.*, 2013).

Ascending genital tract infections and chorioamnionitis are risk factors for PTB(Goldenberg *et al.*, 2006, 2008). Chorioamnionitis is common in HIV infected women and can be more severe compared to uninfected women (Goldenberg *et al.*, 2006; D'costa, Khadke and Patil, 2007; Schuetz *et al.*, 2011; Ategeka *et al.*, 2019; Obimbo *et al.*, 2019). Bacterial Vaginosis is common in women with HIV infection and is also risk factor for PTB delivery (Hay, Lamont, *et al.*, 1994; Taha, 1999; Leitich *et al.*, 2003; Slyker *et al.*, 2014). Co-infection with sexually transmitted infections including Syphilis, Hepatitis C virus, Herpes Simplex virus, Gonorrhea and Chlamydia have also been associated with increased risk of PTB in HIV infected women(Lambert *et al.*, 2000; Ravizza, Martinelli and Bucceri, 2007; Kreitchmann *et al.*, 2014; Slyker *et al.*, 2014; Burnett, Loucks and Lindsay, 2015; Shava *et al.*, 2019). Malarial infection is a large contributor to infection associated preterm birth in low income countries as are other viral infections such as cytomegalovirus both of which have been demonstrated as important risk factors in HIV infected women(McDonald *et al.*, 2019; Moraka *et al.*, 2019),

1.2 Antiretroviral therapy and PTB

There is an increasing body of evidence that suggests treatment with cART does not improve rates of PTB in HIV infected women and is associated with higher rates of PTB. This was first described by Lorenzi et al in 1998 who reported a high PTB rate of 33% in HIV infected women from Switzerland receiving PI-based cART(Lorenzi, V. M. Spicher, Laubereau and Al, 1998). This led to several other HIV cohort studies publishing data on prematurity rates in association with antiretroviral therapy with a range of different results. For a summary of all the studies exploring the effect of ART on PTB to date, presented by world region, see table 1.

1.2.1 European data

Observational data from European studies such as the European Collaborative Study ((ECS) data from nine countries)(Thorne, Patel and Newell, 2004; Townsend *et al.*, 2010; Bagkeris *et al.*, 2015), national surveillance registers from the UK(Townsend, M., *et al.*, 2007) and Italy (Ravizza, Martinelli and Bucceri, 2007) and prospective cohort studies from Germany, Austria(Grosch-Woerner *et al.*, 2008) and Spain(Bertran Bellon Cano *et al.*, 2004) have reported increased rates of PTB in association with cART. To date, 11 analyses have been published that have explored the association between cART and PTB in Europe, compared with no ART or earlier prophylactic strategies e.g. ZDV mono, all of which have shown a significant association, see table 1 for details of study designs and individual outcomes.

The risk of PTB in women who received cART was twice that seen with no ART(Lorenzi, V. M. Spicher, Laubereau and Al, 1998; Thorne *et al.*, 2000; Rudin *et al.*, 2011). Similarly PTB rates for pregnant women taking cART were between 2-5 fold greater than the rates observed in those who took ZDV mono or dual NRTIs, in whom rates were similar to the general population(Thorne, Patel and Newell, 2004; Martin and Taylor, 2007; Townsend, M., *et al.*, 2007; Grosch-Woerner *et al.*, 2008; Sibiude *et al.*, 2012; Short *et al.*, 2014; Bagkeris *et al.*, 2015).

1.2.2 North American data

Contemporaneously observational data from North America, where background rates of PTB are higher, has been less conclusive regarding the association between cART and PTB. To our knowledge there has been eight analyses (four from the Women and Infants Transmission study cohort (WITS)) directly comparing cART use with no ART or either ZDV mono or other NRTI-based regime in heterogeneous comparison groups that have included uninfected and HIV infected pregnant women.

Results from the WITS (1990-2000) indicated there was no increased risk of PTB comparing cART use with no therapy or ZDV mono(Cooper *et al.*, 2002; Tuomala *et al.*, 2002). Subsequent results from this cohort, which included data up to 2002, concluded that antiretroviral therapy use was associated with improved obstetric outcomes(Tuomala *et al.*, 2005). This paper did however demonstrate a 8-fold risk of PTB in women receiving cART which did not include ZDV late in pregnancy (after 32 weeks gestation) whereas there was a protective effect of antiretroviral therapy that included ZDV (ZDV mono or cART), aOR 0.53 (95%CI 0.34-0.83) (Tuomala *et al.*, 2005).

Later the American Pediatric Spectrum of Disease Study (cohort data from eight sites) reported an increased risk of PTB with PI-based cART compared with dual-NRTI (aOR 1.21 (95% CI 1.04-1.40))(Schulte *et al.*, 2007).

The Surveillance Monitoring for ART Toxicities (SMARTT) study, again included the WITS cohort, in combination with women recruited to IMPAACT P1025, and showed HIV infected pregnant women initiating PI-based cART in the first trimester were at greater risk of PTB compared to HIV infected pregnant women taking no ART (aOR 1.55 (95% CI 1.16–2.07)(Watts *et al.*, 2013).

The three remaining studies have used uninfected pregnant women receiving no ART as the comparison group, all with different findings. In 2012 Brown et al published a case control study that demonstrated HIV infected pregnant women had nearly three fold the risk of PTB compared to uninfected pregnant women

(aOR 2.83 (95% CI 1.22-6.54)) and also showed women receiving cART had four fold the risk of PTB compared to women not receiving cART (aOR 4.02 (95% CI 1.27-12.72)). Equally in Gagnon et al.'s recent Canadian matched cohort analysis HIV infected women receiving PI-based cART had twice the rate of PTB compared to women receiving non PI -based ART (including ZDV mono) (OR 2.1 (95% 1.0-4.6)), however this was no longer significant when adjusted for race (aOR 1.2 (95% CI 0.4-3.6))(Gagnon *et al.*, 2016).

Conversely in Duyrea et al.'s retrospective cohort study the highest rates of PTB were observed in HIV infected pregnant women receiving no ART (21%) compared with PI based cART (14%), and with any other ART (including ZDV mono) (13%)(Duryea *et al.*, 2015). The protective association of PI based cART did not remain significant after correction for ethnicity, age, and duration of diagnosis, viral load and CD4 cell count (aOR 0.9 (95%CI 0.5-1.5).

1.2.3 Low and middle-income country data

There is increasing high quality data from low and middle-income countries where the burden of disease is greatest. Fifteen analyses (including 3 RCTs, 8 prospective cohort and 4 retrospective cohort studies) were identified that compared cART use in pregnancy against a variety of comparator groups including uninfected pregnant women and HIV infected pregnant women receiving no ART or NRTI-based ART.

Of the twelve observational studies, all but five have shown an association of cART with PTB, see table 1. The National International Site Development Initiative (NISDI) Perinatal study prospective cohort (2002-2005) comprising data from Argentina, Brazil, and Mexico and the Caribbean compared cART with NRTI-only therapy (either ZDV mono or dual-NRTI) and showed no increased risk of PTB with cART (Szyld *et al.*, 2006). However a more recent analysis drawing data from the same NISDI cohort (2002-2007) in addition to women from the Longitudinal Study in Latin American Countries (LILAC) study protocol found that HIV infected pregnant women receiving cART at conception were at significantly higher risk of PTB than those who were not taking cART (aOR 1.4

(95% CI 1.0-1.9)). A further smaller cohort study from Brazil also demonstrated a higher rate of PTB in women receiving cART(13%) compared to dual-NRTI (9%) see table 1(Machado *et al.*, 2009).

Data from African countries has been more conflicting. Most of the cohort and RCT data has been collected in eastern (Kenya, Malawi, Mozambique, Tanzania, Uganda) and southern (Botswana, South Africa, Zambia and Zimbabwe) Africa, see table 1. There have been three studies that have recruited women from West Africa (Burkina Faso, Cameroon and Nigeria)(Kesho Bora study, 2011; Ikpim *et al.*, 2016; Njom Nlend *et al.*, 2016).

Of the five cohort studies undertaken in East Africa, three have shown an increased risk of PTB (1.2-5.6 fold) in association with cART compared to either no ART or ZDV mono(Van Der Merwe *et al.*, 2011; J. Chen *et al.*, 2012a; Li *et al.*, 2016). Two cohort studies conducted in Malawi have shown a protective effect of non-PI-based cART compared to no ART(Marazzi *et al.*, 2011) and more recently no difference in PTB rates between HIV infected and uninfected mothers(Dadabhai *et al.*, 2019).

The one cohort study from Cameroon, West Africa demonstrated a trend towards an association between cART and PTB compared to ZDV mono (aOR 1.9 (95%CI 0.9-3.7), p=0.06)(Njom Nlend *et al.*, 2016). Conversely another West African cohort study from Nigeria demonstrated a reduction in PTB rates with non PI-based cART (4%) compared to no ART (17%), p< 0.0001(Ikpim *et al.*, 2016).

Less data are available for Asian countries with just four studies identified. One Indian study has shown a 3-fold increased risk of PTB with cART compared to no therapy (Darak *et al.*, 2013) whilst another more recent Indian study has shown a protective effect of cART (unadjusted OR 0.23 (95% CI 0.07-0.79) and NRTI only ART (unadjusted OR 0.30 (95% CI 0.10-0.91) compared to no treatment(Dadhwal *et al.*, 2017). There have been two Chinese studies to date, the first compared PTB rates in HIV infected pregnant women receiving non PI-

based cART (12%) compared with ZDV mono (3%) and no ART (4%), demonstrating a trend towards higher PTB rates for women receiving cART (Yu *et al.*, 2012). The second study was a retrospective case note review comparing cART use with no ART and demonstrated that early cART (pre- conception or initiated in first trimester) was associated with significantly higher rates of PTB (aOR 2.92 (95%CI 1.56–5.44))(Wang *et al.*, 2018).

1.2.4 Randomised Controlled Trials

There have now been three Randomised Controlled Trials (RCTs) from Sub Saharan Africa comparing cART with either ZDV mono or triple NRTIs. In each study, HIV infected women who did not need treatment for their own health (CD4+ count>200 cells/mL) were randomized to either PI-based cART (Lopinavir/ritonavir-based) or NRTI-only therapy (ZDV mono) plus single dose Nevirapine (Kesho Bora study(Kesho Bora study, 2011), PROMISE)(M. Fowler *et al.*, 2016) or triple-NRTI (Mma Bana study)(Powis *et al.*, 2011)). The primary outcome was prevention of MTCT of HIV infection during the antenatal and postnatal period (which included up to 6 months of breast feeding). PTB was evaluated as a secondary outcome. These data should be less susceptible to bias however in spite of this the studies have reached different conclusions.

The first, the Kesho Bora study, which was undertaken in Burkina Faso, Kenya, and South Africa did not find any significant difference in rates of PTB comparing Lopinavir (LPV)/ZDV/3TC (400mg LPV, 100mg ritonavir dose) (13%) and ZDV mono (11%)(Kesho Bora study, 2011). The Kesho Bora study protocol limited recruitment to women with a CD4 count >200 cells/mcL but <500 cells/mcL and initially started treatment from 34 -36 weeks, however halfway through the trial, earliest treatment initiation changed from 34 to 28 weeks gestation. One can argue however that if treatment was initiated later, there was little time for an effect to be observed on PTB risk. This trial did however show a higher rate of MTCT with ZDV mono (11%) compared to LPV/r/ZDV/3TC (6%) p=0.02.

In the second RCT, the Mma Bana study in Botswana, women with a CD4 count >

200 cells/mcL started treatment from 26 weeks gestation. LPV/r/ZDV/3TC (400mg LPV, 100mg ritonavir dose) was associated with a 2-fold risk of PTB (aOR 2.02 (95% CI 1.26-3.27) compared to triple-NRTI (ABC/3TC/ZDV), with no excess risk of MTCT of HIV (0% vs. 2%, p=0.42)(Powis *et al.*, 2011).

A similar association between LPV/r based cART and PTB was found in the most recently published RCT: The PROMISE Study, undertaken in India and six South Eastern African countries: Malawi, South Africa, Tanzania, Uganda, Zambia, and Zimbabwe (M. Fowler *et al.*, 2016). This study randomized women with CD4 counts >350 cells/mcL to either LPV/r/ZDV/3TC, LPV/r/FTC/tenofovir (TDF) or ZDV mono plus single dose Nevirapine. Treatment was initiated after 14 weeks up to and including term. LPV was given at the standard 400mg dose with 100mg ritonavir twice daily until the third trimester when it was increased to LPV-600mg, ritonavir -150mg twice daily, resulting in a total ritonavir dose of 300mg. PTB rates were lowest in women receiving ZDV mono (14%), followed by LPV/r FTC/TDF (19%) and LPV/r /ZDV/3TC (20%), p=0.09. More striking was the excess moderate-severe PTB (<34 weeks) observed in the LPV/r FTC/TDF arm (6%) compared to 3% in both LPV/r /ZDV/3TC and ZDV mono arm, p=0.04. There was also significantly higher mortality in the first week of life in neonates exposed to FTC/TDF in utero compared to ZDV based ART, p<0.0001. The rates of early MTCT in this trial were lower for cART (0.5%) vs. ZDV mono (1.8%).

Table 1 Summary of studies exploring the effect of cART on PTB

Reference and study period	Mother-child pairs (n)	Design	ART class (n)	PTB rate (%)	Treatment comparison	aOR	(95% CI)	PTB Association
European data								
(Lorenzi, V. Spicher, *et al.*, 1998) AIDS (1996-1998)	37	Prospective cohort **Mo-CHiV**	PI cART 16 Dual NRTI 21 ZDV mono 112 (Swiss Neonatal HIV study ref) No cART 452(Swiss Neonatal HIV study ref)	25 29 17 14	PI cART vs. no cART	2.30	1.17–7.10	PI cART
(Thorne *et al.*, 2000) AIDS (1986-2000)	3920	2 Prospective cohorts **ECS & MoCHiV**	cART 289 ---PI cART 101 ---Non PI cART 108 ZDV mono 555 No ART 2891	24 29 22 17 16	PI cART Non-PI cART ZDV mono vs. No ART	2.6 1.82 1.03 1	1.43-4.75 1.13-2.92 0.70-1.50	PI cART
(Bertran Bellon Cano *et al.*, 2004) Med Sci Monit (1997-2000)	126	Prospective cohort	78 cART ---PI cART 74 ---non PI cART 6 Dual NRTI 41 ZDV mono 46 No ART 16	18 }10 - -	- cART + Dual NRTI vs. no ART			cART +Dual NRTI
(Thorne, Patel and Newell, 2004) AIDS (1986-2004)	4372	Prospective cohort **ECS**	cART 757 ---New cART 446 ---Pre-conception cART 321 ZDV mono+ Dual NRTI 568 No ART 944	26 - - 16 -	- New cART Pre-conception cART vs. ZDV mono + dual NRTI No ART	1.88 2.05 1 1.01	- 1.34-2.65 1.43–2.95 0.71-1.41	cART
(Townsend, Cortina-Borja, *et al.*, 2007) AIDS (1990-2005)	4939	National surveillance register **NSHPC**	cART 3384 ZDV mono 904 Dual NRTI 157 No ART 494	14 10 - 16	cART vs. ZDV mono + dual NRTI	1.51	1.19-1.93	cART
(Grosch-Woerner *et al.*, 2008) HIV Med (1995-2001)	183	Prospective cohort	cART 75 ZDV mono 76 Dual NRTI 32	39 26 44	PI cART Non-PI cART Dual NRTI vs. ZDV mono	3.40 0.89 1.57 1	1.13-10.2 0.38-2.12 0.38-1.28	PI cART
(Rudin *et al.*, 2011) HIV Med (1986-2007)	1180	Prospective cohort	cART 409 ZDV mono + Dual NRTI 147 No ART 624	24 20 15	cART ZDV mono + Dual NRTI vs. no ART	2.46 1.75 1.00	1.41–4.28 0.85–3.62	cART

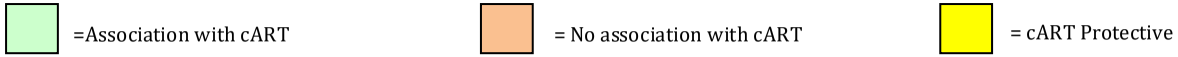
(Sibiude *et al.*, 2012) CID (1990–2009)	10 402	Prospective cohort	cART 6738 ZDV mono 2975 Dual NRTI 1664 Sub-analysis ---PI cART 1253 ---Boosted PI 1066 ---Non Boosted PI 187	15 10 11 14 14 9	cART vs. dual NRTI/ZDV mono Boosted PI cART vs. non boosted PI cART	1.69 2.03	1.38–2.07 1.06–3.89	Boosted PI cART
(Short *et al.*, 2014) HIV Medicine (1996-2010)	331	Prospective cohort	cART 246 ---PI cART 96 ZDV mono 65 No ART 8	15 23 6 13	New PI cART vs. ZDV mono	5.00	1.49–16.79	New PI cART
(Bagkeris *et al.*, 2015) Lancet 2015 (2000-2012)	8884	Prospective cohort **ECS**	Total Pre-conception 252 Post-conception 7030 cART 2949 ---PI cART 260 ZDV mono 4396 No ART 1497	9	cART vs. ZDV mono no cART vs. ZDV mono	1.64 2.94	1.14-1.73 2.43-3.57	cART
North American data								
(Cooper *et al.*, 2002) JAIDS (1990-2000)	1542	Prospective cohort **WITS**	cART 250 ZDV mono 710 No cART 396	17 16 21	cART vs. ZDV mono	-		NSIG
(Tuomala *et al.*, 2002) NEJM (1990-1998)	3266	7 prospective cohort studies inc **ACTG 076 & 185,** **WITS & PACTS**	cART 533 ---PI cART 37 ZDV mono 1590 No ART 1143	15 18 16 20	cART PI cART vs. ZDV mono	1.08 1.45	0.71-1.62 0.81-2.50	NSIG
(Tuomala *et al.*, 2005) AIDS (1990-2002)	2543	Prospective cohort **WITS**	cART 588 ZDV mono 932 Dual NRTI 258 No ART 751	Not stated	Late ART without ZDV Late ART containing ZDV	7.86 0.53	1.39-44.58 0.34-0.83	Late use ART without ZDV
(Schulte *et al.*, 2007) Peds (1989-2004)	11231	Prospective cohort **PSD**	cART 2563 ---PI cART 782 ZDV mono 2621 Dual NRTI 2312 No ART 2565	18 17 17 14 21	PI cART vs. dual NRTI	1.21	1.04-1.40	PI cART
(Brown *et al.*, 2012) AIDS Res Human Retro (2000-2009)	71 HIV+ 71 HIV-	Case control study	cART 57 ---PI cART 40		HIV + vs. HIV- controls cART vs. no ART	2.83 4.02	1.22-6.54 1.27-12.72	cART

(Watts *et al.*, 2013) JID (2007-2010)	1869	**SMARTT** study: 2 prospective cohorts **IMPAACT P1025 &WITS**	cART 1663 ---PI cART 1319 ZDV mono/Dual NRTI 144 No ART 62	21 19	1st trimester PI cART vs. no ART	1.55	1.16–2.07	PI cART
(Duryea *et al.*, 2015) ID in Obs Gynae (1984-2014)	1004	Retrospective cohort	PI cART 597 Non PI ART (inc ZDV mono and dual NRTI) 230 No ART 177 P	14 13 21	Non PI ART vs. no ART No comparison with PI presented	0.9	0.5-1.5	NSIG lower PTB rate with ART both PI and non PI compared to no ART
(Gagnon *et al.*, 2016) Eur J Ob Gynae & Rep Bio (2007-2012)	97 HIV 288 non HIV (1:3)	Matched prospective cohort	HIV ---PI cART 74 HIV- controls	20 9	HIV vs. HIV- controls PI cART vs. non PI cART (inc ZDV mono)	1.4 1.2	(0.5-3.6) (0.4-3.6)	NSIG
Middle Income country data								
(Szyld *et al.*, 2006) AIDS (2002-2005)	681	Prospective cohort **NISDI**	cART 587 ---PI cART 330 ZDV mono+ Dual NRTI 94	9 11 6	PI cART vs. ZDV mono + dual NRTI	1.1	0.5-2.8	NSIG
(Machado *et al.*, 2009) STDS (1996-2006)	696	Prospective cohort	cART 305 PI cART 193 ZDV mono 179 Dual NRTI 212	- 16 12 8	Pre-conception cART vs. dual NRTI	5.06	1.5-17.06	cART
(Kreitchmann *et al.*, 2014) BJOG (2002-2007)	1392	2 Prospective cohort studies **NISDI Perinatal & LILAC**	Pre conception cART 550 Post-conception cART 842 No ART	20	Pre conception cART vs. no ART at conception	1.4	1.0-1.9	cART
Low Income country data								
(Dadabhai *et al.*, 2019) JAIDS (2016-2017)	1299	Matched prospective cohort	HIV cART 614 HIV – controls 685 Pre-conception 299 1st-2nd trimester 257 third trimester 58	10 9 11 9 12	HIV infected vs. uninfected	1.10	0.73-1.65	NSIG
(Wang *et al.*, 2018) Int J Gynecol Obstet 2009-2011	731	Retrospective cohort	cART 559 ---LPV/r based 265 ---EFV based 104 ---NVP based 190 no cART 17	 16 8 14 8	early cART (pre- conception or initiated in 1st trimester) vs. no cART Late cART (2nd or third trimester) vs. no cART	2.82 1.05	1.47–5.44 0.52–2.11	cART
(Dadhwal, MD *et al.*, 2017) IMJA (2002-2015)	212 HIV+ 238 HIV-	Prospective cohort	HIV + ---cART 80 ---Dual NRTI 97 ---no ART 31 HIV-	9 6 8 23 7	HIV+ vs. HIV- cART vs. no ART ZDV mono/dual NRTI vs. no ART	1.27 0.23 0.30	0.65-2.48 0.07-0.79 0.10-0.91	cART and NRTI only ART protective

(M. G. M. Fowler *et al.*, 2016) NEJM (2011-2014) (Sebikari *et al.*, 2019) JAIDS	3333	**PROMISE RCT** CD4>350 cells/mcL initiation >14 weeks up to term	1497 LPV/r/ZDV/3TC 419 LPV/r/ FTV/TDF 1507 ZDV mono + sdNVP (ref)	21 19 13	LPV/r/ZDV/3TC vs. ZDV mono p<0.001 LPV/r/FTC/TDF vs. ZDV mono p=0.09 LPV/r/FTC/TDF vs. LPV/r/ZDV/3TC p=0.77	1.82 1.77 0.97	1.47-2.26 1.29-2.43 0.72-1.31	PI cART, TDF-based vs. ZDV based high rate of very preterm <34weeks (6%) aOR 3.14 (1.77-5.55) p=0.04 & infant mortality (4%) in first week of life p=0.001
(Li *et al.*, 2016) J Infect Dis. (2002-2011)	3314	Prospective cohort	cART 1094 ZDV mono 1768 No ART 452	29 27 -	PI cART vs. ZDV mono EFV cART vs. NVP cART	1.23 1.45	1.04-1.47 1.01-2.07	PI cART EFV based
(Njom Nlend *et al.*, 2016) PLOSone (2008-2011)	760	Retrospective cohort Post-conception	cART 481 ---NNRTI based 472 ---PI based 9 ZDV mono 279	10 6	cART vs. ZDV mono	1.9	0.9-3.7	Trend cART p=0.06
(Ikpim *et al.*, 2016) Tropical Doc (2006-2010)	258 HIV 257 HIV-	Retrospective case note review	HIV ---cART 181 (purportedly non PI although no details provided) ---No cART HIV neg control	13 4 17 7	HIV+ vs. HIV – cART vs. no cART	1.72 0.20	0.82-3.59 0.10-0.52	cART protective
(Darak *et al.*, 2013) AIDS Pt Care STDS (2008-2012)	512	Prospective cohort	cART 192 ZDV mono 324	25 13	cART vs. ZDV mono	3.35	1.52-7.38	cART
(Yu *et al.*, 2012) Chinese Med J (2006-2009)	194	Prospective cohort	cART 139 sdNVP+/- ZDV mono/Dual NRTI 25 no ART 39	12 4 3	cART sdNVP+/- ZDV mono/Dual NRTI no ART ref	4.05 1.21 1	0.52-31.61 0.07-20.35	NS high PTB rate with cART
(J. Chen *et al.*, 2012b) JID (2009-2011)	9504 HIV 22609 non HIV	Retrospective cohort	cART 2942 PI cART 312 ZDV 3290	24 14	Post-conception cART vs. ZDV mono	1.4	(1.2-1.8)	cART
(Powis *et al.*, 2011) JID (2006-2008)	530	**Mma Bana RCT** CD4>200 cells/mcL	267 LPV/r/ZDV/3TC 263 ZDV/3TC/ABC	21 12	PI cART vs. triple NRTI	2.03	1.26-3.27	PI cART

		26-34 weeks at initiation						
(Kesho Bora study, 2011) Lancet (2005-2008)	805	**Kesho Bora RCT** CD4>200 cells/mcL 28-36 weeks at initiation	401 LPV/r/ZDV/3TC 404 ZDV mono	13 11	PI cART vs. ZDV mono	-	-	NSIG
(Marazzi *et al.*, 2011) AIDS 2011 (2005-2009)	1340	Retrospective cohort **DREAM**	Non PI cART 1330 No ART 10	9 70	Non-PI cART vs. no ART	0.15	0.14–0.19	Non PI cART protective
(Van Der Merwe *et al.*, 2011) J Int AIDS Soc (2004-2007)	1630	Retrospective cohort	cART 1397 PI cART 419 No ART 233	15 6 5	Early (<28/40) non-PI cART vs. no cART Early (<28/40) PI cART vs. no cART	5.6 3.0	2.1-15.2 1.1-8.4	cART (<28/40) NNRTI>PI
International pooled analyses								
(AP Kourtis *et al.*, 2007) AIDS (1984-2004)	20426	Meta-analysis 14 studies	Not stated	-	cART PI cART ZDV mono vs. No ART PI cART vs. non-PI ART	1.13 1.35 0.86 1 1.35	0.79–1.63 1.08-1.70 0.73–1.01 1.08–1.70	PI cART
(Townsend *et al.*, 2010) BJOG (1990-2006)	19585	Pooled analyses 5 studies including: **ECS, NSHPC & PSD**	cART 9005 ZDV mono 4323 Dual NRTI 1481 No ART 4537	15 - 13 -	cART vs. Dual NRTI	1.49	1.19-1.87	cART
(Xiao *et al.*, 2015) BMC Preg Childbirth (Pre 1995-2015)	200,896	Meta-analysis 40 studies	cART numbers not stated HIV - ref		HIV vs. HIV neg ref HIV cART HIV no ART	1.56 1.77 1.54	1.49-1.63 1.55-2.02 1.23-1.93	HIV and cART
(Veroniki *et al.*, 2018) PLOSone (1995-2015)	20576	Meta-analysis 35 studies	Individual cART numbers not stated		IDV/ZDV/3TC vs. ZDV mono LPV/r/ZDV/3TC vs. ZDV mono +sdNVP NVP/ZDV/3TC vs. ZDV mono +sdNVP EFV/ZDV/3TC vs. ZDV mono +sdNVP ZDV mono vs. no ART	108.20 234.30 93.02 208.80 0.00	3.81-7553.00 33.52-2554.00 12.73-1040.00 19.86-3000.00 0.00-0.02	cART

Key: PI=Protease Inhibitor; cART=combination antiretroviral therapy; NRTI= Nucleoside reverse transcriptase inhibitor; ZDV mono=zidovudine monotherapy; sdNVP=single dose Nevirapine; NSIG=non significant; LPV/r= Lopinavir & ritonavir; EFV= Efavirenz; FTC= Emtricitabine; TDF= Tenofovir Disoproxil Fumarate; 3TC= Lamivudine and ABC=Abacavir


=Association with cART
= No association with cART
= cART Protective

1.2.5 Why do these results differ? Pooled analyses results

The discrepancies in the conclusions of these studies led to efforts to combine the data and to further examine the differences in these studies, for summary of meta-analysis undertaken see table 1.

A meta-analysis of observational studies from Europe, North and South America published in and before 2004 (thirteen prospective cohorts and one retrospective study) concluded there was no elevated risk of PTB with cART including PI-based cART compared with no therapy(A. P. A. Kourtis *et al.*, 2007). Interestingly it did show a moderate increase (aOR 1.35 (95% CI 1.08–1.70)) in PTB with PI-based cART compared to non PI-based cART.

A pooled analysis of the ECS and WITS cohort data up to 2006 was undertaken which revealed that the rates of PTB from European and North American studies were indeed different with a combined analysis indicating an association with cART compared to dual-NRTI (aOR 1.49 (95% CI 1.19-1.87))(Townsend *et al.*, 2010). Both analyses proposed the reasons behind the disparate conclusions to be: heterogeneous populations with different background rates of PTB; varying study designs in adjustment for confounders such as race, age, smoking, illicit drug use, alcohol, HIV factors (degree of immunosuppression, indication for treatment), co-infections as well as choice of comparison groups(A. P. A. Kourtis *et al.*, 2007; Townsend *et al.*, 2010).

In 2015 Xiao et al. published the largest meta-analyses in this field which included data on 200,896 infants born to HIV infected mother from 40 studies (including Africa and Asian studies)(Xiao *et al.*, 2015). Xiao concluded that HIV infection increase odds of PTB 1.6 fold compared to pregnant women without HIV infection. This OR was only modestly increased to 1.8 with use of cART, see table 1. In 2018 Veroniki et al.'s network meta-analysis (NMA) of 35 studies explored individual drug regime effects of congenital malformations and adverse birth events e.g. PTB(Veroniki *et al.*, 2018). The authors identified ZDV mono + sdNVP was associated with a reduction in PTB risk compared to no therapy +/-

planned caesarian section (NMA OR 0.00 (95% CI 0.00-0.02)), whereas both PI and NNRTI-based cART regimes increased risk of PTB (100-200 fold) compared to ZDV mono + sdNVP, see table 1. These more recent meta-analyses provide further weight to the link between cART and increased PTB and the protective effect of NRTI only therapy, particularly ZDV mono, through more current international data.

1.2.6 The effect of individual ART drug classes

Several investigators have hypothesised that a specific class of drugs, the protease inhibitors, drives the association between cART and PTB. This class of ART was one of the first to be used in combination antiretroviral therapies and is the one with the most available data on safety outcomes in pregnancy(Gulick *et al.*, 2002; Hammer *et al.*, 2002; Pasley *et al.*, 2013). For a summary of all published studies to examine the effect of the PI class compared to non-PI-based cART on PTB see table.2.

One of the cohorts from the WITS study was the first to highlight this association, with an elevated risk with PI-based cART compared to non PI-based ART (aOR 1.8 (95% CI 1.1–3.0))(A. Cotter *et al.*, 2006). Two other American studies have reported an increased risk of PTB with PI-based cART (aOR 1.2-1.6) versus non PI-based ART or no ART(Schulte *et al.*, 2007; Watts *et al.*, 2013). There has also been one study comparing PTB rates between two different PIs (LPV and Atazanavir (ATV) that identified a non-statistically higher rate of PTB with LPV (21%) compared to ATV (16%), aOR 1.14 (95%CI 0.75-1.72)(Rough *et al.*, 2018). However there have been another four cohort studies from N. America showing no statistical difference in PTB rates above those observed with non PI-based ART(Tuomala *et al.*, 2002; K Patel *et al.*, 2010; Dola *et al.*, 2012; Gagnon *et al.*, 2016).

Data from Europe is more conclusive with nine of ten cohort studies showing high rates of PTB in women receiving PI-based cART (1.6-5 fold) compared to non PI ART, no ART and the general population, see table 2 for references. There has been one recent study from Holland comparing PI based cART to NNRTI-

based cART that showed no difference in rates of PTB between these drug classes(Snijdewind *et al.*, 2018). Two UK studies have compared the role of specific PI drugs in cART regimes: LPV, that has been widely used in pregnancy, with newer PIs including ATV (Perry *et al.*, 2016; Favarato *et al.*, 2018) and showed similar rates of PTB across the PI class.

The role of boosted PI concentrations in the high observed rates of PTB, by co-administration of another PI called ritonavir usually given at a concentration of 100mg per day, has been examined by one Canadian and one French study (Sibiude *et al.*, 2012; Kakkar *et al.*, 2015). These studies have demonstrated that PI-based cART regimes containing ritonavir increase PTB risk two fold compared unboosted cART regimes indicating that the excess PTB risk may be driven by the practice of ritonavir boosting.

Much of the recent data exploring the association between PI-based cART and PTB has originated from African countries. Two RCTs, Mma Bana and PROMISE have shown higher rates of PTB with LPV/r based cART (in the region of two fold) compared with NRTI only therapy (ABC/3TC/ZDV and ZDV mono, respectively)(Powis *et al.*, 2011; M. Fowler *et al.*, 2016). Notably PROMISE used a higher dose of both LPV (1200mg) and ritonavir (300mg) than standard in the third trimester, which may account for some of the excess PTB see in women receiving LPV/r regimes, possibly by increasing TDF exposure. Contrariwise the Kesho Bora RCT did not find a significant increase risk comparing LPV/r ZDV/3TC cART with ZDV mono and the PROMOTE RCT did not find a difference between LPV/r ZDV/3TC and EFV/ZDV/3TC(Kesho Bora study, 2011; Koss *et al.*, 2014). Stringer et al.'s combined analysis of 3 RCTs comparing PI-based cART with NNRTI based cART and triple-NRTIs showed high rates of PTB across all groups (38%, 28% and 38% respectively). The higher rate of PTB in women receiving PI-based cART compared to NNRTI cART did not reach statistical significance in multivariate analysis (aOR 1.35 (95% CI 0.67-2.71))(Stringer *et al.*, 2018).

Much of the African cohort data compares PI-based cART to specific cART regimes including EFV and NVP based NNRTI cART, see table 2. There have been two studies that have compared LPV/ZDV/3TC to EFV based regimes which have found LPV/r regimes are associated with higher PTB(Rebecca Zash *et al.*, 2017; Wang *et al.*, 2018). Matoba et al. did not find any difference in PTB rates between PI: Nelfinavir based cART compared to NNRTI: NVP based cART(Masaba *et al.*, 2018). Recently the Tsepamo study and DOLPHIN2 RCT have compared two non PI class regimes: NNRTI: EFV-based cART against INSTI: DTG-based cART in pregnancy and found no differences in PTB rates between these two ART classes(Zash, Jacobson, *et al.*, 2018; Khoo, Kintu and Malaba, 2019).

Meta-analyses have been more helpful with all three to date, each including at least 20,000 maternal-infant pairs, estimating an increased risk of PTB with PI-based cART regimes over non PI -based regimes of 1.4-2.5 fold (A Kourtis *et al.*, 2007; Mesfin, Kibret and Taye, 2016; Veroniki *et al.*, 2018).

Table 2 Summary of studies exploring effect of ART Class on PTB

Reference and study period	Mother-child pairs (n)	Design	ART class (n)	PTB rate (%)	Treatment comparison	aOR	(95% CI)	PTB Association
European data								
(Thorne *et al.*, 2000) AIDS (1986-2000)	3920	2 Prospective cohorts **ECS & MoCHiV**	cART 289 ---PI-cART 101 --- non PI cART 188 ZDV mono 555 No ART 2891	24 29 22 17 16	PI cART Non-PI cART ZDV mono vs. No ART	2.6 1.82 1.03 1	1.43-4.75 1.13-2.92 0.70-1.50	PI cART
(Ravizza, Martinelli and Bucceri, 2007) JID (2001-2006)	419	National surveillance register	cART 366 ---PI cART* 167 ---Non-PI cART 199 No ART 53	 32 18	PI cART vs. Non-PI +No ART	2.81 1	1.46-5.39	PI cART
(Grosch-Woerner *et al.*, 2008) HIV Med (1995-2001)	183	Prospective cohort	cART 75 ZDV mono 76 Dual NRTI 32	39 26 44	PI cART Non-PI cART Dual NRTI vs. ZDV mono	3.40 0.89 1.57 1	1.13-10.2 0.38-2.12 0.38-1.28	PI cART
(Sibiude *et al.*, 2012) CID (1990–2009)	10 402	Prospective cohort	cART 6738 ZDV mono 2975 Dual NRTI 1664 Sub-analysis ---PI cART 1253 ---Boosted PI 1066 ---Non Boosted PI 187	15 10 11 14 14 9	cART vs. dual NRTI/ZDV mono Boosted PI cART vs. non boosted PI cART	1.69 2.03	1.38–2.07 1.06–3.89	Boosted PI cART
(Lopez *et al.*, 2012) AIDS (1986- 2010)	519 HIV 1038 non-HIV	Matched cohort	cART 298 ---PI cART 178 ---non PI cART 153 No ART 221	-	PI cART vs. non PI cART	Iatrogenic		PI cART with Iatrogenic
(Short *et al.*, 2014) HIV Medicine (1996-2010)	331	Prospective cohort	cART 246 ---PI cART 96 ---NNRTI cART 137 ZDV mono 65 No ART 8	15 23 12 6 13	New PI cART vs. ZDV mono PI cART vs. NNRTI cART p=0.04	5.00	1.49–16.79	Post conception PI cART PI>NNRTI
(Perry *et al.*, 2016) HIV Med 2016 (2007-2012)	493	Retrospective cohort	LPV/r 306 ---Pre-conception 82 ---Post-conception 224 ATV/r 187 ---Pre-conception 96 ---Post-conception 91	14 12 12 13 13 21	Pre-conception LPV/r cART vs. ATV/r cART Post-conception LPV/r cART	1.13 1.87	0.43-2.98 0.93-3.75	No difference in PI regimes, higher PTB rate than general UK population

					vs. ATV/r cART p=0.08			
(Favarato *et al.*, 2018) AIDS (2007-2015)	6073	National surveillance register **NSHPC**	HIV Pre-conception 2983 Post-conception 3090 PI cART 4184 ---LPV/r cART 2368 ---Other PI cART 1816 NNRTI cART 1889	10 12 10 9	Pre-conception & CD4 <350 cells/mcL ---LPV/r cART ---Other PI cART ---NNRTI cART -ref CD4> 350 cells/mcL ---LPV/r cART ---Other PI cART ---NNRTI cART -ref	 2.01 2.05 1.00 1.64 0.98 1.00	 1.03-3.91 1.07-3.89 - 1.08-2.47 0.66-1.46	Pre-conception , particularly LPV/r, CD4 <350
(Snijdewind *et al.*, 2018) PLOSone (1997-2015)	1392	Prospective cohort **ATHENA**	HIV HIV- ref PI cART 928 NNRTI cART 438	15 5 14 17	NNRTI cART vs. PI cART	1.30	0.95-1.77 p=0.11	NSIG
North American data								
(Tuomala *et al.*, 2002) NEJM (1990-1998)	3266	7 prospective cohort studies inc **ACTG 076 &185,** **WITS & PACTS**	cART 533 ---PI cART 37 ZDV mono 1590 No ART 1143	15 18 16 20	cART PI cART vs. ZDV mono PI cART vs. non-PI cART	1.08 1.45 1.80	0.71-1.62 0.81-2.50 0.94-3.43	NSIG
(A. M. Cotter *et al.*, 2006) JID (1990-2002)	1337	Prospective cohort	cART 507 ---PI cART 134 ---non PI cART 373 ZDV mono 492 No ART 338	30 37 27 24 26	PI cART vs. non-PI cART	1.8	1.1–3.0	PI cART
(Schulte *et al.*, 2007) Peds (1989-2004)	11231	Prospective cohort **PSD**	cART 2563 ---PI cART 782 ---Non PI cART 1781 ZDV mono 2621 Dual NRTI 2312 ref No ART 2565	18 17 18 17 14 21	PI cART vs. dual NRTI Non PI cART vs. dual NRTI No ART vs. dual NRTI	1.21 crude OR 1.37 1.16	1.04-1.40 (1.10–1.50) (1.02–1.32)	PI cART
(Kunjal Patel *et al.*, 2010) JID (2002-2008)	777	Prospective cohort	cART 760 ---PI cART 558 ---non PI 219	 18 13	PI cART vs. non-PI ART	1.29	0.77-2.15	NSIG

			ZDV mono 6 Dual NRTI 11					
(Dola *et al.*, 2012) J Perinat Med (1999 to 2003)	143	Retrospective cohort	cART 64 ---PI cART 53 ---non PI cART 11 ZDV mono 63	PI cART 14 Non-PI ART 21	PI cART vs. non-PI ART	0.63	0.25 – 1.59	NSIG
(Watts *et al.*, 2013) JID (2007-2010)	1869	**SMARTT** surveillance study: **Included women from WITS & IMPAACT P1025**	cART 1663 ---PI cART 1319 ---Non PI 160 ---triple NRTI 193 ZDV mono/Dual NRTI 144 No ART 62	21 19 17 5 13 31	1st trimester PI cART vs. no ART in 1st trimester 1st trimester NNRTI cART vs. no ART 1st trimester triple NRTI vs. no ART	1.55 1.34 0.84	1.16–2.07 0.78–2.30 0.37–1.91	PI cART
(Kakkar *et al.*, 2015) JIAS (1988-2011)	525	Retrospective cohort	PI cART ---Boosted PI 144 ---Unboosted PI 220 ref NNRTI or NRTI cART 166 ZDV mono 77 No cART 59	 19 11 9 12 25	Boosted vs. un boosted PI NNRTI/NRTI based cART vs. unboosted PI No cART vs. un boosted PI	2.17 0.67 1.50	1.05-4.51 0.27-1.63 0.33-6.78	Boosted PI
(Duryea *et al.*, 2015) ID in Obs Gynae (1984-2014)	1004	Retrospective cohort	PI cART 597 Non PI ART (inc ZDV mono and dual NRTI) 230 No ART 177 P	14 13 21	Non PI ART vs. no ART No comparison with PI presented	0.9	0.5-1.5	NSIG lower PTB rate with ART both PI and non PI compared to no ART
(Gagnon *et al.*, 2016) Eur J Ob Gynae & Rep Bio (2007-2012)	97 HIV 288 non HIV (1:3)	Matched prospective cohort	HIV ---PI cART 74 HIV- controls	20 9	HIV vs. HIV- controls PI cART vs. non PI cART (inc ZDV mono)	1.4 1.2	0.5-3.6 0.4-3.6	NSIG
(Rough *et al.*, 2018) NEJM (2007-2016)	4646	**SMARTT** surveillance study: **& IMPAACT P1025**	LPV/r/FTC/TDF ATV/r/FTC/TDF LPV/r/ZDV/3TC	21 16 19	LPV/r/FTC/TDF vs. ATV/r/FTC/TDF	1.14	0.75-1.72	No difference in PI regimes, higher PTB rate than general US population NSIG
Middle Income country data								
(Szyld *et al.*, 2006) AIDS (2002-2005)	681	Prospective cohort **NISDI**	cART 587 ---PI cART 330 ZDV mono+ Dual NRTI 94	9 11 6	PI cART vs. ZDV mono + dual NRTI	1.1	0.5-2.8	NSIG
(Cecchini *et al.*, 2011) Medicina (1997-2006)	204	Prospective cohort	cART 200 ---PI cART 62 no ART 4	-	PI cART vs. non-PI ART	2.67	1.15-6.18	PI cART

(Delicio *et al.*, 2018) Reprod Health (2000-2015)	787	Retrospective cohort	Overall NNRTI cART 141 NVP based 138 EFV based 3 PI cART 579 LPV/r based 428 NFV based 135 ATV/r based 26 Other PI 14	22 25 - 24 21 20 - 	NNRTI vs. PI cART NVP based LPV/r based NFV based ATV/r based	0.79 1 0.98 0.87 0.81	0.53-1.16 0.67-1.45 0.53-1.35 0.32-2.06	NSIG ART class but high rates of adverse birth outcomes
Low Income country data								
(Masaba *et al.*, 2018) AIDS (2003-2009)	384	**KiBS** Phase IIB open label CD4>250 cells/mcL (26-32 weeks)	HIV cART 177 NVP/ZDV/3TC 207 NFV/ZDV/3TC	18 20 16	NVP/ZDV/3TC vs. NFV/ZDV/3TC	RR 5.5	-2.3 - 13.4	NSIG
(Wang *et al.*, 2018) Int J Gynecol Obstet (2009-2011)	731	Retrospective cohort	cART 559 ---LPV/r based 265 ---EFV based 104 ---NVP based 190 no cART 17	 16 8 14 8	LPV/r based cART NVP based cART EFV based cART ref	2.40 2.07 1	1.07-5.37 0.91-4.70	LPV/r based cART
(Stringer *et al.*, 2018) PLOSOne	253	3 RCT **HTN052** **ACTG A5208** **ACTG A5175** Preconception cART only	NNRTI cART 127 PTB % live births/79 PI cART 118 PTB % live births/76 Triple NRTI 8 PTB % live births/5	28 46 38 59 38 60	PI cART vs. non-PI cART NNRTI cART vs. non- NNRTI cART PI cART vs. NNRTI cART	1.46 0.92 1.35	0.75-2.85 0.49-1.72 0.67-2.71	High rates of adverse birth outcomes, highest with PI cART
(R Zash *et al.*, 2017) JAMA Paediatr 2017 (2014-2016)	11932	Prospective surveillance	cART 10592 ---conceived on 5780 EFV/FTV/TDF 2503 NVP/TDF/FTC 775 NVP/ZDV/3TC 1403 LPV/r/FTC/TDF 237 LPV/r/ZDV/3TC 169	 21 19 25 24 29	NVP/ZDV/3TC vs. EFV/FTV/TDF LPV/r/ZDV/3TC vs. EFV/FTV/TDF	1.15 1.36	1.01-1.29 1.06-1.75	LPV/r/ZDV/3TC
(M. G. M. Fowler *et al.*, 2016) NEJM (2011-2014) (Sebikari *et al.*, 2019) JAIDS	3333	**PROMISE RCT** CD4>350 initiation >14 weeks up to term	1497 LPV/r/ZDV/3TC 419 LPV/r/ FTV/TDF 1507 ZDV mono + sdNVP (ref)	21 19 13	LPV/r/ZDV/3TC vs. ZDV mono p<0.001 LPV/r/FTV/TDF vs. ZDV mono p=0.09 LPV/r/FTV/TDF vs. LPV/r/ZDV/3TC p=0.77	1.82 1.77 0.97	1.47-2.26 1.29-2.43 0.72-1.31	PI cART, TDF-based vs. ZDV based high rate of very preterm <34weeks (6%) aOR 3.14 (1.77-5.55) p=0.04 & infant mortality

								(4%) in first week of life p=0.001
(Li *et al.*, 2016) J Infect Dis. (2002-2011)	3314	Prospective cohort	cART 1094 ZDV mono 1768 No ART 452	29 27 -	PI cART vs. ZDV mono EFV based vs. NVP based cART	1.23 1.45	1.04-1.47 1.01-2.07	PI cART EFV based
(Koss *et al.*, 2014) JAIDS (2009-2012)	356	**PROMOTE** Open label RCT	LPV/r/ZDV/3TC 177 EFV/ZDV/3TC 179	15 16	LPV/r/ZDV/3TC vs. EFV/ZDV/3TC	1.12	0.63-2.00	NSIG
(Powis *et al.*, 2011) JID (2006-2008)	530	**Mma Bana RCT** CD4>200 26-34 weeks at initiation	267 LPV/r/ZDV/3TC 263 ZDV/3TC/ABC	21 12	PI cART vs. triple NRTI	2.03	1.26-3.27	PI cART
(Kesho Bora study, 2011) Lancet (2005-2008)	805	**Kesho Bora RCT** CD4>200 28-36 weeks at initiation Burkina Faso, Kenya, and South Africa	401 LPV/r/ZDV/3TC 404 ZDV mono	13 11	PI cART vs. ZDV mono	-	-	NSIG
(Van Der Merwe *et al.*, 2011) J Int AIDS Soc (2004-2007)	1630	Retrospective cohort	cART 1397 PI cART 419 No ART 233	15 6 5	Early (<28/40) non-PI cART vs. no cART Early (<28/40) PI cART vs. no cART	5.6 3.0	2.1-15.2 1.1-8.4	cART (<28/40) PI and NNRTI
(D.K. *et al.*, 2011) JAIDS (2003-2009)	326	Retrospective cohort	cART 326 EFV based 203 NVP based 123	10 13	EFV based cART vs. NVP based cART	-	-	NSIG
International pooled analyses								
(AP Kourtis *et al.*, 2007) AIDS (1984-2004)	20426	Meta-analysis 14 studies	Not stated	-	cART PI cART ZDV mono vs. No ART PI cART vs. non-PI ART	1.13 1.35 0.86 1 1.35	0.79–1.63 1.08-1.70 0.73–1.01 1.08–1.70	PI cART
(Mesfin, Kibret and Taye, 2016) Reprod Health (2002-2013)	23490	Meta-analysis 10 studies	Individual details not given		PI cART vs. non-PI cART	1.51	1.07-1.96	PI cART
(Veroniki *et al.*, 2018) PLOSone	20576	Meta-analysis 35 studies	Individual cART numbers not stated		LPV/r/ZDV/3TC vs. EFV/3TC/d4T	0.32	0.03-2.99	LPV/r based cART >NVP based cART

(1995-2015)	LPV/r/ZDV/3TC vs. NVP/ZDV/3TC	2.51	1.00-6.39	
	NVP/ZDV/3TC vs. EFV/3TC/d4T	0.12	0.01-1.27	

Key: PI=Protease Inhibitor; cART=combination antiretroviral therapy; NRTI= Nucleoside reverse transcriptase inhibitor; ZDV mono=zidovudine monotherapy; LPV/r= Lopinavir & ritonavir; ATV/r= Atazanavir &ritonavir; NNRTI= Non-nucleoside reverse transcriptase inhibitor ; NSIG= non-significant; EFV= Efavirenz; sdNVP= single dose Nevirapine; NFV= Nelfinavir; FTC= Emtricitabine; TDF= Tenofovir Disoproxil Fumarate; 3TC= Lamivudine; ABC=Abacavir and d4T=Stavudine.

=Association with PI cART = No association with PI cART =Comparison within PI class

1.2.7 Does pre-conception cART increase risk of PTB

Timing of treatment initiation with respect to gestation also appears to be critical. Increasingly data from the Europe, Africa and Asia indicate that length of exposure of treatment is key with women conceiving on cART at a higher risk than women initiating treatment during pregnancy, see table 3(Sibiude *et al.*, 2012; Favarato *et al.*, 2018; Wang *et al.*, 2018; Zash, Rough, *et al.*, 2018). The two meta-analyses that have explored timing of cART exposure in relation to PTB risk have also shown that pre conception cART is a stronger risk factor for PTB than cART initiated in the later in pregnancy (A. P. A. Kourtis *et al.*, 2007; O. Uthman *et al.*, 2017).

In different settings cohort studies have shown that initiation of cART is associated with the highest risk. Historical data from our cohort at Imperial College Healthcare NHS Trust showed that in a subgroup of 28 HIV infected pregnant women with CD4 cell count above the level for which concurrent British HIV Association guidelines recommended long-term cART (i.e. > 200 cells/mL pre 2008 and >350 cells/mL post 2008), who could have taken either ZDV mono or cART for PMTCT, the highest rate of PTB was seen in those women who took cART (OR 4.90; 95% CI 1.33– 18.13; P = 0.011)(Short *et al.*, 2014). Another European study showed that post conception cART was associated with a 6 fold risk of iatrogenic rather than spontaneous PTB(Lopez *et al.*, 2012). Other cohort studies, including two from Africa and two from N America, also suggest the women initiating treatment at the highest risk compared to NRTI only ART or no ART (Tuomala *et al.*, 2002; Van Der Merwe *et al.*, 2011; J. Y. J. Chen *et al.*, 2012; Watts *et al.*, 2013).

It has been hypothesised that selection bias towards preconception cART exposure can be created by excluding cases who experienced PTB prior to treatment initiation from the post conception group and thus disproportionally weighting data to show greater rates of PTB in women who conceive on treatment (Stringer *et al.*, 2017).

1.2.8 What is the role of NRTI backbone? Does TDF or ZDV drive severe PTB risk?

Since the results of PROMISE suggesting a signal for an association between TDF and moderate to severe prematurity, with an associated increase in neonatal mortality, there has been interest in examining data regarding NRTI exposure in pregnancy(M. G. M. Fowler *et al.*, 2016).

Three cohort studies, one each from Europe, N. America and Africa have dissected PTB rates by TDF exposure and there has been one large meta-analysis(Nachega *et al.*, 2017; Pintye *et al.*, 2017; Rough *et al.*, 2018; EPPIC Study Group, 2019). EPPIC, the European Pregnancy and Paediatric HIV Cohort Collaboration, compared PTB rates by exposure to ZDV/3TC, ABC/3TC and TDF combined with either 3TC or FTC in a group of 7193 maternal infant pairs. There was no difference in PTB rates 10%, 9% and 11% respectively, nor was there in a difference in rates of severe PTB (<34 weeks) 3%, 3% and 4% respectively.

Equally Rough et al. examined PTB rates in 4646 women infant pairs prospectively recruited to the SMARTT and IMPAACT P1025 in women who received ZDV/3TC in combination with LPV/r (19%) and found similar PTB rates compared to women receiving FTC/TDF/LPV/r (21%) (aOR 0.90 (95%CI 0.60-1.33). Rates of severe PTB rates (<34 weeks) were similar by NRTI exposure: ZDV/3TC/LPV/r (5%) and FTC/TDF/LPV/r (4%).

Authors of an African cohort analysis from two HIV prevention studies (Partners PREP and Partners Demonstration Project) concluded that compared to non TDF NRTI exposure, TDF containing ART regimes were associated with a reduction in PTB rates (aOR 0.34 (95% 0.13-0.85))(Pintye *et al.*, 2017). It should be born in mind when interpreting these results that only 1% of the TDF exposed group took a PI compared to 10% in the non TDF group therefore it is difficult to exclude confounding bias from this known risk factor. The same group compared TDF with AZT exposure in a subgroup of 371 with no PI exposure and did not find a difference in PTB rates (10% vs. 6% respectively, p=0.20)

Meta-analysis of 4 cohort studies including 3027 maternal infant pairs from 4 studies concluded also there was a slightly lower rate of PTB (<37weeks) by TDF vs. non TDF NRTI exposure (aOR 0.90 (95%CI 0.81-0.99) but no difference in severe prematurity (<34 weeks) (aOR 1.08 (95%CI 0.72-1.62))(Nachega *et al.*, 2017).

Prospective data reports to Antiretroviral Pregnancy Registry (1989- 2013) on 12780 maternal infant pairs exposed to ZDV compared to 1904 exposures to non ZDV ART has shown no difference in PTB rates (aOR 1.00 (95%CI 0.87-1.15))(Vannappagari *et al.*, 2016). Restricting comparison to women who were exposed to ZDV containing ART and non ZDV ART in the 1st trimester showed no difference in PTB rate (<37 weeks): 12% for both groups and no difference in severe PTB rate (<32 weeks): 2% for both groups.

1.2.9 Limitations and conclusion from of PTB and cART clinical data

The spectrum of findings presented here is, in part, due to interactions between maternal health status and clinical practice in different settings, in addition to population differences in traditional PTB risk factors. These factors are difficult to completely adjust for in efforts to limit bias in observational cohort studies.

Other methodological issues that may influence the quality of the data include the accuracy of how gestational age is measured and how missing data and study design biases are accounted for in analyses. Bengston et al. reported that manipulation of missing data regarding risk factors and HIV treatment as well and gestational age measurement errors, using Bayesian prior inference modeling, can prevent underestimation of potential associations with PTB(Bengtson *et al.*, 2016).

In many of the studies undertaken in low income countries gestational age is measured by last menstrual period and or symphysis-fundal height (SFH). Where fetal ultrasound is available it may not be undertaken early enough for optimum measurement accuracy due to late presentation to healthcare facilities. Malaba et al. explored the accuracy of these three methods in examining true

PTB rates in a cohort of 1014 uninfected and 773 HIV infected women from a single treatment centre in S. Africa (Malaba *et al.*, 2018). PTB incidence as estimated by LMP was 36%, 17% by SFH and 11% by ultrasound. LMP tended to underestimate gestation age whereas SFH overestimated gestational age, especially in women with high BMIs. HIV infected pregnant women had a higher rate of PTB as estimated by USS compared to uninfected women but not by LMP or SFH. No difference in treatment timing with relation to conception and PTB was observed by any method.

Santosa et al. used 1st trimester USS dating in a prospective S. African study of 229 HIV infected and 404 uninfected pregnant women and identified that HIV infected women had higher rates of PTB (20%) compared to uninfected women (15%), although this was only a trend, p=0.15 (Santosa *et al.*, 2019). The authors also described a non statistically significant higher PTB rate in women who conceived on ART (24%) compared to those who initiated cART post conception (15%), p=0.22. Careful consideration is required interpreting PTB data from analyses where gestational age estimation has not used USS. These data also demonstrate the potential importance of exploring composite poor birth outcomes in low-income countries that may be a surrogate for PTB such as low birth weight.

In spite of conflictions within the literature, there is now mounting evidence that cART, particularly PIs, have a role in the excess risk of PTB in HIV infected women. The association with other drug classes and treatment timing remain less certain. Understanding the underlying mechanism of these observed elevated rates of PTB is key to elucidating the role of cART in this obstetric outcome, addressing its management and identifying optimum cART regimens, in addition to assessing potential toxicity of new antiretrovirals. Furthermore HIV infected pregnant women are an unique group in which to investigate and model immunological causes of PTB, due to PMTCT requiring administration of drugs known to increase risk of PTB, which in any other cohort might be deemed unethical, however the balance of protecting the fetus from HIV infection outweighs concerns regarding PTB.

Table 3 Summary of studies exploring effect of timing of cART exposure, in relation to conception, on PTB

Reference and study period	Mother-child pairs (n)	Design	ART class (n)	PTB rate (%)	Treatment comparison	aOR	(95% CI)	PTB Association
European data								
(Thorne *et al.*, 2000) AIDS (1986-2000)	3920	2 Prospective cohorts **ECS & MoCHiV**	cART 289 ---PI-cART 188 ZDV mono 555 No ART 2891	24 29 17 16	Pre-conception cART vs. No ART	2.17	1.03-4.58	Pre-conception cART
(Thorne, Patel and Newell, 2004) AIDS (1986-2004)	4372	Prospective cohort **ECS**	cART 757 ---New cART 446 ---Pre-conception cART 321 ZDV mono+ Dual NRTI 568 No ART 944	26 - - 16 -	 New cART Pre-conception cART vs. ZDV mono + dual NRTI	 1.88 2.05 1	- 1.34-2.65 1.43–2.95	 cART preconception OR> post but not directly compared
(Boer *et al.*, 2007) BJOG (1997-2003)	143 HIV 196 non-HIV	Case control study **AmRo**	cART 143 HIV- controls 196	16 9	HIV vs. non- HIV cART 1st trimester vs. no cART 1st trimester	2.24 2.24	1.12-4.47 - (p=0.19)	HIV NSIG association with cART in 1st trimester
(Martin and Taylor, 2007) JID (1995-2006)	211	Prospective cohort	cART 75 ZDV mono 52	17 6	Post-conception cART vs. Pre-conception cART + ZDV mono	5.03 1	1.4-17.8	Post conception cART
(Sibiude *et al.*, 2012) CID (1990–2009)	10 402	Prospective cohort	cART 6738 ZDV mono 2975 Dual NRTI 1664 Sub-analysis ---PI cART 1253 ---Boosted PI 1066 ---Non Boosted PI 187	15 10 11 14 14 9	Pre-conception cART vs. post-conception cART	 1.31	 1.11–1.55	 Pre-conception cART
(Lopez *et al.*, 2012) AIDS (1986- 2010)	519 HIV 1038 non-HIV	Matched cohort	cART 298 ---PI cART 178 ---non PI cART 153 No ART 221	-	Post-conception cART vs. pre-conception cART	Iatrogenic 6.16	1.42–26.8	Post-conception cART with Iatrogenic NSIG with sPTB
(Short *et al.*, 2014) HIV Medicine (1996-2010)	331	Prospective cohort	cART 246 ---PI cART 96 ZDV mono 65 No ART 8	15 23 6 13	New PI cART vs. ZDV mono	8.71	2.30–33.06	Post-conception PI cART

(Favarato *et al.*, 2018) AIDS (2007-2015)	6073	National surveillance register **NSHPC**	HIV Pre-conception 2983 Post-conception 3090 PI cART 4184 ---LPV/r cART 2368 ---Other PI cART 1816 NNRTI cART 1889	10 12 10 9	Pre-conception vs. Post-conception Pre-conception & CD4 <350 cells/mcL ---LPV/r cART ---Other PI cART ---NNRTI cART -ref CD4> 350 cells/mcL ---LPV/r cART ---Other PI cART ---NNRTI cART -ref	1.27 2.01 2.05 1.00 1.64 0.98 1.00	1.01-1.61 1.03-3.91 1.07-3.89 - 1.08-2.47 0.66-1.46	Pre-conception, particularly LPV/r, CD4 <350
(Snijdewind *et al.*, 2018) PLOSone (1997-2015)	1392	Prospective cohort **ATHENA**	HIV HIV- ref Pre-conception 550 Post-conception 842 PI cART 928 NNRTI cART 438	15 5 18 13 14 17	Pre-conception cART vs. post-conception cART	1.39	0.99-1.94 p=0.06	Trend pre conception p=0.06
North American data								
(Tuomala *et al.*, 2005) AIDS (1990-2002)	2543	Prospective cohort **WITS**	cART 588 ZDV mono 932 Dual NRTI 258 No ART 751	Not stated	Post-conception without ZDV Post-conception containing ZDV	7.86 0.53	1.39-44.58 0.34-0.83	Post-conception ART without ZDV Not compared with pre-conception
(Watts *et al.*, 2013) JID (2007-2010)	1869	Prospective cohort	cART 1663 ---PI cART 1319 ZDV mono/Dual NRTI 144 No ART 62	21 19	First trimester PI cART vs. no ART	1.55	1.16–2.07	Post-conception PI cART Not compared with pre-conception
Middle Income country data								
(Machado *et al.*, 2009) STDS (1996-2006)	696	Prospective cohort	cART 305 PI cART 193 ZDV mono 179 Dual NRTI 212	- 16 12 8	Pre-conception cART vs. dual NRTI	5.06	1.5-17.06	Pre-conception cART Not compared with post-conception
(Kreitchmann *et al.*, 2014) BJOG (2002-2007)	1483	2 Prospective cohort studies **NISDI Perinatal & LILAC**	Pre conception cART No ART	20	Pre conception cART vs. No ART	1.4	1.0-1.9	Pre-conception cART Not compared with post-conception
(Delicio *et al.*, 2018) Reprod Health (2000-2015)	787	Retrospective cohort	Overall Pre-conception 143 Post- conception 623	22 29 23	Post-conception vs. pre-conception	0.78	0.55-1.10	NSIG

			NNRTI cART 141 NVP based 138 EFV based 3 PI cART 579 LPV/r based 428 NFV based 135 ATV/r based 26 Other PI 14	 25 - 24 21 20 -				but high rates of adverse birth outcomes
Low Income country data								
(Santosa *et al.*, 2019) AIDS (2013-2016)	229 HIV 404 non-HIV	Prospective cohort	109 with details on timing Pre-conception 38 Post- conception 71	 24 15	p=0.022			NSIG but high rates of PTB with pre-conception cART
(Malaba *et al.*, 2018) Ann Epidemiol (2014-2016)	1787	Prospective cohort	Pre-conception 336 Post-conception 353	12 15	Pre-conception cART vs. Post-conception cART	0.69	0.33-1.47	NSIG
(Zash, Rough, *et al.*, 2018) J Ped Infect Dis Soc (2013-2016)	3384	Prospective surveillance	EFV/FTV/TDF 2882 Pre-conception 502 Post-conception 0-7 weeks 97 8-13 weeks 613 14-19 weeks 1622 20-21 weeks 550		Pre-conception Post-conception 0-7 weeks 8-13 weeks 14-19 weeks 20-21 weeks(ref)	1.33 1.28 1.10 1.08 1	1.04-1.70 0.84-1.95 0.86-1.41 0.88-1.34 ref	Pre-conception EFV/FTV/TDF vs. late 2nd trimester exposure
(Wang *et al.*, 2018) Int J Gynecol Obstet 2009-2011	731	Retrospective cohort	cART 559 ---LPV/r based 265 ---EFV based 104 ---NVP based 190 no cART 17	 16 8 14 8	early cART (pre- conception or initiated in 1st trimester) vs. no cART Late cART (2nd or third trimester) vs. no cART	2.82 1.05	1.47–5.44 0.52–2.11	Pre-conception/1st trimester cART
(Sebitloane and Moodley, 2017) Niger J Clin Pract (2011-2014)	1461	Retrospective cohort	HIV-infected 1159 ---Pre-conception 312 ---Post-conception 423 ---ZDV mono + sdNVP 424 Uninfected 302	25 29 22 24 17	HIV vs. uninfected Pre-conception vs. uninfected Pre-conception vs. post conception Pre-conception vs. ZDV mono (PTB<34weeks)	1.65 1.72 1.24 1.85	1.17-2.33 1.33-3.01 0.88-1.75 1.19-2.90	Pre-conception cART
(Li *et al.*, 2016) J Infect Dis. (2002-2011)	3314	Prospective cohort	cART 1094 --- Pre conception 582 --- Post conception 512 ZDV mono 1768 No ART 452	29 38 26 27 -	Pre-conception vs. ZDV mono Post-conception vs. ZDV mono	1.24 0.85	1.05-1.47 0.70-1.02	Pre-conception cART Not directly compared with post-conception

(Njom Nlend *et al.*, 2016) PLOSone (2008-2011)	760	Retrospective cohort	Post-conception cART 481 ---NNRTI based 472 ---PI based 9 ZDV mono 279	10 6	Post-conception cART vs. ZDV mono	1.9	0.9-3.7	Trend post conception cART p=0.06
(Aniji *et al.*, 2013) SA J HIVMED (2008-2009)	245	Retrospective cohort	Pre-conception 76 Post-conception 169	21 24	Pre-conception vs. post-conception p=0.348	-	-	NSIG high rates of adverse birth outcomes
(Van Der Merwe *et al.*, 2011) J Int AIDS Soc (2004-2007)	1630	Retrospective cohort	cART 1397 PI cART 419 No ART 233	15 6 5	Early (<28/40) non-PI cART vs. no cART Early (<28/40) PI cART vs. no cART	5.6 3.0	2.1-15.2 1.1-8.4	Post-conception cART (<28/40) NNRTI>PI Not compared with pre-conception
(J. Chen *et al.*, 2012b) JID (2009-2011)	9504 HIV 22609 non-HIV	Matched cohort study	cART 2942 PI cART 312 ZDV 3290	24 14	Post-conception cART vs. ZDV mono	1.4	(1.2-1.8)	Post-conception cART Not compared with pre-conception
International pooled analyses								
(AP Kourtis *et al.*, 2007) AIDS (1984-2004)	20426	Meta-analysis 14 studies	Not stated	-	cART PI cART ZDV mono vs. No ART PI cART vs. non-PI ART Pre-conception+1st trimester cART vs. 2nd +third	1.13 1.35 0.86 1 1.35 1.71	0.79–1.63 1.08-1.70 0.73–1.01 1.08–1.70 1.09–2.67	Pre-conception cART
(O. O. A. Uthman *et al.*, 2017) Lancet HIV (1986-2012)	19189	Meta-analysis 11 studies	Pre-conception 9443 Post-conception 7773		Pre-conception vs. post-conception	1.2	1.01-1.44	Pre-conception cART

Key: PI=Protease Inhibitor; cART=combination antiretroviral therapy; NRTI= Nucleoside reverse transcriptase inhibitor; ZDV mono=zidovudine monotherapy; LPV/r= Lopinavir & ritonavir; NNRTI= Non nucleoside reverse transcriptase inhibitor ; NSIG= non-significant

=Association with pre-conception cART

= Association with post-conception cART

=No association with ART timing

1.3 Mechanism of PTB

The main mechanisms through which PTB is triggered can be broadly divided into: inflammation; placental abruption; premature uterine distension and maternal-fetal stress**.** The mechanism by which cART increases PTB rates has not been elucidated. Several potential mechanisms have been hypothesised which are currently under evaluation including: abnormal placental vascularity and structure (D'costa, Khadke and Patil, 2007; Baurakiades *et al.*, 2011; Conroy *et al.*, 2017; Mohammadi *et al.*, 2018; Obimbo *et al.*, 2019); antiretroviral interaction with hormonal axes(Papp *et al.*, 2015; Siou *et al.*, 2016; Mohammadi *et al.*, 2018; Esemu *et al.*, 2019) and dysregulation of the maternal immune system by HIV and antiretrovirals inducing inflammation at the foetal-placental unit (Fiore *et al.*, 2006; Faye *et al.*, 2007; Pornprasert *et al.*, 2009). Here we will discuss the evidence to support an inflammatory cause.

1.3.1 Immunology of pregnancy and labour

The Th1/Th2 cytokine paradigm of pregnancy was first coined in 1993 by Wegmann and colleagues(Wegmann *et al.*, 1993). The hypothesis being that pregnancy is a Th2 dominated cytokine state to enable tolerance of a semi allogenic fetus (paternal alleles) which is latterly reversed to Th1, integral to the process of triggering labour(Sykes *et al.*, 2012). Current thinking has advanced this concept to a maternal foetal immune system that changes according to the stage of pregnancy(Mor, Aldo and Alvero, 2017). Pregnancy begins at conception where a Th1/Th17 pro-inflammatory environment is necessary for implantation followed by a Th2/ immune-regulatory/tolerance phase, which facilitates fetal growth that ultimately ends in the Th1/pro-inflammatory process of labour.

1.3.2 T helper cytokine classification

Cytokines can be classified according to which CD4+T helper cell subset they are secreted by and interact with. Many cytokines have several functions and do not fit neatly into one classification. Th1 cytokines are typically pro-inflammatory, classically known as regulators of cellular immunity, their target cells are macrophages and they are important to intracellular pathogen responses(Abbas, Lichtman and Pillai, 2014); Th2 cytokines were first described as important for

humeral immunity and atopy but are also immune-regulatory and anti-inflammatory in their actions; and Th17 cytokines assist the recently defined Th17 T helper cell that are important to mucosal immunity, particularly in effecting neutrophils responses to extracellular pathogens.

1.3.3 Placentation/growth phase

The growth /tolerance phase is believed to be facilitated by this shift in Th2/immune-regulatory cytokines, changes in the expression of MHC molecules on trophoblast cells, down regulation of antigen presentation, alongside alteration of the spectrum of immune cells populating the uterus and its lymphatic system(Hunt, 2006; Erlebacher, 2013). The principle local immune cells involved in successful fetal trophoblast invasion of the decidua and spiral arterioles are believed to be decidual macrophages, tolerogenic dendritic cells, NK cells and T regulatory cells(PrabhuDas *et al.*, 2015; Tsuda *et al.*, 2019).

1.3.4 Labour phase

Pro-inflammatory/Th1 cytokine responses are thought to be important in triggering labour, both term and preterm, by: mediating cervical ripening; reducing amniotic membrane rupture threshold; increasing myometrial contractility through the recruitment of neutrophils and macrophages and through up regulation of MMPs and prostaglandins(Peltier, 2003; Challis *et al.*, 2009).

1.3.5 Cytokine function and role in labour

1.3.5.1 IL-1 (Pro-inflammatory/Th1/Th17)

IL-1 is a pro-inflammatory cytokine that is predominantly expressed by tissue macrophages but in the female genital tract it is also produced by monocytes, NK cells, lymphocytes, neutrophils, fibroblasts and epithelial cells (Robertson *et al.*, 2012). IL-1 is a 17.5 kDa peptide that has two forms: α and β. In addition there is an inhibitory analogue IL- 1 Receptor Antagonist (RA), which is a competitive antagonist to IL-1. IL-1 was previously called lymphocyte-activating factor which gives a clue to its action in the immune system. It promotes differentiation and

activation of Th-1 and Th-17 helper T cells(Abbas, Lichtman and Pillai, 2014). Its effects are mediated locally on a cellular level and systemically as an acute phase protein via its action on the liver and hypothalamus. IL-1 is believed to be up-regulated by various stimuli including bacterial antigens via Toll Like Receptors (TLR) (Challis *et al.*, 2009).

In the context of labour, it has been elucidated that IL-1 has several downstream effects including inducing prostaglandin expression through activation of the cyclo-oxygenase-2 (COX-2) enzyme at the cervix, myometrium and fetal membranes(Todd *et al.*, 1996; Brown *et al.*, 1998; Schmitz *et al.*, 2003; Mohan, Loudon and Bennett, 2004). IL-1 can also up-regulate MMPs expression at the cervix, amnion and myometrium, causing detachment of the placenta, cervical ripening and reduces threshold for membrane rupture(Peltier, 2003). IL-1 induces myometrial expression of receptors for corticotrophin-releasing hormone (CRH) and oxytocin (Markovic *et al.*, 2007; Terzidou, 2007) and up-regulates oxytocin production by myometrial cells(Friebe-Hoffmann, Chiao and Rauk, 2001).

1.3.5.2 IL-6 (Pro-inflammatory Th1/Th2/Th17)

IL-6 is another pro-inflammatory cytokine expressed by monocytes, macrophages, NK cells, decidual endothelial cells, cervical epithelial cells and fibroblasts(Young *et al.*, 2005; Robertson *et al.*, 2012). IL-6 is a 26kDa peptide, formally known as B-cell differentiation factor. Like IL-1, it can act locally on a cellular level or systemically as an acute phase protein. Initially believed to be a Th-1 cytokine, has also been classified as Th-2 because it has been shown to have some immune-regulatory functions(Sykes *et al.*, 2012). More recently its role in promoting the differentiation of Th-17 cells has been described(Abbas, Lichtman and Pillai, 2014). It is one of the main cytokines induced by bacterial antigen Lipopolysaccharide (LPS) and its production is also stimulated by IL-1 and TNF-α (Robertson *et al.*, 2012).

IL-6 has several actions that may be involved with the initiation of labour which include: increasing expression of oxytocin and oxytocin receptors at the myometrium(Friebe-Hoffmann, Chiao and Rauk, 2001; Rauk *et al.*, 2001), promotion of CRH release from the placenta ((Papatheodorou *et al.*, 2013), inhibiting differentiation of T regulatory cells and promoting differentiation of macrophages from PBMCs(Nakashima *et al.*, 2010; Saito *et al.*, 2010; Robertson *et al.*, 2012).

1.3.5.3 IL-8 (Chemokine/Pro-inflammatory)

IL-8 is a pro-inflammatory chemokine (regulator of leucocyte activation and migration). It is an 8.5kDa glyco-protein that was originally described as macrophage-derived neutrophil chemotactic factor. In addition to macrophages, it is also produced by monocytes, choriodecidual endothelial cells, amnion and cervical epithelial cells and cervical fibroblasts(Mohan, Loudon and Bennett, 2004; Young *et al.*, 2005; Robertson *et al.*, 2012). Its production is stimulated by LPS, IL-1β and TNF-α(Elliott, 2002; Mohan, Loudon and Bennett, 2004).

IL-8 assists the initiation of labour by causing an infiltration of leucocytes to the gestational tissues, namely neutrophils and macrophages to the cervix where they are key to initiating ripening (dilation and effacement) to the uterus where they act on the lower segment to initiate contractility, and to the fetal membranes where they induce downstream mediators of ECM re-modelling(Osman *et al.*, 2003; Peltier, 2003; Mohan, Loudon and Bennett, 2004; Gomez-Lopez *et al.*, 2010).

1.3.5.3 IL-12 (Th1/Pro-inflammatory)

IL-12 is a pro-inflammatory cytokine, produced by macrophages and antigen presenting cells, that drives T helper cells towards the Th1 subtype, promotes differentiation of NK cells and regulates Th1 IFN-γ secretion. It is a 70kDa peptide.

Far less is known about the mechanistic role of IL-12 in pregnancy. It has been shown that decidual mononuclear cells IFN-γ output can be up-regulated by IL-

12(Negishi *et al.*, 2011) thus inducing a Th1 cytokine shift. It is believed that LPS driven IL-12 up-regulation of NK cytotoxicity is inhibited by progesterone and that this dampening mechanism may contribute to normal term outcomes(Par *et al.*, 2003). Conversely, IL-12 can induce NK cells to produce TNF-α, which is known to facilitate production of prostaglandins, although this has yet to be demonstrated in late pregnancy.

1.3.5.4 TNF-α (Th1/Pro-inflammatory)

TNF-α is a major systemic and local inflammatory mediator. It is a 17kDa peptide produced by macrophages within the placenta, fetal membranes, myometrium and cervix but also by cervical epithelial cells and decidual stromal cells(Young *et al.*, 2005). TNF-α is stimulated by LPS and other pro-inflammatory cytokines and its actions largely overlap with the IL-1 system(Robertson *et al.*, 2012).

TNF-α has many actions including increasing production of prostaglandins in the myometrium via COX-2 induction, which in turn can increase concentrations of MMPs, thus facilitating myometrial contractions. TNF-α also induces IL-8 production which in turn causes cervical ripening by inducing neutrophil infiltration of the cervix(Sykes *et al.*, 2012). It also regulates progesterone stimulating receptors thus inhibiting progesterone release(Pandey, Chauhan and Awasthi, 2017).

1.3.5.5 IL-10 (Th2/immune-regulatory)

IL-10 is both an immune-regulatory and anti-inflammatory cytokine. It was initially described as the cytokine synthesis inhibitory factor (Fiorentino, Bond and Mosnmann, 1989). It is a 90 kDa peptide. The source in pregnancy is believed to be a mixture of cell types including trophoblast cells, uterine NK cells, monocytes and T regulatory cells (Thaxton and Sharma, 2010). Uterine T regulatory cells are likely to be peripheral in origin migrating towards hCG hormone(Alexander *et al.*, 2009) and PBMCs isolated from whole blood in pregnant women are capable of secreting IL-10 even in the absence of stimulation(Denney *et al.*, 2011). IL-10 regulates and moderates the effects of

pro-inflammatory cytokines and equally can be stimulated by LPS(Simpson, Keelan and Mitchell, 2004).

IL-10 is increasingly recognized for its role in immune tolerance during the placentation/growth phase of pregnancy. There are several downstream effects of IL-10 in pregnancy including: inhibition of COX-2 expression in the placenta and thus reduction in prostaglandin production(Hanna *et al.*, 2006); blocking of IL-1β and TNF-α down regulation of prostaglandin synthesis dehydrogenase in trophoblast cells which metabolises prostaglandins(Pomini, Caruso and Challis, 1999). In addition IL-10 can reduce the concentration of Il-1β at the fetal membranes(Brown *et al.*, 1998).

1.3.5.6 IL-17 (Th17/pro-inflammatory)

IL-17 secreting T cells (Th17) are present during pregnancy in the uterus and peripheral blood although the ratio of Th17 cells to total CD4+ cells is low(Nakashima *et al.*, 2010). Although the role of Th17 secreting cells in healthy pregnancy is unclear, it is known as an inflammatory cytokine(Saito *et al.*, 2010). IL-6 and TGF-β are cytokines that regulate the Th17/T regulatory cell lineages but in opposing fashion.

1.3.5.7 NFkB: the labour associated gene

Many of the actions of pro-inflammatory cytokines in pregnancy are mediated via transcription factor NF-κB in positive feed forward loops (Lindström and Bennett, 2005). At the myometrium and fetal membranes, IL-1β, IL-6 and TNF-α all induce NF-κB release downstream of their receptors, in turn, the genes for each of these cytokines contains an NF-κB binding site in the transcription promotor (Mohan, Loudon and Bennett, 2004).

NF-κB is a transcription factor that is known for its role in inflammatory pathways both in response to infection (via toll like receptors) and in response to non-infectious triggers. It is responsible for up-regulating expression of many labour associated proteins. Labour associated proteins up-regulated by these pro-inflammatory cytokines include chemokine IL-8, MMPs, COX-2 and oxytocin

receptors(Lindström and Bennett, 2005)(Christiaens *et al.*, 2008)(Terzidou, 2007).

1.4 Systemic cytokine environment through human pregnancy

Knowledge of the cytokine milieu in human pregnancy is the result of cytokine concentration studies of maternal plasma/serum, amniotic fluid and cervicovaginal fluid in addition to stimulation of maternal PBMCs, ex vivo tissue explant and mouse models(Robertson *et al.*, 2012). Below is a summary the pertinent data to systemic cytokine concentrations in pregnancy.

Makhseed and colleagues were amongst the first to measure plasma cytokine concentrations longitudinally through pregnancy in 30 women delivering at term and 30 delivery preterm and found the Th1/pro-inflammatory cytokine IFN-γ increased from second trimester to delivery as did immune-regulatory IL-10(Makhseed *et al.*, 2003). Curry and colleagues also assessed cytokine concentrations through pregnancy in 1274 women and found IFN-γ, IL-12 and TNF-α increased from early to mid- pregnancy whereas immune-regulatory GM-CSF decreased over this period (Curry *et al.*, 2008). In addition IL-6 increased from 25 weeks on wards.

Denney et al. explored cytokine concentrations in un-stimulated and stimulated peripheral blood mononuclear cells (PBMCs) from 45 pregnant women and found the converse to other authors with a reduction in pro-inflammatory IFN-γ, IL-1β, IL-6 and TNF-α across gestation but no change in Th2/immune-regulatory IL-4 and a decrease in anti-inflammatory IL-10 although stimulation of PBMCs with PHA revealed the potential of these cells to increase their IL-10 secretion over gestation(Denney *et al.*, 2011).

Other studies of PMBCs using stimulation systems, which arguably have less resemblance to the physiological environment, have shown both increases and decreases in Th1/pro-inflammatory cytokines and increases in immune-regulatory IL-4, IL-10 and GM-CSF in normal pregnancy(Austgulen *et al.*, 1994;

Marzi *et al.*, 1996; Matthiesen *et al.*, 1998; Vassiliadis *et al.*, 1998; Raghupathy *et al.*, 2000).

1.4.1 Cytokines and PTB

More data exists exploring associations of systemic cytokines and poor birth outcomes including recurrent abortions, preterm birth and pre-eclampsia with some excellent reviews published (Wei, Fraser and Luo, 2010; Sykes *et al.*, 2012; Lucaroni *et al.*, 2018).

One of the first studies to consider systemic inflammatory markers in association with asymptomatic preterm birth was the National Institute of Child Health and Human Development Preterm Prediction Study lead by Goldenburg and colleagues. They explored many different markers from different biological fluids and found that a plasma G-CSF concentration >75th centile was predictive of PTB(Goldenberg *et al.*, 2001).

In addition to characterizing plasma cytokines in normal pregnancy in the Danish Birth Cohort, Curry et al. explored mid pregnancy plasma cytokine levels from women who experienced PTB, stratified into early (24-29 weeks, n=61), moderate (30-33 weeks, n=278) and late PTB (34-36 weeks, n= 334) and 1125 women who delivered at term (Curry *et al.*, 2007). Curry found that plasma IFN-γ and IL-6 concentrations >75th percentile were associated with late PTB and plasma IFN-γ >90th percentile were associated with moderate PTB.

Makhseed and colleagues' 2003 study of stimulated PBMCs, presented above, also showed that women who had PTB had higher plasma IL-6 at delivery as opposed to women delivering at term (Makhseed *et al.*, 2003). Vogel et al measured serum and CVF inflammatory markers early in the second trimester in 62 pregnant women with a prior history of PTB(Vogel *et al.*, 2007). They identified that women who went on to deliver preterm had higher serum concentrations of Th1 cytokines: IL-1β, IL-2, IL-8, IL-12, IL-18 and TNF-α alongside higher Th17 inducing pro-inflammatory cytokine IL-6 and higher concentrations of immune-regulatory cytokines: IL-5, GM-CSF and TGF-β.

More recently Sorokin et al. explored mid pregnancy serum cytokine

concentrations as part of a secondary analysis of an RCT (n=475) examining the use of steroids for PTB prevention in women with a past history of PTB (Sorokin *et al.*, 2010). Sorokin and colleagues identified that IL-6 and CRP concentrations >90th centile were associated with severe PTB (<32 weeks).

In summary studies of plasma and PBMCs from the second and third trimester of pregnancy have largely shown that systemic cytokine balance is likely to be more complex than a simple Th1/Th2 dichotomy and studies correlating systemic cytokines with birth outcome have consistently shown plasma IL-6 is associated with PTB.

1.4.2 HIV PTB Th1/Th2 reverse shift hypothesis

Historically one of the most popular hypotheses for HIV associated PTBwas that cART causes a reverse shift in pregnancy-associated cytokine changes at the maternal fetal interface to a pro-inflammatory Th1 predominant profile(Fiore *et al.*, 2006). The natural history of untreated HIV infection sees a shift in cytokine secretion by peripheral blood mononuclear cells (PBMCs) towards Th2 predominant profiles whilst initiation of cART in non-pregnant adults results in a Th2 to Th1 cytokine shift(Barcellini *et al.*, 1994; Marzi *et al.*, 1996; Klein *et al.*, 1997; Clerici *et al.*, 2002). This led to the theory that the same reverse pro-inflammatory Th1 cytokine shift in HIV infected pregnant women on, or initiating treatment, may drive a lower threshold for the trigger of labour in these women, see pictorial representation in figure1 below.

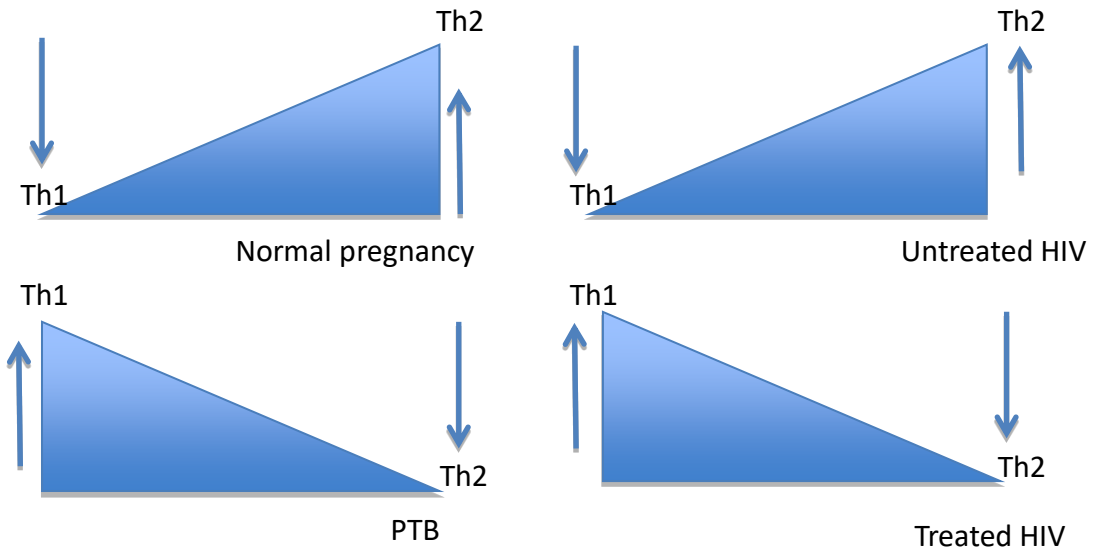


Figure 1 Cytokine shift hypothesis
Diagram to demonstrate hypothesised parallel drawn between the Th1 cytokine shift observed in preterm birth l with cytokines changes observed in treated HIV infection.

1.4.3 Peripheral cytokines network in HIV and PTB

Fiore et al were the first to produce systemic data on Th1 and Th2 cytokines in HIV infected pregnant women compared by ART exposure(Fiore *et al.*, 2006). Fiore et al. measured Th1 interleukin 2 (IL-2) and Th2 IL-10 production from peripheral blood mononuclear cells (PBMCs) obtained from 49 HIV infected pregnant women during each trimester (three time points). Just over half (53%) of women conceived on cART. They demonstrated an increasing IL-2/IL-10 ratio across the trimesters (Th1 shift), a reduced median IL-10 in women receiving cART (n=26, 22 (85%) receiving PI-based cART) compared to those who did not (n=23) and higher IL-2 slope values were associated with PTB.

In a study that predates Fiore et al., Abrams and colleagues measured cytokine concentrations in maternal plasma from 572 pregnant women in Malawi, of whom 400 were HIV infected (Abrams *et al.*, 2004). They showed that plasma IL-8 and IL-6 were higher in women who experienced PTB. In a nested sub-study, in which 92% were HIV infected, chorioamnionitis (CAM) was strongly associated with PTB. There was also a trend towards maternal plasma IL-6 being higher in cases of CAM. These data indicated a Th1/inflammatory shift in systemic cytokine response to local inflammation at the fetal placental unit in HIV-infected

pregnant women experiencing PTB. The research discussed above led to the following objectives:

1.4.4 Objectives 1

- To characterize plasma pro-inflammatory and immune-regulatory cytokine profiles in a larger more immunologically matched group of HIV-1 infected and uninfected pregnant women across the second and third trimester
- To compare plasma cytokines across gestation and how these differ by HIV status, cART (drug class, timing of exposure) and gestational age at delivery.

1.5 Cellular Immune activation in HIV infected pregnant women

Persistent inflammation is driven by cellular immune activation, a known consequence of chronic HIV infection not fully reversed by ART(Hileman and Funderburg, 2017), therefore it is important to also consider other markers of immune activation, namely peripheral monocyte subtypes and ratios.

1.5.1 Markers of T cell activation in HIV infection master class

When a naïve (CD3+ CD45RA+) T helper cell in a lymphoid organ encounters HIV antigen presented by a MHC molecule that its T cell receptor (TCR) recognises (CD8+ with MHC Class I and CD4+ with MHC Class II), the T cell is "activated" to become an effector cell. The activated T cell then receives a secondary signal via the constitutively expressed cell surface CD28 and other molecules, and can then undergo clonal expansion under the influence of interleukin-2 (IL-2), formerly known as T cell growth factor, and other cell specific cytokine signals which drive the activated cell into a particular effector type. For Th1 helper cells this is IL-12 and IFN-γ, for Th2 the driving cytokine is IL-4, for Th17 this is either IL-1, IL-6 or TGF-β and for T regulatory cell s this is TGF-β (Abbas, Lichtman and Pillai, 2014). When CD4+ and CD8+ cells are activated, they typically express CD25, CD38 and HLA-DR(Kestens, 2008).

In untreated HIV infection CD4+ T cell numbers decline as the infection progresses through: direct killing by viral budding and cell apoptosis, the host's cytolytic immune response to the virus infected, activated CD4 T cells and innocent bystander cells; as well as longstanding damage to the precursor cells in the thymus and bone marrow. This results in a reducing pool of naïve and resting memory CD4+ T cell (Hazenberg *et al.*, 2000; Hunt and Deeks, 2006). HIV specific CD8+ T cells continue to proliferate in an effort to control the infection, both through their cytotoxic capacity and their ability to secrete anti HIV proteins such as RANTES and MIP-1(Cao *et al.*, 2016). This results in a reduction in CD4/CD8 ratio(Serrano-Villar *et al.*, 2013; Utay and Hunt, 2016). The orchestrated cellular and adaptive immune response to HIV infection is unable to effectively clear the virus so, in the presence of continued antigenic HIV virus and cytokine stimulation, both CD4+ and CD8+ T cells remain activated.

In addition to measuring percentage of T cells expressing surface markers of activation (CD38, HLA-DR) other accepted markers of immune activation include high absolute and percentage CD8+ T cells, a low CD4/CD8 ratio (<1) and increased soluble CD14, neopterin and β2-microglobulin (as markers of monocyte/macrophage activation) (Taylor *et al.*, 1989; Liu *et al.*, 1998; Sandler *et al.*, 2011; Utay and Hunt, 2016). Continued activated immune response can be also measured indirectly through inflammatory cytokines such as IL-2, IL-6 and TNF-α (Fahey *et al.*, 1998). More recently there has been an emphasis on measuring other biomarkers of persistent inflammation as a surrogate measure of immune activation such as C reactive protein and the coagulation cascade(Kuller *et al.*, 2008; Neaton *et al.*, 2010; Rodger *et al.*, 2010).

1.5.2 Milieu of peripheral mononuclear cells in HIV in pregnancy and the effect of ART

Studies of HIV infected pregnant women describing T cell activation to date have not specifically evaluated the effect of cART nor associations with birth outcome.

In a study from the era prior to cART Mikyos et al. examined immunologic activation marker during pregnancy in 99 HIV infected (67% received ZDV monotherapy) and 46 uninfected pregnant women: serial measurement of lymphocyte phenotype and serum activation molecules including β2 microglobulin, neopterin and TNF-α (Mikyas *et al.*, 1997). Median absolute CD8+ T cell counts were higher and CD4+ cell counts were lower in HIV infected pregnant women than uninfected pregnant women and HIV infected pregnant women had higher percentage of CD8+HLA-DR+ at all time points $p<0.0001$. No birth outcome data were presented.

Ono et al. characterised T cell phenotypes and plasma IL-7 (a cytokine important for T cell development and activation) in 44 HIV infected, 45 uninfected pregnant women and 20 uninfected non pregnant women(Ono *et al.*, 2008). All HIV-infectedwomen received cART, 29 received PI-based cART and 15 received an NNRTI-based cART. HIV infected mothers had a higher mean absolute CD8+ T

cell count (800 cells/mcL) compared to uninfected pregnant women (385 cells/mcL) and non-pregnant women (362 cells/mcL), p=0.02. These HIV infected pregnant women also had a higher percentage of activated CD4+CD38+ (12%) and CD8+CD38+ cells (15%) compared to uninfected pregnant women (9% CD4+ CD38+, 10% CD8+CD38+) and non-pregnant controls (9% CD4+CD38+, 6% CD8+CD38+). There was also a trend towards a higher plasma concentration of IL-7 in HIV infected pregnant women (7 pg/ mL) compared to uninfected pregnant women (5 pg/mL) and non-pregnant women (4 pg/mL), p=0.066. No birth outcomes were described.

Truong and colleagues explored T cell phenotypes, β2 microglobulin, neopterin and TNF-α plasma concentrations in 96 HIV infected pregnant women and 28 uninfected women through the third trimester and delivery(Truong *et al.*, 2010). Of the HIV infected pregnant women, 11 received no ART, 49 received ZDV mono and 36 received PI-based cART. Truong et al. noted an elevation in absolute CD8+ cells in the third trimester of pregnancy in HIV infected women either not on ART or receiving ZDV mono but not in those who received cART with a PI (nelfinavir or ritonavir). β2 microglobulin, neopterin and TNF-α plasma concentrations were higher in HIV infected women during the third trimester through to delivery but were not compared by ART exposure. Birth outcome data was not presented.

Kolte et al. presented data for both plasma cytokines and peripheral immune cell subtypes in their study of 20 HIV infected and 16 uninfected pregnant women. HIV infected pregnant women were all treated with cART, 12 conceived on cART, 18 were treated with a PI as the third agent and 10 received ZDV mono (Kolte *et al.*, 2011). HIV infected women had higher percentages of CD4+ CD38+HLA-DR+ T cells and CD8+CD38+HLA-DR+ T cells than uninfected pregnant controls (p=0.002 and 0.007 respectively). A reduction in percentage of CD4+ CD38+HLA-DR+ T cells was observed across pregnancy in HIV women receiving cART (predominantly PI-based), p=0.03. HIV infected women also had lower levels of Th2/immune-regulatory cytokines: IL-4, TGF-β and immune-regulatory IL-10 but no difference in Th1/pro-inflammatory: IFN-γ, IL-1β and Th17: IL-6, IL-10 and TGF-β increased during pregnancy. No data on prematurity were available.

Hygino and colleagues performed T cell culture and measured production of Th1/pro-inflammatory cytokines: IFN-γ, IL-1β, and TNF-α and Th2/immune-regulatory cytokines: IL-4, IL-10 and TGF-β from PBMCs obtained in the third trimester from 25 HIV infected pregnant women and 25 infected non pregnant women and 10 uninfected pregnant women. All HIV infected women initiated ART post conception, 15 women received PI-based cART and 4 ZDV mono. Hygino et al. found that the most concentrated cytokine in supernatant from stimulated T cells was immune-regulatory IL-10 in pregnant women, with HIV infected pregnant women having lower levels compared to uninfected pregnant women. They also demonstrated a trend towards higher pro-inflammatory Th1 cytokines (IFN-γ and TNF-α) release from PBMCs obtained from HIV pregnant women compared to uninfected pregnant women. No cytokine production was detectable from un-stimulated cells. No data was presented on PTB but infants born to HIV infected women had a tendency to a lower birth weight compared to uninfected pregnant women ($p = 0.056$).

Bento and et al. studied 16 uninfected pregnant women and 58 HIV pregnant women divided into those with 'controlled' HIV (n=32) or 'non controlled' HIV (n=26) according to whether women had plasma HIV copies under the limit of detection (<80 copies RNA/mL) at delivery (Bento *et al.*, 2009). Only 23% of 'non controlled' women received ART during pregnancy due to presentation at delivery compared to 82% of women with 'controlled' HIV. ART comprised either ZDV mono or the NNRTI nevirapine in combination with dual NRTI (containing ZDV). All women had a CD4 count >400 cells/mcL but 'non controlled' women had a lower mean count compared to 'controlled' (597 cells/mcL vs. 907 cells/mcL). All HIV infected pregnant women received ZDV during delivery. Immune activation (measured by T cell proliferation and pro-inflammatory cytokine production) was lower in HIV pregnant women in the 'controlled' group compared to the 'non-controlled' women and higher plasma concentrations of immune-regulatory cytokines (IL-4 and IL-10) were measured in 'controlled' and uninfected pregnant women. These data show a predominant immune-regulatory profile in pregnancy, especially in HIV infected women receiving ZDV. Associations with birth outcome could not be made within the

constraints of the study design but the 'non controlled' HIV group had the highest Th1 cytokines: IFN-γ, IL-1β and TNF-α and the lowest birth weight.

Together these studies suggest that there is a down regulation but not elimination of HIV associated immune activation with cART in pregnancy and a potential immune-regulatory effect of ZDV especially as ZDV mono has limited effect on HIV viral load.

1.5.3 Do T regulatory cells have a role in PTB?

The Th1/pro-inflammatory HIV PTB concept can be expanded to describe reductions in T regulatory cells and associated immune-regulatory/Th2 cytokines. Kolte et al. were able to demonstrate percentage T regulatory cells ($CD4+CD25+CD127^{low}FOXP3+$) were inversely associated with percentage of CD4+ CD38+HLA-DR+ T cells p=0.03 and that in uninfected pregnant women the $CD4+CD25+CD127^{low}FOXP3+$ T cell subset expanded in the second trimester. Hygino et al identified the T cell producing IL-10 in their study to be CD4+CD25+FoxP3- and observed that this subset of cells was significantly expanded in healthy pregnancies in HIV infected women.

Bento and colleagues were also able to demonstrate that the subset of T cells secreting IL-10 could regulate Th1 cytokine secretion in T cell culture (Bento *et al.*, 2009). They postulated that ZDV could be modulating in vivo production of IL-10 and that T cells producing IL-10 could improve fetal tolerance. Bento et al.'s suggestion that ZDV could modulate IL-10 production is support by data from Pornprasert and colleagues who examined placentas from 61 HIV infected women and found women who had a longer duration of ART exposure with ZDV during the pregnancy more frequently had detectable IL-10 in their placentas (Pornprasert *et al.*, 2009).

Richardson et al. explored different T regulatory cell subsets and plasma cytokines in 20 HIV infected and 18 uninfected pregnant women(Richardson and Weinberg, 2011). HIV infected pregnant women, all of whom received cART during pregnancy and four who conceived on cART, had lower percentage of various T regulatory cell subsets including: CD4+CD25+FoxP3+cells,

CD4+CTLA4+ and CD8+ CTLA4+, during late pregnancy compared to early pregnancy and uninfected women. Conversely an increase in other T cells subsets during late pregnancy was observed, namely CD4+IL-10+ and CD4+TGFβ+ cells, similar to the finding by Hygino and colleagues of an expansion of IL-10 producing T cells during pregnancy. Plasma concentrations of Th1 cytokines: IFN-γ, IL-1, IL-8 and TNF-α were higher in HIV infected women compared to uninfected women as were Th2: IL-4 and immune-regulatory IL-10 and all were positively associated with the percentage of T regulatory cells. No differences were observed between early and late pregnancy and again associations with birth outcome were not explored.

Interpretation of these data show that systemic cytokine balance is likely to be more complex than a simple Th1/Th2 dichotomy. We suggest a move away from the Th1/Th2 shift hypothesis and propose a general up-regulation of cytokine signaling in the context of altered regulatory T cell populations in HIV infected pregnant women. It remains unclear if ART induces a pro-inflammatory profile or if certain ART e.g. ZDV can induce a more immune- regulatory cytokine profile which is favorable for term delivery.

1.5.4 Pilot data: Dysregulation of cytokines hypothesis

Supernatant from un-stimulated PBMCs was collected from 51 HIV-1 infected and 12 uninfected pregnant women at five time points during pregnancy (12, 20, 28, 34 and 38 weeks) at Imperial College Healthcare NHS Trust between 2007-2011. We performed chemiluminescent multiplex immunoassays to characterise concentrations of pro-inflammatory cytokines: IL-1β, IL-2, IL-6, IL-8, IL-12, GM-CSF, IFN-γ and TNF-α and the immune-regulatory cytokine, IL-10. Antiretroviral therapy was categorised as cART (PI-based (n=22) or NNRTI-based (n=17)) or NRTI-only (ZDV mono (n=5) or triple-NRTI (n=7)). Mean cytokine concentrations, median slope values; mixed ANOVA and linear regression analysis were used to compare cytokines by HIV status, therapy and PTB.

HIV-1 infected and uninfected pregnant women were of similar age. Baseline antenatal CD4 count did not differ by therapy group: cART group= 390 cells/mcL

(range 170-1520) vs. NRTI-only group= 492 cells/mcL (range 260-810) p=0.18. Five HIV-1 infected women (all on cART) and one uninfected woman had a PTB, p=0.86.

Mean plasma concentration of Th1, Th17 and immune-regulatory IL-10 cytokines were significantly higher at week 20 in HIV-1 infected pregnant women compared to uninfected pregnant women, $p<0.04$. There were no consistent trends in Th1 or Th17 cytokines concentrations between cART versus NRTI-only ART exposed women. Reduced plasma IL-10 concentrations were observed in women initiating PI-based cART at weeks 20-34 (3-15 fold, minimum mean concentration 31 pg/mL, $p<0.02$), compared to those initiating NRTI-only regimes. There was significant main effect of treatment regimen on IL-10
$F(1,21) = 12$, $p<0.002$, partial eta squared=0.372).

There was a negative linear correlation for IL-10 over gestational age in NRTI-only treated women ($r=-0.26$ $p=0.04$), not observed for cART ($r=0.04$ $p=0.31$). CD4 T cell count and plasma HIV RNA were not correlated with IL-10 concentration. Lower plasma IL-10 concentrations were observed in women experiencing PTB (4-13 fold, minimum mean concentration 14 pg/mL, $p<0.03$) compared to term birth at weeks 12-28(Short *et al.*, 2012; Short and Taylor, 2014)

IL-10 inhibits the release of Th1 cytokines and thus acts as a break in the pathway that triggers labour. These data are the first to show differences in this immune –regulatory cytokine in plasma by treatment exposure and gestational age at delivery in women with HIV-1 infection, adding weight to the plausibility of an inflammatory mechanism. The research discussed above led to the following objectives:

1.5.5 Objectives 2

- To characterize peripheral T cell markers of immune activation in a group HIV-1 infected and uninfected pregnant women across the second and third trimester

- To compare cellular immune activation across gestation and how these differ by HIV status, cART (drug class, timing of exposure) and gestational age at delivery.

1.6 Local Female Genital Tract Inflammation

Plasma cytokines and T cell activation markers provide information on systemic inflammation, which is a diluted and indirect version of interplaying immune networks at the maternal fetal interface and in the gestational tissues. In order to study local immune mechanisms, an accessible acceptable representative biological fluid is the ideal. Female Genital Tract (FGT) fluid can provide information on both the cervicovaginal environment but also the upper reproductive tract. It can be obtained safely and simply without the need for pregnant women to undergo invasive procedures such as amniocenteses or chorionic villus sampling that could put the pregnancy at risk.

1.6.1 Female Genital Tract cytokines during pregnancy

There are limited data on FGT fluid cytokines during pregnancy pre labour however collectively most evidence points to dynamic changes in the FGT IL-1 system across pregnancy and labour.

Kutteh and colleagues sampled cervical mucous during each trimester of pregnancy in 36 healthy women and found that IL-1β increased significantly from the first to second trimester and remained elevated in the third trimester(Kutteh and Franklin, 2001).

Conversely Donders et al. explored cervicovaginal lavage (CVL) cytokines during pregnancy in 30 pregnant women comparing them with 62 non pregnant healthy controls and found similar IL-1β concentrations between groups, lower IL-1RA concentrations throughout pregnancy and a greater proportion of pregnant women had undetectable IL-6 and IL-8 (Donders *et al.*, 2003).

A recent large longitudinal study (n=242) sampling cervicovaginal fluid (CVF) during each trimester revealed a significant increase in IL-1α, IL-8, IL-10 and MMP-8 across gestation whereas TNF-α decreased(K. B. Ashford *et al.*, 2018). CVF IL-1β was higher in women who subsequently delivered preterm.

1.6.2 Female Genital Tract cytokines during labour

Term and preterm labour are believed to share common initiation pathways. Imseis and colleagues explored vaginal cytokines in 72 pregnant women comparing levels in labouring and non-laboring women and identified that IL-1β and IL-6 were elevated in labour (Imseis *et al.*, 1997).

Heng's group further characterised the IL-1 system in pregnancy and labour by obtaining serial CVF samples from 65 women during mid pregnancy (weeks 25-26) and late pregnancy (>36 weeks). They measured IL-1α, IL-1β and IL-1RA concentrations and identified that in the two weeks prior to labour, CVF IL-1α, IL-1β increased and IL-1RA decreased(Heng *et al.*, 2014).

1.6.3 Female Genital Tract cytokines and PTB

PTB has been consistently associated with elevated CVF IL-6, which lead to exploration of this Th17 associated cytokine as a biomarker for the prediction of both threatened and asymptomatic preterm labour (PTL), and prelabour premature rupture of membranes (PPROM). Lockwood et al. were some of the first to explore the predictive capacity of IL-6 concentrations in cervical and vaginal fluid in 161 asymptomatic pregnant women and whilst they found 3-4 fold elevated concentrations of this cytokine in women who had PTL compared to term, it had a poor sensitivity and intermediate specificity as a biomarker in this cohort(Lockwood *et al.*, 1994).

The National Institute of Child Health and Human Development Preterm Birth Prediction Study is one of the largest case control studies to examine the predictive capacity of IL-6 for spontaneous PTL to date(Goepfert *et al.*, 2001). Goepfurt and colleagues compared CVF IL-6 in 125 women who delivered by PTL (<35 weeks) compared to those who delivered at term (>37 weeks) and found that women who had PTL had higher CVF IL-6 concentrations which was highly significant at week 24. IL-6 has also been demonstrated to have a high predictive capacity for microbial invasion of the amniotic cavity with PPROM(Jun *et al.*, 2000; Cobo *et al.*, 2014; Kacerovsky *et al.*, 2018).

FGT IL-1 and IL-8 have also been correlated with PTL in several studies to date. Tanaka et al. were amongst the first to correlate CVL IL-1β and IL-8 with PTL in pregnant women and showed that CVL IL-1β and IL-8 were significantly higher in women in with PTL and term labour compared to women at matching time-points who were not in labour. They were also able to show significant correlations between cervical dilation and CVL IL-1β and IL-8 and also demonstrated that these two cytokines were highly correlated in CVL(Tanaka *et al.*, 1998).

Cervical fluid IL-1β, IL-6 and IL-8 cytokine concentrations were evaluated by Discacciati et al. in 45 pregnant women at term not in labour and 37 women in PTL(Discacciati *et al.*, 2011). Women in PTL had statistically higher concentrations of IL-6 and IL-8 in their cervical fluid and a trend towards higher IL-1β. FGT fluid IL-8 has also been identified as a marker of microbial invasion of the amniotic cavity with PPROM(Kacerovsky *et al.*, 2015; Jung *et al.*, 2017)

Genc et al. demonstrated that in women colonised with abundant vaginal anaerobic bacterial spp. e.g. *G. vaginalis, Prevotella spp.* and other gram negative rods, the ratio of IL-1β to its antagonist IL-1RA increases risk of PTL (Genc *et al.*, 2004). IL-1β has also been shown to correlate with fetal fibronectin (fFN), a biomarker for PTL used for screening in clinical practice(Amabebe *et al.*, 2018).

1.6.4 Female Genital Tract cytokine PTB biomarkers performance compared to the current clinical biomarkers

To date, individual cervical cytokines have shown inferior performance in predicting PTB compared to cervical ultrasound and fFN. A 2009 National Institute for Health Research (NIHR) Health Technology Assessment systematic review of screening methods to predict PTB concluded that cervical length measurement on ultrasound and CVF fFN had the greatest like hood ratios for predicting threatened and asymptomatic PTL compared to other biomarkers including CVF and amniotic IL-6 and IL-8(Honest *et al.*, 2009).

Holst et al. evaluated 27 different immune proteins in CVF from 89 pregnant women with threatened PTL and found that individual cytokines were inferior in predicting delivery within 7 days compared to the predictive capacity of a model combining CVF IL-6, IFN-γ, MCP-1 and cervical length (AUC 0.91)(Holst *et al.*, 2009). This work and that of others suggest that multiple biomarkers used in combination can improve predictive performance(Thorsen *et al.*, 2001; Holst *et al.*, 2009).

A more recent study of 136 women with threatened PTL <34 weeks sampled CVF for IL-1β, IL-6 and IL-8 measurement prior to cervical length ultrasound and found that IL-1β and IL-8 were associated with delivery within 7 days(Jung *et al.*, 2016). The addition of IL-8 determination to cervical length measurement improved specificity of screening to identify women who did not deliver within 7 days but did not improve sensitivity.

1.6.5 Female Genital Tract fluid cytokines in HIV infected women

CVF cytokines have been measured in women at risk of HIV infection and HIV infected non-pregnant women. The majority of the studies to date have been performed on CVL with normal saline, obtained by various methods.

1.6.5.1 Pre cART

One of the first studies in the pre cART era was undertaken by Belec and colleagues (Bélec *et al.*, 1995). Belec measured CVL and serum IL-1β, IL-6 and TNF-α concentrations in 50 HIV infected and 45 uninfected women and identified that HIV infected women had higher CVL cytokine concentrations than uninfected controls and the highest concentrations of CVL cytokines were in women with advanced HIV disease (stage IV/AIDS). In addition, the authors demonstrated that cytokine expression (mRNA) occurred locally in CVL and thus the presence of cytokines could not be explained by transudation alone.

Another study from the pre cART era was undertaken in the WITS cohort by Sha et al.(Sha *et al.*, 1997). CVL IFN-γ, but not IL-1β, IL-2, IL-6, IL-10 and TNF-α, were significantly higher in the 16 HIV infected women than the five uninfected high

risk women. There was also a trend towards an inverse correlation between CD4+ cells count and IFN-γ concentration.

1.6.5.2 Post cART

Crowley-Nowick et al. were amongst first studies to present FGT cytokine data from HIV infected women in the era of cART (Crowley‐Nowick *et al.*, 2002). Cervical fluid was collected with Polyvinyl acetyl (PVA) sponges from 122 adolescents, 82 of whom were HIV infected and 43% of which were receiving cART. HIV infected women had higher FGT fluid concentrations of IL-12 and IL-10. Cytokine concentrations did not differ by race.

Zara et al. explored how local CVL cytokines associated with HIV shedding in 60 HIV infected women, of whom 83% were on cART (Zara *et al.*, 2004). IL-1β was significantly associated with both CVL HIV RNA and proviral DNA. In addition cART was associated with higher CVL IL-6 and lower TNF-α. Increasing duration of cART was also associated with higher IL-6 (rho=0.22, p=0.03).

In the Centre for the AIDS Programme of Research in South Africa (CAPRISA) 002 -Acute Infection Study, CVL samples were collected from 44 women with HIV primary infection and 29 uninfected women(Bebell *et al.*, 2008). Primary HIV infection was associated with higher concentrations of CVL IL-6, IL-10 and IL-12 and CVL IL-1β, IL-6 and IL-8 inversely correlated with systemic CD4 count. In a longitudinal analysis of this cohort which included 49 HIV infected women in acute infection, 22 of whom had samples pre seroconversion, Roberts et al. identified that elevated cytokine concentrations did not differ relative to pre infection concentrations, some women maintained higher CVL concentrations and others did not(Roberts *et al.*, 2012). CVL cytokine concentrations were however elevated in women who had detectable genital HIV RNA and lower blood CD4 cells counts.

To date, no study has evaluated the use of menstrual soft cups for use in collecting female genital tract fluid (CVF) in HIV infected pregnant women, nor has any study used this method for exploring CVF cytokines in pregnancy. This

sampling method should provide a more functional picture of the local CVF cytokine milieu as it is undiluted and thus contains more elements of the local immune system (cells, mucin, bacteria etc.). For further background on CVF collection methods, see chapter 2.1. The research discussed above led to the following objectives:

1.6.6 Objective 3:

- To optimise collection methods of CVF in pregnant women for cytokine concentration exploration, to enable analysis of cytokines in an accessible biological fluid that more closely represents the fetal-placental unit, with less dilution of important biological trends
- To characterise local CVF pro-inflammatory and immune-regulatory cytokine profiles in HIV-1 infected and uninfected pregnant women across the second and third trimester
- To compare local markers of inflammation across gestation and how these differ by HIV status, cART (drug class, timing of exposure) and gestational age at delivery.

1.7 Vaginal microbiota of pregnancy and PTB

In order to consider local inflammation in the female genital tract and ascending infections as a cause of PTB, consideration must be given to the local bacteria colonizing this region. Bacterial vaginosis/vaginal dysbiosis is prevalent in women from Sub-Sahara Africa and common women with HIV infection(van de Wijgert and Jespers, 2017). Cervicovaginal bacteria, particularly highly diverse communities including *Gardnerella* and *Prevotella spp.* are strongly correlated with pro-inflammatory cytokine concentrations(Anahtar *et al.*, 2015). Bacterial vaginosis is more pro-inflammatory in pregnant women compared to non-pregnant women(Beigi *et al.*, 2007) and it has long been known that bacterial vaginosis confers an increased risk of PTB(Hay, Lamont, *et al.*, 1994).

Culture free genomic sequencing technologies have enabled great advancements in characterization of bacteria in the FGT(Van De Wijgert *et al.*, 2014).16S rRNA

sequencing techniques have revealed that term pregnancies are associated with increasing numbers *of Lactobacillus (L. crispatis, L. jensensii)* and reducing bacterial diversity(Romero *et al.*, 2014). PTB is associated with *L. iners*, and dysbiosis: the presence of diverse obligate and facultive anaerobes e.*g. Gardnerella vaginalis (G. vaginalis), Atopobium vaginae, Prevotella spp., Sneathia spp., Megasphaera spp., etc.* (DiGiulio *et al.*, 2015; L. Kindinger *et al.*, 2016; L. M. Kindinger *et al.*, 2016). *L. iners* dominated microbiota are less stable during pregnancy compared to *L. crispatus* and more likely to transition to abnormal non *lactobacillus* dominated microbiota(Verstraelen *et al.*, 2009).

1.7.1 Vaginal microbiota of women with or at risk of HIV

Vaginal polymicrobial dysbiosis is also prevalent in women with, or at risk of, HIV infection (Spear *et al.*, 2008; Hummelen *et al.*, 2010; Pépin *et al.*, 2011; Borgdorff *et al.*, 2014; Chervenak *et al.*, 2018; Bayigga *et al.*, 2019). The associated high vaginal pH can inhibit immunological antiviral proteins (Hearps *et al.*, 2017). It is a risk factor for sexual transmission of HIV and other infections e.g. Human Papilloma Virus, Herpes Simplex Virus(Borgdorff *et al.*, 2014; Reimers *et al.*, 2016; Gosmann *et al.*, 2017; Bayigga *et al.*, 2019).

In women already infected with HIV, this dysbiotic state can enhance HIV shedding in CVF (Cu-Uvin *et al.*, 2001; Borgdorff *et al.*, 2014). Cross-sectional studies of mixed methodologies have revealed that women with HIV infection have greater vaginal microbial diversity and reduced abundance of operational taxonomic units (OTUs) assigned to the lactobacillus genera compared to those with uninfected women(Spear *et al.*, 2008; Pépin *et al.*, 2011; Schellenberg *et al.*, 2011) . On a species level, some studies have demonstrated high level of colonization with *G. vaginalis* and *L. iners* in HIV infected women (Hummelen *et al.*, 2010; Dols *et al.*, 2012; Borgdorff *et al.*, 2014; Mehta *et al.*, 2015). Other studies have shown other mixed anaerobes such as *Mycoplasma hominis, Prevotella bivia, Dialister spp., Atopobium spp.* to be highly abundant in HIV infected women (Sha *et al.*, 2004; Hummelen *et al.*, 2010; Pépin *et al.*, 2011; Benning *et al.*, 2014). Conversely, a recent longitudinal analysis of the US

Women's Interagency HIV Study (WIHS) demonstrated no association of community state type by HIV status (Mehta *et al.*, 2015).

African studies of women at high risk of HIV infection have demonstrated a high prevalence of *L. iners* compared to other *lactobacillus spp.*(Jespers *et al.*, 2015, 2017). The contribution of ethnicity, distinct from HIV serostatus, has been addressed by one small sub-study that compared women from North America and Rwanda and demonstrated a similar prevalence of most major bacterial genera with the exception of *Megasphaera* which was more prevalent in HIV infected women from North America and *Mycoplasma* which was more common in HIV infected Rwandan women(Benning *et al.*, 2014).

1.7.3 Vaginal microbiota in HIV, the role of ART

Recent sub-analysis of the large CAPRISA 004 pre –exposure prophylaxis RCT has revealed that high bacterial diversity (including the presence of G. vaginalis and *Prevotella spp*.) is associated with reduced efficacy of PREP and an increased incidence of HIV infections (Klatt *et al.*, 2017). Klatt et al. suggest that *Gardnerella spp.*, *Prevotella spp., Mobiluncus spp.,* and *E.coli* may metabolise TDF based on in vitro experiments showing reduced drug concentrations in supernatant of co-cultures of HIV target cells and organism however they also reported data showing concomitant increase in intracellular TDF in *Gardnerella* organisms (Klatt *et al.*, 2017).

Donahue Carlson et al. did not replicate an inverse relationship between anaerobic species and CVF TDF concentrations in a study of HIV infected women taking oral ATV/r with FTC/TDF(Carlson *et al.*, 2017). They found TDF and ATV FGT fluid concentrations were highest in the intermediate diversity state and lowest in both the low diversity (lactobacillus dominant) and high diversity (mixed anaerobes) states. In this study, both the high and low diversity states had the lowest prevalence of Gardnerella vaginalis, possibly the result of clinical BV being an exclusion criteria.

1.7.4 Vaginal microbiota of HIV infected pregnant women

Some of these studies included pregnant women in small numbers (Borgdorff et al., 2014; Taha et al., 1999) but to date only one West African retrospective case control study from the pre cART era has explored the vaginal microbiota of a cohort of HIV infected pregnant women with bacteria 16S rRNA pyro sequencing (Frank *et al.*, 2012). Frank et al. characterised the vaginal microbiota of 68 HIV infected women at 36-38 weeks gestation, median CD4 count 520 cells/mcL (IQR 383–704), 25 of who received ZDV mono(Frank *et al.*, 2012). Authors identified four vaginal community clusters using 16S rRNA pyro sequencing: 30 women had *Lactobacillus spp*. predominant vaginal microbiotas (VMB), of which the majority were *L. iners* (77%), 24 women had *Gardnerella Vaginalis spp.* predominant VMB of which 19% also had *L. iners* carriage, 8 women had *Staphylococcal spp.* predominant VMB and 2 women had low *Lactobacillus* carriage with high abundance of other anaerobic spp. such as *Prevotella spp*., *Sneathia spp.* and *Peptostreptococcus.* HIV infected women who transmitted HIV to their infant antepartum had a higher relative abundance of *G. vaginalis* OR 1.7 (95% CI 1.2 to 2.4), p = 0.004. This study indicates that pregnant women may have similar prevalence of vaginal diverse anaerobic spp. and *L. iners* to HIV infected non pregnant women and thus may help explain the high prevalence of inflammatory FGT cytokine profiles in these women which may in turn increase risk of PTB. The research discussed above led to the following objectives:

1.7.5 Objective 4:

- To characterise vaginal microbiota in a group HIV-1 infected and uninfected pregnant women longitudinally with targeted 16S rRNA gene illumima sequencing in the post cART era
- To explore differences by HIV status, cART type and timing as well as correlations with local inflammation and gestational age at delivery.

1.8 Cervicovaginal fluid proteome

The proteome is a collection of proteins expressed by an organism, cell or in a biological or anatomical compartment. CVF forms a protective barrier in the lower female genital tract where it lubricates and hydrates the underlying mucosa. It contains cervical/endometrial mucus, vaginal epithelial transudate,

epithelial cells and leucocytes, commensal bacteria and has its own 'proteome' comprising of many immune proteins of different innate and adaptive immune functions. These immune proteins include: mucins, antibodies (IgG and IgA), cytokines, chemokines, growth factors, antibacterial proteins and anti-viral proteins. (Zegels *et al.*, 2010; Chappell *et al.*, 2014)

Knowledge of the FGT fluid proteome and transcriptome has advanced rapidly over the past twenty years with the application of proteomic antibody based high throughput multiplex technologies, antibody independent separation methods in tandem with mass spectroscopy, RNA seq and more recently single cell transcriptomics (Romero *et al.*, 2006; Vento-Tormo *et al.*, 2018). These techniques have been applied to pregnancy to identify biomarkers for PTB and in efforts to identify proteins important in the mechanisms behind HIV transmission and protection(Zegels *et al.*, 2010). Characterization of what constitutes normal expression must be undertaken first (Romero *et al.*, 2006; Zegels *et al.*, 2010).

1.8.1 Cervicovaginal fluid proteome in pregnancy

Dasari and colleagues were the first to undertake proteomic characterisation of the whole CVF in pregnancy collected with posterior vaginal fornix Dacron swabs(Dasari *et al.*, 2007). They used a combined fractionation approach of multi-dimensional liquid chromatography (2D-LC) and one dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D SDS-PAGE) with mass spectrometry. Peaks were cross-referenced with protein databases. The authors generated forty fractions from seven pregnant women during the second trimester, two pooled samples from three individuals for the 2D-LC and one individual with 1D SPS-PAGE. Using this strategy 77 proteins unique to CVF compared to serum and amniotic fluid were identified, 32% with putative roles in metabolism and 22% with immune response and defense related functions. The immune proteins were subdivided into three main types: pro-inflammatory, anti- inflammatory, and antimicrobial proteins (AMPs).

Pro-inflammatory proteins identified included immunoglobulins, complement, Vitamin D binding protein (a macrophage activating factor precursor), myeloperoxidase (MPO), MMP-9 and calgranulins. Anti-inflammatory proteins included IL-1 receptor antagonist (IL-1RA) and leucocyte elastase inhibitor. Antimicrobial proteins included Lipocalins, α-defensins and β-defensins, kallikreins and lactotransferrin. The remaining proteins identified had functions attributed to cell differentiation (11%), transport (9%), cell organization (8%), enzyme regulation (6%), signal transduction (3%), and cell proliferation (3%).

1.8.2 CVF Proteomic studies in women experiencing preterm birth

The same group also compared protein spectra between five women who experienced sPTB without signs of infection, five women who had PTL without birth and five controls(Pereira *et al.*, 2007). They used the same peptide fractionation of 2D-LC but the complementary method was two-dimensional SPS –PAGE followed by mass spectrometry. This time the authors undertook semi quantitative analysis of both differential abundance of SPS-PAGE protein spots and counts of spectral proteins peaks identified by mass spectrometry between cases and controls. Periera et al. identified 205 CVF proteins, 25% of which were assigned as metabolic in function, 23% were immune response proteins and 18% were characterised as transport proteins. Comparing sPTB with controls, 30 differentially expressed proteins were identified including: S100A7, FFN, Vitamin D binding protein, insulin-like growth factor binding protein (IGF-BP), calgranulin C, α1 anti-trypsin and transferrin.

Since these studies, advances in protein separation techniques and sensitivity and resolution of mass spectrometry have led to an exponential growth in identification of CVF proteins(Zegels *et al.*, 2010). However with this increase in absolute numbers of proteins, so has the proportion of non-functional variable proteins. This makes it more challenging to identify meaningful differences between disease and non-disease states to identify important mechanistic differences or potential biomarkers. It is highly likely that immune proteins work in concert and no one single protein is characteristic of preterm birth.

1.8.3 Current CVF protein biomarkers available for screening for preterm birth

CVF proteins whose identification and validation as a biomarker for PTB has reached current clinical use are fFN, CRP and Insulin Related Growth Factor Binding Protein (IRGF BP). FFN has a sensitivity of between 22-95% and specificity of 65-98% in asymptomatic women and sensitivity of 20-90% and specificity of 40-84% in women with symptoms of labour (Honest *et al.*, 2009; Heng *et al.*, 2015; Lucaroni *et al.*, 2018). CRP performance is inferior with a sensitivity of between 30-85% and specificity of 70-75%(Honest *et al.*, 2009). IRGF BP is not superior with a sensitivity between 10-85% and specificity between 45-100%(Conde-Agudelo and Romero, 2016; Lucaroni *et al.*, 2018)(Honest *et al.*, 2009). Clearly there is much scope for improvement in biomarkers, especially in their sensitivity. Two or more biomarkers used in combination can improve screening performance(Holst *et al.*, 2009; Jung *et al.*, 2016).

1.8.4 Cervicovaginal fluid proteome in women with HIV infection

Ghosh et. al. measured known antimicrobial proteins in CVL from thirty-two HIV infected (CD4 count >350 cells/mcL, ART naïve) and fifteen uninfected non pregnant women and explored their anti HIV activity on target cell infection(Ghosh *et al.*, 2010). Antimicrobial β-defensin-2, trappin-2/elafin, SLPI and MIP-3α were identified in CVL from both HIV infected and uninfected women with β-defensin-2 being three-fold higher in infected women. Both β-defensin-2 and MIP-3α displaying anti HIV activity when correlated with % inhibition of target cell infection.

HIV prevention trials have provided further insight into CVF antimicrobial proteins associated with HIV transmission or protection. In an HTPN035 sub study, vaginal swabs were analysed for immune proteins from 8 African HIV seroconverters and 24 women who remained uninfected(Dezzutti *et al.*, 2012). . Immune proteins identified by multiplex proteome array with beads or ELISA included: IL-1β, IL-2, IL-6, IL-7, MIP-1α, MIP-1β, lactoferrin, SLPI, β-

defensin 1–3 and α-defensin 1–3. Women who seroconverted were more likely to have detectable vaginal concentrations of β-defensin-2 prior to infection.

As part of CAPRISA002 Roberts et al. explored FGT fluid inflammatory proteins in 49 South African women with acute HIV infection and their associations with CVL and plasma HIV RNA concentrations with longitudinal follow up(Roberts *et al.*, 2012). At both 6 weeks and 1 year post infection CVL HIV RNA concentrations positively correlated with the following immune proteins: IL-1α, IL-1β, IL-6, IL-8, MCP-1, MIP-1β, G-CSF, RANTES, Eotaxin and CX3CL1. This work suggests either that FGT inflammation up regulates HIV shedding or conversely HIV has a direct up-regulatory effect on immune proteins in the FGT.

In another CAPRISA002 sub-study, Archary et al. compared the measurement of 48 different FGT fluid immune proteins obtained with the instead soft cup (whole CVF) and by CVL in 40 HIV infected women (ART naïve, median CD4 558 cells/mcL (IQR 448–734))(Archary *et al.*, 2015). All innate and adaptive immune proteins were detectable in CVF, which included: IgG, cytokines (IL-1α, IL-1β, IL-1RA, IL-8, IL-12p70, IL-18, LIF, MIF), chemokines (CXCL-1, CXCL-7, CXCL-9, IP-10, CXCL-12, MCP-1, MCP-3, RANTES) and growth factors (G-CSF, GM-CSF). Fewer of these immune proteins were detectable in CVL and thus the authors concluded that whole CVF was superior for analyzing FGT fluid soluble immune mediators.

In yet another CARPISA002 sub-study, Madan et al. characterised *E.coli* inhibitory activity, α -defensins, β-defensins and SLPI in CVL from 17 HIV seroconverters and 39 women who remained uninfected (Pellett Madan *et al.*, 2015). Madan demonstrated that all of these proteins were identified in lavage samples from both infected and uninfected women but greater CVL β-defensin-1, SLPI and *E.coli* inhibitory activity was associated with HIV seroconversion. In primary HIV infection α-defensins 1–3 increased significantly and were correlated with plasma HIV viral load set point highlighting the association between genital and systemic immune compartments.

1.8.5 Inflammatory CVF proteome and associated immune cells in women at high risk of HIV infection

Arnold et al. characterised the FGT fluid proteome associated with elevated cytokine expression in CVL samples from Kenyan female sex workers women (n=96)(Arnold *et al.*, 2016). For proteomic exploration CVL were separated by reverse phase liquid chromatography and analysed by mass spectrometry. Fifty-three proteins were identified that associated with elevated FGT cytokines (defined as at least three cytokines in the upper quartile from TNF-α, IL-1α, IL-1β, IL-8, MIP-α, MIP-β, or RANTES, n=28). The signature factors that differentiated mucosal inflammation included neutrophil proteases (MMP-8, MMP-9, lysozyme C), neutrophil chemotaxis and adhesion (s100a9) and proteins important for leukocyte migration whereas anti-proteases such as SLPI and serine protease inhibitors were decreased.

In a microbicide feasibility trial sub-study Francis et al. characterised forty-five FGT soluble innate and adaptive immune proteins in CVL from one hundred women at high risk of HIV from Tanzania(Francis *et al.*, 2016). Their aim was to explore modulation of these proteins by the menstrual cycle, contraception, and intravaginal practices. Multiplex bead immunoassays were used to quantify concentrations of analytes which, in addition to immunoglobulin, cytokines, chemokines and growth factors, included antimicrobial proteins: SLPI, Elafin, s100a8, α-defensins 1-3 and β-defensins 1-3, all of which were detectable. Interestingly vaginal douching with soap did not have a significant effect on immune activation biomarkers but using a cloth to cleanse the vagina, a prevalent practice in this region, and using hormonal contraception did. This group were also able to correlate these immune markers with cervical neutrophil abundance and presence of lymphocytes. Notably neutrophils were positively associated with: IL-1α, IL-1β, IL-6, IL-8, α-defensins, β-defensin2, MIP-1β, IP-10, RANTES, IgA and IgG and lymphocytes with: IL-1β, IL-6, IL-8, MIP-1β, MCP-1, IgA and IgG. This illustrates the complex networks of immune cells and signaling proteins likely to be at play in the face of enhanced inflammation and antigenic stimuli.

1.8.6 CVF proteome in pregnancy and HIV infection

To our knowledge no data exist for the whole CVF proteome in women with HIV infection who are pregnant. Cu-Uvin's group recently explored the effect of pregnancy on antimicrobial proteins (AMPs) with known anti HIV action(Hughes *et al.*, 2016). CVF from forty pregnant and thirty-seven non-pregnant uninfected women were obtained by lavage on three occasions during the menstrual cycle or during each trimester for pregnancy. AMPs that were measured and detected in both pregnant and non-pregnant women included: Elafin, β-defensin-2 & 3, RANTES, Lactoferrin, MIP-3α, SLPI and Cathelicidin. Pregnant women expressed less β-defensin-2, Lactoferrin and MIP-3α during the second and third trimester. Elafin correlated with protection against HIV infectivity of target cells in pregnant women whereas there was a trend towards enhanced HIV infectivity with β-defensin-2 & 3, Lactoferrin and Cathelicidin during pregnancy. These data demonstrate that the FGT proteome contains innate immune proteins that are active against HIV and are modulated by pregnancy.

In a recent study Morrison et al. measured concentrations of ten known cervical immune biomarkers in CF collected with an endocervical swab from a large number of HIV infected and uninfected non pregnant and uninfected pregnant women (n=943)(Morrison *et al.*, 2018). This study was nested in the human contraception and HIV acquisition study (HC-HIV) that recruited women in Uganda and Zimbabwe to identify cervical proteins associated with HIV seroconversion. Proteins that were differentially expressed between HIV exposed women who seroconverted and those who remained uninfected were: RANTES and β-defensin-2 (increased) and SLPI (decreased). Pregnant women (n=63) had higher IL-8 and SLPI in cervical fluid compared to non-pregnant women but were not analysed by HIV status.

The Vaginal Biomarkers Study explored the effect of HIV and pregnancy individually on FGT immunological markers and molecular vaginal microbiota in Sub-Saharan African women and associations with HIV infection risk(Kyongo *et al.*, 2015). Women were recruited from Kenya, South African and Rwanda (n=430), CVL samples were obtained and twelve soluble immune proteins were

measured with multiplex ELISAs which included: IL-1RA, IL-1α, IL-1β, IL-6, IL-8, IL-12p70, MIP-1β, IP-10, G-CSF, GM-CSF, Elafin and SLPI. HIV infected women (n=30, cART >6 months, CD4> 350 cells/mcL) and pregnant women (n=60) had exclusive FGT inflammatory profiles. HIV infected women had the most distinct pro-inflammatory profiles with elevated concentrations of IL-1β, IL-6, MIP-1β and IL-8 compared to the uninfected reference group, irrespective of whether they had bacterial vaginosis (Nugent score 7-10). Pregnant women from South Africa had higher IL-1α, and pregnant women from Kenya had lower Elafin compared to the non-pregnant reference group, again this association was seen after controlling for Nugent score. Women with BV were shown to have higher concentrations of pro-inflammatory CVF cytokines as expected. Pregnant women within this analysis were uninfected and thus these data show that individually these patient groups display differential CVF proteome secretion profiles, which may have an additive effect when explored in HIV infected pregnant women.

1.8.7 Proteins of interest common to HIV infection and PTB

Some of these CVF immune mediators identified in women with or at risk of HIV infection have also been characterised in association with PTB such as inflammatory cytokines/chemokines: IL-1, IL-6, IL-8, MCP-1, MIP-3α, and RANTES(Laudanski *et al.*, 2014; Heng *et al.*, 2015; Amabebe *et al.*, 2018). Increased expression of AMPs: β-Defensins, Lactoferrin, Elafin, SLPI have also been associated with PTB(Goldenberg *et al.*, 2000; Itaoka *et al.*, 2015; Varrey *et al.*, 2018). The overlap of immune proteins in these two groups demonstrates the utility of an exploration of the CVF proteome in pregnant women with HIV infection to identify important proteins in our understanding of PTB pathogenesis, that could be validated as biomarkers or targets for treatment. The research discussed above led to the following objectives:

1.8.8 Objectives 5

- To characterise the CVF Proteome of HIV-1 infected women using a directed approach
- To identify differentially abundant proteins by comparing groups of women by ART exposure and prematurity
- To discover novel pathogenic proteins involved in mechanism of PTB syndrome and potential PTB biomarkers
- To quantify standard clinical screening tools for PTB, cervical length and fFN in this group that could be compared to immune proteins of interest and by ART exposure

1.9.1 Aim:

The aim of the following body of work is to characterize immunological mechanisms underlying the elevated PTB risk observed in HIV-1 infected pregnant women and define the role of antiretroviral therapy in this risk. The ultimate goals are to enable improved PTB risk models and PTB management in HIV-1 infected women and to achieve a greater understanding of the immunology behind this adverse birth outcome relevant to all women and infants.

1.9.2 Hypotheses

Systemic inflammation

1.9.2.1 T cell activation markers

1. Peripheral cellular markers of immune activation in pregnancy will differ by HIV status
2. Peripheral cellular markers of immune activation will associate with gestational age at sampling, gestational age at delivery and prematurity.
3. Peripheral cellular markers of immune activation will differ by ART drug class exposure and timing in relation to conception

1.9.2.2 Plasma Cytokines

1. Plasma cytokines in pregnancy will differ by HIV status
2. Plasma cytokines will change during the second and third trimesters, with an increase in pro-inflammatory cytokines towards the end of pregnancy in order to trigger labour.
3. Changes in pro-inflammatory cytokines will be accompanied by change in immune-regulatory cytokines during the second and third trimester in the same direction to regulate their action towards tolerance.
4. Plasma pro-inflammatory cytokines will correlate with gestational age at delivery and premature birth
5. Plasma immune-regulatory cytokines will decrease with the onset of labour.

6. Plasma cytokines will differ by ART drug class exposure and timing of initiation of cART in relation to conception

Local inflammation

1.9.2.3 CVF cytokines

1. CVF cytokines in pregnancy will differ by HIV status
2. CVF cytokines will change during the second and third trimesters with an increase in pro-inflammatory cytokines
3. Changes in pro-inflammatory cytokines will be accompanied by change in immune-regulatory cytokines during the second and third trimester in the same direction to regulate their action towards tolerance.
4. CVF pro-inflammatory cytokines positively correlate with gestational age at delivery and premature birth
5. CVF cytokines will differ by ART drug class exposure and timing in relation to conception

1.9.2.4 Vaginal Microbiota

1. The vaginal microbiota of pregnant women with HIV-1 infection will differ from uninfected pregnant women
2. The vaginal microbiota of women with HIV-1 infection who deliver preterm will be different from those who have term deliveries.
3. Community state type (CST), bacterial genera and species will associate with gestational age at delivery
4. HIV-1 pregnant women's CST and vaginal bacterial diversity will be stable through pregnancy
5. Exposure to ART at conception will result in a different vaginal microbiota compared to women who initiate ART during pregnancy.

1.9.2.5 Cervicovaginal Proteome

1. HIV-1 infected pregnant women will have a distinct FGT proteome compared to uninfected pregnant women
2. This FGT proteome will include inflammatory cytokines and chemokines.

3. The FGT proteome of HIV-1 infected women will differ by ART timing (pre and post conception)
4. The FGT proteome of HIV-1 infected women will differ by ART class (PI and NNRTI based)
5. The FGT proteome of HIV-1 infected women who delivery preterm will differ from those women who deliver at term.

1.9.2.6 Genital-plasma cytokine gradients and immune compartment correlations

1. CVF cytokines will correlate with plasma cytokines
2. A genital-plasma cytokine gradient will exist
3. Genital-plasma cytokine gradient will correlate with gestational age at sampling, gestational age at delivery and ART exposure
4. Plasma cytokine concentrations will correlate with peripheral blood mononuclear cell subtypes
5. CVF cytokine concentrations will correlate with peripheral blood mononuclear cell subtypes
6. Genital-plasma cytokine gradient will correlate with peripheral blood mononuclear cell subtypes
7. CVF cytokine concentrations will correlate with local vaginal polymorphonuclear cell (neutrophil) count
8. CVF cytokines will correlate with bacterial diversity and species.

Chapter 2 Methods and materials

2.1 Optimisation of female genital tract fluid collection for cytokine analysis in pregnant women

A pilot study comparing cervical fluid and cervicovaginal fluid collection methods was conducted prior to the main study to choose the most appropriate method. This study is therefore present first.

2.1.1 Background

The study of FGT fluid to further understand the molecular aetiology of obstetric complications and sexually transmitted infections is a growing field(Castle *et al.*, 2004; Nguyen *et al.*, 2005; Wei, Fraser and Luo, 2010; Dezzutti *et al.*, 2011; Archary *et al.*, 2015), FGT fluid comprises of cervical mucous/fluid (CF) and vaginal secretions or a combination of the two (cervicovaginal fluid (CVF)). FGT fluid is an accessible and safely collected biological fluid without the need for invasive procedures. In addition to enabling the study of the local vaginal immune compartment, it can also provide information about the cervix and upper reproductive tract. It has been postulated that cervical fluid is a more pure immunological representation of the higher genital tract whereas CVF represents a mixture of secretions of the cervix and vagina, possibly two immunologically distinct anatomical sites.

FGT fluid has been utilised in a multitude of assays including the characterization of cytokines and other immune proteins, biomarker discovery through high throughput 'omic' techniques, microbiome studies and drug levels with potential for many more (Zegels *et al.*, 2010). In obstetrics, cytokines concentrations of IL-1β, IL-6, IL-8 and TNF-α concentration in FGT fluid, collected by a variety of methods, have been evaluated as correlates of for PTB (Tanaka *et al.*, 1998; Goepfert *et al.*, 2001; Oncley, Horst and Atd, 2004; Goldenberg, Goepfert and Ramsey, 2005; Wei, Fraser and Luo, 2010; Discacciati *et al.*, 2011). The optimum FGT fluid for use in obstetric studies, specifically PTB research, has not been

established. The most suitable method will also depend on the proteins of interest, the volume of fluid required for the intended experimental techniques, balanced with the acceptability of the method to patients.

Several systems have been developed to collect FGT fluid including: CVL, polyester swabs, cervical wicks and ophthalmic sponges under direct visualization of the vagina and cervix using a speculum. Ophthalmic sponges collect a mean volume of 32 mcL, are highly absorbent, have a low binding affinity for protein and superior performance in recovery of cytokines from CF compared to polyester tipped swabs(Lieberman *et al.*, 2008). Less invasive methods for the collection of CVL with sterile saline solution and self-inserted menstrual blood collection cups. Aside from differing collection sites, these methods offer different recovery of FGT fluid volumes and immunological proteins(Marks *et al.*, 2012; Chappell *et al.*, 2014; Jaumdally *et al.*, 2018). Self-inserted menstrual Instead™soft cups are easy to use and offer a mean collection of 0.5mL of undiluted CVF (range 0.1–1.5 mL)(Boskey *et al.*, 2003). There has been increasing use of menstrual Instead™soft cups in the HIV prevention field with excellent performance in quantifying immune mediators including cytokines(Price *et al.*, 2011; Shukair *et al.*, 2013; Archary *et al.*, 2015; Cosgrove *et al.*, 2016; Francis *et al.*, 2016). In addition, CVF collected by Instead™ soft cups have been used with mass spectrometry technologies to identify vaginal mucosal proteins with anti HIV activity (Venkataraman *et al.*, 2005).

Obstetric studies have traditionally used lavage of the cervix and upper vagina for CVL collection or polyester swabs for CF collection however this produces large volumes of dilute CVL and small volumes of CF respectively (7, 13, 15, 17, 18). There is limited data on the use of ophthalmic sponges and menstrual cups (MC) in obstetric studies. In addition, there has been no consensus method for normalisation of cytokine concentration with some studies presenting data adjusted for sample dilution based on weight and some controlling for total protein(Quesnel *et al.*, 1997; Rohan *et al.*, 2000; Snowhite *et al.*, 2002; Castle *et al.*, 2004; Lieberman *et al.*, 2008; Dezzutti *et al.*, 2011; Marks *et al.*, 2012). Here both normalisation methods are presented and evaluated for their effectiveness

with each technique. This was the first study to compare these methods directly and to evaluate their use in pregnant women(Short *et al.*, 2018).

Objective:

- To optimize collection methods for CVF in pregnant women for cytokine concentration exploration, to enable analysis of cytokines in an accessible biological fluid that more closely represents the fetal-placental unit, with less dilution of important biological trends

In addition to the main hypotheses three method specific hypotheses were generated:

2.1.2 Hypotheses

- Menstrual soft cups will collect a greater volume of FGT fluid compared to PVA sponges in pregnant women

- The cytokine concentration in FGT fluid collected by MC will be greater than that extracted from in PVA sponges

- Menstrual soft cups will provide an acceptable method to collect FGT fluid in pregnant women

2.1.3 Pilot study design

2.1.3.1 Study subjects

18 healthy uninfected pregnant women were included in this sub-study of parent study (13/LO/0107: The immunological basis of preterm delivery). Details of recruitment and exclusion criteria are provided in Chapter 2.2.1.

2.1.3.2 Sample collection

Women underwent FGT fluid sampling using two methods: 1. a CF sample with a PVA swab under speculum guidance and 2. a CVF sample with a Instead™ soft cup. See below in figure 2.

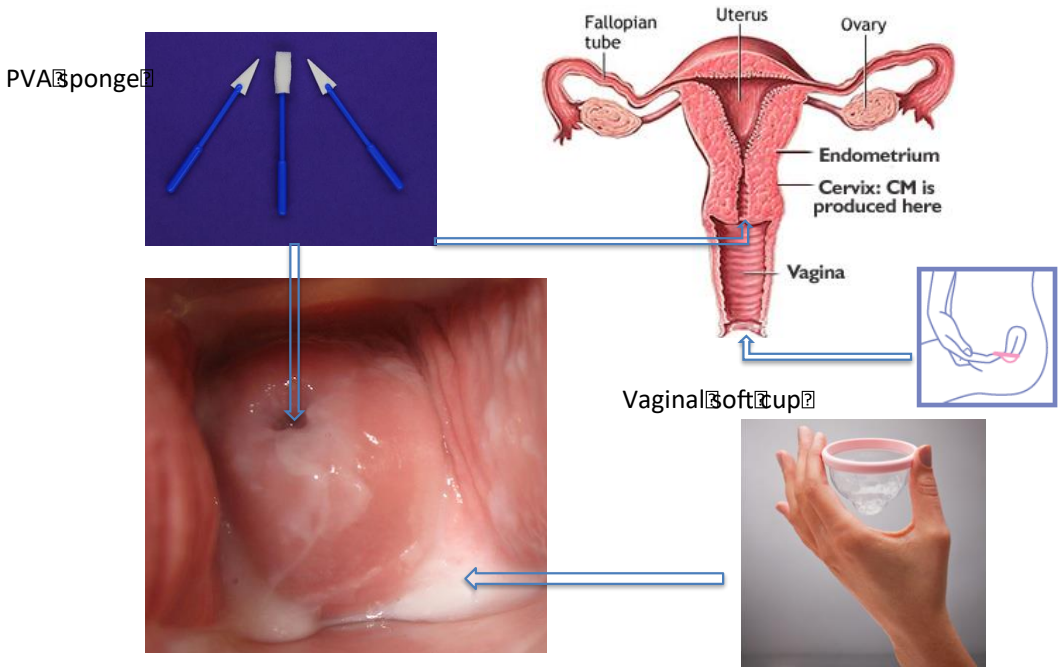


Figure 2 Diagram of CVF collection techniques

2.1.3.3 CVF Collection protocol

A visual representation of the procedures for CVF collection are demonstrated in figure 2. Firstly participants underwent a sterile speculum examination in order to visualize the cervical os, posterior vaginal fornix and lateral vaginal wall. A cervical fluid sample was obtained from the cervical os using a pre weighed Eyetec™ PVA sponge (Network Medical Ltd., Yorkshire, UK), held in place for 1 minute held in a sterile surgical needle holder. The PVA sponge was then

replaced in a pre-weighed and labeled 15mL sterile conical plastic tube. Simultaneously a polyester tipped swab was placed in the posterior fornix for 10 seconds and then replaced in the fFN specimen collection kit's buffer solution (Hologic, Massachusetts, USA), to immerse the swab tip. The collection kit was refrigerated at 2° to 8°C until analysis. Two high lateral vaginal wall swabs were performed using a plastic inoculation loop to test vaginal pH (Litmus test paper strip) and prepare dry vaginal slides for gram staining and a BBL™ Culture Swab™ MaxV Liquid Amies swab (BD, Oxford, UK) for bacterial 16S rRNA sequencing.

Participants were then given a pre-weighed menstrual Instead™ soft cup (Evofem Ltd., CA, USA) and sterile 50mL plastic sterile conical tube to self-collect the CVF sample. They received instructions from the research team on how to insert and remove the cup and were also provided with the option of clinician insertion. The cup was left in situ for a minimum of 5 minutes, removed and placed into the sterile 50mL plastic conical tube. Post collection weights were obtained for both CF swab and CVF cup containing conical tubes which were then placed on ice and stored at -80°C within 4 hours until ready for extraction These procedures enabled an the initial sub-study for comparison of PVA sponges and menstrual Instead™ soft cup for the collection of CVF for cytokine measurement in pregnant women to take forward for the remaining planned investigations.

FGT fluid sampling occurred at three sequential mid trimester time points: 12.0-21.9, 22.0-26.9 and 27.0-31.9 weeks gestation. Matched plasma samples were obtained at each time point. For details of EDTA blood and FGT fluid processing see section 2.2.5.1 and section 2.2.5.2.

2.1.3.4 Protocol for cytokine multiplex chemi-luminscent assays

Multi-spot chemi-luminescent Pro-inflammatory Panel 1 (human) cytokine assays (V-plex, Meso Scale Discovery™ (MSD), Maryland, USA) were used to measure 10 cytokines in plasma, CF and CVF (IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8,

IL-10, IL-12p70, IL-13 and TNF-α) according to manufacturer's instructions, detailed below.

Sample dilution

Eluted CF samples and neat plasma samples were used for the cytokine chemi-luminscent assay. CVF was diluted 1 in 2 with extraction buffer containing protease inhibitors by adding 100mcL of CVF to 100mcL of extraction buffer in a 1.5mL sterile plastic microtube. Extraction buffer constituents are detailed in section 2.2.5.2. All samples were run in duplicate. Matched plasma, CF and CVF samples were run on the same plate to limit any inter-plate variability

Standard curve generation

Samples for a eight-point calibrator curve in duplicate were prepared from reconstituted multi-analyte lyophilized calibrator. Samples were diluted down 4 fold, by transferring 100mcL of the concentrated sample into 300mcL of diluent 2, and mixed by vortexing. This was repeated five times starting at the neat solution until 7 different calibrator dilutions were made. The eighth point of the curve was made a zero by using diluent 2. Each concentration of analyte was LOT specific and the code was entered into the MSD DISCOVERY WORKBENCH™ software (Maryland, USA). The sensitivity of these assays range from 0.02-938 pg/mL.

Assay Procedure

50 mcL of standard or diluted sample (CF, CVF or plasma) were added to each well of the 96 well V-plex plate, which was then sealed and placed on a plate shaker (300-1000 rpm) at room temperature for 2 hours. The plate was then washed by adding and removing 150mcL PBS 0.05%Tween (PBS-T) 3 times following which 25mcL of MSD SULFO-TAG™ detection antibody solution (Maryland, USA) was added to each well. The plate was sealed then placed on the plate shaker for 2 hours at room temperature. The plate was then repeatedly

washed and 150mcL of read buffer was added prior to being read on the MSD-SECTOR Imager (Maryland, USA).

Data from the plates were analysed using MSD DISCOVERY WORKBENCH™ software version 4 and cytokine concentrations (pg/mL) were calculated using plate LOT specific standard curves for individual cytokines. Where sample cytokine concentrations did not fall within the standard curve they were re run with a suitable dilution of 1 in 4 or 1 in 10.

2.1.3.5 Protein measurement

Total protein concentrations in CF and CVF were estimated using the Bicinchoninic acid (BCA) method (Thermo Scientific). Samples were diluted at 1 in 50 or 1 in 100 in extraction buffer, depending if the final concentration fell in the standard curve, working range of which was 20-2000mcg/mL.

The standard curve was made by diluting 1mL ampule of 2mg/mL Albumin Standard to final concentrations of 2000, 1500, 1000, 750, 500, 250, 125, 25, 0 mcg/mL = blank according to manufacturer's instructions. Samples and standards were run in duplicate with a total volume per well of 25 mcL.

A working reagent made up by mixing 50mL reagent A with 1mL reagent B. 200mcL of working reagent was added to each well. The plate was sealed and placed on the plate shaker for 30 seconds. The plate was then incubated at 37°C for 30 minutes before it was measured at 562nm absorbance on a plate reader.

A standard curve was prepared by plotting the average absorbance measurement for each BSA standard concentration minus the average absorbance measurement of the blank standard replicates. SoftMax Pro™ version 5 was used to generate best-fit standard curves from which the protein concentration of each sample was interpolated (mcg/mL).

2.1.3.6 Statistical Analysis

Primary cytokine data was multiplied up according to sample dilution. Plasma values remained unchanged. CF samples cytokine values were multiplied by the dilution factor obtained from the weight based dilution calculation, see extraction protocol section 2.1.3.4. CVF values were multiplied by the dilution with which they were run, mostly by 2 but when IL-8 concentrations were outside the range of the standard curve and re run at 1 in 4 or 1 in10 dilution, the cytokine output data were then multiplied up by 4 or 10 respectively.

Categorical variables were described in numbers and % and continuous variables summarized with median and interquartile ranges (IQR) and compared with the Kruskal–Wallis test. Cytokine concentrations were normalised to both sample dilution and total protein (expressed as a ratio of pg cytokine/mg protein). The correlation of cytokine concentration with specimen weight pre and post normalisation and associations with potential known confounders was calculated with Spearman's correlation co-efficient. Bonferroni correction was made for multiple comparisons (0.05/10) therefore a p value <0.005 was deemed significant. Analyses were performed using SPSS (version 24).

2.1.4 Results

2.1.4.1 Study subjects

53 samples collected between October 2013 and July 2014 from 18 healthy uninfected pregnant women were included in the analysis. For patient demographics, see table 4. Women were predominately caucasian, married, nulliparous, non-smokers with healthy range BMIs.

Table 4 Participant characteristics and clinical data

Characteristic	Value
Age (median years (IQR))	34.0 (30.0-34.0)
Ethnicity n (%) Caucasian Black Asian Latin Other	 15 (83) 0 2 (11) 1 (6) 0
Relationship Status n (%) Married Cohabiting Single	 14 (88) 4 (22) 0
BMI (median (IQR))	23 (20-24)
Smoking status n (%) Smoker Non smoker	 2 (22) 16 (88)
Parity (median (IQR))	0 (0-1)
Intercourse in preceding 24 hours n	0
Practice of vaginal douching n	0
Vaginal pH	4.1 (3.8-4.6)
Bacterial vaginosis current n	4/18 women in total[1] 8/50 samples[2]
Candida current n	4/18 women in total 5/50 samples
Preterm delivery n (%)	1 (5)

[1]These were different women to those in whom BV was identified

[2]50 corresponding vaginal microscopy samples were available

2.1.4.2 Collection method and sample volume

Genital tract fluid weights and dilutions are shown in Table 5. CVF is a more viscous secretion than CF but can be easily handled with a positive displacement pipette. The MC method enabled collection of greater volume of secretions than PVA sponges. For example CVF: median 0.5g, when diluted 1 in 2 with 100mcL extraction buffer produces approximately 5 x 200mcL aliquots, total 1000mcL versus CF: median 0.07g, gives a 1 in 10 dilution [(0.07+0.6g)/0.07=10] in 600 mcL of extraction buffer. The MC method of CVF collection has a more constant

dilution factor (2.0-10.0) compared to a large range of dilution factors (1.3-61.0) observed with the process of elution of CF from the PVA sponge.
Anecdotally participants reported that they found both sampling methods acceptable but expressed that it would be advantageous if speculum examination were avoidable.

2.1.4.3 Total Protein

Total protein concentrations in CVF and CF are shown in table 5 and Figure 3. CVF protein concentrations are 30 fold greater than observed in CF (Median CVF protein 71731 mcg/mL (IQR 56489-109967) versus CF 2396 mcg/mL ((IQR 1822-3124), p<0.0001)).

Table 5 Median specimen weight and dilution factors of genital tract secretions by sampling method

Secretion type/method	Weight/g (IQR)	Range/g	Dilution factor (IQR)	Range of dilutions
CF by PVA sponge	0.07 (0.05-0.12)	0.01-0.54	9.6 (6.0-13.0)	1.3-61.0
CVF by MC	0.54 (0.35-0.82)	0.20-1.90	2.0 (2.0-4.0)	2.0-10.0

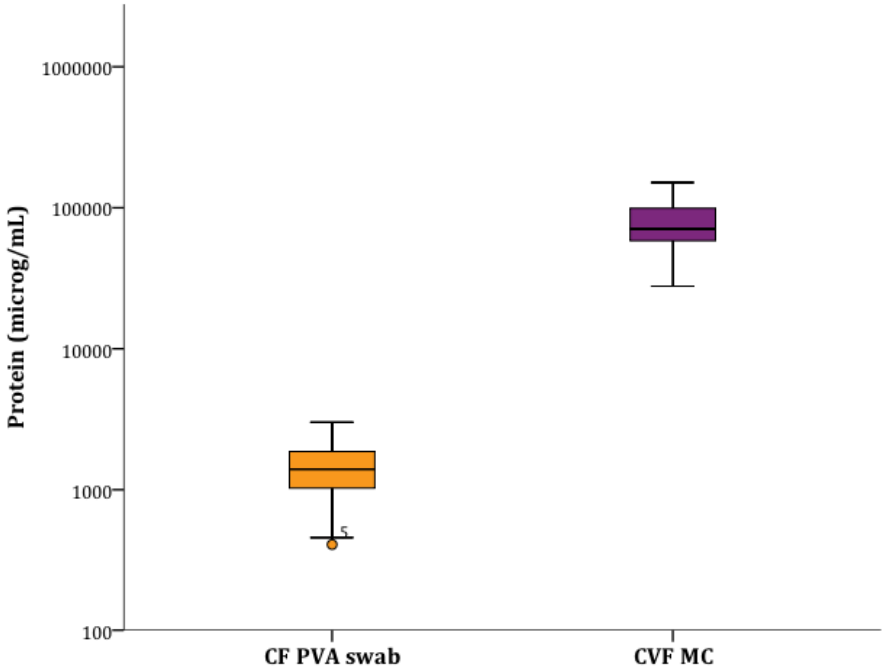


Figure 3 Boxplot of protein concentrations by female genital tract fluid and collection method
Demonstrating higher median protein concentrations in CVF compared to CF.

2.1.4.4 Normalisation of cytokine concentrations

The effect of normalising female genital tract cytokine concentrations to sample volume (dilution) or total protein concentrations is shown in tables 6 and 7. CVF cytokine concentrations adjusted to either sample dilution or total protein were independent of sample weight with the exception of IL-1β and IL-6, however these associations with sample weight were removed by correction for multiple analyses. Conversely, CF cytokine concentrations were inversely associated with PVA sample weight after both volume and total protein adjustment, see table 7. From henceforth cytokine concentrations will be presented in pg/mL normalized to sample volume.

Table 6 Effect of normalisation method on the correlation of CVF cytokine concentration and specimen weight

Cytokine	Normalisation method	Median (IQR)	Spearman's (ρ)	P value
Pro-inflammatory				
IL-1β	As measured (pg/mL)	58 (20-443)	-0.401	0.004
	Dilution (pg/mL)	116 (44-1441)	-0.365	0.008
	Protein (pg/mg)	2 (1-14)	-0.351	0.013
IL-6	As measured (pg/mL)	8 (3-40)	-0.348	0.012
	Dilution (pg/mL)	24 (8-119)	-0.325	0.02
	Protein (pg/mg)	0.3 (0.1-1.6)	-0.359	0.01
IL-8	As measured (pg/mL)	621 (141-3170)	-0.281	0.046
	Dilution (pg/mL)	2636 (281-17248)	-0.252	0.075
	Protein (pg/mg)	202 (36-453)	-0.264	0.067
TNF-α	As measured (pg/mL)	0.6 (0.2-1.6)	-0.166	0.243
	Dilution (pg/mL)	2 (0-5)	-0.168	0.238
	Protein (pg/mg)	0.02 (0.006-0.07)	-0.179	0.212
Immune-regulatory				
IFN-γ	As measured (pg/mL)	3 (1-6)	-0.293	0.037
	Dilution (pg/mL)	6 (3-20)	-0.241	0.089
	Protein (pg/mg)	0.1 (0.04-0.3)	-0.217	0.130
IL-2	As measured (pg/mL)	0.5 (0.3-0.9)	-0.258	0.068
	Dilution (pg/mL)	1 (0.6-3.7)	-0.198	0.164
	Protein (pg/mg)	0.02 (0.006-0.04)	-0.252	0.077
IL-4	As measured (pg/mL)	0.1 (0.06-0.4)	-0.272	0.053
	Dilution (pg/mL)	0.4 (0.1-1.1)	-0.250	0.077
	Protein (pg/mg)	0.005 (0.002-0.11)	-0.208	0.147
IL-10	As measured (pg/mL)	0.7 (0.3-1.7)	-0.119	0.407
	Dilution (pg/mL)	3 (1-6)	-0.098	0.496
	Protein (pg/mg)	0.02 (0.01-0.09)	-0.119	0.411
IL-12	As measured (pg/mL)	0.2 (0.1-0.7)	-0.048	0.739
	Dilution (pg/mL)	0.6 (0.2-3.3)	-0.016	0.909
	Protein (pg/mg)	0.007 (0.003-0.05)	-0.042	0.770
IL-13	As measured (pg/mL)	4 (2-7)	-0.210	0.136
	Dilution (pg/mL)	12 (5-35)	-0.137	0.336
	Protein (pg/mg)	0.1 (0.05-0.4)	-0.193	0.180

Table 7 Effect of normalisation method on the correlation of CF cytokine concentration and specimen weight

Cytokine	Normalisation method	Median (IQR)	Spearman's (ρ)	P value
Pro-inflammatory				
IL-1β	As measured (pg/mL) Dilution (pg/mL) Protein (pg/mg)	35 (13-87) 1352 (178-5036) 18 (6-36)	-0.098 -0.397 -0.285	0.493 0.004 0.045
IL-6	As measured (pg/mL) Dilution (pg/mL) Protein (pg/mg)	5 (2-20) 252 (55-1375) 3 (1-13)	-0.145 -0.370 -0.246	0.311 0.008 0.085
IL-8	As measured (pg/mL) Dilution (pg/mL) Protein (pg/mg)	789 (421-1909) 34491 (6553-119938) 325 (155-812)	-0.130 -0.496 -0.123	0.384 0.0001 0.397
TNF-α	As measured (pg/mL) Dilution (pg/mL) Protein (pg/mg)	0.4 (0.2-1.0) 14 (4-60) 0.1 (0.1-0.5)	-0.029 -0.601 -0.448	0.841 0.0001 0.001
Immune-regulatory				
IFN-γ	As measured (pg/mL) Dilution (pg/mL) Protein (pg/mg)	2 (1-4) 48 (9-327) 1 (1-2)	-0.214 -0.391 0.426	0.132 0.005 0.002
IL-2	As measured (pg/mL) Dilution (pg/mL) Protein (pg/mg)	0.4 (0.2-0.7) 9 (3-42) 0.2(0.0-0.5)	-0.167 -0.528 -0.385	0.241 0.0001 0.006
IL-4	As measured (pg/mL) Dilution (pg/mL) Protein (pg/mg)	0.1 (0.0-0.2) 2 (0-6) 0.04 (0.01-0.1)	-0.210 -0.333 -0.375	0.139 0.057 0.007
IL-10	As measured (pg/mL) Dilution (pg/mL) Protein (pg/mg)	0.6 (0.4-1.6) 29 (6-87) 0.3 (0.1-1.0)	-0.409 -0.574 0.558	0.003 0.0001 0.0001
IL-12	As measured (pg/mL) Dilution (pg/mL) Protein (pg/mg)	0.3 (0.2-0.6) 10 (1-43) 0.1 (0.06-0.3)	-0.202 -0.302 -0.368	0.155 0.031 0.09
IL-13	As measured (pg/mL) Dilution (pg/mL) Protein (pg/mg)	5 (3 -11) 168 (48-636) 2 (1-5)	-0.331 -0.471 -0.538	0.0018 0.0001 0.0001

2.1.4.5 Cytokine concentrations by biological fluid type

All measured cytokines were detectable in both FGT fluid types and plasma, see table 6 and table 7. The cytokine profile in CF and CVF displayed very similar ranking with high concentrations pro-inflammatory cytokines: IL-8, IL-1β, IL-6 and TNF-α. Of the immune-regulatory cytokines IL-13 was observed in the highest concentration in both FGT fluids.

For all cytokines median concentrations were 7-17 fold higher in CF compared to CVF, $p<0.0001$. A lower proportions of samples <lower limit of detection (LLOD) were observed with CF compared to CVF, see table 8.

Plasma cytokine concentrations were generally lower than observed in female genital tract fluid (most notably pro-inflammatory cytokines: IL-8, IL-1β, IL-6 and immune-regulatory: IL-13), see figure 4. Similar concentrations were observed between plasma and CVF for TNF-α and IFN-γ. A similar percentage of plasma samples contained detectable cytokines compared to FGT samples, see table 9.

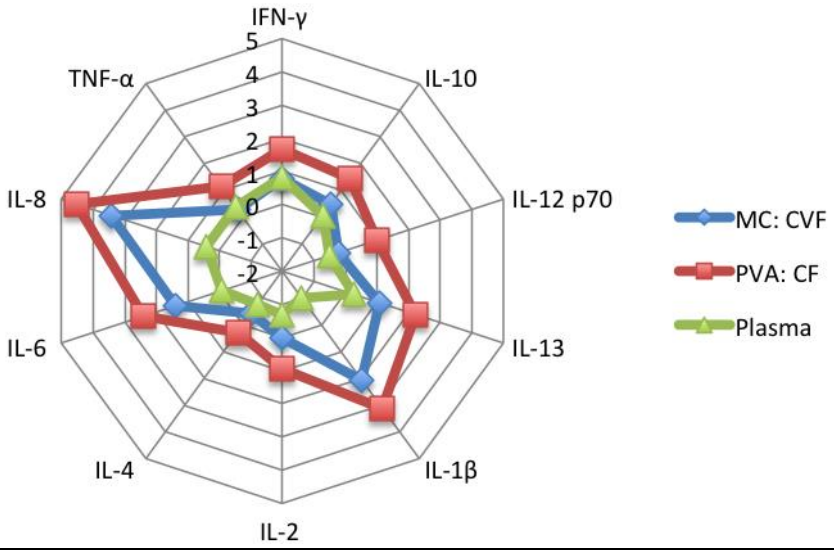


Figure 4 Spider chart to demonstrate log 10 median cytokine concentrations /pg/mL by biological fluid type
Demonstrating the highest concentrations of cytokines were observed in CF, followed by CVF and the lowest concentrations were observed in plasma of uninfected pregnant women

Table 8 Comparison of cytokine detectability in female genital tract fluid and plasma

	PVA: CF	MC: CVF	Plasma
Cytokine (pg/mL)	**% Detectable**		
Pro-inflammatory			
IL-1β	100	100	100
IL-6	98	100	96
IL-8	100	100	100
TNF-α	96	94	100
Immune-regulatory			
IFN-γ	96	100	100
IL-2	100	98	94
IL-4	88	90	92
IL-10	100	98	100
IL-12	90	85	88
IL-13	96	88	83

High vaginal pH was associated with both pro-inflammatory and immune-regulatory FGT cytokine concentrations in pregnant women

Correlations between cytokine concentration and vaginal pH were explored in both biological fluids. In CF, higher pH was positively correlated with all measured cytokines: IFN-γ (r=0.414, p=0.003); IL-10 (r=0.387, p=0.005); IL-12 (r=0.360, p-0.009); IL-13 (r=0.308, p=0.028); IL-1β (r=0.360, p=0.009); IL-2 (r=0.367, p=0.008); IL-4 (r=0.368, p=0.008); IL-6 (r=0.334, p=0.017); IL-8 (r=0.359, p=0.013) and TNF-α (r=0.329, p=0.022). In CVF, IFN-γ (r=0.329, p=0.018) and IL-1β (r=0.384, p=0.005) were positively correlated with pH. After correction for multiple comparisons only CF IFN-γ, IL-10 and CVF IL-1β remained significantly positively associated with vaginal pH.

Vaginal polymorphonuclear leucocytes were correlated with both pro-inflammatory and immune-regulatory FGT cytokines in pregnant women

Correlations between total polymorphonuclear leucocyte count per high power field on vaginal microscopy and FGT fluid cytokine concentration were analysed. In CF all measured cytokines, with the except of IL-2, were positively correlated with increasing leucocyte count per high power field: IFN-γ (r=0.358, p=0.011);

IL-4 (r=0.382, p=0.006); IL-10 (r=0.331, p=0.019); IL-12 (r=0.373, p-0.008); IL-13 (r=0.299, p=0.035); IL-1β (r=0.334, p=0.009); IL-6 (r=0.276, p=0.05); IL-8 (r=0.346, p=0.019) and TNF-α (r=0.302, p=0.033). In CVF only IL-8 was significantly correlated with leucocyte count (r=0.351, p=0.012). There was a trend towards a positive correlation between leucocyte count and IFN-γ (r=0.239, p=0.094) and IL-1β (r=0.266, p=0.062). These associations did not withstand correction for multiple comparisons.

Bacterial vaginosis was associated with higher median CF IL-1β, IL-13 and IFN-γ

The presence of BV was associated with higher concentrations of CF IL-1β (4117 pg/mL (IQR 1383-5364) versus 799 pg/mL (126-4222) p=0.05) and IL-13 (367 pg/mL (IQR 252-782) versus 134 pg/mL (IQR 35-393) p=0.04) compared to women with normal vaginal flora, notwithstanding correction for multiple comparisons. There was a trend towards a higher median IFN-γ in the FGT fluid of women with BV compared to women with normal vaginal flora: CF 269 pg/mL (IQR 41-646) versus 27 pg/mL (IQR 8-275) p=0.056; CVF 13 pg/mL (IQR 5-33) versus 4 pg/mL (IQR 2-15) p=0.092.

Vaginal candida associated with higher median FGT fluid pro-inflammatory and immune-regulatory cytokines

The presence of vaginal candida was associated with higher CVF concentrations of IL-12 (13 pg/mL (IQR 2-30) versus compared to women with no candida (1 pg/mL (IQR 0-3)), p=0.027. This trend was also observed between the presence of candida and IFN-γ (354 pg/mL (IQR 8-1674) versus 6 pg/mL (IQR 3-17)), p=0.081, IL-1β (6322 pg/mL (IQR 515-11086) versus 109 pg/mL (IQR 45-1055)), p=0.081 and IL-8 (34415 pg/mL (IQR 8968-36420) versus 1870 pg/mL (IQR 296-8788)), p=0.075. These associations did not withstand correction for multiple comparisons.

The presence of vaginal candida was associated with higher CF concentrations of IFN-γ (558 pg/mL (IQR 266-83308)) compared to women without candida (45 pg/mL (IQR 9-269)), p=0.05, IL-2 (295 pg/mL (IQR 46-1682) versus 8 pg/mL (IQR 2-23)), p=0.027, IL-12 (86 pg/mL (IQR 26-1420) versus 9 pg/mL (IQR 2-

30)), p=0.039 and TNF-α (122 pg/mL (IQR 36-2202) versus 13 pg/mL (IQR 4-43)), p=0.05. A similar trend was seen for IL-13 (863 pg/mL (IQR 323-5328) versus 158 pg/mL (IQR 48-382)), p=0.059 and IL-8 (295306 pg/mL (IQR 58056-1208132) versus 29999 pg/mL (IQR: 6617-70560)), p=0.077. These associations did not withstand correction for multiple comparisons.

2.1.5 Discussion

In this study, the first to evaluate FGT fluid collection methods in pregnancy, we found both PVA sponges and MC to be valid methods of collection for the measurement of cytokines with all the multiplex cytokines being measurable in each fluid. The highest concentrations of Th1 cytokines: IL-1β, IL-6 and IL-8 observed in both fluid types are similar in profile to the literature for FGT fluid from pregnant women (Tanaka *et al.*, 1998; Kutteh and Franklin, 2001; Simhan *et al.*, 2005; Beigi *et al.*, 2007; Nenadić and Pavlović, 2008; Ryckman *et al.*, 2009; Dadhwal, MD *et al.*, 2017).

CVF sampling by MC enables collection of high fluid volumes suitable for multiple assays, with the opportunity to collect undiluted samples, making it more useful for high dimensional 'omic' studies. The option of self-sampling is also attractive. Pregnant women in this study achieved comparable sample weights (median 0.5g) to studies in non-pregnant women despite relatively short retention of the MC (minimum 5 minutes, maximum 30 minutes) (Boskey *et al.*, 2003). These figures add to the paucity of data on the optimal time for MC retention with one study in non-pregnant HIV infected women demonstrating a median sample weight of 0.31g with a minimum retention time of 60 minutes and another in uninfected non-pregnant women achieving a median sample weight of 0.5g with a retention time of 5 seconds(Boskey *et al.*, 2003; Jaumdally *et al.*, 2018).

Conversely CF sampling by PVA swabs demonstrated the highest recovery of cytokines both in final concentration and number of samples above the LLOD. This reflects the fact that the cervix/fetal placental unit may be the source of many of the cytokine producing cells and vaginal secretions dilute measurements in the CVF. However CF volumes are small and necessitate elution from the PVA sponge with extraction buffer introducing potential for confounding errors. Wider ranges of cytokine measurements were observed in the CF, which is likely to be the product of the variability in weight dependent dilution factors. Normalisation of CF cytokine concentrations to protein concentrations does not remove this weight dependent effect however CVF

cytokine concentrations were largely independent of weight and protein concentration. The precision of volume dilution with CVF and reduced susceptibility to sample weight bias make this method advantageous.

The elevated concentrations of CVF pro-inflammatory cytokines: IFN-γ and IL-12 observed in the presence of vaginal candidiasis mirror current knowledge of a IFN-γ CD4 Th1 adaptive and phagocytic candidacidal response induced by antigen presenting cells under the control of IL-12 and is in keeping with this inflammatory vaginitis (Romani L. 1999; Yano 2012). These data demonstrate the use of CVF cytokine measurement in detecting biological plausible mechanisms.

The observed association between FGT fluid IL-1β and bacterial vaginosis has been described in the literature(Beigi *et al.*, 2007; Jaumdally *et al.*, 2017). Bacterial vaginosis is a known risk factor for preterm delivery(Hay, Lamont, *et al.*, 1994). Whilst it is clear from these data that it does not appear to stimulate the same level of pro-inflammatory response in the genital tract of these HIV-1 uninfected pregnant women compared to candida however this shift in normal vaginal flora may generate a level of immunogenicity.

In addition to being the first to demonstrate the utility of these FGT fluid collection methods in pregnant women, other strengths of this study include the evaluation of different normalization methods (weight (based on accurate individual sample pre and post collection measurements) and protein concentration) and correlates of cytokines concentration e.g. pH, leucocyte counts and presence of bacterial vaginosis and candidiasis.

Limitations of this work include the potential confounding in order of sampling in that collection of CF sample with the PVA sponge first may have an impact of subsequent collection of CVF with the MC, potentially reducing the measurable cytokine concentrations by in the subsequent sample. This was not reported to have a negative impact in a recent study by Jaumdally et al. (Jaumdally *et al.*,

2018). Some women preferred provider insertion of the MC and the data was not available to compare recovery between insertion techniques.

2.1.6 Conclusion

In summary both methods presented are acceptable and robust in pregnant women however given the high number of planned immunological assays and the reduction in potential dilution errors the Instead™ soft cup method was taken forward for the rest of the main study.

2.2 Main study clinical Design and clinical assessments

2.2.1 Study Design

A prospective observational study.

Ethics

This study was approved by the South East Coast RES Committee (The immunological basis of preterm delivery: 13/LO/0107).

Sample size calculation

The sample size was calculated using the pilot plasma IL-10 data for HIV-1 infected pregnant women, at week 34, who initiated PI-based cART versus initiated non PI-based cART (61 pg/mL SD=82 versus 318 pg/mL, SD=116). A target of 33 patients in each group had an 80% chance of detecting a minimal two fold difference in mean plasma IL-10 concentration, at a 5% level of significance, between antiretroviral treatment strategies (using unpaired t test, based on the lower mean 61 pg/mL).

In addition a FGT proteomic study, utilising SELDI-TOF Mass Spectrometry to identify a novel protein with a role in labour in cervical fluid, had 20 patients per group (Brown *et al.*, 2006) therefore 33 in each group should be sufficient to ensure 20 patients with samples from at least the time points. This sample size

was felt to be realistic based on known numbers of HIV-1 pregnant women receiving antenatal care in the areas across London covered by the London HIV Perinatal Research Group (LHPRG) network.

2.2.2. Recruitment and eligibility

Women were identified at booking visits (12-14 weeks) from HIV specialist and general antenatal clinics at 11 sites across London: Saint Mary's Hospital Paddington; Queen Charlotte's and Chelsea Maternity Hospital, Chelsea and Westminster Hospital; Northwick Park Hospital; Homerton University Hospital; North Middlesex Hospital; St Thomas' Hospital; Royal Free Hospital; Barnet Hospital; Woolwich Queen Elizabeth Hospital and University Hospital Lewisham. This collection of hospitals has been termed the 'Pan London HIV PTB Research Network'.

Inclusion criteria

All women: Pregnant, > 16 years and able to give written informed consent.
HIV-1 infected women: CD4 cell count > 350 cells/mcL.

Exclusion criteria

Multiple pregnancies, in vitro fertilization, recreational drug use, co-morbidities requiring immunosuppressive/modulating treatment, patients unable to give written, informed consent

2.2.3 Participants

- Uninfected pregnant women (n=32)
- HIV-1 infected women (n=76) were purposely sampled to the following groups:
    1. HIV-1 infected pregnant women conceiving on PI-based cART (n=22)
    2. HIV-1 infected pregnant women conceiving on non PI- based cART (n=37)
    3. HIV-1 infected pregnant women initiating PI-based cART (n=7)
    4. HIV-1 infected pregnant women initiating non PI- based cART (n=10)

2.2.4 Clinical assessments and data collection

2.2.4.1. Visit schedule

Six time points: 12.0 – 21.9 weeks, 22.0-26.9 weeks, 27.0-31.9 weeks, 32.0-36.9 weeks and 37 weeks or later, up to and including delivery and a post-partum sample 4-8 weeks post-delivery. This schedule was designed to include a pre cART initiation sample for those who started cART in pregnancy and a sample 2-4 weeks post cART initiation.

2.2.4.2 Data Collection

Subject demographics, cART regimen, timing and adherence, medical and obstetric risk factors for PTB, intrapartum management, maternal complications and birth outcome were collected from participants and their medical notes. CD4 cell count and HIV RNA level, full blood count, biochemical profile measurement and STI screening were undertaken in accordance with routine clinical care and data were recorded from hospital pathology databases.

2.2.4.3 Biological sample collection

Details of biological sample collection and timings are summarized in table 9. Phlebotomy was performed using non-touch aseptic technique according to NHS trust guidelines. 8mL of EDTA whole blood were transported to the research laboratory at room temperature within 4 hours for plasma and PBMC separation and 4mL went to Imperial College Healthcare NHS Trust pathology laboratories for outsourced Flow Cytometry analysis of T cell activation. CVF sampling was undertaken using menstrual soft cups followed by two self-taken blind vaginal swabs. The first using a plastic inoculation loop to test vaginal pH (Litmus test paper strip) and prepare dry vaginal slides for gram staining and the second was a BBL™ Culture Swab™ MaxV Liquid Amies swab (BD, Oxford, UK) for bacterial 16S rRNA sequencing. FGT sampling was undertaken at the first three time-points.

Table 9 Study visit schedule and biological sample collection

TIME BASED VISITS							
ANTEPARTUM							POSTPARTUM
	SCREEN BOOKING 12-14 WEEKS	12.0-21.9 WEEKS (pre treatment if initiating cART)	22.0-26.9 WEEKS (2-4 weeks post treatment)	27.0-31.9 WEEKS (8 weeks post treatment)	32.0-36.9 WEEKS	37 WEEKS (up to and including delivery)	4-8 WEEKS POST PARTUM
BLOOD							
Total Blood Volume	X	12 mL (3x 4mL EDTA)	12 mL (3x 4mL EDTA)	12 mL (3x 4mL EDTA)	12 mL (3x 4mL EDTA)	12 mL (3x 4mL EDTA)	12 mL (3x 4mL EDTA)
CERVICOVAGINAL FLUID							
CVF	X	Speculum+ PVA swab Instead™ Soft cup	Speculum+ PVA swab Instead™ Soft cup	Speculum+ PVA swab Instead™ Soft cup	X	X	X
Infection screening	X	Vaginal Microscopy Microbiome swab	Vaginal Microscopy Microbiome swab	Vaginal Microscopy Microbiome swab	X	X	X
Fetal fibronectin measurement	X	X	√	√	X	X	X
CERVICAL LENGTH							
TV USS	X	X	√	√	X	X	X

EDTA: Ethylenediaminetetraacetic acid; CVF: Cervicovaginal Fluid; PVA: Polyvinyl acetyl; TV USS: Transvaginal ultrasound scan

2.2.4.4 Trans-vaginal ultrasound scan of cervical length

The clinician performed a TV USS after the FGT fluid sampling as manipulation of the cervix can cause the release of fFN. A cervical length (CL) measurement was taken by trans-vaginal ultrasound scan with the participant in supine position, with an empty bladder, avoiding placing excess pressure on the cervix during the scan. A length of <25 mm at ≤24 weeks gestation was defined as a short cervix that required referral to the NHS preterm birth clinic for intervention(Iams *et al.*, 1996).

2.2.4.5 Fetal fibronectin measurement

The fFN 10Q Analyzer (Hologic, Massachusetts, USA) monoclonal antibody assay was used to measure fFN in CVF according to manufacturer's instructions. Briefly, the specimen transport tube was brought to room temperature and gently mixed before the polyester swab was removed and disposed of retaining the buffer mixture in the tube.

200 mcL of the buffer containing the patient's CVF was added to the application well of the rapid fFN cassette when prompted by the Hologic 10Q Analyzer. The sample flowed from an absorbent pad in the cassette across a nitrocellulose membrane containing monoclonal anti-fetal fibronectin antibody conjugated to blue microspheres. The blue conjugated sample then flowed across part of membrane containing polyclonal anti-human fibronectin capture antibodies, which fixed the fibronectin-conjugate complexes. The remaining sample then flowed through an area of membrane containing polyclonal IgG capture antibody, which fixed unbound conjugate (control). After 20 minutes the intensities of the test line and control line were interpreted with the 10Q Analyzer. Fetal fibronectin concentration was reported in ng/mL, between thresholds of 10 and 200ng/ mL. A value of 50ng/mL or above is deemed to be a positive result with a 97% negative predictive value for sPTB <34 weeks(Abbott *et al.*, 2013).

2.2.4.6 Vaginal flora assessment by light field microscopy

Vaginal dry slides were prepared by applying a thin smear of CVF obtained from the lateral vaginal wall from the plastic inoculation loop onto a pre labeled glass microscope slide (frosted side up). The slide was then allowed to air dry and stored in a microscope slide box before undergoing the gram staining procedure in box batches.

Light field microscopy was undertaken to classify vaginal flora using Hay/Ison's criteria (Ison and Hay, 2002). Polymorphonuclear leucocyte count was graded by ordinal scale: 0, 1-5, 6-10, 11-20, 21-30, and 31+ per high-powered field over an average of 3 fields. The presence of clue cells, fungal spores and hyphae, and sperm were also recorded.

2.2.5 Sample processing and storage for cytokines, proteomics and microbiome

2.2.5.1 Plasma separation protocol

Two x 4 mL EDTA tubes of whole blood were spun at 800g, no brake, for 5 minutes at 4°C Plasma was carefully aspirated and 2-3 x 1 mL aliquoted into standard 2mL plastic microtube and transferred immediately to a - 80°C freezer. The remaining cells were retained for PBMC isolation (not detailed here).

2.2.5.2 Female Genital Tract fluid extraction protocols

The protocol for cervical fluid extraction from PVA sponges and CVF processing from the menstrual Instead™ soft cup are described below, the first step in the proceedure is to prepare the extraction buffer.

Protease inhibitor buffer for Female Genital Tract fluid extraction and dilution

One vial of Calbiochem™ protease inhibitor cocktail set 1 (Merck, Darmstadt, Germany) was reconstituted in in 1 ml sterile distilled H_20 per vial (100 X solution) and vortexed for 30 seconds at room temperature to dissolve completely. 10% sodium azide solution was made by adding 1g of sodium azide into a sterile 15 mL plastic conical tube with 10mL of sterile distilled water and stored at 2- 8^0C. 1ml (100 x) protease cocktail I (prepared as above) was added with 20mcL 10% sodium azide solution to 100mls with 1XPBS solution with 1.5g NaCl (0.25M final concentration). This solution was then filter sterilised using a 0.22mcM filter unit into a sterile reagent bottle and stored at 2- 8^0C. This buffer was based on methods described by Castle et al. (Castle *et al.*, 2004).

PVA sponge cervical fluid extraction

PVA sponges were thawed for a maximum of 30 minutes on ice. 300mcL of extraction buffer was pipetted into a Spin-X™ tube (Sigma-Aldrich, Merck, Germany). The PVA sponge was removed from the 15mL plastic conical tube with sterile forceps and place into the top chamber of the Spin-X™ tube. In order to seal the tube the handle of the spear was cut off with clean scissors. The Spin X tubes were then centrifuges at 13,000rpm for 15 minutes at 4^0C. Providing all the extraction buffer had passed through to the bottom chamber on visual

inspection, the process was repeated with an addition 300mcL of extraction buffer. If not all the buffer passed into the bottom chamber then the filters where exchanged and the Spin-X™ tubes were re-spun for a further 5 minutes. The 600mcL of eluted PVA CF was then frozen at -80^{0}C until use. This method was based on methods described by Castle et al.(Castle *et al.*, 2004).

Dilution calculation

The dilution factor of PVA CF samples was calculated using the following formula: [(*x* - *y*) + 0.6 g of extraction buffer] / (x - y)], where *x* equals the weight of the PVA sponge + falcon post collection and y is the weight of the PVA sponge and falcon pre collection. The density of extraction buffer =1.005g/mL(Rohan *et al.*, 2000).

Soft cup Cervicovaginal fluid extraction

The 50mL plastic conical tubes containing the MC was thawed for a maximum of 30 minutes on ice and centrifuged for 15 minutes at 1300rpm, 4°C to pool the CVF at the bottom of the 50mL plastic conical tube. The CVF was divided into 100µL aliquots using a positive displacement pipette (Rainin c10-100, Mettler-Toledo, Ohio, USA) and stored in 1.5mL plastic microtubes. The MC was re-spun if significant CVF remained on the cup or in the 50mL plastic conical tube. CVF was diluted 1 in 2 with the addition of 100mcL of extraction buffer (Cosgrove *et al.*, 2016). The CVF was stored at -80°C until further testing.

2.3 Measurement of Systemic Inflammation

2.3.1 Participants

76 HIV -1infected pregnant women and 32 uninfected women from the main study provided EDTA whole blood for PBMC and plasma samples at up to five time points through gestation (12.0 – 21.9, 22.0-26.9, 27.0-31.9, 32.0-36.9 weeks and 37 weeks or later, up to and including delivery) as per main study visit schedule see table 4. A total of 362 blood samples from the 108 women were available for analysis.

2.3.2 Plasma Cytokines

Multi-spot chemi-luminescent cytokine assays (V-plex, MSD, Maryland, USA) were used to measure 10 pro-inflammatory and immune –regulatory cytokines (IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNF-α), in duplicate undiluted plasma samples for full description see methods section 2.1.3.4.

2.3.3 T cell activation markers

PMBC activation markers (CD3+, CD4+, CD8+, CD25+ and HLA-DR+) were measured by Beckman Coulter Navios Flow Cytometer (Beckman Coulter, CA, USA) in the Imperial College Healthcare NHS Trust Pathology laboratories. CD3+CD4+and CD3+CD8+ absolute counts and percentages were reported in addition to percentages of these subsets expressing CD25 and expressing HLA-DR i.e. CD4+CD25+cells, CD8+CD25+cells, CD4+HLA-DR+cells and CD8+HLA-DR+cells. The reference ranges, generated by ICHNT Pathology, were: CD3+CD4+CD25+ (15.7%-34.9%), CD3+CD4+HLA-DR+ (4.2%-13.6%), CD3+CD8+CD25+ (4.6%-10.9%) and CD3+CD8+HLA-DR+ (5.7%-38.2%).

2.3.4 Analysis

See statistical analysis section 2.7

2.4 Measurement of Inflammation in Cervicovaginal Fluid

2.4.1 Participants

48 HIV -1infected pregnant women and 27 uninfected women from the main study provided CVF samples at up to three points through gestation (12.0 – 21.9, 22.0-26.9 and 27.0-31.9 weeks) as per main study visit schedule see table 9. A total of 188 CVF samples were available.

2.4.2 Cervicovaginal Cytokines

Multi-spot chemi-luminescent cytokine assays (V-plex, MSD) were used to measure 10 pro-inflammatory and immune –regulatory cytokines (IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNF-α) in duplicate CVF samples, diluted 1 in 2 in extraction buffer (or 1 in 4 or 1 in 10, depending on concentration falling with in standard curve), for full description see methods section 2.1.3.4.

2.4.3 Analysis

See statistical analysis section 2.7

2.5 Vaginal Microbiome sub study

2.5.1 Sub study design

Longitudinal cohort study of vaginal microbiota in HIV-1 infected pregnant women across the second and third trimester with second trimester cross-sectional comparative data available for uninfected pregnant women.

2.5.2 Participants

This is a sub study of 53 HIV-1 infected women from our main prospective cohort study (REC 13/LO/0107) with 22 uninfected healthy term controls for whom vaginal microbiota sample data were already available from an alternative prospective cohort study (REC 14/LO/0328) undertaken at Imperial College Healthcare NHS Trust(Brown *et al.*, 2018). Participant recruitment and clinical data collection is described in section 2.2.1.

2.5.3 Sample collection

Blind vaginal BBL ™ Culture Swab™ MaxV Liquid Amies swabs (BD, NJ, USA) for microbiota analysis were obtained from HIV-1 infected pregnant women at three times points across the second and third trimester: 12.0-22.9 weeks, 23.0-26.9 weeks and 27.0-31.9 weeks. Uninfected control samples were obtained at one second trimester time period: 19-25 weeks (Brown *et al.*, 2018).

For HIV-1 infected pregnant women both bacterial swab and matching CVF collected by menstrual soft cup for cytokine analysis were available, as well as vaginal dry slide gram staining, see table 9 and sample collection protocol, section 2.1.3.3. and section 2.2.4.3.

2.5.4 Preparation of nucleic acids for sequencing

2.5.4.1 DNA extraction

To ensure maximal DNA purification from difficult to lyse gram positive and some gram-negative bacteria two pretreatment stages were undertaken. Bacterial cell wall disruption was effected using a combination of enzymatic digestion and mechanical disruption. BBL ™ Culture Swab™ swabs were thawed on ice and bacterial cells were re-suspended in transport buffer (Amies liquid medium) by vortexing the tip of the swab in its container. The transport buffer and swab are enveloped in absorptive foam, which was then carefully removed and transferred into a sterile 5mL plastic syringe using a sterile pipette tip. The foam was squeezed with the syringe plunger several times to ensure the majority of the buffer containing bacterial cells was squeezed out into a sterile 2 mL mini tube. The volume obtained in the receiving plastic 2mL mini tube was usually between >500mcL, if less than 500mcL then an additional 500mcL of PBS was added to the foam containing syringe which was then re-plunged as described above. The 2mL mini tube was then centrifuged at 10000g for 10 minutes, the Amies liquid medium is discarded and the cell pellet is suspended into 150mL of PBS. For each extraction batch, a clean swab was used as a negative control.

2.5.4.2 Enzymatic digestion

Bacterial cell wall lysis was initiated by adding a cocktail of enzymes to the cell buffer solution, all of which were kept on ice until use. To each 2mL mini tube containing re suspended cells in 150mL PBS, the following was added; 50 mcL of lyzosyme (10 mg/mL) (Sigma-Aldrich, Merck), 6 mcL of mutanolysin (25,000 U/mL) (Sigma-Aldrich), 3 mcL of lysostaphin (4,000 U/mL in sodium acetate) (Sigma-Aldrich), and 41 mcL of TE50 buffer (10 mM Tris HCL and 50 mM EDTA, pH 8.0)(Ravel *et al.*, 2011). Each 2mL mini tube was then incubated at 37°C in a water bath for 1 hour

2.5.4.3 Mechanical disruption of membrane

Sample mini tubes were then double sealed with Parafilm™ (Bemis™, NE, USA) and underwent additional mechanical disruption by bead beating. This was performed by using 100mg of acid bleached and rinsed silica beads (0.1mm diameter) for 2 min at 50Hz at room temperature in a Tissue Lyser LT (Qiagen, Hilden, Germany). The 2mL tubes were then briefly spun to pellet out the silica beads and the lysate was carefully aspirated into a new sterile 2 mL tube.

2.5.4.4 Purification of bacterial DNA

Bacterial DNA was then extracted using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Briefly, 20 mcL of proteinase K was added to each lysate post bead beating. Then 100 mcL of Buffer VXL with 1 mcg carrier RNA was added and the tube was incubated at room temperature for 15 minutes. 350 mcL of buffer ACB was then added and the sample was mixed by vortexing. The sample mixture was then transferred to a QIAamp mini column placed in a 2 mL collection tube, which was sealed and centrifuged for 1 min at 8000 rpm. The mini column (now containing the bacterial DNA in its membrane) was then transferred to a clean 2 mL collection tube. 600 mcL of buffer AW1 was added and the mini column and underlying tube were spun again at 8000 rpm for 1 min. The mini column was transferred to a clean 2 ml collection tube once more and 600 mcL of buffer AW2 was added. The mini column and underlying tube were spun again at 8000 rpm for 1 min.

The mini column was transferred to a clean 1.5 ml collection tube. Then 150 mcL of elution buffer AVE was added and allowed to incubate at room temperature for 1 min. The mini column and final underlying tube were spun at 14000 rpm for 1 min and the 150 mcL elution buffer with bacterial DNA was recovered in the underlying 1.5mL tube. The DNA solution was stored at -80°C before further analysis.

2.5.4.5 DNA quality confirmation

The integrity of the extracted bacterial DNA in samples and control was confirmed by PCR amplification using the universal primers: Forward 28F GAGTTTGATCNTGCCTCAG-3' and Reverse 338R 5'-TGCTGCCTCCCGTAGGAGT-3' (Frank *et al.*, 2008) and DNA electrophoresis using a 100bp DNA ladder, see figure 5. The PCR mix for each reaction (total 50mcL) comprised of 5mcL of DNA sample with 10mcL 5x Buffer, 1mcL 10mMdNTP, 1mcL 10pmol/mcL Forward Primer, 1mcL 10pmol/mcL Reverse Primer, 0.25mL of Taq Polymerase (One*Taq*™, New England BioLab, Massachusetts, USA) and 31.75mL of DNase/RNase free water.

The following conditions were inputted into a Gene Amp® 9700 PCR Thermocycler (Applied Biosciences™, Thermo Fisher Scientific, Massachusetts, USA): 5 minutes at 95°C to initiate denaturing of the DNA, followed by 30 cycles of: 30 second 95°C (denaturing temperature), 30 seconds 56°C (annealing temperature) and 90 seconds 72°C (sequence elongation temperature), then 7 minutes at 72°C for final elongation, after this the PCR machine held samples at 4°C. The DNA and control samples, along with 6x loading dye, were then loaded in a 1% Agarose gel within Tris-acetate-EDTA (TAE) running buffer with a 100bp DNA ladder at each edge, across 140V, until the DNA bands had been electrophoresed away from the loading wells and the bands were distinctly visible. The gel was then carefully removed and imaged with a UV camera to confirm presence or absence of bacterial DNA band in each lane.

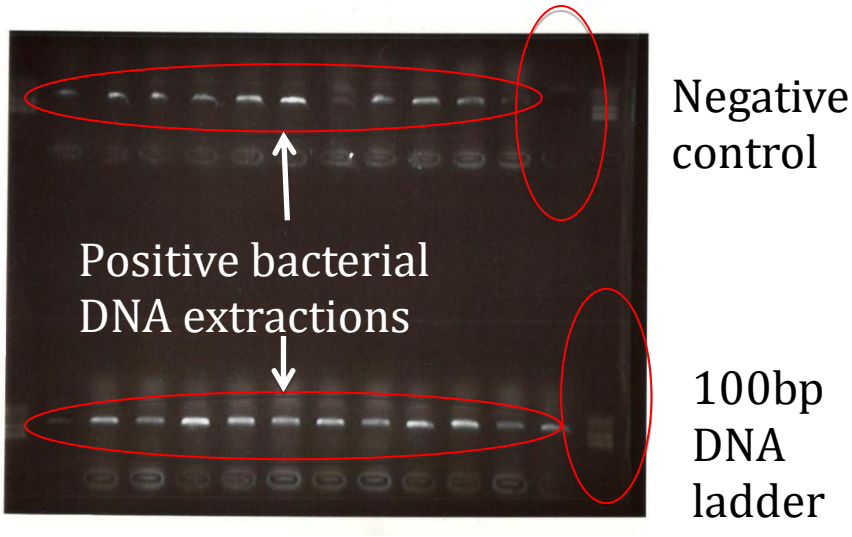


Figure 5 Confirmatory PCR bacterial DNA Electrophoresis

2.5.5. 16S rRNA sequencing

The V1-V2 hypervariable regions of the 16S rRNA gene were amplified with a fusion primer set that includes four different 28F primers chosen to improve detection of *Bifidobacteriales* and a 388R primer (Frank *et al.*, 2008). The 28F-YM forward primer (5′-GAGTTTGATCNTGGCTCAG-3′) was mixed in a ratio of 4:1:1:1 with 28F *Borrellia* (5′-GAGTTTGATCCTGGCTTAG-3′), 28F *Chloroflex* (5′-GAATTTGATCTTGGTTCAG-3′) and 28F *Bifido* (5′-GGGTTCGATTCTGGCTCAG-3′) (RTL Genomics Amplicon Diversity Assay List). The forward primers were constructed with an illumina™, SD, USA) i5 adapter (5′-AATGATACGGCGACCACCGAGATCTACAC-3′), an 8-base-pair (bp) bar code and primer pad (forward, 5′-TATGGTAATT-3′). The 388R reverse primer (5′-TGCTGCCTCCCGTAGGAGT-3′) was constructed with an illumina™ i7 adapter (5′-CAAGCAGAAGACGGCATACGAGAT-3′), an 8-bp bar code, a primer pad (reverse, 5′-AGTCAGTCAG- 3′). The pair end multiplex sequencing was performed on an Illumina™ MiSeq™ platform at RTL Genomics Research and Testing Laboratory (Lubbock, TX, USA).

2.6. Vaginal microbiome analysis

2.6.1 16S rRNA Sequence analysis

The MiSeq SOP pipeline and software package Mothur were used to analyse sequences from two runs (HIV-1 infected and uninfected) (Kozich *et al.*, 2013).

Highly similar amplicons were aligned into operational taxonomic units (OTUs) using the kmer searching method and the Silva bacterial database (www.arb-silva.de/). All OTUs had an alignment of ≥97%. Classification was then performed using the Ribosomal Database Project (RDP) reference sequence files and the Wang method (Wang *et al.*, 2007). The RDP Multi Classifier script was used for determination of OTUs (phylum to genus) and species level taxonomies were determined using USEARCH (Edgar, 2010). To normalize the results we subsampled our data to a proportion of the smallest OTU read count (1750). Diversity indices (NP Shannon, Inverse Simpson, and Species observed (sOB) were calculated using the vegan package within R.

The Statistical Analysis of Metagenomic Profiles (STAMP) software package was used to explore differences in the vaginal microbiota at a genus and species level(Parks and Beiko, 2010). Hierarchical clustering analysis using furthest neighbour linkage was performed with a clustering density threshold of 0.75. Samples were classified into six CSTs: I (*L crispatus* dominant), II (L. gasseri), III (*L. iners*), IIIB (Diverse, moderate *lactobacillus spp.* and low levels of obligate anaerobes), IV (Diverse, high proportions of *Atopobium, Gardnerella, Prevotella* spp. and others), and CST V (*L. jensensii*), adapted from clusters described by Gajer et al. (Gajer *et al.*, 2012).

2.6.2 Cross-sectional cohort data

A cross section of second trimester samples from HIV-1 infected and uninfected women was used to explore differences by HIV status. Principle component analysis (PCA) was used to explore β diversity between groups and identify common clusters. Differences in mean proportions of bacterial genera and species were calculated using the Welsh test and corrected using Benjamini-Hochberg False Discovery Rate. Categorical variables were compared by the Fisher Exact test and differences between continuous variables were calculated by the Mann Whitney U test. Comparisons across multiple groups were made using the Kruskal Wallis test. Correction for multiple testing was performed using the Bonferroni principle.

Within the HIV-1 infected pregnant women, comparison in CST and species were made by delivery outcome. Linear discriminant analysis with effect size (LEfSe) was undertaken to explore if preterm birth were associated with any combination of differentially abundant vaginal species compared to term births but did not reveal any specific bacterial signature. The relationship between bacterial species and gestational age at delivery was explored using hierarchical multiple linear regression analysis in SPSS (version 24) for Mackintosh. The model was adjusted for maternal age, BMI and ethnicity, log transformed bacterial abundance was inputted as the second model factor, and patient ID was modeled as a random effect. Missing case data was excluded in a pair wise fashion. Differences in species by ART treatment exposure: at conception versus initiated in second trimester; NRTI exposure during pregnancy with FTC/TDF versus ABC/3TC were explored by the Welsh test.

2.6.3 Longitudinal cohort data

For women who had two or more samples, CST stability was examined pictorially by plotting CST type as a function of sample collection time point with the corresponding Inverse Simpson Index. The total number of CST transitions by baseline CST classification was compared using the Kruskal Wallis test. Mean gestational age at delivery was compared by number of CST transitions (0,1, and 2) using the Kruskal Wallis test.

2.6 Cervicovaginal Fluid Proteome Sub Study

2.6.1 Experiment designs

For experiment 1 and 2, each pooled sample group was contributed to by ten individual cases, further details of which are provided in results chapter 7. Groups constituted: uninfected pregnant women, HIV-1 infected women conceiving and continuing on PI-based cART; HIV-1 infected women conceiving and continuing on NNRTI/INSTI cART and HIV-1 infected pre-treatment initiation (i.e. no ART).

For experiment 3, each pooled sample group was contributed to by five individuals. The groups constituted of women who delivered at term and preterm, with further details in chapter 7. All CVF samples were obtained in the second trimester, with sampling gestations presented in chapter 7. Where possible cases were matched for age, ethnicity, BMI and vaginal CST.

Directed protein antibody arrays were performed according to the manufacturer's instructions (Proteomic Profiler™ Human XL Cytokine, R&D Systems, Oxford, UK) to identify novel immune proteins and differences in protein expression between groups.

2.6.2 Protein array

2.6.1 Sample dilution

CVF dilution required for protein array experiments was modeled on optimum CVF dilution to obtain CVF IL-8 concentrations by chemi-luminescence within the range of standard curve and thus a dilution of 1 in 100 was trialed using uninfected samples. A dilution of 1 in 100 enabled several abundant proteins to be identified using x-ray film and cassette exposed for 1 minute, therefore was used for the remaining experiments.

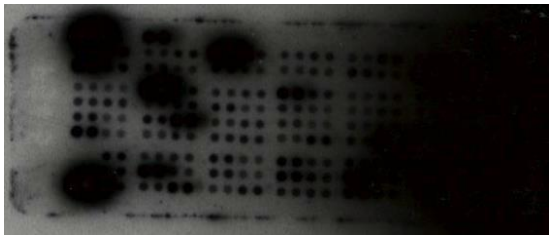


Figure 6 X-ray of protein array membrane incubated with CVF 1 in 100 dilution

In the absence of specific manufacturer's instructions for CVF, pooled samples were made up to a volume of 200mcL as suggested in the instructions for other biological bodily fluids e.g. plasma, breast milk and urine. All addition dilutions were done with an extraction buffer containing protease inhibitor cocktail (Calbiochem, Merk). For details of preparation of extraction buffer see CVF storage methods 2.2.5.2. CVF samples frozen at a 1 in 2 dilution with extraction buffer were diluted by adding 10mcL of sample to 490mcL of extraction buffer to make 500mcL sample at 1 in 100 dilution. In experiment 1 and 2, 20mcL of the 1 in 100 dilutions of CVF were pooled from each case (n=10) to make a total volume of 200mcL. Where less than 10 cases were available for the pre-conception cART group (n=7), 28.5mcL of each sample diluted 1 in 100 were pooled to make 200mcL total volume. For experiment 3, 40 mcL of CVF at 1 in 100 dilution were pooled for each case (n=5) to make a total volume of 200mcL.

2.6.2 Array procedure

Care was taken when handling the protein array film with clean gloved hands, with mouth shut and eye protection to prevent in advertent protein contamination from sweat, saliva and tears. Any manipulated of the array film was undertaken with blunt sterile tweezers.

Background blocking

Extraneous binding sites on the protein array film outside of the capture antibody spots were initially blocked blocking by immersing the arrays in 2mL of blocking buffer (Buffer 6) in 50 mL plastic tubes on a roller mixer for 1 hour. 200mcL of pooled sample at 1 in 100 dilution was added to 1300mcL of Array buffer 6 to make a total volume of 1.5mL to incubate with the protein array film. The blocking buffer was then aspirated from each arrays and the 1500mcL pooled CVF (+ Array Buffer 6) sample for each group was added to the 50mL plastic tube of the respective array film and incubated overnight at 2-8 °C on a roller mixer. Each array film was removed from its 50mL plastic tube with sterile tweezers and placed in a clean 50mL plastic tube with 20 mL of 1X Wash Buffer (made according to manufacturer's instructions) and replaced on the roller mixer for 10 minutes. The 1X Wash Buffer was then aspirated and discarded and a further 20mL of IX Wash Buffer was added and the 50mL plastic tube with array was replaced on the roller mixer for a further 10 minutes. The wash procedure was then repeated for a total of three washes.

Biotinylated Detection Antibody binding

30 mcL of Detection Antibody Cocktail was added to 1.5 mL of 1X Array Buffer 4/6 (made according to manufacturer's instructions). 1.5 mL of diluted Detection Antibody Cocktail was added to a clean 50mcL plastic tube, one for each array film. Each array film were carefully transferred into the diluted Antibody Cocktail using sterile tweezers, taking care to allow the excess Wash Buffer to drain away and incubated on the roller mixer for 1 hour. Each array film was then washed three times as described in previous paragraph.

Streptavidin Horseradish Peroxidase

1X Streptavidin-HRP was made by adding 5mcL of 2000X Streptavidin-HRP to 9995mcL of Array Buffer 6. 2mL of 1X Streptavidin-HRP was added to a clean 50mL plastic tube, once for each array. Each array film was carefully removed from the Antibody Cocktail with sterile tweezers allowing excess wash buffer to drain off and transferred to the clean 50mL plastic tube containing 1X Streptavidin-HRP and replaced on the roller mixer for 30minutes at room temperature. The array films were then washed three times as described previously.

Chemi-luminescent detection

Without interruption each protein array film was removed from its plastic tube with care and placed on the bottom sheet of a clear plastic folder 10 x 12 cm that was open on three sides with the identification number facing up and the top sheet was replaced. Blotting the edges of the plastic wallet with a clean paper towel absorbed excess wash buffer. Chemi Reagent Mix was prepared immediately prior to use (according to manufacturer's instructions) and 1mL was pipetted evenly over each membrane. The top plastic sheet was carefully replaced and smoothed out on a paper towel to prevent air bubbles and absorb excess Chemi Reagent Mix. The array films plus Chemi Reagent Mix were incubated for 1 minute.

2.6.4 Imaging acquisition

The protein array membranes were then placed face down with identification numbers at the bottom of transmission mode C-DiGiT™blot scanner (LI-COR Biosciences UK Ltd, Cambridge, UK). Images were obtained using the Image Studio™ Software version 5 (LI-COR Biosciences) on high sensitivity setting. Resolution of images was adjusted by modulating the brightness and contrast to obtain optimum digital visibility of individual spots. These images were then saved as 16 bit.tif files to be exported and analysed in Image Quant TL version 8 (GE Healthcare Life Science, Bucks, UK).

2.6.5 Analysis

An array template grid of 10 x 24 small spots, one duplicate blot for each protein, was constructed in Image Quant TL version 8. Each grid spot with a positive pixel value was adjusted to insure it was wide enough to incorporate all pixels for that blot but not to overlap a duplicate blot or a different protein spot. Background pixel density was calculated for each protein spot duplicate by using a spot edge average for that pair. This was then subtracted from the total pixel density for individual spots and an average density of the duplicates was calculated. Spots were flagged as present at readings of 10 pixels or more. The signal values were then exported into Excel version 14.6.7 for Macintosh. Protein analyte mean pixel density (arbitrary units) for each patient group was semi-quantitatively compared visually by bar charts generated in Excel.

2.7 Statistical analysis

2.7.1 Patient Characteristics

Categorical variables were described in numbers and % and continuous variables summarized with median and interquartile ranges (IQR). Categorical variables were compared by the Fisher Exact test and differences between continuous variables were calculated by the Mann Whitney U test. Fetal fibronectin values were correlated with Spearman's rank test. Analyses were performed using SPSS (version 24) for Mackintosh.

2.7.2 Cytokines and T cell activation markers

Continuous variables summarized with median and IQR. Cytokine concentrations were log transformed to conform to normality assumptions. Cytokine ratios between pro-inflammatory and immune-regulatory cytokines were calculated. Second trimester cytokine slopes values between time points 1 and 3 were calculated for both CVF and plasma and third trimester plasma cytokine slopes were calculated between time points 3 and 5.

Comparisons of continuous variables between two groups were made by Mann Whitney U tests and comparisons across multiple groups were made using the Kruskal Wallis test. Spearman's rank test was used to explore correlations between cytokines and T cell activation markers and gestational age at sampling or delivery. The relationship between cytokines, cells and gestational age at delivery were modeling using mixed linear regression in SPSS. The models were adjusted for both mixed categorical and continuous potential confounding covariates: maternal age, BMI, ethnicity, and sampling time. T cell activation markers or log transformed cytokine concentrations were inputted as the second model factor, and patient ID was modeled as a random effect. Missing case data was excluded in a pair wise fashion.

2.7.3 Plasma–genital CVF cytokine gradients and immune compartment correlations

Genital-plasma cytokine gradients were calculated by dividing CVF cytokine concentrations by plasma cytokine concentrations for each time point. A value of >1 indicates that the cytokine was more concentrated in CVF compared to plasma. Ratios were compared by HIV status, across gestational age and correlated with gestational age at delivery and prematurity. Plasma-Genital cytokine ratios were also correlated with T cell subsets.

PMBC subsets and bacterial species abundance were correlated with log transformed CVF and plasma cytokine concentrations using Spearman's rank test. Vaginal polymorphonuclear leucocyte counts were converted to ordinal values (0=1; 1-5=2; 6-10=3; 11-20=4; 21-30=5, and 31-40=6) and correlated with log transformed CVF and plasma cytokine concentrations using Spearman's rank correlation test. Bonferroni correction for multiple comparisons (0.05/10) deemed a p value <0.005 as the threshold for significance. Due to the small sample, interesting results with a p value above threshold were not overlooked, as this was an exploratory analysis therefore maximum use of the data for hypothesis generation was made to ensure no potentially important trends were dismissed. It must be born in mind that this approach does increase risk of type 1 error.

Chapter 3. Clinical characteristics of the patient cohort

3.1 Participant characteristics

Participant characteristics are summarised in table 10. HIV-1 infected pregnant women were non-significantly older than uninfected women, 35 years vs. 33 years, p=0.092. HIV-1 infected pregnant women had a higher BMI than uninfected pregnant women, 27 vs. 22 respectively, p<0.0005. There was a low rate of smoking (<10%) in both groups. The ethnic diversity of HIV-1 infected pregnant women was significantly different from uninfected women with a larger proportion of black race (83% vs. 6%, p<0.0005) and lower proportion of Caucasian (9% vs. 66%, p<0.0005) and other races.

HIV-1 infected pregnant women were more likely to be multiparous (66% vs. 34%, p=0.004) compared to uninfected women and to have a known risk factor for PTB (20% vs. 13%, p=0.021). The most common risk factors for PTB in HIV-1 infected women were: prior history of PTB n=15, six HIV-1 infected women had a history of cervical surgery (e.g. Lower Loop Excision of the cervical Transformation Zone (LLETZ) for cervical intraepithelial neoplasia) or cervical cerclage for short cervix in pregnancy, five women had a history of hypertension, four had diabetes mellitus and three had fibroids. HIV-1 infected women had a higher rate of planned and emergency sections compared to uninfected women with a lower rate of vaginal deliveries, see table 10.

3.2 Preterm birth rate higher in HIV-1 infected women

HIV-1 infected pregnant women had a marginally lower median gestational age at delivery and higher rate of preterm birth compared to uninfected women, see table 10. Infants born to HIV-1 infected mothers had a lower birth weight than those born to uninfected mothers although this did not reach statistical significance.

Of the nine preterm deliveries to HIV-1 infected women: three had spontaneous preterm labour (SPTL) at 31, 32 and 33 weeks, one had PPROM at 34 weeks and

five had caesarean sections undertaken for medical grounds (two for intra uterine growth restriction (IUGR) at 31 and 32 weeks and three for foetal distress at 35, 36 and 36 weeks). Of the two preterm deliveries to uninfected women, one was PPROM at 36/40 and the other was induced at 35 weeks for IUGR.

There was also one stillbirth in the HIV-1 infected group at 39 weeks of unknown cause and one termination at 21 weeks in the uninfected group for bilateral renal agenesis.

Table 10 Study participant characteristics

Characteristic	HIV-1 infected pregnant women n=76	Uninfected pregnant women n=32	p value
Maternal age, Median (range)	35 (21-43)	33 (20-42)	0.092
Ethnicity, n (%) Caucasian Black Other	 7 (9) 63 (83) 6 (8)	 21 (66) 2 (6) 9 (28)	<0.0005*
BMI, kg/m^2 (IQR)	27 (22-35)	22 (21-25)	<0.0005*
Smoker, n (%) Yes No	 3 (4) 73 (96)	 3 (9) 29 (91)	0.261
Mode of delivery n (%) Vaginal Planned C Section Emergency C Section Missing	 23 (35) 17 (26) 26 (39) 9	 23 (77) 4 (13) 3 (10) 1	0.001
Parity, n (%) Nulliparous Multiparous Missing	 26 (34) 48 (66) 2	 21 (66) 11 (34) 0	0.004
PTB risk factors, n (%) Yes No	 15 (20) 61 (80)	 4 (13) 28 (87)	0.021
Prior PTB, n Cervical surgery Fibroids HTN Diabetes Other	15 6 3 5 4	1 2 1 0 1 1 hypothyroidism	
Cervical length at 22-26 weeks /mm Median (IQR)	32 (29-34)	30 (28-32)	0.274
FFN Median (IQR) ng/mL	--	7 (5-10)	
Median gestational age at delivery, weeks (IQR) Missing, n	39 (38-40) 6	40 (39-41) 1	0.009*
Birth outcome, n (%) Term Preterm Other	 60 (87) 9 (13) 1 IUD	 28 (90) 2 (6) 1 termination (anomaly)	0.159
Median birth weight /grams (IQR) Missing, n	3080 (2700-3300) 41	3200(3100-3500) 8	0.149

3.3 Antiretroviral drug exposure in HIV-1 infected pregnant women

A greater proportion of HIV-1 infected pregnant women conceived on ART as compared to started ART during pregnancy (76% vs. 24%), see table 11. The most common third drug belonged to the PI, or NNRTI class, 37% and 39% respectively. INSTI was the third drug for 17% of women. Of the 18 women who initiated treatment during pregnancy: 7 (39%) received INSTI based regimens, 7 (39%) received a PI and 4 (22%) received triple NRTI (ABC/3TC/ZDV). FTC/TDF were the NRTIs used in combination with a third drug in 64% of women with the remaining taking ABC/3TC.

Table 11 Timing and type of ART exposure during pregnancy

ART at conception, n (%)	
Yes	58 (76)
No	18 (24)
Median ART exposure /weeks (range)	161 (0-1116)
ART class, n (%)	
PI based	28 (37)
NNRTI based	30 (39)
INSTI based	13 (17)
CCR5I based	1
Triple NRTI (inc ZDV)	4
NRTI backbone, n	
FTC/TDF	47 (64)
ABC/3TC	27 (36)
Missing	2

PI= Protease inhibitor; NNRTI= Non-Nucleoside Reverse Transcriptase Inhibitor; INSTI= Integrase Nucleus Strand Transfer Inhibitor; CCR5I= Chemokine Receptor 5 Inhibitor; NRTI= Nucleoside Reverse Transcriptase Inhibitor; ZDV= Zidovudine; FTC=Emtricitabine; TDF= Tenofovir; ABC= Abacavir; 3TC= Lamivudine

3.4 Cervical length measurement at 22-26 weeks was normal for both HIV-1 infected and uninfected women

Median cervical length measured between week 22 and 26 were similar for HIV-1 infected (32 mm) and uninfected pregnant women (30 mm) and in the normal range, see table 10. A short cervix (<25mm) was identified in 2/14 HIV-1 infected pregnant women, both of whom went on to have a cervical cerclage in the local NHS premature birth clinic. One women, who had a history of cervical surgery and PTB, went on to deliver at 31.6 weeks and the other, who did not have a history of PTB, delivered at 37.6 weeks. None of the 26 uninfected pregnant women who underwent cervical length measurement had a cervical length <25 mm.

A second measurement of cervical length was obtained in 24 uninfected and 2 HIV-1 infected women at 27-31 weeks, due to the low number measurements from HIV-1 infected women, no meaningful comparisons can be made.

3.5 Fetal fibronectin measurements at 22-24 weeks in uninfected pregnant women were with in normal range and did not correlate with CVF measurements

Fetal fibronectin measurement at the posterior fornix was assessed in the initial pilot study optimizing collection of CVF in pregnant women in a subgroup of 18 uninfected pregnant women and found to be in the normal range (<50ng/mL), see table 10. Fetal fibronectin concentrations were measured in 12 CVF samples (diluted 1 in 4) from time points for which there was a matching validated value obtained with the Hologic™fFN kit. The CVF fFN values were then correlated with Hologic™fFN kit values and found not to be significantly associated, see figure 7 (rho=-0.31, p=0.334).

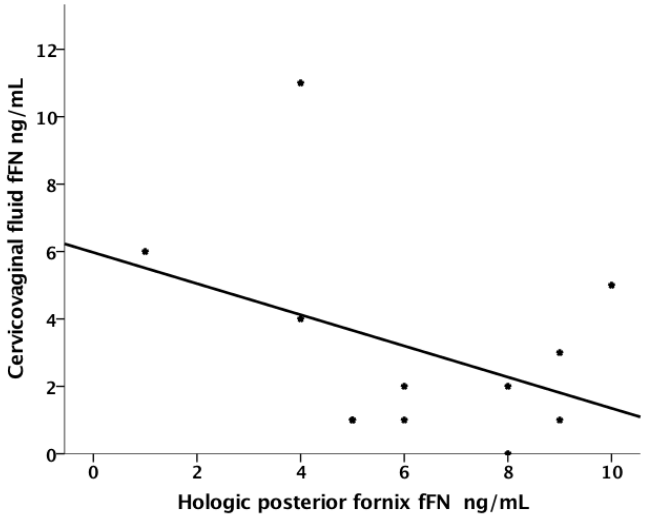


Figure 7 Correlation of Fetal fibronectin concentrations obtained by two different methods
Demonstrating no correlation between CVF fFN obtained by Dacron swab of the posterior vaginal fornix and tbe menstrual cup method, Spearman's rho=0.31, p0.334.

CVF collection with the soft cup was then adopted as the optimal collection methods and thus as FFN measurement could not be reliably extrapolated from CVF measurements, this part of the study was not continued.

3.6 Summary of results

- HIV-1 infected pregnant women had a non-significantly higher rate of PTB than uninfected women and a non-significant lower birth weight
- HIV-1 and uninfected pregnant women comprised of different racial diversity, with more HIV-1 infected women being of black ethnicity and more uninfected women being caucasian or asian
- There was a high prevalence of traditional risk factors for PTB represented with in the HIV-1 infected group e.g. prior PTB, LLETZ of the cervix
- Over half (5/9) of all PTB to HIV-1 infected women was indicated on medical grounds, 4/9 were spontaneous and just one was the result of PPROM
- Most HIV-1 infected women conceived on ART, with a similar number receiving a three drug combination with a Protease Inhibitor or a Non-Nucleoside Reverse Transcriptase Inhibitor and a smaller proportion receiving an Integrase Inhibitor.

3.7 Conclusions and interpretation

The HIV-1 infected pregnant women in this study are not optimally matched for comparison with the uninfected pregnant women. HIV-1 pregnant women demonstrate several factors that increase their background risk of PTB including black ethnicity, high BMI, past history of PTB(Goldenberg *et al.*, 2008). This may explain why these women exhibit higher rates of PTB, distinct from the role of antiretrovirals, and makes it more challenging to identify additional differences by drug exposure and timing in this heterogeneous group.

The high prevalence black ethnicity within the HIV-1 infected pregnant women reflects both the local population that the clinics serves and the majority ethnicity of women of reproductive age living with HIV worldwide (Short *et al.*, 2014; World Health Organization, 2015a; unaids.org, 2018). The low prevalence of black ethnicity with in the uninfected pregnant women reflects the ethnicity of women attending general antenatal clinics at the main recruitment NHS trust(MacIntyre *et al.*, 2015) and a change in practice to move "low risk" antenatal clinics into the community during the recruitment to this study, therefore limiting the available control women. In spite this there was a reasonable proportion of non-Caucasian women represented with in the uninfected group and comparisons can still be made of mechanistic factors between groups adjusted for ethnicity and other underlying risks for PTB.

The high rate of iatrogenic/medically induced PTBs observed in the HIV-1 infected pregnant women is similar to that previously described in historic data from our cohort and that of others(Lopez *et al.*, 2012; Short *et al.*, 2014) and will be discussed in more detail in later chapters.

The majority of HIV-1 pregnant women in this study conceived on antiretroviral therapy, reflecting trends in national HIV care as women are living longer and choosing to expand their families in the knowledge that extremely low rates of mother to child transmission are achievable with optimal management of HIV during pregnancy and birth(Raffe *et al.*, 2017; Gilleece *et al.*, 2018).

The antiretroviral therapies received during pregnancy with in this group of women includes equal numbers of women receiving PI-based cART and NNRTI-based cART. During the course of the study increasing numbers of women received INSTI-based regimes reflecting current prescribing practices(Bailey *et al.*, 2018). There were a small number of women in the study who received newer ART regimes with fewer available data in pregnancy. Two women received the NNRTI: Rilpivirine and two women received Dolutegravir, a newer INSTI which has subsequently had concerned raised around the rate of neural tube defects in women conceiving on this regimen in the Tsepamo Botswana birth defect surveillance study (Dorward *et al.*, 2018). This lead to the recommendation by the WHO in May 2018 that where alternative agents are available, DTG should avoided in women who express the desire to conceive(World Health Organization, 2018c). This highlights the benefits of consecutively recruiting prospective cohort studies such as this one in exploring safety and toxicity concerns around the use of newer antivirals in pregnancy and underlying mechanisms. The 2019 WHO guidelines have since recommended DTG as the first line treatment for all populations including pregnant women and those of childrearing potential with the caveat that in women and adolescent girls of childbearing potential the quality of evidence is still low and that EFV-based cART is a safe and effective alternative. They state that DTG appears to be safe when started later in pregnancy after the period of risk of neural tube defects (second trimester onwards) based on newer RCT evidence (Dolphin-1/2) (Khoo, Kintu and Malaba, 2019)

Regardless of the limitations of any comparison with uninfected pregnant women, this is the largest study of both systemic and local immunological factors in HIV-1 infected pregnant women.

Chapter 4. Systemic inflammation

4.1. Introduction

Studies of plasma and PBMCs in pregnancy have largely shown that systemic cytokine balance is likely to be more complex than a simple Th1/Th2 dichotomy. Similar pro-inflammatory plasma and FGT cytokine profiles, namely IL-1, IL-6, IL-8 and TNF-α have been demonstrated to be elevated in term labour as are increased in preterm labour, with the most evidence for Th17 inducing pro-inflammatory cytokine IL-6(Álvarez-De-La-Rosa *et al.*, 2000; Turhan, Karabulut and Adam, 2000; Murtha *et al.*, 2006). The lack of consistent identifiable pattern in systemic cytokine expression in pregnant HIV infected women, as in uninfected women, may be due to both study design and heterogeneity in ART exposure and timing. It remains unclear if ART induces a Th1/pro-inflammatory dominated profile or if certain ART e.g. ZDV can induce a more Th2/immune-regulatory cytokine profile which is favourable for term delivery.

We examine peripheral markers of T cell activation and plasma cytokines in HIV-infected and uninfected pregnant women, purposely sampled by ART exposure, and explore associations with gestational age at delivery.

Chapter objectives

- To characterize peripheral T cell markers of immune activation in a group HIV-1 infected and uninfected pregnant women across the second and third trimester
- To compare cellular immune activation across gestation and how these differ by HIV status, cART (drug class, timing of exposure) and gestational age at delivery.
- To characterize plasma pro-inflammatory (IFN-γ, IL-1β, IL-2, IL-8, IL-12 and TNF-α) and immune-regulatory cytokine (IL-4, IL-6, IL-10 and IL-13) profiles in a larger more immunologically matched group of HIV-infected and uninfected pregnant women across the second and third trimester
- To compare plasma cytokines across gestation and how these differ by HIV status, cART (drug class, timing of exposure) and birth outcome.

4.2 T cell activation Results

4.2.1 Baseline immunophenotype

HIV-1 infected pregnant women had a lower absolute CD4+ count at study entry compared to uninfected mothers, see table 12 (632 vs. 930 cells/mcL, p<0.005) and a similar CD8+ T cell count (665 vs. 603 cells/mcL, p=0.09). Both % activated (HLA-DR+) CD4+ and CD8 +T cells were significantly higher at baseline in HIV-1 infected women, see table 12, p<0.0005. There was a non-significant trend towards a higher baseline CD4+CD25+ % in HIV-1 infected women compared to uninfected women at baseline and no difference in CD8+CD25+% between groups.

Table 12 Participant characteristics at study baseline

Characteristic	HIV-1 infected pregnant women	Uninfected pregnant women	p value
CD4+ cell count at entry /mcL, median (IQR)	632 (495-751)	930 (732-1058)	<0.0005*
CD8+ cell count at entry /mcL, median (IQR)	665 (526-891)	603 (439-728)	0.089
HIV viral load at entry copies/mL, median (range)	<20 (<20-18522)	NA	-
CD4+CD25+%	25.5 (19.0-30.0)	22.5 (19.0-28.0)	0.337
CD4+HLA-DR+%	8.0 (5.0-10.0)	5.0 (4.0-6.8)	<0.0005*
CD8+CD25+ %	5.5 (5.0-7.0)	6.0 (5.0-7.8)	0.440
CD8+HLA-DR+ %	26.0 (18.0-34.8)	13.0 (8.0-19.5)	<0.0005*

4.2.2 T cell activation was higher throughout gestation in HIV-1 infected pregnant women compared to uninfected pregnant women.

Absolute and % CD4+ T cells were significantly lower throughout pregnancy in HIV-1 infected women, p<0.003, see figure 8. Conversely, CD8+% were higher in HIV-1 infected pregnant women compared to uninfected pregnant women, p<0.0005.

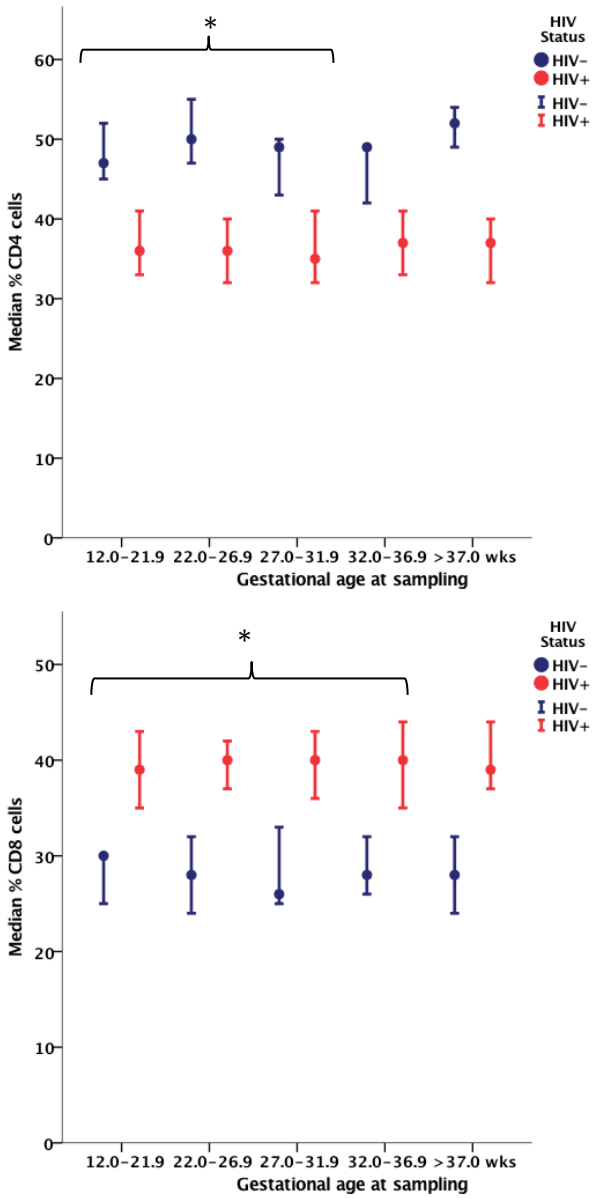


Figure 8 Percentage of CD4+ T cells and CD8+ T cells across gestation by HIV status
Demonstrating higher CD8+% T cells and lower CD4+ % and in HIV-1 infected pregnant women compared to uninfected pregnant women,* = p<0.0005; Ψ = p<0.005; Ŧ =p<0.05.

Activated CD4+HLA-DR + and CD8+ HLA-DR + T cells were significantly higher across all time-points in HIV-1 infected compared to uninfected pregnant women, p<0.005, see figure 9.

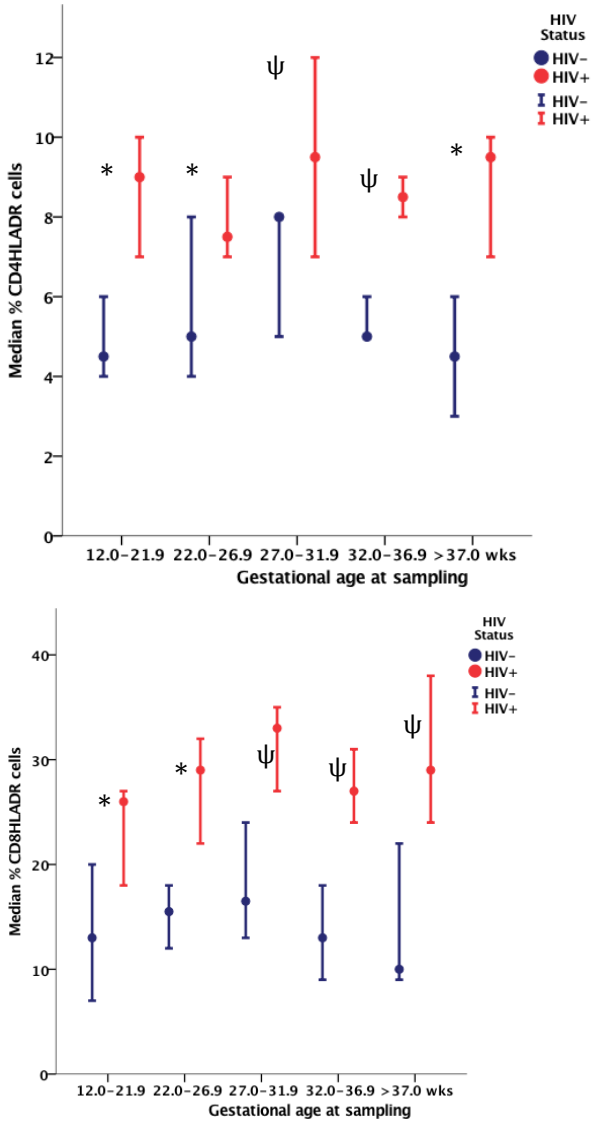


Figure 9 CD4+CD8+ T cell activation markers across gestation by HIV status
Demonstrating higher % CD4+ and % CD8+ T cell activation in HIV-1 infected pregnant women compared to uninfected, * = p<0.0005; Ψ = p<0.005; Ŧ =p<0.05.

In addition, CD4/CD8 ratios were lower in HIV-1 infected women throughout the period of sampling, p<0.0005, see figure 10. There was a non-significant trend towards higher CD4+ CD25+ and CD8+ CD25+ cells in HIV-1 infected women, see figure 11.

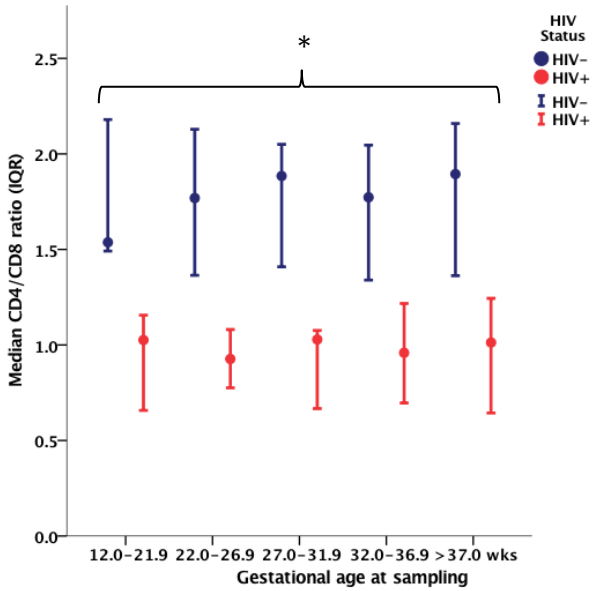


Figure 10 CD4/CD8 cell ratios across gestation by HIV status
Demonstrating lower CD4/CD8 T ratios across pregnancy in HIV-1 infected pregnant women compared to uninfected, = p<0.0005; Ψ = p<0.005; Ŧ =p<0.05.

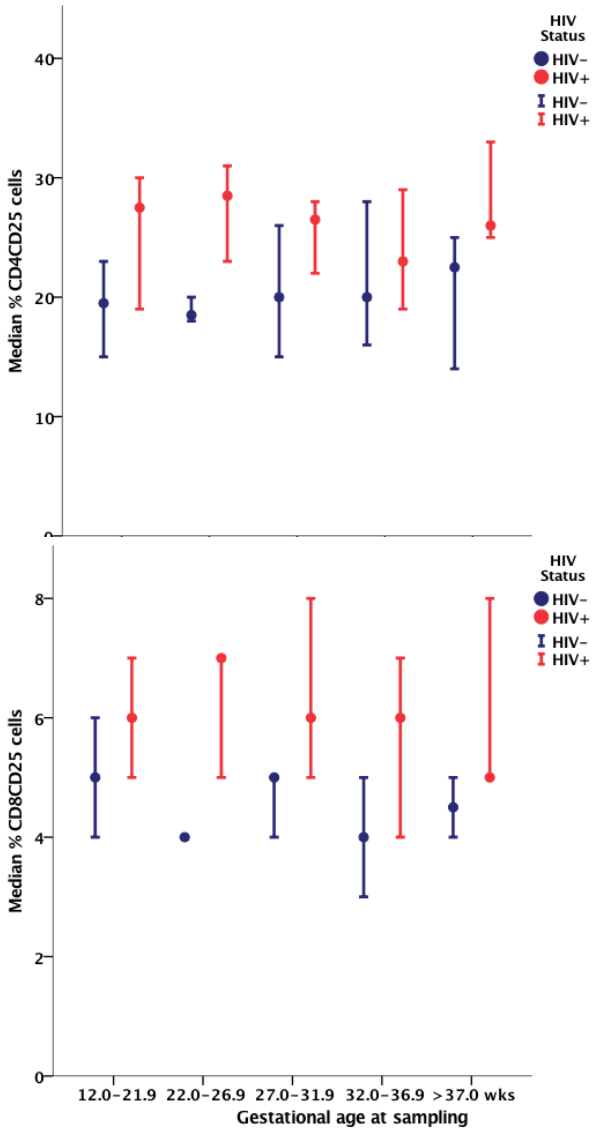


Figure 11 Percentage of CD4+CD25+ T cells and CD8+CD25+ T cells across gestation by HIV status, Demonstrating a trend towards a higher % CD25+ expression on CD4+ and CD8+ T cells in HIV-1 infected compared to uninfected pregnant women, * = p<0.0005; Ψ = p<0.005; Ŧ =p<0.05.

Correlations between T cell activation markers and the gestational age at sampling, including all women, were explored. CD4+CD25+ % was positively correlated with increasing gestation of pregnancy (rho=0.11, p=0.04) when all groups were included in the analysis. Considering HIV-1 infected women alone, there remained a trend toward this correlation (rho=0.12, p=0.071). No other correlations between T cell markers and gestation of sampling during pregnancy were observed.

4.2.3 T cell activation was inversely associated with gestational age at delivery.

Associations between T cell markers and gestational age at delivery were explored in all women combined to obtain maximum power. There was a negative association between absolute and CD8+%, CD4+ HLA-DR+% and CD8+ HLA-DR+% and gestational age at delivery, see table 13.

Positive correlations were observed between gestational age at delivery and CD4+ % and CD4/CD8 ratio, see table 13. The significance of these observations was not retained when analysing HIV-1 infected women alone, with the exception of absolute CD8+ T cell count (rho =-0.13, p=0.042) and CD8+HLA-DR+ % (rho=-0.15, p=0.004).

Table 13 Spearman's correlation coefficient for T cell markers and gestational age at delivery

T cell subtype	Spearman's rho	p value
CD4+%	0.114	0.030
Absolute CD8+	-0.178	0.001
CD8+%	-0.181	0.001
CD4+HLA-DR+%	-0.127	0.022
CD8+HLA-DR+%	-0.134	0.016
CD8+CD25+%	0.099	0.074
CD4/CD8 ratio	0.149	0.004

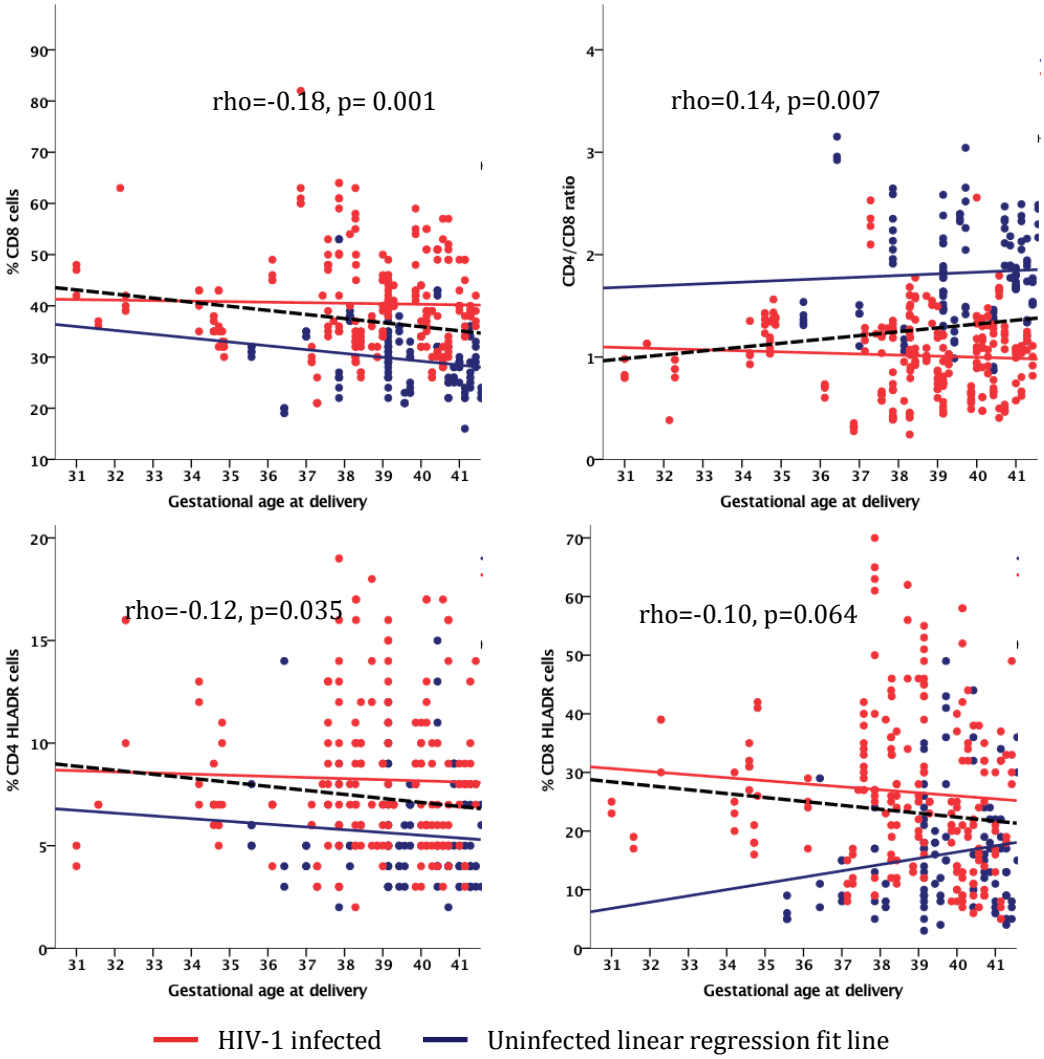


Figure 12 Scattergraphs of CD4+ CD8+ T cell markers against gestational age at delivery /weeks Demonstrating a positive correlation between CD4/CD8 ratio and gestation age at delivery and negative correlations between gestational age at delivery and markers of Immune activation CD8%, CD8HLADR+% and CDD4HLADR+%, rho=Spearman's correlation co-efficient and ----- =linear regression fit line for both HIV-1 infected and uninfected pregnant women combined

Next, hierarchical linear regression, adjusted for BMI, age, ethnicity and sampling time, was performed to evaluate the predictive capacity of peripheral T cell markers for gestational age at delivery, see table 14. This revealed an inverse relation with total and CD8+ %, CD4+HLA-DR+ % and a trend towards a relationship with CD8+HLA-DR+ %, see figure 12 above. A positive relation with CD4/CD8 ratio and gestational age at delivery was also observed. The significance of these observations was not retained when analysing HIV-1 infected women alone.

Table 14 Linear regression of T cell markers with gestational age at delivery adjusted for gestational age at sampling

T cell subtype	Fold change	Estimate	Standard Error	F value	Q value
Absolute CD8+	7.56	-0.14	7.55	4.14	0.006
CD8+ %	11.60	-0.18	9.83	5.80	0.001
CD4+HLA-DR+%	4.48	-0.12	0.09	2.48	0.035
CD8+HLA-DR+%	3.47	-0.10	0.34	2.05	0.064
CD4/CD8 ratio	7.28	0.14	0.01	3.74	0.007

4.2.4 Preterm Birth was associated with lower absolute CD4+ T cell count and higher CD8+%

Median absolute CD4+ T cell count was higher in all women delivering at term compared to preterm (739 vs. 576 cells/mcL, p=0.002). The same women had a lower median CD8+% compared to those delivering preterm (35% vs. 40%, p=0.036). No difference was observed in markers of T cell activation between these groups.

The same observation was made comparing term and preterm deliveries to HIV-1 infected women alone. Women who delivered at term had a greater median absolute CD4+ T cell counts compared to those who delivered preterm (632 vs. 532 cells/mcL, p=0.024) and a lower median CD8+ % (39% vs. 43%, p=0.021).

4.2.5 Women initiating ART after conception display higher T helper cell activation throughout pregnancy

Next, T cell activation markers were compared by ART exposure, comparing HIV-1 infected pregnant women who conceived on ART with those who initiated ART

post conception. Median T cell activation markers at the five different sampling times during pregnancy were compared by ART exposure see figure 13. Women who initiated ART post conception had a trend towards a lower CD4+% T cells and higher absolute and CD8+% T cells compared to those who conceived on ART although this did not reach statistical significance.

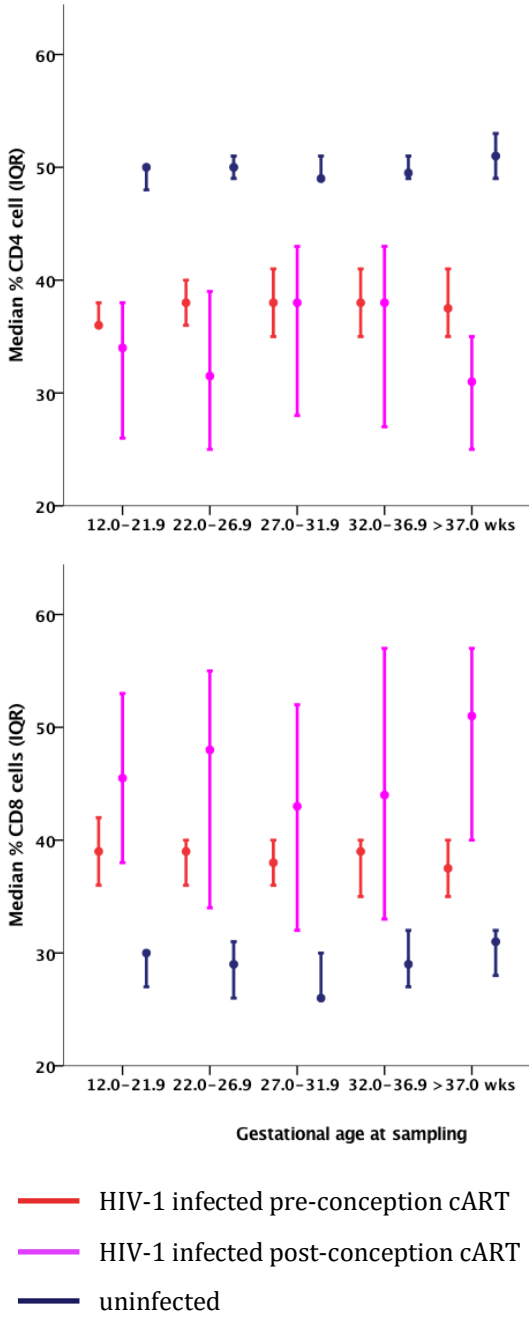


Figure 13 Median CD4+ CD8+ T cell percentages across gestational age by ART exposure in relation to conception
Demonstrating a trend towards a higher %CD4+ T cells and lower %CD8+ T cells in pregnant women conceiving on cART compared to those who initiated cART in pregnanc, which is sustained across gestation

Higher median % activated CD4+ and CD8+ (HLA-DR+) cells were observed in women who initiated ART post conception compared to those who conceived on ART, p<0.05 across gestation, see figure 14.

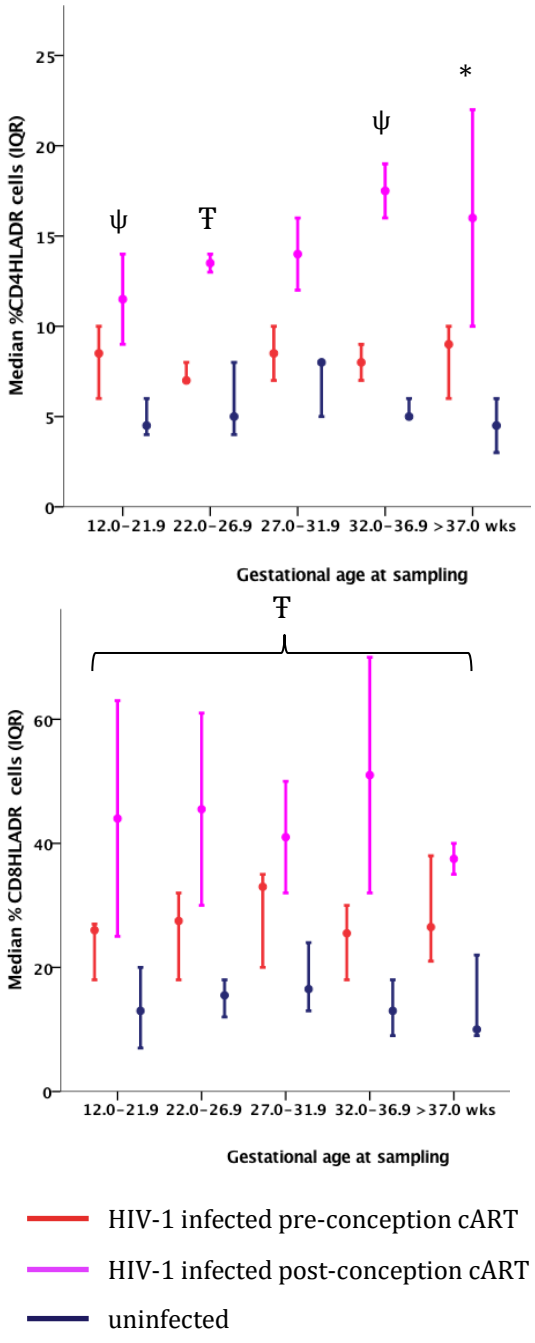


Figure 14 Median CD4+ CD8+ HLA-DR+ T cell percentage across gestational age by ART exposure in relation to conception
Demonstrating higher HLA-DR+ T cell activation in those women who initiated cART in pregnancy compared to those who conceived on cART, which is sustained across gestation, * = p<0.0005; Ψ = p<0.005; Ŧ =p<0.05.

These women also had a lower CD4+CD25+%, CD8+CD25+ % and CD4/CD8 ratios than women conceiving on ART but this did not reach statistical significance at all time points, see figure 15.

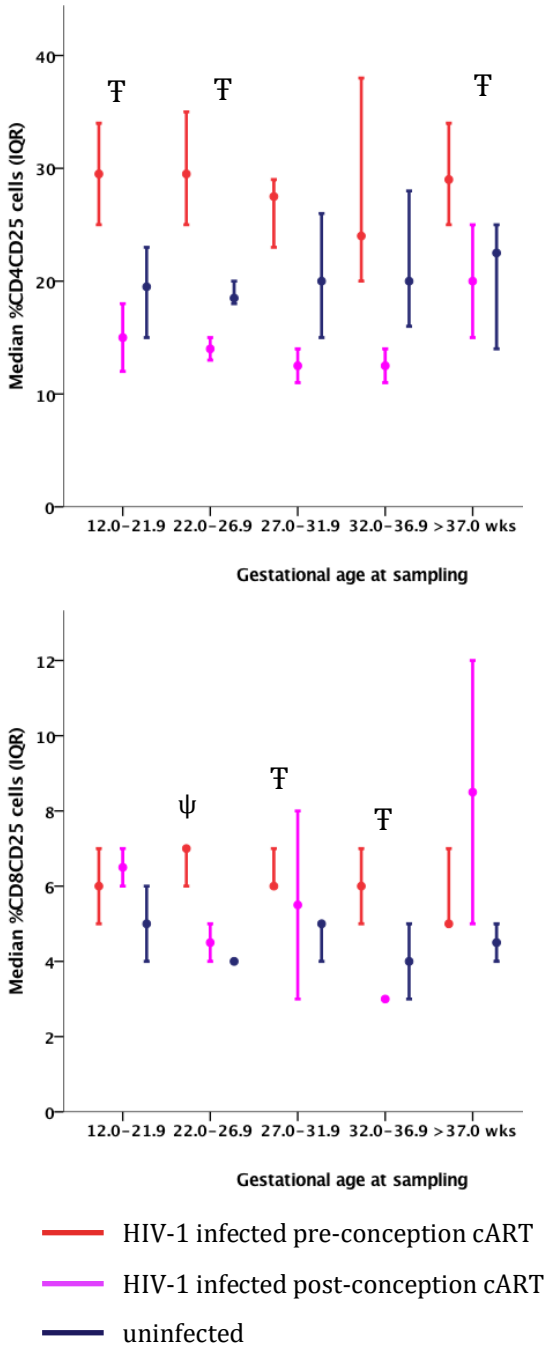


Figure 15 Median CD4+CD8+CD25+ T cell percentages across gestational age
Demonstrating a higher percentage expression of CD25+ on CD4+ T cells and CD8+ T cells in pregnant women conceiving on cART compared to those who initiated cART in pregnancy,* = p<0.0005; Ψ = p<0.005; Ŧ =p<0.05

T cell activation markers were then correlated with time on ART (weeks), see table 15. There was a significant positive association between length of time on ART and CD4%, CD4+CD25+%, CD8+CD25+% and CD4/CD8 ratio. There was an inverse correlation between length of time on ART and CD8% and % activated (HLA-DR+) CD4 and CD8 cells.

Table 15 Spearman's correlation coefficient for T cell markers and log 10 ART exposure /weeks

T cell subtype	Spearman's rho	p value
CD4+%	0.286	<0.0005
CD8+%	-0.162	0.025
CD4+ HLA-DR+%	-0.263	0.016
CD4+ CD25+%	0.185	0.001
CD8+ HLA-DR+%	-0.244	<0.0005
CD8+ CD25+%	0.331	0.001
CD4/CD8 ratio	0.219	0.002

Hierarchical linear regression analysis of log^{10} ART exposure with T cell markers adjusted for gestational age at sampling revealed relationships in the same polarity that were observed in the correlation analysis with the exception of the CD4/CD8 ratio which was no longer retained significance, see table 16.

Table 16 Linear regression of T cell markers with log^{10} ART exposure/ weeks adjusted for gestational age at sampling

T cell subtype	Fold change	Estimate	Standard Error	F value	Q value
CD4+%	6.82	0.19	0.84	3.45	0.01
CD8+%	2.92	-0.13	0.92	2.01	0.089
CD4+HLA-DR+%	13.2	-0.28	0.36	6.71	<0.0005
CD4+ CD25+%	3.89	0.15	0.89	2.26	0.05
CD8+ HLA-DR+%	10.0	-0.24	1.37	5.35	0.002
CD8+ CD25+%	11.8	0.26	0.28	5.92	0.001
CD4/CD8 ratio	0.3	0.04	0.04	0.17	0.578

4.2.6 Systemic immune activation during pregnancy alters by exposure to different classes of ART

Median T cell activation markers in HIV-1 infected women through pregnancy by exposure to different ART classes (PI, NNRTI and INSTI based) are presented in Figure 16. Significant differences were observed between these treatment groups in absolute CD4+ T cells and CD4+%, CD8+% and % activated (HLA-DR+) CD4+ and CD8+ T cells but not CD4+CD25+% and CD8+CD25+% or CD4/CD8 ratio. Comparing women receiving PI-based ART to non PI -based regimes, the PI group displayed a trend for lower CD4+%, CD4+CD25+% (except at time point 4) and higher absolute CD8+ T cells, CD8+% and CD8+HLA-DR+% (except at time point 2) compared to women receiving NNRTI-based ART.

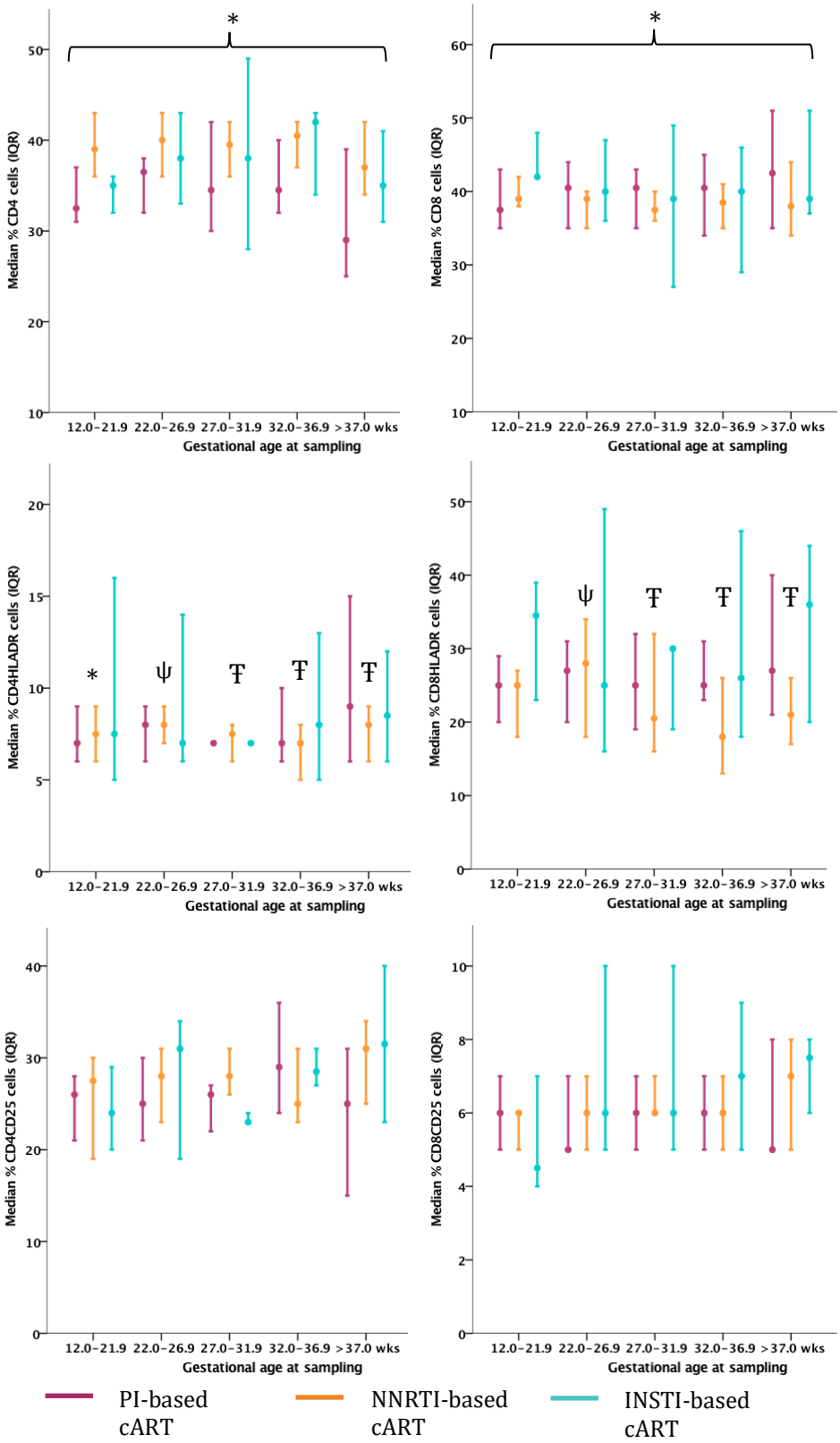


Figure 16 CD4+ CD8+ T cell activation markers across gestation by ART class
Demonstrating differences in % of CD4+ CD8+ T cells and % HLA-DR+ expression between ART types, specifically lower %CD4+ T cells and higher %CD8+ T cells and HLA-DR expression in CD8+ T cells were observed in pregnant women receiving PI-based cART compared to pregnant women receiving NNRTI-based cART which was sustained across gestation, * = p<0.0005; Ψ = p<0.005; Ŧ =p<0.05

There was a trend towards lower CD4/CD8 ratios in the PI group compared to women receiving either NNRTI or INSTI based ART from 22 weeks onwards, see figure 17. These associations did not maintain significance in the post hoc analysis, presented in Table 17.

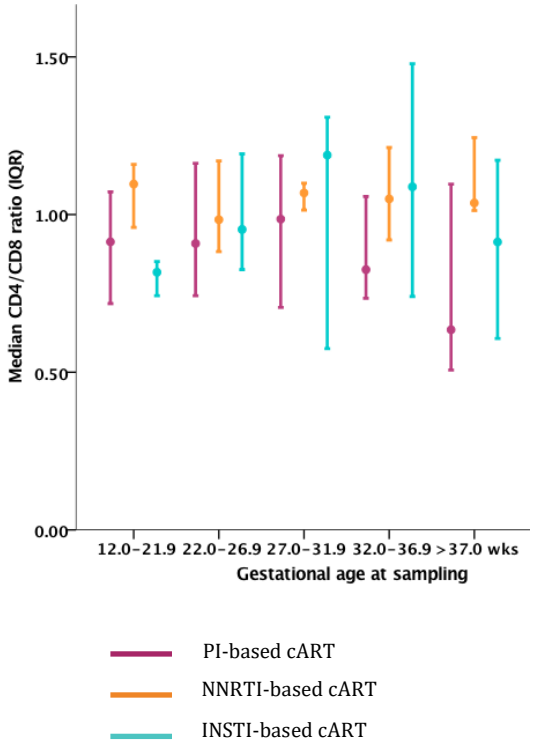


Figure 17 CD4/CD8 ratio across gestation by ART class
Demonstrating a trend towards a lower CD4/CD8 ratio in pregnant women receiving PI-based cART compared to pregnant women receiving NNRTI-based cART which is sustained across gestation

Table 17 Post hoc analysis comparing median measurements of T cell subtypes by ART class

NS= p value >0.2

Class of therapy	PI vs. non PI p value					NNRTI vs. non NNRTI p value					INSTI vs. non INSTI p value				
Gestational age /weeks	12.0-21.9	22.0-26.9	27.0-31.9	32.0-36.9	≥37 wks	12.0-21.9	22.0-26.9	27.0-31.9	32.0-36.9	≥37 Wks	12.0-21.9	22.0-26.9	27.0-31.9	32.0-36.9	≥37 wks
Total CD4	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
% CD4	0.085	NS	0.138	0.056	0.081	0.009	NS	0.131	0.165	0.080	NS	NS	NS	NS	NS
Total CD8	NS	NS	NS	NS	0.031	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
% CD8	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.137	NS	NS	NS	NS
%CD4 HLA-DR+	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
%CD8 HLA-DR+	NS	NS	NS	NS	NS	0.134	NS	NS	0.093	0.075	NS	0.071	NS	NS	NS
CD4/CD8 ratio	NS	NS	NS	0.167	0.123	0.091	NS	NS	NS	0.160	0.169	NS	NS	NS	NS

4.2.7 Peripheral T cell activation is inversely associated with %CD4+CD25+ cells in HIV-1 infected and uninfected women

Correlation analysis was performed between T cell phenotypes for HIV-1 infected parturients, see table 18. Activated CD8+HLA-DR+ cells were positively correlated with CD4+HLA-DR+% and CD8+%, $p<0.0005$ and inversely correlated with CD4+% and CD8+CD25+%, $p<0.03$. Activated CD4+ HLA-DR+ cells were also positively correlated with both CD8+HLA-DR+% and CD8+%, $p<0.0005$ and inversely correlated with CD4+ % and CD4+CD25+%. On exploring these correlations in uninfected women, there was a positive correlation between CD8+ HLA-DR+% and CD4+ HLA-DR+% and a negative correlation between CD8+HLA-DR+% and %CD4+CD25+% but not between CD4+HLA-DR+% and CD4+CD25+%, see table 19.

Table 18 Correlations between T cell phenotypes in HIV-1 infected pregnant women

T cell phenotype	CD8+HLA-DR+%		CD4+HLA-DR+%	
	rho	p value	rho	p value
CD4+%	-0.43	<0.0005	-0.43	<0.0005
CD8+%	0.46	<0.0005	0.46	<0.0005
CD4+CD25+%	0.06	0.41	-0.15	0.02
CD8+CD25+%	-0.14	0.03	-0.07	0.28
CD4+HLA-DR+%	0.78	<0.0005	-	-
CD8+HLA-DR+%	-	-	0.78	<0.0005

Table 19 Correlations between T cell phenotypes in uninfected pregnant women

T cell phenotype	CD8+HLA-DR+%		CD4+HLA-DR+%	
	rho	p value	rho	p value
CD4+%	-0.02	0.83	-0.05	0.62
CD8+%	-0.07	0.48	0.07	0.49
CD4+CD25+%	-0.10	0.27	0.19	0.04
CD8+CD25+%	0.18	0.06	0.50	<0.0005
CD4+HLA-DR+%	0.55	<0.0005	-	-
CD8+HLA-DR+%	-	-		

4.2.8 Summary of T-cell activation results

1. Immune activation of circulating CD4+ and CD8+ T cells as measured by HLA-DR% expression was higher in HIV-1 infected pregnant women than uninfected pregnant women throughout pregnancy

2. The percentage of activated T cells correlated inversely with gestational age at delivery when considering all women, HIV-1 infected and uninfected. CD8+HLA-DR+ % remained inversely correlated with gestational age at delivery in the analysis of HIV-1 infected pregnant women alone.

3. HIV-1 infected pregnant women delivering preterm had a lower median CD4+ T cell count and higher CD8+% than women delivering at term but no statistical difference in activated T cells was observed.

4. Initiating ART during the pregnancy was associated with higher levels of activation in circulating T-cells (HLA-DR+) and lower CD4+ CD25+% compared to HIV-1 infected women who conceived on ART.

5. The duration of ART exposure was inversely correlated with circulating activated T cells during pregnancy and positively correlated with CD4+%, CD4+CD25+% and CD4/CD8 ratio.

6. Women receiving PI-based ART displayed a trend towards a higher % of activated CD8+HLA-DR+ T cells and lower CD4/CD8 ratios compared to women receiving NNRTI-based ART, however this was not statistically significant.

4.3 Systemic plasma cytokine concentrations Results

4.3.1 Plasma cytokine concentrations in HIV-1 infected women are generally lower than in uninfected pregnant women

Median plasma cytokine concentration across all measured time points by HIV status are presented in Figure 18. Plasma cytokine concentrations were higher in uninfected pregnant women compared to HIV-1 infected women with the exception of IL-2, $p<0.01$. The highest concentration of all the measured plasma pro-inflammatory cytokines during pregnancy for both groups was IFN-γ (uninfected median=7.50 pg/mL (IQR 5.16- 12.60) vs. HIV-1 infected median =5.44pg/mL (IQR 2.25-9.09), $p <0.0005$), followed by IL-8 (uninfected median= pg/mL (IQR) vs. HIV-1 infected median =pg/mL (IQR), $p <0.0005$, TNF-α (uninfected median=2.33 pg/mL (IQR 1.58- 3.41) vs. HIV-1 infected median =1.63 pg/mL (IQR 1.21-2.51), $p <0.0005$). Of the plasma immune-regulatory cytokines, IL-13 (uninfected median=3.41 pg/mL (IQR 1.38- 6.11) vs. HIV-1 infected median =1.48 pg/mL (IQR 0.82-3.09), $p <0.0005$) and IL-10 (uninfected median=0.74 pg/mL (IQR 0.39- 1.73) vs. HIV-1 infected median =0.32 pg/mL (IQR 0.21-0.46), $p <0.0005$) were measured at the highest concentrations for both groups.

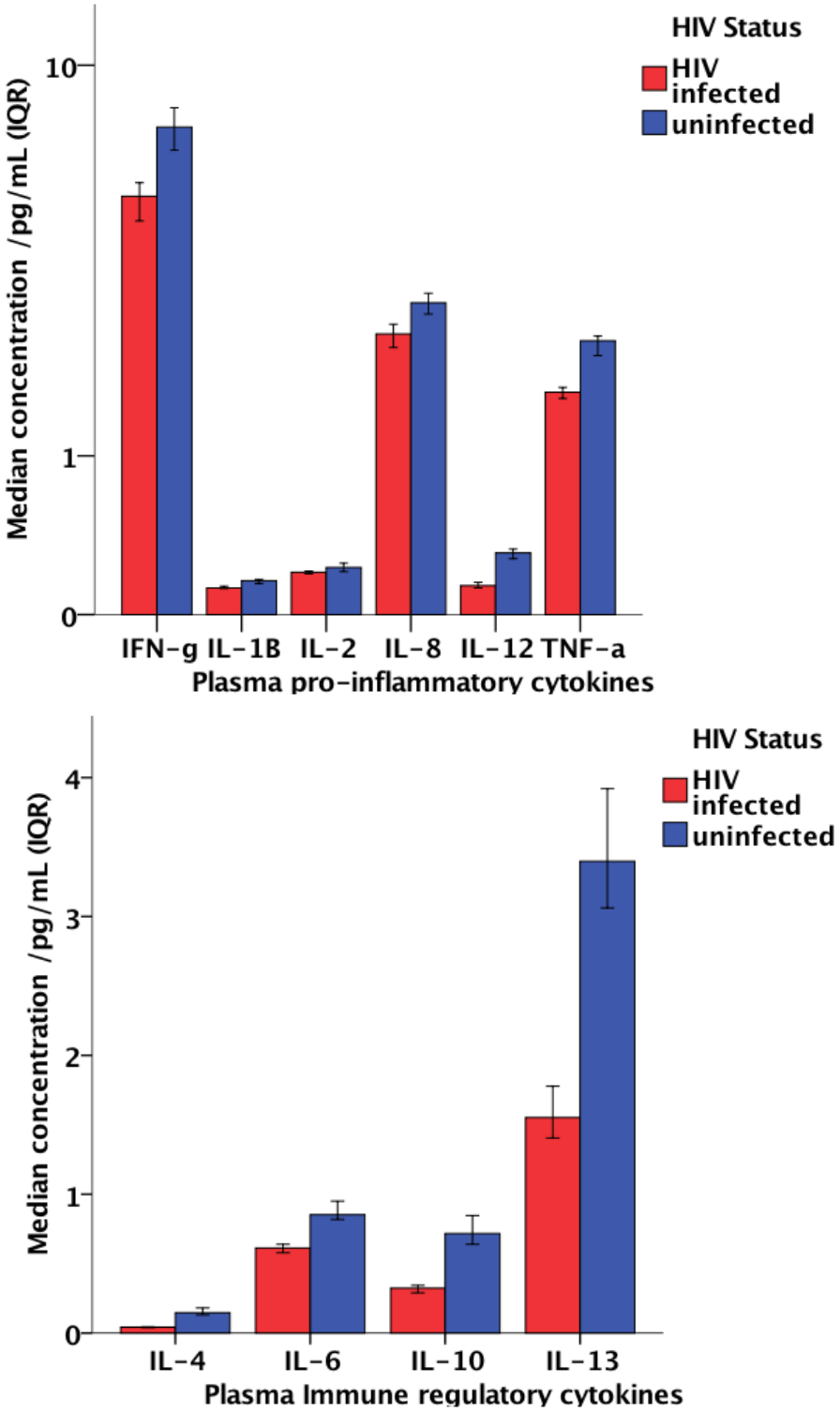


Figure 18 Median plasma cytokine concentration across pregnancy by HIV status

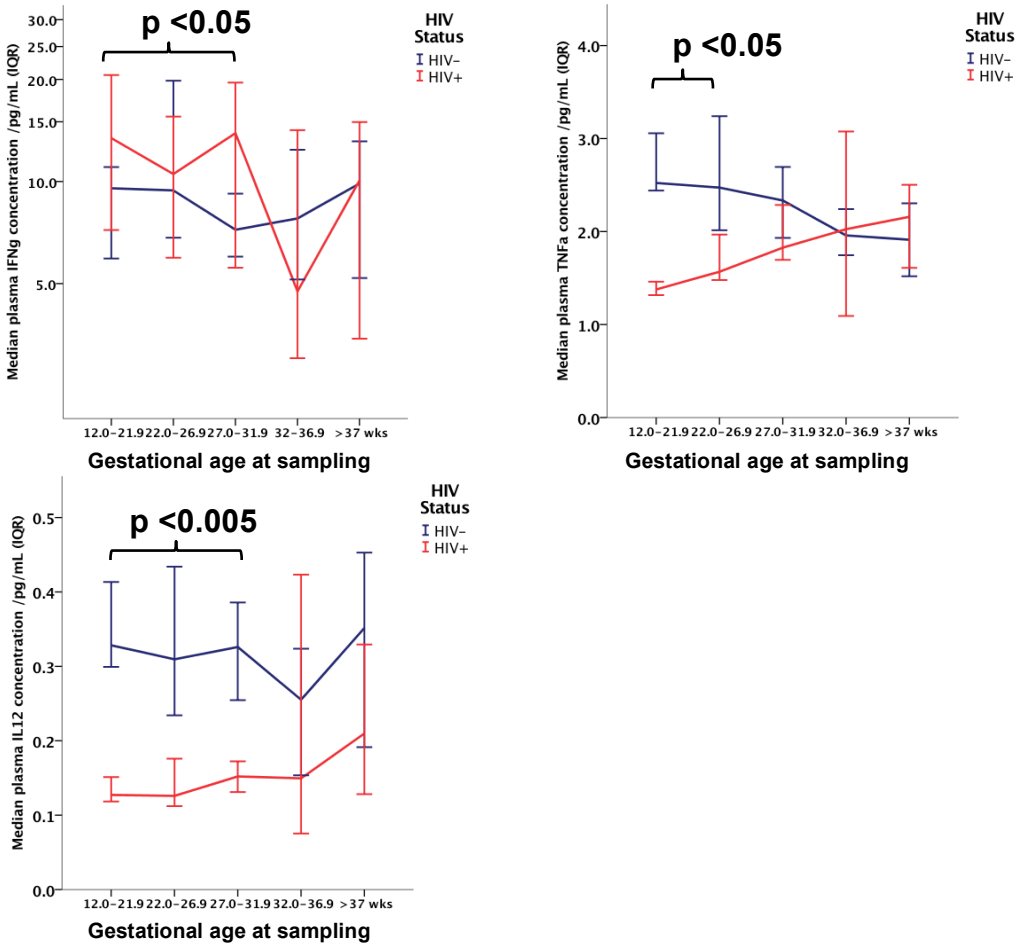


Figure 19 Plasma pro-inflammatory cytokine concentrations across pregnancy by HIV status
Demonstrating a lower pro-inflammatory plasma cytokine concentrations in HIV-1 infected pregnant women compared to uninfected pregnant women across gestation

4.3.2 HIV-1 infected women displayed higher plasma pro-inflammatory IFN-γ concentrations than uninfected pregnant women to week 32 and lower IL-12 and TNF-α during the second and third trimesters.

Considering differences between HIV-1 infected and uninfected women across the second and third trimester at each of the five sampling times, revealed fewer statistical differences in cytokines. HIV-1 infected women had a higher median IFN-γ during the second trimester up to week 32 compared to uninfected women ($p<0.05$) see figure 19. This changed from week 32 so that uninfected women had higher plasma IFN-γ concentrations although this did not reach statistical significance by HIV status. Conversely, TNF-α plasma concentrations were lower in HIV-1 infected women than uninfected women during the second trimester ($p<0.05$) and increased during pregnancy to week 32 where they exceeded the level observed in uninfected pregnant women, see figure 19, again this did not

reach statistical significance. IL-12 plasma concentrations were lower in HIV-1 infected women compared to uninfected women throughout pregnancy although this was only significant up to week 32.

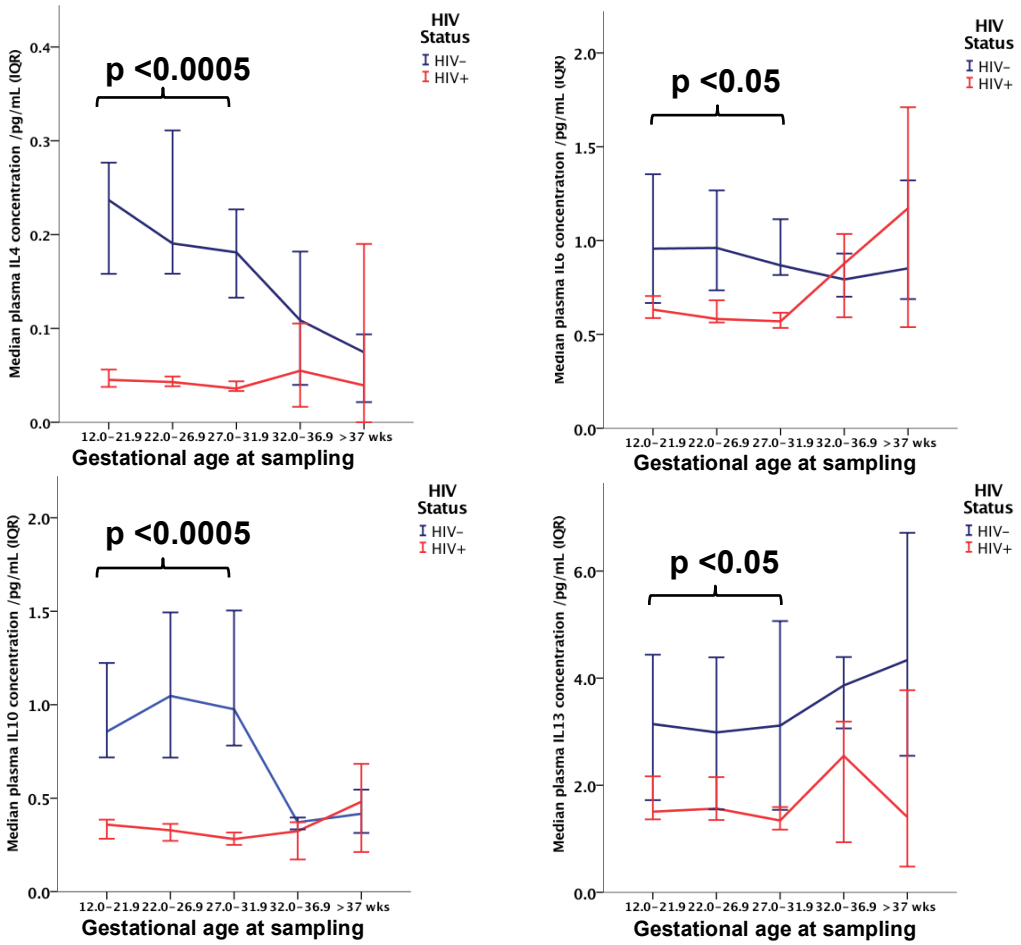


Figure 20 Plasma immune -regulatory cytokine concentrations across pregnancy by HIV status
Demonstrating a lower immune -regulatory plasma cytokine concentrations in HIV-1 infected pregnant women compared to uninfected pregnant women across gestation

4.3.3 HIV-1 infected pregnant women had lower immune-regulatory plasma IL-4, IL-6, IL-10 and IL-13 concentrations up to week 32.

Plasma immune-regulatory cytokines IL-4, IL-6, IL-10 and IL-13 were significantly lower in HIV-1 infected compared to uninfected pregnant women up to 32 weeks, $p<0.05$, see figure 20. IL-6 plasma concentrations increased from week 32 in HIV-1 infected women but this was not significant compared to uninfected women. No other significant differences in patterns of plasma cytokine expression by HIV status were observed across gestation.

4.3.4 Plasma pro-inflammatory and immune-regulatory cytokines are significantly intercorrelated in HIV-1 infected women.

Cytokine intercorrelations were explored in HIV-1 infected pregnant women. Th1 pro-inflammatory cytokines TNF-α, IL-1β and IL-12 were highly inter correlated and also positively correlated with immune-regulatory Th2 IL-4 and IL-13, see table 20. Pro-inflammatory chemokine IL-8 was positively correlated with Th1 cytokines: TNF-α, IL-1β, IL-12 and with the T regulatory cytokine IL-10. Th17 inducing cytokine IL-6 positively correlated with pro-inflammatory Th1 (IFN-γ, IL-1β, IL-2, TNF-α), Th2 (IL-4, IL-13) and T regulatory (IL-10) cytokines.

Table 20 Plasma cytokine intercorrelations in HIV-1 infected pregnant women

rho= spearman's correlation coefficient, * p<0.05, **p<0.0005

	IFN-γ		IL-1β		IL-2		IL-8		IL-12		TNF-α		IL-4		IL-6		IL-10		IL-13	
	rho	**P**	**rho**	**p**	**rho**	**p**	**rho**	**p**	**rho**	**p**	**rho**	**p**	**rho**	**p**	**rho**	**p**	**rho**	**p**	**rho**	**p**
IFN-γ	1.00	-	0.16*	0.04	0.17*	0.03	0.09	0.26	0.01	0.91	-0.16	0.03	0.00	0.99	0.27*	**	0.06	0.42	-0.01	0.92
IL-1β	0.16*	0.04	1.00	-	0.12	0.13	0.39	**	0.35*	**	0.20*	0.007	0.33*	**	0.15*	0.05	0.12	0.11	0.29*	**
IL-2	0.17*	0.03	0.12	0.13	1.00	-	0.04	0.61	0.26*	0.001	0.31*	**	-0.08	0.27	0.18*	0.02	0.10	0.19	0.26*	**
IL-8	0.09	0.26	0.39*	**	0.04	0.61	1.00	-	0.25*	**	0.26*	0.001	0.09	0.25	0.00	0.99	0.13	0.09	0.47*	**
IL-12	0.01	0.91	0.35*	**	0.26*	0.001	0.25*	0.001	1.00	-	0.37*	**	0.37*	**	0.08	0.33	0.24*	0.001	0.12	0.11
TNF-α	-0.16*	0.03	0.20*	0.007	0.31	**	0.26*	0.001	0.37*	**	1.00	-	0.16*	0.03	0.20*	0.007	0.24*	0.001	0.30*	**
IL-4	0.00	0.99	0.33*	**	-0.08	0.27	0.09	0.25	0.37*	**	0.16*	0.03	1.00	-	0.30*	**	0.47*	**	0.68*	**
IL-6	0.27*	**	0.15*	0.05	0.18*	0.02	0.00	0.99	0.08	0.33	0.20*	0.007	0.30*	**	1.00	-	0.43*	**	0.37*	**
IL-10	0.06	0.42	0.12	0.11	0.10	0.19	0.13	0.09	0.24*	0.001	0.24*	0.001	0.47*	**	0.43*	**	1.00	-	0.49*	**
IL-13	-0.01	0.92	0.29*	**	0.26*	**	0.12	0.11	0.47*	**	0.30*	**	0.68*	**	0.37*	**	0.49*	**	1.00	-

4.3.5 There was a positive correlation between TNF-α and gestational age at sampling in HIV-1 infected pregnant women

Exploring the association between gestational age at sampling and log^{10} plasma cytokine concentration in all women revealed a positive correlation with TNF-α (rho=0.14, p=0.02) and IL-13 (rho=0.13, p=0.027). Considering these associations in HIV-1 infected pregnant women alone, demonstrated stronger positive correlation with TNF-α and gestational age at sampling (rho=0.23, p=0.002) but did not retain significance with IL-13, see figure 21. There were no other significant relationships between other cytokines and gestational age at sampling when considering gestational age as a continuum.

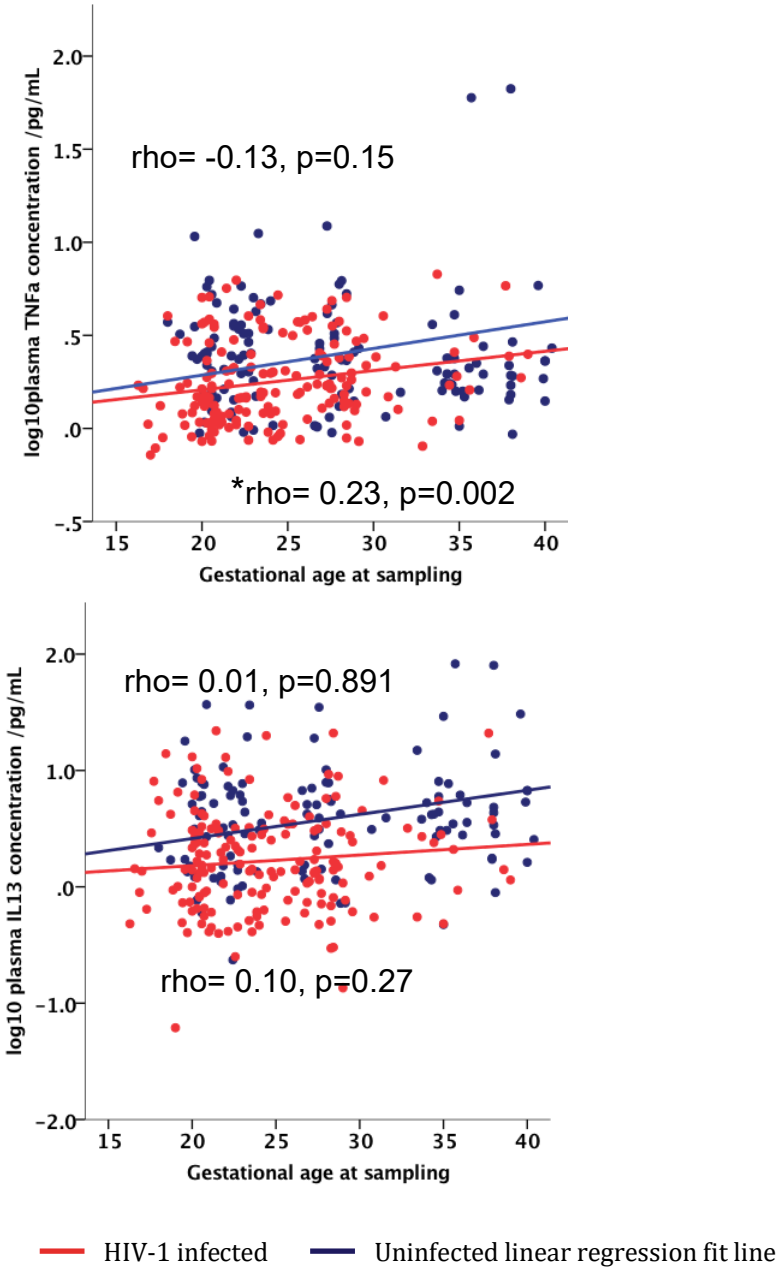


Figure 21 Scatter graphs of log[10] plasma cytokine concentrations against gestational age at sampling by HIV status

Demonstrating a significant correlation between gestational age at sampling and TNF-α in HIV-1 infected pregnant women, no significant correlations were observed in uninfected women or with IL-13, rho=Spearman's correlation co-efficient

4.3.6 Pro-inflammatory IL-12 is positively correlated with gestational age at delivery in HIV-1 infected pregnant women.

The relationship between log[10] plasma cytokine concentrations and gestational age at delivery were then explored with spearman's correlation coefficient. There was a positive association with IL-12 (rho=0.28, p<0.0005) when considering all women. The association between gestational age at delivery and

pro-inflammatory IL-12 remained significant in HIV-1 infected pregnant women (rho=0.20, p=0.01).

Next, hierarchical linear regression, adjusted for sampling time, was performed to evaluate the predictive capacity of log^{10} plasma IL-12 cytokine concentration on gestational age at delivery, see figure 22. Plasma IL-12 was the only cytokine that remained a significant predictor of gestational age at delivery in all women (β=0.26, R^2= 0.070, SE= 1.96, Fold change=19.4, F value=11.5, p<0.0005) and in HIV-1 infected women (β=0.20, R^2= 0.043, SE= 2.11, Fold change=6.93, F value= 4.66, p=0.009).

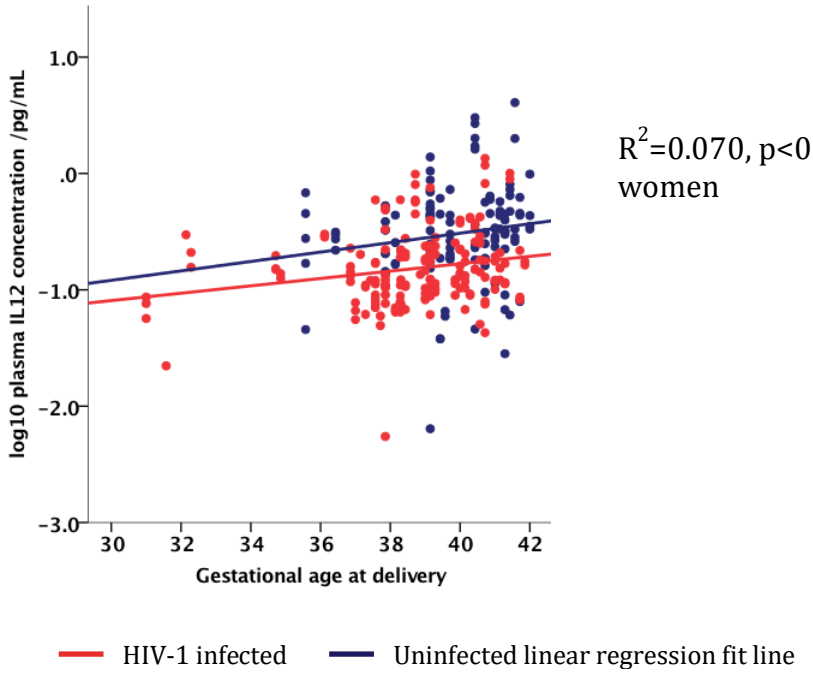


Figure 22 Scatter graphs of log 10 plasma IL-12 against gestational age at delivery by HIV status
Demonstrating a significant correlation between gestational age at delivery and plasma IL-12 in all women (R^2= 0.070, p<0.0005) and HIV-1 infected pregnant women (R^2= 0.070, p<0.0005).

Median cytokine concentration by prematurity was explored. There were no significant differences in median cytokine concentrations by term versus premature births in HIV-1 and uninfected women.

4.3.7 Women who conceived on ART had higher plasma Th1: IL-12 and Th2: IL-4 and IL-13 cytokine concentrations compared to those who initiated ART in pregnancy.

Women who initiated ART during pregnancy had lower plasma IL-12, IL-4 and IL-13 concentrations up to week 32 than those women who conceive on ART ($p<0.05$), see figure 23. No other differences in plasma cytokines were observed between groups by timing of ART exposure.

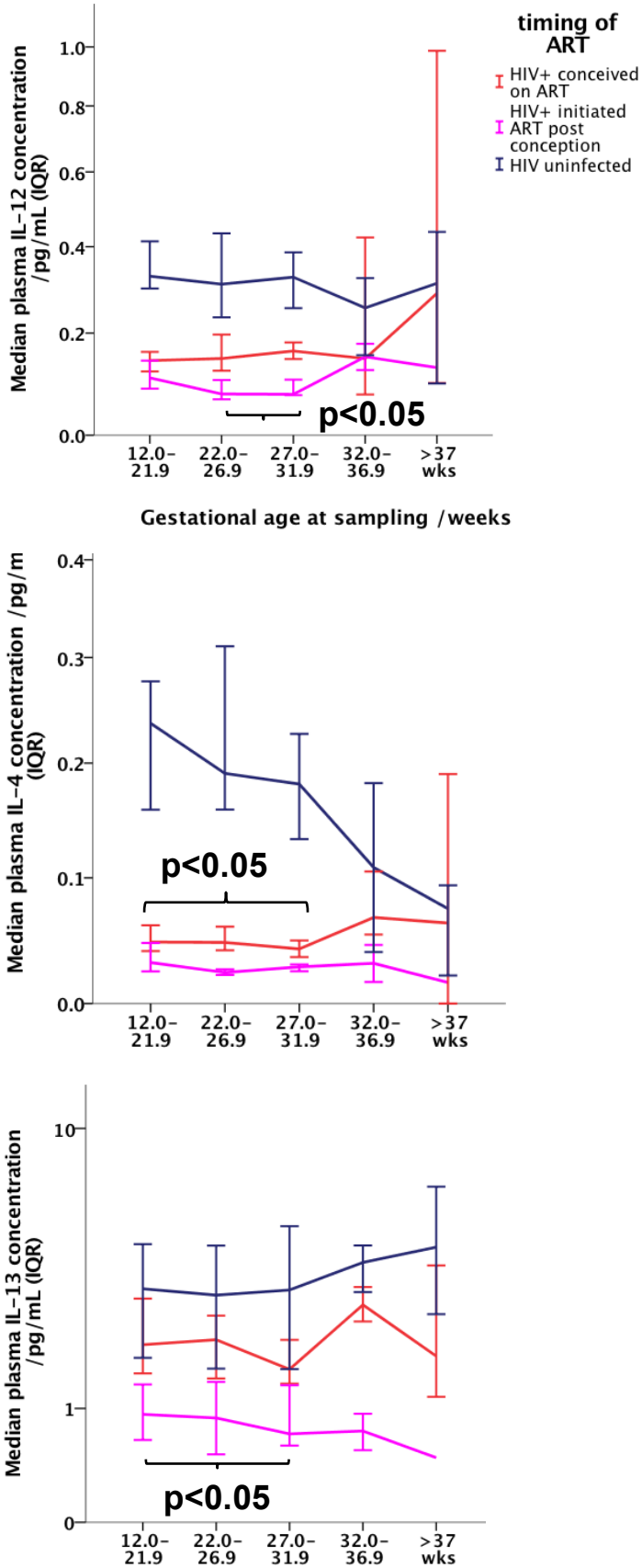


Figure 23 Plasma cytokine concentrations by timing of ART in relation to conception in HIV-1 infected women

Demonstrating a lower plasma concentration of IL-4, IL-12 and IL-13 in women initiating cART in pregnancy upto week 32, $p<0.05$.

4.3.8 Relationship between plasma cytokines and length of ART exposure

The length of ART exposure in weeks was then correlated with plasma cytokine concentrations (both log^{10} transformed). Pro-inflammatory IL-12 and immune-regulatory IL-4 and IL-13 were significantly associated with ART exposure, see table 21. No other significant correlations between ART and the other cytokines were identified.

Hierarchical linear regression, adjusted for sampling time, was performed to evaluation the predictive capacity of log^{10} ART exposure on plasma cytokine concentrations, see table 22. ART exposure significantly predicted IL-4, IL-12 and IL-13 concentration during pregnancy.

Table 21 Spearman's correlation coefficient for log^{10} cytokine concentrations and log plasma cytokine concentrations and log^{10} ART exposure /weeks

Cytokine	Spearman's rho	p value
IL-4	0.32	<0.0005
IL-12	0.32	<0.0005
IL-13	0.32	<0.0005

Table 22 Linear regression of plasma cytokine concentration with log^{10} ART exposure /weeks adjusted for gestational age at sampling

Cytokine	Fold change	Estimate	Standard Error	F value	Q value
IL-4	13.1	0.32	0.43	6.70	<0.0005
IL-12	5.0	0.20	0.32	2.58	0.028
IL-13	14.9	0.33	0.41	7.59	<0.0005

4.3.9 NNRTI exposed women had higher plasma concentrations of Th2 cytokines: IL-4, IL-10 and IL-13 than PI and INSTI exposed women and lower Th1: IL-2 and TNF-α but this did not remain significant when comparing across pregnancy

Plasma cytokine concentrations through pregnancy were compared by third class of drug: PI, NNRTI and INSTI. There was no statistical difference in cytokine concentrations between groups when compared across pregnancy, for examples see figure 24.

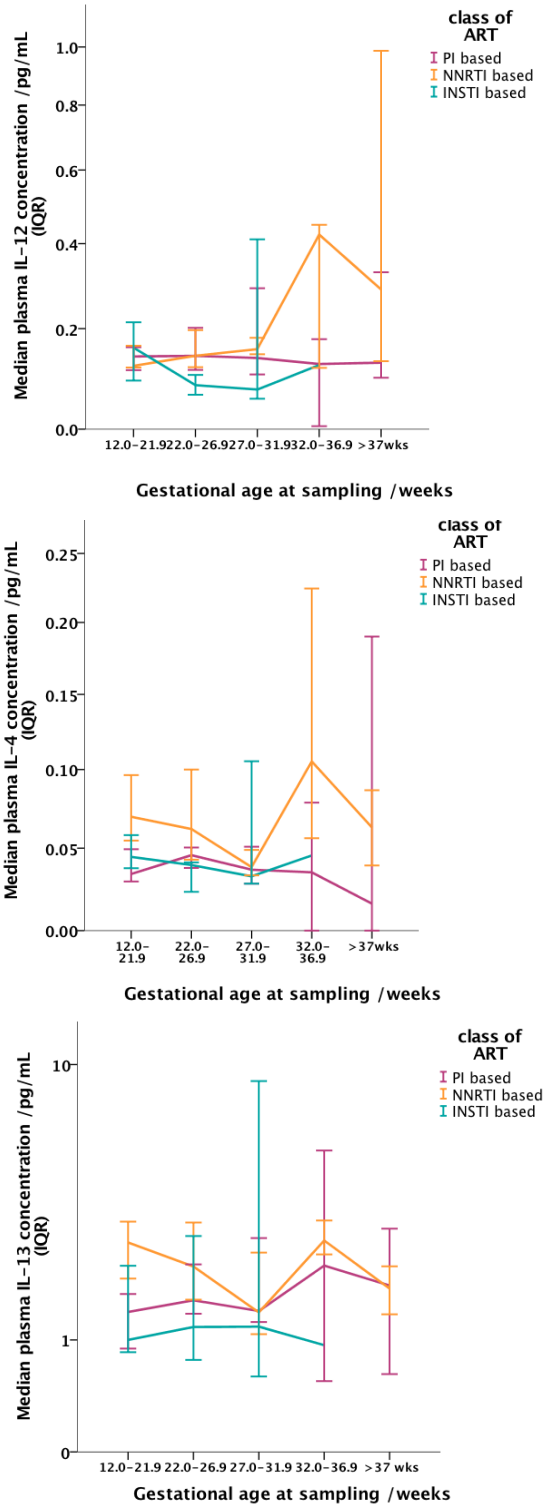


Figure 24 Median plasma cytokine concentrations across pregnancy by third ART class drug exposure

Demonstrating no significant difference in plasma IL-4, IL-12 and IL-13 across gestation by third drug.

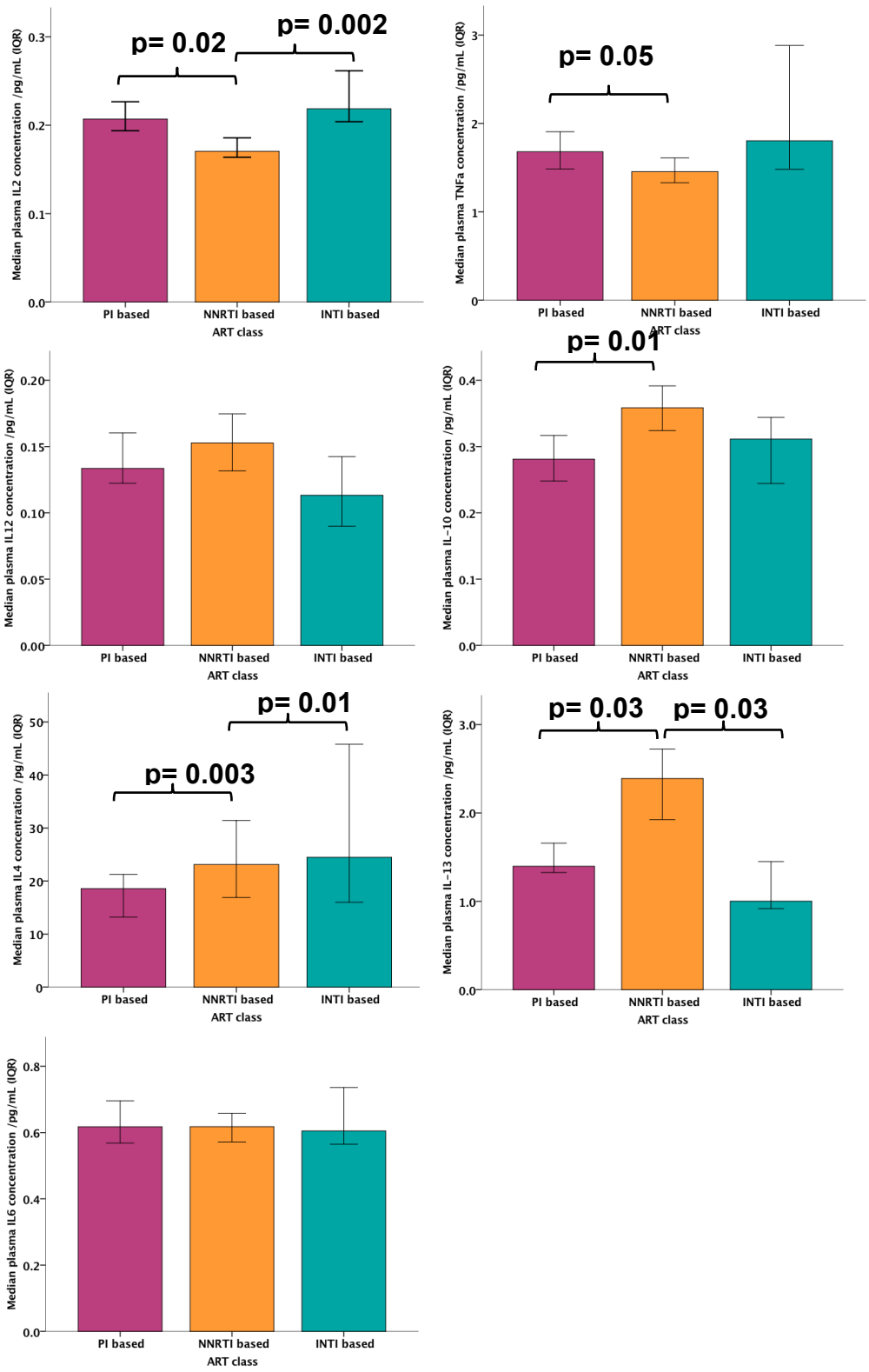


Figure 25 Total median plasma cytokine concentration by third drug exposure
Demonstrating higher group median immune-regulatory cytokine concentrations (IL-4, IL-10 and IL-13) and lower pro-inflammatory IL-2 and TNF-α in women receiving NNRTI-based cART vs. PI-based cART.

When cytokine measurements were considered across all three classes of third drug, NNRTI exposed women had higher overall median plasma concentrations of Th2 immune-regulatory IL-4, IL-10, IL-13 and lower Th1 pro-inflammatory IL-2 and TNF-α than either those receiving PI or INSTI based therapy, there were no differences in other measured cytokines including IL-6 and IL-12, see figure 25. There were no statistical differences between the PI and INSTI exposed women. No difference in plasma cytokine concentrations by NRTI backbone FTC/TDF or Abacavir (ABC)/3TC were observed.

4.3.10 Women receiving PI based cART have more Th1/Th17 predominant cytokine profiles compared to women who received NNRTI based cART in the third trimester

The ratios of Th1/Th2 and Th17/Th2 cytokines were explored across gestation by ART exposure, see figure 26. The cytokine ratios that were different on visual inspection by third ART drug were Th1: IL-2, TNF-α and Th17: IL-6 divided by Th2: IL-4, IL-10 and IL-13. There was a trend towards the highest Th1/Th2 and Th17/Th2 ratios in women receiving INSTI or a PI as their third ART drug, than NNRTIs, with HIV uninfected pregnant women having the lowest ratios (ie Th2 predominant).

Post hoc comparisons of plasma cytokine ratios according to the third ART class were undertaken. Compared to women treated with NNRTIs, significantly higher ratios (i.e. Th1/Th17 balanced systemic cytokine environment) were observed in those who receiving PIs during pregnancy, mainly at study baseline (week 12-22) and in the third trimester (week 32 to 37). The trend towards higher ratios in women receiving INSTIs compared to NNRTIs did not reach statistical significance.

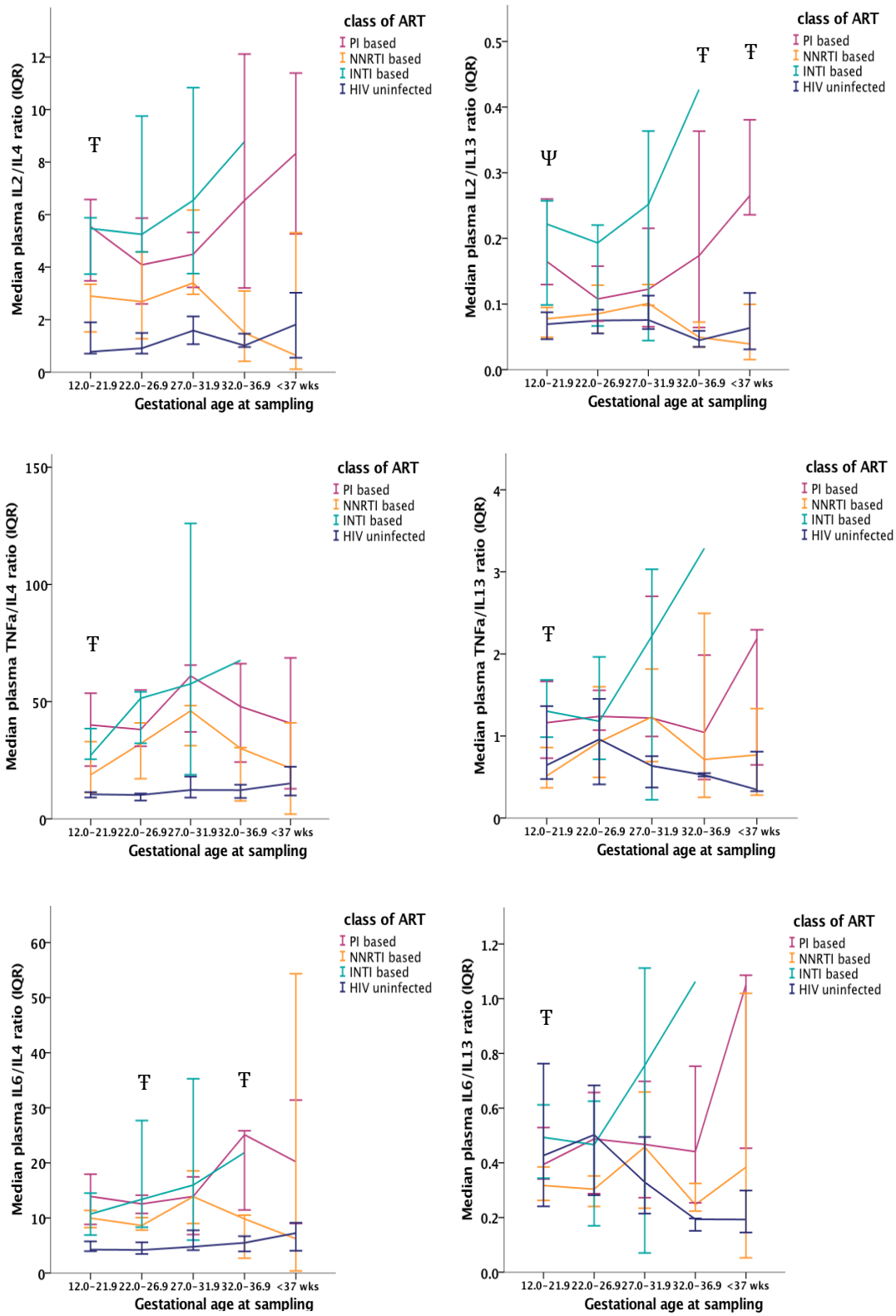
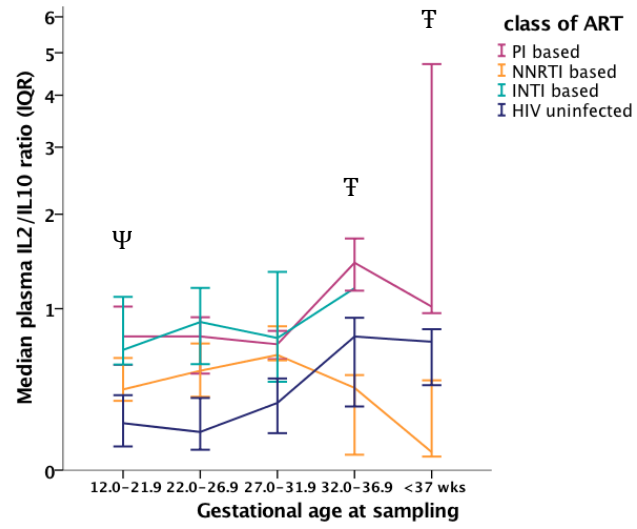
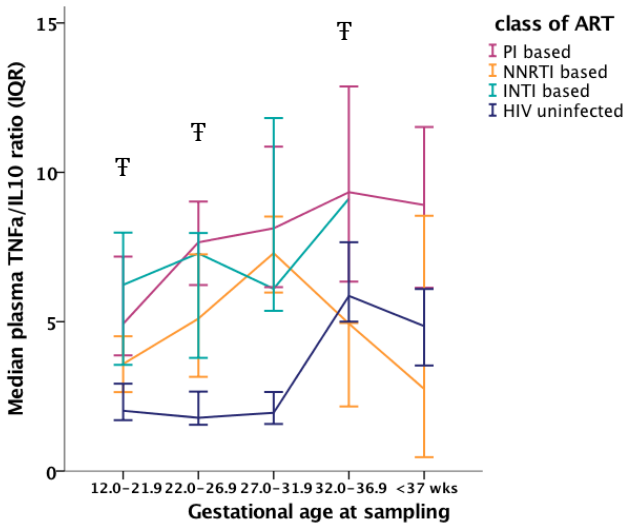


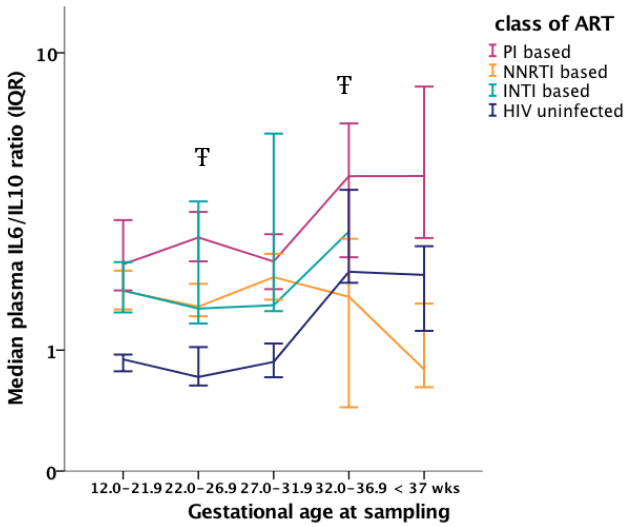
Figure 26 Th1/Th2 and Th17/Th2 plasma cytokine ratios through pregnancy by HIV status and ART exposure

Demonstrating a trend towards Th2 biased ratios with Th1 and Th17 cytokines in women receiving NNRTIs vs. PIs as their third ART drug from week 32,,* = p<0.0005; Ψ = p<0.005; Ŧ =p<0.05.

Figure 26 cont







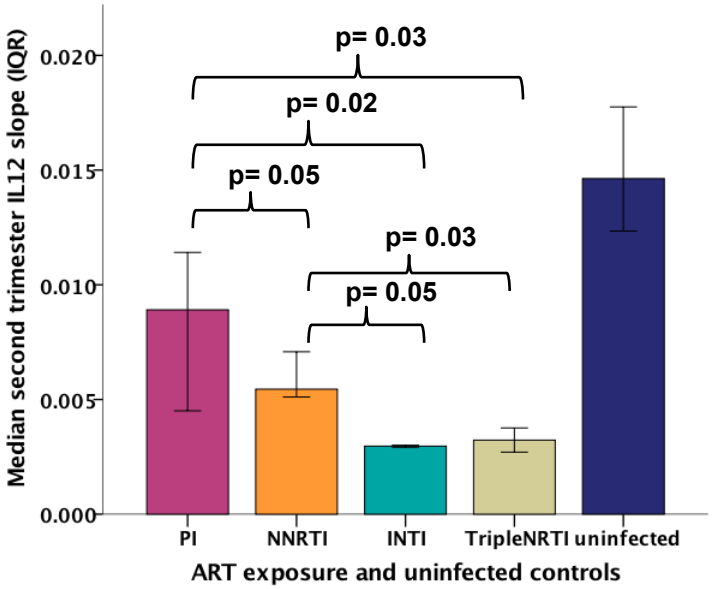
4.3.11 Changes in Th1/Th2 cytokine ratios and slopes during pregnancy differ by third ART drug with PIs associated with the greatest Th1 shift in the second and third trimester compared to NNRTIs.

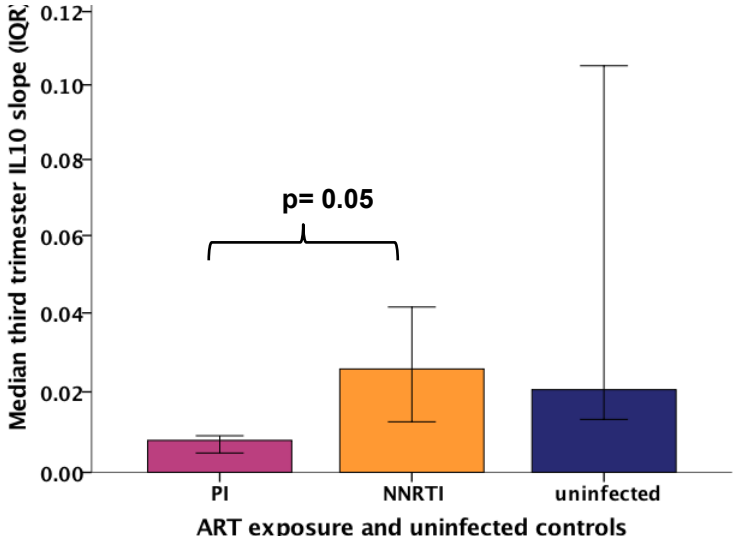
Exploring changes in Th1/Th2 ratios with gestational age at sampling, revealed a generalised pattern that HIV-1 infected pregnant women receiving an NNRTI (and also uninfected women) have a decreasing Th1/Th2 and Th17/Th2 ratios from week 32 to 37 (Th2 shift) whereas women receiving a PI or INSTI as their third drug have increasing ratios (Th1/Th17 shift).

To explore this observation further cytokine slopes were calculated for individual women for two time periods: visit 1 up to visit 3 (approximates to second trimester) and visit 3 up to visit 5 (third trimester, week 32 to 38 weeks or delivery).

Median second trimester plasma cytokine slopes were compared by third drug class, see figure 27. The only plasma cytokine slope that differed by ART exposure was IL-12. IL-12 slope (increasing positive values equating to Th1 shift) was highest in women receiving PIs compared to women receiving NNRTIs, $p<0.05$, with the lowest IL-12 shift observed in women receiving INSTIs and triple NRTIs (including ZDV), $p<0.03$.

Median third trimester plasma cytokine slopes were compared between PIs and NNRTI exposed women (other classes could not be compared due to low sample numbers). Women receiving PI based cART had higher IL-2 slopes (Th1 shift) and lower IL-10 (Th2) compared to women receiving NNRTIs in the third trimester, $p<0.05$, see figure 27.





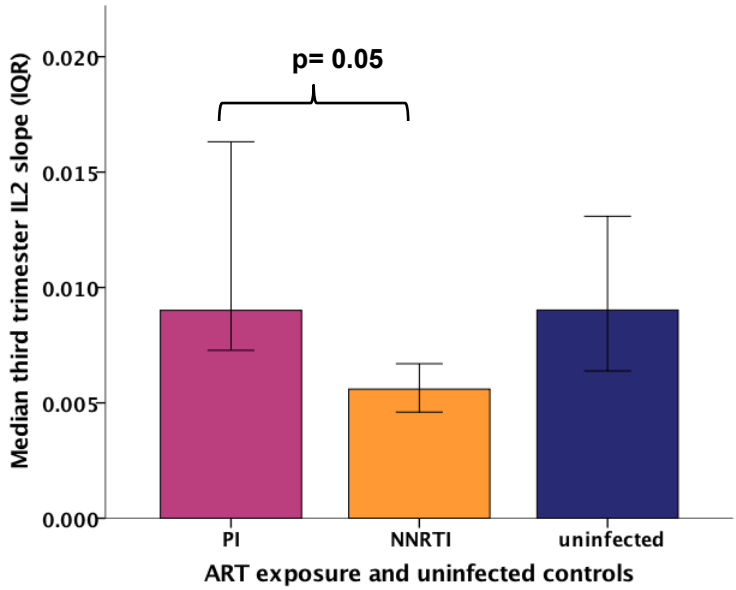


Figure 27 Median cytokine slopes by trimester and ART exposure
Demonstrating in women receiving PIs vs. NNRTIs, an increased second trimester L-12 slope (Th1 shift), $p<0.05$, and in the third trimester a higher IL-2 slopes (Th1 shift) and lower IL-10 (Th2), $p<0.05$.

4.3.12 Summary of plasma cytokine results

1. HIV-1 infected women have lower plasma cytokine concentrations compared to uninfected women throughout pregnancy.

2. In HIV-1 infected women, Th1 cytokine: TNF-α increased with advancing gestational age of sampling and the Th1 cytokine IL-12 was positively associated with gestational age at delivery.

3. Plasma cytokine concentrations in HIV-1 infected pregnant women are highly inter-correlated. Specifically Th1: IL-12 and TNF-α were highly inter-correlated and in addition significantly associated with Th2 cytokines:IL-4 and IL-13. Th17 cytokine: IL-6 was also correlated with Th1 cytokines: IL-2 and TNF-α and Th2 cytokines: IL-4, IL-10 and IL-13.

4. HIV-1 infected women who conceived on cART had higher plasma IL-12, IL-4 and IL-13 concentrations compared with those who initiated ART in pregnancy. Duration of ART exposure in weeks positively correlated with the same cytokines in liner regression analyses demonstrating a dose response effect.

5. Women receiving PI based cART had higher Th1: IL-2 and TNF-α plasma concentrations than women receiving NNRTIs and lower Th2: IL-4, IL-10 and IL-13, this pattern did not reach significance when comparing across all time points.

6. Women receiving PIs had: higher Th1 and Th17/Th2 ratios up to week 32; a greater median Th1:IL-12 slope during the second trimester; and a higher median Th1: IL-2 slope and lower immune-regulatory IL-10 in the third trimester up to delivery, compared to women receiving NNRTIs. These are all indicators of an overall Th1 shift in women receiving PIs during pregnancy compared to those receiving NNRTIs.

4.4 Conclusions and interpretation

The data show that despite systemic reduction in T helper cells in HIV-1 infection, these women still display significant peripheral CD4+ and CD8+ T cell activation in pregnancy compared to uninfected pregnant women with higher CD4 counts. The highest T cell activation was observed in women initiating cART in pregnancy and longer cART exposure in weeks was associated with an overall reduction in T cell activation and increase in %CD4+CD25+ cells (likely to be T regulatory cells). The association between ART and a reduction in immune activation has been described previously(Watts *et al.*, 2009; Hileman and Funderburg, 2017) as has the inverse association between activated T cells and CD4+CD25+ cells (Hunt *et al.*, 2008; Richardson and Weinberg, 2011).

Whilst women who initiated cART during this pregnancy displayed higher immune activation, women who conceived on cART had the highest IL-12 concentrations with the duration of cART exposure positively associated with IL-12 concentrations. These data support the hypothesis that treated HIV infection in pregnancy is associated with Th1 cytokine profile. These data add to those presented by Fiore et al who were one of the first to demonstrate a Th1 shift in HIV-1 infected pregnant women(Fiore *et al.*, 2006) and the first to identify this pattern of IL-12 expression in HIV-1 pregnant women.

Pregnant women with HIV-1 who received a PI compared to an NNRTI as the third ART drug had higher plasma Th1/Th2 cytokine ratios and a higher median IL-12 slope value (steeper increase in IL-12 concentrations over gestation) in the second trimester. These differences were observed for the second trimester up to week 32 however comparing the same women during the third trimester revealed a greater slope in Th1 cytokine IL-2 and lower slope in anti-inflammatory IL-10. Fiore et al also identified changes in IL-2 and IL-10 in women receiving ART during pregnancy but were not powered to show differences by third agent(Fiore *et al.*, 2006). Women receiving PIs also had a trend towards more immune activation than women receiving NNRTIs in further support of the pro-inflammatory effect of these drugs in pregnancy.

These changes could potentially lower the threshold for starting labour in this group of women and supports the hypothesis that PI-based cART is associated with a greater pro-inflammatory/Th1 cytokine shift than non PI -based regimes. The lower third trimester IL-10 slope identified in women receiving a PI may help explain why this group experience higher rates of PTB(Powis *et al.*, 2011; Fowler *et al.*, 2017; Favarato *et al.*, 2018) and widens our understanding of the role of this anti-inflammatory cytokine in pregnancy(Thaxton and Sharma, 2010). The main source of IL-10 in HIV-1 infected pregnant women may be T regulatory cells(Bento *et al.*, 2009; Hygino *et al.*, 2012). Bento et al. hypothesise that ZDV alone may enhance IL-10 production and thus may explain why traditionally this group has the lowest rates of PTB, the converse could be true of PIs(Bento *et al.*, 2009; Short *et al.*, 2012; Short and Taylor, 2014)

Immune activation (%CD8+HLA-DR+ cells) was inversely associated with gestational age at delivery where as Th1 cytokine IL-12 was associated with increasing gestational age at delivery. These findings likely reflect two sides of the Th1 pro-inflammatory trigger of labour. Firstly there was an increase in the Th1 cytokine IL-12 in plasma pre delivery which could be preparation for the inflammatory initiation of labour. IL-12 has been shown to increase in plasma from mid to late pregnancy(Szereday, Varga and Szekeres-Bartho, 1997; Curry *et al.*, 2007; Arababadi *et al.*, 2012) and is thought to induce/associate with pro-inflammatory cytokines in the placenta (El-Shazly *et al.*, 2004; Negishi *et al.*, 2011).

Secondly systemic immune activation, which is generally a consequence of underlying infectious trigger (HIV virus, microbial antigen), was exaggerated in women who go on to deliver preterm. Immune activation has been observed at the maternal fetal interface, the cervix and the myometrium in labour and preterm labour(Osman *et al.*, 2003; Young *et al.*, 2005; Sykes *et al.*, 2011; Hunter *et al.*, 2016). Prakesh and colleagues have demonstrated that PBMC CD4/CD8 ratios reflect those observed in the cervix from the same women and cervical T cells have higher activation markers than PBMCs(Prakash *et al.*, 2001; Prakash, Patterson and Kapembwa, 2001). It is possible that activated peripheral T cells

migrate to the gestational tissues and along with resident T cell immune activation, forward the initiation of labour through downstream inflammation.

Another theme that is evident from these data is that plasma cytokine concentrations are highly correlated so that Th1 cytokines correlate with Th2 cytokines. Indeed, it was observed that in women who conceived on ART, high IL-12 plasma concentrations were echoed with increases in Th2 cytokines IL-4 and IL-13. This is likely to be part of the regulatory or self-limiting mechanisms controlling inflammation during pregnancy as has been observed previously in whole blood from pregnant women (with and with out HIV infection) (Holmes *et al.*, 2003; Makhseed *et al.*, 2003; Frank, Buchmann and Schackis, 2004; Halonen *et al.*, 2009; Denney *et al.*, 2011; Richardson and Weinberg, 2011; Ferguson *et al.*, 2014; K. Ashford *et al.*, 2018).

In summary HIV-1 infected pregnant women demonstrate evidence of increases in immune activation and systemic pro-inflammatory cytokines IL-12 and TNF-α concentrations across pregnancy. This coincides with reciprocal upregulation of the percentage of T cells expressing CD4+CD25+ and immune-regulatory cytokines suggesting a innate physiological mechanism to limit inflammation. Women receiving PI based cART showed a trend towards a pro-inflammatory shift in cytokine ratios and slopes. These data indicate that this group of women have more exaggerated increases in immune activation/inflammation across gestation, possibly compounded by ART and thus are at high risk of inflammation triggered PTB.

Chapter 5 Local cervicovaginal cytokines

5.1 Introduction

Local cytokine production at the maternal fetal interface, the myometrium and cervix is more informative in characterising physiological mechanisms in pregnancy, partition and poor birth outcomes than systemic concentrations.

To date, no study using MC to collect CVF have compared cytokine concentrations in HIV-1 infected to uninfected women, nor has any study used this method for exploring FGT cytokines in pregnancy. This sampling method should provides a more functional picture of the local cervicovaginal cytokine milleau as it is undiluted and thus contains more elements of the local immune system (cells, mucin, bacteria etc) and is less prone to sampling processing and normalisation errors for the measurement of FGT biomarkers (Short *et al.*, 2018).

The aim of this study was to explore local CVF cytokine profiles in HIV-1 infected and uninfected pregnant women and how these differ by cART (drug class, timing of exposure) and birth outcome.

Chapter objectives:

- To characterise local CVF pro-inflammatory (IFN-γ, IL-2, IL-8, IL-12, TNF-α) and immune-regulatory cytokine (IL-6, IL-4, IL-10 and IL-13) profiles in HIV-1 infected and uninfected pregnant women across the second and third trimester
- To compare local markers of inflammation across gestation and how these differ by HIV status, cART (drug class, timing of exposure) and gestational age at delivery.

5.2 Results

5.2.1 Cervicovaginal cytokine concentrations in HIV-1 infected pregnant women are elevated compared to uninfected pregnant women

When all CVF cytokines samples were analysed together it is clear that HIV-1 infected women have much higher concentrations of all cytokines both pro-inflammatory and immune-regulatory, see figure 28. IL-8 was measured at the highest concentration, followed by IL-1β, IL-13, IL-2, TNF-α, IL-6, IFN-γ, IL-10, IL-12 and IL-4, $p<0.0005$. CVF cytokine concentrations were also significantly elevated in HIV-1 infected women across gestation in the longitudinal analysis see figure 29 and 30.

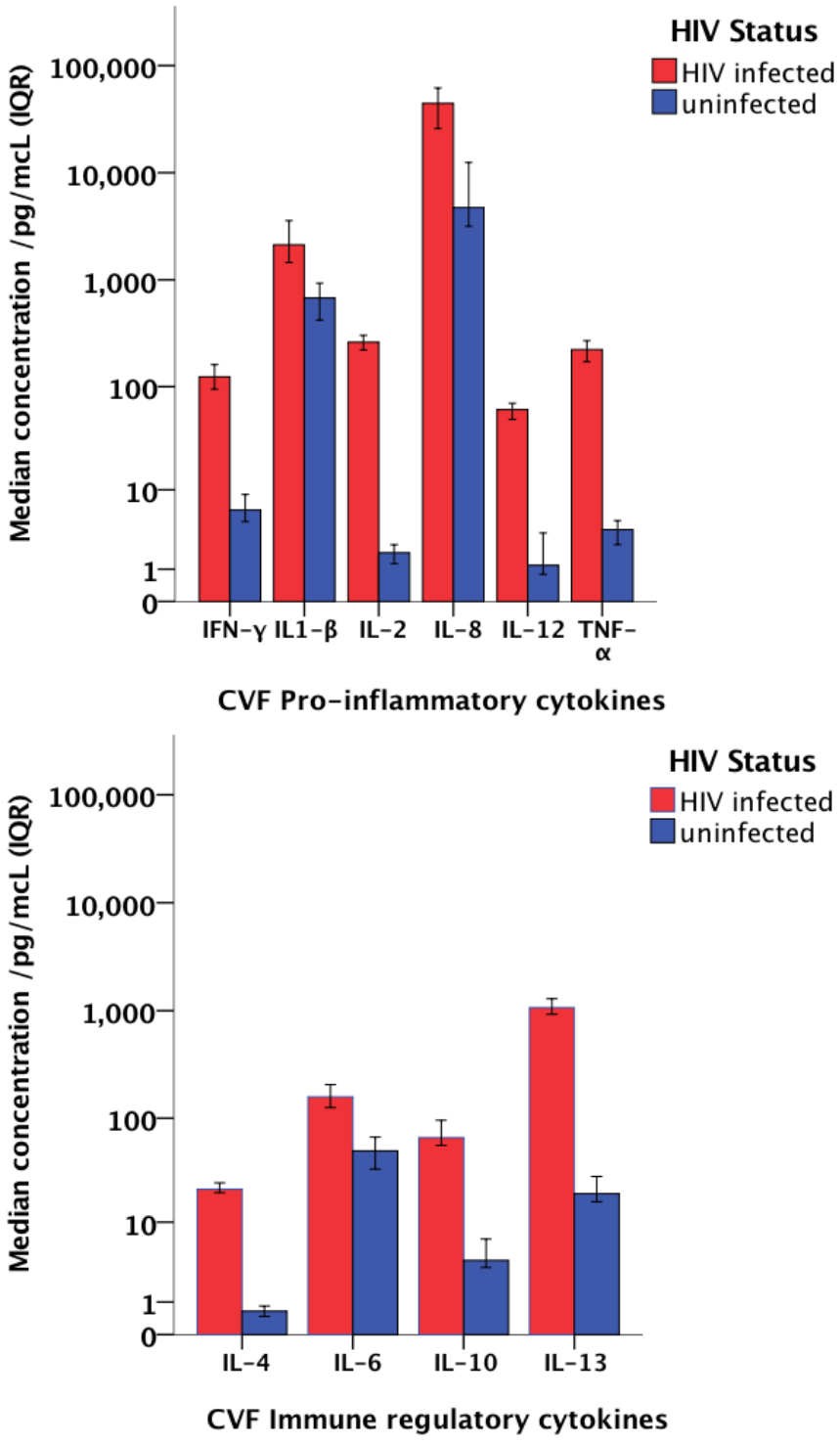


Figure 28 CVF cytokine concentration by HIV status

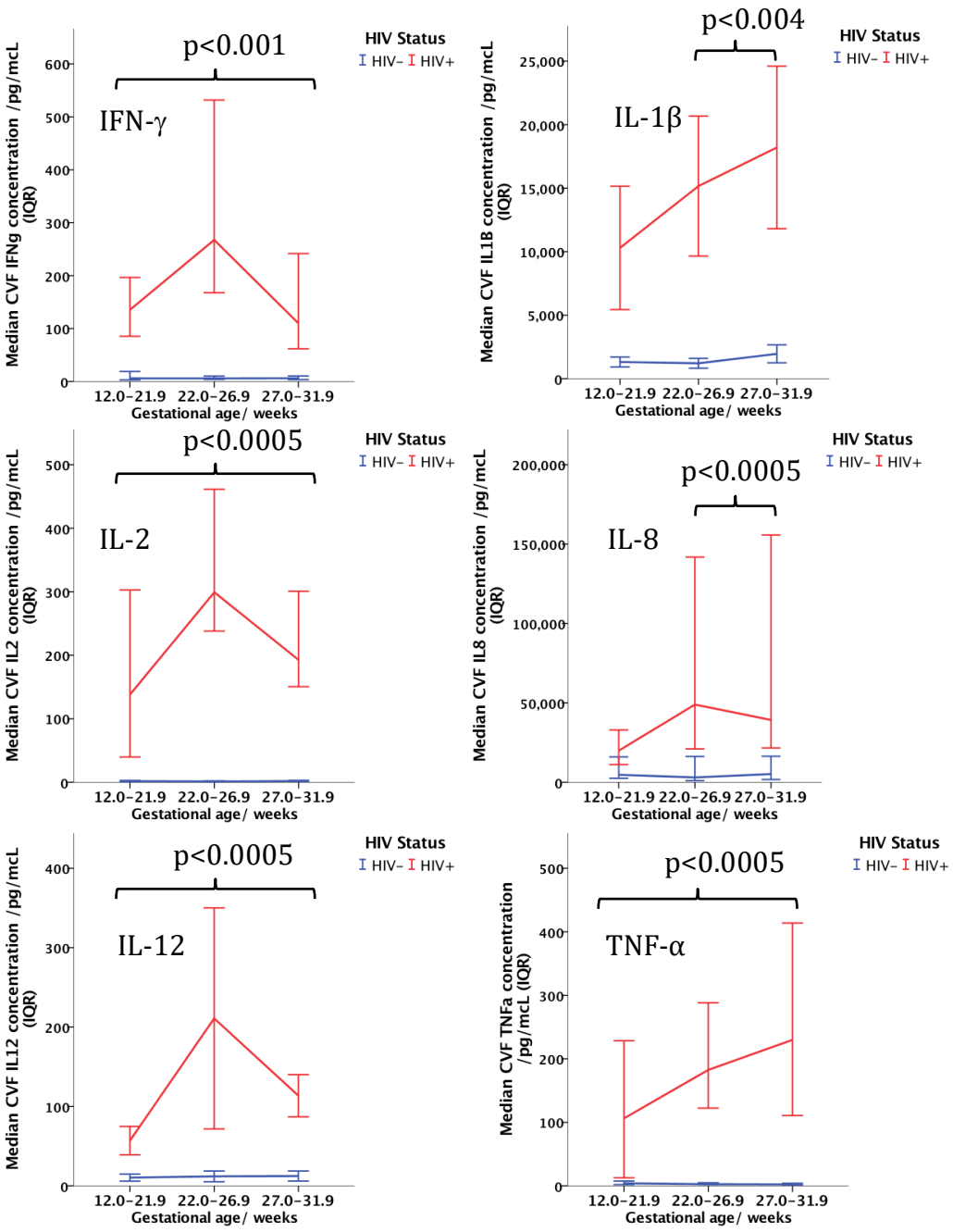


Figure 29 CVF pro-inflammatory cytokines across gestation by HIV status
Demonstrating higher pro-inflammatory CVF cytokine expression in HIV-1 infected women vs. uninfected pregnant women.

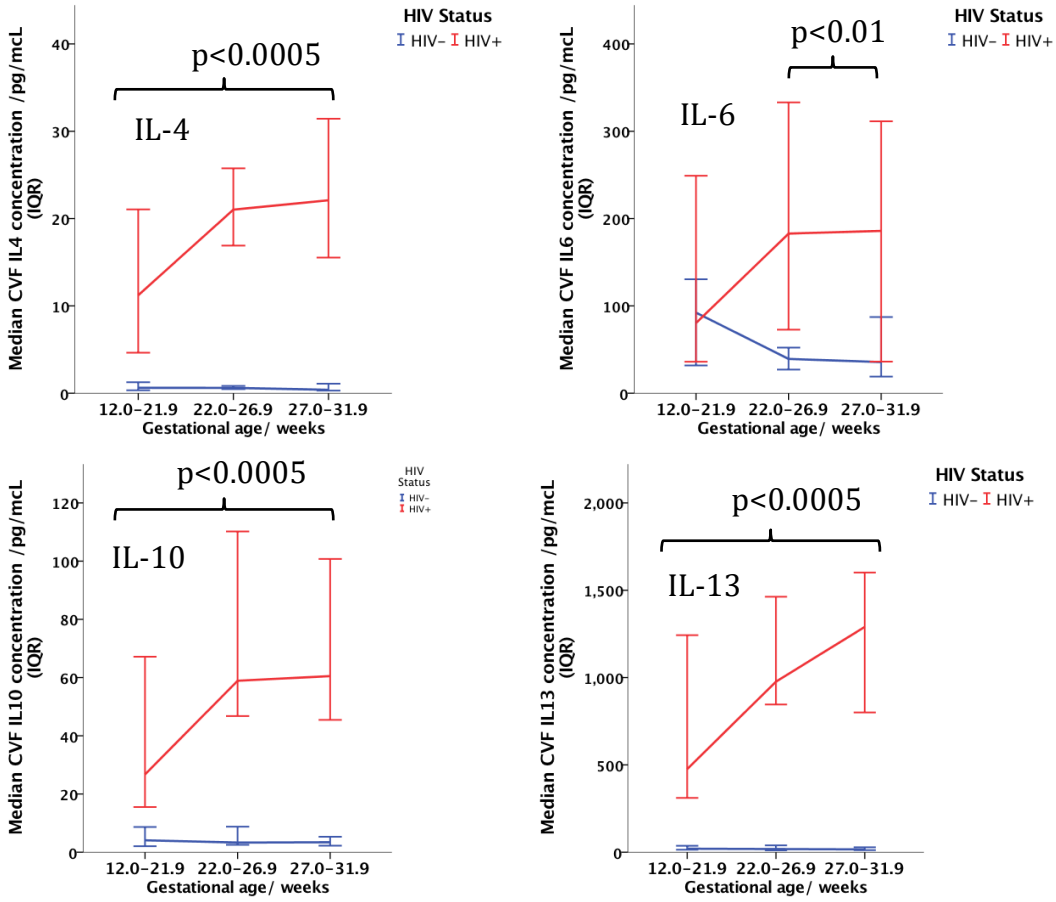


Figure 30 CVF immune -regulatory cytokine across gestation by HIV status
Demonstrating higher immune -regulatory CVF cytokine expression in HIV-1 infected vs. uninfected pregnant women.

5.2.2 Trends in CVF cytokines across gestation

On visual inspection, CVF pro- inflammatory cytokines: IL-1β, IL-2, IL-8, IL-12 and TNF-α concentrations increased across gestation in HIV-1 infected women whereas concentrations of these cytokines were stable in uninfected women. Th17 IL-6 concentrations increased during pregnancy in HIV-1 infected women whereas IL-6 concentrations decreased in uninfected women. Concentrations of all measured immune-regulatory cytokines: IL-4, IL-10 and IL-13 also increase during pregnancy in HIV-1 infected women whereas they remained stable in uninfected women.

5.2.3 Th1: IL-12 and Th2: IL-13 are associated with gestational age at sampling in HIV-1 infected pregnant women

Correlations between CVF cytokines with gestational age at sampling were explored. Firstly in all women combined revealed no significant associations. However considering HIV-1 infected pregnant women alone revealed a significant association between gestational age at sampling with IL-12 (rho=0.29, p=0.004) and IL-13 (rho=0.21, p=0.035), see figure 31. No other significant correlations were identified, including IL-6, and no association between CVF cytokine and gestational age at sampling was identified in uninfected women.

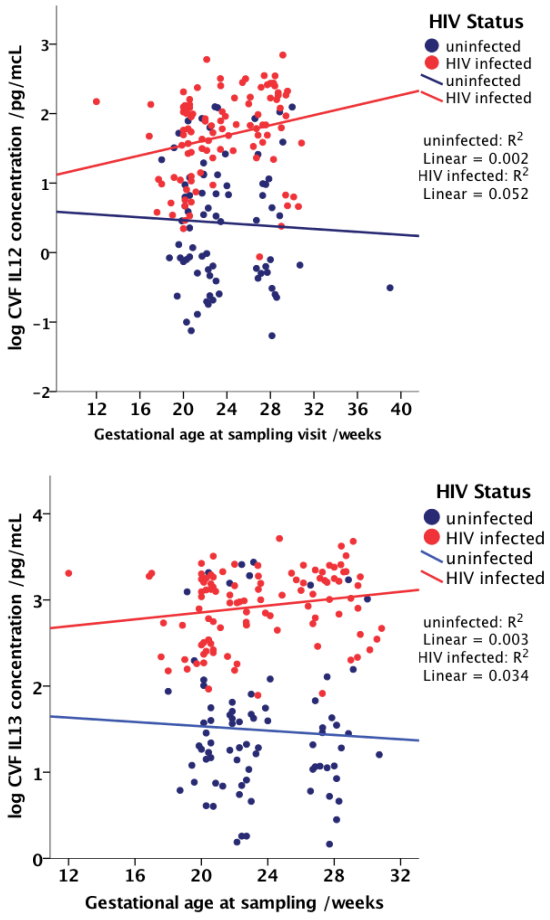


Figure 31 Scattergraph of CVF cytokines Th1: IL-12 and Th2: IL-13 against gestational age at sampling with individual linear regression fit lines for HIV-1 infected and uninfected women
Demonstrating a significant association between gestational age at sampling with CVF IL-12, rho=0.29, p=0.004, and IL-13, rho=0.21, p=0.035 in HIV-1 infected pregnant women.

5.2.4 Th1/Th17/Th2 ratios across gestation

The only CVF Th1/Th17/Th2 ratio that correlated with gestational age at sampling was IL-2/IL-10 with a positive rho value indicating a Th2 shift (rho=0.21, p=0.032), see figure 32.

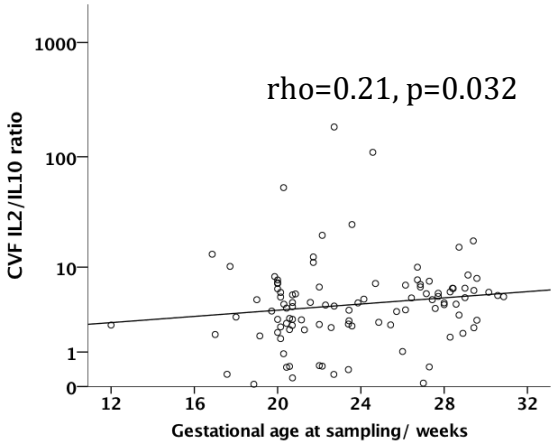


Figure 32 Scattergraph of CVF IL-2/IL-10 ratio across gestation
Demonstrating a positive correlation between CVF IL-2/IL-10 ratio (Th1 shift) and gestational age at sampling, Spearman's rho=0.21, p=0.032, --------- fit line =linear regression.

5.2.5 Lack of association between CVF cytokines and gestational age at delivery or PTB

On correlation analysis no significant association between CVF cytokines and gestational age at delivery was identified either in HIV-1 infected and uninfected pregnant women considered combined or separately. No difference in median CVF cytokine concentrations by prematurity were observed.

5.2.6 CVF cytokine concentration did not differ by ART exposure in relation to conception

CVF cytokine concentration was explored by ART exposure in relation to conception, see figure 33 and 34. No statistical differences in CVF cytokine concentration by ART exposure were observed. A trend towards a higher pro-inflammatory median cytokine concentration in women initiating ART post conception can be observed with the exception of IL-2 and IL-12 which were higher in the preconception exposure group. Immune-regulatory CVF cytokine concentrations followed the same upward trend in both groups.

When ART exposure in weeks was correlated with CVF cytokine concentration the only significant correlation was an inverse correlation between IFN-γ concentration and ART exposure (rho=-0.24, p=0.036).

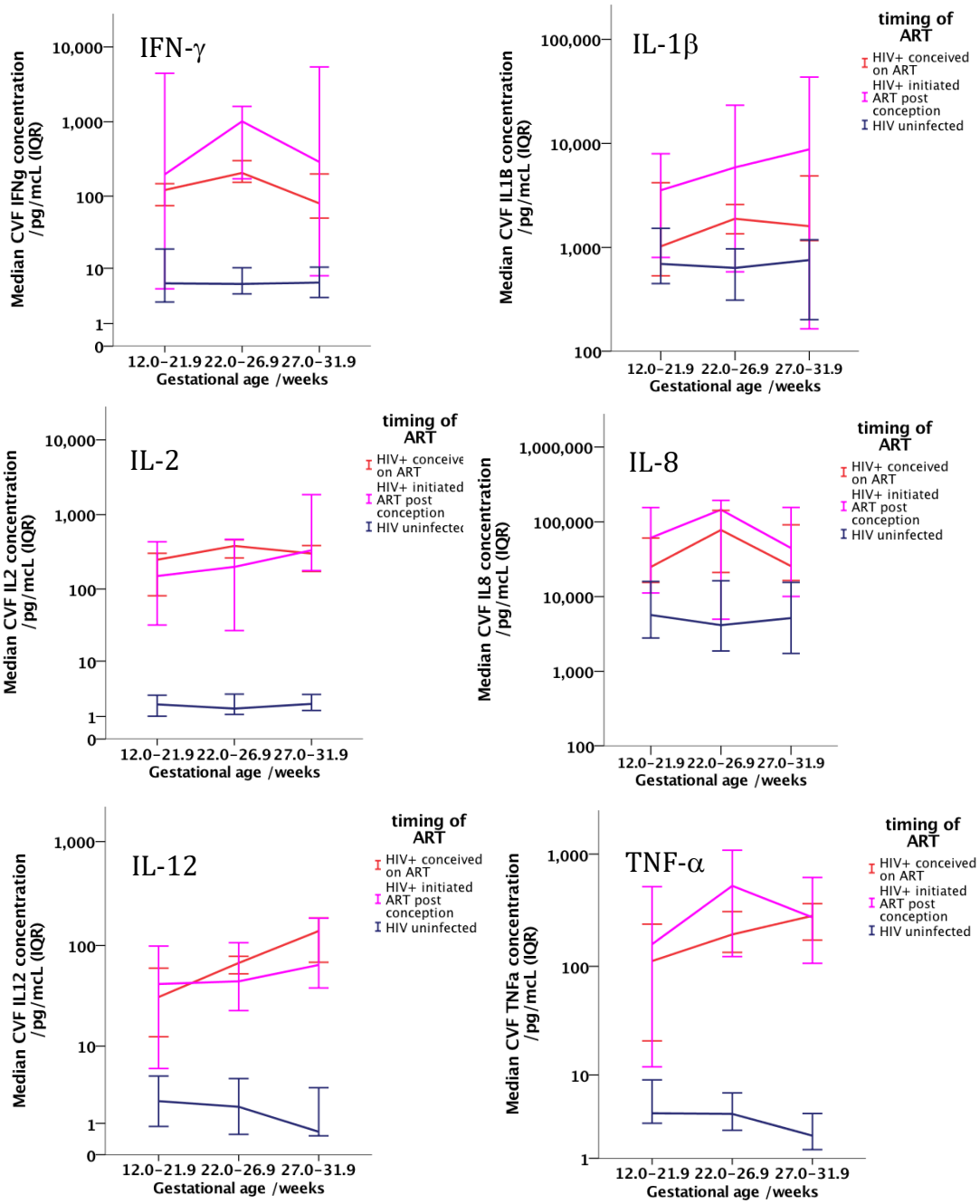


Figure 33 CVF pro-inflammatory cytokine concentration by ART exposure
Demonstrating a trend towards a higher pro-inflammatory median cytokine concentration in women initiating ART post conception with the exception of IL-2 and IL-12 where the convserse trend is apparent.

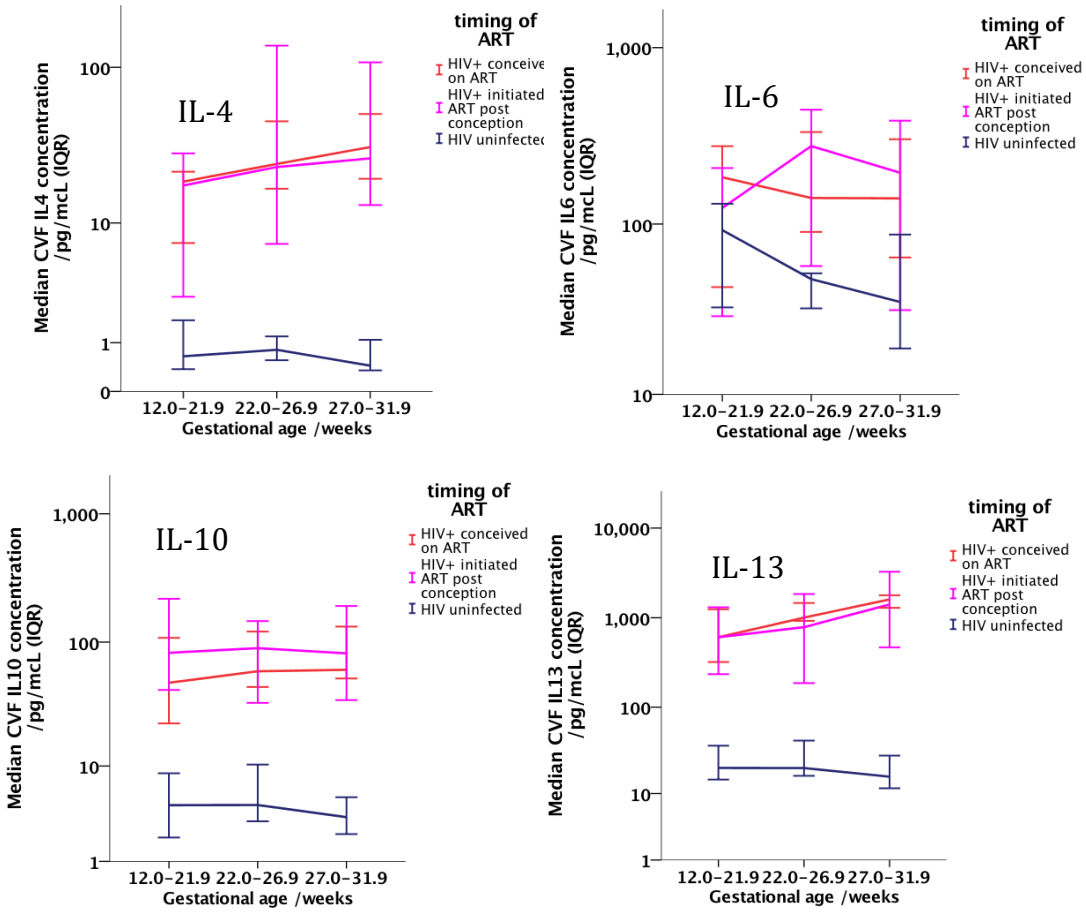


Figure 34 CVF immune-regulatory cytokine concentration by ART exposure
Demonstrating a trend towards a higher CVF IL-6 (Th17) and anti-inflammatory IL-10 (Th2) cytokine concentration in women initiating ART post conception.

5.2.7 CVF cytokine concentration did not differ by class of ART

No statistical difference was observed in median CVF cytokine concentration across gestation by third ART drug class comparing PIs, NNRTIs and INSTIs, see figure 35 and 36. Next, median slope values were compared by third ART drug, which did not show a statistical difference.

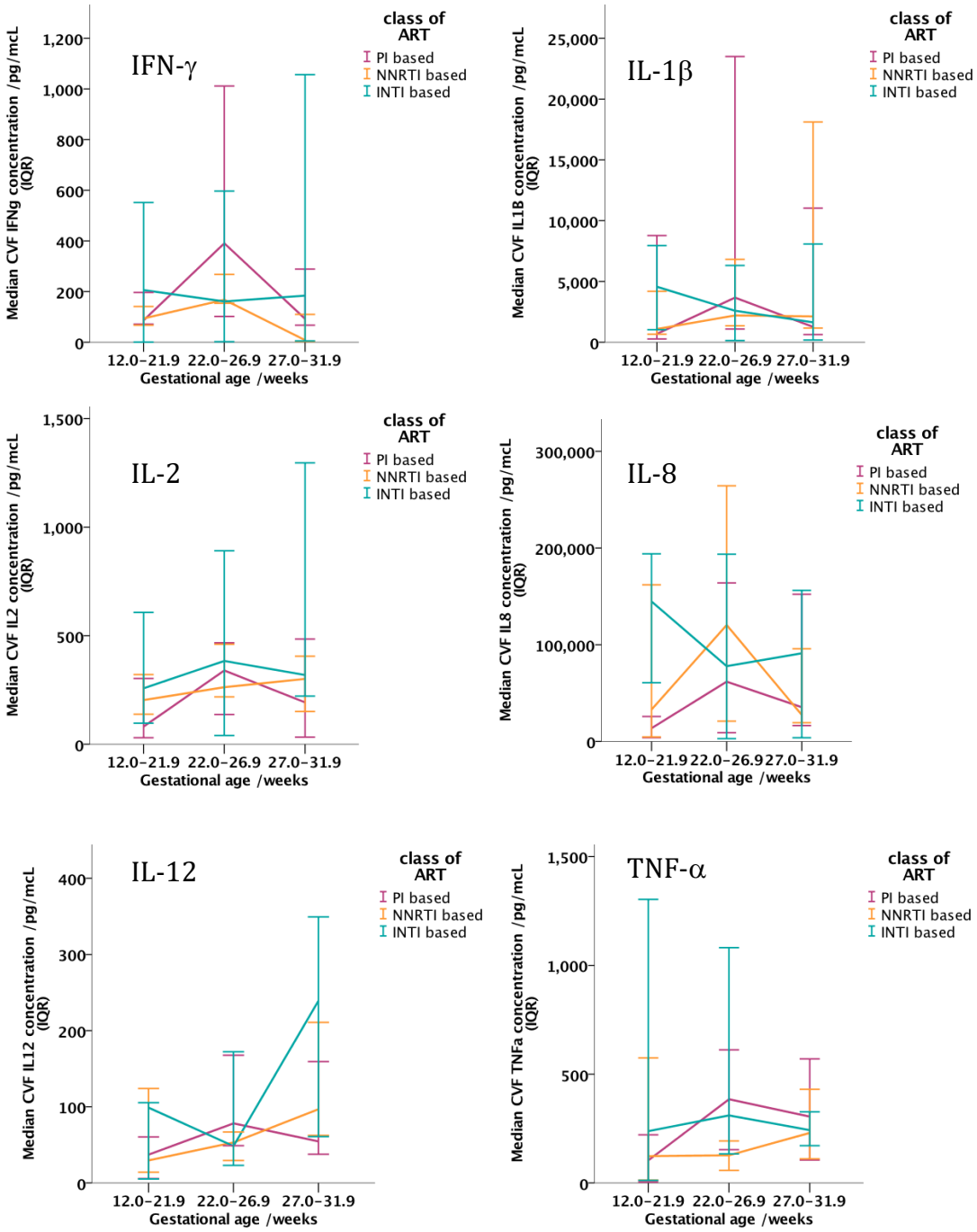


Figure 35 Pro-inflammatory cytokine across gestation by ART class
Demonstrating no statistical difference in pro-inflammatory CVF cytokines by third drug class.

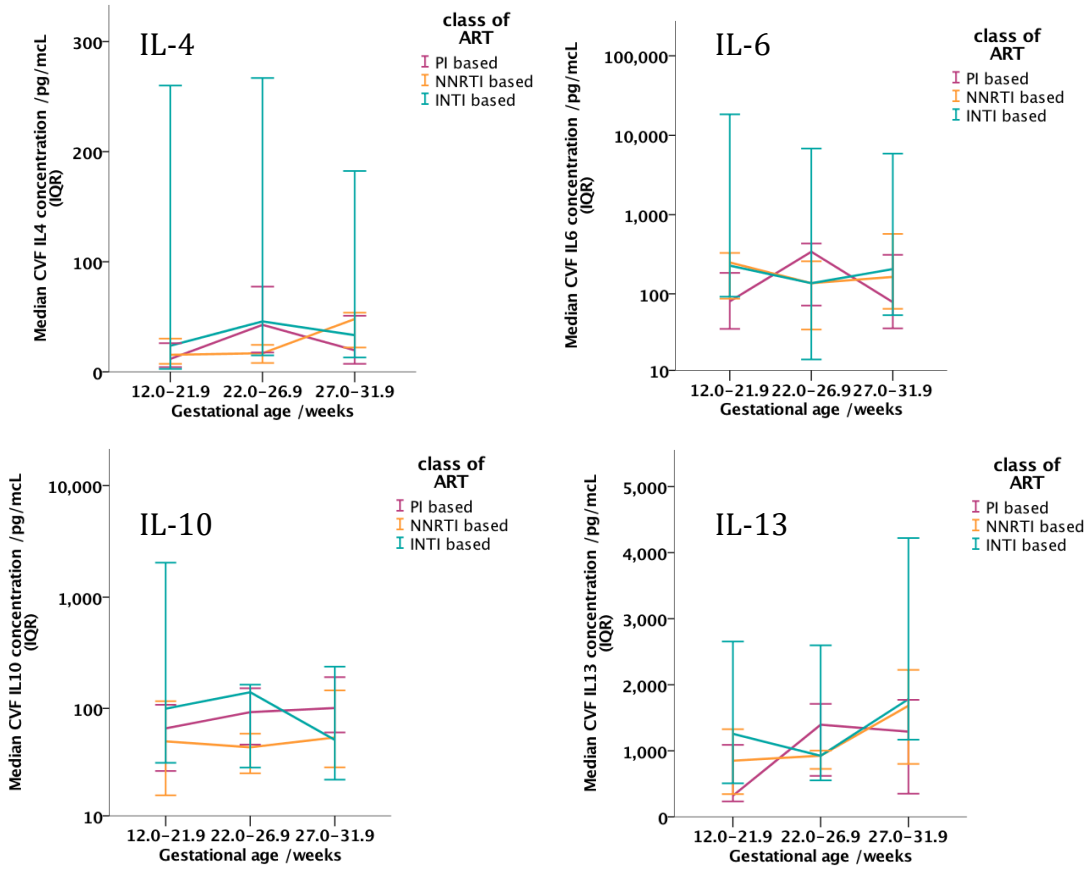


Figure 36 Immune-regulatory cytokines across gestation by ART class
Demonstrating no statistical difference in pro-inflammatory CVF cytokines by third drug class.

5.2.8 CVF cytokines were highly inter-correlated

With the exception of IFN-γ, all other measured cytokines were highly inter-correlated, see table 23. Of note highly inter-correlated (rho>0.7, p<0.0005) inflammatory CVF cytokines were: IL-1β with IL-8 and IL-6; IL-2 with IL-8 and TNF-α. Immune-regulatory cytokine IL-13 was highly correlated with pro-inflammatory cytokines IL-2, IL-12 and TNF-α. IL-4 was also inter-correlated with pro-inflammatory IL-12.

Table 23 CVF cytokine inter-correlations in HIV-1 infected pregnant women

rho= spearman's correlation coefficient,* p<0.05, **p<0.005,***p<0.0005

CVF cytokine	IFN-γ		IL-1β		IL-2		IL-8		IL-12		TNF-α		IL-4		IL-6		IL-10		IL-13	
	rho	p	rho	p	rho	p	rho	P	rho	p	rho	p	rho	p	rho	p	rho	p	rho	p
IFN-γ	1	-	**0.40**	***	0.09	0.374	**0.60**	***	0.15	0.123	**0.24**	*	0.03	0.745	**0.32**	**	0.16	0.098	0.05	0.654
IL-1β	**0.40**	***	1	-	**0.22**	*	**0.83**	***	**0.43**	***	**0.67**	***	**0.43**	***	**0.74**	***	**0.31**	**	**0.46**	***
IL-2	0.09	0.374	**0.22**	*	1	-	**0.25**	***	**0.67**	***	**0.56**	***	**0.63**	***	**0.31**	**	**0.56**	***	**0.72**	***
IL-8	**0.60**	***	**0.83**	***	**0.83**	***	1	-	**0.37**	***	**0.56**	***	**0.25**	*	**0.67**	***	**0.22**	*	**0.52**	***
IL-12	0.15	0.123	**0.43**	***	**0.67**	***	**0.37**	***	1	-	**0.76**	***	**0.84**	***	**0.57**	***	**0.66**	***	**0.80**	***
TNF-α	**0.24**	*	**0.67**	***	**0.76**	***	**0.61**	***	**0.76**	***	1	-	**0.66**	***	**0.61**	***	**0.24**	***	**0.77**	***
IL-4	0.03	0.745	**0.31**	***	**0.63**	***	**0.25**	*	**0.84**	***	**0.66**	***	1	-	**0.49**	***	**0.63**	***	**0.69**	***
IL-6	**0.32**	**	**0.74**	***	**0.31**	**	**0.67**	***	**0.57**	***	**0.67**	***	**0.49**	***	1	-	**0.34**	**	**0.53**	***
IL-10	0.16	0.098	**0.31**	**	**0.56**	***	**0.22**	*	**0.66**	***	**0.56**	***	**0.63**	***	**0.34**	**	1	-	**0.54**	***
IL-13	0.05	0.654	**0.46**	***	**0.72**	***	**0.52**	***	**0.80**	***	**0.77**	***	**0.69**	***	**0.53**	***	**0.54**	***	1	-

5.3 Summary of CVF Cytokine results

1. All measured CVF cytokine concentrations were higher in HIV-1 infected pregnant women than in uninfected pregnant women

2. CVF IL-12 (pro-inflammatory), IL-13 (immune-regulatory) concentrations and IL-2/IL-10 ratio increased during pregnancy in HIV-1 infected pregnant women

3. CVF cytokine concentrations were not associated with gestational age at delivery or prematurity.

4. There was a trend towards higher CVF concentrations in women initiating ART post conception (except IL-2 and IL-12) and an inverse correlation between length of ART exposure in weeks and CVF IFN-γ concentration.

5. ART type did not have a significant effect on CVF cytokine concentration.

6. CVF cytokines were highly inter-correlated

5.4 Conclusions and interpretation

HIV-1 infected pregnant women had higher CVF cytokine concentrations of both pro-inflammatory and immune-regulatory compared to uninfected pregnant women. Higher FGT cytokines in HIV-1 infected compared to uninfected women has been described in both pre cART literature(Bélec *et al.*, 1995; Sha *et al.*, 1997) and post cART(Crowley‐Nowick *et al.*, 2002; Kyongo *et al.*, 2013). To our knowledge this is the first work to demonstrate this phenomenon in pregnancy and the first with CVF obtained using the MC method. Recent work evaluating CVF collected using MCs for cytokine measurement in HIV-1 infected women have not made a comparison to uninfected women nor collected samples in pregnancy(Archary *et al.*, 2015; Jaumdally *et al.*, 2018).

It is possible the higher CVF cytokine concentrations are a direct effect of the HIV on the local mucosal immunology as evidenced by the trend that women initiating treatment in pregnancy, and therefore had a detectable viraemia during the sampling period, had higher concentrations of CVF cytokines. The numbers of women in this group and thus the number of corresponding plasma viral load samples were too low to perform meaningful correlation analysis to support this theory, in addition CVF HIV viral load was not measured as part of this work. Zara et al were able to demonstrate in HIV-1 infected non pregnant women that FGT IL-1β was significantly correlated with CVF HIV viral load(Zara *et al.*, 2004). Roberts and colleagues were also able to demonstrate elevated FGT cytokines in women with primary HIV-1 infected with detectable HIV-1 RNA in their genital tracts(Roberts *et al.*, 2012).The inverse correlation between length of time on ART and CVF IFN-γ concentration, the antiviral cytokine, also supports this theory as the longer time on ART should equate to a lower HIV viral load/ stimulation and thus a dampened type II IFN secretion by NK cells and lymphocytes. These data support a role of the HIV virus in either directly or indirectly up-regulating FGT cytokines.

The observation that the CVF Th1 pro-inflammatory cytokine IL-12 increases during the second and third trimesters in HIV-1 infected women is a novel finding. This mirrors the finding that plasma IL-12 is associated with gestational

age at delivery although the same association was not significant for CVF IL-12. Whilst elevated IL-12 concentrations in CVF in pregnancy has not previously been described, the concentration of this cytokine in amniotic fluid and placentas has been studied. El-Shazly and colleagues have demonstrated elevated concentrations of IL-12 in placentas from women who delivered preterm compared to term(El-Shazly *et al.*, 2004). Lemancewicz et al. also identified higher amniotic fluid IL-12 concentrations in women who delivered preterm compared to term(Lemancewicz *et al.*, 2001). Combo et al. explored endometrial IL-12 concentrations in women experiencing recurrent pregnancy loss and identified that IL-12 was higher in women who miscarried compared to women with proven fertility(Comba *et al.*, 2015).

IL-12p70, the subtype measured in this analysis, is usually produced by macrophages, monocytes and other antigen presenting cells(Liu *et al.*, 2005). It has previously been shown that monocytes in pregnancy are pre primed to produce IL-12(Sacks, Redman and Sargent, 2003). It is possible that peripheral monocytes and local macrophages are both primed to produce IL-12 in these HIV-1 women in response to HIV virus or LPS or another stimuli. The effect of IL-12 or other cytokines on local and systemic cell populations has not been the focus of this work. Lee et al. have demonstrated that IL-12 is critical for up-regulating IFN-γ producing NK cells in cord blood (Lee *et al.*, 1998). Decidual mononuclear cell IFN-γ production in response to LPS is also regulated by IL-12(Negishi *et al.*, 2011). It is conceivable that this cytokine is the signature of local up-regulation of Th1 helper cell and or NK cell recruitment/activation to the gestational tissue in pregnant women and that this increases susceptibility to preterm birth.

Another theme that is evident here, as in the previous chapter, is the dual up-regulation of immune-regulatory cytokines alongside pro-inflammatory cytokines, with both CVF Th1: IL-12 and Th2: IL-13 increasing across second and third trimesters. This has been increasingly documented in studies examining whole blood cytokines in pregnancy, term and preterm labour but is a relatively novel finding in CVF.

In one of the largest longitudinal studies of FGT cytokines in pregnancy to date, Ashford and colleagues described both elevated CVF pro-inflammatory cytokine and IL-10 concentrations in women delivering preterm compared to term, regardless of gestational age at sampling(K. Ashford *et al.*, 2018). Dutt and colleagues identified that cervical IL-10 increased during the second and third trimesters, in both caucasian and non-caucasian women but did report their data on pro-inflammatory cytokines(Dutt, Raker and Anderson, 2015). Non-Caucasian women demonstrated the highest concentrations of IL-10 and this group of women also displayed the greatest vaginal bacterial diversity. These data support our observation of increasing local Th2/ immune-regulatory cytokines across the last 2 trimesters of pregnancy.

Dubicke and colleagues evaluated mRNA and protein expression of pro and anti-inflammatory cytokines in cervical biopsies from women in preterm labour, term labour, non-labouring and non-pregnant women and found that in addition to Th1:IL-1α and IL-1β mRNA upregulation, IL-10 and Th2: IL-13 mRNA were also upregulated in labour. In addition IL-4 protein concentrations were higher in biopsies from women laboring preterm.

Our data showing up-regulation of CVF IL-13 with increasing gestational age suggest local dysregulation of this Th2/anti-inflammatory cytokines at in the lower genital tract mucosa during pregnancy in HIV-1 infected women. Subtle and specific local modulation of pro and anti-inflammatory cytokine network in HIV-1 infected women is further supported by our finding that IL-2/IL-10 ratio increases during pregnancy.

It should be noted that we were not able to make any associations between local CVF cytokine concentrations and antiretroviral class. The numbers of women and samples in this study were too small to identify a class effect on overall cytokine level.

These data highlight that Weggman and Fiore's description of a Th2 cytokine shift in pregnancy and Th1 up-regulation in association with adverse pregnancy outcomes is over-simplified (Wegmann *et al.*, 1993; Fiore *et al.*, 2006). Our limited sample size meant we were not able to make any meaningful comparisons by birth outcome however our data suggest both Th1 and Th2 CVF cytokines are up-regulated in HIV-1 infected women during the second and third trimesters of pregnancy. It is possible that this joint Th1/Th2 up-regulation keeps the fetal-maternal immune system in a state of quiescence, supporting fetal growth. This fine balance in CVF cytokines may reach a threshold at which one or more cytokines predominate in the local environment nearer to point of delivery, which is not captured by these data. However, the up-regulation of CVF cytokines in HIV-1 infected women indicates that the lower FGT immune system is more activated than in uninfected women and that this in itself may reduce the threshold for the trigger of labour. Our results suggest but cannot confirm that this up-regulation may in part be due to a direct HIV effect but CVF cytokine remain elevated even in women receiving effective ART and thus ART does not correct FGT inflammation.

Chapter 6. Vaginal Microbiota results

6.1 Introduction

Ascending genital infection is one of the most important causes of preterm birth, with evidence of bacterial invasion of the fetal membranes and associated inflammation in amniotic fluid being identified in upwards of 80% of women delivering at <32 weeks (Goldenberg *et al.*, 2008). Despite the fact that bacterial vaginosis is common in HIV and a known risk factor for PTB(Hay, Lamont, *et al.*, 1994; Taha *et al.*, 1999), there is a paucity of data on the microbiome of HIV -1 infected pregnant women. Indeed histological evidence of chorioamniontis in placentas from HIV-1 infected women is common(Goldenberg *et al.*, 2006). Many microbiological studies exploring the aetiology of PTB predated culture independent technologies and thus potentially underestimate the role of infection. There is now an increasing understanding of role of the microbiome in the mechanisms underlying PTB with the use of targeted bacterial gene sequencing. In this chapter we set out to describe the vaginal microbiota of a cohort of UK HIV-1 pregnant women using 16S rRNA sequencing and explore associations with ART exposure and prematurity.

Chapter objectives:

- To characterise vaginal microbiota in a group HIV-1 infected and uninfected pregnant women longitudinally with targeted 16S rRNA gene illumima sequencing in the post cART era
- To explore differences by HIV status, cART type and timing, and prematurity.

6.2 Study participant characteristics

In this sub-study of women consenting to genital tract sampling, 16S rRNA bacterial sequencing was undertaken on vaginal swabs from 53 of the HIV-infected and 22 uninfected pregnant women.

The median age of HIV-1 infected pregnant women (34 years, range 21-42) was similar to that of uninfected women (32 years, range 26-39), p=0.06). HIV-1

infected women were more likely to be black 81% (43/53) and less like to be asian 5% (2/53) or caucasian 9% (5/53) than the uninfected women 23% (5/22), (41% 9/22) (36% (8/22) respectively (all comparisons $p<0.0001$). HIV-1 infected women were non-smokers with a mean BMI that was overweight (28 (range 18-44)). Comparative data for BMI and smoking were not available for the uninfected women.

The mean CD4 count of the HIV-1 infected women was within the normal range (668 cells/mm^3 (range 356-1505)). Most of these women had a fully supressed infection with plasma HIV <50 copies/mm^3 at the time of sampling (79% (42/53)).

The majority of HIV-1 infected pregnant women had conceived on cART (n=41), see figure 37. The NNRTI Efavirenz with the nucleotide analogue Tenofovir and the nucleoside analogue Emtricitabine was the most prescribed regimen, see supplementary table 1 for detailed drug regimen details. Twelve women initiated cART during this pregnancy, with similar numbers initiating a Protease nhibitor (PI) based (n=5) and Integrase-based (non PI) (n=4) regimens, see figure 37.

All babies born to uninfected women were delivered at term whereas six HIV-1 infected women delivered preterm. Of these two had spontaneous vaginal deliveries at 32 and 35 weeks gestational age. The remaining four had emergency caesarian sections; two for fetal distress at 35 and 36 weeks and two for intra-uterine growth restriction at 31 and 32 weeks. There was also one stillbirth at 34 weeks

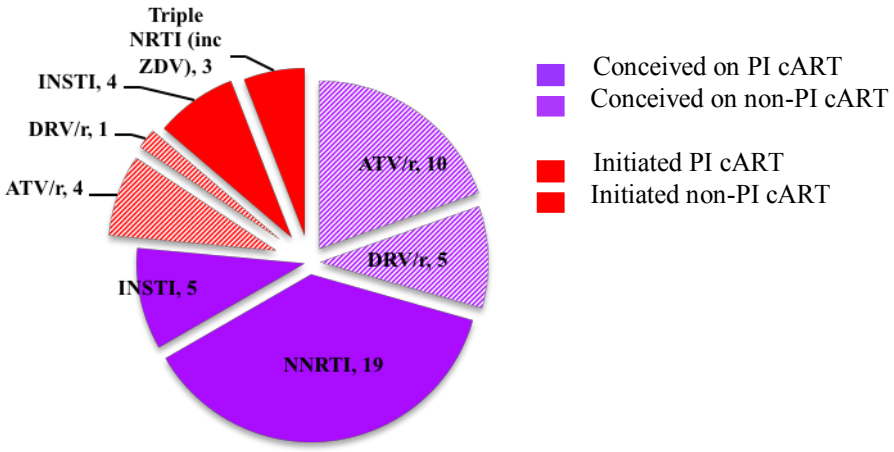


Figure 37 Pie chart to show study participant antiretroviral exposure and timing
PI=Protease Inhibitor; cART= combination antiretroviral therapy; ATV/r= Atazanavir/ritonavir; DRV/r= Darunavir/ritonavir; NNRTI= non-nucleoside reverse transcriptase inhibitor; INSTI= integrase strand transfer inhibitor; NRTI= nucleoside reverse transcriptase inhibitor; ZDV=zidovudine;
Demonstrating the majority of women conceived on cART, with fewer women initiating cART in pregnancy. The majority of women conceivied on an NNRTI (Efavarinz or Nevirapine), followed by conceiving on a PI (ATV/r or DRV/r) with similar numbers conceiving on INSTIs (Raltegravir or Dolutegravir) and initiating a PI and intiating an INSTI.

6.3 Results

6.3.1 The vaginal microbiota of HIV-infected pregnant women is different from uninfected pregnant women during the second trimester

Primary Component Analysis revealed three main clusters of vaginal bacterial colonization: *L. iners*; *L. crispatus* dominant and *G. vaginalis* dominant, see figure 38. The mean number of sequences per sample for HIV-1 pregnant women was 28,692 (range 8,015-206,599) and for uninfected pregnant women 14,974 (range 1,750-27,111). HIV-1 infected pregnant women had significantly greater microbial diversity compared to uninfected women and a greater number of species was observed, see figure 38. At a species level, even HIV-1 infected pregnant women delivering at term had significantly lower proportions of *L. crispatus* and higher proportions of species associated with dysbiotic vaginal microbiomes compared to uninfected term deliveries, see figure 39.

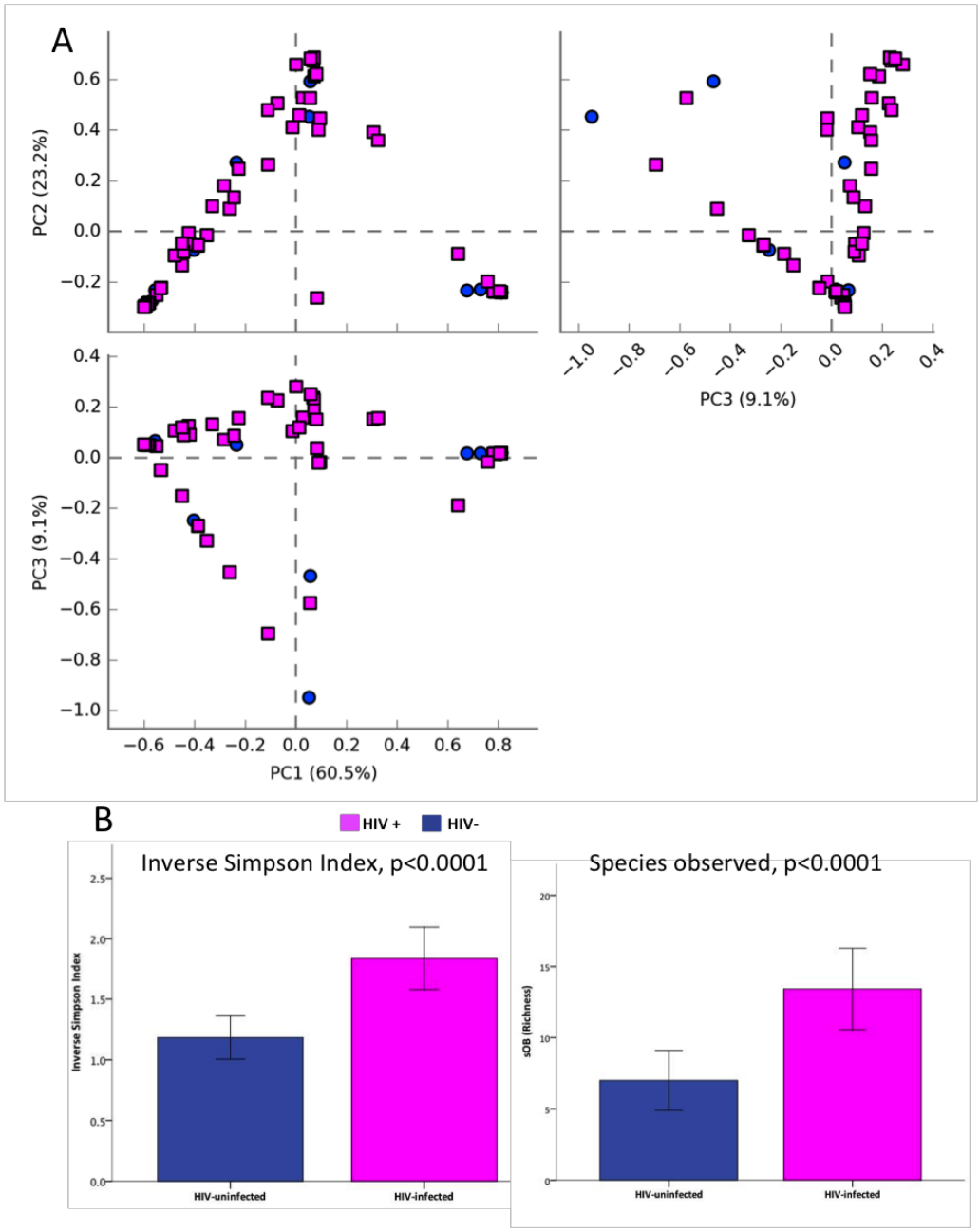


Figure 38 Diversity of vaginal microbiota by HIV status

A: PCA demonstrating β diversity of vaginal microbiota.
The majority of microbial diversity between samples (84%) is explained by PC1 and PC2. Within the *L. crispatus* dominant cluster, HIV uninfected women are the majority representatives where as, within the *G. vaginalis* dominant cluster, samples from HIV-infected women are significantly more represented.

B: Bar chart to demonstrate mean α diversity (NP Shannon Index) and mean sample richness (sOB)

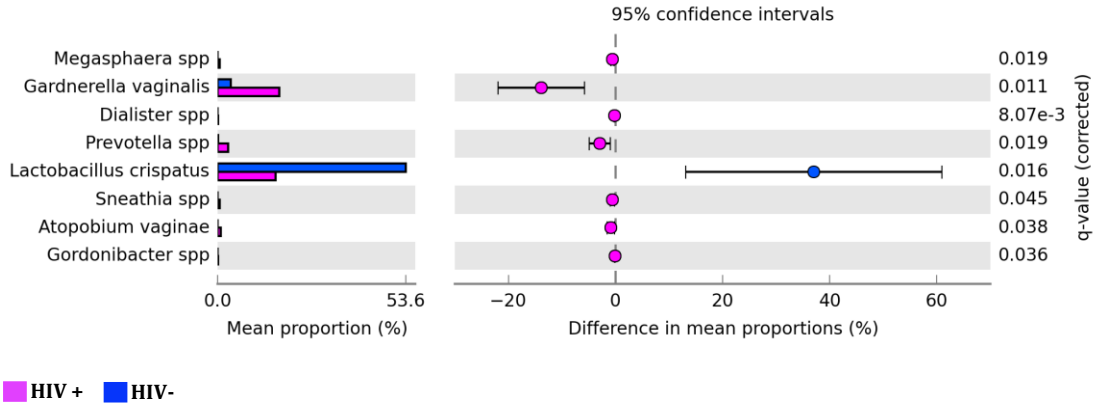


Figure 39 Extended error bar plot of mean proportions of vaginal bacterial species by HIV status

HIV-1 infected pregnant women demonstrate lower proportions of *L crispatus* sequences in their vaginal microbiota compared to uninfected women and higher proportions of *G. vaginalis*, *Prevotella spp., Atopobium vaginae*, Sneathia spp., *Dialister spp.*, Gordinobacter spp., and *Megasphaera spp.*

Using hierarchical clustering, six CST groups could be identified consistent with those previously described(Gajer *et al.*, 2012), see figure 40. Within CST I there are similar numbers of self-reported Black and Caucasian women, whereas within CST III, the majority ethnicity was Black. All ethnicities were represented in CST IV however there no Caucasian women had either CST IIIB or CST V, p=0.031, see figure 40.

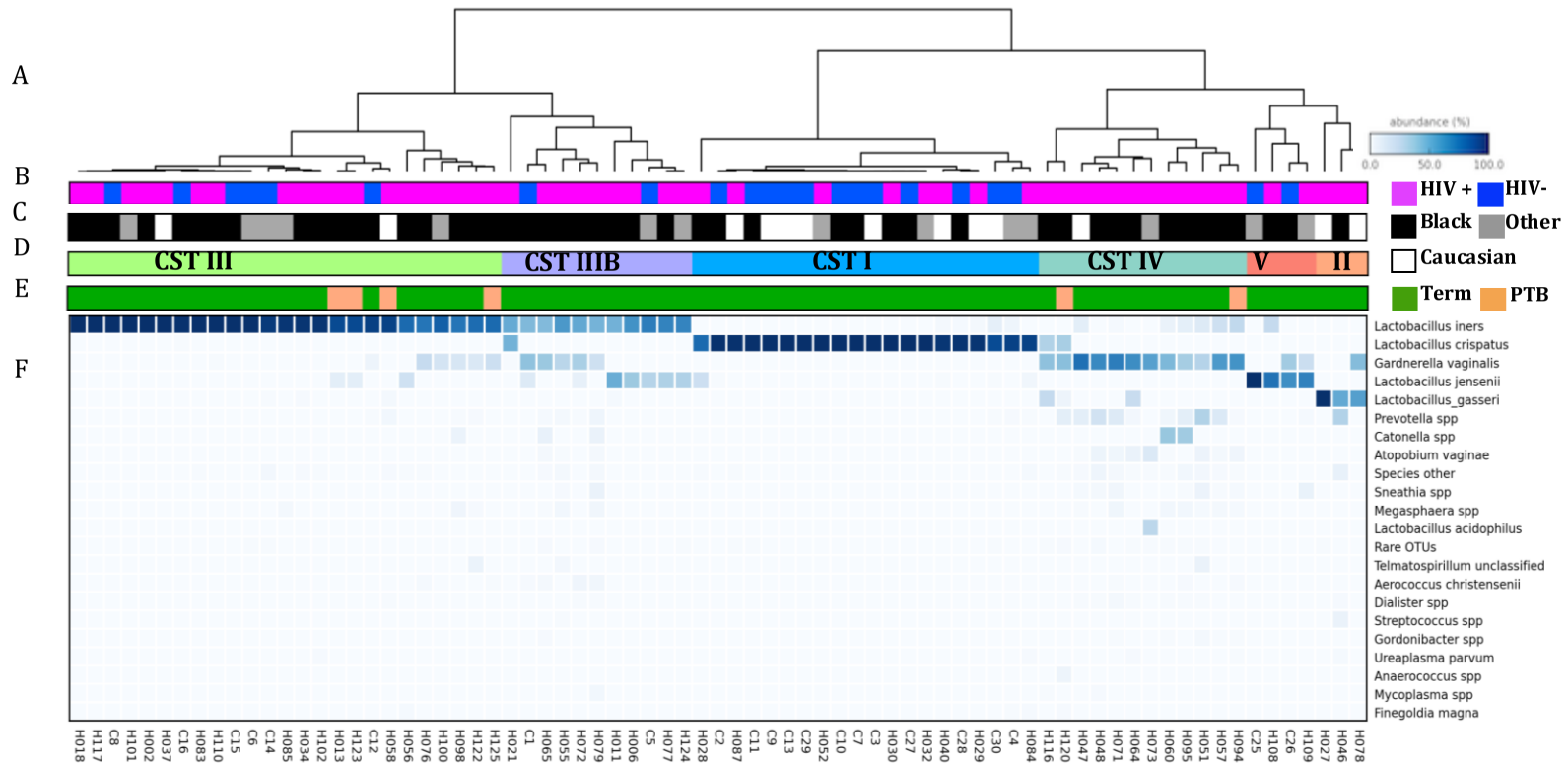


Figure 40 Bacterial species composition of vaginal community state types (CST) from pregnant women during the second trimester

(A) Dendrogram using furthest neighbour linkage of microbial species data shows that vaginal microbiota can be clustered into 6 major groups. (B) HIV status. (C) Ethnicity (D) CST types (E) Prematurity (F) Heat map of relative species abundances of vaginal bacteria.

The predominant CST observed in HIV-1 infected women was III (36%), followed by IIIB (17%) and IV (23%), see figure 41. The majority of uninfected pregnant women's vaginal microbiota were classified as CST I, with none assigned to CST II or IV, p=0.003.

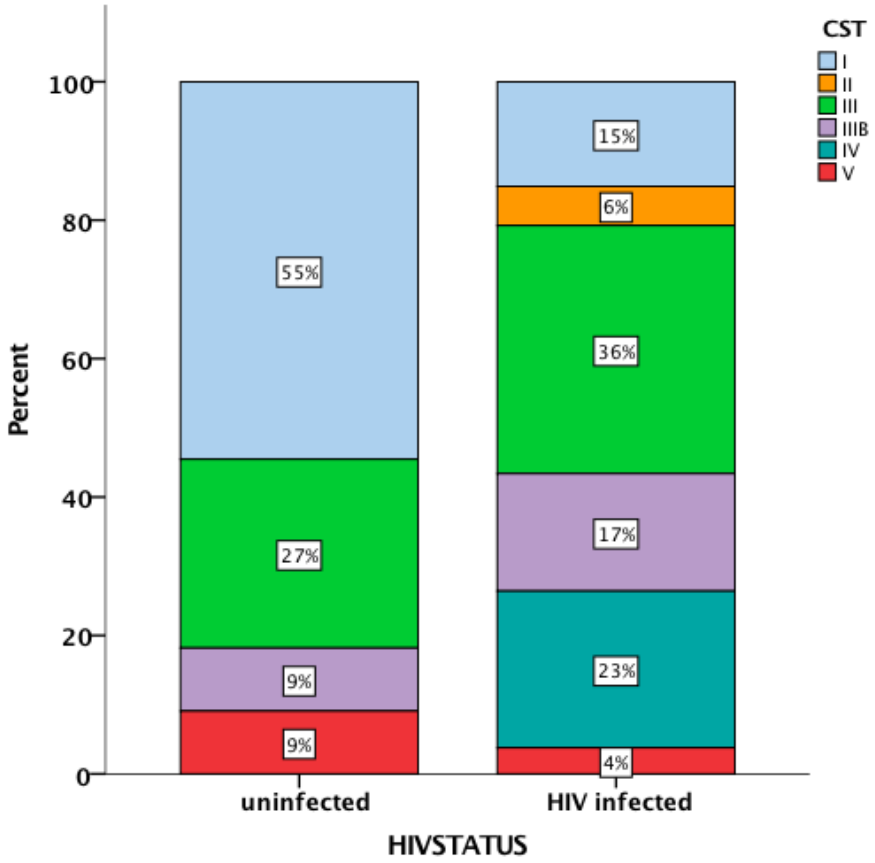
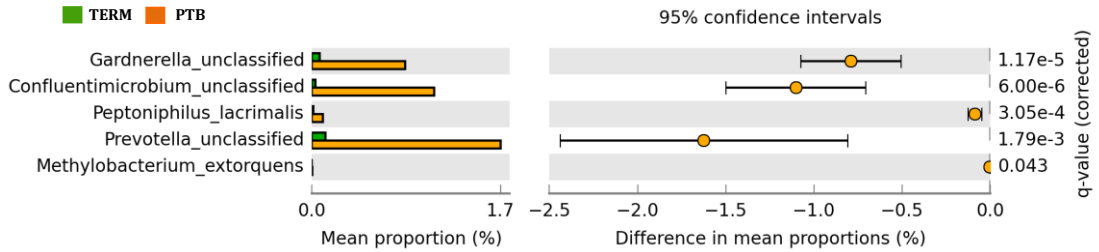


Figure 41 Pie charts to demonstrate CST assignment by HIV status
Demonstrating the predominant CST group in uninfected women is CST I (55%) where as in HIV-1 infected women the most common CSTs were *L. iners* dominant: III (36%) + IIIB (17%) =53%, no uninfected women had the diverse microbiota group IV where as 23% of HIV-1 infected women had this CST.

6.3.2 Preterm birth is associated with increased proportions of vaginal Gardnerella species and other mixed anaerobes

In HIV-1 infected women delivering preterm compared to those delivering term, at a species level, PTB was associated with increased relative abundance of *Gardnerella spp.*, *Peptoniphilus spp.* and *Confluentmicrobacterium spp*. ($p<0.0001$) and *Prevotella spp*. ($p<0.001$), see figure 42. All PTBs occurred in women assigned to CST III and IV, see figure 42.

A



B

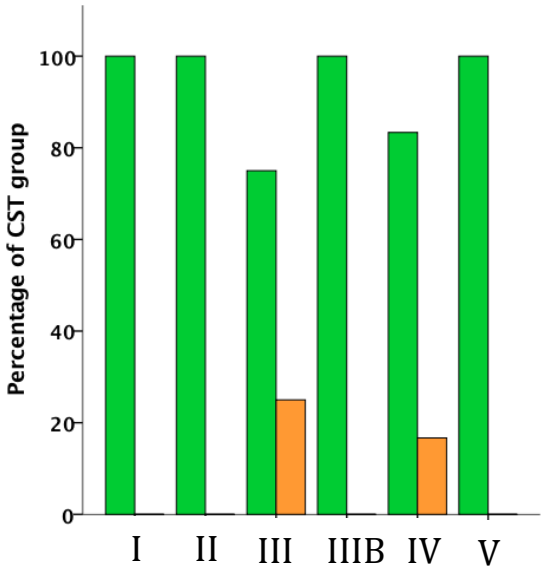


Figure 42 (A) Extended error bar plot comparing mean proportions of vaginal bacterial species from HIV-1 infected pregnant women who delivered at term v preterm
Demonstrating HIV-1 infected women experiencing PTB had high relative abundance of *Gardnerella* ($p=0.00001$) and *Prevotella* species ($p=0.002$) sequences than those delivering at term.
(B) Bar chart to show delivery outcome by CST
Demonstrating all PTBs in HIV-1 infected women occurred in CST III and IV.

26% of women in CST III delivered preterm, and 14% of women in CST IV.

6.3.3 Mixed anaerobes are associated with gestational age at delivery

Linear regression modeling against gestational age at delivery, adjusted for ethnicity, maternal age and BMI, demonstrated that mixed anaerobes: *Prevotella spp. Sneathia spp*. and *Dialister spp*. are inversely associated with gestational age at delivery, see table 24. *L. crispatus* also remained in the model with a positive correlation with gestational age at delivery.

Table 24 Regression of species against gestational age at delivery after adjustment for age, BMI and ethnicity

Species	Fold Change	Estimate	Std. Error	F value	Q value
Lactobacillus_crispatus	4.38	0.34	0.16	2.09	0.04
Lactobacillus_iners	2.78	-0.27	0.16	-1.67	0.09
Lactobacillus_jensenii	3.09	-0.29	0.16	-1.76	0.08
Lactobacillus_gasseri	2.85	-0.43	0.26	-1.69	0.09
Gardnerella_vaginalis	3.25	-0.29	0.16	-1.8	0.07
Prevotella_spp.	5.55	-0.52	0.22	-2.36	0.02
Catonella_spp	1.79	-0.85	0.63	-1.34	0.24
Atopobium_vaginae	0.05	-0.1	0.46	-0.21	0.83
Megasphaera_spp.	0.02	-0.51	0.86	-0.59	0.56
Lactobacillus_acidophilus	13.73	3.04	0.82	3.71	0.17
Snaethia_spp.	7.45	-1.24	0.45	-2.73	0.01
Aerococcus_christensenii	1.85	-0.72	0.53	-1.36	0.18
Telmatospirillum_ unclass	0.9	-1.77	1.86	-0.95	0.41
Dialister_ spp.	7.62	-1.27	0.46	-2.76	0.01
Anearococcus_spp.	1.48	-0.83	0.68	-1.22	0.26
Gordinbacter _spp.	2.69	-1.29	0.79	-1.64	0.12
Mycoplasma_spp	3.5	2.18	1.17	1.87	0.09
Finegoldia_magna	1.29	-0.61	0.54	-1.14	0.26
Ureaplasma_parvum	0.004	0.04	0.58	0.06	0.95
Streptococcus_spp.	NA	NA	NA	NA	NA

cART at conception associated with vaginal Gardnerella spp. and Ureaplasma parvum

In a cross-sectional analysis of samples obtained between 12-21 weeks, women conceiving on cART had a higher relative abundance of *Gardnerella spp.*, *Atopobium vaginae, L.gasseri, Snaethia spp., Ureaplasma parvum and Bifidobacterium breve* than women who initiated cART in pregnancy (of which only one had initiated cART by this time point), $p < 0.025$. Following correction

using the Benjamini-Hochberg FDR method, only *Ureaplasma parvum and Gardnerella spp.* remained associated with cART use at conception (p=0.009), see figure 43. There was a trend towards an increase proportion of *Gardnerella spp*. in women exposed to FTC/TDF compared to ABC/3TC, which was lost following correction, see figure 44.

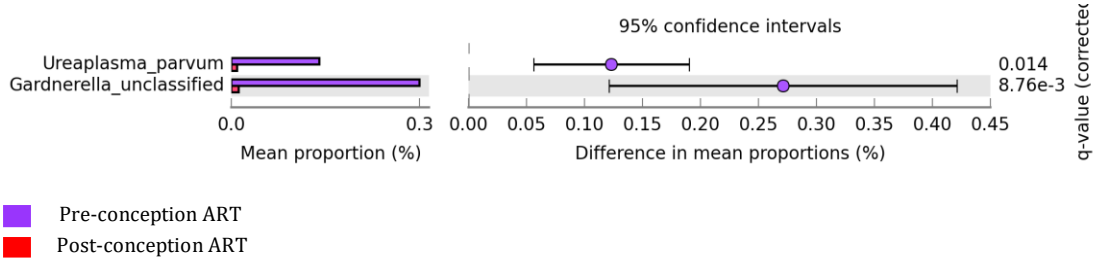


Figure 43 Extended error bar plot comparing mean proportions of vagina bacterial species from HIV-1 infected pregnant women conceiving on cART compared to those women pre cART
Demonstrating that women conceiving on cART have signficantlly higher relative abundance of *Gardnerella* species sequences (p=0.009) and *Ureoplasma parvum* (p=0.01) compared to women initiating cART in pregnancy

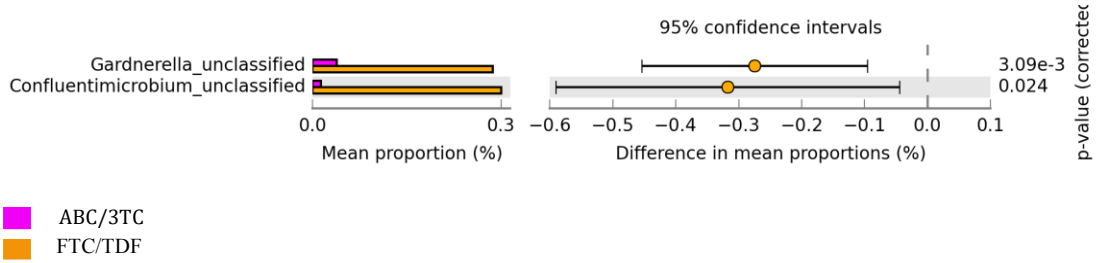


Figure 44 Extended error bar plot comparing mean proportions of vaginal species from HIV-1 infected pregnant women exposed to TDF as the third antiretroviral drug compared to those women with a non TDF third drug
Demonstrating that women receiving FTC/TDF have a higher relative abundance of *Gardnerella* species sequences (p=0.003) compared to women receiving ABC/3TC in pregnancy.

6.3.4 CST I was the most stable and CST IIIB was the least stable during pregnancy

Thirty-nine HIV-1 infected women had two or more consecutive vaginal samples, see figure 45. Women who were classified as CST I or CST V on their first sample, remained in the same community state throughout the sampling period. CST IIIB was the least stable (1/5) with transitions to I, III, IV and V. Whilst there were three cases where CST IV reverted from high diversity to lower diversity CST III, more cases (8/11) remained in this high diversity community state type, most of which demonstrated an increase in α diversity (Inv simpson index) as pregnancy progressed (figure 45). Vaginal microbiota assigned to CST III were either stable

(10/13) or transitioned to IIIB or II and those assigned to CST II were either stable (2/4) or transitioned to III or IV. The number of transitions significantly differed by CST assignment, see figure 45, p=0.03.

6.3.5 CST instability correlates with reducing gestational age at delivery

There was a trend towards a greater number of CST transitions with decreasing mean gestation age at delivery. Women experiencing no CST transitions delivered at 39.0 weeks, women with one transition delivered at 38.6 weeks and women who had two CST transitions delivered at 36.1 weeks, p=0.093.

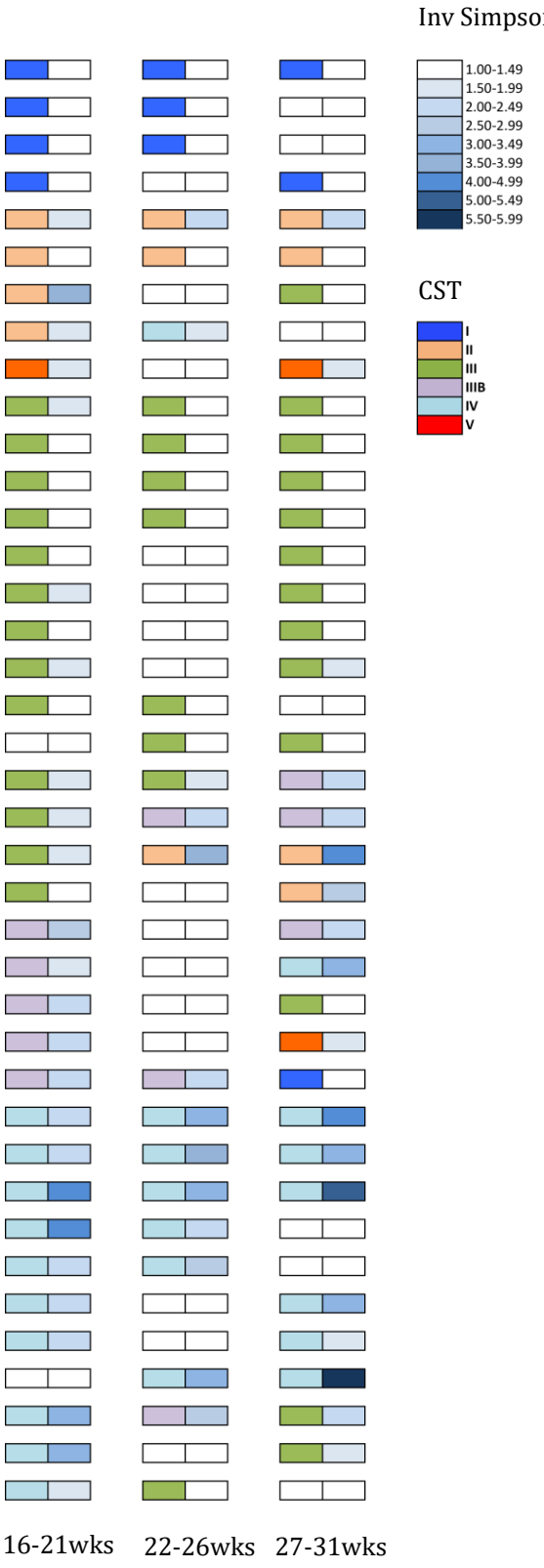


Figure 45 Vaginal community state type profiles throughout pregnancy in a UK HIV-1 infected pregnant cohort

Each sample was assigned a CST indicated by the coloured rectangles with corresponding Inverse Simpson Indices presented (white- low diversity and dark blue- high diversity). The number of transitions significantly differed by CST assignment with CST I demonstrating the most stability during pregnancy; CST IIIB was the least stable transitioning to other CSTs including III and IV; and the majority of women with CST IV remained in this diverse community type with a trend towards increased diversity within the community structure over gestation.

6.3.6 Correlates with clinical diagnostics

6.3.6.1 Strong agreement with clinical bacterial vaginosis and high diversity community state types

Matching gram stained vaginal slides were available for 21 samples from HIV-1 infected pregnant women. Vaginal flora were characterised with light microscopy using the Hay-Ison scoring system(Ison and Hay, 2002). Bacterial vaginosis (grade 3) was identified in 7/21 samples (33%) and from these 6/7 (86%) were clustered as high diversity vaginal microbiotas types (IIIB and IV) on 16S rRNA sequencing demonstrating good agreement.

6.3.6.2 High pH is correlated with Gardnerella species and bacterial diversity

Corresponding pH data were available for 57 samples. pH was associated with increasing α diversity index (NP Shannon index) (r=0.26, p=0.05) and there was a trend towards a positive correlation between pH and number of species observed (Richness) (r=0.23, p=0.089).

pH was positively associated with increasing proportions of *G. vaginalis* (r=0.38, p=0.04). There was a trend towards lower pH with increasing proportions of *L. crispatus* (r=-0.23, p=0.089). There was no significant association between pH and other individual species.

6.4 Summary of vaginal microbiota results

- HIV-1 infected pregnant women had a different vaginal microbiota, with greater bacterial diversity, compared to uninfected pregnant women
- HIV-1 infected pregnant women had reduced *L.crispatus* collonisation compared to uninfected pregnant women in whom it was the most abundant species. *L. crispatus* dominant community state type (I) was observed in 55% of uninfected pregnant women compared to 15% of HIV-1 infected pregnant women.
- The most abundant bacterial species in HIV-1 infected pregnant women was *L. iners* with 36% of HIV-1 infected pregnant women having an *L. iners* dominant community state type (III), followed by 21% of women with *L. iners* mixed (with *Gardnerella*, other anaerobic species or *Lactobacillus spp.*) community state type (IIIB) and 21% with diverse anaerobic species.
- CST III was the least stable across pregnancy whereas CST I was the most stable.
- PTB associated with increased abundance of *Gardnerella* and *Prevotella spp.* and only occurred in women with vaginal CSTs III, IIIB and IV.
- Abundance of *Prevotella, Sneathia* and *Dialister spp.* was inversely associated with gestational age at delivery and *L. crispatus* was positively correlated with gestational age at delivery
- Length of cART exposure, especially pre-conception, was associated with increased *Gardnerella spp.* abundance and a trend towards FTC/TDF exposure being associated with increasing *Gardnerella spp.* abundance compared to ABC/3TC.

- Vaginal pH was strongly associated with bacterial diversity and abundance of *Gardnerella vaginalis.*

6.5 Conclusions and interpretation

The overall objective of this study was to better understand the pathogenesis of pre-term birth in HIV-1 infected pregnant women, the rate of which is particularly high.. The vaginal microbiota differs between HIV-1 infected and

uninfected pregnant women by diversity, genera, species and CST. *L. iners* and diverse anaerobes, particularly *G. vaginalis* and *Prevotella spp*. are highly prevalent in the HIV-1 infected compared to the uninfected women whereas the latter have a higher mean abundance of *L. crispatus*. Thus HIV-1 infected women are at high risk for PTB with an underlying ascending infectious trigger (Witkin, 2015). It should also be noted that there were twice the number of sequence reads from each HIV-1 sample compared to those from uninfected pregnant women suggesting a greater bacterial load in the HIV-1 infected women however this was not formally quantified.

We were able to demonstrate that *Gardnerella spp*. and *Prevotella spp.* were associated with PTB in the HIV-1 infected women. Exploring the more sensitive dependent factor of gestational age at delivery, *Gardnerella spp., Sneathia spp*., and *Dialister spp*. were associated with earlier delivery whereas *L. crispatus* was associated with later delivery. This is one of the first studies to demonstrate these previously characterised associations in HIV-1 infected pregnant women.

Second, the vaginal microbiome during pregnancy is considered to be more stable and less diverse, with increasing numbers of *Lactobacillus spp.,* however longitudinal study of our HIV-1 infected pregnant women revealed high instability especially with CST II, III and IV (Romero *et al.*, 2014). These data are consistent with earlier work in both pregnant and non-pregnant women that has shown the *L. crispatus* dominant CST is the most stable and *L. iners* and *L. gasseri* dominant CSTs are more likely to transition to higher diversity CSTs, that associate with PTB (Verstraelen *et al.*, 2009; Kindinger *et al.*, 2017). This stability is thought to be the result of *L. crispatus* ability to produce the L' lactic acid isomer unlike *L. iners* which produces only the D' isomer(Witkin *et al.*, 2013). L' lactic acid is thought to have the ability to activate innate immune responses that are protective against sexually transmitted infections eg. HPV, HIV (Borgdorff *et al.*, 2014; Reimers *et al.*, 2016; Hearps *et al.*, 2017). Of note, following treatment for clinical BV (most aligned with CST IV) in HIV-1 infected non pregnant women, the vaginal microbiota does not fully restore and commonly shifts to *L.*

iners dominant (CST III) which can co-exist with BV- associated bacteria whereas these are toxic to *L. crispatus*(Hummelen *et al.*, 2010).

Finally, we have explored the relationship between antiretroviral therapy and pre-term birth. Conceiving on cART is associated with increased abundance of *Gardnerella spp*. and *Ureoplasma parvum* compared to those women initiating cART in pregnancy, suggesting that duration of exposure to ART may be vital to its role in PTB. No differences by drug class were observed however a trend toward higher *Gardnerella spp.* carriage in women exposed to TDF with FTC was observed which has not previously been described. These data are at odds with Klatt et al.'s recent sub-analysis of CAPRISA 004 showing topical vaginal TDF concentrations negatively correlate with *Gardnerella spp.* and their in vitro work suggesting that *Gardnerella spp.* may metabolise TDF (Klatt *et al.*, 2017). It is possible that TDF is fueling the *Gardnerella,* or that at lower concentrations it provides a selective pressure for *Gardnerella* and other anaerobic spp and at higher concentrations (such as application of a local topical gel) it is bactericidal. In Donahue Carlson's study of genital systemic drug concentrations in women taking oral ATV/r with FTC/TDF, where prevalence of *Gardnerella spp.* was very low as clinical BV was an exclusion criteria (Carlson *et al.*, 2017), genital drug concentrations reflected Gardnerella abundance. These data could be interpreted as antiretrovirals providing nutrients or selection pressure for the local bacterial species and thus would be congruous with our findings that oral TDF exposure was associated with an increase in *Gardnerella spp.*

It is known that some antiretrovirals: ZDV, ATV and EFV have antimicrobial action against *Bacillus spp.*(Shilaih *et al.*, 2018), therefore we propose certain antiretroviral combinations introduce a selection pressure to the vaginal microbiota, possibly against a specific *lactobacillus spp*. or alternately a fuel for anaerobic species. In turn this would facilitate the generation of biofilms including *Gardnerella* and other species which are difficult to treat/eliminate with single course antibiotics(Machado *et al.*, 2016). Repeated referral for assessment and treatment of BV can encourage maintenance of a more healthy

vaginal microbiota, and thus improve PTB risk in an HIV-1 infected and uninfected population(Mehta *et al.*, 2015).

One criticism of this work could be the higher percentage of Black women in the HIV-1 infected compared to uninfected group, an ethnicity previously associated with *L. iners* carriage and dysbiosis in pregnancy(Hyman *et al.*, 2014). However, our linear regression model included ethnicity, maternal age and BMI, and was still able to reveal an additional association of diverse anaerobic species with earlier gestational age at delivery. Inclusion of more risk factors and confounders into the regression model could have enabled increased model fit and reduce any bias. Ideally the uninfected women would be better matched for ethnicity however increasingly during the study period there was a shift for healthy women to be cared for in community antenatal clinics, limiting recruitment from the hospital antenatal setting.

The study of PTB in HIV-1 infected women, who have high rates of PTB and who are necessarily exposed to drugs that have been associated with increased PTB, is an ideal clinical model in which to study PTB mechanisms as these may be exaggerated in comparison to uninfected women. This may be why we have been successful in identifying several associations with earlier gestational age at delivery with classifications down to species level, including abundance at the minority level, in a small group of HIV-1 infected women, some of which have been demonstrated in the general population.

Chapter 7. Cervicovaginal immune proteins in HIV-1 infected pregnant women

7.1 Introduction

The FGT proteome of pregnant women has been described, with specific focus on labour and PTB. Equally the FGT proteome of HIV-1 infected and high-risk uninfected women has been increasingly explored to define factors that are protective against HIV transmission. To date, there has been no study that has undertaken large-scale characterization of FGT immune proteins in HIV -1 infected pregnant women or investigated the role of novel immune proteins and birth outcome in these women.

In this chapter directed exploration of a panel of 48 immune proteins (cytokines, chemokines, AMPs and growth factors) in FGT of HIV-1 infected pregnant women will be presented and semi quantitative comparisons made according to ART exposure and prematurity.

Chapter objectives:

- To characterise the CVF Proteome of HIV-1 infected women using a directed approach
- To identify differentially abundant proteins by comparing groups of women by ART exposure and prematurity
- To discover novel pathogenic proteins involved in mechanism of PTB syndrome and potential PTB biomarkers

7.2 Results of Experiment 1 - Comparison of second trimester CVF protein arrays between uninfected pregnant women and HIV-1 infected pregnant women conceiving on ART

Experiment 1 compared CVF protein arrays from CVF obtained during the second trimester (weeks 17-22) pooled into three groups, presented in table 25:

1. Uninfected n=10 (A1-10)
2. HIV-1 infected
    a. Conceiving on PI-based cART n=10 (B1-10)
    b. Conceiving on NNRTI/INSTI cART n=10 (C1-10)

Characteristics of women who were included in the pooled samples for each group are presented in table 25. Within this CVF proteomic sub study, the demographic split by HIV status mirrored that seen in the main study, with the majority of uninfected women being caucasian (8/10) and the majority of HIV-1 infected women, from both cART exposure groups, being of Black ethnicity (8/10 in each group). HIV-1 infected pregnant women in this sub study were of similar age to the uninfected pregnant women (p=0.5) and had a BMIs that were in the overweight range compared to uninfected pregnant women who had normal BMIs, see table 25.

HIV-1 infected pregnant women had lower CD4 counts than uninfected pregnant women (p=0.001), see table 25. Vaginal bacterial community state types (CST) were not available for the uninfected women as these women were not included in the microbiota analysis in chapter 6. CST III (*Lactobacillus iners* dominant), IIIB (*Lactobacillus iners* mixed) were the predominant CSTs in all groups, reflective of the microbiota communities seen in the whole HIV-1 infected study cohort. There were slightly more women with CST I (*Lactobacillus crispatus* dominant) in the pooled group conceiving on NNRTI/INSTI based cART (n=3) compared to women conceiving on PI-based cART or no ART (n=1 in each).

7.2.1 CVF immune protein expressed at either high abundance or low abundance were observed in both HIV-1 infected and uninfected pregnant women

CVF protein expression captured by the proteomic protein array could be classified as high abundance and low abundance. In order not to dismiss potentially important proteins that may be expressed at lower abundance, protein expression was subdivided into those whose presence was detected at a density of more or less than two thousand pixels using the LIQOR transmission mode scanner.

Table 25 Experiment 1 and 2 individual patient demographics

Case	Age	BMI	Ethnicity	GA at sample / wks	GA at delivery /wks	cART regime	CD4 count cells/mcL	CST
Uninfected								
A1	29	21	Caucasian	20.1	39.1	-	892	-
A2	25	23	Caucasian	20.3	41.1	-	1316	-
A3	38	24	Other	20.6	37.9	-	1199	-
A4	33	25	Caucasian	20.3	39.1	-	938	-
A5	30	22	Caucasian	19.7	40.7	-	1415	-
A6	34	19	Caucasian	20.6	40.7	-	889	-
A7	35	31	Caucasian	20.3	39.7	-	935	-
A8	43	20	Asian	20.1	UK	-	745	-
A9	32	24	Caucasian	20.7	41.6	-	1649	-
A10	32	20	Caucasian	19.4	39.1	-	995	-
Mean	**33**	**23**	**NA**	**20.2**	**39.9**	-	**1097**	**-**
HIV-1 conceived on PI-based cART								
B1	36	23	Asian	17.0	40.4	ATV/r FTC/TDF	641	IV
B2	36	31	Black	20.7	39.0	ATV/r FTC/TDF	657	I
B3	30	28	Black	19.1	UK	ATV/r FTC/TDF	760	-
B4	34	24	Black	20.0	34.9	DRV/r ABC/3TC	541	IV
B5	21	22	Black	20.0	39.3	ATV/r FTC/TDF	843	IIIb
B6	42	22	Other	19.7	40.3	DRV/r FTC/TDF	951	IIIb
B7	27	31	Black	20.4	36.3	ATV/r FTC/TDF	392	-
B8	35	34	Black	20.0	41.1	ATV/r FTC/TDF	417	III
B9	40	25	Black	20.3	39.9	DRV/r FTC/TDF	474	III
B10	20	29	Black	20.4	39.1	ATV/r FTC/TDF	437	III
mean	**32**	**27**	**NA**	**19.8**	**38.9**	**NA**	**611**	
HIV-1 conceived on NNRTI/INSTI cART								
C1	35	22	Black	20.6	36.8	EFV/FTC/TDF	624	III
C2	32	24	Black	22.3	41.3	NVP/ABC/3TC	607	III
C3	42	20	Asian	20.7	40.7	EFV/ABC/TDF	632	III
C4	38	35	Other	20.4	39.3	EFV/FTC/TDF	1331	I
C5	37	30	Black	20.4	38.4	EFV/FTC/TDF	494	I
C6	30	24	Black	17.7	40.1	EFV/FTC/TDF	532	IV
C7	36	28	Black	22.1	39.0	EFV/FTC/TDF	862	III
C8	28	18	Black	20.7	41.0	NVP/ABC/3TC	830	I
C9	35	40	Black	21.6	38.9	RPV/ FTC/TDF	710	IIIB
C10	38	21	Black	19.9	40.1	RAL/ ABC/3TC	632	IIIB
mean	**35**	**26**	**NA**	**20.6**	**39.6**	**NA**	**725**	
HIV-1 pre cART initiation								
D1	40	21	Black	20.4	40.7	-	312	III
D2	35	29	Other	16.9	37.3	-	1255	III
D3	30	29	Black	20.3	37.9	-	721	IV
D4	37	30	Black	23.8	38.3	-	1505	I
D5	35	34	Black	17.6	38.3	-	832	IV
D6	30	27	Black	20.3	40.3	-	520	III
D7	37	UK	Black	21.1	38.3	-	332	IIIB
mean	**35**	**29**	**NA**	**20.1**	**38.7**	**NA**	**782**	

7.2.2 Pro-inflammatory proteins, anti-microbial proteins, regulators of extracellular matrix and upper FGT proteins were detected in high abundance in the CVF

Identified immune proteins detected in the CVF could be divided into functional themes depending on their known actions: pro-inflammatory, anti-microbial proteins, regulators of the extracellular matrix and endothelial cell growth factors. Functional classification and roles of proteins identified are presented in the discussion in table 28. Proteins that are known to be expressed in the upper FGT or gestational tissues were also identified in CVF, see table 28.

7.2.3 Similar CVF protein profiles were observed in both HIV-1 infected and uninfected pregnant women during the second trimester

There was significant overlap in the CVF protein expression profiles of HIV-1 infected and uninfected women, see figure 46. The most abundant proteins common to both women were: antimicrobial proteins- Trefoil factor 3 (TFF3), Lipocalin-2, Myeloperoxidase (MPO) and inflammatory proteins associated with macrophage and neutrophil recruitment, activation and auto-regulation: IL-8, Macrophage Migration Inhibitory Factor (MIF), C-X-C Motif Chemokine Ligand 1(CXCL-1), Chitinase 3 like 1 protein (CHI3L1), Resistin, and IL-1 Receptor Antagonist (IL-1RA). Extracellular matrix collagenase: matrix metalloproteinase 9 and its inducer protein EMMPRIN were also highly abundant. Proteins known to be expressed in the upper FGT and gestational tissues were also identified including: Resistin, Dipeptidyl-peptidase IV (DDPIV), SERPINE1, and Sex Hormone binding globulin (SHBG).

Lower abundance proteins expressed in both HIV-1 infected and uninfected pregnant women were Vitamin D Binding Protein (Vit D-BP), and another protein associated with macrophage recruitment: Monocyte chemo-attractant protein (MCP-1).

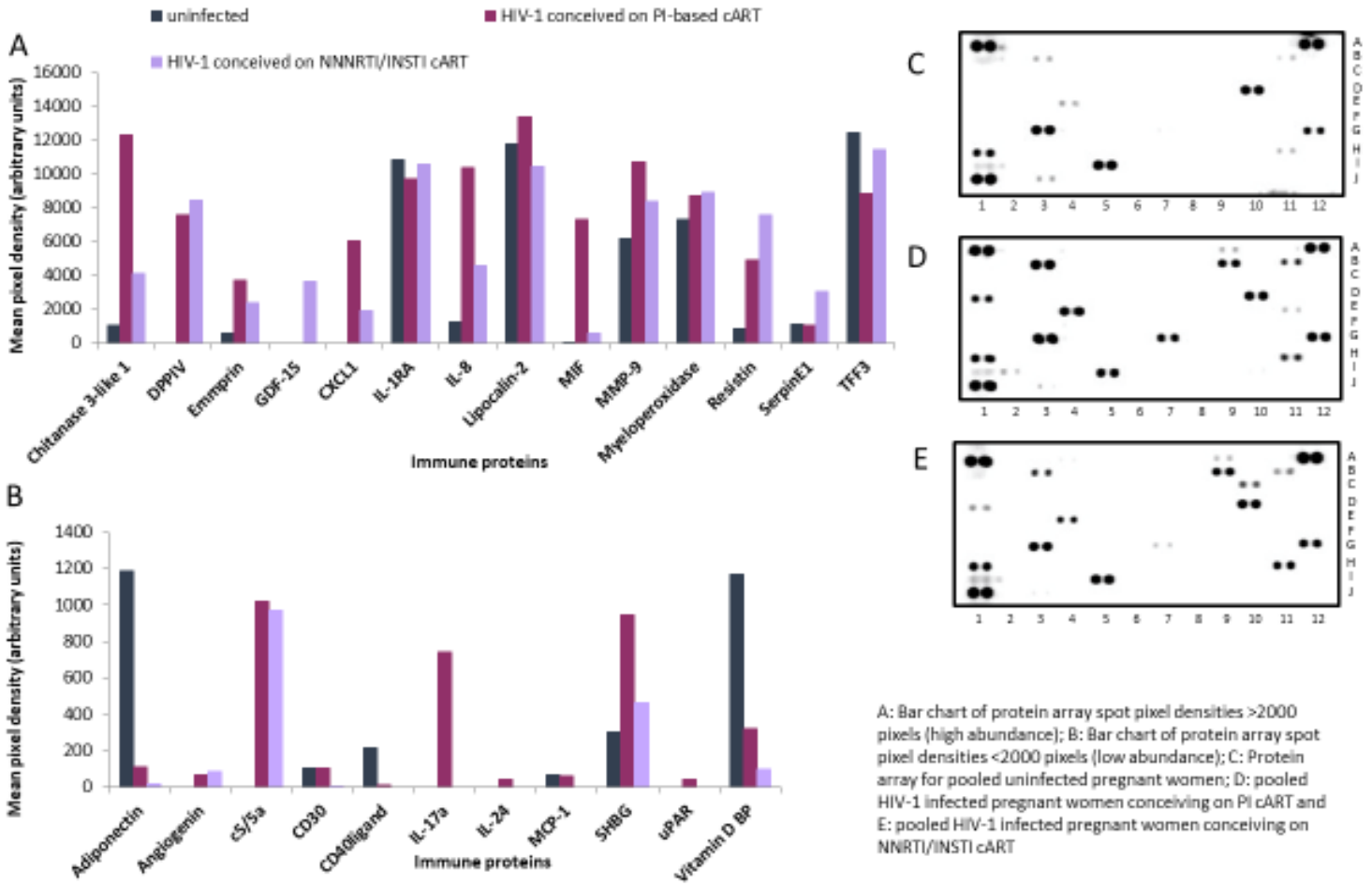


Figure 46 Experiment 1 Second trimester CVF proteomic profile of uninfected women compared to HIV-1 infected women who conceived on cART

Table 26 List of immune proteins and corresponding spot position on protein array

	1	2	3	4	5	6	7	8	9	10	11	12
A	REF	Adiponectin	Aggrecan	Angiogenin	Angiopoietin-1	Angiopoietin-2	BAFF	BDNF	C5/C5a	CD14	CD30	REF
B	-	CD40L	CHI3L1	CFD	CRP	EGF-CFC	Cystatin C	DKK-1	DPPIV	EGF	EMMPRIN	-
C	-	CXCL-5	Endoglin	Fas-Ligand	FGF-2	FGF-7	FGF-19	Fit-3 Ligand	G-CSF	GDF-15	GM-CSF	-
D	CXCL-1	GH	HGF	ICAM-1	IFN-γ	IGFBP-2	IGFBP-3	IL-1α	IL-1β	IL-1RA	IL-2	IL-3
E	IL-4	IL-5	IL-6	IL-8	IL-10	IL-11	IL-12p70	IL-13	IL-15	IL-16	IL-17A	IL-18 BPa
F	IL-19	IL-22	IL-23	IL-24	IL-27	IL-31	IL-32α/β/γ	IL-33	IL-34	IP-10	CXCL-11	Kallikrein 3
G	Leptin	LIF	Lipocalin-2	MCP-1	MCP-3	M-CSF	MIF	MIG	MIP-1α/β	MIP-3α	MIP-3β	MMP-9
H	MPO	Osteopontin	PDGF-AA	PDGF-AB/BB	Pentraxin-3	CXCL-4	RAGE	RANTES	RBP4	Relaxin-2	Resistin	SDF-1α
I	Serpin E1	SHBG	IL-1R4	TARC	TFF3	TfR	TGF-α	THBS1	TNF-α	uPAR	VEGF	-
J	REF		Vit-D BP									Neg control

BAFF: B-cell activating factor; BDNF: Brain-derived Neurotropic Factor; C5/C5a: Complement Component C5/C5a; CD40L: CD40 ligand; CHI3L1: Chitinase 3-like 1; CFD: Complement Factor D; CRP: C-Reactive Protein; EGF-CFC: epidermal growth factor-CFC; DKK-1: Dickkopf-1; DPPIV: Dipeptidyl-peptidase IV; EGF: Epidermal Growth Factor; EMMPRIN: Extracellular Matrix Metalloproteinase Inducer; CXCL-5: C-X-C Motif Chemokine Ligand 5; FGF: Fibroblast Growth Factor; Fit-3 Ligand: FMS-like tyrosine kinase *3 ligand;* G-CSF: Granulocyte colony stimulating factor; GDF-15: Growth/differentiation factor 15; GH: Growth Hormone; HGF: Hepatocyte Growth Factor; ICAM-1:Intercellular Adhesion Molecule-1; IGFBP: Insulin Like Growth Factor Binding Protein; IL-1RA: Interleukin-1 Receptor Antagonist; IL-18BPa Interleukin 18 binding protein Isoform a; IP-10: Interferon gamma-induced protein-10, LIF: Leukemia inhibitory factor; MCP: Monocyte chemo attractant protein; MIF: Macrophage migration inhibitory factor; MIG: Monokine induced by gamma interferon; MIP: Macrophage inflammatory protein; MMP: Matrix metalloproteinase ;MPO: Myeloperoxidase; PDGF: Platelet-derived growth factor; RAGE: receptor for advanced glycation end-products; RANTES: Regulated on activation, normal T cell expressed and secreted; RBP: Retinol binding protein; SDF-1: stromal cell-derived factor-1; Serpin E1: Serine proteinase inhibitor E1; SHBG: Steroid Hormone Binding Globulin; IL-1R4: Interleukin 1 Receptor 4; TARC: Thymus and activation regulated chemokine; TFF3: Trefoil factor-3; TfR: Transferrin receptor protein; TGF-α: Transforming growth factor alpha; THBS1: Thrombospondin-1; uPAR: Urokinase receptor; VEGF: Vascular Endothelial Growth Factor and Vit-D BP: Vitamin D binding protein.

7.2.4 HIV -1 infected pregnant women had greater abundance of immune proteins associated with macrophage and neutrophil infiltration and neutrophil myeloperoxidase compared to uninfected pregnant women

There was an overall trend of higher abundance of inflammatory proteins associated with macrophage and neutrophil recruitment and activation: CHI3L1, IL-8, MIF and CXCL-1, Complement component C5/C5a and Resistin in HIV-1 infected pregnant women compared to uninfected women, see figure 46. No CXCL-1, C5/C5a and minimal MIF was identified in the pooled CVF array of uninfected pregnant women.

There was also a trend towards greater abundance of the neutrophil produced antimicrobial enzyme MPO in HIV-1 infected pregnant women compared to uninfected pregnant women but not of two other antimicrobial proteins: TFF3 and Lipocalin-2 which appeared to be more abundant in uninfected pregnant women. Vitamin D-BP was also more highly abundant in uninfected pregnant women.

7.2.5 HIV -1 infected pregnant women had greater abundance of proteins associated with extracellular matrix remodeling compared to uninfected women

Protein matrix metalloproteinase 9 and its inducer EMMPRIN were more abundant in HIV-1 infected pregnant women than uninfected pregnant women.

7.2.6 Proteins known to be secreted by the placenta were more abundant in HIV-1 infected women

Upper FGT proteins associated with gestational tissues, most commonly the placenta: DPPIV, Angiogenin and Growth/differentiation factor 15 (GDF-15) were more abundant in CVF from HIV-1 infected women. DPPIV, GDF-15 and Angiogenin were not identified on the CVF array of pooled uninfected women. SHBG, produced mainly by the liver but also by the ovaries and placenta, was observed at higher abundance in HIV-1 infected women compared to uninfected pregnant women.

7.2.7 Effect of cART class in HIV-1 infected pregnant women (PI versus NNRTI/INSTI) - Women conceiving on PI based cART displayed a higher abundance of immune proteins associated with macrophage and neutrophil recruitment and activation compared to pregnant women conceiving on NNRTI/INSTI based cART

CVF protein profiles in women conceiving on PI- based cART displayed higher abundance of CHI3L1, CXCL-1, IL-8, MIF and IL-17 compared to and NNRTI/INSTI based cART in experiment 1. Similar concentrations of antimicrobial proteins MPO and TFF3 were observed between groups although there was a trend towards higher Lipocalin-2 expression in women conceiving on PI-based cART. Women conceiving on PI-based cART demonstrated higher CVF Vit D-BP and SBHG concentrations compared to women conceiving on NNRTI/INSTI based cART.

7.3 Results of Experiment 2: HIV-1 infected women conceiving on ART compared to HIV-1 infected pregnant women pre cART initiation

Experiment 2 compared CVF protein arrays from CVF obtained during the second trimester (weeks 17-22) pooled into four groups, presented in table 25:

1. Uninfected n=10 (A1-10)
2. HIV-1 infected
    a. Conceiving on PI-based cART n=10 (B1-10)
    b. Conceiving on NNRTI/INSTI cART n=10 (C1-10)
    c. pre cART initiation n=7 (D1-7)

7.3.1 The same functional themes and individual proteins observed in Experiment 1 were identified in Experiment 2.

The most abundant CVF proteins in both HIV-1 infected and uninfected women in this experiment were inflammatory immune proteins: IL-8, CHI3L1, Resistin, CXCL-1; anti-microbial proteins: TFF3, Lipocalin-2 and MPO and extracellular

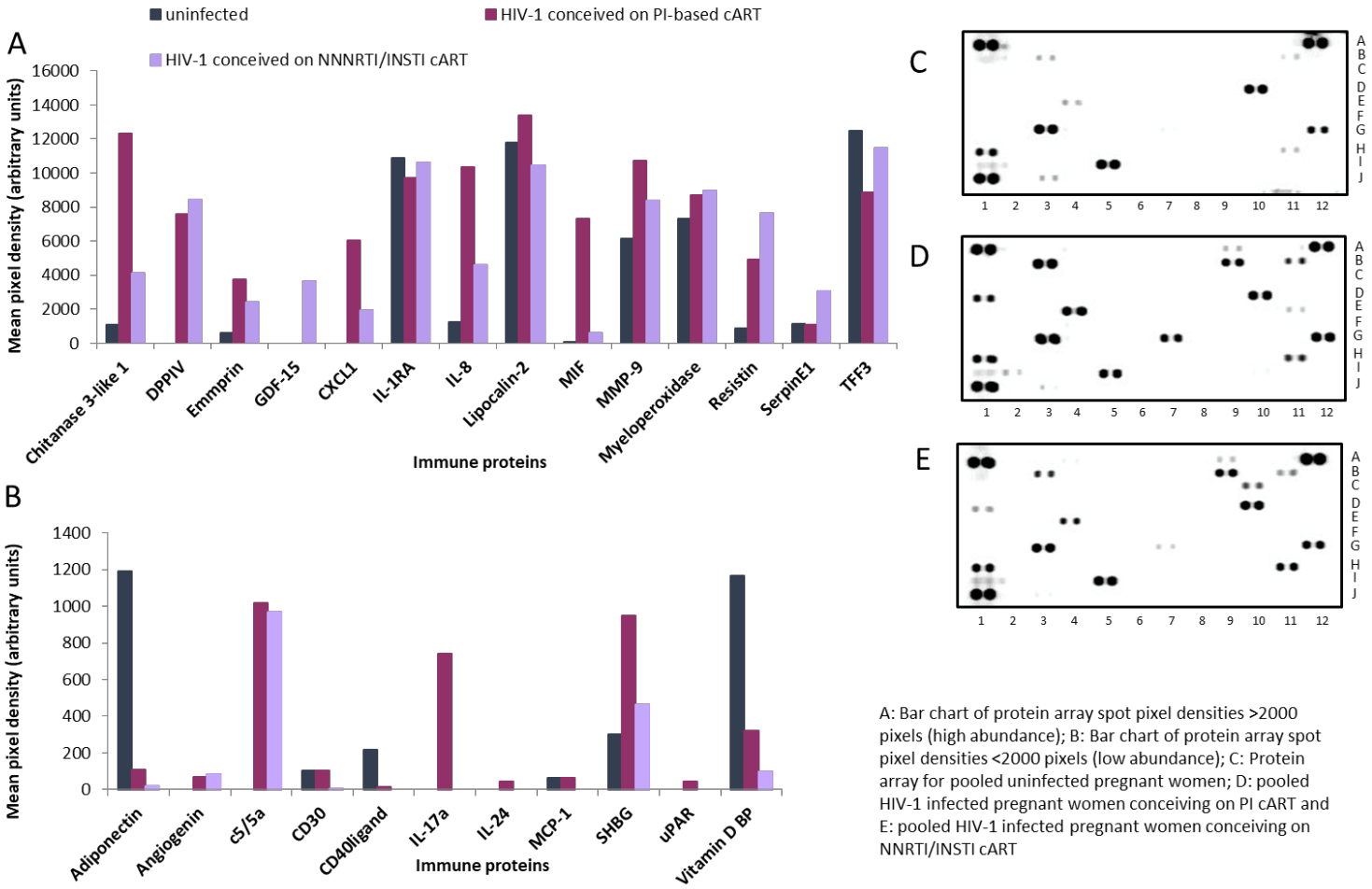
matrix regulators: EMMPRIN and MMP9, see figure 47. The following placenta derived proteins were identified at high abundance: Growth/differentiation factor 15 (GDF-15), SERPINE1, uPAR, Vascular Endothelial Growth Factor (VEGF) and SHBG.

A greater number of immune proteins were identified in the CVF at lower abundance in experiment 2. Proteins identified at low abundance in the second experiment that were not identified in experiment 1 included inflammatory cytokines IL-1a, IL-16, IL-17, IL-24, MIP-3β, placental derived Dickkopf-1 (DKK-1), Endoglin and ECM factors: Aggrecan: Osteopontin and Pentraxin3.

7.3.2 HIV-1 infected pregnant women had higher inflammatory, ECM modifying and gestational tissue secreted proteins compared to uninfected pregnant women as seen in experiment 1

Pregnant women with HIV-1 again displayed greater abundance of inflammatory proteins specifically associated with macrophage and neutrophil recruitment and activation: C5/C5a; CHI3L1; CXCL-1; IL-1α; IL-8, MIF, MIP-3α/β; Resistin, and IP-10. Extra cellular matrix modifying proteins were also more abundant in HIV-1 infected pregnant women compared to uninfected specifically: MMP-9, EMMPRIN, SERPINE1, uPAR, Aggrecan and Osteopontin.

Gestational tissue proteins secreted from the placenta or foetus: DPPIV, DKK-1, GDP-15, Endoglin and VEGF were nearly exclusively identified in CVF from HIV-1 infected women. A far greater number of proteins identified at low abundance were observed in HIV-1 infected pregnant women compared to uninfected pregnant women.



A: Bar chart of protein array spot pixel densities >2000 pixels (high abundance); B: Bar chart of protein array spot pixel densities <2000 pixels (low abundance); C: Protein array for pooled uninfected pregnant women; D: pooled HIV-1 infected pregnant women conceiving on PI cART and E: pooled HIV-1 infected pregnant women conceiving on NNRTI/INSTI cART

Figure 47 Experiment 2 Second trimester CVF proteomic profile of HIV-1 infected women conceiving on ART compared to HIV-1 infected pregnant women pre cART initiation compared to uninfected pregnant women

7.3.3. HIV-1 infected pregnant women who had not yet commenced cART in this pregnancy had similar functional CVF protein profiles to those women who conceived on cART

The same CVF proteins were identified in HIV-1 pregnant women who had not yet commenced cART in pregnancy in comparison to those women who conceived on cART. Inflammatory protein associated with macrophage and neutrophil recruitment and activation were up regulated eg. IL-8, IL-17, CXCL-1, MIF, Resistin and CHI3L1. These women also displayed a high abundance of GDF-15, a regulator of macrophage activation, similar to women conceiving on NNRTIs/INSTIs, which were both higher than observed for both HIV-1 women conceiving on PI-based cART and uninfected pregnant women.

7.3.4 CD40 ligand, Aggrecan, MIP-3α, DPPIV and Kallikrein 3 were differentially upregulated in CVF of HIV-1 infected women not taking cART and moderately higher ECM modifiers: MMP9 and uPAR, and higher VEGF abundance was observed compared to all other groups

Proteins identified as significantly up regulated in women pre cART initiation compared to all other groups were CD40 ligand, MIP-3α; Kallikrein 3, Aggrecan and DPPIV. HIV-1 infected pregnant women who were yet to initiate cART also demonstrated a trend towards higher expression of ECM regulators: MMP9 and uPAR and higher Endothelial Growth Factor: VEGF than women conceiving on cART and uninfected women.

7.3.5 Women who conceived on PI-based cART the highest abundance of inflammatory proteins compared to women conceiving of NNRTI/INSTI based cART and uninfected pregnant women, along with women not yet initiating cART having similar abundance profile for some CVF ECM modifying proteins

Inflammatory immune proteins: CHI3L1, IL-8; IL-1α, IL-16, IL-17, IL-24, CXCL-1, ICAM-1, IP-10 and MIP-3β were most abundant in pooled CVF from women who conceived on PI-based cART compared to all other groups. SHBG was most highly expressed in women receiving PI-based cART compared to all other

groups. ECM modifiers: MMP-9, EMMPRIN, SERPINE1 and Osteopontin were most highly expressed by both women conceiving on PI-based cART and HIV-1 infected pregnant women pre cART initiation.

Antimicrobial proteins: MPO and Lipocalin-2 were moderately more abundant in HIV-1 infected women conceiving on PI-based cART compared to other groups of HIV-1 infected pregnant women.

Vascular endothelial protein Endoglin was also up-regulated in women conceiving on PI-based cART, more than in HIV –infected women not taking cART and more than in uninfected women but not as high as observed in women conceiving on NNRTI/INSTI based cART.

7.4 Results of Experiment 3: HIV-1 infected women delivering at term compared to those who delivered preterm

Experiment 3 compared CVF protein arrays from CVF obtained during the second trimester (weeks 17-22) pooled into two groups, presented in table 25:

1. HIV-1 infected
    a. Delivered >37 weeks (TERM DELIVERY) n=5 (T1-5)
    b. Delivered <37 weeks (PRETERM DELIVERY) n=5 (P1-5)

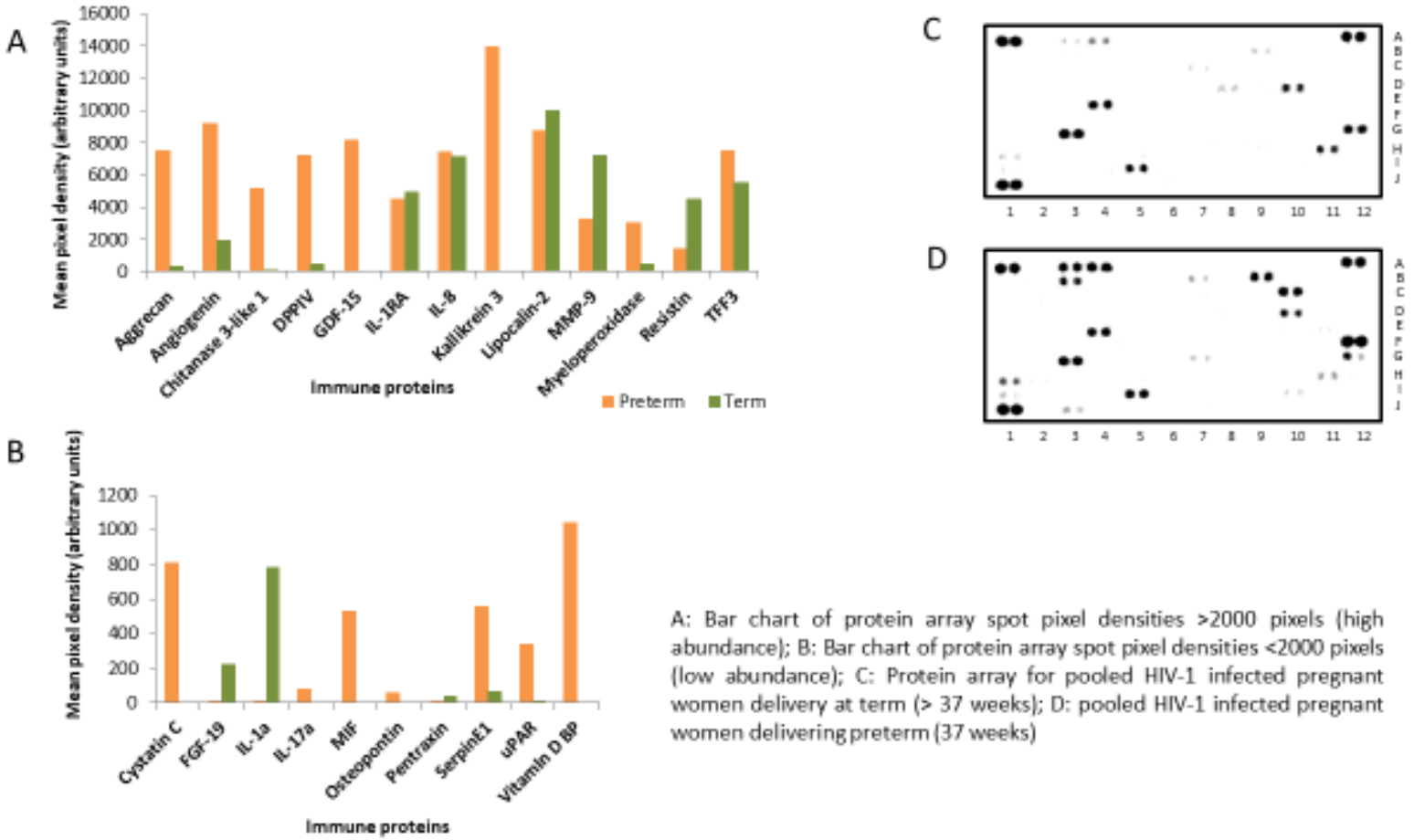
<u>The groups were matched on demographic characteristics</u>
Characteristics of women who were included in the pooled samples for each group are presented in table 27. Women in both groups were matched in demographics as much as possible within the constraints of available samples. Mean maternal age (32 years), mean BMI (25), ethnicity (Black) and mean gestational age at sampling (20 weeks) were the same between groups. The majority vaginal microbiota CST for both groups was III/IIIB. Mean CD4 counts of each group were >500 and similar between groups, p=0.82. Mean gestational age for term deliveries was 40 weeks and for preterm deliveries 35 weeks. Three women from the Term group had vaginal deliveries and two women had

caesarian sections. Most (four) of the women who delivered preterm <37 weeks had emergency caesarians for fetal distress, one woman had a spontaneous preterm birth.

Table 27 Demographics of term and preterm delivery cases included in Experiment 3

Case	Age	BMI	Ethnicity	CD4 count cells /mcL	GA at sampling /wks	GA at delivery /wks	MODE	CST	cART regime
Term									
T1	35	34	Black	392	20.0	41.0	EMCS for FD	III	RPV/FTC/TDF
T2	34	18	Black	832	20.1	38.3	PLCS	IV	ATV/r/ FTC/TDF
T3	33	UK	Black	396	19.8	41.7	SVD	-	ATV/r/ ABC/3TC
T4	37	24	Black	421	20.0	41.0	SVD	IIIB	RAL/FTC/TDF
T5	21	22	Black	846	20.0	39.2	SVD	IIIB	ATV/r/ FTC/TDF
Mean	**32**	**25**	**NA**	**577**	**20.0**	**40.2**	**NA**	**-**	**NA**
Preterm delivery									
P1	35	22	Black	624	20.6	36.8	EMCS for FD	III	EFV/FTC/TDF
P2	34	28	Black	356	20.1	32.1	EMCS for FD	III	DRV/r/ FTC/TDF
P3	28	21	Black	819	19.4	34.7	SPTL	IV	ATV/r/ FTC/TDF
P4	34	24	Black	541	20.0	34.9	EMCS for FD	IIIB	DRV/r/ABC/3TC
P5	27	31	Black	392	20.4	36.3	EMCS for FD	-	ATV/r/ FTC/TDF
Mean	**32**	**25**	**NA**	**546**	**20.1**	**35.0**	**NA**		**NA**

EMCS: Emergency Caesarian Section; FD: Fetal Distress; PLCS: Planned Caesarian Section; SVD: Spontaneous Vaginal Delivery; SPTL: Spontaneous Preterm Labour; RPV: Rilpivirine; FTC: Emtricitabine; TDF: Tenofovir Disoproxil Fumarate; ATV: Atazanavir; r: Ritonavir boost; ABC: Abacavir; 3TC: Lamivudine; RAL: Raltegravir; EFV: Efavarinz; DRV: Darunavir.



A: Bar chart of protein array spot pixel densities >2000 pixels (high abundance); B: Bar chart of protein array spot pixel densities <2000 pixels (low abundance); C: Protein array for pooled HIV-1 infected pregnant women delivery at term (> 37 weeks); D: pooled HIV-1 infected pregnant women delivering preterm (37 weeks)

Figure 48 Experiment 3 second trimester CVF proteomic profile of HIV-1 infected women delivering at term v preterm

7.4.1 Women delivering preterm had higher abundance of inflammatory immune proteins associated with neutrophil and macrophage activation in their CVF during the second trimester than women delivering at term.

Second trimester pooled CVF from women who subsequently delivered < 37 weeks, regardless of whether iatrogenic or spontaneous labour, had higher abundance of proteins associated with inflammation, specifically macrophage and neutrophil activation factors: IL-8, IL-17a, CHI3LI, MIF and Vitamin D BP, see figure 48. Whereas anti-inflammatory IL-1RA was marginally lower in abundance in women who went on to deliver preterm. Macrophage regulator GDF-15 was only observed in the preterm group in this experiment. Conversely Resistin, a macrophage-produced protein was reduced in CVF from women who subsequently delivered preterm compared to term.

7.4.2 Women delivering preterm had higher abundance of neutrophil MPO in their CVF during the second trimester than women delivering at term and exclusive expression of antimicrobial protein Cystatin C at low abundance.

Neutrophil antimicrobial protein MPO was significantly elevated in women who went on to deliver preterm as was β-defensin inducing TFF3. Antimicrobial protein Cystatin C and Kallikrein were also exclusively identified in the CVF pooled from these women and was not seen in women delivering at term. Antimicrobial Lipocalin-2 was similar in both groups, but minimally higher in women delivering at term.

7.4.3 Proteins that are likely to derive from gestational tissues were higher in second trimester CVF in women who subsequently delivered preterm

Proteins that may be secreted from the placenta such as: Angiogenin, GDF-15, SERPINE1 and DPPIV and endothelial growth factors associated with embryogenesis: VEGF and FGF-19; were more abundant in women who went on to deliver preterm compared to term.

7.4.4 ECM protein MMP-9 was not unregulated in CVF during the second trimester in women who delivered preterm compared to term

ECM modifying proteins: Aggrecan, SERPINE1, uPAR and Osteopontin were up-regulated in second trimester CVF in HIV-1 infected women who ultimately

delivered preterm compared to term but up regulation of MMP9 was not observed in the pooled samples

7.5 Summary of FGT Proteome results

- HIV-1 infected pregnant women had similar CVF protein profiles to uninfected pregnant women which could be divided according to function:
    - Factors associated with recruitment, activation and regulation of innate immune cells (neutrophils and macrophages)
    - Antimicrobial proteins
    - ECM regulating factors
    - Endothelial growth factors and hormones

- Several proteins known to be secreted in the gestational tissues were identified in CVF

- In general, most of these CVF proteins were more abundant in HIV-1 infected pregnant women compared to uninfected pregnant women

- HIV-1 infected pregnant women initiating cART in pregnancy had high abundance of inflammatory proteins associated with activation of neutrophils and macrophages e.g. IL-8, IL-17, CXCL-1, MIF, MIP-3α, MMP-9 and CHI3L1. These CVF proteins were more abundant than in uninfected women and HIV-1 infected women conceiving on NNRTI/INSTI but mostly not as high as seen in women conceiving on PI-based cART

- Women pre cART initiation had exclusive up-regulation of adaptive immune cell associated protein: CD40 Ligand (T cell activation)

- Women pre cART initiation also had the highest levels of ECM modifiers: MMP-9, uPAR and Aggrecan compared to all other groups with CVF from women conceiving on PI-based cART having a marginal reduction in comparative abundance followed by women receiving NNRTI/INSTI based cART with uninfected women having the lowest abundance.

- Antimicrobial proteins MPO and Lipocalin were most abundant in women conceiving on PI-based cART where as TTF3 was more abundant in HIV-1 infected women conceiving on no ART or NNRTI/INSTI –based cART.

- Neutrophil and macrophage associated proteins: IL-8, IL-17a, CHI3LI, MPO, MMP-9, MIF and Vitamin D BP were expressed at a greater quantity in second trimester CVF pooled from HIV-1 infected women who subsequently delivered preterm compared to term whereas IL-1RA (pro-inflammatory IL-1 antagonist) was reduced in women who delivered preterm compared to term.

- Cystatin C is an AMP that was exclusively up-regulated in pooled second trimester CVF from HIV-1 infected women who delivered preterm.

- ECM associated proteins Aggrecan and SERPINE1, uPAR and Osteopontin were unregulated in pooled CVF from HIV-1 infected pregnant women who delivered preterm compared to term but the same increase was not observed with MMP-9.

- Gestational tissue associated proteins: Angiogenin, GDF-15, DPPIV, SERPINE1, VEGF and FGF-19 were also more highly expressed in pooled second trimester CVF from HIV-1 infected women who delivered preterm compared to term.

Table 28 Table of immune protein functions and HIV-1 interactions

Protein	Function /origin	Known HIV-1 interaction	Reference
Inflammatory proteins associated with macrophage and neutrophil function			
IL-1α	Also known as alarmin, it is an initiator of inflammation. It is a chemo attractant for neutrophils and can activate expansion of CD4 T cells. Induces cell adhesion molecules on leukocytes and vascular endothelium. Mainly produced by monocytes, macrophages, and neutrophils. Known to be expressed in CVF, amnion and chorion. Up regulated in PTB in association with IL-1β and IL-1RA.	gp120 up regulates IL-1α in endometrial epithelial cells tat and nucleocapsid up regulate IL-1α in T cells vpr up regulates IL-1α in monocyte derived dendritic cells	(Lee and Park, 2009; Barrero *et al.*, 2013; Nazli *et al.*, 2013; Heng *et al.*, 2014; Zahoor *et al.*, 2015; Nadeau-Vallée *et al.*, 2016; Pandey, Chauhan and Awasthi, 2017; Hadley *et al.*, 2018)
IL-8	Chemokine secreted by neutrophils, induces chemotaxis of neutrophils to sites of infection. Also induces MMP-9. Identified in amniotic fluid and CVF in response to intra amniotic infection. Up regulated in cervix, decidua and amnion and in term and preterm labour. Has been assessed as a biomarker for PPROM and PTB.	gp120 up regulates IL-8 in endometrial epithelial cells gp120 up regulates IL-8 in monocyte derived macrophages nef up regulates IL-8 in dendritic cells tat up regulates IL- 8 secretion by T cells	(Cicala *et al.*, 2002; Messmer *et al.*, 2002; Osman *et al.*, 2003; Ekman-Ordeberg and Dubicke, 2012; Johnson *et al.*, 2013; Nazli *et al.*, 2013; Kacerovsky *et al.*, 2015; Jung *et al.*, 2017; Hadley *et al.*, 2018)
IL-17	Pro-inflammatory Th17 T cell cytokine, induces IL-6 production and COX-2. Important to mucosal immunity, induces neutrophil activation and inflammation. Also induces β defensin production by epithelial cells. Elevated in association with chorioamnionitis and PTB	tat up regulates IL-17 secretion by T cells Th17 producing cervical mononuclear cells deplete in HIV-1 infection, not restored by cART	(Ito *et al.*, 2010; Saito *et al.*, 2010; McKinnon *et al.*, 2011; Johnson *et al.*, 2013; Masson, Salkinder, *et al.*, 2015; Caruso *et al.*, 2019)
MIF	Pro-inflammatory cytokine produced by macrophages, monocytes, T cells and endothelial cells. It induces macrophage activation and production of IL-1β, IL-6 and TNF-α. Antagonises the effects of glucocorticoids. Present in placenta and amnion. Associated with PTB	Contained in persistent extracellular vesicles with nef, correlate with CD14+ monocytes and may be involved in persistent immune activation	(Ietta *et al.*, 2002; Calandra and Roger, 2003; Pearce *et al.*, 2008; Bevilacqua *et al.*, 2014; Lee *et al.*, 2016; Zhu and Yang, 2018)
CXCL-1	Chemokine also known as GRO-a, secreted by monocytes and macrophages and induce chemotaxis of neutrophils. Has been shown to be both up regulated in intra amniotic infected and down regulated in PTB	CXCL-1 is up regulated in vaginal cells by gp120 Contained in persistent extracellular vesicles with nef, correlate with CD14+ monocytes and may be involved in persistent immune activation	(Hsu *et al.*, 1998; Fanibunda *et al.*, 2011; Laudanski *et al.*, 2014; Lee *et al.*, 2016)

C5/C5a	Part of the innate immune complement cascade, important pro-inflammatory mediator. Mainly produced by the liver but also by macrophages. Acts of both innate and adaptive immune cells. Causes cytokine release from leucocytes, adhesion molecule expression, chemotaxis, de granulation of neutrophils, and phagocytosis. Causes macrophages to release MMP-9 and induce PTB.	c5 binds gp120	(Süsal *et al.*, 1996; Manthey *et al.*, 2009; Vaisbuch *et al.*, 2010; Gonzalez *et al.*, 2011)
CHI3L1	Chitin is found in bacterial cell walls and this chitin binding protein is expressed by macrophages. It involved in inflammation and antibacterial responses, mediating downstream functions of IL-13. CHI3L1 also has a role in ECM remodeling. Shown to be up regulated in PTB.	Macrophage expression of CHI3L1 up regulated by vpr	(Lee *et al.*, 2011; Barrero *et al.*, 2013; He *et al.*, 2013; McDonald *et al.*, 2015; Paquette *et al.*, 2018)
IP-10	Interferon gamma-induced protein 10 is a chemokine produced by monocytes, macrophages, dendritic and T and B cells. Chemo attraction of dendritic cells, NK cells and T cells.	Up regulated by gp120, tat, vpr and gag	(Cicala *et al.*, 2002; Liu *et al.*, 2011; Rasaiyaah *et al.*, 2013; Kukkonen *et al.*, 2014; Zahoor *et al.*, 2015)
Vitamin D BP	Binder of Vitamin D and sequester of actin. Associated with macrophage activation, leukocyte C5a-mediated chemotaxis. Predictor of intra- amniotic infection and PTB.	-	(Gomme and Bertolini, 2004; Liong *et al.*, 2013; Jang *et al.*, 2016)
Resistin	Produced by macrophages, PBMCs and placental cells. Induced by pro-inflammatory cytokines and up regulated in pregnancy. Associated with Endothelial dysfunction. Associated with intra-amniotic infection and inflammation. Inducer of NFκB. Associated with PTB.	gp41 up regulates Resistin by PBMCs	(Nien *et al.*, 2007; Kusanovic *et al.*, 2008; Romero *et al.*, 2010; Denner *et al.*, 2013)
Immune -regulatory			
GDF-15	Member of the TGF-β family also known as macrophage inhibiting cytokine 1 and placental transformation growth factor is a stress response protein. Produced by macrophages, endothelial cells, vascular smooth muscle cells and placental trophoblasts. It is a regulator of macrophage activation and is induced by both IL-1β and TNF-α. Shown to be up regulated in pre-eclampsia.	-	(Bootcov *et al.*, 1997; Lawton *et al.*, 1997; Sugulle *et al.*, 2009; Adela and Banerjee, 2015)
IL-1RA	IL-1 antagonist, regulates action of IL-1 by competing for the IL-1 receptor. Expressed by most cells but particularly	Up regulated by tat and and gp120	(Reinhold *et al.*, 1996; Jelicic *et al.*, 2013; Heng *et al.*, 2014; Kyongo *et al.*, 2015)

	leucocytes, bioavailability far in excess of IL-1a and IL-1b. Regulates inflammatory response, reduction in ratio of IL-1/IL-RA associates with bacterial vaginosis and PTB.		
DPPIV	Serine peptidase that cleaves other immune proteins, eg RANTES, IP-10 to regulate T cell migration and activation. Also involved in glucose metabolism. Highly expressed in placenta and lymphocytes	Enhanced apoptosis of HIV-1 infected cells. tat inhibits enzyme	(Jacotot *et al.*, 1996; Mentlein, 1999; Lorey *et al.*, 2003; Barreira Da Silva *et al.*, 2015; Casrouge *et al.*, 2018)
Adiponectin	Anti-inflammatory adipocytokine. Shown to be protective of term birth and down regulated in PTB and intra amniotic infection.	Down-regulated by tat in preadipocytes	(Mazaki-Tovi *et al.*, 2009; Díaz-Delfín *et al.*, 2012; Vieira *et al.*, 2017; Mierzyński *et al.*, 2018)
AMPs and inducers			
MPO	Neutrophil lysosomal peroxidase, stored in granules and released as a first line antimicrobial, generates reactive oxygen species, highly up regulated in response to bacterial pathogens. Up-regulated in response to intra amniotic infections.	tat up regulates MPO in T cells	(Hampton, Kettle and Winterbourn, 1998; Gravett *et al.*, 2004; Park *et al.*, 2007; Klein *et al.*, 2008; Romero *et al.*, 2010)
Lipocalin-2	Also known as neutrophil gelatinase-associated Lipocalin. An iron sequesting, bacteriostatic protein that co-localises with LPO in neutrophil granules. It also forms a complex with MMP-9 to prevent its degradation, also interacts with Cystatin C and SLPI. Induced by IL-1, IL-17 and TNF-α. Identified in CVF.	Has been shown to be decrease in HIV-1 infection and be restored with cART with a positive correlation with neutrophils.	(Klein *et al.*, 2008; Landrø *et al.*, 2008; Zegels *et al.*, 2010; Chakraborty *et al.*, 2012; Moschen *et al.*, 2017)
TFF3	Trefoil factor 3, secretory protein associated with Intestinal mucosal membrane. Inducer of β defensin AMPs and down regulated IL-8 and IL-6 secretion from intestinal epithelial cells. Shown to increase in maternal plasma through normal pregnancy.	-	(Barrera, Sanchez and Gonzalez, 2012; Romero *et al.*, 2017)
Cystatin C	Cysteine protease inhibitor that binds complement and modulated neutrophil action. Cystatin family also known to stabilize MMP-9 and induce NO release from IFN-γ activated macrophages. Highly expressed in amniotic fluid, also identified in serum and CVF. Associated with pre-eclampsia.	-	(Foster and Lyall, 2006; Shah *et al.*, 2009; Zegels *et al.*, 2009; Ochieng and Chaudhuri, 2011; Raymond and Peterson, 2011; Romero *et al.*, 2017; Mao *et al.*, 2019)

MIP-3α/β	Chemotactic to monocytes, neutrophils and T cells (including Th17 CD4+ helpers). MIP-3β expression is increased in CVL from women with elevate pro-inflammatory cytokines and carriage of anaerobic bacterial species and suppressed by vaginal lactic acid. Has shown to be share some homology to the β defensing AMPS and can be induced by the AMP Lactoferrin. Expressed in decidua and cervix, relatively suppressed during healthy pregnancy. Associated with preterm birth	Anti HIV protein MIP-3β expression is increased in the cervix in early HIV-1 infection	(Ghosh *et al.*, 2010; McKinnon *et al.*, 2011; Laudanski *et al.*, 2014; Yarbrough, Winkle and Herbst-Kralovetz, 2015; Arnold *et al.*, 2016; Hughes *et al.*, 2016; Hearps *et al.*, 2017; Hadley *et al.*, 2018)
Kallikrein 3	Serine protease, also known as PSA, present in CVF. Activates AMPs and causes epithelial desquamation to slough off bacterial. Also a marker of recent sexual activity.	-	(Gallo *et al.*, 2006; Yoon *et al.*, 2007; Muytjens *et al.*, 2016)
ECM factors			
MMP-9	Zinc dependent metalloproteinase responsible for cleaving ECM proteins including collagens and fetal fibronectin. Contained in neutrophil tertiary granules released in response to IL-8 and CXCL-1. Identified in amnion, chorion, decidua, myometrium and cervix. Important in placentation, embryogenesis, fetal membrane rupture and cervical remodeling prior to labour. Associated with intra-amniotic infection, PPROM and PTB.	gp120 up regulates MMP-9 in vaginal epithelial cells tat up regulates MMP-9 expression in monocytes	(Athayde *et al.*, 1998; Kumar *et al.*, 1999; Maymon *et al.*, 2000; Chakrabarti, 2005; Sorokin *et al.*, 2010; Fanibunda *et al.*, 2011; Heng *et al.*, 2012; Hadley *et al.*, 2018)
EMMPRIN	Membrane bound and soluble inducer of Matrix metalloprotainases. Produced during embryogenesis, present in placental, uterine, cervical and vaginal cells. Regulated by D isomer of lactic acid in vagina.	tat suppressed expression in HeLa cells	(Waldeck *et al.*, 2009; Dang *et al.*, 2013; Lee *et al.*, 2013; Witkin, 2015)
Aggrecan	Proteoglycan that is an integral part of cartilage ECM. Homology to glycosaminoglycans found in the placenta. May have a role in pre-eclampsia.	-	(Achur *et al.*, 2000; Warda *et al.*, 2008)
SERPINE1	Serine peptidase inhibitor also known as plasminogen activator inhibitor type 1. Prevents ECM degradation during implantation and placentation. Expressed in the placenta.	-	(Ye *et al.*, 2017; Hadley *et al.*, 2018)

	Up regulation associated with pre-eclampsia and recurrent early pregnancy loss.		
Osteopontin	Phosphorylated glycoprotein that modulates interactions with ECM collagen, fibronectin, integrins and is a substrate of MMP-9. Also up regulated at sites of inflammation and can act as a soluble Th1 cytokine: inducing chemotaxis and activation of macrophages, dendritic and T cells (particular Th1/Th17). Up regulates expression of IL-12 and IFN-γ. Expressed by trophoblast cells.	-	(Lund, Giachelli and Scatena, 2009; Wu, Liu and Xie, 2015)
Fetal-placental GF and hormones			
VEGF	Potent vascular endothelial growth factor, secreted by placenta and during embryogenesis, up regulated in PTB and implicated in PPROM	Up regulated by gp120 and tat	(Benelli *et al.*, 2002; Cicala *et al.*, 2002; Chaiworapongsa *et al.*, 2009; Johnson *et al.*, 2013; Paquette *et al.*, 2018)
Endoglin	Endothelial cell glycoprotein, part of the TGF-β complex. Expressed by placental trophoblasts. Purportedly anti-angiogenic. Associated with iatrogenic and spontaneous PTB.	gp 120 up regulates expression of Endoglin in B cells	(Chaiworapongsa *et al.*, 2009; Mijal *et al.*, 2012; Jelicic *et al.*, 2013; McDonald *et al.*, 2015)
Angiogenin	Formation of blood vessels in placenta and fetus, inducer of VEGF, also found in amniotic fluid in association with intra amniotic infection and has been identified as a potential biomarker for PTB.	Contained in persistent extracellular vesicles with nef, correlate with CD14+ monocytes and may be involved in persistent immune activation	(Spong *et al.*, 1997; Yoon *et al.*, 2001; Pavlov *et al.*, 2003; Lee *et al.*, 2016)
SHBG	Binding protein of oestrogen. Secreted mainly by liver but also by ovaries and placenta. Constituently expressed by T cells causes internalization of oestrogen as part of lymphocyte signaling. Identified as a predictive biomarker for PTB	-	(Krupenko, Avvakumov and Strel'chyonok, 1990; Saade *et al.*, 2016; Balogh *et al.*, 2019)

7.6 Conclusions and interpretation

Targeted immune factor exploration of the CVF proteome in HIV-1 infected and uninfected women revealed several high and low abundance proteins common to each group. Themes of protein functions included: inflammatory proteins involved in macrophage and neutrophil and T cell recruitment and activation; regulators of these immune cells and pathways; ECM modifiers; and growth factors and proteins involved in endothelial function and angiogenesis. Further details of individual protein function, grouped by themes are presented in table 28.

Several of these proteins have previously been observed to be up regulated in association with PTB, see table 28 (including references). These include cytokines and chemokines: IL-1, IL-8, IL-17, MIF, MIP-3, CXCL-1 and other inflammatory proteins: CHI3L1; complement component C5/C5a; Resistin and Vitamin D binding protein. Antimicrobial binding proteins and their inducers identified in this sub study, that have been shown to be deregulated in pregnancy, included: MPO, Lipocalin 2, and TFF3. ECM regulator MMP-9, identified in abundance in pooled CVF from both groups, is also known to be up-regulated during labour and preterm birth, for references see table 28.

Pooled samples from HIV–1 infected pregnant women appeared to contain many of these proteins in greater abundance than CVF from uninfected pregnant women based on pixel density from the arrays. This mirrors the findings of studies in non-pregnant women that have shown greater concentrations of IL-1, IL-RA, IL-8 and IP-10 in FGT fluid from HIV-1 infected women compared to uninfected women (Kyongo *et al.*, 2015; Masson, Passmore, *et al.*, 2015).

Herold et al. were able to demonstrate higher CVF IL-1β concentrations in women with higher plasma HIV RNA copies (>10000 copies/mL)(Herold *et al.*, 2013) suggesting that the virus itself contributes directly to the up-regulation of these inflammatory proteins. The observation of greater concentrations of inflammatory IL-1α and IL-8 in CVF from both high and low risk uninfected

pregnant women compared with respective risk uninfected non-pregnant women is also in synchrony with our results and implies an additive effect of pregnancy on up regulation of these immune responses(Anderson *et al.*, 2013; Kyongo *et al.*, 2015; Morrison *et al.*, 2018).

Screening CVF from HIV-1 infected and uninfected pregnant women has identified novel AMPs for these groups. To the authors knowledge, whilst Lipocalin 2, TFF3 and Cystatin C are recognized AMPs in CVF they have not previously been described in pregnant women(Zegels *et al.*, 2009). MPO has long been recognised as a neutrophil AMP but has not been widely studied in CVF in the context of pregnancy. The identification of AMP Kallikrein 3, also known as PSA, could be the result of recent sexual activity or local expression(Gallo *et al.*, 2006; Muytjens *et al.*, 2016). Although women were asked about recent sexual activity and samples were ideally not taken within 48 hour of intercourse, responses may not be accurate and sperm were seen on microscopic examination of more than one sample.

Studies of the effect of pregnancy on the more widely recognized CVF AMPS in high risk women have shown a reduction in β defensins, Elafin and MIP-3 and an increase in SLPI(Anderson *et al.*, 2013; Kyongo *et al.*, 2015; Morrison *et al.*, 2018). Comparing the effect of HIV on AMPs in non-pregnant women has shown an up regulation in α/β defensins and SLPI(Ghosh *et al.*, 2010; Dezzutti *et al.*, 2012; Masson, Passmore, *et al.*, 2015; Pellett Madan *et al.*, 2015). Although Archary et al. did not have a comparison group, they did identify some overlapping cytokines and chemokines in CVF obtained from HIV-1 infected non pregnant women (by the same MC method) namely: CXCL-1, IP-10, MIF and MCP-1(Archary *et al.*, 2015). These data support the observation that HIV-1 infected pregnant women have higher concentrations of AMPs than uninfected pregnant women. Interestingly the one exception to this was the relatively unexplored inducer protein of β defensins: TTF3 which was more abundant in uninfected pregnant women.

It is of great interest that the one of the main mediators of preparation of the cervix for labour, MMP-9, is up regulated in HIV-1 infected women, alongside several proteins with known interactions: EMMPRIN, Lipocalin 2, Osteopontin, CXCL-1, IL-8 and Cystatin C. The discovery of abundant MMP9 network proteins in the CVF of these women suggest that this may be a key downstream pathway in effecting the excess risk of PTB observed in these women.

The ability to identify several proteins with similar functions likely to be working in concert also applied to innate and adaptive CVF immune networks. Many of the overlapping proteins identified have central roles in effecting neutrophil responses (IL-8, IL-17, MPO, Lipocalin 2, CXCL-1, MIP-3, MMP-9) and macrophage responses (C5/C5a, IL-1, CXCL-1, MIF, MIP-3α, CHI3L1, IP-10, Resistin and Vitamin D BP) to pathogenic stimuli such as anaerobic vaginal bacteria. Macrophages and T cells are also key to the adaptive immune response and proteins indicating T cell activation (CD40 Ligand, IP-10, IL-17, MIP-3, and DPPIV) were also identified.

Local production of these proteins is most likely but it is possible the identification of these proteins is the result of extravasation. In a study of the CVF proteome by Tang et al., nearly half of all proteins identified were classified as plasma proteins including complement, SERPINs and Vitamin D BP. Tang et al. suggest this is due to permeability of genital tract mucosa in the context of inflammation. Authors did also qualify polymorphonuclear leukocytes in the CVL samples as average or overabundant and found a greater abundance of plasma proteins in the group with overabundant polymorphonuclear leukocytes, which neither confirms nor refutes this hypothesis. We cannot make any definitive conclusions about the cellular or transudate origins of the proteins identified in the CVF within this sub study however these results suggest local activation of neutrophils and macrophages (and possibly T cells) in the FGT of these pregnant women. The observation that signaling from these immune cell networks are enhanced in the context of HIV-1 infection may confer increased risk of inflammation driven PTB.

Semi quantitative comparison of protein arrays between women who conceived on cART and those that initiated cART after the second trimester sampling point suggest higher levels of inflammatory proteins associated with neutrophil and macrophage interaction prior to cART initiation (IL-8, IL-17, CXCL-1, MIF, MIP-3α, MMP-9 and CHI3L1) compared to pregnant women conceiving on NNRTI/INSTI based cART and uninfected pregnant women but not in those women who conceived on PI-based cART who had the highest abundance (with the exception of MMP-9, high in both groups). Women who initiated cART in pregnancy were the only group in whom T cell activation marker CD40 Ligand was detected. This is likely to be the result of active virus expression within the FGT although this cannot be confirmed as HIV RNA was not measured in CVF.

The fact that pooled CVF inflammatory protein profiles in women conceiving on NNRTI/INSTI were more similar to uninfected women compared to much higher abundance of immune proteins in women taking PI-based ART could possibly be as a direct result of the drug class's mechanism of action, which could be explored further in vivo. Women receiving PI-based cART also had the highest abundance of neutrophil associated antimicrobial proteins MPO and Lipocalin. It could be hypothesised that this is due to an increased number of neutrophils, induced by enhanced FGT inflammation, although this cannot be confirmed. Whilst it is possible that the antigenic stimuli from the vagina differ by cART class exposure such a difference was not demonstrated by our vaginal microbiota sub study.

MMP-9 the neutrophil associated ECM modifier with known roles in preparation for labour was most abundant in women conceiving on PI-based cART and HIV-1 women yet to initiate cART. This may be due to enhanced inflammatory cytokines in these groups upstream of MMP-9 release from tertiary neutrophil granules(Chakrabarti, 2005). MMPs induce cervical remodeling as well as reduce threshold for fetal membrane rupture and initiation of contractions(Peltier, 2003; Lindström and Bennett, 2005). It should be noted that the pooled samples used for the CVF protein arrays were obtained in the second trimester so maybe in advance of normal physiological changes in MMP-9 levels. However higher

overall MMP-9 levels may reduce the threshold for the trigger of labour in these groups who traditionally have higher rates of PTB, particularly marked in our cohort (Short *et al.*, 2014).

Comparing HIV-1 infected pregnant women delivering preterm to those who delivered at term in a small number of individuals (n=5 in each group), higher abundance of neutrophil and macrophage associated proteins: IL-8, IL-17a, CHI3LI, MPO, MIF and Vitamin D BP were expressed in women who subsequently delivered preterm compared to term, whereas IL-1RA (pro-inflammatory IL-1 antagonist) was reduced. Supporting a downstream inflammatory mechanism involving neutrophils and macrophages in the FGT underlying some of the increased risk of PTB observed in these women. The upstream trigger of this could be both HIV per se and vaginal bacterial dysbiosis, possibly modulated by cART.

Interestingly the same up regulation was not observed for MMP-9. This may be due to the timing of the CVF sampling in the second trimester being too early to see an effect or because of a difference in mechanisms behind iatrogenic PTB for fetal distress, responsible for four out of the five cases included in the preterm group. Many studies of PTB have different definitions of spontaneous PTB, some excluding iatrogenic causes. Whilst other studies have specifically found differences in immune proteins between iatrogenic PTB and term deliveries (Parry *et al.*, 2014; Chervenak *et al.*, 2018). This indicates that whilst there are different subgroups of the preterm birth syndrome, there may be some common pathological mechanism. An excess risk of both spontaneous and iatrogenic PTB in HIV infected pregnant women in association with cART has been described(Lopez *et al.*, 2012).

In spite of no obvious trend in MMP-9 expression, ECM protein Osteopontin was increased in the preterm group compared to term. Osteopontin is a substrate for MMP-9 that interacts with fetal membrane protein- fibronectin and has Th1 cytokine effects(Wu, Liu and Xie, 2015). In addition, two ECMs known to be associated with pre-eclampsia- Aggrecan, and SERPINE1(Achur *et al.*, 2000;

Warda *et al.*, 2008; Ye *et al.*, 2017) were elevated in the preterm group. This indicates that local inflammation may modulate ECM structures possibly in placenta, cervix and fetal membranes, preparing for labour. Vascular endothelial proteins expressed in placental and foetal vasculature: Angiogenin, VEGF and FG-19 were also detected at an increased abundance in pooled CVF from HIV-1 infected women experiencing PTB, four out of five cases were due to foetal distress, compared to term births. It could be speculated that there may be some underlying aberrant pathology in the placenta increasing risk of foetal distress that was subclinical as none of these women were diagnosed with hypertension or pre-eclampsia.

A relatively novel AMP, Cystatin C, which was also up regulated in the pooled CVF from HIV-1 infected women delivering preterm, was also identified. Cystatin C, a cysteine protease inhibitor that modulates inflammation via its action on complement, neutrophils and macrophages as well as stabilizing MMP-9, has previously been identified in both amniotic fluid, CVF and serum (Shah *et al.*, 2009; Zegels *et al.*, 2009; Bastek and Elovitz, 2013; Romero *et al.*, 2017; Mao *et al.*, 2019). Cystatin C has been shown to be elevated in association with pre-eclampsia (Raymond and Peterson, 2011; Bastek and Elovitz, 2013). To our knowledge, only one study has explored is role in CVF as a biomarker for PTB in uninfected pregnant women in CVF and did not find a difference between women experiencing PTB and controls(Shah *et al.*, 2009).

Limitations and Strengths

It should be acknowledged that despite the protein array containing antibodies for several immune proteins known to be present in CVF of HIV-1 infected non pregnant women and uninfected pregnant women (RAGE, RANTES, sCD14, IGF-BP, IL-1β, G-CSF, GM-CSF, MIP-1, LIF and CRP), we did not detect them(Zegels *et al.*, 2010; Archary *et al.*, 2015). Neither did we identify cytokines known to be quantifiable by chemiluminescence, demonstrated in chapter 5. The reason behind this is likely to be the dilution chosen to appreciate semi quantitate differences in protein expression between groups (1 in 100), whilst enabling comparison between proteins of high abundance, this dilution will have reduced

sensitivity to identify proteins expressed at lower and possibly highly variable quantities(Zegels *et al.*, 2010). The targeted exploratory protein array used also did not include capture antibodies to many of the immune proteins previous observed in CVF including the α/β Defensin AMPs, SLPI and TGF-β thus we are unable to describe their expression in this group of women.

Another limitation that should be considered is the method of pooling samples for protein exploration. The theory behind this approach is the summative signal of proteins will identify only the factors that are abundantly and differentially expressed between pooled groups, minimizing the error introduced by influence of non-significant factors that are highly expressed by one or two individuals. This approach may overcome power issues of small sample sizes. However, such approach has been shown to potentially reduce sensitivity in identifying significant outlying proteins i.e. those variably expressed at lower concentrations in individuals that are the result of true biological interactions(Sadiq and Agranoff, 2008).

Another factor that may bias results from CVF protein studies is the effect of vaginal cleansing practices, which can be prevalent in certain African and Caribbean cultures, which has previously been identified as a factor that can modify cervical vaginal proteins(Kyongo *et al.*, 2015; Arnold *et al.*, 2016; Jespers *et al.*, 2017). Many of the HIV-1 infected pregnant women who participated in this study, identified with the aforementioned cultures. All women were asked about vaginal cleansing practices however and no participants reported it.

An advantage of using whole CVF collected by a MC for directed exploration of immune proteins is that this biological fluid should contain all factors that work together in the true biological setting therefore providing a more accurate snap shot of in vivo physiology. This enables more accurate delineation of protein networks as it accounts for all interactions including vaginal microbiota. Another advantage of CVF, which can be drawn from the data presented here, is that many proteins were identified that are known to be expressed by upstream gestational tissues such as the placenta, eg. Angiogenin, DPPIV, SERPINE1, VEGF

and GDF-15. This therefore is a proof of concept that CVF can be informative about not just the cervicovaginal environment but also the upper genital tract in a way that is non-invasive.

In conclusion, we have presented data that demonstrates up-regulated interrelated CVF inflammatory and anti-microbial proteins, likely associated with activation of neutrophils and macrophages in the lower FGT in HIV-1 infected pregnant women compared to uninfected women. These immune proteins were influenced by cART exposure, particularly up-regulated in association with PI-based cART and were more abundant in women who delivered preterm compared to term. These immune factors are likely to be working in concert to increase the rates of PTB seen in this patient group. Formal quantification of these proteins is now required on an individual case basis to explore and characterize these protein networks. Any potential biomarker or biomarkers for PTB could be correlated with local immune cells and further on, validated in a larger cohort.

Chapter 8. Genital-plasma cytokine gradients and immune compartment correlations

8.1 Background: Correlations between local and systemic cytokines, peripheral blood mononuclear cells and vaginal bacteria

The link between immune compartments in terms of HIV expression has been studied in the context of viral suppression on ART and risk of transmission at mucosal surfaces(Fox and Fidler, 2010). The concept of distinct immune compartments or "privileged" immune sites is also an important consideration for cure strategies and organ pathology (i.e. the HIV reservoir)(Barton, Winckelmann and Palmer, 2016; Wong and Yukl, 2016). Usually, expression of HIV virus at the female genital mucosa correlates with plasma viraemia (Cu-Uvin *et al.*, 2006). However, the phenomenon of female genital HIV shedding during full suppression of HIV in plasma has been described(Cu-Uvin *et al.*, 2010), as has detectable HIV in breast milk(Myer *et al.*, 2017). The presence of HIV virus at the genital mucosa is dependent on several factors including ART exposure, inflammation, bacterial dysbiosis, concurrent sexually transmitted infection, mucosal integrity and immune cells (King *et al.*, 2017). This local immune network may be influenced by systemic effects i.e. peripheral HIV viraemia (Roberts *et al.*, 2012; Herold *et al.*, 2013), peripheral cytokines/chemokines and PBMCs(Bebell *et al.*, 2008; Roberts *et al.*, 2012).

Associations between local and plasma cytokines

Studies of genital cytokine expression in non-pregnant women who were either HIV infected or at high risk for HIV infection have predominantly revealed higher concentration of cytokines in cervicovaginal lavage compared to plasma. A study of cytokine and chemokine mucosal/systemic profiles in HIV exposed sex workers from the Punwani Cohort in Nairobi, Kenya found higher genital tract concentrations of IL-1α, IL-1β, IL-RA, IL-6, IL-7 and IL-8 compared to the systemic concentrations.(Lajoie *et al.*, 2012). Subtle differences in gradients were observed in women who were HIV infected compared with uninfected women.

The same group also explored differences in cytokine expression between systemic and genital compartments in sex workers from Benin and identified higher genital mucosal concentrations of TNF-α and IFN-γ in HIV infected compared to uninfected women with the opposite trend found in serum(Lajoie *et al.*, 2008). Lajoie et al. postulate that the differences in compartment expression could be the result of HIV factors, anti-proteases as innate immune proteins regulating cytokine expression as well as differences in immune cells.

Genital-plasma cytokine gradients

The concept of a genital mucosa–systemic plasma cytokine gradient has been developed further with data from the CAPRISA 004 tenofovir gel trial(Liebenberg *et al.*, 2017). Passmore's group explored ratios of genital to plasma cytokine and chemokine concentrations and compared values found in women who were became infected with HIV with those who remained uninfected. Higher genital gradients for four chemokines (interferon-γ- inducible protein 10 (IP-10), macrophage inflammatory protein -1β (MIP-1β), IL-8 and monocyte chemotaxis protein-1 (MCP-1)) were associated with HIV acquisition. No measured cytokines were associated with HIV acquisition however the raw data for the direction of mucosal gradients were not presented. Passmore et al. hypothesise that the positive gradients observed for chemokines but not cytokines may suggest increased immune cell chemotaxis to genital mucosa.

In an earlier analysis of the CAPRISSA 004 cohort, Masson et al. defined participants has having an elevated genital inflammatory profile as ≥5 of 9 measured inflammatory cytokines above the 75th percentile and found no difference in plasma cytokines comparing women with and without genital inflammation(Masson, Passmore, *et al.*, 2015). In addition, no correlation between genital and plasma cytokines were identified in either CAPRISSA 004 analysis. Conversely, in their pre cART paper, Belec et al. identified a correlation between CVL and serum for TNF-α ($r=0.42$, $p<0.005$) and IL- 6 ($r=0.33$, $p<0.03$) but not for IL-1β. (Bélec *et al.*, 1995).

The genital-plasma gradient in HIV-1 infected pregnant women on cART has not been studied, neither have correlations between genital and plasma cytokine concentrations.

Associations between cytokines and immune cells

Local innate and adaptive immune cells of pregnancy masterclass

Maternal decidual macrophages regulate the principle component of the local innate immunity, the uterine NK cell, to ensure a favourable environment for the fetal trophoblast invasion at implantation (PrabhuDas *et al.*, 2015). Antigen presentation at the maternal fetal interface is strictly controlled through unique MHC Class expression on trophoblast cells and down regulation of antigen presentation on uterine dendritic cells which do not migrate into the lymphatic system(Mor, Aldo and Alvero, 2017; Menzies, 2018).

Adaptive immunity is also regulated through CD4+ T helper cell phenotype shifting from Th1 to immune-regulatory Th2 (discussed in detail in introduction)(Sykes *et al.*, 2012) and an up regulation of T regulatory cells (Drayson *et al.*, 2004; Sasaki *et al.*, 2004). Dysregulation of uterine NK cells and decidual T regulatory cells has been implicated in infertility, miscarriage and preterm birth (Piccinni, 2010; Gomez-Lopez *et al.*, 2014; Mor, Aldo and Alvero, 2017). In addition Th1 helper cell responses are associated with preterm birth and miscarriage (Makhseed *et al.*, 2003; Sykes *et al.*, 2012; Gomez-Lopez *et al.*, 2014).

Local FGT cytokines and peripheral blood mononuclear cells

The association between circulating peripheral blood mononuclear cells (PBMCs) and genital tract inflammation is unknown. It has recently been demonstrated that antigen presenting cells, the key link between innate and adaptive immunity, are able to migrate within genital tissues in both HIV infected and uninfected women and that the same cell subsets are observed in whole blood from these women(Shey *et al.*, 2016). Cervical dendritic and langerhans cell migration in the cervix is enhanced by inflammatory cytokines,

particularly in HIV infected compared to uninfected women(Shey *et al.*, 2016). It has been suggested that the genital mucosal-systemic plasma gradient may be a good surrogate marker for the migration of peripheral mononuclear cells to the genital mucosa where they can influence the local immune environment (Lajoie *et al.*, 2012; Liebenberg *et al.*, 2017).

Bebell and Roberts demonstrated inverse correlations between peripheral blood CD4+ T cells and local inflammation in the female genital tract in HIV infected women with the highest CVL cytokine concentrations seen in women with the most advanced immunosuppression (Bebell *et al.*, 2008; Roberts *et al.*, 2012). Conversely in recent work by Caruso et al. peripheral CD4+ CD161+ lymphocytes (known for their gut homing potential) were positively correlated with cervical Th17 cytokines production(Caruso *et al.*, 2019).

These data indicate that peripheral mononuclear cells are likely to migrate to the genital tract in response to and to influence inflammation within this compartment. This is a relatively novel concept in research into HIV infection and no data are available in pregnancy.

Vaginal bacteria, local FGT cytokines and cellular immune response

In a study defining normal levels of FGT soluble and cellular biomarkers in 30 Causasian women, Kyongo et al. characterised CVL cytokines, chemokines, endocervical leucocytes as well as quantifying a number of bacterial species(Kyongo *et al.*, 2012). Women with *Lactobacillus crispatus* carriage had reduced total T cells and a lower percentage expressing CD4+ HLA-DR+. Monocytes (CD14+) constituted 3% total endocervical cells, of which 52% were CD4+ CCR5+ cells. T cells (CD3+) made up 1% of endocervical cells, of which 61% were CD4+ CCR5+. HLA-DR+ expression, was identified in 46% of all monocytes and 16% of T cells. Women with the highest percentages of T cells (CD3+) had higher concentrations of IL-1RA, GM-CSF and Elafin.

Anahtar et al. further defined the effect of cervicovaginal bacteria in modulation of the inflammatory responses in the female genital tract in a group of uninfected young South African women, exploring associations between bacteria, cytokines, chemokine and both local and peripheral leucocytes(Anahtar *et al.*, 2015). High diversity bacterial communities were associated with elevated pro-inflammatory cytokines, which in turn were correlated with higher numbers of activated endocervical CD4+ T cells (CCR5+ CD38+HLA-DR+). No association with activated peripheral CD4+ T cells was observed.

In the Females Rising through Education, Support and Health (FRESH) study, Gosmann and colleagues replicated the findings of Anahtar et al. in a larger cohort of uninfected South African women(Gosmann *et al.*, 2017). This elegant work characterised that women with high diversity bacterial communities in their FGT had elevated pro-inflammatory cytokines (IL-1α, IL-1β, IL-8, IL-17, IL-23 and TNF-α) and chemokine: MIP-1α, MIP-1β and increased activated cervical CD4+ T cells (CCR5+ CD38+HLA-DR+). Of note when comparing women that became HIV infected with those who remained uninfected, there was no additional difference in cytokine concentrations within bacterial community types suggesting that it is the bacterial communities that are the predominant influence over FGT inflammation. This finding echoes the results of a much earlier study, using gram staining to characterize the vaginal microbiota, that concluded bacterial vaginosis and not HIV infection was primarily responsible for elevated pro-inflammatory vaginal cytokines(Mitchell *et al.*, 2009).

This chapter will explore correlations of local FGT cytokines with peripheral cytokines, local vaginal polymorphonuclear cells (likely to be neutrophils/granulocytes), vaginal bacterial diversity and species, peripheral mononuclear cells and markers of peripheral cellular immune activation in HIV - 1 infected and uninfected pregnant women. This will enable further elucidation of the local immune network in these women and how this is influenced by systemic inflammation.

Objectives

- To characterize the genital-plasma cytokine gradient in HIV-1 infected pregnant women and how this is associated with ART exposure and prematurity
- To correlate local polymorphonuclear leucocytes with local FGT inflammation
- To correlated PBMCs and cellular markers of immune activation with local CVF and systemic plasma cytokines
- To explore associations between vaginal bacteria and local FGT inflammation

8.2 Cytokine Results

8.2.1 CVF cytokines are present in higher concentration than in plasma in all women

The concentrations of cytokines in CVF are higher than the same cytokines in plasma in both HIV-1 infected and uninfected pregnant women, with the greatest concentration differences between mucosal and systemic compartments observed for HIV-1 infected women as illustrated in the radar chart see figure 49.

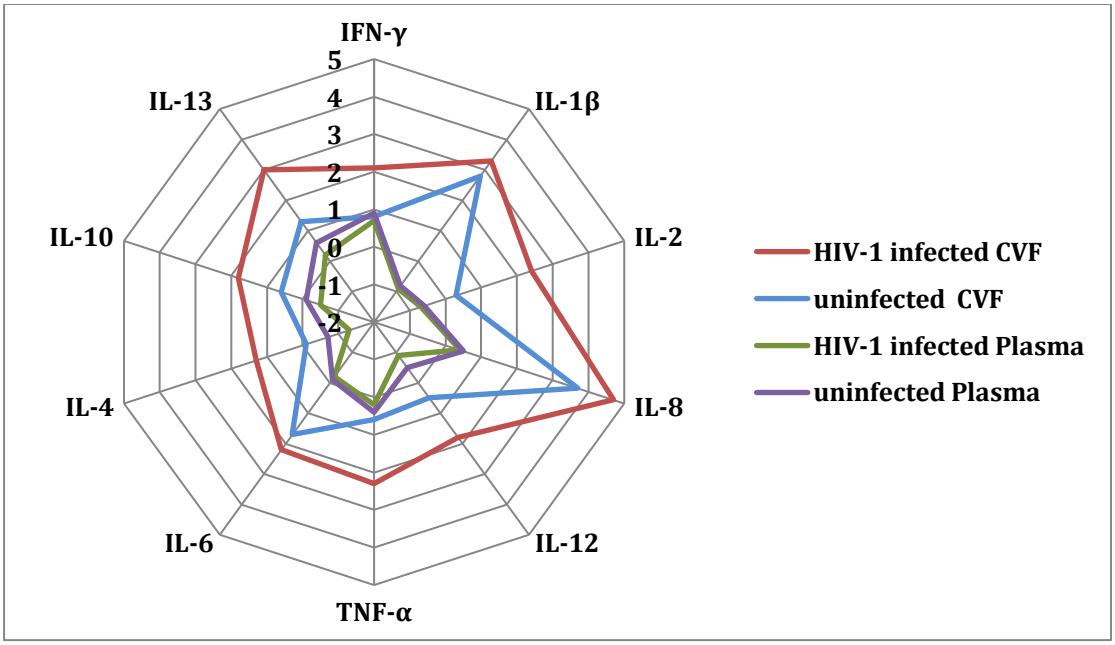


Figure 49 A radar chart to demonstrate log [10] pro-inflammatory and immune-regulatory cytokine concentration by genital and systemic compartment and HIV status
Demonstrating a higher CVF concentration and lower plasma concentration of all cytokines in HIV-1 infected pregnant women compared to uninfected pregnant women.

8.2.2 A genital-plasma cytokine gradient was observed for all measured cytokines in HIV-1 infected pregnant women, with the greatest gradients observed for IL-1β and IL-8 in both HIV -1 infected and uninfected women

Genital-plasma gradients were greater in HIV-1 infected pregnant women compared to uninfected women, $p<0.0001$, see table 29. In rank order, the greatest gradients observed in HIV-1 infected women were for pro-inflammatory cytokine IL-1β and chemokine IL-8, followed by IL- 2, IL-13, IL-12, IL-4, IL-6, IL-10, TNF-α and IFN-γ. In uninfected pregnant women IL-1β and IL-8 genital-plasma gradients were the greatest followed by IL-6. The calculated gradients for other measured cytokines in uninfected women were of much smaller magnitude, see radar chart and table 29. The genital-plasma gradient of IL-1β was nine-fold higher in HIV-1 infected pregnant women than in uninfected women, the IL-8 gradient was three-fold higher and the IL-6 gradient was five-fold higher. The difference in other cytokine gradients by HIV status was much higher due to the low values obtained for uninfected women, for example IL-2 genital-plasma gradient in HIV-1 infected pregnant women was over two hundred times the magnitude of uninfected pregnant women (1068 compared to 5), see table 29.

Table 29 Median genital-plasma cytokine gradients during second trimester by HIV status

Cytokine gradient Median (IQR)	HIV-1 infected	Uninfected	p value
IFN-γ	63 (9-468)	1 (0-4)	<0.0001
IL-1β	17294 (5076-115968)	5675 (663-14654)	<0.0001
IL-2	1068 (325-2304)	5 (3-29)	<0.0001
IL-8	15788 (4347-64995)	1812 (219-7160)	<0.0001
IL-12	464 (86-949)	4 (1-28)	<0.0001
TNF-α	99 (11-316)	1 (0-8)	<0.0001
IL-4	353 (97-9790	2 (1-6)	<0.0001
IL-6	262 (50-742)	49 (9-143)	<0.0001
IL-10	152 (61-467)	2 (0-20)	<0.0001
IL-13	579 (226-1328)	6 (1-34)	<0.0001

8.2.3 Pro-inflammatory and immune-regulatory genital-plasma cytokine gradients increased through the second trimester in HIV-1 infected pregnant women.

Considering all women there was no association between genital-plasma gradients and gestational age at sampling. However, in HIV-1 infected women several genital-plasma gradients were significantly correlated with gestational age at sampling, notably IFN-γ, IL-2, IL-12 and IL-4 and IL-13, see table 30. No association between genital-plasma cytokine gradient and gestational age at delivery was observed. No differences in gradients by prematurity were seen.

Table 30 The association between genital-plasma cytokine gradients and gestational age at sampling/ weeks in HIV-1 infected pregnant women

Genital-plasma gradient cytokine	Spearman's correlation co-efficient, rho	p value
Pro-inflammatory		
IFN-γ	**0.24**	**0.03***
IL-1β	0.11	0.29
IL-2	**0.24**	**0.02***
IL-8	0.15	0.68
IL-12	**0.22**	**<0.01***
TNF-α	0.10	0.34
Immune-regulatory		
IL-6	0.06	0.57
IL-4	**0.30**	**<0.01***
IL-10	0.17	0.12
IL-13	**0.26**	**<0.01***

8.2.4 CVF cytokines correlated with immune-regulatory plasma cytokines in HIV-1 infected pregnant women

Correlations between CVF cytokines and plasma cytokines were explored, see table 31. There was a general trend for immune-regulatory CVF cytokines to be

associated with plasma immune-regulatory cytokines, predominantly IL-13. CVF IL-4 was associated with plasma IL-13 (p=0.02), CVF IL-10 was associated with IL-13 (p=0.04) with a trend also seen with IL-4 (p=0.09) and CVF IL-13 demonstrated a trend towards an association with IL-4 (p=0.12), IL-13 (p=0.07) and a significant negative correlation with IFN-γ (p=0.04).

Certain pro-inflammatory CVF cytokines were also associated with plasma immune-regulatory cytokines: CVF IL-1β with plasma IL-10 (p=0.02); CVF IL-12 with plasma IL-13 (p=0.02) and CVF TNF-α with IL-10 (p=0.02), IL-13 (p=0.01) and a trend with IL-4 (p=0.09).

Table 31 CVF cytokine correlations with plasma cytokines in HIV-1 infected pregnant women

rho= spearman's correlation coefficient,* p<0.05, bold italics p<0.15

CVF	IFN-γ		IL-1β		IL-2		IL-8		IL-12		TNF-α		IL-4		IL-6		IL-10		IL-13	
Plasma	rho	P	rho	p	rho	p	rho	p	rho	p	rho	p	rho	p	rho	p	rho	p	rho	p
IFN-γ	***0.17***	***0.116***	0.03	0.785	-0.11	0.291	0.06	0.620	-0.12	0.264	-0.07	0.547	-0.12	0.274	-0.01	0.356	-0.05	0.664	**-0.22***	**0.039**
IL-1β	0.11	0.304	0.06	0.551	0.02	0.867	*0.16*	*0.157*	-0.07	0.535	0.04	0.682	0.11	0.965	-0.06	0.573	0.08	0.450	-0.02	0.879
IL-2	-0.04	0.711	-0.07	0.510	-0.03	0.753	0.04	0.740	-0.03	0.758	-0.04	0.687	-0.01	0.964	-0.13	0.225	-0.02	0.885	-0.07	0.516
IL-8	*0.15*	*0.158*	***0.17***	***0.113***	-0.04	0.713	***0.19***	***0.097***	-0.13	0.234	0.06	0.567	-0.02	0.829	0.06	0.553	-0.05	0.663	-0.03	0.800
IL-12	-0.04	0.686	-0.02	0.849	-0.03	0.772	-0.04	0.750	0.07	0.523	-0.01	0.963	0.05	0.637	0.04	0.250	0.09	0.409	-0.01	0.918
TNF-α	-0.05	0.624	-0.09	0.419	-0.15	0.173	-0.07	0.521	0.16	0.371	0.02	0.862	***0.17***	***0.111***	-0.09	0.392	-0.02	0.827	0.01	0.989
IL-4	-0.06	0.586	0.09	0.385	0.09	0.425	0.10	0.395	0.13	0.220	***0.18***	***0.088***	*0.17*	*0.126*	0.08	0.440	***0.18***	***0.090***	***0.17***	***0.119***
IL-6	0.07	0.496	0.03	0.815	-0.04	0.708	0.12	0.293	0.04	0.685	0.10	0.353	0.06	0.365	0.12	0.265	0.13	0.215	-0.07	0.537
IL-10	0.12	0.284	**0.25***	**0.019**	0.05	0.647	0.13	0.264	0.15	0.170	**0.25***	**0.021**	*0.15*	*0.156*	0.12	0.250	0.07	0.502	0.10	0.352
IL-13	-0.11	0.304	*0.15*	*0.159*	*0.14*	*0.166*	0.12	0.285	**0.26***	**0.014**	**0.27***	**0.010**	**0.26***	**0.015**	0.04	0.709	**0.22***	**0.036**	***0.20***	***0.067***

8.3 T-cell activation results

8.3.1 T-cell activation correlates with plasma cytokines

Percentage of CD3+CD4+ cells and CD4/CD8 ratio were positively associated with both pro-inflammatory and immune-regulatory cytokines. Percentage of CD3+CD8+ cells and markers of peripheral T cell activation (CD4+HLA-DR+% and CD8+HLA-DR+%) were inversely associated with plasma immune-regulatory cytokine concentrations

Initial analysis of T cell activation marker correlations with plasma cytokine concentrations was performed for HIV-1 infected and uninfected pregnant women combined, see table 32. Absolute CD3+CD4+ cell count and CD3+CD4+% cells were positively correlated with pro-inflammatory cytokines: IL-1β, IL-8 and IL-12. CD4/CD8 ratio positively correlated with IL-8, IL-12 and TNF-α.

Absolute CD3+CD4+ cell count, CD3+CD4+ % and CD4/CD8 ratio were positively correlated with immune-regulatory cytokines: IL-4, IL-6, IL-10 and IL-13. Conversely, absolute CD3+CD8+ cell count, CD3+CD8+%, CD4+HLA-DR+% and CD8+HLA-DR+ % were inversely associated with immune-regulatory cytokines: IL-4, IL-6, IL-10 and IL-13.

Table 32 Correlations between plasma cytokines and whole blood T cell subtypes in all pregnant women

*=p<0.005

T cell subtype	Pro-inflammatory cytokines												Immune-regulatory cytokines							
	IFN-γ		IL-1β		IL-2		IL-8		IL-12		TNF-α		IL-4		IL-6		IL-10		IL-13	
	rho	p	Rho	p	rho	p	rho	p	rho	p	rho	p	rho	p	rho	p	rho	p	rho	p
Absolute CD4+	0.02	0.749	0.10	0.086	**-0.14**	**0.026**	**0.17**	**0.005**	**0.14**	**0.024**	0.10	0.102	**0.30**	*	**0.13**	**0.035**	**0.27**	*	0.09	0.14
CD3+CD4+%	0.03	0.595	0.11	0.080	-0.04	0.547	0.10	0.098	**0.20**	**0.001**	0.09	0.145	**0.33**	*	**0.14**	**0.018**	**0.36**	*	**0.16**	**0.009**
Absolute CD8+	0.09	0.900	-0.01	0.874	-0.06	0.339	0.07	0.274	**-0.12**	**0.053**	-0.03	0.577	-0.11	0.073	-0.04	0.496	**-0.19**	**0.02**	**-0.15**	**0.017**
CD3+CD8+%	-0.01	0.894	-0.08	0.212	0.06	0.372	-0.08	0.198	**-0.18**	**0.003**	**-0.13**	**0.039**	**-0.26**	*	**-0.14**	**0.025**	**-0.18**	**0.003**	**-0.14**	**0.003**
CD4+CD25+%	**-0.14**	**0.032**	0.02	0.743	0.05	0.424	-0.10	0.123	-0.08	0.211	-0.07	0.320	0.01	0.879	-0.12	0.073	**-0.14**	**0.034**	-0.03	0.648
CD4+HLA-DR+%	0.10	0.136	0.04	0.571	**0.18**	**0.006**	-0.03	0.603	0.00	0.989	0.05	0.468	**-0.17**	**0.009**	-0.12	0.063	-0.22	*	-0.05	0.463
CD8+CD25+%	0.02	0.782	-0.10	0.126	0.08	0.191	0.00	0.997	0.07	0.293	0.03	0.645	0.05	0.453	-0.04	0.551	0.04	0.585	0.06	0.325
CD8+HLA-DR+%	0.32	0.623	0.03	0.631	**0.14**	**0.029**	-0.12	0.098	0.03	0.668	0.02	0.807	**-0.15**	**0.021**	**-0.13**	**0.041**	**-0.23**	*	-0.04	0.531
CD4/CD8 ratio	0.04	0.538	0.10	0.113	-0.05	0.407	0.10	0.097	**0.22**	*	**0.12**	**0.046**	**0.33**	*	**0.17**	**0.005**	**0.39**	*	**0.18**	**0.003**

8.3.2 Complex correlations between T-cell activation and plasma cytokines in HIV-1 infected pregnant women

Markers of peripheral T cell activation (%CD4+HLA-DR+ and % CD8+HLA-DR+) and CD3+CD8+ % were positively correlated with pro-inflammatory IFN-γ and IL-2 plasma concentrations. Percentage of CD3+CD4+ cells and CD4/CD8 ratio are inversely associated with pro-inflammatory cytokines: IFN-γ and IL-2.

Exploring cytokine correlations in HIV-1 infected pregnant women alone, revealed significant positive associations between pro-inflammatory IFN-γ, IL-2, and IL-12 with percentage of CD4+HLA-DR+ cells. Significant correlations were also seen between percentage of CD8+HLA-DR+ cells and pro-inflammatory IFN-γ, IL-2 and IL-12, see figures 50-52.

Other significant associations identified were that IFN-γ and IL-2 were positively correlated with total CD3+ CD8+ T cells and CD3+ CD8+ % and inversely correlated with total CD3+ CD4+ T cells and CD3+ CD4+ % and with CD4/CD8 ratio, see figures 50&51.

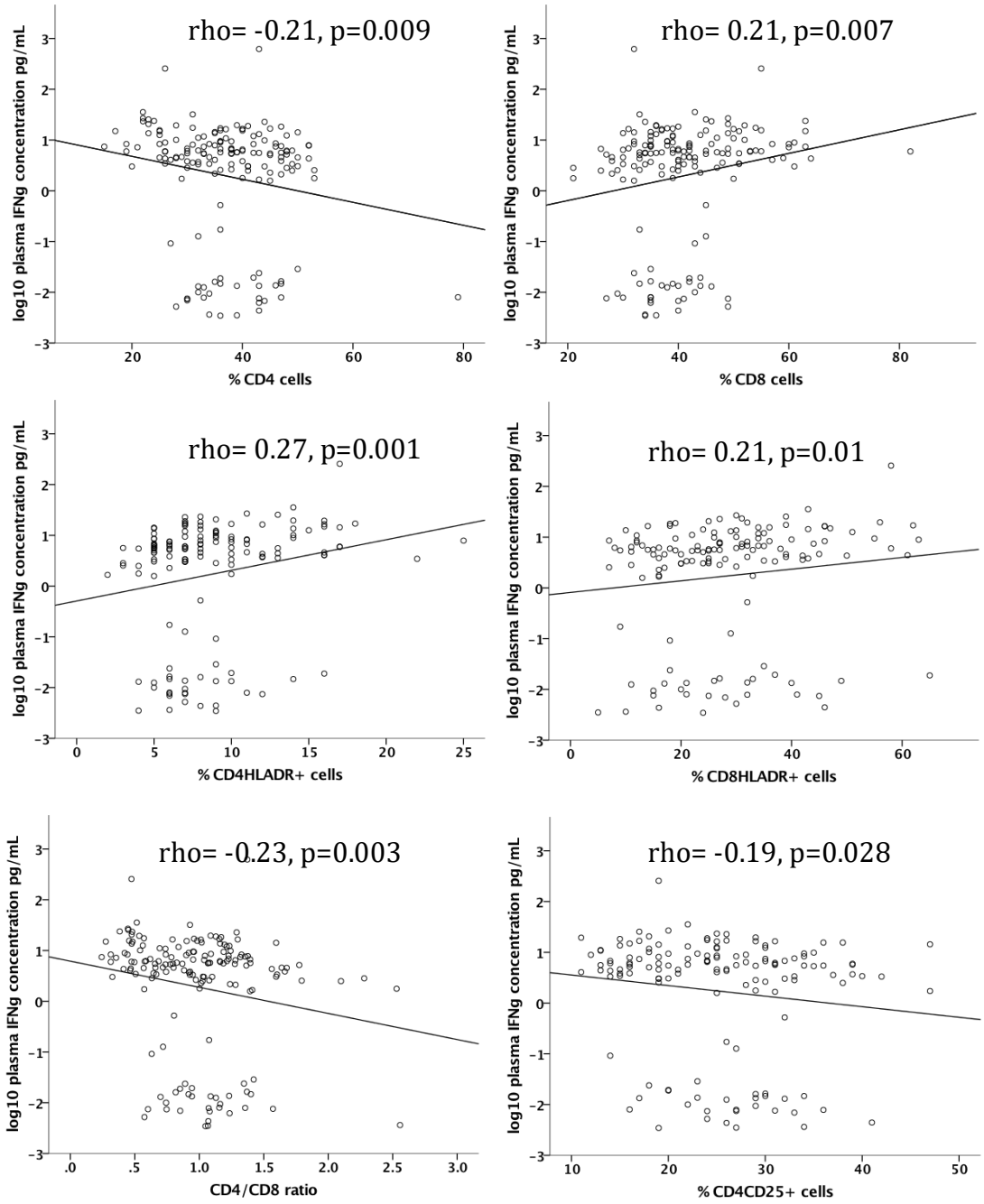


Figure 50 Scatter of CD4+ and CD8+ T cell markers against plasma pro-inflammatory IFN-γ cytokine concentrations in HIV-1 infected pregnant women

Demonstrating positive correlations between plasma IFN-γ concentrations and cellular markers of immune activation: %CD8+, %CD4+HLAR-DR+ and %CD8+HLAR-DR+ and inverse correlations with markers of immune restoration: %CD4, CD4/CD8 ratio and immune regulation: %CD4+CD25+ T cells subsets, rho= Spearman's correlation coefficient and ----- fit line =linear regression.

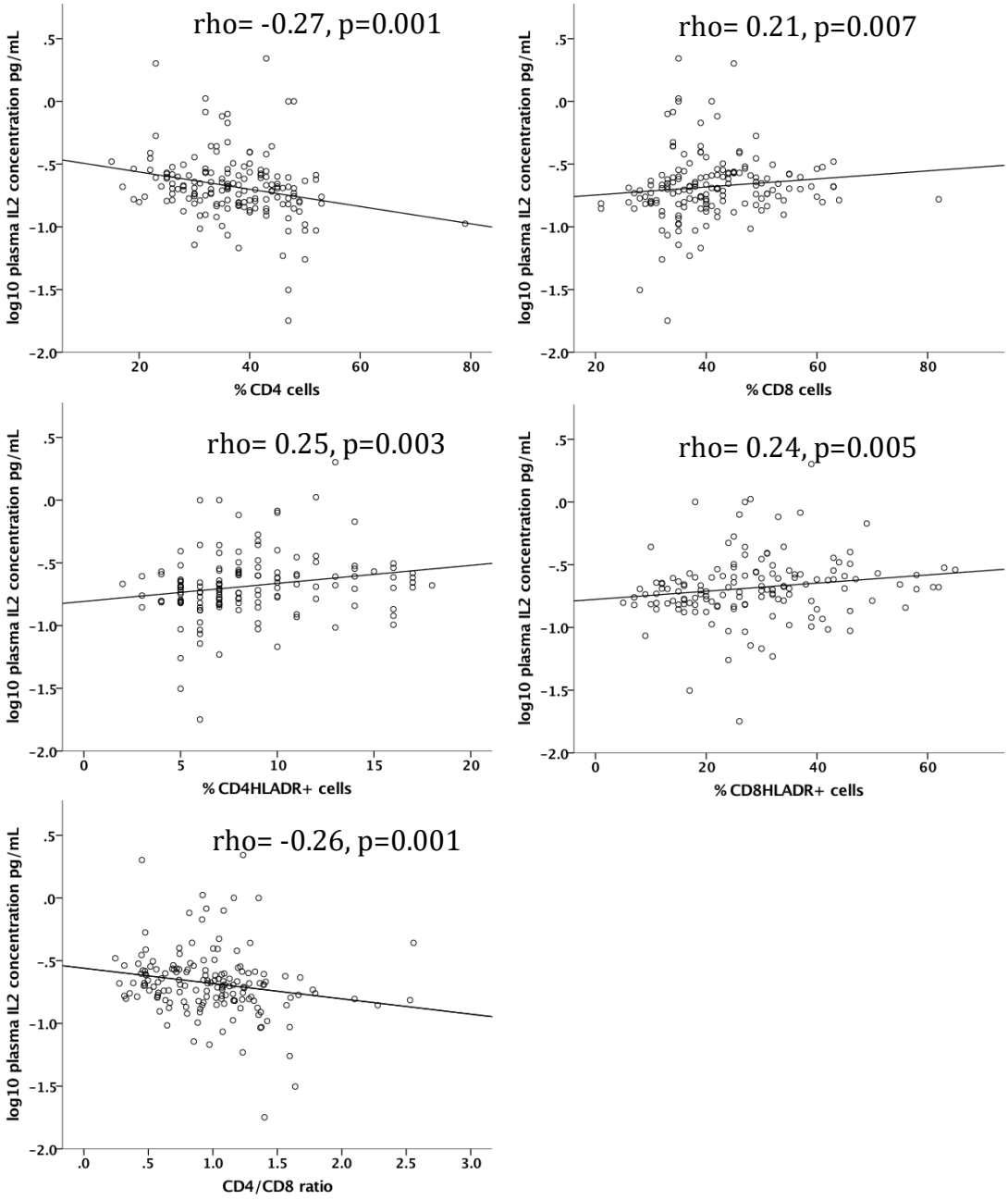


Figure 51 Scatter of CD4+ and CD8+ T cell markers against plasma pro-inflammatory IL-2 cytokine concentrations in HIV-1 infected pregnant women

Demonstrating positive correlations between plasma IL-2 concentrations and cellular markers of immune activation: %CD8+, %CD4+HLAR-DR+ and %CD8+HLAR-DR+ and inverse correlations with markers of immune restoration: %CD4, CD4/CD8 ratio, rho= Spearman's correlation coefficient and ----- fit line =linear regression.

II: IL-12

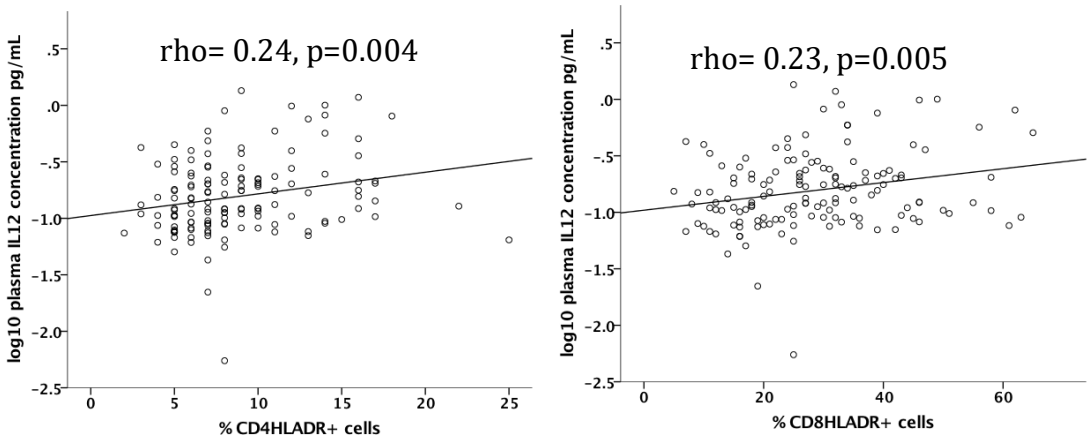


Figure 52 Scatter of CD4+ and CD8+ T cell markers against plasma pro-inflammatory IL-12 cytokine concentrations in HIV-1 infected pregnant women
Demonstrating positive correlations between plasma IL-12 concentrations and cellular markers of immune activation: %CD4+HLAR-DR+ and %CD8+HLAR-DR+, rho= Spearman's correlation coefficient and ----- fit line =linear regression

In addition, the percentage of CD8+ HLA-DR+ cells significantly, positively correlated with the plasma concentration of the pro-inflammatory cytokine TNF-α (rho=0.18, p=0.034), see figure 53.

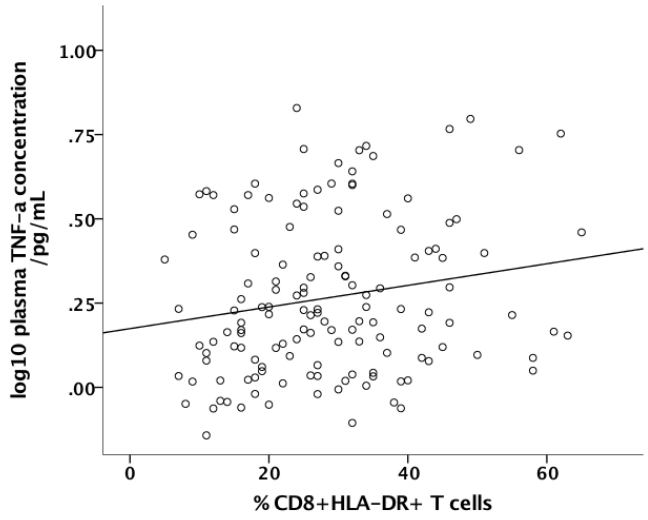


Figure 53 Scatter of the percentage CD8+ HLA-DR+ T cells against plasma pro-inflammatory TNF-α cytokine concentrations in HIV-1 infected pregnant women
Demonstrating a positive correlation between TNF-α and activated CD8+ T cell subsets, Spearman's rho=0.18, p=0.034 and ----- fit line =linear regression.

8.3.3 Relationship of T-cell activation with Immune-regulatory cytokines

Absolute CD3+CD4+ cell counts and the CD4/CD8 ratio positively associate with immune-regulatory IL-4 plasma concentrations whereas percentage of CD8+ cells inversely correlates with plasma IL-4 concentration

Immune-regulatory IL-4 was positively correlated with absolute CD3+CD4+ cell counts and with CD4/CD8 ratio but inversely correlated with absolute CD3+CD8+ cell count and was not associated with CD4+HLA-DR+ % and CD8+HLA-DR+ %, see figure 54.

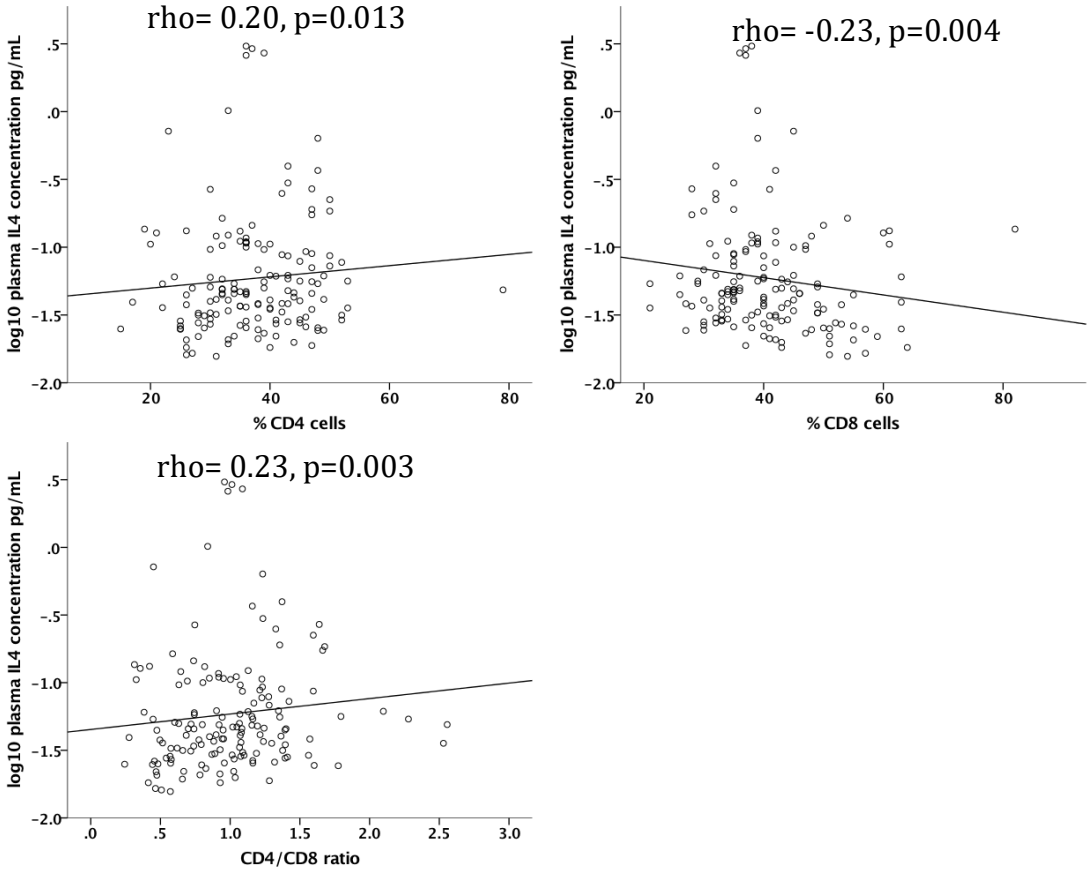


Figure 54 Scatter of CD4+ and CD8+ T cells and CD4/CD8 ratios against plasma immune-regulatory IL-4 concentrations in HIV-1 infected pregnant women
Demonstrating negative correlations between plasma IL-4 concentrations and cellular markers of immune activation: %CD8 and positive correlations with markers of immune restoration: %CD4 and CD4/CD8 ratio, rho= Spearman's correlation coefficient and ----- fit line =linear regression

Percentage of CD4+CD25+ cells was inversely associated with pro-inflammatory IFN-γ (rho=-0.19, p=0.028) and IL-8 (rho=-0.19, p=0.0021), see figure 55.

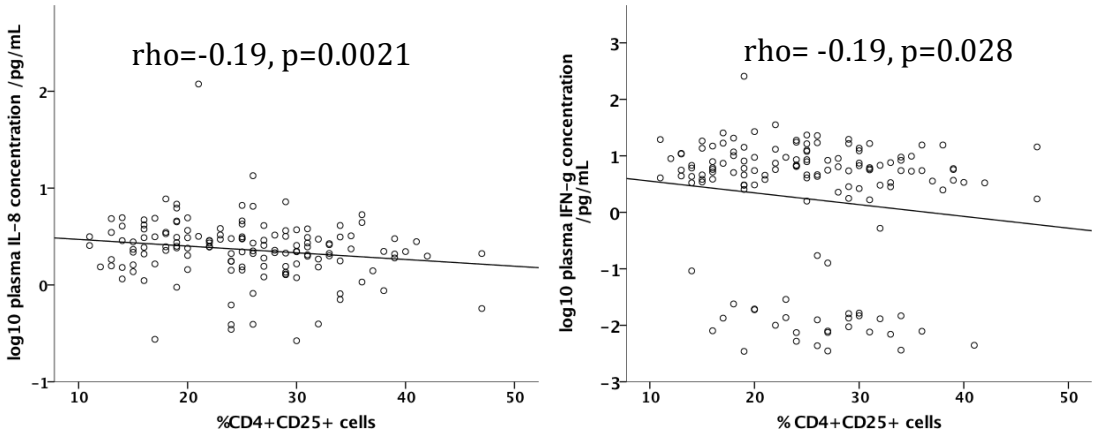


Figure 55 Scatter of % CD4+ CD25+ T cells against plasma pro-inflammatory IFN-γ and IL-8 concentrations in HIV-1 infected pregnant women
Demonstrating a negative correlation between these pro-inflammatory cytokine/chemokines, rho= Spearman's correlation coefficient and ----- fit line =linear regression

8.3.4 Relationship between CVF cytokines and circulating T-cells

8.3.4.1 Results considering all women

Peripheral T cell activation was positively associated with local cervicovaginal pro-inflammatory and immune-regulatory cytokine concentrations

Considering the two main groups (HIV-1 infected and uninfected pregnant women) together peripheral blood mononuclear cell subsets correlated with CVF cytokine concentrations, see table 33. Absolute CD3+CD4+ cell count, percentage of CD3+CD4+ cells and the CD4/CD8 ratio were inversely associated with all measured CVF cytokine concentrations whereas CD3+CD8+ cells positively correlated with all measured CVF cytokines.

Pro-inflammatory cytokines

Activated (HLA-DR+) CD4+ and CD8+ cells correlated positively with cervicovaginal pro-inflammatory cytokines: IL-2, IL-8, IL-12 and TNF-α. The percentage of CD4+CD25+ cells inversely correlated with pro-inflammatory IL-1β and the percentage of CD8+CD25+ cells inversely correlated with pro-inflammatory IL-1β and IL-8.

Immune-regulatory cytokines

A positive correlation was also observed between activated (HLA-DR+) CD4+ and CD8+ cells with CVF immune-regulatory: IL-4, IL-10 and IL-13 concentrations.

Each of absolute CD3+CD4+ cell count, percentage of CD3+CD4+ cells, and the CD4/CD8 ratio were inversely associated with all measured CVF cytokine concentrations whilst the percentage of CD3+CD8+ cells positively correlated with all measured CVF cytokine.

Table 33 Correlations between CVF cytokines and whole blood T cell subtypes in all pregnant women

T cell subtype	Pro-inflammatory cytokines												Immune-regulatory cytokines							
	IFN-γ		IL-1β		IL-2		IL-8		IL-12		TNF-α		IL-4		IL-6		IL-10		IL-13	
	rho	p	rho	p	rho	P	rho	p	rho	p	rho	p	rho	p	rho	p	rho	p	rho	p
Absolute CD4+	-0.18	0.021	-0.19	0.014	-0.40	*	-0.35	*	-0.37	*	-0.34	*	-0.37	*	-0.11	0.158	-0.36	*	-0.45	*
% CD3+CD4+	-0.28	*	-0.26	0.001	-0.43	*	-0.34	*	-0.40	*	-0.38	*	-0.45	*	-0.17	0.033	-0.43	*	-0.47	*
Absolute CD8+	0.15	0.057	0.15	0.056	0.18	0.018	0.09	0.274	0.18	0.024	0.15	0.058	0.20	0.009	0.17	0.027	0.18	0.021	0.19	0.013
% CD3+CD8+	0.24	0.002	0.28	*	0.39	*	0.34	*	0.38	*	0.35	*	0.38	*	0.30	*	0.35	*	0.42	*
%CD4+CD25+	-0.02	0.773	-0.25	0.002	0.11	0.193	-0.12	0.160	0.06	0.492	-0.04	0.620	0.09	0.258	-0.18	0.033	0.09	0.270	0.10	0.219
%CD4+HLA-DR+	0.07	0.404	0.11	0.190	0.21	0.013	0.19	0.023	0.22	0.007	0.26	0.002	0.24	0.003	0.05	0.516	0.21	0.012	0.27	0.001
%CD8+CD25+	-0.12	0.160	-0.18	0.030	0.02	0.796	-0.15	0.069	-0.05	0.532	-0.10	0.214	-0.01	0.952	-0.14	0.094	-0.05	0.579	-0.01	0.911
%CD8+HLA-DR+	0.11	0.182	0.06	0.443	0.19	0.021	0.18	0.038	0.20	0.016	0.19	0.023	0.23	0.006	0.06	0.495	0.20	0.013	0.24	0.003
CD4/CD8 ratio	-0.27	*	-0.28	*	-0.46	*	-0.35	*	-0.43	*	-0.40	*	-0.46	*	-0.25	0.001	-0.43	*	-0.49	*

8.3.4.2 Results considering only HIV-1 infected pregnant women -

In HIV-1 infected pregnant women peripheral CD4+CD25+ % was positively associated with local cervicovaginal IL-10 concentrations and inversely associated with local pro-inflammatory cytokines: IL-1β, IL-8 and TNF-α**.** Considering HIV-1 infected pregnant women alone, CD4% and the CD4/CD8 ratio were positively correlated with IL-2 (%CD4: rho=0.22, p=0.035, CD4/CD8: rho=0.21, p=0.038). There were no associations between HLA-DR+ T cells and cytokines however CD4+CD25+% was significantly inversely associated with local pro-inflammatory cytokines: IL-1β (rho=-0.36, p=0.001), IL-8 (rho=-0.25, p=0.037) and TNF-α (rho=-0.28, p=0.015) and positively correlated with anti-inflammatory IL-10 (rho=0.24, p=0.036), see figure 56.

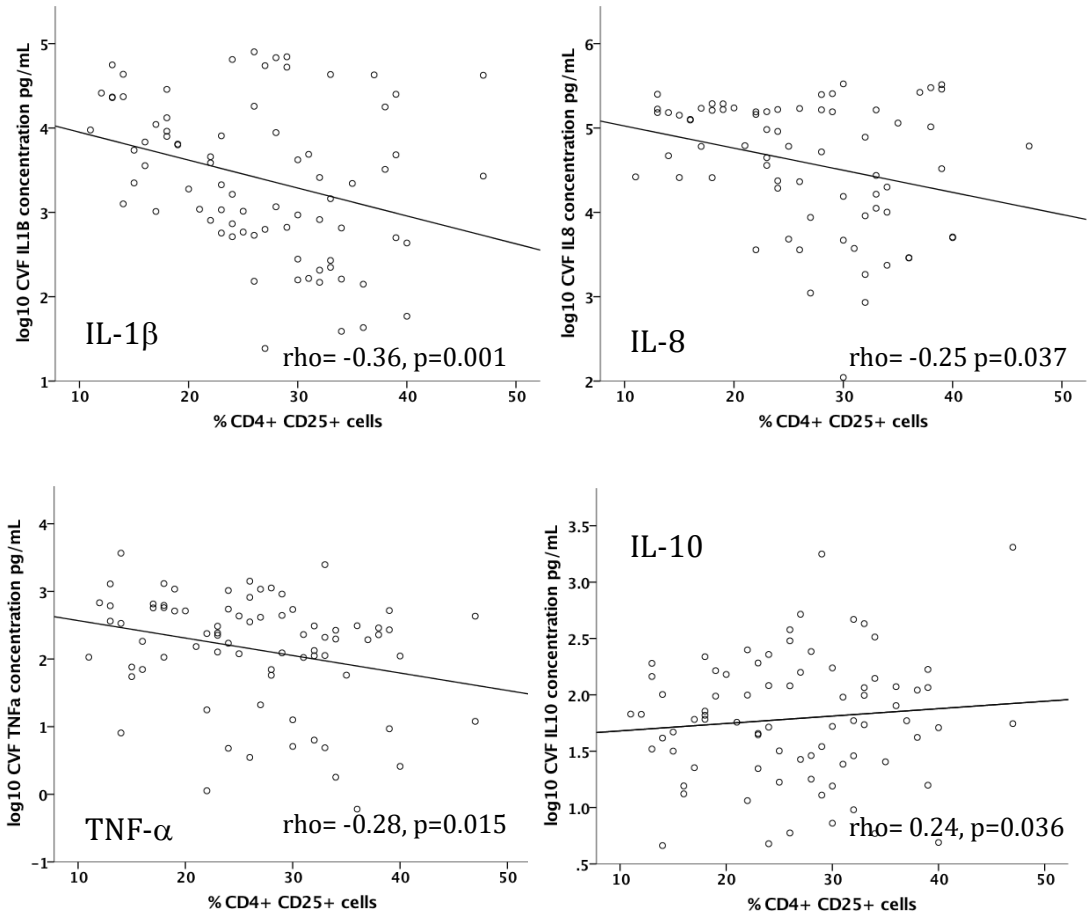


Figure 56 Scatter of peripheral blood CD4+CD25+ % against CVF IL-1β, IL-8, IL-10 and TNF-α cytokine concentrations in HIV-1 infected pregnant women
Demonstrating invserse correlations between CD25+ CD4+ T cell subsets and pro-inflammary cytokines: IL-1β, IL-8 and TNF-α and a positive correlation with immune regulatory IL-10, rho= Spearman's correlation coefficient and ----- fit line =linear regression.

Percentage of CD8+CD25+ T cells were also inversely associated with the pro-inflammatory cytokines: IL-1β (rho=-0.25, p=0.027) and TNF-α (rho=0.22, p=0.05) and positively associated with anti- inflammatory IL-10 (r=0.53, p<0.0005), see figure 57.

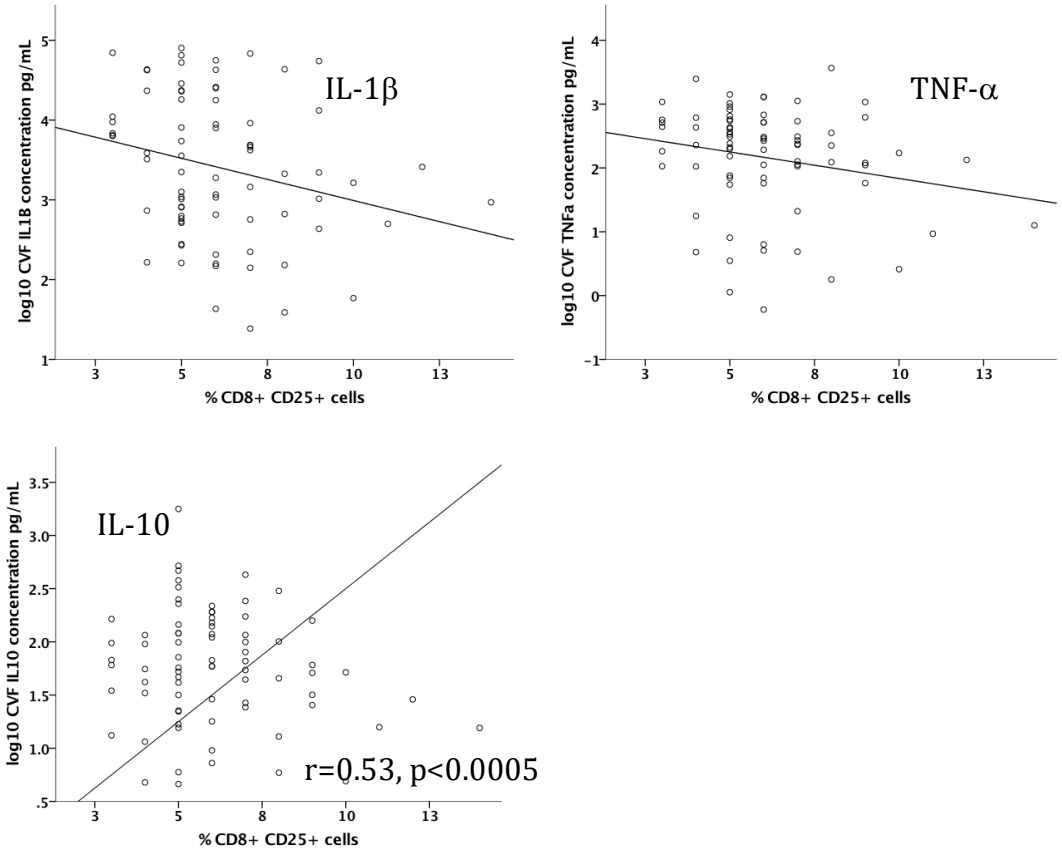


Figure 57 Scatter of peripheral blood %CD8+CD25+ T cells against CVF IL-1β, IL-10 and TNF-α cytokine concentrations in HIV-1 infected pregnant women
Demonstrating invserse correlations between CD25+ CD8+ T cell subsets and pro-inflammary cytokines: IL-1β and TNF-α and a positive correlation with immune regulatory IL-10, rho= Spearman's correlation coefficient, r=Pearson's correlation coefficient and ----- fit line =linear regression.

8.3.5 Relationship between genital-plasma cytokine gradient and T-cell activation

When considering all women activated peripheral T cells (HLA-DR+) were positively associated with both pro-inflammatory and immune-regulatory cytokine gradients and CD25+ peripheral T cells were negatively associated with the gradient of pro-inflammatory IL-1β

Considering all women in the analysis, plasma-genital ratios for both pro-inflammatory and immune-regulatory cytokines correlated inversely with absolute and percentage CD3+CD4+ cell counts and CD4/CD8 ratios, see table 34. Positive correlations were observed between cytokine gradients and percentage CD3+CD8+ cells and markers of T cell immune activation (CD4+HLA-DR+% and CD8+HLA-DR+ %), see table 34.

Table 34 The association between genital-plasma cytokine gradients and PBMCs subsets and CD4/CD8 ratio in all women

Genital-plasma gradient cytokine	Absolute CD3+CD4+ T cell		% CD3+CD4+ T cell		Absolute CD3+CD8+ T cell		% CD3+CD8+ T cell		CD4/CD8 ratio	
	rho	p value	rho	p value	rho	p value	rho	p value	rho	p value
Pro-inflammatory										
IFN-γ	-0.24	0.003*	-0.33	<0.0001*	0.19	0.023*	0.29	<0.0001*	-0.34	<0.0001*
IL-1β	-0.16	0.05*	-0.26	0.002*	0.19	0.021*	0.29	<0.0001*	-0.30	<0.0001*
IL-2	-0.39	<0.0001*	-0.42	<0.0001*	0.18	0.027*	0.37	<0.0001*	-0.42	<0.0001*
IL-8	-0.37	<0.0001*	-0.36	<0.0001*	0.13	0.127	0.35	<0.0001*	-0.38	<0.0001*
IL-12	-0.36	<0.0001*	-0.37	<0.0001*	0.15	0.066	0.33	<0.0001*	-0.38	<0.0001*
TNF-α	-0.38	<0.0001*	-0.38	<0.0001*	0.15	0.071	0.35	<0.0001*	-0.39	<0.0001*
Immune-regulatory										
IL-4	-0.42	<0.0001*	-0.51	<0.0001*	0.27	0.001*	0.43	<0.0001*	-0.52	<0.0001*
IL-6	-0.16	0.055	-0.23	0.004*	0.24	0.003*	0.35	<0.0001*	-0.38	<0.0001*
IL-10	-0.45	<0.0001*	-0.50	<0.0001*	0.24	0.003*	0.42	<0.0001*	-0.50	<0.0001*
IL-13	-0.40	<0.0001*	-0.45	<0.0001*	0.24	0.003*	0.39	<0.0001*	0.46	<0.0001*

Table 35 The association between genital-plasma cytokine gradients and cellular markers of immune activation in all women

Genital-plasma gradient cytokine	% CD4+HLA-DR+ T cells		% CD8+ HLA-DR+ T cells		% CD4+ CD25+ T cells		% CD8+ CD25+ T cells	
	rho	p value	Rho	p value	rho	p value	rho	p value
Pro-inflammatory								
IFN-γ	0.09	0.297	0.16	0.063	0.08	0.342	-0.4	0.617
IL-1β	0.11	0.223	0.07	0.402	**-0.20**	**0.019***	**-0.19**	**0.027***
IL-2	0.15	0.081	0.14	0.106	0.07	0.457	-0.05	0.552
IL-8	**0.19**	**0.028***	**0.18**	**0.038***	-0.06	0.521	**-0.18**	**0.045***
IL-12	**0.24**	**0.006***	0.17	0.051	0.04	0.646	-0.14	0.122
TNF-α	**0.23**	**0.006***	**0.18**	**0.035***	-0.02	0.862	-0.12	0.158
Immune-regulatory								
IL-4	**0.27**	**0.003***	**0.25**	**0.005***	0.10	0.275	-0.07	0.441
IL-6	0.17	0.056	**0.18**	**0.036***	-0.07	0.413	-0.09	0.318
IL-10	**0.26**	**0.003***	**0.25**	**0.004***	0.15	0.083	-0.06	0.519
IL-13	**0.23**	**0.012***	**0.20**	**0.026***	0.11	0.200	-0.05	0.559

The opposite association was seen with CD4+CD25+%, which was inversely correlated with IL-1β and percentage of CD8+CD25+%, which were inversely correlated with both IL-1β and IL-8, see table 34.

<u>When considering only HIV-1 infected pregnant women pro-inflammatory IL-2 genital-plasma gradient was positively correlated with percentage CD3+CD4+ cells and the CD4/CD8 ratio and inversely correlated with percentage CD4+HLA-DR+ cells</u>

Correlations between genital-plasma cytokine gradients and PMBCs were explored in HIV-1 infected pregnant women. Interestingly the associations between cytokine gradients and CD3+CD4+ cells and CD4/CD8 ratios in HIV-1 infected women were fewer and changed polarity. The retained significant correlations were percentage CD3+CD4+ cells with pro-inflammatory IL-2 gradient (rho=0.28, p=0.01) and immune-regulatory IL-13 (rho=0.23, p=0.04). CD4/CD8 ratios were positively correlated with IL-2 plasma genital gradient (rho=0.25, p=0.02). No associations were observed with CD8 cell counts.

Genital-plasma IL-1β cytokine gradient correlated negatively with percentage CD4+CD25+ and percentage CD8+ CD25+ in HIV-1 infected women and positively correlated with plasma HIV viral load.

Correlations with T cell immune activation markers and genital-plasma cytokine gradients in pregnant women with HIV-1 infected were explored. Similar associations were observed as were seen in the analysis of all women, but fewer retained statistical significance. IL-1β was inversely associated with CD25+ peripheral T cell subsets (CD4+CD25+; rho=-0.30, p=0.003; CD8+CD25+; rho=-0.26, p=0.03). IL-2 was inversely associated with activated (HLA-DR+) CD4+ T cell subsets (CD4+HLA-DR+; rho=-0.28, p<0.01). No correlations between cytokine gradients and percentage CD8+HLA-DR+ cells were observed.

Plasma viral load was positively correlated with genital-plasma IL-1B gradient (r=0.23, p=0.03). No difference in genital-plasma cytokine gradients by ART exposure or ART class were observed.

8.4 Vaginal leucocyte concentration is associated with CVF cytokine concentrations

Twenty-one samples had paired high vaginal light field microscopy. The relationships between total leucocyte count per high power field on vaginal microscopy and cytokine concentrations were explored. Leucocyte count was significantly correlated with pro-inflammatory IL-1β (rho= 0.506, p=0.023) and there was a trend towards an association between pus cell count and IFN-γ (rho=0.423, p=0.063).

8.5 The vaginal microbiota differs with CVF cytokine patterns

<u>High bacterial diversity and mixed anaerobes are associated with CVF pro-inflammatory cytokines</u>

Matching CVF cytokine data were available for 79 of the 117 vaginal microbiota samples from HIV-1 infected women. High CVF bacterial diversity and increased number of species within subjects was associated with increased concentrations of IL-1β, see figure 58.

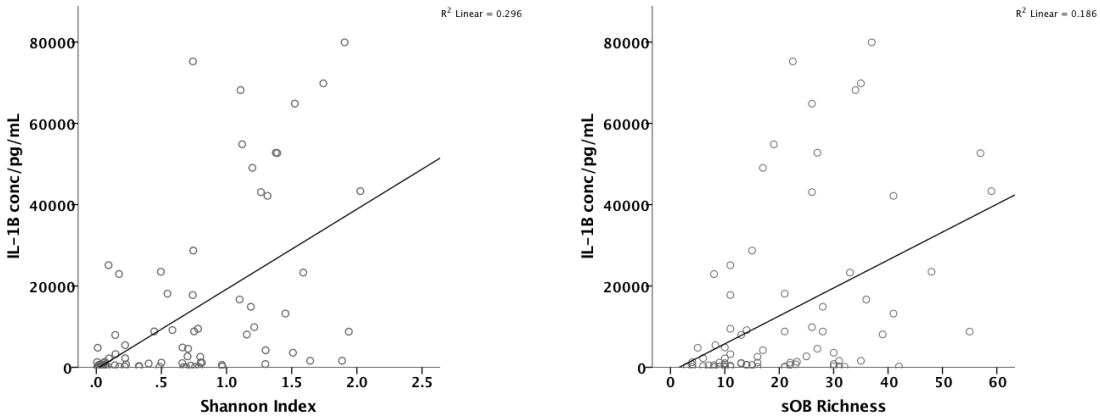


Figure 58 Scatter plots to demonstrate the positive correlation between IL-1β and NP Shannon Index (α diversity) and Richness (sOB)
---- fit line =linear regression

Mean proportion of anaerobic species (*Atopobium vaginalis*, *Dialister spp., Gardnerella spp.,* and *Prevotella spp.)* was positively correlated with higher concentrations of CVF pro-inflammatory cytokines: IFN-γ, IL-1β, and TNF-α, see table 36.

Table 36 Associations between vaginal microbiota and cervicovaginal pro-inflammatory cytokines during the second trimester

r = pearson's correlation co-efficient.

Cytokine	IL-1β		IL-8		IL-12		IFN-γ		TNF-α	
	r	p	r	p	r	p	r	p	r	p
L. iners	-0.293	0.009	-0.061	0.618	-0.044	0.701	-0.200	0.078	-0.165	0.145
L. crispatus	-0.178	0.124	0.085	0.486	-0.032	0.778	-0.113	0.320	-0.186	0.101
Gardnerella spp.	0.550	0.0001	-0.020	0.872	0.069	0.544	0.276	0.014	0.311	0.005
L. jensenii	0.549	0.0001	0.019	0.875	0.071	0.536	0.272	0.015	0.370	0.001
L. gasseri	0.085	0.455	0.014	0.907	0.143	0.210	0.197	0.082	0.078	0.495
Catonella spp.	0.102	0.373	-0.023	0.851	-0.078	0.494	-0.045	0.695	0.276	0.014
Prevotella spp.	0.474	0.0001	0.059	0.628	0.282	0.012	0.414	0.0001	0.414	0.0001
Atopobium vaginalis	0.484	0.0001	0.022	0.860	0.069	0.543	0.532	0.0001	0.259	0.021
Megasphaera spp.	-0.004	0.974	-0.058	0.636	-0.190	0.094	-0.086	0.452	-0.046	0.684
Snaethia spp.	0.257	0.022	0.027	0.823	-0.104	0.359	-0.045	0.695	0.050	0.664
Ureaplasma parvum	0.025	0.824	-0.017	0.818	0.101	0.377	0.146	0.198	0.078	0.492
Aerococcus christensenii	0.347	0.002	0.052	0.670	0.076	0.587	-0.002	0.988	0.288	0.018
Dialister spp.	0.614	0.0001	0.077	0.531	0.413	0.0001	0.674	0.0001	0.442	0.0001

Pro-inflammatory cytokine concentrations of IFN-γ, IL-1β, IL-8 and TNF-α were significantly higher in CST IIIB, IV and II compared to other CSTs, see figure 59. Within this small group, no associations between cytokine concentrations and gestational age at delivery were observed.

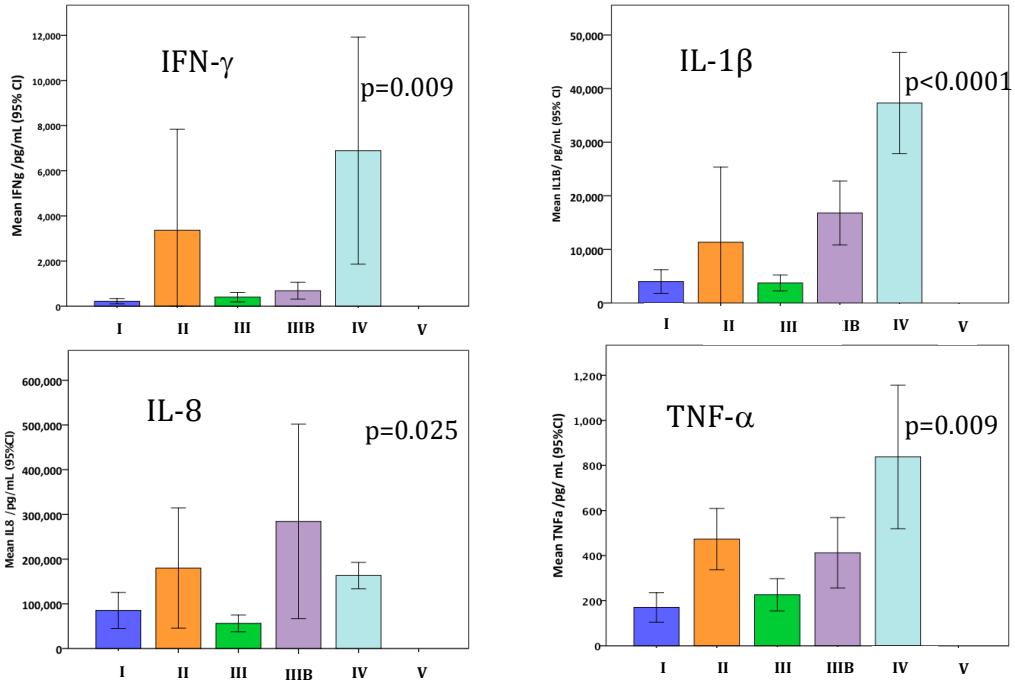


Figure 59 Bar charts to demonstrate mean cytokine concentration by CST during the second trimester.
Demonstrating significantly higher pro-inflammatory cytokine expression: IL-1β, IL-8, IFN-γ and TNF-α in women with advsere CST IIIb and IV compared to CST I and III.

8.6 Summary of Complex Correlation Results

1. Plasma IL-13 concentrations were highly inter-correlated with immune-regulatory CVF cytokines: IL-4 and IL-10 and there was a trend with CVF IL-13 in HIV-1 infected pregnant women
2. A positive genital-plasma cytokine gradient (i.e. >1) existed for all measured cytokines in both HIV-1 infected and uninfected pregnant women, with genital mucosal cytokine concentrations being up to 100,000 fold (IL-1β) higher than those observed in the plasma.
3. The genital-plasma cytokine gradient in HIV-1 infected pregnant women was significantly greater than in uninfected women.
4. In rank order, the greatest gradients were observed in HIV-1 infected women and were for pro-inflammatory cytokine IL-1β and chemokine IL-8, followed by IL- 2, IL-13, IL-12, IL-4, IL-6, IL-10, TNF-α and IFN-γ.
5. Genital-plasma cytokine gradients (IFN-γ, IL-2, IL-12, IL-4 and IL-13) increased with advancing gestational age at sampling in HIV-1 infected women. No association between cytokine gradient and gestational age at delivery was observed.
6. Peripheral T cell activation (% CD4+HLA-DR+ T cells and %CD8+HLA-DR+ T cells) in HIV-1 infected pregnant women was positively correlated with plasma concentrations of pro-inflammatory cytokines IFN-γ, IL-2, IL-12 and TNF-α.
7. In HIV-1 pregnant women, peripheral CD4+CD25+ cells percentage was inversely correlated with pro-inflammatory CVF cytokines: IL-1β, IL-8 and TNF-α concentration
8. In HIV-1 pregnant women, immune-regulatory CVF IL-10 concentration was positively associated with peripheral CD4+CD25+ T cell percentage.
9. Genital-plasma IL-1β gradient was inversely correlated with peripheral CD4+CD25+ T cell and CD8+CD25+ T cell percentage in HIV-1 infected pregnant women and positively correlated with plasma HIV-1 viral load
10. Increasing bacterial diversity was positively correlated with CVF IL-1β concentration.

11. Diverse anaerobic species including *Gardnerella, Prevotella, Sneathia, Dialister spp.* and *Atopobium vaginalis* were positively correlated with pro-inflammatory CVF cytokines: IL-1β, IL-12, IFN-γ and TNF-α.

8.7 Conclusions and interpretation

The positive direction of the measured genital-plasma cytokine gradients reflects that pro-inflammatory and immune-regulatory cytokines are "mucosally biased" and concentrate in the lower FGT. The observation that these gradients are greater in women with HIV-1 infection suggests that a factor (either locally driven or systemically) is causing an increase in production of cytokines in the female genital tract of these pregnant women.

It is interesting that cytokine gradients of pro-inflammatory IL-1β and neutrophil chemokine IL-8 in HIV-1 infected pregnant women are the most marked. It is possible that these gradients reflect an increased production of local chemokines and cytokines by monocytes/macrophages in the lower FGT inducing migration of neutrophils. The induction of this type of immune response may be the result of antigenic stimulation and or dys-regulated innate and adaptive immunity in the context of HIV-1 viral infection. Indeed, plasma IL-8 and CVF IL-8 are significantly correlated in these women and there was a trend towards a positive association between plasma IL-8 and CVF IL-1β.

The increase in genital-plasma cytokine gradient observed with gestational age could be artifact, the result of the systemic haemodilution through increasing circulating volume, artificially decreasing measured plasma cytokines and thus calculated ratios. The effect of haemodilution on plasma cytokine measurement in pregnancy is unknown but likely to have a minimal effect in the context of significant genital inflammation. Indeed, this observation may reflect increasing cell migration and inflammation across gestation in preparation for labour. The fact that this is enhanced in HIV-1 infected pregnant women may explain why this group of women experience higher rates of PTB than uninfected women.

Within these data we have demonstrated a positive association between IL-1β and vaginal polymorphonuclear cells. These cells are highly likely to be neutrophils but these observations are based on light field microscopy on a subset of samples and the cells have not been formally immuno-phenotyped. The local population of leucocytes has not been fully characterised in all these

women. Therefore, we cannot draw firm conclusions about the cellular origin of IL-1β nor the innate and adaptive leucocyte networks, including macrophages and T cells that exist within the local FGT.

Non-invasive cytobrush cervical cell collection used in conjunction with flow cytometry has been demonstrated to be an effective technique to immunotype endocerivcal leucocytes(Prakash, Patterson and Kapembwa, 2001; Liebenberg *et al.*, 2011). Of interest, CD4/CD8 ratios in the cervix have been shown to reflect those observed in PBMCs from the same women(Prakash, Patterson and Kapembwa, 2001). Arnold, Anahtar and Gosmann have also demonstrated the association between pro-inflammatory cervical cytokines and increased cervical CD4+ T cells in uninfected non pregnant women at high risk for HIV(Anahtar *et al.*, 2015; Arnold *et al.*, 2016; Gosmann *et al.*, 2017). This knowledge affirms our hypothesis that the elevated FGT inflammation observed in HIV-1 infected pregnant women is likely to be accompanied by influxes of local innate and adaptive leucocytes.

When adaptive immune associations between peripheral mononuclear cells were explored in all pregnant women, positive associations between activated peripheral CD4+HLA-DR+ T cells and CD8+HLA-DR+ T cells and local CVF pro-inflammatory (IL-2, IL-8, IL-12 and TNF-α) and CVF immune-regulatory cytokines (IL-4, IL-10 and IL-13) were identified. These findings suggest that activated peripheral T cells may directly or indirectly influence local genital cytokine production. The positive association identified between activated T (HLA-DR+) cells and pro-inflammatory and immune-regulatory genital-plasma cytokine gradients adds further weight to this hypothesis and may be a signal that these gradients may influence the migration of activated T cells to the local mucosa(Liebenberg *et al.*, 2017). The migration of T cells to the lower female genital tract could then augment the local production of these cytokines directly from T cells or through interactions with macrophages (classical or non-classical activation) and other local cytokine producing cells.

Peripheral CD4+HLA-DR+ T cells and CD8+HLA-DR+ T cells were inversely associated with plasma immune-regulatory cytokines (IL-4, IL-6 and IL-10). These data indicate that activated peripheral T cells negatively influence the production systemic immune-regulatory cytokines. This action could be due to decreased cytokine secretion by these cells or possibly through modulation of other cells which produce these cytokines such as T regulatory cells in the case of IL-10(Trinchieri, 2007) and Th-2 helper cells in the case of IL-4(Brown, 2014). A reduction of systemic immune-regulatory cytokines may facilitate both local and systemic inflammatory responses.

When relationships between activated (HLA-DR+) T lymphocytes and local and systemic cytokines were explored in HIV-1 infected women alone not all of the associations discussed previously were reproduced and some new ones were revealed. This is likely to be due to the fact that HIV-1 pregnant women are immunologically distinct, their relative reductions in absolute CD3+CD4+ cells and the smaller sample size will also reduce statistical power.

Percentage of HLA-DR+ T cells positively correlated with pro-inflammatory plasma cytokines (IFN-γ, IL-2 and IL-12) in HIV-1 infected pregnant women, which was not observed in the combined analysis. These cytokine profiles are the signature of Th1 CD4+ helper cell proliferation and recruitment and activation of macrophages(Abbas, Lichtman and Pillai, 2014; Duque and Descoteaux, 2014). This finding also indicates that further characterization of both peripheral and local T cell subsets and macrophages is warranted to elucidate their contribution to genital inflammation in HIV-1 infected pregnant women.

Nkwanyana et al.'s cytobrush study findings would also support the hypotheses that the elevated CVF cytokine are the result of enhanced numbers and activation of T cells and antigen presenting cells/macrophages in the lower FGT of HIV infected non-pregnant women(Nkwanyana *et al.*, 2009). They found that CVF IL-1β, IL-12 and TNF-α were associated with increased cervical CD3+ T

cells. Neutrophils were significantly associated with IL-1β, IL-6, IL-8 and IL-10 and TNF-α and APC numbers were associated with IL-12 and TNF-α.

Complementary to these data are the associations identified in this body of work with the CD25+ T cell subsets. When correlations were explored in all women, plasma IFN-γ, the Th1 signature pro-inflammatory cytokine, was inversely correlated with percentage of CD4+CD25+ T cells. In addition trends towards a negative correlation with plasma IL-6 (p=0.07) and IL-8 (p=0.12) and percentage CD4+CD25+ T cells were seen, both IL-8 and IL-6 are important inflammatory immune proteins. The negative correlation with plasma IFN-γ (rho=-0.19, p=0.028) and IL-8 (rho=-0.19, p=0.0021) were replicated in analysis of HIV-1 infected pregnant women.

These data suggest that this lymphocyte subset may have a regulatory action on systemic inflammation. It is known that CD4+CD25+FoxP3+ cells have been identified as T regulatory cells. Foxp3, an intracellular fork head transcription factor protein, is an essential protein in the development of T regulatory cells (Sakaguchi, 2005). The IL-2 receptor CD25, is important in the generation and function of T regulatory cells, however it is a less specific cellular marker of T regulatory cells. Therefore, whilst it can be speculated that these CD4+CD25+ cells are T regulatory cells that are regulating the expression of pro-inflammatory cytokines by Th1 helper cells, it cannot be confirmed.

The negative associations between percentages of CD4+CD25+ T cells and CD8+CD25+ T cells and inflammatory CVF cytokines (IL-1β and IL-6) in all women combined and in HIV-1 infected pregnant women alone (IL-1β, IL-8 and TNF-α) indicated that these peripheral T cell subsets may regulate inflammation in the genital tract. There were also negative correlations between IL-1β genital--plasma gradient and percentage CD4+CD25+ T cells and CD8+ CD25+ T cells suggesting fewer of these cells are present in the face of enhanced genital inflammation or are enabling enhanced inflammation in the absence of negative regulation. The positive association between these cells and CVF anti-inflammatory IL-10, a signature cytokine for T regulatory cells, gives further

weight to the conclusion that peripheral CD25+ T cells may have the ability to migrate to and regulate local genital inflammation.

These data could be interpreted in another way, in that fewer CD25+ T cells are observed in the periphery in the context of genital inflammation as a result of up-regulation and migration to this local extravascular tissue site. Indeed, the observation of enhanced or reciprocal up-regulation of immune-regulatory CVF cytokines in the context of genital inflammation could support this, if these cells were involved in the production of these immune-regulatory cytokines.

This hypothesis could be reinforced by the observation in HIV-1 infected pregnant women that plasma immune-regulatory cytokine IL-13 is positively associated with CVF pro-inflammatory cytokines: IL-12 and TNF-α. The actions of these cytokines (IL-12 and TNF-α) are synergistic and are secreted by macrophages to enhance Th1:IFN-γ production(Duque and Descoteaux, 2014). Plasma IL-10 is also positively correlated with CVF IL-1β and TNF-α and plasma IL-4 with CVF TNF-α. These CD25+ T cells, whether Th2 or regulatory in action, could be activated to produce these immune-regulatory cytokines as part of the adaptive regulation of the inflammatory response in the genital tract.
This would be supported by Richardson et al., who identified positive correlations with plasma IL-4 and CD4+CD25+FoxP3+ T cells and IL-10 with CD8+CD25+FoxP3+T cells in HIV infected pregnant women(Richardson and Weinberg, 2011). The associations between percentage CD25+FoxP3+ T lymphocytes and both pro-inflammatory and immune-regulatory cytokines in this study were interpreted as having common pathophysiological trigger. We also found a positive correlation between percentage of peripheral CD4+CD25+ T cells and CD8+CD25+ T cells with CVF immune-regulatory IL-10. Whilst these cells may migrate to local sites, an overall up-regulation of these regulatory cytokines was observed systemically.

Richardson et al. also described the dynamics of T regulatory cells across pregnancy, and found, similar to our data, that HIV infected pregnant women, all of whom received cART (4/20 conceived on cART) had higher frequencies of T

regulatory cells compared to uninfected pregnant women in the second trimester(Richardson and Weinberg, 2011). Richardson's observations differ from ours in that percentage CD4+CD25+FoxP3+ T cells and CD8+CD25+FoxP3 T cells positively correlated with plasma pro-inflammatory cytokines IFN-γ and IL-8, whereas we found a negative correlation with the same cytokines in plasma and CVF.

Kolte et al. characterised T regulatory cell populations in HIV infected and uninfected pregnant women and found that HIV infected women do not undergo the second trimester expansion of CD4+CD25+FoxP3+ T cells that is seen in uninfected women. This was despite all women receiving cART (12/20 of whom conceived on cART). The data from our cohort suggests that frequency of CD4+CD25+ T cells in women who conceive on cART exceeds that seen in uninfected women whereas those who initiate treatment after conception do not reach the levels of this cell subset observed in uninfected women until the third trimester and do not reach the same level as those who conceived on cART during pregnancy. This may be a function of CD4 recovery/reconstitution. This is corroborated by the positive linear association between CD4+C25+ T cells and ART exposure/weeks. A lower percentage of CD4+CD25+ FoxP3 T cells during pregnancy in women who initiate treatment in pregnancy could be a contributing factor to the trend towards an enhanced pro-inflammatory and reduced immune-regulatory CVF cytokine millieu seen in these women.

CD4+FoxP3- cells have been identified as one of the main producers of IL-10 in the T cell culture work of Hygino and colleagues(Hygino *et al.*, 2012) in keeping with our finding that the percentage CD4+CD25+ cells correlates with plasma IL-10. In Hygino's study of 25 HIV infected pregnant women (19 initiated cART, all during pregnancy), 25 uninfected pregnant and 10 HIV-1 infected non pregnant controls, HIV specific IL-10 secreting CD4+FoxP3- cells were increased during pregnancy compared to non-pregnant women but reduced compared to uninfected pregnant women.

T cell culture work performed by Bento et al. advance our knowledge of T regulatory cells in HIV infected pregnant women and the effect of antiretrovirals. They demonstrated that plasma IL-10 concentrations were higher in 32 HIV pregnant women who had undetectable plasma viral loads (<80 copies/mL) and uninfected pregnant women compared to women with detectable HIV viraemia. In addition they observed higher IL-10 concentrations in both plasma and cell supernatants from women on ART (all including ZDV) compared to no ART, irrespective of plasma viral load. They were also able to show that blockade of IL-10 in T cell cultures augmented pro-inflammatory cytokines: IFN-γ, IL-1β and TNF-α secretion. The effect of cART, independent from the effects of HIV shown by this group are interesting. We were not able to reproduce these findings, possibly because it is a specific effect of ZDV, which is not used in current practice but also most of our women conceived on cART whereas all the women in Bento's study initiated cART post conception and these are two distinct groups with different PTB risks.

Recently work has been undertaken to explore the effect of cART on local FGT immunological profiles. Caruso et al. characterised PBMC T regulatory CD4+ T cell subsets, and explored both PMBC and cervical monocytes cytokine and chemokine expression profiles, in 53 HIV infected (32 on cART and 33 cART naïve) and 41 uninfected non pregnant women(Caruso *et al.*, 2019). HIV-1 infected women had greater numbers of activated whole blood T regulatory cells and cART exposure did not effect these numbers. Stimulation of cervical monocytes revealed that in HIV infected women receiving cART, these cells did display greater IL-10 secretion compared to those who were cART naïve, although this was still lower than in uninfected women. Interestingly the authors were able to show that T regulatory chemokine (CCL17) levels in ectocervical swab samples were higher in HIV infected women than uninfected women and positively correlated with IL-10 from cervical monocyte supernatant. These data indicate that it is highly likely that T cell subsets, secreting immune-regulatory cytokines will be present in HIV-1 infected pregnant women and may be the main source of these cytokines in the FGT. It appears that ART may allow

reconstitution of these cell subtype and this potentially enables regulation of the inflammatory response in the FGT.

The cause of increased FGT inflammation in these HIV-1 infected pregnant women is unknown. It is plausible that there is a direct role of HIV-1 in inducing genital inflammation which is supported by the positive correlation observed between IL-1β genital-plasma gradient and HIV-1 plasma viral load. The association between pro-inflammatory cytokines and HIV virus in the FGT has been demonstrated previously(Zara *et al.*, 2004; Bebell *et al.*, 2008; Mitchell *et al.*, 2011; Roberts *et al.*, 2012; Herold *et al.*, 2013). This would tie in with the fact that length of cART exposure is inversely correlated with CVF IFN-γ, the main antiviral cytokine, in these women if we assume that cART exposure positively correlates with genital viral load (untested). It is also highly likely that vaginal bacterial antigenic stimuli has an effect.

We have presented data showing that α diversity, number of species and the presence of anaerobes: *Atopobium spp., Dialister spp., G. vaginalis, Prevotella spp.,* and *Sneathia spp.* are associated with elevated FGT pro-inflammatory cytokines. BV is increasingly recognised as a pro-inflammatory condition (Sturm‐Ramirez *et al.*, 2002; Beigi *et al.*, 2007). Interestingly pregnant women (uninfected) have been shown to elicit higher pro-inflammatory cytokine responses to BV than in non-pregnant women(Beigi *et al.*, 2007).

Replication of the association between diverse anaerobic species with increased FGT inflammation within HIV-1 infected pregnant women suggests ascending infectious/inflammation as one of the underlying triggers of elevated PTB in this group. This hypothesis is also supported by a recent study by Lopez et al. showing high plasma levels of soluble CD14 and liposaccharide-binding-protein (markers of microbial translocation) in the first trimester in HIV infected pregnant women who went on to experience PTB compared to HIV infected women delivering at term and uninfected pregnancies (Lopez *et al.*, 2016).

A recent study by Mitchell et al. also explored LPS and sCD14 in 10 uninfected and 30 HIV infected pregnant women initiating cART in pregnancy, in addition to 16S rRNA quantification of bacterial load by real time PCR. LPS and 16S rRNA declined post cART initiation in pregnancy whereas sCD14 increased across the second and third trimesters. This contrasts with uninfected pregnant women in whom sCD14 was within normal range. These data suggest that whilst cART may partially reverse evidence of microbial translocation in pregnancy, there is evidence of increase in monocyte activation(Mitchell *et al.*, 2018). If activated peripheral monocytes/macrophages were to influx to the FGT during the second half of pregnancy in HIV-1 infected women as the result of bacterial stimulation, this could explain increases in CVF inflammation and increased risk of triggering parturition.

The concept that microbial translocation may be relevant to birth outcome in women with HIV infection is a relatively novel concept but one that is gaining recognition. A nested case-control study from India undertaken as part of the SWEN RCT (comparing single dose NVP with a 6 week extended dose to prevent HIV-1 transmission via breastfeeding) explored plasma markers of acute phase response, intestinal integrity and microbial translocation during pregnancy and associations with PTB in 26 cases and 81 controls (term births) (Shivakoti *et al.*, 2018). In multivariate modeling Shivakoti et al. demonstrated that macrophage activation markers: sCD14 and sCD163 and intestinal barrier dysfunction marker: fatty acid binding protein were associated with PTB in HIV infected women. This study was not powered to look at treatment effect as women were not recruited until 32 weeks and only 11% of women received cART prior to delivery. It is interesting that authors conclude microbial translocation is intestinal in origin when the lower FGT is a bacterial source with a direct route to the fetal placental unit that is anatomically closer.

Chapter 9. Discussion

9.1. Summary

The increase in FGT inflammation in HIV-1 infected pregnant women and the factors that influence this inflammation are the major findings of this body of work. Our results indicate a genital mucosal biased cytokine environment of HIV-1 infected pregnant women, which we hypothesise could lead to enhanced local inflammatory immune responses to stimuli such as HIV or commensal bacteria. Such an exaggerated immune response increases the likelihood that the inflammatory threshold for the trigger of labour could be reached earlier. A pictorial representation of the study findings and how they may work in concert to increase risk of PTB in HIV-1 infected women is shown below in figure 60.

Gestational age at delivery correlated inversely with evidence of peripheral immune activation (CD8+HLA-DR+ T cells) and with the abundance of vaginal anaerobic species: *Prevotella, Sneathia* and *Dialister* in HIV-1 infected pregnant women where as *L. crispatus* is positively associated with gestational age at delivery indicating that these factors are likely to have the greatest effect on birth outcome with this cohort. Although this study was underpowered to explore associations with PTB, we were able to show that women experiencing PTB tended to have higher CD8+% and lower CD4+% of circulating T-cells. In terms of vaginal bacterial species, women experiencing PTB were more likely to be colonized with *Gardnerella and Prevotella spp.* In addition, directed exploration of FGT immune proteins demonstrated a greater abundance of immune proteins associated with neutrophil and macrophage function e.g. IL-8, IL-17a and MIF as well as AMPs e.g. MPO in women experiencing PTB compared with those delivering at term. These data indicate that some of the excess PTB seen in HIV-1 infected pregnant women is likely to be the result of ascending infection and associated inflammatory immune response.

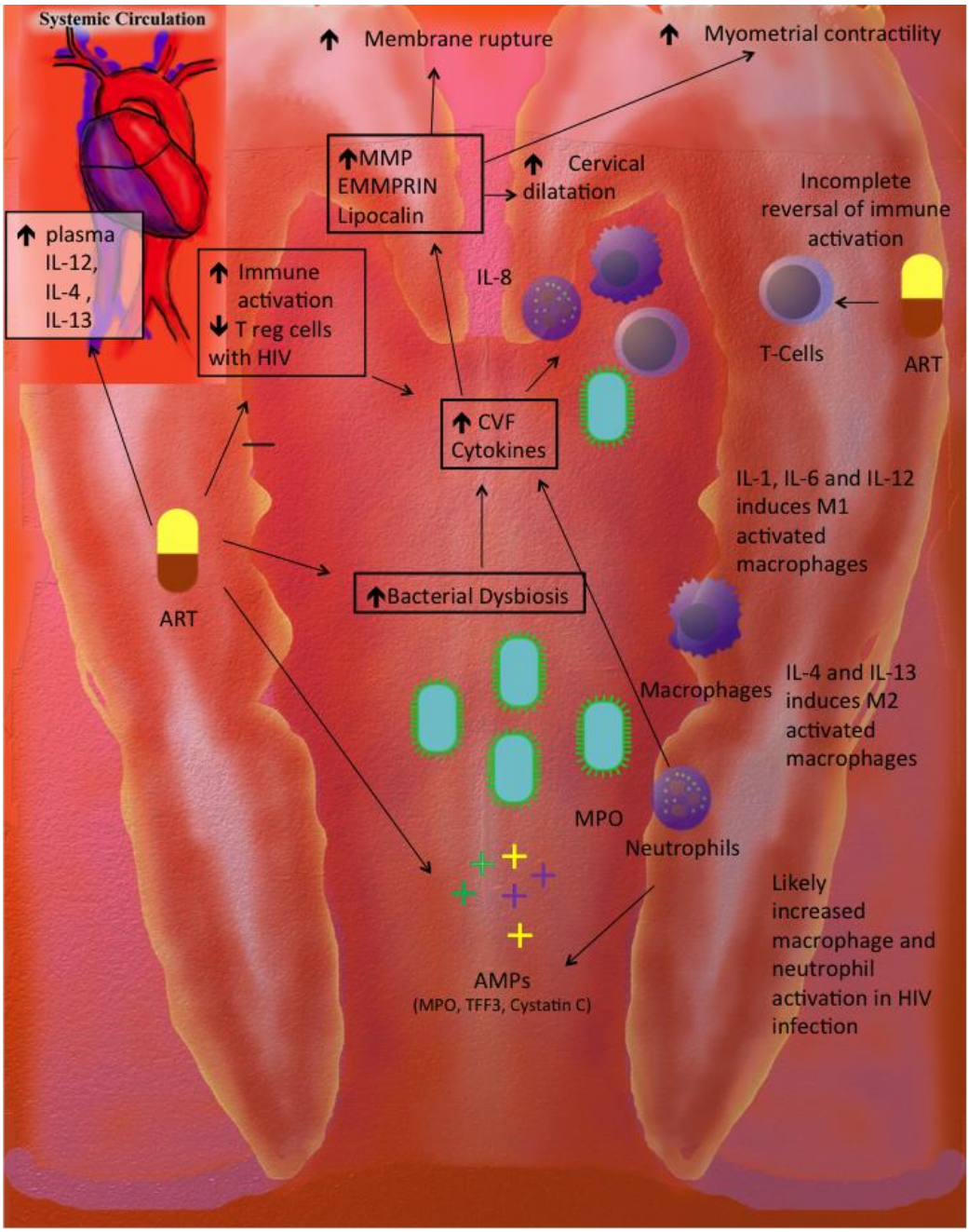


Figure 60 Pictorial representation of suggested inflammatory model of PTB in HIV-1 infection

MMP: Matrix metalloproteinase; EMMPRIN: Extracellular matrix metalloproteinase Inducer; CVF: Cervicovaginal fluid; ART: Antiretroviral therapy; M1: Classically activated macrophage; M2: Alternatively activated macrophage; MPO: Myeloperoxidase; AMPs: Antimicrobial proteins and TFF3: Trefoil factor-3.

Firstly we identified that genital mucosal-plasma cytokine gradients in HIV-1 infected pregnant women were positively associated with peripheral immune activation (CD4+HLA-DR+% and CD8+HLA-DR+%). Secondly we have shown that CVF pro-inflammatory cytokines in HIV-1 infected pregnant women were positively associated with diverse anaerobic vaginal bacterial species. We have

also identified a high abundance of immune factors involved in activation of neutrophils and macrophages and AMPs in HIV-1 infected women compared to uninfected women, with a trend towards the highest concentrations in women receiving PI-based cART. These data support the hypotheses of an anaerobic bacterial antigen driven mechanism stimulating local inflammation in these women, which may be influenced by systemic immune activation and inflammation. The positive correlation identified between CVF IL-1 and local polymorphonuclear cells (likely to be neutrophils) and bacterial diversity in these pregnant women is also supportive. The fact that there is now increasing interest in microbial translocation, as measured by LPS and sCD14, in HIV-1 infected pregnant women and how it associates with immune activation, gut barrier dysfunction and birth outcome is timely (Lopez *et al.*, 2016; Mitchell *et al.*, 2018; Shivakoti *et al.*, 2018). Brenchley et al. have suggested that the origin of microbial antigen is bowel as seen in the natural history of HIV-1 infection(Brenchley *et al.*, 2006; Shivakoti *et al.*, 2018) however we propose that in the case of pregnancy there may be a contribution of microbial antigen from the FGT that has a more direct route of connection between compartments.

The role of cART in modulating this local inflammation may be a mixture of direct and indirect effects. Direct effects of cART are evidenced by the trend towards to the highest CVF cytokine concentrations being found in women initiating cART (with the exception of IL-2 and IL-12) which is reduced by pre-conception cART but not to the levels observed in uninfected pregnant women. This indicates that whilst cART does restore some systemic and local immunity it is not complete. Women receiving pre-conception cART also had the highest abundance of *Gardnerella spp.* indicating that cART may directly modulate the vaginal microbiota of these women, possible through inherent antibacterial action, which is cumulative. In spite of a wealth of research into the intestinal microbiota of HIV-1 infected persons (Brenchley *et al.*, 2006) little value has been placed on a direct action of antiretrovirals on the bacterial communities (Shilaih *et al.*, 2018).

Indirect effects of ART could be mediated by the association of length of time on cART with a reduction in peripheral T cell activation (HLA-DR expression) and an increase in the percentage of CD4+CD25+ cells (possibly T regulatory cells) but not to levels comparable to uninfected women. Peripheral CD4+CD25+ T cells were positively associated with immune-regulatory CVF IL-10 concentrations and correlated inversely with IL-1β, IL-8 and TNF-α and thus cART could indirectly effect local inflammation through increasing the proportion of CD4+CD25+ cells resulting in increased CVF IL-10 and a reduction in IL-1β, IL-8 and TNF-α. Length of time on cART was also associated with increased plasma concentrations of IL-12, IL-4 and IL-13. Plasma IL-13 was also strongly positively correlated with both CVF immune-regulatory cytokines: IL-4, IL-10 and IL-13; and pro-inflammatory CVF cytokine TNF-α and IL-12, both known for their function in macrophage activation (Duque and Descoteaux, 2014). ART could indirectly effect CVF inflammation through its effect on increasing plasma IL-13 concentration. This would fit with the observation that cART restores plasma IL-13 but not to the level seen in uninfected pregnant women thus perhaps the end effect is that balance of CVF pro-inflammatory cytokine ratio to immune-regulatory cytokines is higher in HIV-1 infected v uninfected pregnancy women .

Women receiving PI-based ART displayed a trend towards a higher % of activated CD8+HLA-DR+ T cells and lower CD4/CD8 ratios compared to women receiving NNRTI-based ART, however this did not reach statistical significance. PI-based cART was associated with higher concentrations of immune proteins in CVF (semi quantitative) than found in women receiving non-PI-based cART. Women receiving PI-based cART had lower plasma concentrations of Th2 cytokines: IL-4, IL-10 and IL-13 than women on NNRTI-based cART and higher concentrations of the Th1 cytokines: IL-2 and TNF-α. In addition, during the third trimester, higher plasma IL-2 slope and lower IL-10 slope in women taking PI-based cART v women taking NNRTI cART which means that the increases in IL-2 concentration observed in women receiving PI-based cART and the decline in IL-10 concentration were of greater magnitude than changes observed in women receiving NNRTI cART. These finding indicate that the protease inhibitor class of

ART may be associated with more persistent immune activation including a pro-inflammatory plasma cytokine shift during the second half of pregnancy compared to NNRTI-based ART. The finding that the PI class may be more inflammatory than NNRTIs in its effects on systemic and local immune function may help explain why traditionally these agents have been associated with the highest rates of PTB.

To truly understand the underlying immune mechanisms at the fetal placental unit greater knowledge of local immune cells and their function and how this is influenced by cART and local pathogens is desirable. Ahmed et al. characterised activated ectocervical leucocytes in ten HIV infected and ten uninfected non pregnant women using immunohistochemistry(Ahmed *et al.*, 2001). Only two of the HIV infected women received cART and these women were mostly profoundly immunosuppressed with a median absolute CD4+ cell count of 38 cells/mcL (range 25-858). Higher absolute numbers of epithelial macrophages were observed in HIV infected women compared to uninfected women. A greater proportion of the macrophages identified in the HIV infected women were of the antigen presenting T cell 'inducing' (RFD1+RFD7-) and T cell 'suppressor' (RFD1+RFD7+) than effector/phagocytic (RFD1-RFD7+) phenotypes (Tormey *et al.*, 1997; Ahmed *et al.*, 2001). HIV infected women also had a significant increase in both stromal and epithelial activated T cells (percentage CD4+HLA-DR+ and CD8+HLA-DR+ cells) and lower CD4/CD8 ratio than uninfected women. Clusters of CD4+ CD45RO+ T cells associated with macrophages or dendritic cells just below the basement membrane of the endocervical epithelium were observed suggesting antigen presentation and activation of adaptive immunity across the genital mucosal barrier. This is also reinforced by Passmore's group who have demonstrated that antigen presenting cells from cervical explants obtained from HIV infected women, are activated by cytokines, and they suggest that this is likely to be a response to pathogenic genital infections and TLR agonism(Shey *et al.*, 2015, 2016).

Macrophage differentiation induced by immune –regulatory cytokines IL-4 and IL-13, both of which were higher in the CVF from HIV-1 infected pregnant

women compared to uninfected pregnant women in our study, has more recently been classified as 'alternatively activated' M2 polarised macrophages that secret IL-10 and TGF- β and are anti-inflammatory in effect (Abbas, Lichtman and Pillai, 2014; Duque and Descoteaux, 2014). 'Classically activated' M1 polarised macrophages are induced by IFN-γ, produce IL-1β and IL-12 and are microbicidal and pro-inflammatory (Duque and Descoteaux, 2014), although this dichotomy is thought by some to be outdated and oversimplified(Martinez and Gordon, 2014). Based on these classifications, and the finding of both elevated pro-inflammatory and immune-regulatory CVF cytokines in HIV-1 infected pregnant women, it could be hypothesised that both M1 and M2 macrophages may have a role in the FGT that may be important in effecting increased susceptibility to preterm birth.

Recently an alternative approach has been taken to measuring local immune activation in the FGT in women at high risk of HIV infection. Francis et al. explored protein expression profiles in 100 women at risk of HIV infection over one menstrual cycle within a microbicide feasibility study(Francis *et al.*, 2016). CVL samples were obtained at week 1 and week 4 and the relationship of immune proteins with total neutrophils from the CVL cell pellet examined. Positive linear correlations were identified between neutrophil counts and pro-inflammatory cytokines IL-1α, IL-1β, IL-6 and chemokines IL-8, GM-CSF and TGFβ. Detection of lymphocytes were dichotomized as present or absent in order to explore association with cytokines and presence of lymphocytes was found to associate with IL-1β, IL-6 and G-CSF. The cytokine signatures observed with different cells types are not specific but the fact that CVF IL-1β and IL-6 were up-regulated in the women in our study, alongside the finding of multiple neutrophil and macrophage associated proteins in the CVF of HIV-1 infected pregnant women substantiates our hypothesis that neutrophils and lymphocytes are up-regulated in the FGT of HIV-1 infected pregnant women.

9.2 Limitations and Strengths

A strength of this work is being able to compare cytokine concentrations in CVF collected with the soft cup with cytokine concentrations in whole blood. This method enables an "in vivo" snap shot of immune networks as any factors that usually influence the immune response will be adjusted for automatically as part of normal physiology.

Our small sample numbers have not enabled sufficient power to meaningfully explore ART exposure as a contributor to FGT inflammation nor elucidate significant associations between FGT inflammation and PTB in these HIV-1 infected pregnant women. Since designing the study in 2013, many changes in antiretroviral prescribing practices have occurred. Initially we had hoped to focus of women initiating cART comparing PI-based cART with non-PI-based cART, including where appropriate triple NRTIs (ZDV/ABC/3TC), in order to obtain pre and post ART samples. In reality the number of women that initiate cART in pregnancy has reduced significantly in recent years with most women conceiving on cART, some with their second child(Peters *et al.*, 2017). Those that do initiate in pregnancy may have been diagnosed through antenatal screening and have advanced disease and were excluded from the study on the basis of CD4 or were struggling with the new diagnosis and the stresses in their personal lives were not conducive to taking part in research. Thus the majority of women in the study conceived on treatment.

Since the start of the study there were several changes in the British HIV Association Treatment Guidelines so that cART was recommended for women at any CD4 count, that short term cART for PMTCT was no longer recommended and all women were advised not to stop cART after pregnancy, especially those with an intention to breastfeed, similar to guidelines from the World Health Organisation(World Health Organization, 2015b; Churchill *et al.*, 2016; Gilleece *et al.*, 2018). In the UK, first-line recommended cART regime for initiation in pregnant adults has broadened from 2012 when boosted PIs were the first line (often older PI: Lopinavir /ritonavir as it was historically the most prescribed third drug regimen in pregnancy), to inclusion of NNRTIs in the 2014 update and

then to named drugs, the newer PI: Atazanavir /ritonavir and NNRTI: Efavarinz, in 2018(Taylor *et al.*, 2012, 2013; Gilleece *et al.*, 2018). INSTIs: Raltegravir and the newer Dolutegravir are recommended alternatives alongside newer PI: Darunavir and NNRTI: Rilpivirine.

During the duration of this study fewer PIs were prescribed and increasingly women were receiving NNRTIs and INSTI based regimes. Whilst we have not ultimately captured the women we were aiming to target, we have captured immune data from women receiving newer ART drugs. Data on immune response is an important tool in assessing the safety of these newer regimes with regards inflammation and birth outcome. The HIV PTB study has recently reopened to phase 2 of recruitment where we will include women with any CD4 count to ensure a range is included but primarily to maximize the number of women we can recruit. This will enable us to overcome some of the power issues by adding to our current cohort but equally enable us to explore the immune effect of different antiretroviral prescribing practices as they evolve.

Another potential criticism of the work presented here is the definition of PTB used included both spontaneous and iatrogenic labour. Whilst one could argue that these are different phenotypes of the preterm birth syndrome, there may be common pathways to both. Whilst a previous study by Lopez et al. concluded that cART use was associated with iatrogenic and not spontaneous PTB(Lopez *et al.*, 2012), their most recent study found systemic evidence of microbial translocation in HIV infected pregnant women who went on to experience either iatrogenic or spontaneous PTB (Lopez *et al.*, 2016).

The mismatching of ethnicity between HIV-1 infected and uninfected pregnant women has been discussed in chapter 3.7 and chapter 6.5. To summarise, the imbalance of black ethnicity between groups potentially introduces racial bias which could contribute to the high rate of PTB observed in the HIV-1 infected group. Where possible ethnicity has been adjusted for in the statistical modelling presented in this body of work to limit any confounding introduced by this racial skew. The HIV PTB study is now focusing on recruiting more

ethnically matched uninfected pregnant controls to reduce this potential bias. The ideal study design to account for any PTB confounders such as ethnicity would be a case matched control study or an RCT. Case matching may be possible in future analysis if we increase study numbers particularly of black uninfected pregnant women. We also plan a similar analysis in CVF samples obtained from an RCT of two different non PI-based cART regimes in the third trimester of pregnancy (EFV or DTG) undertaken in South Africa and Uganda which will enable us to explore the effect of geographical location (Southern Africa, East Africa and the UK) and ethnicity on the vaginal microbiota and FGT inflammation (Dolphin2). Whilst this racial imbalance can be considered a limitation it is also strength. In spite being recruited in the UK many of the Black HIV-1 infected women in this study were born in Sub-Saharan Africa, which potentially widens the relevance of our results. UNAIDS estimated that in 2018 there were 1 million pregnant women living with HIV in east and southern Africa of which 93% received antiretroviral therapy (unaids.org, 2018). Further elucidation of the infectious triggers to PTB in HIV-1 infected women, the interaction of antiretroviral therapy, and its management are imperative to reduce its global impact

9.3 Future directions

Quantifying some of these novel immune and ECM protein candidates in this group of women already known to be associated with PTB such as MMP or MPO, and proteins not previously associated with PTB, including Chitinase 3-like 1 protein, is the next step for this work. Once novel protein targets were characterised in this cohort, we would measure such proteins in a future larger cohort (powered to look at PTB as primary outcome), exploring associations with ART, gestational age at delivery and prematurity. The aim would be to identify one or a selection of biomarkers to construct a clinically useful model that may predict which women are at greatest risk of PTB and in whom tailored management can then be pursued.

Another avenue for further work is further characterization of local immune cells and PBMCs. PBMCs were obtained as part of the methodology of this study and T regulatory cells could be formally characterised with more specific cell markers such as foxP3 and CD127. The menstrual soft cup collects cellular material from the FGT and the technique could be optimized for collection of cells such as T lymphocytes, monocytes and neutrophils for flow cytometry or other validated techniques such as cytobrushes could be used. This would enable further characterization of local immune networks and the influence of T regulatory cells in these women.

The antimicrobial action of antiretrovirals should be studied to determine the MIC of these compounds on important bacterial species such as *G. vaginalis* or the *Lactobacillus* species common to HIV-1 infected women and which are associated with transition to diverse anaerobic bacterial communities and PTB (*L. iners*) (Verstraelen *et al.*, 2009; Hummelen *et al.*, 2010).

9.4 Impact

Understanding the inflammatory impact of cART in pregnancy is an innovative approach to assessing cART toxicity, which can be taken forward in assessing potential of new regimens for use in pregnancy. Ultimately a tailored treatment approach may be key. Currently we do not recommend changing treatment during pregnancy if a women has already conceived on ART as it is important not to cause unnecessary potential adverse drug reactions or viral rebound. It would be possible however to advise on the least inflammatory cART regime in pregnant women initiating cART post-conception. In addition frequent genital sampling and treatment to ensure favourable vaginal microbiota may reduce vaginal bacterial diversity, bacterial vaginosis and PTB. Previous research has largely shown treatment of BV in pregnancy has not improved birth outcome but this was based on microscopy diagnostics and not in HIV-1 infected pregnant women(Brocklehurst *et al.*, 2013). Timing of antibiotic treatment of vaginal dysbiosis may be key. Hay et al. recommended that eradication treatment should commense no later than the start of the second trimester in order to prevent late

miscarriage and PTB(Hay, Morgan, *et al.*, 1994). It has been shown repeated referral for assessment and treatment of BV can encourage maintenance of a more healthy vaginal microbiota, and thus improve PTB risk in an HIV infected and uninfected population(Mehta *et al.*, 2015). There is increasing interest in vaginal probiotics in establishing healthy lactobacillus communities in the vagina, primarily to reduce risk of transmission but these approaches have been proven to be effective and safe (Happel *et al.*, 2018). The effect of probiotics on PTB risk is more controversial but there is some evidence to show that they may be beneficial in preventing early PTB<32 weeks(Othman, Neilson and Alfirevic, 2007; Yang *et al.*, 2015; Kirihara *et al.*, 2018).

Evidence for the optimum clinical tool to screen HIV-1 infected women and identify those at highest risk of PTB is lacking. Fetal fibronectin measurement at week 28 has shown to be predictive of PTB in HIV infected women (Goldenberg *et al.*, 2007). Price et al. found no correlation between FGT HIV viral concentration and cervical length in a study of HIV infected pregnant women from Lusaka and found that the proportion of women with a short cervix <25 mm was similar in pregnant women with or without HIV infection(Price *et al.*, 2019). Cervical cerclage and progesterone are both effective targeted management options for PTB(Iams, 2014). Improvements in screening and management of PTB in HIV-1 infected women are warranted. Progesterone has been shown to be acceptable intervention in HIV infected pregnant women and results are awaited regarding its efficacy(Siou *et al.*, 2016; Wong *et al.*, 2017). The trajectory of this work is exciting and combined with improved screening tools could make an important impact in reducing this complication.

9.5 Conclusion

In summary these data suggest that local activation of both innate immune cells and systemic activation of adaptive immune cells occurs in the context of genital inflammation, which may be enhanced in women with dysbiotic vaginal bacteria and in women with HIV-1 infection. The mechanism both through which this immune activation and inflammation increases the risk of PTB and how HIV and

it's treatment contribute to this process remains to be determined and this will be the focus of the next phase of my research.

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10. Appendix

Publications

specific combinations included AZT and 3TC alone (1), or in combination with raltegravir (3), nelfinavir (1), lopinavir/ritonavir (1) or NVP (1). None of the infants were infected.

Conclusion: The majority (80%) PHIV women in the CMIS cohort were resistant to all of the drugs currently recommended for neonatal prophylaxis for prevention of perinatal HIV transmission. While the risk of transmission of drug resistant virus is not clear, these results suggest that for infants of PHIV women at higher risk of transmission due detectable viral load, alternatives to the currently recommended regimens for newborns may be necessary.

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The vaginal microbiome of HIV–infected pregnant mothers: associations with local inflammation and gestational age at delivery

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Background: Bacterial vaginosis is a known risk factor for preterm birth. In spite the high rate of PTB experienced by HIV-infected mothers, few data are available about the vaginal microbiome of HIV-infected women during pregnancy. We sought to describe the microbiome in a group of HIV-infected pregnant women and explore associations with local cytokine environment and gestational age at delivery.

Materials and Methods: A prospective observational multi-site study. Vaginal and cervicovaginal fluid (CVF) were obtained using swabs and menstrual soft cups from HIV-infected and uninfected mothers (Exclusion criteria: <350 cells/mm³, multiple or in-vitro pregnancy and injecting drug user) at 3 time points (12-22, 23-26, 27-31 weeks). MiSeq sequencing of 16S rRNA gene amplicons was used to characterise the vaginal microbiome. Multiplex chemiluminescent assays were used to measure CVF cytokine concentrations. Multivariate modelling was performed to explore associations with bacterial genus/species, CVF cytokine concentrations and clinical data.

Results: HIV-infected mothers (n=53) had a median age of 35, 81% were of Black ethnicity and 14% had PTB. In comparison, HIV-uninfected mothers (n=30) had a median age of 33 and 50% were Caucasian. HIV-infected mothers delivering at term had higher abundance of Gardnerella (18% versus 3% p=0.003) and Prevotella genera (4% versus 0.1% p=0.002) and lower proportions of Lactobacillus species (70% versus 93% p=0.009) compared to uninfected mothers. The predominant vaginal community state type (CST) of HIV-infected pregnant women was III (L. iners dominant) 55% (n=23), 20% (16) were CST IV (high diversity, anaerobic), 13% (7) were CST I (L. crispatus dominant) and 2% (1) were CST II (L. gasseri dominant).

Amongst HIV-infected women, PTB was associated with increased proportions of anaerobes: Gardenerella, Peptoniphilus, Aerococcus, Gordinobacter (p<0.0001), Megasphaera (p=0.02) and Prevotella species (p=0.03) compared with term birth. Bi-directional switching of CST type during the second trimester between IV to III was associated with lower gestational age at delivery (p=0.007).

Matching CVF cytokine data were available for 79 of the 117 vaginal microbiome samples. Sequence read counts of CST IV associated anaerobes were positively correlated with higher concentrations of CVF proinflammatory cytokines: IFNγ (Gardnerella spp., Atopobium vaginae and Prevotella spp., p<0.02); IL-1β (Gardnerella spp., Atopobium vaginae, Prevotella spp. and Snaethia spp., p<0.0001); IL-8 (Atopobium vaginae and Prevotella spp., p<0.01) and TNFα (Gardnerella spp., Atopobium vaginae, Prevotella spp. and Snaethia spp., p<0.03). Within this small group, no associations between cytokine concentrations and gestational age at delivery were observed.

Conclusion: Presence of diverse bacterial communities within the vaginal microbiota in HIV-infected mothers during the second trimester is associated with PTB. The associated local proinflammatory cytokine profile may reflect the pathogenic contribution of these organisms to the early trigger of labour.

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Research paper

Optimising the collection of female genital tract fluid for cytokine analysis in pregnant women

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ABSTRACT

Introduction: To better understand the immunology of pregnancy, study of female genital tract fluid (FGF) is desirable. However the optimum method of collection of FGF in pregnant women for immunological methods, specifically cytokine measurement, is unknown.

Methods: A prospective study of HIV-uninfected pregnant women comparing two methods of FGF collection: polyvinyl acetal sponge collection of cervical fluid (CF) and menstrual cup collection of cervicovaginal fluid (CVF). Samples were collected at 3 time points across the second and third trimesters: 14–21, 22–25 and 26–31 weeks. Multiplex chemi-luminescent assays were used to measure: IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13 and TNF-α. Optimal methodology for cytokine normalisation (sample weight, volume and total protein) was explored.

Results: All cytokines were measurable in both fluid types. IL-1β, IL-8 and IL-6 were detected at the highest concentrations (ranking order CF > CVF > plasma). CVF collection was simpler, provided the largest volume of sample (median 0.5 g) with the potential for undiluted usage, and allowed for self-insertion. CF cytokine concentrations were intrinsically associated with sample weight and protein concentration however CVF cytokines were independent of these.

Conclusion: Both methods of collection are robust for measurement of FGF cytokines during pregnancy. We recommend CVF collection using a menstrual cup as a viable option in pregnant women for high dimensional biological techniques.

1. Introduction

The study of female genital tract fluid (FGF) to further understand the molecular aetiology of obstetric conditions (e.g. preterm birth) and sexually transmitted infections (e.g. Human Papilloma Virus (HPV) and Human Immunodeficiency Virus (HIV)) is an expanding field (Wei et al., 2010; Castle et al., 2004; Nguyen et al., 2005; Dezzutti et al., 2011; Archary et al., 2015). FGF comprises of cervical mucous/fluid (CF) and vaginal secretions or a combination of the two (cervicovaginal fluid (CVF)). An advantage of this fluid type is that, in addition to studying the local vaginal immune compartment, it can also provide information about the upstream uterine and cervical environment (Dasari et al., 2007; Zegels et al., 2010). Another benefit is that it is easily obtained without the need for invasive procedures. This fluid has been used for a multitude of assays including the characterization of cytokines and other immune mediators, biomarker discovery through high dimensional biological 'omic' techniques, microbiome studies and drug levels, with potential for many more (Castle et al., 2004; Snowhite et al., 2002; Walter et al., 2011; Romero et al., 2006; Goldenberg et al., 2005; Amabebe et al., 2016; Kindinger et al., 2016; Parsons et al., 2014; Pereira et al., 2007; Price et al., 2011). In the context of pregnancy, the study of the FGF cytokine milieu has enabled the identification of IL-1β, IL-6, IL-8 and TNF-α as correlates of preterm delivery (Wei et al., 2010; Goldenberg et al., 2005; Tanaka et al., 1998; Discacciati et al., 2011; Goepfert et al., 2001; Jun et al., 2000).

Several systems have been developed to collect female genital tract secretions including: cervicovaginal lavage (CVL), polyester swabs, cervical wicks and ophthalmic sponges. Obstetric studies have traditionally used lavage for obtaining CVF samples or polyester swabs for CF collection however this produces large volumes of dilute CVF and small volumes of CF respectively (Walter et al., 2011; Pereira et al., 2007; Tanaka et al., 1998; Goepfert et al., 2001; Jun et al., 2000). Ophthalmic polyvinyl acetal (PVA) sponges are highly absorbent, have low binding affinity for protein and superior performance in recovery of

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cytokines from CF compared to other techniques (Lieberman et al., 2008). A novel technique using menstrual soft cups has emerged which enables larger volumes of undiluted CVF to be collected without a speculum and with the option of self-sampling (Boskey et al., 2003). There has been increasing use of this technique in the HIV-prevention field with excellent performance in quantifying immune mediators including cytokines (Archary et al., 2015; Price et al., 2011; Shukair et al., 2013; Cosgrove et al., 2016). To date neither ophthalmic sponges nor menstrual cups have been used in obstetric studies. The aim of this study was to evaluate and compare these methods for use in pregnant women. In addition, there is no consensus method for normalisation of cytokine concentration with some studies presenting data adjusted for sample dilution based on weight and some controlling for total protein (Castle et al., 2004; Dezzutti et al., 2011; Snowhite et al., 2002; Lieberman et al., 2008; Rohan et al., 2000; Quesnel et al., 1997; Marks et al., 2012). Here we present both methods and evaluate the effectiveness for each technique.

2. Methods and materials

2.1. Study subjects

Pregnant women attending routine antenatal booking appointments were invited to participate in this prospective observational study at St Mary's Hospital, London. This study was approved by the South East Coast RES Committee (13/LO/0107: The immunological basis of preterm delivery). Written informed consent was obtained enabling clinical data analysis and sample collection. Exclusion criteria were: multiple or in-vitro fertilization pregnancy, injecting drug use, HIV infection, current *Chlamydia trachomatis* or *Neisseria gonorrhoeae* infection.

2.2. Sample collection

Women were asked to provide EDTA whole blood and undergo FGF sampling by two methods at the three sequential mid trimester time points: 14–21, 22–25 and 26–31 weeks gestation (this sampling frame corresponds with routine antenatal clinic visits and the schedule of the parent study (13/LO/0107). Firstly, a speculum examination was performed enabling direct visualization of the cervical os to obtain a CF sample using an opthalmic PVA sponge (Eyetec™, Network Medical Ltd.), held in place for 1 min, with a sterile surgical needle holder. The PVA sponge was then replaced in a pre-weighed and labelled 15 mL sterile conical tube. A plastic loop was then used to obtain a smear from the lateral vaginal wall for microscopic and pH examination (Litmus test paper strip). Dry mounted glass slides were gram stained to enable evaluation of leucocyte cell count per high power field (in five cell increments), presence of *candida* and identify *Bacterial Vaginosis* by Hay Ison's Criteria (Ison & Hay, 2002; BASHH, 2012). Where clinical history indicated, *Chlamydia trachomatis* and *Neisseria gonorrhoeae* testing was performed (Nucleic acid amplification (AMPLICOR, Roche)). Women then underwent self or clinician insertion of a menstrual cup (MC) (Instead™ SoftCup, Evofem Ltd) to obtain cervicovaginal fluid (CVF). Correct vaginal placement of MC results in a lack of appreciation of the devise in situ, which was confirmed for each woman. The MC was removed at the end of the women's clinical appointment (minimum 5 min, maximum 30 min) and placed in a pre-weighed and labelled 50 mL sterile conical tube.

2.3. Specimen processing

EDTA whole blood was separated into plasma and peripheral blood mononuclear cells by sucrose density centrifugation within 4 h of sampling. Post collection weights were obtained for both CF swab and CVF cup containing conical tube tubes which were then placed on ice and stored at −80 °C within 4 h until ready for extraction.

2.4. CF

CF was extracted from the PVA sponge as previously described (Castle et al., 2004). To summarise, the PVA sponge was thawed on ice then 300 µL of extraction buffer (500 µL 1× protease cocktail I (Calbiochem®) + 10µl 10% Sodium Azide solution +0.75 g NaCl, final volume made to 50mls with 1× PBS solution, filter sterilised) was added to the PVA sponge in a Spin-x centrifuge filter unit (Costar, MA). This was spun at 13,000 rpm for 15 min at 4 °C, to elute the cytokines into the lower chamber. This process was repeated with another 300 µL of extraction buffer solution. The final 600 µL of CF was stored in 1.5 mL eppendorfs and frozen at −80 °C until further testing.

The dilution factor was calculated using the following formula: [(x − y) + 0.6 g of extraction buffer] / (x − y)], where x equals the weight of the PVA sponge + conical tube post collection and y is the weight of the PVA sponge and conical tube pre collection. The density of extraction buffer = 1.005 g/mL (Rohan et al., 2000).

2.5. CVF

The MCs were thawed for a maximum of 30 min on ice and centrifuged for 15 min at 400 g and 4 °C to pool the CVF at the bottom of the conical tube. The CVF (cells and supernatant) was divided into 100 µL aliquots into 1.5 mL eppendorfs using a positive displacement pipette (Rainin c10–100, Mettler Toledo). CVF was diluted 1 in 2 with the addition of 100 µL of extraction buffer (Castle et al., 2004; Cosgrove et al., 2016). The MC was re-spun if any CVF remained on the cup or in the conical centrifuge tube. The CVF was stored at −80 °C until further testing.

2.6. Cytokine measurement

Multi-spot chemi-luminescent assays (V-plex, Meso Scale Discovery® (MSD)) were used to measure 10 cytokines in plasma, CF and CVF (IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13 and TNF-α) according to the manufacturer's instructions. The sensitivity of these assays range from 0.02–938 pg/mL and 25 µL sample volume is required per reaction. Eluted CF samples, CVF samples (1 in 2 dilution) and neat plasma were analysed. All samples were run in duplicate. Matched plasma, CF and CVF samples were run on the same plate to limit any inter-plate variability. Data from the plates were analysed using MSD DISCOVERY WORKBENCH® version 4 and cytokine concentrations (pg/mL) were calculated using plate specific standard curves for individual cytokines. Where sample cytokine concentrations did not fall within the standard curve they were re run after dilution.

2.7. Protein measurement

Total protein concentrations in CF and CVF were estimated using the Bicinchoninic acid (BCA) method (Thermo Scientific ™ Pierce™). Samples were diluted 1 in 50 in extraction buffer and run in duplicate with a total volume per well of 25 µL. SoftMax Pro® version 5 was used to generate standard curves from which the protein concentration of each sample was interpolated (µg/mL).

2.8. Statistical analysis

Categorical variables were described in numbers and % and continuous variables summarised with median and interquartile ranges (IQR) and compared with the Kruskal–Wallis test. Cytokine concentrations were normalised to both sample dilution and total protein (expressed as a ratio of pg cytokine/mg protein). The correlation of cytokine concentration with specimen weight pre and post normalisation and associations with potential known confounders was calculated with Spearman's correlation co-efficient. Bonferroni correction was made for multiple comparisons (0.05/10) therefore a p value <

Table 1
Participant characteristics and clinical data.

Characteristic	Value
Age (median years (IQR))	34.0 (30.0–34.0)
Ethnicity n(%)	
Caucasian	15 (83)
Black	0
Asian	2 (11)
Latin	1 (6)
Other	0
Relationship Status n(%)	
Married	14 (88)
Cohabiting	4 (22)
Single	0
BMI (median (IQR))	23 (20–24)
Smoking status n(%)	
Smoker	2 (22)
Non smoker	16 (88)
Parity (median (IQR))	0 (0–1)
Intercourse in preceding 24 h n	0
Practice of vaginal douching n	0
Vaginal pH	4.1 (3.8–4.6)
Bacterial Vaginosis	4/18 women in total[a]
current n	8/50 samples[b]
Candida	4/18 women in total
current n	5/50 samples
Preterm delivery n(%)	1 (5)

[a] These were different women to those in whom BV was identified.
[b] 50 corresponding vaginal microscopy samples were available.

0.005 was deemed significant. Analyses were performed using SPSS (version 24).

3. Results

3.1. Study subjects

Between October 2013 and July 2014 20 women were recruited. One woman declined speculum examination and another withdrew from the study due to relocation. 53 samples collected from the remaining 18 women were included in the analysis. For patient demographics, see Table 1. Women were predominately Caucasian, married, nulliparous and non-smokers with healthy range BMIs.

Genital tract fluid weights and dilutions are shown in Table 2. CVF is a more viscous secretion than CF but can be easily handled with a positive displacement pipette. Eight times the amount of secretions were collected by the MC compared to PVA sponges and final dilutions were less using the MC. CVF: median 0.5 g, when diluted 1 in 2 with 100 µL extraction buffer produces approximately 5 × 200 µL aliquots, total 1000 µL versus CF: median 0.07 g, gives a 1 in 10 dilution [(0.07 + 0.6 g)/0.07 = 10] in 600 µL of extraction buffer. The MC method of CVF collection has a more constant dilution factor (2.0–10.0) compared to a wider range of dilution factors (1.3–61.0) observed with the process of elution of CF from the PVA sponge.

Anecdotally participants reported that they found both sampling methods acceptable but expressed that it would be advantageous if speculum examination were avoidable.

Table 2
Median specimen weight and dilution factors of genital tract secretions by sampling method.

Secretion type/ method	Weight/g (IQR)	Range/g	Dilution factor (IQR)	Range of dilutions
CF by PVA sponge	0.07 (0.05–0.12)	0.01–0.54	9.6 (6.0–13.0)	1.3–61.0
CVF by MC	0.54 (0.35–0.82)	0.20–1.90	2.0 (2.0–4.0)	2.0–10.0

3.2. Collection method and sample volume

Genital tract fluid weights and dilutions are shown in Table 2. CVF is a more viscous secretion than CF but can be easily handled with a positive displacement pipette. Eight times the amount of secretions were collected by the MC compared to PVA sponges and final dilutions were less using the MC. CVF: median 0.5 g, when diluted 1 in 2 with 100 µL extraction buffer produces approximately 5 × 200 µL aliquots, total 1000 µL versus CF: median 0.07 g, gives a 1 in 10 dilution [(0.07 + 0.6 g)/0.07 = 10] in 600 µL of extraction buffer. The MC method of CVF collection has a more constant dilution factor (2.0–10.0) compared to a wider range of dilution factors (1.3–61.0) observed with the process of elution of CF from the PVA sponge.

Anecdotally participants reported that they found both sampling methods acceptable but expressed that it would be advantageous if speculum examination were avoidable.

3.3. Total protein

Total protein concentrations in undiluted CVF are higher than observed in CF (median CVF: 71731 µg/mL (IQR 56,489–109,967) versus CF: 19310 µg/mL (IQR 14,245–33,215), $p < 0.0001$).

3.4. Normalisation of cytokine concentrations

The effect of correcting female genital tract cytokine concentrations to sample dilution or normalising to total protein concentration is shown in Tables 3 and 4. CVF cytokine concentrations adjusted to either sample dilution or total protein were independent of sample weight

Table 3
Effect of normalisation method on the correlation of CVF cytokine concentration and specimen weight.

Cytokine	Normalisation method	Median (IQR)	Spearmans (ρ)	P value
Pro-inflammatory				
IL-1β	As measured (pg/mL)	58 (20–443)	−0.401	0.004
	Dilution (pg/mL)	116 (44–1441)	−0.365	0.008
	Protein (pg/mg)	2 (1–14)	−0.351	0.013
IL-6	As measured (pg/mL)	8 (3–40)	−0.348	0.012
	Dilution (pg/mL)	24 (8–119)	−0.325	0.02
	Protein (pg/mg)	0.3 (0.1–1.6)	−0.359	0.01
IL-8	As measured (pg/mL)	621 (141–3170)	−0.281	0.046
	Dilution (pg/mL)	2636 (281–17,248)	−0.252	0.075
	Protein (pg/mg)	202 (36–453)	−0.264	0.067
TNF-α	As measured (pg/mL)	0.6 (0.2–1.6)	−0.166	0.243
	Dilution (pg/mL)	2 (0–5)	−0.168	0.238
	Protein (pg/mg)	0.02 (0.006–0.07)	−0.179	0.212
Immuno-regulatory				
IFN-γ	As measured (pg/mL)	3 (1–6)	−0.293	0.037
	Dilution (pg/mL)	6 (3 − 20)	−0.241	0.089
	Protein (pg/mg)	0.1 (0.04–0.3)	−0.217	0.130
IL-2	As measured (pg/mL)	0.5 (0.3–0.9)	−0.258	0.068
	Dilution (pg/mL)	1 (0.6–3.7)	−0.198	0.164
	Protein (pg/mg)	0.02 (0.006–0.04)	−0.252	0.077
IL-4	As measured (pg/mL)	0.1 (0.06–0.4)	−0.272	0.053
	Dilution (pg/mL)	0.4 (0.1–1.1)	−0.250	0.077
	Protein (pg/mg)	0.005 (0.002–0.11)	−0.208	0.147
IL-10	As measured (pg/mL)	0.7 (0.3–1.7)	−0.119	0.407
	Dilution (pg/mL)	3 (1–6)	−0.098	0.496
	Protein (pg/mg)	0.02 (0.01–0.09)	−0.119	0.411
IL-12	As measured (pg/mL)	0.2 (0.1–0.7)	−0.048	0.739
	Dilution (pg/mL)	0.6 (0.2–3.3)	−0.016	0.909
	Protein (pg/mg)	0.007 (0.003–0.05)	−0.042	0.770
IL-13	As measured (pg/mL)	4 (2–7)	−0.210	0.136
	Dilution (pg/mL)	12 (5–35)	−0.137	0.336
	Protein (pg/mg)	0.1 (0.05–0.4)	−0.193	0.180

Table 4
Effect of normalisation method on the correlation of CF cytokine concentration and specimen weight.

Cytokine	Normalisation method	Median (IQR)	Spearmans (ρ)	P value
Pro-inflammatory				
IL-1β	As measured (pg/mL)	35 (13–87)	−0.098	0.493
	Dilution (pg/mL)	1352 (178–5036)	−0.397	0.004
	Protein (pg/mg)	18 (6–36)	−0.285	0.045
IL-6	As measured (pg/mL)	5 (2−20)	−0.145	0.311
	Dilution (pg/mL)	252 (55–1375)	−0.370	0.008
	Protein (pg/mg)	3 (1−13)	−0.246	0.085
IL-8	As measured (pg/mL)	789 (421–1909)	−0.130	0.384
	Dilution (pg/mL)	34,491 (6553–119,938)	−0.496	0.0001
	Protein (pg/mg)	325 (155–812)	−0.123	0.397
TNF-α	As measured (pg/mL)	0.4 (0.2–1.0)	−0.029	0.841
	Dilution (pg/mL)	14 (4–60)	−0.601	0.0001
	Protein (pg/mg)	0.1 (0.1–0.5)	−0.448	0.001
Immuno-regulatory				
IFN-γ	As measured (pg/mL)	2 (1–4)	−0.214	0.132
	Dilution (pg/mL)	48 (9–327)	−0.391	0.005
	Protein (pg/mg)	1 (1–2)	0.426	0.002
IL-2	As measured (pg/mL)	0.4 (0.2–0.7)	−0.167	0.241
	Dilution (pg/mL)	9 (3–42)	−0.528	0.0001
	Protein (pg/mg)	0.2 (0.0–0.5)	−0.385	0.006
IL-4	As measured (pg/mL)	0.1 (0.0–0.2)	−0.210	0.139
	Dilution (pg/mL)	2 (0–6)	−0.333	0.057
	Protein (pg/mg)	0.04 (0.01–0.1)	−0.375	0.007
IL-10	As measured (pg/mL)	0.6 (0.4–1.6)	−0.409	0.003
	Dilution (pg/mL)	29 (6–87)	−0.574	0.0001
	Protein (pg/mg)	0.3 (0.1–1.0)	0.558	0.0001
IL-12	As measured (pg/mL)	0.3 (0.2–0.6)	−0.202	0.155
	Dilution (pg/mL)	10 (1–43)	−0.302	0.031
	Protein (pg/mg)	0.1 (0.06–0.3)	−0.368	0.09
IL-13	As measured (pg/mL)	5 (3−11)	−0.331	0.0018
	Dilution (pg/mL)	168 (48–636)	−0.471	0.0001
	Protein (pg/mg)	2 (1–5)	−0.538	0.0001

with the exception of IL-1β and IL-6, however these associations with sample weight were removed by correction for multiple analyses. Conversely, CF cytokine concentrations were inversely associated with PVA sample weight after both dilution and total protein adjustment, see Table 4. From henceforth cytokine concentrations will be presented in pg/mL corrected for sample dilution.

3.5. Cytokine concentrations by biological fluid type

All measured cytokines were detectable in both genital fluid types and plasma, see Fig. 1. The cytokine profile in CF and CVF displayed very similar ranking with high concentrations of pro-inflammatory cytokines: IL-8, IL-1β, IL-6 and TNF-α. Of the immune regulatory cytokines IL-13 was observed in the highest concentration in both genital tract fluids. For all cytokines median concentrations were 7–17 fold higher in CF compared to CVF, $p < 0.0001$.

Plasma cytokine concentrations were generally lower than those observed in female genital tract fluid (most notably pro-inflammatory cytokines: IL-8, IL-1β, IL-6 and immune regulatory: IL-13), see Fig. 1. Similar concentrations were observed between plasma and CVF for TNF-α and IFN-γ.

A similar percentage of plasma samples contained detectable

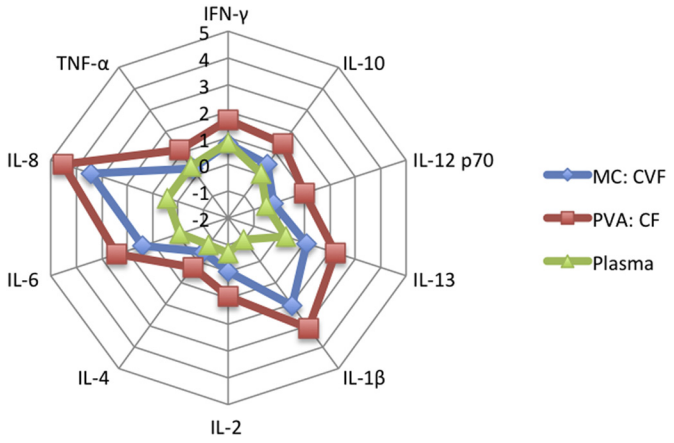

Fig. 1. Spider chart to demonstrate log 10 median cytokine concentrations/pg/mL by biological fluid type.

Table 5
Comparison of cytokine detectability in genital tract fluid and plasma.

Cytokine (pg/mL)	PVA: Cervical Fluid	MC: Cervicovaginal Fluid	Plasma
	% Detectable	% Detectable	% Detectable
Pro-inflammatory			
IL-1β	100	100	100
IL-6	98	100	96
IL-8	100	100	100
TNF-α	96	94	100
Immuno-regulatory			
IFN-γ	96	100	100
IL-2	100	98	94
IL-4	88	90	92
IL-10	100	98	100
IL-12	90	85	88
IL-13	96	88	83

cytokines compared to FGF samples, see Table 5.

3.6. Effect of vaginal pH and leucocytes on cytokine concentrations

Correlations between cytokine concentration and vaginal pH were explored in both biological fluids. In CF, higher pH was positively correlated with all measured cytokines: IFN-γ ($r = 0.414$, $p = 0.003$); IL-10 ($r = 0.387$, $p = 0.005$); IL-12 ($r = 0.360$, p −0.009); IL-13 ($r = 0.308$, $p = 0.028$); IL-1β ($r = 0.360$, $p = 0.009$); IL-2 ($r = 0.367$, $p = 0.008$); IL-4 ($r = 0.368$, $p = 0.008$); IL-6 ($r = 0.334$, $p = 0.017$); IL-8 ($r = 0.359$, $p = 0.013$) and TNF-α ($r = 0.329$, $p = 0.022$). In CVF, IFN-γ ($r = 0.329$, $p = 0.018$) and IL-1β ($r = 0.384$, $p = 0.005$) were positively correlated with pH. After Bonferroni correction only CF IFN-γ, IL-10 and CVF IL-1β remained significantly positively associated with pH.

Next correlations between total leucocyte count per high power field on vaginal microscopy and FGF cytokine concentration were analysed. In CF all measured cytokines, with the except of IL-2, were positively correlated with increasing leucocyte count per high power field: IFN-γ ($r = 0.358$, $p = 0.011$); IL-4 ($r = 0.382$, $p = 0.006$); IL-10 ($r = 0.331$, $p = 0.019$); IL-12 ($r = 0.373$, p −0.008); IL-13 ($r = 0.299$, $p = 0.035$); IL-1β ($r = 0.334$, $p = 0.009$); IL-6 ($r = 0.276$, $p = 0.05$); IL-8 ($r = 0.346$, $p = 0.019$) and TNF-α ($r = 0.302$, $p = 0.033$). In CVF only IL-8 was significantly correlated with leucocyte count ($r = 0.351$, $p = 0.012$). There was a trend towards a positive correlation between leucocyte count and IFN-γ ($r = 0.239$, $p = 0.094$) and IL-1β ($r = 0.266$, $p = 0.062$). These associations did not withstand Bonferroni correction.

3.7. BV associated with higher median CF IL-1β, IL-13 and IFN-γ

The presence of BV was associated with higher concentrations of CF IL-1β (4117 pg/mL (IQR 1383–5364) vs 799 pg/mL (126–4222) p = 0.05) and IL-13 (367 pg/mL (IQR 252–782) vs 134 pg/mL (IQR 35–393) p = 0.04) compared to women with no BV, not withstanding Bonferroni correction. There was a trend towards a higher median IFN-γ in the FGF of women with BV compared to those without: CF 269 pg/mL (IQR 41–646) vs 27 pg/mL (IQR 8–275) p = 0.056; CVF 13 pg/mL (IQR 5–33) vs 4 pg/mL (IQR 2–15) p = 0.092.

3.8. Vaginal candida associated with higher median CF and CVF TH^1 AND TH^2 cytokines

The presence of vaginal candida was associated with higher CF concentrations of IFN-γ (558 pg/mL (IQR 266–83,308)) compared to women without candida (45 pg/mL (IQR 9–269)), p = 0.05, IL-2 (295 pg/mL (IQR 46–1682) vs 8 pg/mL (IQR 2–23)), p = 0.027, IL-12 (86 pg/mL (IQR 26–1420) vs 9 pg/mL (IQR 2–30)), p = 0.039 and TNF-α (122 pg/mL (IQR 36–2202) vs 13 pg/mL (IQR 4–43)), p = 0.05. A similar trend was seen for IL-13 (863 pg/mL (IQR 323–5328) vs 158 pg/mL (IQR 48–382)), p = 0.059 and IL-8 (295,306 pg/mL (IQR 58056–1,208,132) vs 29,999 pg/mL (IQR: 6617–70,560)), p = 0.077. These associations did not withstand Bonferroni correction.

In CVF, the presence of candida was associated with higher median cytokine concentrations of IL-12 (13 pg/mL (IQR 2–30)) compared to women with no candida (1 pg/mL (IQR 0–3)), p = 0.027. This trend was also observed between the presence of candida and IFN-γ (354 pg/mL (IQR 8–1674) vs 6 pg/mL (IQR 3–17)), p = 0.081, IL-1β (6322 pg/mL (IQR 515–11,086) vs 109 pg/mL (IQR 45–1055)), p = 0.081 and IL-8 (34,415 pg/mL (IQR 8968–36,420) vs 1870 pg/mL (IQR 296–8788)), p = 0.075. These associations did not withstand Bonferroni correction.

No significant differences in cytokine concentrations between second and third trimester time points were observed by either collection method, data not shown.

4. Discussion

In this study we found both PVA sponges and the menstrual cup to be valid methods of collection for the measurement of FGF cytokines with all the multiplex cytokines being measureable in each fluid. The highest concentrations of TH^1 cytokines: IL-1β, IL-6, IL-8 and TNF-α observed in both fluid types are similar in profile to the literature for FGF from pregnant women (Kutteh & Franklin, 2001; Simhan et al., 2005; Nenadic & Pavlovic, 2008; Ryckman et al., 2009; Beigi et al., 2007).

CVF sampling by MC enables collection of high fluid volumes suitable for studies demanding multiple assays, with the opportunity to collect undiluted samples, making it useful for eliminating concerns about low-level contaminants/confounders that could be added during sample preparation. The option of self-sampling is also attractive and offers the potential for multiple sampling over pregnancy without the use of a speculum. Pregnant women in this study achieved comparable sample weights (median 0.5 g) to studies in non-pregnant women despite relatively short retention of the MC (Boskey et al., 2003). These figures add to the paucity of data on the optimal time for MC retention with one study in non-pregnant HIV-infected women demonstrating a median sample weight of 0.31 g with a minimum retention time of 60 min and another, in uninfected non-pregnant women, achieving a median sample weight of 0.5 g with a retention time of 5 s (Boskey et al., 2003; Jaumdally et al., 2017).

Conversely CF sampling by PVA swabs demonstrated the highest concentrations of cytokines. This reflects the fact that the cervix/fetal placental unit is the likely source of many of the cytokine producing cells and vaginal secretions dilute measurements in the CVF. However CF volumes are small and necessitate elution from the PVA sponge with extraction buffer introducing potential for confounding errors. Wider ranges of cytokine measurements were observed in the CF, which is likely to be the product of the variability in weight dependent dilution factors. Normalisation of CF cytokine concentrations to protein concentrations does not remove this weight dependent effect however CVF cytokine concentrations were largely independent of weight and protein concentration. The precision of volume dilution with CVF and reduced susceptibility to sample weight bias make this method advantageous.

The elevated concentrations of FGF pro-inflammatory cytokines: IFNγ and IL-12 observed in the presence of vaginal candidiasis mirror current knowledge of a IFN-γ CD4 TH^1 adaptive and phagocytic candidacidal response induced by antigen presenting cells under the control of IL-12 and is in keeping with this inflammatory vaginitis (Romani, 1999; Yano et al., 2012). These data demonstrate the potential use of FGF cytokine measurement in detecting biological plausible mechanisms.

The observed association between FGF IL-1β and bacterial vaginosis has been described in the literature (Beigi et al., 2007; Sturm-Ramirez et al., 2000). Bacterial vaginosis is not believed to be an inflammatory condition yet is a known risk factor for preterm delivery. Whilst it is clear from these data that it does not appear to stimulate the same level of pro-inflammatory response in the genital tract of these pregnant women compared to candida, this shift in normal vaginal flora may generate some inflammation.

In addition to being the first to demonstrate the utility of these FGF collection methods in pregnant women, other strengths of this study include the evaluation of different normalisation methods (weight (based on accurate individual sample pre and post collection measurements) and protein concentration) and correlates of cytokines concentration with vaginal pH, leucocyte counts, bacterial vaginosis or candidiasis. This work also explored the changes in cytokine concentrations between the second and third trimester in the same individual.

Limitations of this work include the potential confounding in order of sampling in that collection of CF sample with the PVA sponge first may have impacted on subsequent collection of CVF with the MC, potentially reducing the measurable cytokine concentrations in the subsequent sample. In addition, not all women were tested for GC/CT testing with the potential for undiagnosed genital infection increasing cytokine concentrations however if this were the case, this would be observed across both methods. Some women preferred provider insertion of the MC and the data was not available to compare recovery between insertion techniques. The diagnoses of BV and candida were based on microscopy not culture and the strength of these correlates may be underestimated by the limits of the diagnostic criteria sensitivity. Although comparable to other work in this field, the relatively small sample size may have resulted in loss of statistically significant associations with clinical correlates through correction for multiple comparisons.

5. Conclusion

In summary both methods presented are acceptable and robust in pregnant women. The short collection time, self-insertion and large volumes of CVF collected by the menstrual cup are advantageous, however the choice of method also depends on the immune compartment of interest.

Conflicts of interest

None.

Sources

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booking (≥13 GW) are risk factors associated with SB.

Results: There were 10,316 singleton pregnancies delivered at ≥24GW in 8069 mothers; 75.4% of mothers were born in Sub-Saharan Africa (SSA); 49.4% pregnancies (4915) were conceived on ART. The most common antenatal ART regimens were PI/r-(5454, 55.0%) and NNRTI-based (2427, 24.5%); specific regimen was unknown in 4.6% pregnancies. MTCT was reported in 43 (0.4%) cases. There were 89 (0.9%) SB. Compared to live births (LB) SB were more likely to be male (39/67 [58.2%] vs 5164/10290 [50.2%]), delivered pre-term (median 33 [IQR 27-37] GW vs 39 [IQR 38-40] GW) and to be SGA (34/62 [54.8%] % vs 2208/9982 [21.1%]). Among the 61/89(68.5%) SB and 10000/10316 (96.9%) LB with data on congenital abnormalities, 9/61 (14.8%) and 285/10000 (2.9%) had congenital abnormalities respectively. Multivariate analysis suggested significant risk factors associated with SB were antenatal CD4 count ≤350cells/μL (IRR 1.73, 95%CI 1.05, 2.86), mother being primiparous (IRR 1.85, 95%CI 1.10, 3.12), older (IRR for age>36 years vs age<28 years: 4.12, 95%CI 1.49, 11.35) and originating from SSA (IRR for SSA vs Europe/Western Countries: 3.26, 95%CI 1.07, 9.95) or other world region (IRR for Other vs Europe/Western Countries: 5.59, 95%CI 1.46, 21.48). Type of antenatal ART regimen, conceiving on ART, delivery year, IDU history and late antenatal booking were not significantly associated with SB.

Conclusions: Despite continued declines in MTCT rates over this period (2007-2015) and increases in the proportion of women conceiving on ART and delivering with suppressed viral load, the SB rate (0.9%) did not decline and remained consistently higher in HIV-positive women than in the general population (0.5% in England/Wales for the same period). Limitations included lack of data on some important risk factors for SB (e.g. maternal BMI, socio-economic status, smoking), missing data for some SB infants and limited ability to classify SB as antepartum or intrapartum. To further understand the circumstances and risk factors for SB in HIV-infected women, the NSHPC plans to undertake an audit of pregnancies ending in SB (following established methodology used in an ongoing audit of cases in which MTCT occurred).

Abstract 80

The Immunological basis of preterm delivery in HIV infection: an exploration of systemic immune activation

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Background: We hypothesise that elevated rates of preterm delivery (PTD) observed in HIV-infected pregnant mothers, are likely to be driven by changes in immunological tolerance of the feta-placental unit, reflected by high levels of systemic immune activation. This study sought to characterise immunological profiles of HIV uninfected and infected mothers throughout gestation, compare by timing and class of combination antiretroviral therapy (cART) and to explore correlations with gestational age at delivery.

Materials & Methods: A multi-site, prospective, observational study of HIV-infected and uninfected mothers. Exclusion criteria: CD4 count <350cells/mm3, multiple or in-vitro fertilisation pregnancy, injecting drug use. PBMCs were isolated at 5 time points (14-21, 22-25, 26-31, 32-37, ≥38 weeks). Flow cytometry was performed for T-cell surface markers: CD4, CD8, HLA-DR and CD25. Median (IQR) were calculated for each lymphocyte sub-population and compared by group using Mann-Whitney U test. Spearman's correlation coefficient was used to evaluate correlations with gestational age at delivery.

Results: Since November 2013 31 HIV uninfected and 63 HIV-1 infected pregnant women were recruited. Of the latter, 23 received Protease Inhibitor (PI)-based cART, 5 of whom initiated cART in the second trimester and 40 received non-PI cART, 7 of who initiated cART in the second trimester (3ABC/3TC/ZDV, 2RAL/FTC/TDF, 2DOL/ ABC/3TC).

Preterm Delivery. Eight HIV-infected women (13%) and two uninfected (7%) delivered <37weeks. Of the HIV-infected mothers: four had preterm prelabour rupture of membranes

(PPROM) and four were induced two for intra-uterine grouth restriction (IUGR) and two for fetal distress. One uninfected woman had PPROM and the second was induced for IUGR.

T-cell activation. Median baseline CD4+ cell count, cells/mm3 (%), was lower in HIV-1 infected pregnant women than uninfected pregnant women 611 (IQR 495-710) (36%) v 941 (IQR 863-1147) (50%), p<0.0001. There was no difference in baseline CD8+(%) cell count between infected and uninfected women (669 (IQR 525-871) (40%) v 603 (IQR 440-760)(30%), p=0.16). Median CD4/CD8 ratio was lower across gestation in HIV-1 infected mothers compared to uninfected (1.0 (IQR 0.9-1.2) v 1.8 (IQR 1.4-2.2), p<0.0001). Median baseline CD8+HLADR+ was 26% (IQR 16-35) in HIV-1 infected women compared to 14% (IQR 8-20), p=0.001 in uninfected women. Activated CD8+ cells (%HLA-DR+) were ≥1.5 fold higher in HIV-infected women across gestation, p<0.01.

Mothers who initiated cART post-conception had lower CD4/CD8 ratios throughout pregnancy 0.52 (IQR 0.44-0.99) compared with those who conceived on cART 1.04 (IQR 0.76-1.22),p<0.0001 and higher frequency of activated CD8 cells (HLADR+) 34% (IQR 25-44) v 24% (IQR 15-33), p<0.0001. No significant differences were observed by PI-exposure pre and post-conception. CD8+ activation was higher in women conceiving on PI-cART compared to non PI-cART (27% (IQR 19-33) v 21% (IQR13-33), p=0.036).

Gestational age at delivery correlated positively with CD4/CD8 ratio (r=0.235, p<0.0001) and inversely with total CD8 (r=-0.195, p=0.001), CD8% (r=-0.261, p<0.0001 and %CD8+HLADR+ Tcells (r=-0.141,p=0.02).

Conclusions: High levels of systemic T-cell activation are observed in HIV-infected mothers especially untreated, persist despite pre-conception cART, particularly in those conceiving on PI-cART. The correlation with gestational age at delivery suggests underlying immune dysregulation contributes to excess PTD observed with HIV infection.

Abstract 81

Adverse pregnancy outcomes among HIV-positive pregnant women receiving antiretroviral therapy in Kenya: early results from a cluster randomized behavioral intervention study.

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Introduction: The scale up of life-long antiretroviral treatment (ART) for all pregnant and breastfeeding women living with HIV (WLWH) has the potential to facilitate elimination of mother-to-child transmission (MTCT) and improve the health of WLWH. However, there are growing concerns about adverse pregnancy outcomes (APO) for women on ART.

Methods: A total of 537 pregnant WLWH enrolled in the Mother-Infant Visit Adherence and Treatment Engagement (MOTIVATE) study in three high HIV prevalence counties in southwestern Kenya between January 2015 to March 2017 were included. Baseline perinatal mortality in the region is 28 per 1000 births. This study is a cluster-randomized trial with a 2X2 factorial design testing the impact of community mentors, text messages, both interventions, or standard of care on retention in care and antiretroviral treatment (ART) adherence among HIV-positive pregnant/postpartum women. Per Kenya national guidelines viral load of <1000 copies/ml is considered virally suppressed. Women with an adverse pregnancy outcome (miscarriage, stillbirth, neonatal death, infant death, preterm delivery, or low birth weight) by March 31, 2017 were compared with women with live birth at least 30 days postpartum without adverse outcomes using univariate and multivariate analysis. Logistic regression analysis was conducted accounting for

EXPERT REVIEWS

Antiretroviral therapy and preterm birth in HIV-infected women

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The use of combination antiretroviral therapy for the prevention of mother to child transmission of HIV infection has achieved vertical HIV transmission rates of <1%. The use of these drugs is not without risk to the mother and infant. Pregnant women with HIV-infection are at high risk of preterm birth (PTB <37 weeks), with 2–4-fold the risk of uninfected women. There is accumulating evidence that certain combinations are associated with higher rates of PTB that others or no antiretroviral treatment. Understanding the pathogenesis of PTB in this group of women will be essential to target preventative strategies in the face of increasing HIV prevalence and rapidly expanding mother-to-child-transmission prevention programmes.

KEYWORDS: antiretroviral therapy • HIV-infection • lopinavir • preterm birth • prevention • protease inhibitors • risk factors • ritonavir • zidovudine

The use of antiretroviral therapy in pregnancy

The ability to prevent mother-to-child transmission (MTCT) of HIV infection can be counted among medicine's greatest successes. The use of combination antiretroviral therapy (cART) during pregnancy reduces HIV MTCT from 25–30% [1,2] to <1% in the both resource poor and rich settings [3,4]. Since the widespread introduction of cART (3 or more drug classes, cART) in 1996, dramatic improvements have been observed in morbidity and mortality for people with living with HIV infection. An adult who is diagnosed early and who initiates cART according to current guidelines can anticipate near normal life expectancy [5].

To date, the nucleoside analog reverse transcriptase inhibitor (NRTI) zidovudine (ZDV) remains the only antiretroviral therapy (ART) licensed for use in pregnancy since its efficacy for prevention of MTCT (8%) was demonstrated in the AIDS Clinical Trial Group 076 study compared with women who received no therapy (25%) [1]. The benefit of prelabor cesarian section (PLCS) in addition to ZDV was shown in the European Mode of Delivery study where women who were randomized to PLCS with ZDV achieved rates of MTCT of 0.8% compared with women receiving ZDV who had a normal vaginal delivery (4.3%) [6]. With the roll out of cART in 1996, increasing numbers of women have conceived on and initiated these regimens for maternal health during pregnancy. Observational data have demonstrated that cART is highly efficacious in the prevention of MTCT. From 2000 to 2006, UK National Surveillance data have shown that there were only three transmissions in the 2202 women (0.1%) who received cART and achieved full viral suppression (plasma HIV concentration <50 copies/μl), and there were no transmissions in women who received ZDV plus PLCS [3]. In women with fully suppressed plasma HIV, rates of MTCT were similar in women who delivered by PLCS or normal vaginal delivery (0.7%) [3]. The widespread use of cART in pregnancy, while unlicensed, has made it possible for these women to choose their preferred mode of delivery, and more recently, in resource-limited settings, the option of breastfeeding while taking cART [7]. Current international and national guidelines for the management of HIV infection in pregnancy are summarized in TABLE 1. Antiretroviral regimen options depend on whether the mother needs cART for her own health (CD4+ cell count <350 cells/l) or solely requires treatment to prevent HIV MTCT. In USA and UK guidelines, ZDV monotherapy with a PLCS remains an

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Table 1. Current international and national guidelines for the management of HIV infection in pregnancy.

Guideline	Conceived on cART	When to start for health	What to start	When to start for PMTCT alone	What to start	To continue?	Mode of delivery	Infant feeding	Infant PEP
WHO 2013	Continue	<500 cells/μl	First line: NNRTI-based cART	>500 cells/μl	First line: NNRTI based	Option B. Cessation 1 week post breastfeeding cessation	NVD	6 months exclusive Breast feeding	NVP od 6 weeks
	Avoid d4T		Ideal: EFV + TDF+ 3TC/FTC		Ideal: EFV + TDF + 3TC/FTC	Option B+ Lifelong		or Formula fed	If formula fed can have ZDV bd 6 weeks
			Second line: PI based		Second line: PI based				
			Ideal: LPV/r or ATV/r		Ideal: LPV/r or ATV/r				
EACS 2013	Continue Switch from EFV if before 8 weeks	<350 cells/μl	PI-based cART: LPV/r or SQV/r or ATV/r	>350 cells/μl	PI-based cART: LPV/r or SQV/r or ATV/r	Not stated	NVD unless detectable VL 34–36 weeks	Not stated	Not stated
BHIVA 2012	Continue	<350 cells/μl	PI-based cART: LPV/r or ATV/r	>350 cells/μl	PI-based cART: LPV/r or ATV/r	Consider if CD4 350–500 cells/μl	NVD unless VL >50 copies/ml at 36 weeks	Formula feeding	ZDV bd 4 weeks if maternal VL <50 copies/ml
	Avoid d4T/ddI		or triple NRTI cART ((ZDV/3TC/ABC) VL <100,000) or ZDV mono (VL<10,000)		or NNRTI based cART: NVP or EFV + TDF/FTC or ZDV/3TC or ABC/3TC	Stop post partum if CD4 >500 cells/μl	or ZDV mono		cART for 4 weeks if maternal VL >50 copies/ml
NIH 2012	Continue	<500 cells/μl	PI-based cART: LPV/r or ATV/r	>500 cells/μl	PI-based cART: LPV/r or ATV/r	Consider if CD4 >500 cells/μl	NVD unless VL >1000 copies/ml near delivery	Formula feeding	ZDV bd 6 weeks
	Avoid d4T/ddI		or NNRTI-based cART: NVP or EFV (avoid EFV in first trimester) + ZDV/3TC		or NNRTI-based cART: NVP or EFV (avoid EFV in first trimester) + ZDV/3TC or ZDV mono (VL <1000)				If no maternal cART give ZDV+NVP

3TC: Lamivudine; ABC: Abacavir; ATV/r: Atazanavir/ritonavir; BHIVA: British HIV Association; cART: Combination antiretroviral therapy; d4T: Stavudine; ddI: Didanosine; EACS: European AIDS Clinical Society; EFV: Efavirenz; FTC: Emtricitabine; LPV/r: Lopinavir/ritonavir; mono: Monotherapy; NIH: National Institutes of Health; NNRTI: Nonnucleoside reverse transcriptase inhibitor; NVD: Normal vaginal delivery; NVP: Nevirapine; PEP: Post exposure prophylaxis; PI: Protease inhibitor; PMTCT: Prevention of mother-to-child transmission; SQV/r: Saquinavir/ritonavir; TDF: Tenofovir; VL: Viral load; WHO: World Health Organization; ZDV: Zidovudine.

option for women with high CD4+ cell counts (>350 cells/μl) who can stop therapy after delivery. In the full 2010 WHO guidelines on antiretroviral drugs for treating pregnant women, this regime is a recommended option (Option A) for the prevention of HIV infection in infants [8]. Although Option A was not included in the most recent WHO guideline update on antiretroviral drugs for treating and preventing HIV infection, it is still used in many countries [7].

Despite this efficacy, antiretroviral therapies are not without potential risk to mothers and infants. Reported side effects therapy are class dependent: The NRTIs have been associated with anemia [9,10], lactic acidosis [11] and mitochondrial dysfunction [12]; Stevens–Johnson syndrome and hepatotoxicity have been described with the non-nucleoside reverse transcriptase inhibitors, particularly nevirapine [9,13]; reported adverse effects associated with protease inhibitors include gestational diabetes [14–17], hypertriglyceridemia [18] and adverse obstetric outcomes such as preeclampsia [19,20], intrauterine death [20,21], low birthweight [16,22–24] and preterm birth (PTB) (gestational age <37 weeks) [22,25–33]. *In utero* exposure to NRTIs has been associated in infants with anemia [34], neutropenia [35] and mitochondrial dysfunction [36], while more recently, treatment with the protease inhibitors lopinavir/ritonavir has been linked with neonatal adrenal suppression [37]. The focus of this perspective will be a review of the evidence for a role of ART in HIV-associated PTB.

The importance of preterm birth

Worldwide the incidence of PTB varies from 6% of all births in Europe, 11% in the USA and 9–18% in African countries [38]. It is the leading cause of neonatal mortality accounting directly for approximately 1 million or 27% of neonatal deaths annually [38]. The consequences of PTB are a spectrum that depends not only on gestational age at delivery but also the available neonatal support facilities.

In resource-rich settings, very preterm infants, born before 32 weeks gestation, have increased rates of mortality and cerebral palsy [39,40]. In the same setting, moderate-to-late preterm infants, born between 32 and 36 weeks gestation, have comparable survival rates to infants born after 37 weeks gestation. However, these infants have increased risk of recurrent hospital admissions, respiratory problems, later behavioral problems and having an IQ < 85 [41,42].

In resource-poor settings, where 85% of PTBs occur, neonatal mortality rates are high for all preterm infants [38]. A recent meta-analysis of East African data estimates rates of neonatal mortality for very and moderate preterm infants (24–34 weeks) to be 47% [43].

HIV-associated preterm birth

HIV infection, *per se*, is a risk factor for PTB. Rates of PTB in HIV-infected women, in the absence of HIV treatment (15–18%), are higher than in uninfected women (6–9%) [31,44,45] and appear to be linked to the degree of immunosuppression. Women with lower CD4+ cell counts and more advance clinical disease (CDC stage C) are at greater risk of PTB [21,27,30,32,46–48]. Detectable HIV-1 viremia is also correlated with increased risk [49–51]. These associations could theoretically lead to antiretroviral treatment indication bias that needs to be considered when interpreting the results of observational studies exploring associations of PTB.

HIV-infected women also have a high prevalence of established risk factors for PTB, which may be intermediate factors in the association with HIV/ART. Within this population, the following risk factors have been shown to increase risk of PTB: increasing maternal age [46,52–54]; black ethnicity [10,27,55]; single relationship status [56]; smoking [57–59]; alcohol use during pregnancy [60]; illicit drugs, for example cocaine, opiates and marijuana [27,54,58,61–63]; past history of PTB [24,51,60]; hypertension and pre-eclampsia [10,19,24,56,59]; anemia [50,59] and low maternal weight gain [21,55,64].

Ascending genital tract infections and chorioamnionitis are risk factors for PTB [65,66]. Chorioamnionitis is common in HIV-infected women and can be more severe compared with HIV-uninfected women [48,66–68]. Coinfection with Hepatitis C virus, Herpes Simplex virus, Cytomegalovirus and Malaria have also been associated with increased risk of PTB in HIV-infected women [26,60,69].

The role of antiretroviral therapy

PTB rates in HIV-infected women have not declined since the advent of effective ART. In 1998, a group in Switzerland published data reporting a PTB rate of 33% in women receiving cART [9]. This led to several other HIV cohort studies publishing data on prematurity rates in association with ART with a range of different results.

European data

Observational data from European studies such as the European Collaborative Study (ECS data from nine countries) [70], national surveillance registers from the UK [47] and Italy [26] and cohort studies from Germany, Austria [25] and Spain [71] have reported increased rates of PTB in association with cART. The risk of PTB in women who received cART was between two and fivefold greater than the rates observed in those who took ZDV monotherapy, in whom rates were similar to the general population [25,47,72,73], and twice the rates seen with no treatment [9,31,44]. Two other cohort studies from the Netherlands [45] and Spain [74] have demonstrated a twofold risk with cART compared with no ART; however, these results did not reach statistical significance in multivariate analysis. In a study from Poland that did not find an association of cART with PTB, the overall PTB rate was high (17%) [75].

North American data

Contemporaneously, observational data from North America, where background rates of PTB are higher, concluded that there were no association between cART and PTB. Results from the Women and Infants Transmission study (WITS, 1990–1998) indicated that there was no increased risk of PTB

comparing cART use with no therapy [76]. Subsequent results from this cohort, which included data up to 2002, concluded that ART use was associated with improved obstetric outcomes. This paper did, however, demonstrate an eightfold risk of PTB in women receiving cART without ZDV late in pregnancy (after 32 weeks gestation), whereas there was a protective effect of ART that included ZDV (ZDV monotherapy or cART) [10].

Later, the American Pediatric Spectrum of Disease Study (cohort data from eight sites) reported an increased risk of PTB with PI-based cART compared with Dual NRTI (adjusted odds ratio [aOR]: 1.21; 95% CI: 1.04–1.40) [27]. More recently, the Surveillance Monitoring for ART Toxicities (SMARTT) study also showed women initiating PI-based cART in the first trimester were at greater risk compared with those taking no ART (aOR: 1.55; 95% CI: 1.16–2.07) [77].

Low & middle-income country data

There is increasing high-quality data from low- to middle-income countries where the burden of disease is greatest. These observational cohort studies and randomized control trials have again used a variety of comparator groups.

The National International Site Development Initiative Perinatal study comprising data from Argentina, Brazil and Mexico and the Caribbean compared cART with NRTI only therapy (either ZDV monotherapy or dual NRTI) and showed no increased risk of PTB with cART [78]. Contrary to these results, a subsequent cohort study from Brazil showed a higher rate of PTB in women receiving cART (13%) compared with dual NRTI (9%) [79].

The largest matched cohort study to address the comparison of cART with ZDV monotherapy is from Botswana and has demonstrated a 1.4-fold increased risk of PTB with new cART versus ZDV monotherapy [59]. Two further African cohort studies comparing non-PI cART with no ART have shown different results. One study from South Africa concluded non-PI cART increased PTB risk sixfold [32], whereas the Drug Resource Enhancement against AIDS and Malnutrition study from Malawi and Mozambique concluded that non PI-based cART is protective [80].

Limited data are available for Asian countries. An Indian study has shown a threefold increased risk of PTB with cART compared with no therapy [21], while in China, a trend toward higher PTB rates for women requiring treatment for their own health with non-PI cART of 12% compared with treatment for prevention of mother-to-child transmission (PMTCT) with ZDV monotherapy (4%) has been reported [63].

Why do these results differ?

The discrepancies in the conclusions of these studies led to efforts to combine the data and to further examine the differences in these studies. A meta-analysis of observational studies from Europe, North and South America published in and before 2004 (13 prospective cohorts and 1 retrospective study) concluded that there was no elevated risk of PTB with cART or PI-based cART compared with no therapy [29]. Interestingly, it did show a moderate increase (aOR: 1.35; 95% CI: 1.08–1.70) in PTB with PI-based cART compared with non-PI cART. A pooled analysis of the ECS and WITS cohort data up to 2006 was undertaken which revealed that the rates of PTB from European and North American studies were indeed different with a combined analysis indicating an association with cART compared with Dual NRTI (aOR: 1.49; 95% CI: 1.19–1.87) [81]. Both analyses proposed the reasons behind the disparate conclusions to be: heterogeneous populations with different background rates of PTB; varying study designs in adjustment for confounders such as race, age, smoking, illicit drug use, alcohol, HIV factors (degree of immunosuppression, indication for treatment), coinfections as well as choice of comparison groups [29,81].

Randomized control trial data

In recent years, the first data from randomized control trials have been published, both of which were performed in Africa. In each study, HIV-infected women who did not need treatment for their own health (CD4+ count >200 cells/μl) and were randomized to either PI-based cART (Lopinavir/ritonavir with ZDV/lamivudine) or NRTI only therapy (ZDV monotherapy) plus single-dose Nevirapine (Kesho Bora study)/ triple NRTI (Mma Bana study) [22,82]. The primary outcome was prevention of MTCT of HIV infection during the antenatal and postnatal period (which included up to 6 months of breastfeeding). PTB was evaluated as a secondary outcome. These data should be less susceptible to bias; however, in spite of this both studies have reached different conclusions.

In the Mma Bana study, treatment was started from 26 weeks gestation. PI-based cART was associated with a twofold risk of PTB with no excess risk of MTCT of HIV (0% with PI-based cART vs 2% with triple NRTI) [4]. Initially, the Kesho Bora study protocol started treatment from 34 to 36 weeks; however, halfway through the trial treatment initiation changed from 34 to 28 weeks gestation. This trial showed no increased PTB risk with PI-based cART and a higher rate of HIV transmission with ZDV monotherapy (6 vs 11%, p = 0.02). One can argue, however, that if treatment was initiated later, there was little time for an effect to be observed on PTB risk.

Class effect

Protease inhibitors

Several investigators have hypothesized that a specific class of drugs, the protease inhibitors, drives the association between ART and PTB. Interestingly, it was one of the WITS centers from North America that was the first to highlight this association, with an elevated risk with PI-based cART compared with non-PI cART (aOR: 1.8; 95% CI: 1.1–3.0) [28]. As stated previously, the American meta-analysis, despite concluding no overall association between cART and PTB, showed an increased risk of PTB with PI-based versus non-PI cART (aOR 1.4; 95% CI: 1.1–1.7) [10]. The American Pediatric Spectrum of Disease and SMARTT studies have also demonstrated a

moderate increased risk comparing PI-based cART to Dual NRTI and no ART (aOR 1.2 & aOR 1.6) [27,77].

However, two American studies, one single centre from New Orleans and results from the larger international Maternal Pediatric Adolescent AIDS Clinical Trials Group, protocol P1025 observed no increase in PTB rates comparing PI based and non-PI cART [83,84].

Cohort data from Europe and South America and Africa have corroborated this potential class link showing a threefold risk of PTB with PI-based cART versus non-PI therapy [25,26,32,33]. As discussed earlier, African RCT data comparing PI-based cART with either ZDV monotherapy or triple NRTI have been conflicting.

One could hypothesize that women who receive PIs during pregnancy as part of cART have more advanced HIV disease compared with women eligible for ZDV monotherapy or triple NRTI, which may drive the association seen with PIs in some observational studies. To reduce the bias from treatment indication and HIV disease status, we explored the rates of PTB in women who did not require treatment for their own health (CD4+ cell count >200 cells/μl pre-2008 and >350 cells/μl post-2008), and thus were eligible for PI-based cART or ZDV monotherapy. Women who received new PI-based cART were eightfold more likely to experience PTB compared with those who received ZDV monotherapy [73].

Few studies have looked at relationships with individual drugs. It has been suggested that this association is the result of the PI ritonavir, which is coadministered with other PIs to inhibit cytochrome p450 and thus increases therapeutic drug levels. Data from the large Agence Nationale de Recherché sur le SIDA (ANRS) French Perinatal cohort shows women receiving ritonavir-boosted Atazanavir being twice as likely to experience PTB as women receiving unboosted Atazanavir [30]. The American SMARTT study also presented data for specific drug regimens [77]. In this study, the following PIs were associated with increased risk of PTB: saquinavir (aOR: 2.32; 95% CI: 1.26–4.27); ritonavir (aOR: 1.35; 95% CI: 1.03–1.77) and lopinavir/ritonavir (aOR: 1.32; 95% CI: 1.00–1.74). ZDV monotherapy was associated with a significant reduction in the risk of PTB (aOR: 0.62; 95% CI: 0.41–0.92).

Timing

Timing of treatment initiation with respect to gestation also appears to be critical. Some data suggest that the length of exposure of treatment is key with women conceiving on cART at the highest risk. European data from ECS and MoCHIVA women conceiving on cART have twice the risk of women who received no ART [31,70]. The French ANRS perinatal cohort study also demonstrates a moderate increased risk of PTB when comparing exposure from conception compared with initiating treatment in pregnancy (odds ratio [OR]: 1.31; 95% CI: 1.11–1.55) [30]. These results have been duplicated in a Brazilian cohort, which demonstrated that preconception cART was associated with a fivefold risk of PTB compared with all later ART [79].

Other studies have suggested the converse effect with women initiating treatment during pregnancy at the highest risk, perhaps as a result of an immune reconstitution phenomenon. The European AmRo (Academic Medical Center Rotterdam) study demonstrated a twofold risk of PTB with cART use in the first trimester versus no ART. Data from our cohort indicate that women who initiate cART in pregnancy are at the highest risk with a fivefold risk compared with women receiving ZDV monotherapy. The greatest risk was observed in women who initiated short-term cART during pregnancy (5.00; 95% CI: 1.49–16.79) [73]. A recent Spanish study has also shown a sixfold risk of PTB in women initiating cART in the second half of pregnancy with women conceiving on cART not demonstrating a significantly increased risk [71]. These findings are in line with the large Botswanan-matched cohort study, which demonstrated a 1.4-fold risk with women initiating cART in pregnancy compared with ZDV monotherapy [59].

Data from the American SMARTT study have also shown women who initiated PI-based cART in the first trimester to be at the highest risk of PTB, whereas cART exposure later in pregnancy was not associated with increased risk [77]. While not the main conclusion of the American meta-analysis, this study showed a 1.7-fold risk with cART started preconception or in the first trimester compared with the second or third trimester [29]. Compared with no therapy, a retrospective study from South Africa showed a sixfold risk of PTB when women started non-PI cART and threefold risk when starting PI-based cART both before 28 weeks [32].

While there is mounting evidence to demonstrate that cART has a role in the excess risk of PTB in these women, the association with specific drug classes and timing remains uncertain. The choice of comparator group in observational studies will be dictated by the available data and concurrent treatment guidelines. The spectrum of findings presented here is, in part, due to interactions between maternal health status (treatment indication) and clinical practice in different settings, in addition to population differences in traditional PTB risk factors. These factors are difficult to completely adjust for in efforts to limit bias in observational cohort studies.

Intrauterine death

Though not so widely reported, rates of intrauterine death, miscarriage and stillbirth are also high in women taking cART. Data from a Spanish group have shown rates of IUD eightfold higher in women taking cART prior to pregnancy compared with no ART [20]. Compared with ZDV monotherapy and Dual NRTI, the UK National Surveillance of HIV in Pregnancy and Childhood register has demonstrated a trend toward a twofold risk of stillbirth with cART use. Indian data looking at adverse perinatal outcomes including stillbirth and miscarriage showed twofold higher rates in women receiving cART compared with ZDV monotherapy [21].

However, North American stillbirth data show no excess risk with cART [10,28].

Type of preterm birth

The terminology PTB refers to a syndrome, which encompasses several different pathologies. These can be divided into spontaneous PTB, which comprises spontaneous preterm labor (SPTL) and preterm premature rupture of membranes (PPROM), or iatrogenic PTB, where labor is induced or a PLCS is performed as a consequence of maternal or fetal complications. Maternal complications include conditions such as pre-eclampsia, eclampsia and diabetes. Fetal complications include intrauterine growth restriction and fetal distress. While these different pathologies are believed to have common mechanisms that trigger PTB, one can postulate that these should be considered as distinct conditions when analyzing associations with ART.

Few studies to date have made the distinction between iatrogenic and spontaneous PTBs or subdivided these into SPTL and PPROM. A case–control study that evaluated the different types of PTB in HIV-infected and -uninfected women showed that rates of spontaneous PTB were higher in HIV-infected women (32 vs 9%), whereas HIV-uninfected women had higher rates of PTB as a result of iatrogenic causes (21 vs 50%) [54]. This study showed that 79% of all PTB in HIV-infected women was spontaneous PTB, 60% was secondary to SPTL and 40% to PPROM. Within our cohort, 53% of all PTBs were spontaneous and 47% were iatrogenic, the most common cause of iatrogenic PTB was pre-eclampsia [73].

A recent Spanish study defined PTB as either spontaneous or iatrogenic with spontaneous PTB accounting for 70% of all PTB [71]. There was no association with cART and spontaneous PTB; however, the risk of iatrogenic PTB was sixfold when cART was initiated in the second trimester. Spontaneous PTB accounted for 61% of all PTBs in the French ANRS cohort [30]. The rates of iatrogenic PTB in women using PI-based cART were significantly higher in women receiving boosted PI-based cART (6%) compared with unboosted PI-based cART (1%). Iatrogenic PTB was most commonly indicated for pre-eclampsia and intrauterine growth restriction. There was a trend toward a higher rate of spontaneous PTB in women receiving PI-based cART, which was not significant in multivariate analyses.

The American SMARTT study showed that 55% of PTB in women with HIV infection were spontaneous and 44% were iatrogenic. PI-based cART exposure in the first trimester was associated with spontaneous PTB and all causes of PTB but not with iatrogenic causes.

Although the American WITS cohort was the first to evaluate an association of cART with PPROM and SPTL, the actual numbers of SPTL and iatrogenic PTB were not presented. In this large American study, the overall rate of PTB was 18%, 56% of these women experienced PPROM. When examining the association between cART and PTB, the investigators demonstrated late use of cART without ZDV to be associated with an increased risk of PPROM but not of SPTL.

The distinction between these different reasons for PTB and their limited and inconsistent use as primary outcomes in studies to date may, in part, explain the spectrum of results in the current available evidence. It is certainly possible that the underlying mechanism that increases PTB risk drives a specific subtype. Spontaneous PTB seems to be more common, but the latest studies indicate a higher risk of iatrogenic PTB with cART, which cannot be completely explained by increased pre-eclampsia. Different rates of this condition between treatment groups have been observed by some but have not been consistently demonstrated [20,46,85]. Within our cohort, we observed low rates of pre-eclampsia in the pre-cART era (0%) and increased rates since the introduction of cART: 1% in women receiving ZDV monotherapy/dual NRTI but 11% in women taking cART (OR: 15.1; 95% CI: 1–270) [19]. Subanalyses by PTB type or disease association means smaller that observational cohort studies may be underpowered to demonstrate cause and effect.

A large-matched cohort study from Botswana has shown an increased risk of PTB and hypertension in pregnancy with cART compared with all other therapies (OR: 1.34; 95% CI: 1.00–1.77), which may explain part of the excess risk of iatrogenic PTB [59]. Data from a Spanish-matched cohort study have also demonstrated a higher risk of pre-eclampsia and fetal death when women have conceived on cART [20].

Mechanism

The main mechanisms through which PTB is triggered can be broadly divided into: inflammation; placental abruption; premature uterine distention and maternal–fetal stress. The mechanism by which cART increases the risk of PTB has not been elucidated. Several potential mechanisms have been hypothesized, which are currently under evaluation.

Inflammation

One of the most popular hypotheses is that cART causes a reverse in pregnancy-associated cytokine changes at the maternal–fetal interface to a proinflammatory Th1 profile. In HIV-uninfected women, successful term pregnancy requires immune tolerance of the fetal allograft. This is achieved by multiple pathways, which include: a shift from Th1 to Th2 cytokine environment and changes in the expression of MHC molecules at the maternal–placental interface, alongside alteration of the spectrum of immune cells populating the uterus and its lymphatic system [86,87]. Th1 cytokine responses are thought to be important in triggering labor, both term and preterm, by: mediating cervical ripening; reducing amniotic membrane rupture threshold; increasing myometrial contractility through the recruitment of neutrophils, macrophages and upregulation of matrix metalloproteinases and prostaglandins [88].

The natural history of untreated HIV infection is a shift toward a Th2 predominant profile, while initiation of cART in nonpregnant adults results in a Th2 to Th1 cytokine shift [89–91]. This led to the theory that the same reverse Th1 cytokine shift in HIV-infected pregnant women on or initiating treatment, may drive a lower threshold for the trigger of labor in these women.

An Italian group was the first (2001–2002) to produce data on the difference in Th1 and Th2 cytokines in HIV-infected pregnant women according to treatment history [92]. This study evaluated concentrations of IL-2 (T cell growth factor defined as Th1) and IL-10 (cytokine synthesis inhibitory factor, previously defined as a Th2 signature cytokine) from stimulated peripheral blood mononuclear cells in 26 HIV-infected mothers taking cART throughout gestation (22, (85%) receiving PI-based cART) and 23 women receiving no therapy. The authors demonstrated reduced IL-10 concentrations in cART-exposed women compared with no therapy but did not find a difference in IL-10 between term and PTB. However, HIV-infected women experiencing PTB had greater increases in IL-2 across pregnancy compared with women delivering at term, although IL-2 concentrations did not differ by ART exposure.

One large international prospective cohort study performed in 56 sites across America and the Caribbean has evaluated markers of inflammation (IL-6 and CRP) and coagulation (D-dimer) in plasma throughout pregnancy in HIV-infected pregnant women who had a pre-cART CD4+ count >350 cells/μl. The authors explored biomarker associations with a composite outcome of pregnancy complications: pre-eclampsia, hypertension, gestational diabetes, PPROM and PTB. All biomarkers increased from week 30 to delivery. There was a trend toward higher concentrations of IL-6 from 30 weeks gestation to delivery in women who experienced one or more pregnancy complications compared with uncomplicated pregnancy ($p = 0.08$). This study did not specifically explore associations of PTB alone.

Data from 51 pregnant women in our cohort between 2007 and 2011 revealed reduced plasma IL-10 concentrations in women initiating PI-based cART at weeks 20–34 (3- to 15-fold, minimum mean concentration 31 pg/ml, $p < 0.02$) compared with those initiating ZDV monotherapy or triple-NRTI and lower concentration in women experiencing PTB (4- to 13-fold, minimum mean concentration 14 pg/ml, $p < 0.03$) compared with term deliveries at weeks 12–28 [93]. IL-10 inhibits the release of Th1 cytokines and thus acts as a break in the pathway that triggers labor. These data suggest a plausible mechanism behind the elevated rates of PTB seen in women with HIV infection who receive cART. Further evaluations of maternal cytokines are underway locally and as part of the ongoing international RCT: Promoting Maternal–Infant Survival Everywhere protocol 1077 [94].

Immune reconstitution

One explanation for the high rates of PTB might be immune reconstitution. HIV-infected individuals commencing cART can experience symptoms transiently worsening as a consequence of improved CD4+ cell function increasing inflammatory responses to opportunistic infections. This phenomenon is more common in individuals with advanced immunosuppression and can manifest as a clinical deterioration of a range of infections, for example, *Mycobacterium tuberculosis*, *Pneumocystis Jirovecii* pneumonia, Hepatitis and Herpes viruses. During pregnancy, a physiological reduction in CD4+ cell count occurs [95]. Initiation of cART during pregnancy and the resultant increase in CD4+ cell count could result in an immune reconstitution inflammatory syndrome related to fetoplacental antigens. A heightened inflammatory response may explain the observation of increased rates of chorioamnionitis as well as a potential mechanism for the trigger of labor. Thus, in a recent African cohort study of women initiating treatment before 28 weeks, there was a 33% reduction in risk of PTB for every 50 cell/μl increment in baseline CD4+ cell count [32]. The observation of increased rates of pre-eclampsia with cART by some studies is also supportive [19,20].

The placenta

The direct effect of cART on the placenta is an area of increasing research. It has been hypothesized that HIV infection and/or cART disrupt physiological angiogenesis of the placenta that is required for successful implantation and maternal–fetal exchange [96,97]. Incomplete invasion of the uterine spiral arterioles by the trophoblast cells is thought to be the underlying mechanism for pre-eclampsia and is associated with endothelial dysfunction and elevated angiogenic markers [98,99]. Endothelial dysfunction has also been implicated in PTB and intrauterine growth restriction [100–102].

In the Mma Bana, RCT Botswana angiogenic markers and pre-eclampsia were evaluated in HIV-infected women taking PI-based cART or triple NRTI [85]. Despite the low prevalence of pre-eclampsia (1%), there was a trend toward an association with PI-based cART. Factors associated with pre-eclampsia were pretreatment HIV RNA concentration more than 100,000 copies/μl and low placental growth factor (PlGF). Women taking PI-based cART displayed an overall decrease in PlGF 1 month after treatment initiation, whereas in women on triple NRTI, there was a trend toward an increase in PlGF. The association between PlGF and PTB was not reported.

Our group has evaluated plasma markers of endothelial dysfunction in HIV-infected and HIV-uninfected pregnant women [103]. We have been able to demonstrate higher concentrations of plasma leukocyte adhesion molecules (cell surface molecules that enable migration of leukocytes into tissues and blood vessels, associated with inflammation) across gestation in HIV-infected women compared with uninfected. In women initiating PI-based cART with a CD4+ count >350cells/μl, mean plasma vascular adhesion molecule-1 (VCAM-1) concentrations increased from weeks 12 to 20 from 3.3 to 5.9 pg/ml. This effect was not observed in women initiating NRTI-only therapy (either triple NRTI or ZDV monotherapy), or in HIV-uninfected pregnant women. Concentrations of VCAM-1 in women initiating PI-based cART were two- to threefold greater at weeks 20–34 than in women receiving NRTI-only treatment and uninfected women ($p < 0.03$). We were also able to demonstrate twofold higher concentrations of VCAM-1 at weeks 20–28 in women initiating PI-based cART during pregnancy compared with women who conceived on PI-based

cART ($p < 0.003$). VCAM-1 concentrations were higher in women experiencing PTB; however, this did not reach statistical significance. While these data are from plasma, they indicate an underlying difference in vascular endothelial cell activation by treatment exposure and timing, which may reflect placental pathology.

In a Brazilian series of histological examinations of HIV-infected and HIV-uninfected placentas, authors have demonstrated more villous immaturity in HIV-infected women compared with uninfected [104]. This study also evaluated the level of expression of leukocyte adhesion molecules in the placenta by HIV serostatus and cART exposure. They demonstrated higher levels of intercellular adhesion molecule-1 in placentas of HIV-infected compared with uninfected women and elevated intercellular adhesion molecule-1 in placentas of women initiating cART before 36 weeks compared with after 36 weeks.

Preliminary findings from the ongoing study of Angiogenesis and Adverse Pregnancy Outcomes in Women with HIV are that compared with uninfected women, HIV-infected women, the majority of whom received cART, had lower placental weight and increased placental abnormalities including fibrotic lesions, intervillous thrombi, villous immaturity and inflammation [97]. While we know that HIV-infected pregnant women have higher rates of chorioamnionitis and deciduitis, to date, differences by ART exposure have not been evaluated.

Maternal–fetal hypothalamic pituitary axis

The initiation of preterm and term labor is under tight regulation by several hormonal feedback loops, the final common pathway of which is the Maternal–Fetal hypothalamic–pituitary axis. Prematurely, elevated levels of placental corticotrophin-releasing hormone have been demonstrated in women who deliver preterm [105]. There is a school of thought that the cytochrome P450 inhibition of ritonavir may interact with the maternal–fetal hypothalamic–pituitary axis [30], preventing glucocorticoid breakdown and in turn increasing prostoglandin levels, which are vital to cervical ripening and myometrial contractions. To date, there are little data to support this hypothesis. Of interest, is the transient neonatal adrenal dysfunction that has been reported in association with the combination of inutero with neonatal lopinavir/ritonavir exposure. This was most marked in preterm babies. Since only lopinavir/ritonavir is available as a syrup, it is uncertain whether this is a class or compound-specific effect [37].

Another signal of an endocrine action is the effect of a PI-based cART (lopinavir/ritonavir and two NRTIs) in pregnant mice on progesterone production. One group has shown PI-based cART exposure that was associated with lower progesterone levels, lower placental and fetal weight and increased fetal loss compared with placebo, indicating that PI-based cART may have an effect on pregnancy hormones [106]. The APPH study plans to evaluate correlations of progesterone with prematurity in humans.

Expert commentary

The benefit of cART to both mother and child cannot be underestimated, but there seems to have been reluctance in some quarters to acknowledge the association between cART and PTB, perhaps through fear of unnecessarily alarming mothers when prevention of HIV transmission is clearly a higher clinical priority. While the initial evidence for the role of cART was contradictory and controversial, with the ever-expanding evidence, attitudes are changing and the importance of understanding the association recognized.

American and European guidelines continue to advocate the use of PI-based cART during pregnancy, see Table 1. Worldwide PI-based cART is the most prescribed regimen for PMTCT [107]. HIV physicians favor these drugs due to their short half-life, which facilitates the stopping of these therapies and their high genetic barrier to resistance. There are also good surveillance data to show no excess of congenital anomalies [108,109]. However, modeling of UK data has shown that for every 100 HIV MTCTs prevented by cART (including PI-based and non-PI cART) instead of ZDV monotherapy, an additional 63 PTBs occur [110]. Currently, 9.7 million of the 28 million eligible HIV-infected adults worldwide are receiving cART [7]. By the end of 2012, 62% of the 1.5 million HIV-infected pregnant women received ART. This number is increasing year on year, and it is anticipated that coverage will reach 90% by 2015 in line with United Nation's millennium goals 5 and 6 [111]. The dark lining of this silver cloud is the prospect of increased numbers of preterm births if cART is PI based.

Five-year view

The 2012 WHO guideline update has boldly advocated that for areas with limited access to ART, a fixed dose combination should be prescribed for all women in order to simplify what have been previously viewed as complex guidelines [7]. This regimen (Efavirenz with Tenofovir plus either Lamivudine or Emtricitabine) does not include a PI and may, if adopted, lead to a reduction in PTB rates in women with HIV. It is difficult to estimate the number of PTBs that could be prevented by avoiding PI cART. Based on current figures of ART and a PTB risk ratio of 2:1 for PI versus non-PI-based therapy, if all women globally were treated with non-PI cART, we estimate that 150,000 PTBs could be prevented annually.

This WHO guideline update has also included the recommendation that for women who do not need treatment for their own health, continuing treatment after pregnancy should be considered in epidemic settings (Option B+) [7]. This additional recommendation has been driven by several theoretical benefits to HIV transmission and maternal health extrapolated from available data in nonpregnant adults. These include facilitating early exposure to ART in repeated pregnancies and enabling cART exposure during prolonged breasting feeding as well as reducing sexual HIV transmission. Treatment interruptions are also associated with immune activation and possible long-term morbidity [112,113] although this has not been shown in relation to HIV treatment and PMTCT. The effect that

these guidelines will have on prematurity rates in HIV-infected women is hard to predict, especially considering some studies have found that increasing length of cART exposure raises PTB risk. Concerns have already been raised about potential increases in incidence of PTB and low birthweight as well as safety, feasibility, economic and ethical considerations from this nonvalidated guidance [114].

Trends in PTB rates are increasing globally in part due to an increase in iatrogenic causes and multiple gestation pregnancies due to assisted reproductive techniques. Iatrogenic causes of PTB for maternal and fetal indications have been strongly associated with cART and PI-based cART. Since the use of cART in pregnancy was accepted in clinical practice, rates of PTB in HIV-infected women in the developed world have increased and then subsequently stabilized [30,70]. Recent data from ECS and MoCHiV have shown that since clinical guidelines have changed to advocate normal vaginal delivery (where maternal plasma HIV RNA copies are undetectable at 36 weeks) instead of prelabor C-section at 38 weeks, a decline in late PTB (34–36 weeks) has been observed [115]. This is thought to be the result of a reduction in C-sections performed to reduce the intrapartum risk of HIV MTCT. While iatrogenic causes of PTB by prelabor C-Section for prevention of MTCT have declined, it remains that iatrogenic, and to a lesser extent spontaneous, causes of PTB for maternal and fetal indications are associated with cART.

Of interest, is the observation from the ECS and MoCHiV cohorts between 2000 and 2010, rates of moderate–severe PTB (24–34 weeks) have increased from 29 to 44% of all PTBs [115]. UK surveillance data have shown that cART use is associated with a twofold risk of severe PTB (<32 weeks) [47], and within our cohort, 53% of all PTB has been moderate–severe [73]. These high rates of severe–moderate PTBs are concerning as this is the group with the poorest morbidity and mortality outcomes in the HIV-uninfected population.

Compared with term infants, preterm infants born to HIV-infected mothers have higher rates of infant mortality: threefold in a Chinese cohort study [116] and fivefold according to Mma Bana RCT [22]. The latter study also showed higher rates of severe lower respiratory tract infections requiring hospitalization in PTBs. Another Botswanan study has also shown higher rates of neonatal death in PTBs compared with term and in women receiving cART compared with ZDV monotherapy [59].

There is currently a paucity of long-term follow-up data for HIV exposed severe–moderate preterm infants in both resource rich and poor settings. However, the majority of infants born to women living with HIV have minimal access to neonatal services and even small increases in PTB, especially at earlier gestations, are likely to negatively impact global infant mortality and morbidity.

The recent WHO guideline update for the management of HIV-infected women is potentially a positive step forward if PI-based cART is the main driver of the excess risk seen in HIV-infected women. To affect a global shift in prescribing practice away from these regimes, further supportive evidence would be required. To date, there have been no RCTs designed to evaluate PTB as the primary outcome with RCT generated data to date including PTB as a secondary outcome. The IMPAACT promoting maternal–infant survival everywhere trial designed to compare PI-based cART (Lopinavir/ritonavir based) with ZDV monotherapy in women with CD4+ counts >350 cells/l is ongoing and will add to the available data; however, PTB remains a secondary outcome measure [94]. To power a RCT for PTB as a primary outcome, a significant sample size would be required, the likes of which is best recruited in low–middle-income countries where the burden of disease is greatest. Since the WHO updated their guidelines to a fixed dose regimen, the likelihood of obtaining ethical approval and financial support for an RCT comparing different cART regimens will become more difficult. We are likely to continue to depend on safety data from retrospective series in heterogeneous populations dependent on evolving prescribing standards. However, ongoing research into biologically plausible mechanisms underlying the association may provide an ethical approach to expanding the available evidence. As new antiretroviral drugs become available, knowledge of the pathogenesis underlying adverse drug effects is another approach to evaluating of potential side effects, especially in pregnancy.

In an ideal world, the excess PTB risk observed in HIV-infected pregnant women would be minimized. Changes in treatment guidelines may go a long way to facilitate this but will not be a one step solution. Risk reduction should also consider traditional risk factors for PTB and perhaps tailored cART in women with elevated risk of PTB. Within developed countries, the management of women at high risk of PTB will vary according to local services. Many centers run prematurity clinics where women undergo screening to identify their risk of PTB including cervical length measurement, fetal fibronectin measurement and increased surveillance for genitourinary infections as well as being offered lifestyle advice. Where indicated, women receive interventions to reduce risk of PTB including progesterone pessaries, antibiotics and cervical cerclage [117–119]. At present, HIV infection *per se* has not been used as an indicator for such assessment, but one can argue that their elevated risk of PTB justifies more active management where resources allow. Current tools for risk stratification such as fetal fibronectin, phosphorylated insulin-like growth factor-binding protein (phIGFBP-1) and cervical length measurement have not been validated in HIV-infected women, but research is ongoing into their utility [46,120].

Preterm delivery is an arbitrary cutoff in the continuum of gestational age at delivery with lesser but more numerous benefits as maturity at delivery increases. Therefore, shifts in prescribing practice and or modifiable traditional PTB risk factors during pregnancy which increase the gestational age of delivery closer to 40 weeks would be of benefit both for infant health in line with United Nation's millennium development goal 4 and by reducing pressure on overstretched neonatal and pediatric services worldwide.

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Key issues

- Combination antiretroviral therapy has led to dramatic reduction in mother-to-child transmission of HIV with infant infection rates of <1% achievable.
- Advances in therapy have enabled HIV-infected women to have normal vaginal deliveries and in certain settings, the option of breastfeeding.
- The infants of HIV-infected women have a high risk of being born preterm of which up to 50% are born <34 weeks (moderate–severe prematurity).
- The cause of these high rates of preterm birth (PTB) in HIV-infected women is likely to be multifactorial.
- Several studies implicate protease inhibitors as a cause of preterm birth although no mechanism has been identified.
- Lower rates of PTB, in line with the general population, have been observed with older, now less used treatments such as the nucleoside analog reverse transcriptase inhibitor only therapy including zidovudine monotherapy.
- The current hypothesis is that combination antiretroviral therapy alters the cytokine environment of the fetoplacental unit and reduces the threshold for labor initiation.
- Tailored antiretroviral treatment in HIV women perceived to be at high risk of PTB might be a viable risk reduction strategy.

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SHORT COMMUNICATION

Preterm delivery risk in women initiating antiretroviral therapy to prevent HIV mother-to-child transmission

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Objectives

The aim of the study was to describe the relationship between preterm delivery (PTD; < 37 weeks of gestation) and antiretroviral therapy in a single-centre cohort of pregnant women with HIV infection.

Methods

A retrospective analysis of data for 331 women who received care in a dedicated HIV antenatal clinic between 1996 and 2010 was carried out. Data on first CD4 cell count and viral load (HIV-1 RNA copies/mL) recorded in pregnancy, class and timing of antiretroviral therapy, gestational age at delivery, and risk factors for and causes of PTD were available from a clinical database.

Results

Overall, 13.0% of deliveries were preterm, of which 53% were severe preterm (< 34 weeks of gestation). The lowest rate of PTD was observed in women treated with zidovudine monotherapy (6.2%). Higher rates of PTD were observed in women starting combination antiretroviral therapy (cART) in pregnancy compared with women conceiving while on cART [odds ratio (OR) 2.52; 95% confidence interval (CI) 1.22–5.20; $P = 0.011$]. Of the women who were eligible for zidovudine monotherapy on the basis of CD4 counts and HIV viral load but who were treated with short-term cART to prevent HIV mother-to-child transmission, 28.6% delivered preterm. Women on short-term cART remained at the highest risk of PTD compared with zidovudine monotherapy in multivariate analysis (OR 5.00; 95% CI 1.49–16.79; $P = 0.015$).

Conclusions

The causes of PTD are multiple and poorly understood. The timing of initiation and type of antiretroviral therapy administered during pregnancy appear to contribute to PTD risk. Understanding this association should improve the safety of antiretroviral therapy in pregnancy without increasing the risk of transmission.

Keywords: antiretroviral therapy, HIV, pregnancy, preterm delivery, protease inhibitor

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Introduction

Whether exposure to antiretroviral therapy (ART) during pregnancy is associated with preterm delivery (PTD; gestational age < 37 weeks) has been a contentious issue. The association was first reported in a Swiss cohort (1996–1998) [1] and has since been supported by the analysis of observational data from a large pan-European cohort [2,3] as well as several independent studies from European centres [4–6]. In data from North America such an association is less clear. In the Women and Infants Transmission Study, in which the overall PTD rate was 18%, initiation of ART after 25 weeks of gestation was associated with a lower rate of PTD if regimens contained the nucleoside reverse transcriptase inhibitor (NRTI) zidovudine [odds ratio (OR) 0.5; 95% confidence interval

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(CI) 0.3–0.8] and a higher rate of PTD if regimens were zidovudine-sparing (OR 7.9; 95% CI 1.44–44.6) [7]. In their multivariate analysis, the Pediatric Spectrum of HIV Disease study found PTD to be associated with no ART and also with protease inhibitor (PI)-based triple therapy [combination ART (cART) with at least three drugs], symptomatic HIV disease, Black race and maternal illicit drug use [8]. The authors of a meta-analysis of studies mostly conducted prior to 2000 concluded that ART was not associated with overall risk of PTD but did report a statistically significant, 1.7-fold relative risk of PTD for cART started pre-conception or in the first trimester [9]. The largest surveillance study to date ($n = 33148$; HIV-infected individuals, $n = 9504$) has demonstrated a 1.2-fold increased risk of PTD for cART started pre-conception compared with all other groups and a 1.4-fold increased risk for cART initiated in pregnancy compared with zidovudine [10]. Two large randomized controlled trials in Africa, designed to evaluate ART to prevent HIV mother-to-child transmission during pregnancy and exclusive breast feeding in women with CD4 lymphocyte counts > 200 cells/μl, also reported PTD rates. The Mma Bana study showed a 2-fold higher PTD rate with PI-based cART *vs*. triple NRTI (21% *vs*. 11%, respectively; $P = 0.003$) in women starting ART from 26 weeks onwards [11]. The Kesho Bora study showed no difference comparing PI-based cART *vs*. zidovudine monotherapy (ZDVm) (13% *vs*. 11%, respectively; $P = 0.39$); however, in this study treatment was not initiated until 34–36 weeks, and was thus less likely to impact on gestational age at delivery [12].

The question is not necessarily which data are correct but why they differ. The answer may lie in the subjects or the therapy. Not all types of ART may be associated with PTD, with a higher risk suggested for PI-based cART [4] and some data showing an association with ritonavir boosting [13]. However, biases are inevitable; for example, at some centres PI-based cART may be preferred for women with more advanced HIV infection. The timing of initiation of therapy in relation to the pregnancy may also be important. Both pre-conception cART [3,10] and initiation of therapy during pregnancy [5] have been associated with the PTD risk. However, particularly where, or when, guidelines for initiating cART outside of pregnancy had a lower threshold than now, the contribution of advanced HIV infection *per se* to these risks is difficult to measure.

In the present study, we have attempted to reduce the potential biases by focusing on women who, according to the concurrent British HIV Association guidelines (CD4 cell count > 200 cells/μL until 2008 and > 350 cells/μL thereafter [14–17]; HIV viral load < 10 000 HIV-1 RNA copies/mL plasma), were eligible for treatment with ZDVm [in combination with a pre-labour Caesarean section (PLCS)] to reduce the risk of HIV mother-to-child transmission but chose to initiate a short-term course of cART as they preferred the option of a vaginal delivery.

Methods

Three hundred and thirty-one HIV-positive pregnant women were managed by a single, multidisciplinary team at St Mary's Hospital from 1996 to the end of July 2010. Anonymized linked routine clinical data pertaining to the mother, including classical risk factors for PTD, and the delivery were prospectively collected and entered into a password-protected database. The database was interrogated for all deliveries up to the end of July 2010 for ethnicity, parity, multiple pregnancies, Centers for Disease Control and Prevention (CDC) disease stage, immunovirological parameters, injecting drug use, history of assisted reproduction, type of ART, timing of ART, birth outcome and gestational age. The use of anonymized data collected during routine clinical care to audit outcomes is a recognized element of clinical governance. This was confirmed by the host institute Joint Research Office and by an independent local research ethics committee.

Data were analysed in SPSS version 19.0 for Macintosh (SPSS, Chicago, IL). Continuous variables were analysed using the *t*-test and categorical variables using the χ^2 test. ORs were calculated for univariate associations with PTD. Bonferoni adjustment was made for multiple comparisons; a *P*-value < 0.0125 was deemed significant. Factors associated with PTD with a *P*-value of < 0.1 were included in a multivariate logistic regression using a backward logistic regression method.

Results

Of the 331 women, 78% were Black African and 94% had become HIV-infected through heterosexual intercourse. The median gestational age at first antenatal visit was 13 weeks + 3 days. The median age at delivery was 32.2 years and the median parity was 1; these variables did not differ by gestational age at delivery. Only 11.5% of women gave a history of any AIDS-defining illness. At baseline (the first antenatal visit during this pregnancy), the median CD4 lymphocyte count was 360 cells/μL [interquartile range (IQR) 227–510 cells/μL] and the median viral load was 1466 (IQR 49–13 436) copies/mL plasma, and 123 women (37.2%) had a viral load < 50 HIV copies/mL plasma.

Eight women (2.4%) took no therapy prior to labour, 65 (19.6%) were treated with ZDVm, seven (2.1%) were treated with two nucleoside reverse transcriptase inhibitors (NRTIs) only and 131 (39.6%) conceived while on cART and

Table 1 Preterm delivery rates according to treatment type

					Median values at first antenatal clinic visit	
Treatment	***n***	**PTD**	**%**	**95% CI**	**CD4 count (cells/μL)**	**HIV RNA (copies/mL plasma)**
Nil	8	1	12.5	0–49.2	370	15713
ZDVm	65	4	6.2	0.2–12.1	450	2648
Dual NRTI	7	0	0	–	150	24689
Triple NRTI	5	0	0	–	551	1041
Short-term cART	59	15	25.4	14.0–36.9	350	10200
Pre-conception cART	131	13	9.9	4.7–15.1	395	< 50
New continuous cART	56	10	17.9	12.0–35.0	150	23430

ART, antiretroviral therapy; cART, combination antiretroviral therapy (not including triple NRTI); CI, confidence interval; dual NRTI, two nucleoside reverse transcriptase inhibitors; PTD, preterm delivery; triple NRTI, three nucleoside reverse transcriptase inhibitors; ZDVm, zidovudine monotherapy.

continued it during the pregnancy. One hundred and twenty women (36.2%) initiated cART during the pregnancy either for their own health ($n = 56$, including two women who did so prior to first attending the antenatal clinic), in which case they continued post-partum (new continuous cART), or solely to prevent mother-to-child transmission of HIV [with either a PI or nevirapine as the third drug (short-term cART; $n = 59$) or with a fixed dose combination of three NRTIs only (abacavir, lamivudine and ZDV; $n = 5$)], in which case they discontinued therapy after delivery. The crude PTD rates are presented in Table 1.

There were five twin pregnancies, of which four delivered preterm; two of these women were taking ZDVm, one was on short-term cART (behave CD4 count 390 cells/μL; viral load > 10 000 copies/mL) and two were on cART started pre-conception. One of these mothers conceived through intra-cytoplasmic sperm injection, the only case of assisted reproduction in the cohort. In women for whom data on smoking status were available, 13% were current smokers. There was no difference in PTD by smoking history (current smokers compared with nonsmokers: OR 1.44; 95% CI 0.32–6.57; $P = 0.632$). Only three women in the cohort had a history of recreational drug use – all delivered at term.

There were six (1.8%) intra-uterine deaths (IUDs) at gestational ages 27 weeks + 2 days to 40 weeks + 5 days, all in women on cART. Four were in women on new continuous cART, one in a woman on cART started pre-conception and one in a woman on short-term cART. There were two second-trimester miscarriages (both at 22 weeks), one in a woman not receiving therapy and one in a woman on new continuous cART. There were 328 live-born infants. Forty-three women delivered before the completion of the 36th week of gestation (13.0%); of these, 23 (53%) delivered prior to 34 weeks of gestation (range 26 weeks to 33 weeks + 6 days) and 11 (26%) delivered prior to 32 weeks of gestation (range 26 weeks + 4 days to 31 weeks + 4 days).

The majority of cases of PTD were accounted for by preterm spontaneous rupture of membranes (15) or labour (6) or pre-eclampsia (10). Of the remainder, early delivery was attributable to maternal health (four women), antepartum haemorrhage (two women), fetal conditions (intrauterine growth restriction and hydrops fetalis) (two women) and uncomplicated twin pregnancy (two women). In two cases, PLCS was planned for term but performed early, pre-labour, pre-rupture of membranes, as part of the individualized prevention of mother-to-child transmission (PMTCT) strategy for women with unsuppressed HIV infection.

In univariate analysis, baseline HIV viral load in this pregnancy and category of treatment but not maternal age, ethnicity, parity, disease status or first CD4 lymphocyte count recorded in pregnancy were associated with PTD. The OR for PTD with any cART exposure (pre-conception, long-term new continuous or short-term cART) in pregnancy compared with NRTI-only therapy (ZDVm, dual NRTI or triple NRTI) was 3.33 (95% CI 1.15–9.66; p 0.02).

Next, the relationship between the timing of initiation of therapy and PTD was explored. Comparing only cART initiated in pregnancy (new continuous and short-term cART) with NRTI-only therapy, the risk of PTD was increased (OR 5.07; 95% CI 1.69–15.20; $P = 0.002$). Initiating any cART during pregnancy (short-term or long-term) was also associated with a significantly increased risk of PTD compared with cART commenced prior to conception (OR 2.52; 95% CI 1.22–5.20; $P = 0.011$). Evaluating PTD risk in women who fulfilled the criteria for long-term cART (CD4 cell count < 200 cells/μL pre-2008 and < 350 cells/μL post-2008), the difference comparing women starting new continuous cART and women who had commenced cART pre-conception was not significant (OR 1.97; 95% CI 0.81–4.82; $P = 0.13$).

The high rates of PTD observed in women who, having CD4 T-lymphocyte counts above the recommended level for starting long-term cART, thus initiated cART only to prevent mother-to-child transmission (25.4% with

short-term cART compared with 6.2% with ZDVm; OR 5.20; 95% CI 1.62–16.74; $P = 0.003$) suggest that the type of therapy, rather than the indication for therapy, was important. We therefore identified the subgroup of 28 women who would have been eligible for ZDVm, by concurrent British HIV Association (BHIVA) guidelines, but chose short-term cART and the option of a vaginal delivery. Eight women (28.6%) delivered preterm between 28 weeks + 2 days and 36 weeks + 3 days of gestation. Compared with ZDVm prescribed according to the same criteria ($n = 53$), this risk was significant in univariate analysis (OR 4.90; 95% CI 1.33–18.13; $P = 0.011$).

Given that the concurrent guidelines recommended starting ZDVm at 28 weeks, the timing of initiation of therapy was examined and no difference was observed between short-term cART (mean 28.6 weeks) and ZDVm (mean 29.2 weeks; p 0.49). Differences in baseline antenatal CD4 T-lymphocyte count (mean 425 *vs*. 466 cells/μL, respectively; $P = 0.31$) and baseline plasma HIV viral load (mean 3712 *vs*. 2528 copies/mL, respectively; $P = 0.09$) in the short-term cART group compared with the ZDVm group did not reach statistical significance.

Finally, the question of whether individual components of cART may be implicated in PTD was addressed. PTD occurred in 16 of 137 women treated with nonnucleoside reverse transcriptase inhibitor (NNRTI)-based cART (11.7%) and in 22 of 96 women treated with PI-based cART (22.9%; $P = 0.04$). Furthermore, no difference in PTD rate was seen when nevirapine-based cART was compared with PI-based cART by indication for therapy. However, of the 28 patients who were treated with short-term cART but were eligible for ZDVm, 22 received PI-based cART. All eight PTD events occurred in these 22 women (short-term PI therapy *vs*. ZDVm: OR 8.71; 95% CI 2.30–33.06; $P < 0.0001$).

In the multivariate analysis (adjusted for pregnancy baseline plasma HIV viral load, maternal age, parity, ethnicity and pregnancy baseline CD4 lymphocyte count; see Table 2), only use of short-term cART compared with ZDVm (OR 5.00; 95% CI 1.49–16.79; $P = 0.015$) was associated with PTD risk.

Table 2 Multivariate analysis evaluating risk factors for preterm delivery

	OR	95% CI	*P*-value
Age (years)	0.97	0.86–1.10	0.62
Black African	10.70	0.63–183.06	0.11
HIV RNA at baseline (ln copies/mL)	0.66	0.40–1.10	0.11
CD4 count at baseline (cells/μL)	1.00	1.00–1.01	0.35
Short-term cART *vs.* ZDVm	5.00	1.49–16.79	0.015

Baseline refers to first antenatal clinic attendance in this pregnancy.
cART, combination antiretroviral therapy; ln, natural logarithm; OR, odds ratio; ZDVm, zidovudine monotherapy.

Discussion

In the absence of a randomized controlled study powered to evaluate PTD as the primary outcome, a detailed analysis of women eligible for ZDVm but treated with short-term cART for PMTCT reduces confounding. For example, in this clinical setting short-term cART and ZDVm were both commenced at the same mean gestational age (29 weeks). This analysis confirms an association between cART and PTD in our cohort and indicates an association of PTD with PI-based regimens initiated for PMTCT only. However, the risk of PTD was not limited to this healthier group, being also seen in patients with lower CD4 counts initiating long-term therapy for maternal health (17.9%). The overall rate of PTD seen in our unselected HIV-infected cohort (13%), which is similar to the national figure (13.3%) from the National Survey of HIV in Pregnancy and Childhood [6], is higher than expected for the general population (7.7%) [18]. Of women experiencing PTD, 44% were induced for maternal or fetal complications, which is similar to data from the Agence Nationale de Recherche sur le SIDA (ANRS) French Perinatal Cohort, in whom 38% of PTDs were induced [13].

The highest risk of PTD in association with cART was observed with cART initiated during pregnancy (25%). This mirrors the finding of some groups that late use of cART is associated with a 3–7-fold increased risk of PTD [7,19]. Conversely, other groups have demonstrated that pre-conception cART increases PTD risk [3,10,20,21]. It is plausible that initiation of treatment during pregnancy induces an immune activation phenomenon with modulation of the cytokine environment at the fetal–placental unit, reducing the threshold for PTD. Early work in this area has demonstrated reduced maternal plasma anti-inflammatory interleukin (IL)-10 concentrations in women receiving ART compared with no therapy and NRTI-only treatment [22,23]. Given that the mean gestation at initiation of short-term cART was 29 weeks, it is possible that earlier treatment, even in women with good CD4 counts and low viral load, might reduce the risk of PTD if there is a key time when cytokine changes might trigger this event or increase a predisposition.

We have considered the potential for bias in this study and find no evidence that women receiving ZDVm were at lower risk of PTD prior to commencing ART in our cohort: of the five twin pregnancies, two were managed with ZDVm plus PLCS and only one with PI-based short-term cART; of four women receiving cervical sutures, a clear

indication of PTD risk, one was treated with ZDVm, two were on cART at conception and one initiated cART during the pregnancy in accordance with CD4 count and viral load criteria. Conversely, each woman is counselled regarding her treatment options and if she were a higher risk candidate for PTD it might be argued that she would be more likely to initiate a non-PI regime, thus leading to an underestimation of the risk of PTD associated with PI-based cART. Nor is there evidence that our cohort had a high risk of PTD in general: the median gestational age at booking was 13 weeks, we identified only three women with a history of recreational drug use in the cohort, all of whom delivered at term, and current tobacco use was reported by 13% of women, with no difference in rates of PTD comparing smokers and nonsmokers. PTDs associated with IUD were not included in the analysis. However, the frequency of IUD (six of 331; 1.8%) is higher than the 5.1/1000 reported in the UK for 2008, and further investigation of both IUD and late second trimester miscarriage rates in relation to HIV infection and ART is warranted.

More detailed analysis of larger cohorts is imperative to address the relationship between ART, other risk factors and clinical aspects of PTD. This is becoming increasing important with the rollout of cART in resource-limited settings where intensive care facilities for premature babies are not generally available. The difficulty of accurately diagnosing gestational age in resource-limited settings may mask this problem and surrogates such as low birth weight, stillbirth or early infant mortality rates may need to be considered.

We do not advocate any change in recommendations to initiate the treatment of pregnant women with low CD4 counts with cART, nor to avoid the use of cART to prevent mother-to-child transmission when maternal viral load is high and especially in settings where caesarean section may not be a safe option. However, the mechanisms and comparative risks of PTD and severe PTD associated with different treatment strategies and treatment timings need urgent attention, ideally within randomized trials. In summary, despite HIV treatment, rates of PTD overall remain higher than in the general population. Timing and type of treatment are driven by many factors, including knowledge of HIV status, maternal health, CD4 count and viral load and patient preference. Some studies find an association between pre-conception cART and PTD, which suggests an impact of either HIV therapy or prior or persisting immunosuppression, or both. In this study, we have examined for the first time an additional risk of PTD among women with good CD4 counts, initiating cART for the prevention of HIV mother-to-child transmission at the end of the second trimester. While women choosing ZDVm plus PLCS had a risk of PTD no greater than that of the general population, a high risk of PTD was observed in the subgroup who chose cART and a spontaneous vaginal delivery. We postulate that this risk might be a result of cytokine switching and that this merits further investigation. Factors involved in the association between PTD and pre-conception cART are likely to be different.

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Preterm Delivery: The Role of Antiretroviral Therapy

a report by **Charlotte-Eve S. Short**[1] and **Caroline Foster**[2]

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Introduction

Prevention of mother-to-child transmission (PMTCT) of HIV-1 infection with interventions that include the use of antiretrovirals during pregnancy is one of modern medicine's greatest triumphs. The benefit of antiretroviral therapy was first demonstrated in 1994 with the use of zidovudine (ZDV) monotherapy in a non-breastfeeding population compared to placebo in the seminal ACTG 076 study, with a MTCT rate of 8% at 18 months compared to 26% in the placebo group.[1] Full suppression of maternal plasma viral load (VL) with combination anti-retroviral therapy (cART), pre-labour caesarian section (PLCS) and avoidance of breast-feeding can achieve MTCT rates of 0.1% in developed settings.[2] Randomised Controlled Trial (RCT) evidence from resource-poor settings has demonstrated rates of MTCT of <1% in women on protease inhibitor (PI) based cART in pregnancy and throughout breast-feeding.[3]

Remarkably to date ZDV remains the only drug licensed for use in pregnancy. However PMTCT guidelines for resource-limited and rich settings recommend the use of cART in all cases where the mother requires treatment for her own health.[5,6] Guidelines differ where treatment aim is PMTCT alone, (see Table 1); WHO and BHIVA guidelines advocate either: ZDV monotherapy (maternal plasma HIV VL<10,000 copies/mL) or cART,[4,5] US National Institutes of Health (NIH) guidelines state that ZDV monotherapy is controversial but should be considered if woman has VL<1,000 copies/mL.[6] European AIDS Clinical Society (EACS) guidelines recommend cART.[7]

The use of combination therapy in pregnancy is not without potential risk both to mother and infant; adverse drug effects reported in mothers include: lactic acidosis,[8] anaemia,[9,10] gestational diabetes,[11] pre-eclampsia,[12] hepatic dysfunction,[9] haemolytic anaemia, elevated liver enzymes and low platelets – "HELLP syndrome",[12] still birth,[13] and preterm delivery (PTD).[9,14] *In utero* exposure has been associated in infants with anaemia[15] and mitochondrial dysfunction.[16]

This review will examine the evidence for the role of cART in PTD, as defined as birth prior to 37 weeks gestation and the impact on perinatal outcomes in resourced and resource poor settings.

Preterm Delivery and cART

In 1998, Lorenzi *et al.* reported high rate of PTD in 10/30 live births to HIV-infected pregnant women receiving cART; aOR of 2.30 (95% CI, 1.17–7.10) compared with no therapy.[9] This association has subsequently been investigated widely using observational studies, with conflicting results (summarised in Table 2).

European Data

A combined analysis of outcomes from the European Collaborative study (ECS) and Swiss Mother á Child HIV Cohort Study (MoCHiV) (n=3920 mother-child pairs; 23% ART exposed) between 1989-2000, described an association of PTD with PI cART vs. no antiretroviral therapy (aOR of 2.6 (95%CI 1.43-4.57) but no association for ZDV monotherapy (1.03 (95%CI 0.70-1.50)).[17]

Subsequently a large number of mother-child pairs were followed in the National Study of HIV in Pregnancy and Childhood (NSHPC) (n=4939) in the UK and Ireland from 1990-2005. Ninety percent were exposed to antiretrovirals, with an aOR of PTD of 1.51 (95% CI 1.19-1.90) for cART vs. mono/dual nucleoside reverse transcriptase inhibitor (NRTI) therapy.[13]

European cohort data consistently demonstrates a significant association between PTD and cART compared to NRTI therapy and no antiretroviral therapy, with rates of PTD of 14-39% seen in the cART-exposed groups (see Table 2). These rates are higher than rates of PTD generally observed in Europe of 5-9%.[18]

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US Data

Conversely most US studies have failed to report an association between PTD and cART (table 2). Tuomala *et al.* evaluated 3266 mother-child pairs between 1990-1998, from seven heterogeneous studies; two clinical trials and five prospective cohorts.[19] Sixty-five percent of mothers were exposed to antiretrovirals. Comparing cART with monotherapy revealed no association with PTD (aOR of 1.08 (95% CI 0.71-1.62)).

Interestingly separate analyses of two of the contributing cohorts included in this study reached slightly different conclusions. In the Women and Infant Transmission Study (WITS) during 1990-2002 (n=2543), 70% were antiretroviral exposed.[10] The analysis evaluated early and late exposure to antiretrovirals, compared to no therapy. Although all cART use during pregnancy, irrespective of timing was not associated with PTD (aOR 0.98 (95% CI 0.72-1.34)), late use of non-ZDV containing antiretrovirals was associated with PTD in multivariate analysis (aOR 7.9 (95%CI 1.4-44.6)). ZDV monotherapy was protective on univariate analysis (aOR 0.68 (95% CI 0.51-0.9)).

Similarly, Cotter *et al.* presented prospective cohort data from a single centre also included in the first US analysis, exclusively included in the treated group.[20] Of 1337 women followed between 1990 and 2002, 66% were exposed to antiretrovirals in pregnancy. Comparison of PTD rates in women who received cART vs. monotherapy was significant in univariate analysis (OR 1.3 (95% CI 1.0-1.8)) but did not retain significance when adjusted for other risk factors. However analysis of PI-based cART vs. non-PI regimens was significant (aOR of 1.8 (95% CI 1.1-3.0)), and was the first study to specifically implicate PI based regimens in PTD. Further US studies have failed to clarify the association of PTD with cART[21,22] and overall observed rates of PTD for mothers receiving cART from US studies range from 15-30%, see Table 2. General rates of PTD in the US general population are 12-13%.[23]

South American and Caribbean data

Szyld *et al.* presented prospective cohort data from the NISDI Perinatal protocol in Latin American and Carribean countries from 2002-2005 (n=681), all received antiretroviral therapy.[24] The authors reported no excess risk of PTD with cART vs. mono/dual NRTI therapy (aOR for PI-based cART 1.1 (95%CI 0.5-2.8) and non-nucleoside reverse transcriptase inhibitor (NNRTI)-based cART (aOR 0.6 (95%CI 0.2-1.7)). Machado *et al.* evaluated 696 mother-child pairs from a single centre in Brazil (1996-2006); the use of preconception cART was associated with a five-fold risk of PTD,[25] although the study did not directly compare cART and NRTI therapy.

Protease Inhibitor Based cART and PTD

Following Cotter *et al.* several analysis have evaluated the specific role of PI therapy in PTD. A significant association has been replicated by some studies with OR ranging from 1.2-3.4[3,17,22,26,27] but not others.[10,13,19,21,24,28] More recently prospective cohort data from the French Perinatal Group (ANRS) (n=945) collected between 1990-2009, implicated a specific role for ritonavir boosted PI therapy compared to unboosted PIs (aOR of 2.05 (1.06-3.89)).[29] It remains unclear if the higher rates of prematurity are driven by PI-therapy.

Timing of cART Initiation in Pregnancy

A single UK centre cohort study (n=211) showed that initiating cART in pregnancy was associated with the highest risk of PTD compared to those women who conceived on cART or received NRTI therapy alone (aOR of 5.03 (95%CI 1.4-17.8)).[30] A similar finding in the WITS cohort described above, where late use of non-ZDV containing ART associated with PTD (aOR 7.9), when compared to no therapy. This group also showed that late ART that included ZDV, either as mono, dual or cART was protective, (aOR 0.06 (95%CI 0.02-0.18)).[10] Conversely Machado *et al.* and other groups have shown the opposite, that increasing length of exposure, specifically preconception cART exposure, is associated with a two- to five-fold risk of PTD.[25,31]

Guideline	WHO 2010[4]	EACS 2009[7]	BHIVA 2008[5]	NIH 2010[6]
Preconception cART	Continue	Continue Switch from EFV	Continue	Continue Switch from EFV
When to start For health CD4 cell count/ Cells/mm^3	350 or WHO clinical stage 3 or 4	350-500	350	500
What to start	NNRTI based cART	ZDV containing cART	PI or NNRTI cART + ZDV+3TC	PI or NVP if CD4 <250 +2 NRTIs inc ZDV
What to avoid	EFV 1st trimester	EFV 1st trimester NVP initiation Triple NRTI D4T+DDI	EFV 1st trimester D4T+DDI Dual NRTI	EFV 1st trimester D4T+DDI
PMTCT alone Option 1 Option 2 Option 3	ZDV mono LPN/r or EFV based cART ZDV+3TC+ABC	PI or NNRTI + 2 NRTIs inc ZDV	ZDV mono (VL<10000) Boosted PI +ZDV+3TC	cART + 2 NRTIs inc ZDV ZDV mono (VL<1000) ZDV+3TC+ABC (VL<100,000)
Mode of delivery	-	PLCS 38/40 if detectable VL	PLCS 38/40 if delectable VL or ZDV mono	PLCS 38/40 if VL >1000
Intrapartum	Yes Recommendation varies with maternal ART	IV ZDV if VL detectable	IV ZDV if VL detectable	IV ZDV
Infant feeding	Breastfeeding (+PEP) or formula	-	Exclusive formula	Exclusive formula
Infant PEP	Yes	-	Yes	Yes

WHO=World Health Organisation; EACS=European AIDS Clinical Society; BHIVA=British HIV Association; NIH=National Institutes of Health; EFV=Efavarinz; NNRTI=non-nucleoside reverse transcriptase inhibitor; ZDV=Zidovudine; PI=protease inhinitor; NVP=Nevirapine; NRTI=nucleoside reverse transcriptase inhibitor; D4T=Stavudine; DDI=Didanosine; LPN/r=Lopinavir/ritonavir; 3TC=Lamivudine; ABC=Abacavir; VL=viral load; PLCS=pre-labour caesarian section; PEP=post exposure prophylaxis

Table 1. Summary of WHO, European, UK and US Guidelines for PMTCT.

Reference	Study Period	Region	Mother-child Pairs (n)	Design	ART Class (n)	PTD rate (%)	Treatment Comparison	aOR	(95% CI)	PTD Association
Rudin *et al.* HIV Med 2011[49]	1986-2007	Switzerland	1180	Prospective cohort	cART 409 ZDV mono + Dual NRTI 147 No ART 624	24 20 15	cART ZDV mono + Dual NRTI vs no ART	2.46 1.75 1.00	1.41–4.28 0.85–3.62	cART
Townsend *et al.* BJOG 2010[14]	1990-2006	Europe and USA	19585	3 Prospective observational studies	cART 9005 ZDV mono 4323 Dual NRTI 1481 No ART 4537	15 - 13 -	cART vs Dual NRTI	1.49 1	1.19-1.87	cART
Grosch-Woerner *et al.* HIV Med 2008[27]	1995-2001	Germany Austria	183	Prospective cohort	cART 75 ZDV mono 76 Dual NRTI 32	39 26 44	PI cART Non-PI cART Dual NRTI vs ZDV mono	3.40 0.89 1.57 1	1.13-10.2 0.38-2.12 0.38-1.28	PI cART
Townsend *et al.* AIDS 2007[13]	1990-2005	UK	4939	National surveillance register	cART 3384 ZDV mono 904 Dual NRTI 157 No ART 494	14 10 - 16	cART vs ZDV mono + Dual NRTI	1.51	1.19-1.93	cART
Martin *et al.* JID 2007[30]	1995-2006	UK	211	Prospective cohort	cART 75 ZDV mono 52	17 6	New cART vs Pre-conception + ZDV mono	5.03 1	1.4-17.8	New cART
Ravizza *et al.* JID 2007[26]	2001-2006	Italy	419	Prospective cohort	cART 366 ---PI cART* 167 ---Non-PI cART 199 No ART 53	 32 18	PI cART vs Non-PI +No ART	2.81 1	1.46-5.39	PI cART
Boer *et al.* BJOG 2006[28]	1997-2003	Netherlands	143 HIV 196 non-HIV	Case control study (included HIV + 98 (1:2 age matched))	cART 143 HIV- controls 196	16 9	HIV vs non- HIV cART 1st trimester vs no cART 1st trimester	2.24 2.24	1.12-4.47 - (p=0.19)	NSIG
Thorne *et al.* AIDS 2004[31]	1986-2004	Europe	4372	Prospective cohort (included 2279 in logistic regression)	cART 757 ---New cART 446 ---Pre-conception cART 321 ZDV mono+Dual NRTI 568 No ART 944	26 - - 16	 New cART Pre-conception cART vs ZDVmono+dual NRTI No ART	1.88 2.05 1 1.01	- 1.34-2.65 1.43–2.95 0.71-1.41	cART
ECS AIDS 2000[17]	1986-2000	Europe	3920	Prospective cohort	cART 323 ---PI-cART 108 ZDV mono 573 No ART	32 17 16	PI cART Non-PI cART ZDV mono vs No ART Pre-conception cART	2.6 1.82 1.03 1 2.17	1.43-4.75 1.13-2.92 0.70-1.50 1.03-4.58	PI cART Pre-conception cART
Patel *et al.* JID 2010[21]	2002-2008	USA	777	Prospective cohort	cART 760 ---PI cART 558 ZDV mono 6 Dual NRTI 11	PI cART 18 Non-PI ART 13	PI cART vs non-PI	1.29	0.77-2.15	NSIG
Schulte *et al.* Peds 2007[22]	1989-2004	USA	11231	Prospective cohort	cART 2563 ---PI cART 782 ZDV mono 2621 Dual NRTI 2312 No ART 2565	18 7 17 14 21	PI cART vs Dual NRTI	1.21	1.04-1.40	PI cART
Kourtis *et al.* AIDS 2007[32]	1984-2004	International	20426	Meta-analysis	Not stated	Not calculated	cART PI cART ZDV mono vs No ART PI cART vs non-PI ART Pre-conception+1st trimester cART vs 2nd +3rd	1.13 1.35 0.86 1 1.35 1.71	0.79–1.63 1.08-1.70 0.73–1.01 1.08–1.70 1.09–2.67	Pre-conception cART
Cotter *et al.* JID 2006[20]	1990-2002	USA	1337	Prospective cohort	cART 507 ---PI cART 143 ZDV mono 492 No ART 338	30 37 24 26	PI cART vs non-PI ART	1.8	1.1–3.0	PI cART
Tuomala *et al.* AIDS 2005[10]	1990-2002	USA	2543	Prospective cohort	cART 588 ZDV mono 932 Dual NRTI 258 No ART 751	Not stated	Late ART with out ZDV Late ART containing ZDV	7.86 0.53	1.39-44.58 0.34-0.83	Late use ART with out ZDV
Tuomala *et al.* NEJM 2002[19]	1990-1998	USA	3266	2 clinical trials 5 Prospective cohorts	cART 533 ---PI cART 37 ZDV mono 1590 No ART 1143	15 18 16 20	cART PI cART vs ZDV mono	1.08 1.45	0.71-1.62 0.81-2.50	NSIG
Machado *et al.* STDS 2009[25]	1996-2006	Brazil	696	Prospective cohort	cART 305 PI cART 193 ZDV mono 179 Dual NRTI 212	- 16 12 8	Pre-conception cART vs Dual NRTI	5.06	1.5-17.06	Pre-conception cART
Szyld *et al.* AIDS 2006[24]	2002-2005	Latin America & Carribean	681	Prospective cohort	cART 587 ---PI cART 330 ZDV mono+Dual NRTI 94	9 11 6	PI cART vs ZDV mono+Dual NRTI	1.1	0.5-2.8	NSIG
Shapiro *et al.*** NEJM 2010[3]	2006-2008	Botswana	530	RCT CD4>200 26-34 weeks at initiation	267 LPN/r/ZDV/3TC 263 ZDV/3TC/ABC	21 12	PI cART vs triple NRTI	2.02**	1.25-3.27	PI cART

*3rd trimester use ** additional analysis[33]
ART=antiretroviral therapy; PTD=preterm delivery; ZDV mono=Zidovudine monotherapy; vs=versus; Dual NRTI= Dual nucleoside reverse transcriptase inhibitor; PI= Protease inhibitor; NSIG=non significance; LPN/r=Lopinavir/ritonavir; 3TC=Lamuvudine; ABC=Abacavir

Table 2. Summary of most recent analyses for studies evaluating the association between PTD and antiretroviral use in HIV-1-infected pregnancies.

Meta-analyses and Pooled Data

Kourtis *et al.* conducted a large meta-analysis including prospective and retrospective data from the US, Europe and South America (n=20426) from 1984-2004 to answer the question; is cART use in pregnancy is associated with PTD?[32] No overall increased risk of PTD was seen for cART exposure, including dual NRTIs, when compared to no therapy (OR 1.13 (95% CI 0.79-1.63)). However associations with PTD were reported for pre-conception/first trimester cART exposure vs. second/third trimester exposure (OR 1.71 (95% CI 1.09-2.67)) and for PI-based therapy vs. non PI based (OR 1.35 (95% CI 1.08-1.70)).[32] The authors concluded that cART in pregnancy did not increase the overall risk of PTD.

To directly address conflicting results obtained from US and European data Townsend *et al.* published a pooled analysis of the ECS, NSHPC and the US Paediatric Spectrum of HIV Disease project (PSD) (n=19585 mother-child pairs), collated from 1990-2006, 46% having received antiretrovirals.[14] Comparison was made between cART and dual NRTI therapy rather than NRTI monotherapy due to heterogeneity across the cohorts. A significant association between cART and PTD vs. dual NRTI therapy was identified (aOR 1.49 (95%CI 1.19-1.87)) see Table 2.

Why Do These Results Differ?

The data presented demonstrate the disparity of the results in this field and their interpretation. The studies are heterogeneous in their size and methodology including selection of mother-child pairs from clinical trials, prospective cohorts and retrospective case control designs. Outcomes may vary by ethnicity, access to healthcare and prevalence of PTD in the background population.

Use of different comparator groups eg. mono/dual NRTI therapy, grouped non-PI therapy, preconception vs. post conception antiretroviral therapy and no therapy makes extrapolation of data for meta-analysis difficult. Adjustment for confounding factors for PTD such as prior PTD, pre-eclampsia, HIV disease progression, other sexually transmitted infections varied between analyses and may further contribute to the observed differences.

The Mma Bana study was the first RCT to address the impact of a boosted PI (lopinavir-ritonavir plus zidovudine-lamivudine) vs. a triple NRTI based regimen (abacavir-zidovudine-lamivudine) and PTD.[3] cART was initiated from 26-34 weeks gestation in women with CD4 cell counts>200 cell/mm^3 in Botswana (n=530). A two-fold risk of PTD was reported in the PI-based cART arm (aOR 2.02 (95% CI 1.25-3.27)).[33] Preterm delivery was associated with higher rates of infant hospitalisation and mortality.

A Possible Underlying Mechanism

To date, only one study has evaluated a possible underlying mechanism for this association. During term pregnancy in the general population, cytokine balance shifts from Th1 to Th2 and the reverse is associated with PTD.[34] Fiore *et al.* showed that 49 HIV-infected pregnant women, 26 of whom were receiving ART, had a reverse shift pattern from Th2 to Th1 cytokine response across gestation, with and increasing Th1 (IL2)/Th2 (IL10) ratio value across gestation.[35] They were able to demonstrate that IL 10 levels were more likely to decrease across gestation in those receiving cART vs. no therapy and that each unit increase in the IL2 slope was associated with an 8% increase in the risk of premature delivery. The hypothesis that an immunological process underlies the increased rates of PTD in HIV women has yet to be proven but is currently under investigation.

Discussion: Does PTD Matter?

Worldwide an estimated 13 million infants are born before 37 weeks gestation each year, with the burden of PTD, 85% or 11 million cases, seen in low and middle income countries mirroring the burden of HIV disease.[36,37] In this setting at the end of 2009, 51% of women who tested HIV-positive in pregnancy required treatment for their own health, although only 15% received cART.[38] As access to treatment improves, and thresholds for initiating therapy rise to CD4 counts<350 cells/mm^3, increasing numbers of women will conceive on cART, with a rising proportion receiving boosted PIs following first line failure of NNRTI based therapy in resource poor settings.[4] Whilst the enormous benefit of cART, both for maternal health and in PMTCT cannot be underestimated, potential increased rates of PTD are of concern.

Prematurity is the leading cause of neonatal death globally accounting directly for approximately 1 million, or 27% of neonatal deaths annually.[36] In addition prematurity is a major risk factor in deaths due to neonatal sepsis. Whilst globally mortality for children under the age of five has fallen, the proportion occurring during the neonatal period has risen to more than 42%.[36] For survivors, premature delivery results in an increased risk of adverse neurological and respiratory outcomes that frequently persist into later life resulting in significant physical, psychological and economic cost.

In resourced settings whilst survival for infants born after 32 weeks approach those near term, increased rates of mortality and cerebral palsy are reported for those delivered at 28-31 weeks gestation (OR 8.8 95% CI 8.0-9.7).[39] Recent data suggests comparable outcomes for infants born after 28 weeks gestation in some middle income settings, although mortality remains higher than in resourced settings for extreme prematurity (<28 weeks).[40] Even infants born between 34-36 weeks gestation in resourced settings have an increased risk of behavioural problems and of having an IQ<85 when compared to a low risk neonatal population born at more than 37 weeks gestation.[41] Data from resource poor settings is frequently complicated by issues of accuracy in assessing gestational age, however in rural Malawi infants born after 32 and before 37 weeks gestation, dated by ultrasound, had twice the risk of death compared to term infants. For infants born between 24 and 33 weeks mortality was 75%.[42]

Co-infection with malaria and /or Tuberculosis (TB) further complicate maternal and foetal outcomes. The majority of women living with HIV reside in areas where malaria is endemic. Pregnant women are more likely to be infected with malaria with increased risk of adverse neonatal outcomes including prematurity and infantile anaemia that may be further amplified by neonatal exposure to ART.[43] The intertwined epidemics of HIV and TB further increase adverse maternal and neonatal outcomes with increased rates of premature delivery in women with tuberculose disease.[44]

The difficulties in balancing PMTCT of HIV, whilst ensuring optimal outcomes for infants in different settings has been demonstrated for infant feeding in the context of HIV.[45] Whilst reducing postnatal transmission of HIV, formula feeding, the standard of care in resourced settings, has been associated with increased infant mortality and morbidity in both HIV exposed and infected infants when compared to exclusive breast-feeding in resource poor settings.[46] Similarly, for infants born in resourced areas with access to neonatal intensive care, a small increase in rates of PTD may have little or no effect on neonatal outcomes. However the majority of infants born to women living with HIV have minimal access to neonatal services and even a small increase in PTD may have a negative impact on infant mortality and morbidity.

Conclusion

The benefit of antiretroviral therapy in the PMTCT of HIV-1 should not be underestimated, however there is mounting evidence implicating cART in the excess PTD seen in HIV-infected women when compared to mono/dual or triple NRTI therapy. Interpretation of this evidence differs between guideline bodies, with those recommending ZDV monotherapy as an option where PMTCT is the single aim, placing greater weight on the potential adverse effects of cART. Treatment options solely for PMTCT are complex; concerns include efavirenz and tetratagenicity,[47,48] nevirapine and hepatic dysfunction, the NNRTI tail and evolution of resistance and boosted protease inhibitors with glucose intolerance and PTD.[5] The role of triple nucleoside regimens, used uniquely for PMTCT is a potential option for consideration for women with low viral loads.[4,6] Further well-designed clinical studies are required to assess the optimum drug regime for PMTCT, weighing up the risk benefit for both mother and infant that may vary according to their setting.

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