Impact of Injectable Hormonal Contraceptives on Innate Immune Environment in the Genital Tract in Women at High Risk for HIV-1 Infection

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Preface

The experimental work described in this dissertation was performed in the CAPRISA Mucosal Laboratory, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa; University of Cape Town, Cape Town, South Africa and Nortwestern University, Chicago, United States of America. This work was co-supervised by Prof Quarraisha Abdool Karim and Dr JoAnn S. Passmore. This study represents original work by the author and has not been submitted in any form for any degree or diploma to any other university. When the work of others has been used, it is clearly acknowledged in the text.

Sinaye Ngcapu (Student)

Prof Quarraisha Abdool Karim

Dr JoAnn S. Passmore

Declaration

I Sinaye Ngcapu declare that:

(i) The research reported in this thesis, except where otherwise indicated, is my original work.

(ii) This thesis has not been submitted for any degree or examination at any other university.

(iii) This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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Sinaye Ngcapu

Ethics Approval

Full ethics approval, from the Biomedical Research Ethics Committee of the Nelson R. Mandela School of Medicine, University of KwaZulu-Natal (Ref: E111/06 and E013/04) was obtained for this study.

Presentations and Publications

1. **Sinaye Ngcapu**, Tracy Meiring, Lindi Masson, Lise Werner, Lenine Liebenberg, Nigel Garrett, Koleka Mlisana, Carolyn Williamson, Quarraisha Abdool Karim, Salim Abdool Karim, Jo-Ann Passmore. *Presence of Male Partner Semen Influences the Inflammatory and Innate Cytokine Environment in the Female Genital Tract.* HIV Research for Prevention (HIV R4P) Conference 2014, Cape Town, South Africa. (Poster presentation).

2. **Sinaye Ngcapu**, Lindi Masson, Sengeziwe Sibeko, Muki Shey, Natasha Samsunder, Salim Abdool Karim, Quarraisha Abdool Karim, Jo-Ann Passmore. Hormonal contraception use and HIV-1 risk in the context of prevention research*: DMPA and NET-EN dampen immunity in the female genital tract.* AIDS Vaccine Conference. 2013, 7-10 October in Barcelona, Spain. (Poster presentation).

3. **Ngcapu S**, Masson L, Sibeko S, Werner L, McKinnon LR, Mlisana K, Shey M, Samsunder N, Abdool Karim SS, Abdool Karim Q, Passmore J-AS. *Lower concentrations of chemotactic cytokines and soluble innate factors in the lower female genital tract associated with the use of injectable hormonal contraceptive.* Journal of Reproductive Immunology 2015; 110 (2015) 14–21.

Dedication

I dedicate this thesis to **Miss Noludumo "Tsili" Eunice Ngcapu**, my mother and the wind beneath my wings; for her bravery, strength and faith, and to Jesus, I could not have done this without you.

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Abbreviations

μ	Micro
α	Alpha
γ	Gamma
β	Beta
x g	G force
AIDS	Acquired immunodeficiency syndrome
BV	Bacterial vaginosis
CAPRISA	Centre for the AIDS Programme of Research in South Africa
CCR	C-C chemokine receptor
CD	Cluster of differentiation
Chromo	Chromosome
COC	Combined oral contraceptive
CpG	Cytosine-guanine
CTACK	Cutaneous T-cell attracting chemokine
CVL	Cervicovaginal lavages
CVS	Cervicovaginal secretion
DAPI	Diamidino phenylindole
DC	Dendritic cells
DMPA	Depot medroxyprogesterone acetate
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FGF	Fibroblast growth factor
FGT	Female genital tract
FLT3	FMS-like tyrosine kinase 3
FSH	Follicle stimulating hormone
G-CSF	Granulocyte-colony stimulating factor
GCM	Gonorrhea-Chlamydia-Mycoplasma
GM-CSF	Granulocyte macrophage-colony stimulating factor
GRO	Growth related oncogene
H_2O_2	Hydrogen peroxide
HC	Hormonal contraceptive
H&E	Hematoxylin and eosin
HGF	Hepatocyte growth factor
HIV-1	Human immunodeficiency virus
HLA-DR	Human leukocyte antigen-D related
HR	Hazard ratio
HSV	Herpes simplex virus
IFN	Interferon
IDL	Interface description language
IL	Interleukin
IP	Induced protein
IQR	Interquatile range
IRR	Incidence rate ratio

IUD	Intrauterine devices
KLK3	Kallikrein-3
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
MCP	Monocyte chemoattractant protein
MDC	Macrophage-derived chemokine
mDC	Myeloid dendritic cells
MIF	Macrophage migration inhibitory factor
MIG	Monokine induced by gamma-Interferon
MIP	Macrophage inflammatory protein
mm^2	Millimeter squared
MMP	Matrix metalloproteinases
MSM	Marginal structural model
NET-EN	Norethisterone enanthate
NGF	Nerve growth factor
NHLS	National Health Laboratory Service
nM	Nano Molar
OCP	Oral contraceptive pill
OCT	Optimum cutting temperature
OR	Odds ratio
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PDGF	Platelet-derived growth factor
PGE2	Prostaglandin-E2
PL	Parameter logistic
PSA	Prostate specific antigen
qPCR	Real time PCR
RH	Risk hazard
SAS	Statistical Analysis System
sCD40L	soluble cluster of differentiation
SCF	Stem cell factor
SCGF	Stem cell growth factor
SDF	Stromal derived factor
sIL	soluble interleukin
SLP	Secretory leukocyte protease
SIV	Simian immunodeficiency viruses
SRY	Sex-determining region
STI	Sexually transmitted infections
TGF	Transforming growth factor
TIMP	Tissue inhibitors of metalloproteinases
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis inducing ligand

TSPY	Testis-specific protein Y-encoded
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

Abstract

Background: The impact of injectable hormonal contraceptives (HCs) on HIV-1 acquisition risk in HIV-1 negative women represents an important public health issue, as is the risk of transmission from HIV-infected women to their HIV discordant partners. Women using the long-acting injectable depot medroxyprogesterone acetate (DMPA) are reportedly at 2-fold increased risk for HIV-1 infection from epidemiology studies, and at an increased risk of transmitting HIV to their sexual partners should they become infected. The aims of this PhD dissertation were to investigate possible biological mechanisms influencing HIV risk, by evaluating (i) the effects of injectable HC use on soluble immune mediators in the lower reproductive tract of HIV-1 negative women at high risk for HIV-1 acquisition, particularly in response to common sexually transmitted infections (STIs) (Chapter 3); (ii) the impact of recent sexual intercourse [measured by presence of Y-chromosome DNA or prostate specific antigen (PSA)] on these soluble mucosal factors (Chapter 4); and (iii) the *in vivo* and *in vitro* influence of DMPA use on activation status and functional characteristics of circulating dendritic cells (Chapter 5). Following HIV-1 infection, this dissertation investigated the impact of injectable HC use on (iv) vaginal epithelial barrier thickness and HIV-1 target cell density in vaginal biopsies taken from acutely HIV-1 infected women; (v) genital viral loads contributing to risk of HIV-1 transmission to sexual partners during acute infection and (vi) rate of HIV-1 disease progression (Chapter 6).

Methods: For studies involving HIV-1 negative women at risk for HIV-1 infection, Chapters 3 to 5 included 64 HIV-1 uninfected women using injectable HCs (DMPA or NET-EN) and 64 women not using any form of HCs, enrolled into the prospective CAPRISA 002 observational cohort study. The concentrations of 48 cytokines, 5 matrix metalloproteases

(MMPs), and 4 tissue inhibitors of matrix metalloproteases (TIMPs) were measured in cervicovaginal lavage (CVL) using Luminex and ELISA. CVLs were assessed for the presence of semen, measured by presence of Y-chromosome DNA by real-time PCR and prostate specific antigen (PSA) by ELISA, to confirm semen exposure as a surrogate for recent sexual intercourse (Chapter 4). Finally, for studies involving HIV-1 infected women, 49 acutely HIV-1 infected women who became infected during the CAPRISA 004 1% tenofovir microbicide trial were included (36 DMPA users, 5 NET-EN users and 8 controls not using injectable HCs) (Chapter 6). Immunohistochemical staining was used to assess epithelial thickness and target cell densities biopsy specimens. Blood CD4 T cell counts, and viral loads were measured in both CVL and plasma samples during acute infection and in plasma during the first year of infection (as a measure of disease progression). The concentrations of 48 cytokines were measured in CVL from acutely HIV-1 infected women using Luminex.

Results: In Chapter 3, HIV-1 negative women using injectable HCs were found to have significantly lower concentrations of several chemokines (eotaxin, MCP-1, MDC), the adaptive cytokine IL-15, the growth factor PDGF-AA, and the tissue inhibitor of metalloproteinases TIMP-2 (responsible for tissue remodelling) in genital secretions (CVLs) than women not using injectable HCs (controls), after adjusting for condom use, sex work status, marital status, BV and STIs. In Chapter 4, CVLs collected from these women who were positive for PSA (indicating exposure to semen within 72 hours) were shown to have significantly lower concentrations of IL-12p70, sIL-2Rα and IL-2, indicating that certain innate responses in the genital tract were suppressed by exposure to semen. In contrast, concentrations of TIMP-1 and MMP-9, both associated with tissue remodelling in the female

reproductive tract, were higher in CVLs from PSA positive women, after adjusting for confounders.

I*n vitro* and *in vivo* experiments focusing on monocytes and dendritic cells (DCs) in Chapter 5 showed that exogenous addition of DMPA was associated with decreased capacity of circulating myeloid DCs to produce TNF-α and IL-6 in response to Toll-like receptor (TLR)-4 (CpG) and -9 (LPS) agonists. Finally, focusing on acutely HIV-1 infected women, Chapter 6 showed that injectable HC use did not influence vaginal epithelial thickness notably, with women using injectable HCs having a median thickness of 356.9 μm/cc (IQR 302.0-414.2) compared to women using no or non-injectable HC users (361.4; 271.0-409.4). However, the frequencies of target CD4+ T cells in the squamous epithelium from injectable HC users were significantly higher than non-injectable HC users (median of 33 cells/mm^2, IQR 23-74 versus 23 cells/mm^2, IQR 17-43, p=0.028]. Other HIV-1 target cells, like CD68+ macrophages were closer to the surface of the vaginal lumen in HIV-1 acutely infected women using injectable HC compared to non-injectable HC users (median 158 μm, IQR 131.8 – 163.8 versus 97 μm, 81.3 – 117.1; p=0.021), although CD68+ target cell density did not differ between groups. Injectable HC use was not associated with higher genital HIV-1 viral loads or influence the rate of disease progression, measured by CD4 T cell counts at 12 months post-infection or plasma viral load set-point.

Conclusion: Overall, the findings from this dissertation suggest that injectable HC use may have immunosuppressive effects in lower reproductive tract of women, altering the innate immune profile through the suppression of dendritic cell activation and function. The presence of semen should be taken into consideration when investigating biological markers

in the female genital tract, since semen has the capacity to modulate host responses. Although injectable HC use was not found to influence vaginal epithelial thickness in this study, evidence of immune cell regulation for intraepithelial and mucosally-associated CD4+ and CD68+ target cells was found. Together, these findings suggest that more mechanistic studies are urgently needed on DMPA and other HCs (such as NET-EN), using *ex vivo* and *in vivo* models, in order to inform their use at public health level particularly in those settings with high rates of incident HIV infection.

CHAPTER 1

Introduction

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Introduction

1.1 Young women: vulnerability to HIV-1 infection and the epidemic

Almost seventy percent of the 35.3 million people globally infected with HIV-1 reside in sub-Saharan Africa, with women accounting for about 60% of these infections (UNAIDS, 2013). Heterosexual transmission of HIV-1 across the genital mucosa in women is the most common mode of infection (Abdool Karim et al., 2010b; Shattock and Moore, 2003; UNAIDS, 2013). In particular, young women (15–24 years) are much more likely to acquire HIV-1 than older women (Stirling et al., 2008). Young women acquire HIV-1 infection 5-7 years before men and they are eight times more likely to be infected than their male counterparts (Abdool Karim et al., 1992; UNAIDS, 2012).

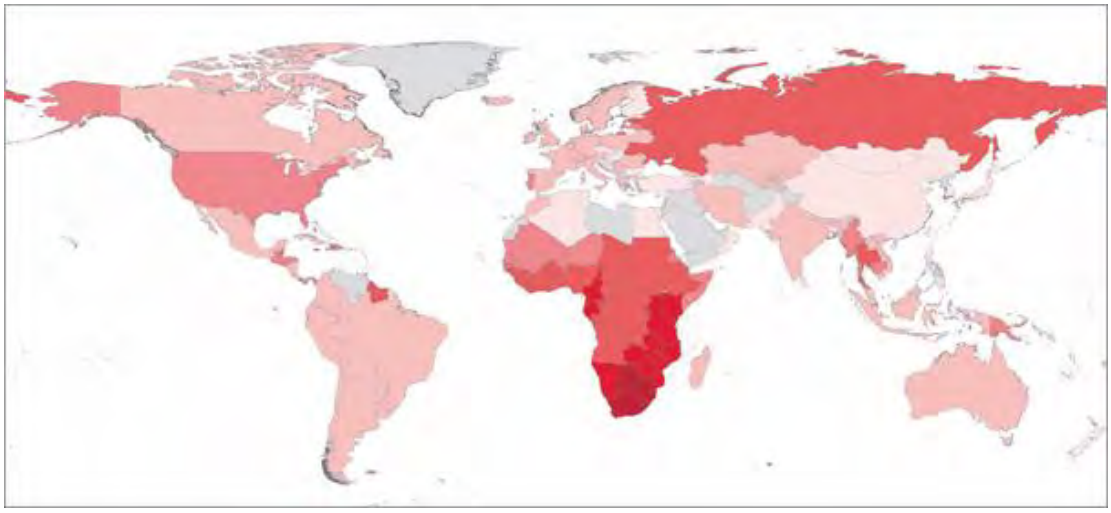


Figure 1.1 Global prevalence of HIV-1 in 2013 according to geographic region. The darker colors indicate regions with higher prevalence. Approximately, 70% of the 35.3 million people infected with HIV-1 worldwide were concentrated in sub-Saharan African countries. *Source: www.unaids.org. UNAIDS Fact sheet, December 2014.*

The unique vulnerability of young women to HIV infection stems from various behavioural and biological factors. While risk for HIV-1 infection may vary from person to person, behavioural factors including unprotected sex, douching after intercourse, sexual relationships with older partners, and a history of sexual abuse or violence are well documented risk factors for HIV-1 infection in young women (Abdool Karim et al., 2014; Chersich and Rees, 2008; Dunkle et al., 2004; Stirling et al., 2008). In addition, fewer years of schooling and lack of food security have also been documented as risk factors driving HIV-1 acquistion in young women who engage in transactional relationships with older men (Abdool Karim et al., 2014; Greif, 2012). Studies in resource-limited settings have demonstrated that sexual relationships with older men can expose young women to an increased risk of HIV-1 infection as they are less likely to be able to negotiate condom use (Gregson et al., 2002; Pettifor et al., 2004).

It is thought that young women become infected with HIV-1 after fewer acts of unprotected sex (Glynn et al., 2001; Pettifor et al., 2005), and several biological risk factors that may predispose women to become infected with HIV-1 have been proposed (Chersich and Rees, 2008). The surface area of the lower reproductive tract exposed during sexual intercourse in women is greater than the reproductive tissue of men, which may increase surface area exposed, time in contact with infectious fluids post-coitus, and exposure of intraepithelial HIV-1 target cells to pathogens (Yi et al., 2013). Microabrasions in the genital tract, in the comparatively more fragile and less keratinized epithelial barrier, may be more common in women than men and provide

a portal for pathogen entry (Stanley, 2009). Cervical ectopy (particularly to younger women) may facilitate increased exposure of HIV-1 target cells to pathogens in the lower reproductive tract during sex (Critchlow et al., 1995). Finally, the presence of co-factors such as sexually transmitted infections (STIs), often asymptomatic and therefore untreated in women, also increase the risk of HIV-1 infection (Cohen, 2004; McKinnon et al., 2011).

1.2 Hormone contraceptives (HCs) and HIV risk in women

Unprotected heterosexual intercourse is the predominant mode of HIV acquisition which also puts young women at risk of unplanned pregnancies (Bearinger et al., 2007; UNAIDS, 2013). HIV-1 is linked to general reproductive health issues in women because many new HIV-1 infections occur during sex (Bearinger et al., 2007). Pregnant women generally have higher HIV-1 prevalence rates than non-pregnant women (39% HIV-1 prevelence in 15-24 year old pregnant women from sub-Saharan Africa compared to 20% in similarly aged non-pregnant women) (Berer, 2004; Department of Health, 2013; Kharsany et al., 2015). Access to safe, effective methods of hormonal contraception (HC) is an essential strategy in women for reducing unwanted pregnancies, and vertical transmission of HIV-1 from infected mothers to their newborn infants, especially in areas of high HIV-1 seroprevalence such as sub-Saharan Africa (Baru, 1993; Berer, 2004).

A number of HC methods are available to prevent unplanned pregnancies, and it is estimated that >150 million women use HC globally, with two thirds of women on combine oral contraceptives (COCs) and a third on long-acting injectable progestin

HCs (Population, 2008; United Nations, 2003). The most commonly used HC in sub-Saharan Africa is depot medroxyprogesterone acetate (DMPA), although use of more modern version of the injectable progestin called norethisterone enanthate (NET-EN) is increasing, especially among young women in South Africa (Department of Health et al., 2007; Sibeko et al., 2011). DMPA use is popular in sub-Saharan Africa because it is cheap, safe, provides contraception that is long acting and highly effective, and does not require a woman to remember to take birth control daily (Department of Health, 2001; Margulies and Miller, 2001). Although the use of injectable HCs has explicit gains in terms of maternal-infant health (WHO, 2015), long acting high-dose progestin formulations (such as DMPA) have also been associated with an increased risk for HIV-1 infection (Baeten et al., 2007b; Heffron et al., 2012; Martin et al., 1998; Morrison et al., 2010). A recent systemic review summarizing findings from several good quality studies (Ralph et al., 2015) and an individual participant data meta-analysis (Morrison et al., 2015) have reported increased HIV-1 risk in women using DMPA and NET-EN compared those not using hormonal contraception. Although the meta-analysis showed increased HIV-1 risk, several individual studies reported no increased risk in women using DMPA compared to non-DMPA using counterparts (Kiddugavu et al., 2003; Kleinschmidt et al., 2007; Myer et al., 2007; Reid et al., 2010; Stringer et al., 2009). Most studies found no evidence that NET-EN use increases women's risk of HIV risk (McCoy et al., 2013; Myer et al., 2007).

1.2.1 Biologic mechanisms by which HCs increase HIV-1 acquisition risk

The mechanism by which injectable HCs may increase risk to HIV-1 infection is not clearly understood. Some studies have found that injectable HCs reduce the integrity and thickness of the genital mucosal barrier in women and the density of intracellular

junction proteins in the stratefied epithelial layer (Chandra et al., 2013; Ildgruben et al., 2003; Miller et al., 2000; Wieser et al., 2001; Wira et al., 2011b). Reduced genital barrier function might facilitate more efficient contact between HIV-1 target cells within the cervicovaginal mucosa and HIV-1 particles entering the vaginal lumen. In non-human primates, high-dose DMPA is commonly used in the simian immunodeficiency virus (SIV) vaginal challenge model, specifically because it thins the vaginal epithelium and significantly increases the efficiency of vaginal transmission (Abel et al., 2004; Marx et al., 1996; Trunova et al., 2006; Wieser et al., 2001). The role of DMPA in increasing risk of HIV-1 infection in women is less clear. Little consensus exists about mucosal epithelial thickness with some reports claiming women who use DMPA have detectably thinner genital epithelial barriers compared to non-DMPA users (Kiddugavu et al., 2003; Myer et al., 2007), while other have found no association between HC use and genital epithelial barrier integrity (Chandra et al., 2013; Mitchell et al., 2014).

DMPA has been reported to alter cervicovaginal mucosal immune responses that could affect mucosal susceptibility to HIV-1 transmission, through increased recruitment of mucosal immune cells of the female genital mucosa (Chandra et al., 2013, Wira et al., 2011; (Ildgruben et al., 2003; Miller et al., 2000; Wieser et al., 2001; Wira et al., 2011b; Wira and Veronese, 2011). Chandra et al. (2013) showed that women using DMPA had significantly higher numbers of activated T cells and macrophages expressing CCR5 compared to their non-DMPA counterparts. In contrast, Mitchell et al. (2014) found no difference in density or numbers of T cells or $CCR5^{+}$ HIV-1 target cells in the lower reproductive tract. I*n vitro* experiments showed that peripheral blood mononuclear cells (PBMCs) from women using DMPA

had higher rates of HIV-1 replication than PBMCs from women not using DMPA, and had higher concentrations of HIV-1 co-receptors CXCR4 and CCR5 on activated T cells (Huijbregts et al., 2013).

DMPA use suppresses both innate and adaptive immune responses and thereby potentially reduces host resistance to the infection with HIV-1 and other pathogens (Huijbregts et al., 2013; Kleynhans et al., 2013). DMPA treatment of PBMCs *in vitro* reduced production of several inflammatory and adaptive cytokines [including interleukin (IL)-6, IL-1β, IL-12p40, IL-12p70, tumor necrosis factor (TNF)-α, TNF-β, granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein (MIP)-1α, interferon (IFN)-γ, IL-2, IL-4, IL-13, IL-17 (Huijbregts et al., 2013)] (Huijbregts et al., 2014). DMPA also reduced the production of IFN-α and TNF-α by plasmacytoid dendritic cells (pDCs) in response to activation by ligands of Toll-like receptors (TLRs)-7, -8 and -9 (Huijbregts et al., 2014). PBMCs from DMPA users stimulated with a common antigen such as BCG (vaccine against tuberculosis) produced significantly lower concentrations of IL-1α, IL-12p40, IL-10, IL-13 and G-CSF compared to non-DMPA users (Kleynhans et al., 2011). Similarly, mice treated with DMPA produced less IFN-γ, IL-6, IL-17, GM-CSF, monocyte chemotactic protein 1 (MCP-1), granulocyte colony-stimulating factor (G-CSF), TNF-α, IL-10, and IL-6 (Kleynhans et al., 2013). Although the *in vivo* and *in vitro* biological effects of DMPA have been investigated, comparatively fewer mechanistic studies have been conducted using NET-EN.

A potential mechanism by which DMPA may decrease expression of cytokines is through its ability to bind to the glucocorticoid receptor complex, as some cytokine genes are regulated by the glucocorticoid receptor. Although some studies have shown a comparatively lower binding affinity of NET to the glucocorticoid receptor compared to DMPA (Koubovec et al., 2005; Schoonen et al., 2000), both are capable of binding with high affinity (Kontula et al., 1983; Koubovec et al., 2005). *Ex vivo* studies also demonstrated that DMPA acts as full to partial agonist of the glucocorticoid receptor, more potent and effective on gene promoter-mediating immune functions than progesterone .

Injectable HCs may also modulate the host environment by altering the vaginal microbiome or susceptibility to bacterial vaginosis (BV) and sexually transmitted infections (STIs), including HPV, *Chlamydia trachomatis, Nessiria gonnorhoeae* (Huijbregts et al., 2013; Kleynhans et al., 2011). The healthy vaginal microbiome is generally dominated by *Lactobacillus* species, which metabolise carbohydrates in the lower reproductive tract to lactic acid, lowering vaginal pH which is inhospitable to pathogens (Ma et al., 2012). When *Lactobacillus* colonization in the reproductive tract declines, the vaginal pH neutralizes, and the risk of HIV-1 infection increases (Cook et al., 1992; Ma et al., 2012). Decreased abundance of *Lactobacillus* spp. may alternatively create an environment that favors the growth of BV-associated organisms (Cook et al., 1992). BV is a highly prevalent dysbiosis in the vagina, characterized by a thin homogeneous white discharge, a vaginal pH >4.5, a positive amine test, and the presence of clue cells microscopically (Amsel et al., 1983; Cook et al., 1992). BV increases risk of HIV-1 infection (Atashili et al., 2008), herpes simplex

virus (HSV) shedding (Cherpes et al., 2005), preterm delivery, and pelvic inflammatory disease (Ness et al., 2005). Microscopy and culture-based studies have suggested that hormonal fluctuations over the course of the menstrual cycle contributes to fluctuation in the vaginal microbiome (Eschenbach et al., 2000). Higher estrogen levels are associated with a lactobacilli-dominated vaginal microbiome, thought to be protective against HIV-1 (van de Wijgert et al., 2013; reviewed by Murphy et al., 2014). The impact of progesterone and its synthetic derivatives DMPA and NET-EN on the vaginal microbiome is less clear.

DMPA use has been associated with increased risk for gonorrhea and chlamydia in US women (Morrison et al., 2004). Conversely, DMPA use was found to reduce risk of BV and *Trichomonas vaginalis* amongst Kenyan sex workers (Baeten et al., 2001). DMPA treatment increased susceptibility to HSV-2 infection 100-fold in the mouse model (Kaushic et al., 2003). Mechanisms that have been suggested to explain this include inhibition of local innate immune responses to HSV-2, increased expression of the HSV-2 entry receptor nectin-1 in a progesterone-dominant environment, or progesterone-associated thinning of the vaginal stratefied epithelium following progesterone treatment (Kaushic et al., 2003). However, a similar relationship between NET-EN and risk for STI infections has not been established.

The mechanisms by which synthetic progesterone-based injectable HCs might increase susceptibility to HIV-1 acquisition are important to investigate, especially in regions where injectable HCs commonly are used and HIV-1 prevelence rates are high, such as sub-Saharan Africa. Because access to effective methods of HC have

substantially reduced maternal and infant mortality rates, through giving women more control over when to have children, negative public perceptions about adverse health risks surrounding a widely used HC could be detrimental to the gains made in improved maternal and child health. Strong, unequivocal evidence needs to be provided and biological mechanisms demonstrated, to definitively inform policy to avoid these unintended negative consequences. The following literature review describes biological mechanisms proposed by which injectable hormonal contraceptive use could influence susceptibility to HIV-1.

1.3 Study Aims and Objectives

The overall aims of this dissertation were to investigate: (i) the biological effect of injectable HCs use on the innate environment in the genital tract of HIV-1 negative women at high risk of HIV-1 infection; and (ii) the impact of injectable HC use on the genital immune environment and epithelial barrier function of HIV-1 infected women and the consequence of these immune changes to their infectiousness to their sexual partners during acute HIV-1 infection.

Rationale

Several HC methods are widely used by South African women to prevent unplanned or mistimed pregnancies, with over 57% of women (aged 15 - 49 years) using long-acting injectable progestin HCs, particularly DMPA (South African Department of Health, 2003). Although the use of DMPA to prevent unplanned pregnancies has explicit gains (WHO, 2010), it has also be associated with an increased risk of HIV-1 infection (Baeten et al. 2007; Martin et al. 1998; Morrison et al. 2010, 2011 and

2012). The precise mechanism by which DMPA may increase risk to HIV-1 infection has not been conclusively demonstrated yet although several studies have suggested that DMPA may reduce integrity and thickness of vaginal and epithelial lining, increase recruitment of HIV-1 target cells to the genital mucosa, and change the vaginal microbiota which may influence HIV-1 risk (Chandra et al. 2013; Ildgruben et al. 2003; Miller et al. 2000; Wieser et al. 2001; Wira et al. 2011). This dissertation focuses on the influence of injectable HC use (DMPA and NET-EN) on risk for HIV-1 infection in the context of a heterosexual epidemic in South Africa; and the potentially confounding effect of injectable HC use in HIV-1 prevention research. If biological plausibility is demonstrable, more informed policy measures could be implemented timeously to avoid these negative consequences.

Specific Objectives 1

To compare concentrations of genital tract soluble immune mediators [including cytokines, chemokines, growth factors, matrix metalloproteinases (MMPs) and tissue-inhibitors of metalloproteinases (TIMPs)] between women using long-acting injectable HCs and women not using HCs, while accounting for BV and common STIs **(Chapter 3)**.

Rationale.

DMPA might increase HIV-1 aquisition risk by changing the inflammatory or chemotactic environment of the genital mucosa so as to influence the recruitment of HIV-1 susceptible immune cells to the mucosa (Ildgruben et al., 2003; Miller et al.,

2000; Wieser et al., 2001; Wira et al., 2011b; Wira and Veronese, 2011). However, *in vitro* treatment of PBMCs with injectable HC was shown to cause reduced production of several inflammatory and adaptive cytokines (Huijbregts et al., 2013). Defining the impact of injectable HCs on female genital tract innate immunity in relation to susceptibility to STIs or BV will provide important insights into biological co-factors influencing HIV-1 risk in women.

Hypothesis.

Injectable HC use is associated with reduced cytokine and soluble factor concentrations in genital secretions compared to non-HC users.

Specific Objective 2

To investigate whether detection of semen in cervicovaginal lavages (CVLs) from high-risk HIV-1 negative women, by assessment of Y-chromosome by PCR and prostate specific antigen (PSA) by ELISA, influenced the cytokine milieu and soluble factors of cervicovaginal secretions **(Chapter 4)**.

Rationale.

The vaginal mucosa immune environment plays a potentially important role in regulating the transmission of STIs, including HIV-1 (Chormont *et al*., 2001). Genital specimens, including CVLs, vaginal and/or cervical swabs, from women have been used to study vaginal mucosa immunity in a number of studies (Bebell et al., 2008;

Belec et al., 1995; Roberts et al., 2012). However, immunological characterization of factors in these genital fluids from sexually active women may be biased by the presence of semen (Silverman *et al*., 1980). Semen contains high concentrations of both inflammatory and anti-inflammatory cytokines and prostaglandins, which may moderate the cytokine environment in the female genital tract (Olivier et al., 2014). Detection of PSA and the Y-chromosome are semen markers that have been used as surrogate indicators for the presence of semen in female genital fluids (Chormont *et al*., 2001). Detecting the presence of semen in genital fluids using these biomarkers provides a tool to objectively assess recent exposure to semen, allowing for objective assessment of unprotected sexual intercourse in HIV-1 prevention trials. In addition, detecting semen also allows evaluation of the confounding effects of semen exposure on the immunological environment in the female genital tract.

Hypothesis.

Presence of semen in cervico-vaginal specimens from sexually active women will influence cytokine concentrations in cervicovaginal fluids.

Specific Objectives 3

To compare the impact of DMPA on innate cell function *in vitro,* to natural progesterone and cortisol following exogenous addition **(Chapter 5).**

Rationale.

In vitro study has shown that addition of exogenous progesterone-derivates such as DMPA decreased TLR-9-induced IFN-α production by plasmacytoid dendritic cells (Hughes et al., 2008). Studies elucidating the basic mechanism/s by which injectable HCs might increase HIV-1 infection could aid the WHO consultants to reach a definitive conclusion on guideline for *Medical eligebility criteria for contraceptive use*, particuraly DMPA.

Hypothesis.

In vitro exogenous addition of HCs to PBMCs from women not using injectable HCs will influence the activation phenotype and suppress cytokine production by monocytes and dendritic cells following TLR stimulation.

Specific Objectives 4

To investigate the influence of DMPA on vaginal epithelial thinning and density of HIV-1 target cells in vaginal biopsies from HIV-1 infected women during acute infection; and implications to their infectiousness to their sexual partners **(Chapter 6).**

Rationale.

DMPA use has been suggested to increase the likelihood of an HIV-1 infected woman infecting her sex partner. The mechanism/s accounting for increased HIV-1 transmission risk in HIV-1 infected women using DMPA remain poorly

characterized. HIV-1 target cell density in the genital mucosa and inflammatory cytokines present in genital secretions has been suggested to influence HIV-1 risk (Li et al., 2009; Masson et al., 2015). HIV-uninfected women with genital inflammation were at 2.9-fold increased risk for HIV-1 infection (Masson et al., 2015). In addition, women who seroconverted had similar levels of genital tract inflammation before they became infected as they had during acute HIV-1 infection (Roberts et al., 2012). Better understanding of the influence of DMPA on recruitment of HIV-1 target cells to the female genital tract, properties of the epithelial barrier that could influence HIV-1 penetration, altered genital cytokine profiles and HIV-1 shedding in vaginal secretions could lead to interventions to disrupt HIV-1 transmission.

Hypothesis.

DMPA use will reduce the thickness of the vaginal epithelial barrier in women, reduce the distance between HIV-1 target cell numbers and the vaginal lumen, reduce pro-inflammatory cytokine concentrations in CVLs, and increase HIV-1 viral loads at the mucosa. Furthermore, DMPA-induced alterations in genital HIV-1 target cell numbers in the epithelium during acute HIV-1 infection will be associated with faster HIV-1 disease progression (as measured by CD4 decline to 350 cells/ml and viral load at 12 months).

CHAPTER 2

Literature Review

CHAPTER 2
Literature Review

2.1 Progestin-only injectable contraceptives

Progestin-only injectable HCs are a highly effective, long-acting and reversible method of contraception. They are exogenous hormones administered by injection, which contain a synthetic progestin hormone, similar to natural progesterone found in women. Currently, there are three types of injectable progestin-only HCs in wide use (Figure 2.1): DMPA (150mg dose in 1 ml injection, Figure 2.1c), depo-subQ provera 104™ (subcutaneous formulation of DMPA, 104mg dose in 0.65ml) and NET-EN (200mg dose in 1 ml; Figure 2.1d) (Bhathena, 2001; Elder, 1984; Fraser and Weisberg, 1981).

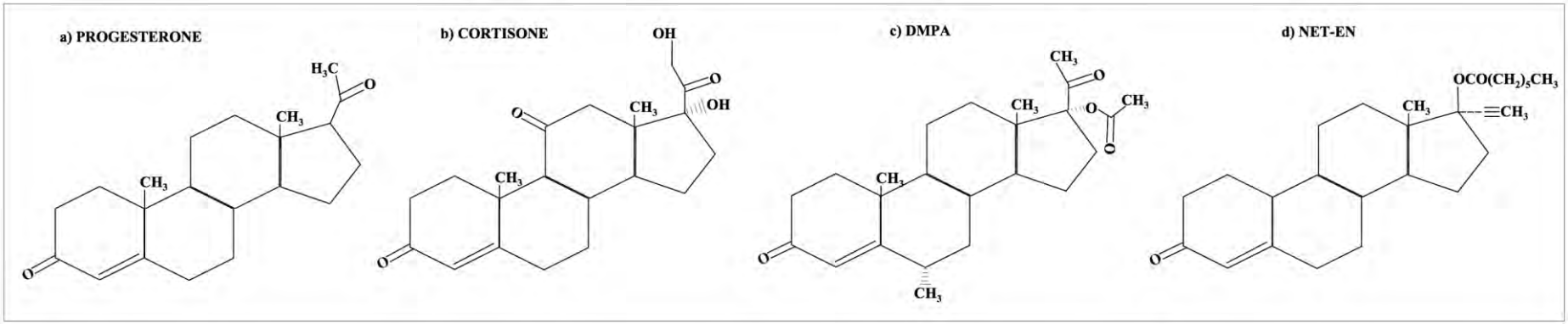


Figure 2.1 The chemical structures of (a) progesterone, (b) cortisol, (c) depot medroxyprogesterone acetate (DMPA) and (d) norethisterone enanthate (NET-EN). The structure of progesterone has 21-carbon precursor ($C_{21}H_{30}O_2$) that are directly altered by enzymes within tissues resulting into different major classes of steroids (Wiebe et al., 2006). For example, the enzyme actions lead indirectly to the corticosteroids (cortisol, $C_{21}H_{28}O_5$). DMPA (24-carbon precursor, $C_{24}H_{34}O_4$) is a synthetic 17-hydroxyprogesterone derivative of progesterone (Fraser and Weisberg, 1981) while NET-EN is a 17C-alpha-ethinyl-17beta-heptanoyloxy-4esterene-3-one synthetic progesterone derivative (Zanartu and Navarro, 1968). Figure drawn by Sinaye Ngcapu.

DMPA and NET-EN differ in cost and frequency of administration, which has implications on budgeting in the health system and patient uptake, respectively. In South Africa and internationally, DMPA is substantially cheaper than NET-EN which is an important consideration from a public health perspective. According to the South African Department of Health Medicine Prices database from the 12th March 2015, a single dose of DMPA (150mg in a 1 ml injection) cost the government R25 (including value added tax) while NET-EN cost more than twice that at R58 per dose (200mg in 1 ml) (http://www.health.gov.za/index.php/single-exit-price-documents). In South Africa, both of these options are available free of charge at national primary health care facilities and are listed in the South African Essential Drugs List (Smit et al., 2001). Internationally, they are also extensively used in reproductive health programs, forming a large proportion of health system expenditure on contraception, especially in resource limited regions (Kaunitz, 1994; Margulies and Miller, 2001). Both of these injectable HC formulations are given by intramuscular injection, and the progestin is then slowly released into bloodstream from the injection site over the course of months.

2.1.1 DMPA

DMPA is a synthetic 17-hydroxyprogesterone derivative of progesterone (Figure 2.1c), administered by intramuscular depot injection as an aqueous microcrystalline suspension (150mg/ml) every three months. It was developed by the UpJohn Company in the late 1950s and was approved as a contraceptive method in many countries in the late 1960s (Babcock et al., 1958; Fraser and Weisberg, 1981). DMPA binds with high affinity to the progesterone receptor expressed by epithelial cells, fibroblast and smooth muscle in the upper female reproductive tract, where it induces secretory transformation of the oestrogen-primed

endometrium, which is then shed (Hatcher et al., 2011).

DMPA gained popularity because of its prolonged 3-month duration of action, achieved by the progestin being slowly released (due to the low solubility of the microcrystals) into the bloodstream from the gluteal or deltoid muscles where it is injected (Mishell, 1996). DMPA can be detected in the bloodstream within 30 minutes after intramuscular injection and increases steadily to effective plasma levels >0.2ng/ml within 24 hours (Ortiz et al., 1977). Plasma DMPA concentrations plateau between 1.0 to 1.5ng/ml for the 3 month duration of the contraceptive treatment but these plasma concentrations then gradually decline to ~0.2ng/ml during the fourth month (Ortiz et al., 1977). The active plasma concentrations of DMPA achieved vary between women, with some women having detectable plasma levels >0.2ng/ml for more than 9 months after a single dose of 150mg/ml (Fraser and Weisberg, 1981; Mishell, 1996). DMPA is highly effective as a form of contraception, with an accidental pregnancy rate of 0.4 per 100 women, with lower efficacy generally only being seen because of inconsistent or incorrect use (Chinnatamby, 1971; Dodds, 1975; Powell and Seymour, 1971).

DMPA primarily provides contraceptive protection by suppressing levels of the follicle stimulating hormone and luteinizing hormone, which lead to inhibition of gonadotropin secretion, thereby inhibiting follicular maturation and ovulation (Hatcher et al., 2011; Mishell, 1996; Mishell et al., 1968; Speroff and Darney, 2010). This action also results in a hypoestrogenic state (Mishell, 1996). DMPA also induces atrophy of the endometrium, by decreasing glycogen content needed to provide energy for the development of the blastocyst after the morula has entered the uterine cavity (Hatcher et al., 2011; Mishell, 1996; Mishell et

al., 1968; Speroff and Darney, 2010). Furthermore, DMPA causes thickening of cervical mucus, making it unfavourable for spermatozoa to migrate to the oviduct to fertilize the egg (Hatcher et al., 2011; Mishell, 1996; Mishell et al., 1968; Speroff and Darney, 2010).

DMPA is a reversible, coitally-independent method of contraception, which takes an average of 4 months for women to return to fertility after DMPA is discontinued (Pardthaisong, 1984). Studies have shown that the use of DMPA during pregnancy or breastfeeding does not adversely affect the duration of lactation, quantity or quality of breast milk or the health and development of nursing infants (Pardthaisong, 1984; Pardthaisong et al., 1992).

2.1.2 Norethisterone Enanthate (NET-EN)

NET-EN is a 17C-alpha-ethinyl-17beta-heptanoyloxy-4esterene-3-one synthetic progesterone derivative (Figure 2.1d), similar to DMPA, developed in 1966 (Zanartu and Navarro, 1968), and now commonly used for contraception. It is expensive compared to DMPA, resulting in more limited roll-out in limited-resourced countries (Smit et al., 2001). NET-EN is a long chain ester of norethisterone prepared in an oily solution that is effective for two months as a contraception (Fotherby et al., 1978; Goebelsmann et al., 1979). The usual dose administered contains 200mg/ml of NET-EN, with the plasma concentrations reaching a peak of 5.5 to 11ng/ml in about 10 days that gradually decreases to 0.8ng/ml at 8 weeks after intramuscular injection (Goebelsmann et al., 1979). NET-EN inhibits ovulation, coinciding with high levels of plasma progesterone (Fraser and Weisberg, 1981; Zanartu and Navarro, 1968). This is followed by premature luteolysis, the structural and functional degradation of the corpus luteum, together with peripheral fertility-inhibiting effect when ovulation-inhibiting effect wears off. The progestogenic effect also cause changes in cervical mucus, tubal function and

endometrium thereby reducing fertility (Fraser and Weisberg, 1981; Zanartu and Navarro, 1968). Like DMPA, the overall pregnancy rate for NET-EN is low, ranging from 0.01–1.49 per 100 women years (Kesseru-Koos et al., 1973). Like DMPA, NET-EN is a reversible method of contraception, but women take an average of 8 months to return to fertility after use (Banerjee et al., 1986). NET-EN use does not adversely affect lactating women but its administration is not advised in the first and early second trimesters of pregnancy (Fine et al., 1963; Karim et al., 1971).

2.2 Link between injectable HCs and risk for HIV-1 acquisition

The potential impact of progesterone-containing injectable HCs (including both DMPA and NET-EN) on HIV-1 acquisition and transmission is an important public health concern, especially in sub-Saharan Africa, where these injectable HCs are popular and HIV-1 prevalence is high. There has been considerable debate about the role of injectable HCs in influencing risk for HIV-1 infection in women. A recent individual participant data meta-analysis of 18 large studies (including 9 cohort studies and 9 randomized controlled trials) concluded an increased risk for HIV-1 infection in women using DMPA [hazard ratio (HR): 1·50, 95% CI 1·24–1·83] although no significant evidence was found for increased HIV-1 risk in women using NET-EN (HR: 1·24, 95% CI 0·84 –1·82), after adjusting for region, marital status, age, number of sex partners, and condom use (Figure 2.2) (Morrison et al., 2015). Similarly, in a recent systematic review, HRs for HIV-1 infection were higher for DMPA (1·40, 95% CI 1·16–1·69) than for NET-EN (1·10, 0·88–1·37) when either method was compared with non-HC use (Ralph et al., 2015).

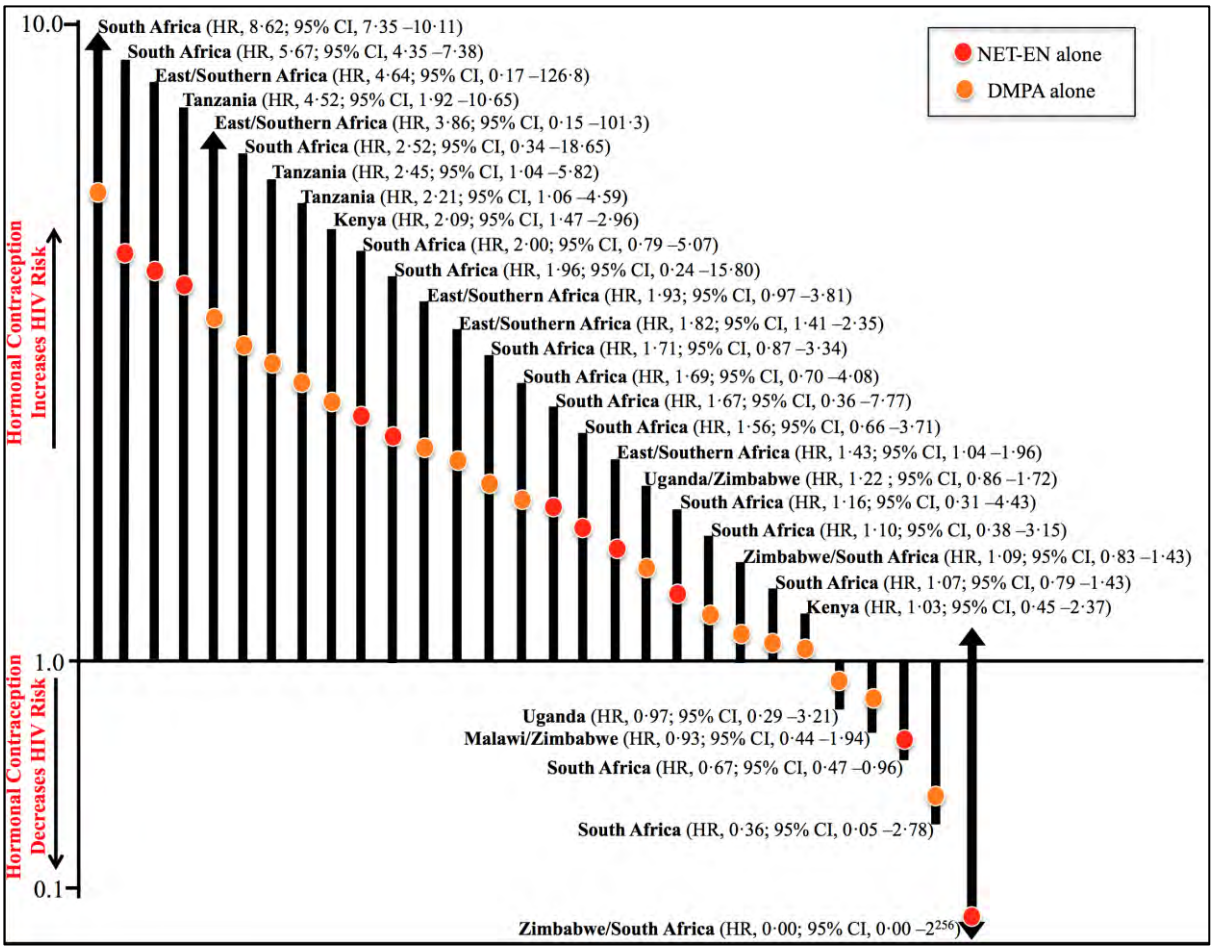


Figure 2.2 Analysis of study-specific risk for HIV-1 infection in women using injectable HCs, including the 18 studies analysed by Morrison et al., (2015). A summary of clinical studies assessing the effects of injectable HCs on HIV-1 acquisition in women compared to non-HC users. Studies that used both Cox proportional hazards (Cox) and marginal structural model (MSM) analyses are also shown. Studies listed above the x-axis show results suggesting that injectable HCs increase risk of HIV-1 infection. Studies listed below the x-axis show results suggesting that injectable HCs decrease risk of HIV-1 infection. HR=hazard ratio. CI = confidence interval. DMPA=depot medroxyprogesterone acetate. NET-EN = norethisterone enanthate. Forest plots were compiled from the following references: (Abdool Karim et al., 2010a; Baeten et al., 2007a; Delany-Moretlwe and Rees, 2010; Heffron et al., 2012; Kapiga et al., 1998; Kaul et al., 2004; Kleinschmidt et al., 2007; Kumwenda et al., 2008; McCormack et al., 2010; McGrath et al., 2014; Morrison et al., 2007; Noguchi et al.,

2015; Padian et al., 2007; Skoler-Karpoff et al., 2008; Vallely et al., 2007; Van Damme et al., 2012; Vandepitte et al., 2011; Watson-Jones et al., 2009). Figure drawn by Sinaye Ngcapu.

Despite epidemiological evidence for increased HIV-1 risk in women using DMPA, many public health officials would argue that the maternal and infant benefits (such as preventing unintended pregnancies with all its harmful biological and social sequelae) for using these HC formulations outweigh these potential moderate although significant risks (WHO, 2012). It is therefore critical to focus research efforts on understanding the biological mechanisms that could contribute to unravelling the epidemiological association between injectable HCs and HIV-1 acquisition. Such basic research into the mechanisms will promote informed policy decisions and could facilitate the promotion of safer alternative modern methods choice of contraception, including intrauterine devices or contraceptives that have a lower impact on the immune system (Huijbregts et al., 2013).

2.3 Mechanisms for increased HIV-1 risk in women using injectable HCs

2.3.1 Influence of injectable HCs on genital epithelial thickness and cell proliferation

It has been suggested that progestin-containing injectable HCs may increase risk to HIV-1 infection by reducing the thickness of the lower genital tract epithelial barrier (Figure 2.3, number 1) (Mauck et al., 1999; Miller et al., 2000). However, the impact of injectable HC use on genital epithelial thickness is contentious, since some have found no change (Bahamondes et al., 2000; Bahamondes et al., 2014; Chandra et al., 2013; Mitchell et al., 2014), while others have reported an increase in epithelial thickness (Ildgruben et al., 2003) or even a slight decrease in squamous epithelial thickness (Mauck et al., 1999; Miller et al., 2000). It is

important to note that some of the measurements reported in these studies were performed on genital tract tissues from women following only short-term (≤ 6 months) (Mauck et al., 1999, Miller et al., 2000) DMPA use, while others were performed on tissues taken from women on long-term use (≥ 3years) (Bahamondes et al., 2000). Chandra and colleagues (2013) reported thinner vaginal squamous epithelium during the late luteal phase and DMPA use as compared to both the follicular and mid-menstrual cycle stages of the menstrual cycle. They also demonstrated that the method used to measure epithelial thickness could introduce >30% intra-observer variation in measurements, and that multiple factors should be considered when determining epithelial thickness: including (1) timing of the biopsy in the menstrual cycle, (2) location of the biopsy (upper versus lower reproductive tract), (3) the ability distinguish non-viable stratum corneum from the viable stratum malighii, and (4) that sophisticated algorithms need to be developed to measure the distance between the basal layer to the lumen epithelial (Chandra et al., 2013; Mauck et al., 1999).

In addition to their potential impact on epithelial thickness, injectable HCs might decrease the integrity of the epithelial barrier by effecting intraepithelial junctions, thereby facilitating closer contact between HIV-1 and mucosal target cells (Figure 2.3, number 2). DMPA has also been reported to alter epithelial cell proliferation, increasing the expression of Ki-67 proliferation marker after 12 weeks of DMPA-treatment (Chandra et al., 2013). Increased proliferation had no effect on cellular proteins involved in protection against microbial invasion, however, and the epithelial structure and integrity was similar in women in the follicular or luteal phase of the menstrual cycle following DMPA treatment (Chandra et al., 2013).

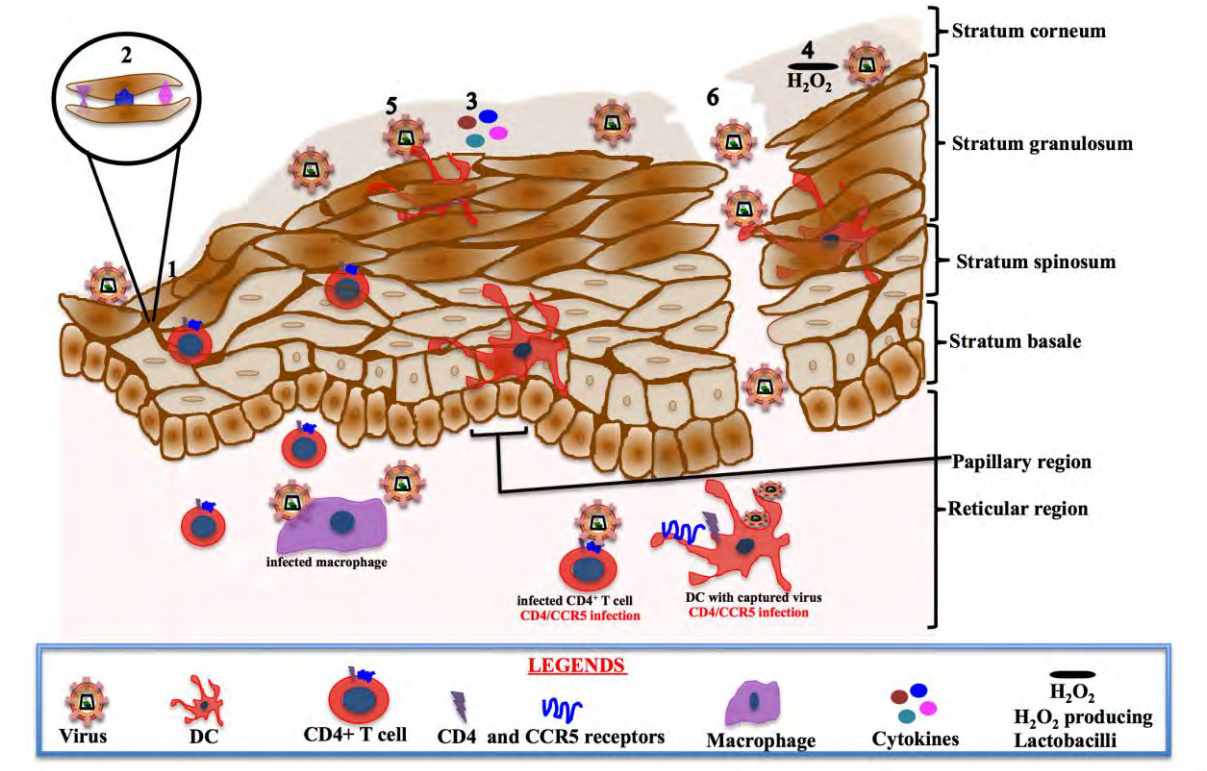


Figure 2.3 Mechanisms by which injectable HCs might contribute to an increased risk of HIV-1 acquisition and transmission include: (1) thinning of the epithelial barrier (including the stratum corneum and granulosa); (2) disruption of tight intercellular junctions, adherens junctions and desmosomes between epithelial cells; (3) changing the inflammatory milieu of the vaginal mucosa; (4) decreased hydrogen peroxide (H_2O_2) producing lactobacilli; (5) modulating recruitment of immune cells [intraepithelial target cells including DC-SIGN$^+$ dendritic cells (DCs), CD4+ T cells and macrophages]; (6) micro-abrasions in the stratified squamous epithelium barrier; and increased acquisition of other STIs (Figure drawn by author). Figure modified from Murphy et al., 2014 (JIR).

2.3.2 Influence of injectable HC use on mucosal immunity

DMPA may alter cervicovaginal immune responses that could influence susceptibility to HIV-1 infection, through modulating the innate environment of the genital tract and recruitment of immune cells to the mucosa (Figure 2.3, number 5) (Chandra et al., 2013; Mitchell et al., 2014; Wira and Veronese, 2011). From vaginal biopsy tissue, a recent study reported that women using DMPA had increased numbers of $CD3^+$, $CD8^+$, HLA-DR^+, $CCR5^+$, $CD45^+$ leukocytes and CD68+ macrophages in the squamous epithelium compared to women not using DMPA (Chandra et al., 2013). In contrast, Brunelli and colleagues (1996) reported women using DMPA had decreased natural killer (NK) cells in blood and $CD8^+$ $CD11b^+$ T-lymphocytes function than women not using DMPA (Brunelli et al., 1996). Similar infiltrations of immune cells were also noted in women not using HCs during a normal menstrual cycle phases during the late luteal phase, with endogenous progesterone inducing the migration of macrophages, increasing immunoglobulin content and cytokine expression within the endometrium (Starkey et al., 1991). In other studies, an increase in the density of cervicovaginal lymphocytes expressing CCR5 was observed in response to increasing progesterone levels (Sheffield et al., 2009), although no difference in HLA-DR^+ and $CCR5^+$ target cells was found in vaginal biopsies after 12 months of DMPA use (Mitchell et al., 2014).

DMPA has also been shown to increase expression of the HIV-1 co-receptors CXCR4 and CCR5 on activated T cells, resulting in increased proliferation of CCR5 and CXCR4-tropic viruses (Huijbregts et al., 2013). An *in vitro* study showed that immune cells treated with progesterone had 10-fold increased expression of CCR5 on $CD14^+$ monocytes/macrophages (Patterson et al., 1998). DMPA also reduced the ability of pDCs to respond to stimulation via TLR-7/8 and TLR-9 (Huijbregts et al., 2013) (Hughes et al., 2008). Progesterone has also

been shown to significantly inhibit proliferation of blood $CD8^+$ T cells and cytokine production by both CD4+ and $CD8^+$ T cells (Vassiliadou et al., 1999). Several studies have shown that the number of antibody-secreting plasma cells at the mucosa, trans-epithelial mucosal transport of immunoglobulins (Igs), and concentrations of mucosal IgG and IgA were down-regulated by progesterone and DMPA in women and non-human primates treated with DMPA (Kutteh et al., 1996; Lu et al., 2002; Lu et al., 2003; Patton et al., 2000). In contrast, higher concentrations of total and antigen-specific IgG secreting cells have been detected in blood after DMPA administration although no differences in plasma IgA and IgM concentrations were observed (Lali et al., 1996).

In macaques vaginal-challenged with SIV, treatment with DMPA enhanced risk for mucosal SIV infection >7-fold (Marx et al., 1996; Trunova et al., 2006; Veazey et al., 2003). In addition to influencing the thickness of the epithelial barrier of macaques, DMPA administration also increased SIV replication, and downregulated cellular immune responses. The dramatic impact of DMPA on SIV acquisition in non-human primates may be exaggerated by the higher doses of DMPA typically used in primates, the way in which viruses are administered (high-dose SIV-culture medium in primates), and the rate of metabolism and circulation of DMPA in these non-human primates after the 3 monthly injection (Hel et al., 2010).

2.3.3 Influence of injectable HC use on innate and soluble factors in the genital tract

HIV-1 infection has been shown to result in productions of inflammatory cytokines that play a central role in determining viral kinetics, influence recruitment of HIV-1 target cells to the mucosa, and development of anti-viral immunity following infection (Stacey et al., 2009;

Roberts et al., 2010; Roberts et al., 2012; Bebell et al., 2008). In addition, pre-existing inflammatory responses, both in the genital mucosa and systemically, are also thought to influence risk of HIV-1 infection in women before they become infected (Masson et al., 2015; Naranbhai et al., 2014).

Several different, partially overlapping, functional classes of cytokines have been described (Abbas and Lichtman, 2007; Charo and Ransohoff, 2006; Connolly et al., 2005). Pro-inflammatory cytokines, such as TNF-β, L-12p40, IL-12p70, IL1α, IL-6, TNF-α, and IL-1β, are involved in recruitment of immune cells from blood into tissue, stimulating their differentiation and activation. Anti-inflammatory cytokines, including IL-1Ra and IL-10, regulate excessive inflammatory reactions, and are produced to counteract the actions of inflammatory cytokines. Chemokines are chemotactic cytokines, including eotaxin, monocyte chemoattractant protein (MCP)-1, macrophage-derived chemokine (MDC), Fractalkine, macrophage inflammatory protein (MIP)-1α, MCP-3, interferon-γ-induced protein (IP)-10, growth related oncogene (GRO), MIP-1β, IL-8, and RANTES, are responsible for recruitment of immune cells to inflammatory sites and damaged tissue. Growth factors, such as interferon (IFN)-α, transforming growth factor (TGF)-α, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF)-AB/BB, and fibroblast growth factors (FGF)-2, are involved in cell growth and proliferation. Hematopoietic cytokines, such as IL-9, FMS-like tyrosine kinase-3 (Flt3L), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage (GM)-CSF, IL-7, and IL-3, are involved in hematopoesis of immune cells from stem cell progenitors. Adaptive cytokines, such as IL-15, IL-5, IL-17, soluble CD40 ligand (sCD40L), IFN-γ, IL-2, IL-4, IL-13, sIL-2Rα, and IFN-α, stimulate growth, differentiation, activation and survival of immune cells.

Changes in hormones during the menstrual cycle, including changes induced by HC use, has been suggested to alter cytokine production in both the upper and lower female reproductive tract (Huijbregts et al., 2013). Use of DMPA has been shown to reduce resistance to microbial invasion by modulating soluble innate mucosal defenses (Figure 2.3, number 3). DMPA was found to inhibit secretion of IFN-γ, IL-2, IL-4, IL-6, IL-12, TNF-α and MIP-1α by activated PBMCs *in vitro* (Huijbregts et al., 2013). DMPA has also been shown to have glucocorticoid receptor (GR) agonistic activity that inhibits the expression of GR-regulated genes (Bamberger et al., 1999; Koubovec et al., 2005; Koubovec et al., 2004). For example, DMPA represses the gene encoding IL-2 in normal human lymphocytes via the GR, which suggests that the GR might mediate the immunosuppressive effects of DMPA in vivo (Bamberger et al., 1999; Koubovec et al., 2005; Koubovec et al., 2004). Cervical cell lines stimulated with both DMPA and TNF-α produced lower levels of RANTES and increased levels in IL-8, while NET-EN stimulation had no effect (Africander et al., 2011). Govender *et al.* (2014) showed that endocervical cells treated with DMPA showed decreased inflammatory responses, marked by lower expression of IL-6, IL-8 and RANTES mRNA, while NET-EN did not (Govender et al., 2014).

In contrast to these studies showing dampening of inflammatory responses, others have suggested that DMPA use may increase expression of RANTES, although decreasing levels of other protective innate factors like the secretory leukocyte protease inhibitor (SLPI) and biodefensin-2 (BD-2) in genital secretions (Morrison et al., 2014). Importantly, this increase in genital RANTES and decreased concentrations of SPLI was also associated with increased risk of HIV-1 acquisition (Morrison et al., 2014).

Others have suggested that DMPA use may dampen immune responses to other common infectious diseases in women, such as tuberculosis. PBMCs from women with tuberculosis who were using DMPA produced lower amounts of IL-1α, IL-12p40, IL-10, IL-13 and G-CSF following stimulation with the mycobacterial antigen BCG compared to non-HC users (Kleynhans et al., 2011). In addition, DMPA using women also had decreased frequencies of circulating monocytes, a key correlate of protection against tuberculosis, compared to non-HC users (Kleynhans et al., 2011). In mice infected with tuberculosis, treatment with DMPA also resulted in down-regulated plasma cytokine levels of TNF-α, IFN-γ, G-CSF, IL-6, IL-10, IL-17 and MCP-1 and increased secretion of IP-10 compared to control mice (Kleynhans et al., 2013).

Matrix metalloproteases (MMPs) are a family of zinc dependent proteases that are required during normal reproductive processes for degradation of specific components of the extra cellular matrix and tissue remodeling in the endometrial compartment of the female genital tract during normal menstruation (Birkedal-Hansen, 1995; Cawston, 1995; Lockwood and Schatz, 1996; Rodgers et al., 1994; Rodgers et al., 1993). MMPs comprise collagenases (enzymes that break down peptide bonds in collagen that includes MMP-1), gelatinases (proteolytic enzymes that hydrolyze gelatin that include MMP-2 and MMP-9), stromelysins (enzymes involved in breakdown of the extracellular matrix that include MMP-3 and MMP-10) and matrilysins (uterine metalloproteinases that are also involved in extracellular matrix degradation that include MMP-7). MMPs are regulated by multiple ways, being activated by other proteases, at the level of gene transcription and by hormones (Vincent et al., 2000). MMPs are inhibited by specific endogenous tissue inhibitors of metalloproteinases (TIMPs)

(Fernandez-Catalan et al., 1998; Gomis-Ruth et al., 1997). Regulation of TIMP expression occurs during tissue development and remodelling (Fernandez-Catalan et al., 1998; Gomis-Ruth et al., 1997). Injectable HCs have been shown to suppress production of epithelial cell-specific MMP-7 and stromal-epithelial specific MMP-3 (Bruner et al., 1995; Osteen et al., 1994; Schatz et al., 1997; Singer et al., 1997). Women using injectable HCs were also found to have decreased TIMP concentrations in their upper reproductive tracts (Vincent et al., 2002). Although not fully understood, changes in the local ratio of MMPs:TIMPs may influence tissue remodeling processes in the reproductive tract, and degradation of extracellular matrix components such as collagen, elastin, fibronectin and laminin by MMP-1, MMP-2 and MMP-9 thus affecting epithelial barrier function upon HIV-1 exposure (Li et al., 2007; Osteen et al., 1994; Vincent et al., 2000, 2002; Zhang et al., 1998; Zhang, 1998).

2.3.4 Susceptibility to bacterial vaginosis (BV) and stability of the vaginal microbiome following injectable HC use

Injectable HCs have been suggested to influence the vaginal microbiome in a way that may increase risk for HIV-1 acquisition (Figure 2.3, number 4) (Atashili et al., 2008; Martin et al., 1999; Miller et al., 2000). H_2O_2-producing lactobacilli species, including *Lactobacillus crispatus, L. jensenii* and *L. gasseri*, are thought to predominate in the healthy vaginal environment and contribute to host defense via metabolism of glycogen to lactic acid, biosurfactants and hydrogen peroxide to maintain the vagina at an acidic pH <5, (Aroutcheva et al., 2001; Boris and Barbes, 2000; Witkin et al., 2007). Low vaginal pH is thought to inhibit CD4 T-cell activation and proliferation and decrease the number of HIV-1 target cells available for infection in the vaginal mucosa (Hill and Anderson, 1992). However, the menstrual cycle affects the vaginal microbial environment. Fluctuations in endogenous

estrogen and progesterone levels around ovulation and menses affect attachment and growth of *Lactobacilli* species to the mucosal epithelium, in addition to influencing cervical mucus secretion, vaginal pH and epithelial cell glycogen content (Miller et al., 2000). Use of DMPA results in a hypo-estrogenic state, which has been associated with reduced H_2O_2-producing lactobacilli colonization, possibly weakening the vaginal epithelial barrier and allowing microbial invasion (Miller et al., 2000). Mitchell et al. (2014) reported that women using DMPA for ~12 months had lower proprotions of H_2O_2-producing *Lactobacilli* species compared to before they started using DMPA (Mitchell et al., 2014). Both Miller et al. (2000) and Mitchell et al. (2014) reported an overall decrease in glycogen content, required for lactobacilli to sustain growth, as well as lactate metabolism in epithelial cells from women using DMPA (Miller et al., 2000; Mitchell et al., 2014).

Decreased glycogen content has also been associated with reduced water content in vaginal epithelial cells, leading to cytoplasmic dehydration and changes in the vaginal microbiota that favour less acid-tolerant bacteria colonization (Miller et al., 2000). BV is a prevalent microbial dysbiosis, characterized by reduced concentrations of commensal *Lactobacilli* species and concomitant overgrowth of anaerobic bacterial species, including *Gardnerella* spp*, Prevotella* spp*,* and *Mobiluncus* spp (Hiller et al., 2008). BV is typically diagnosed using a clinical algorithm including signs and symptoms such as odorous but clear vaginal discharge, a vaginal pH >4.5, a positive amine test, and the presence of clue cells (vaginal epithelial cells with a distinctive stippled appearance as a result of being covered with bacteria in a biofilm) (Amsel et al., 1983; Cook et al., 1992). In addition, BV is also evaluated using Nugent scoring whereby bacterial morphotypes are scored using Gram staining from smears of lateral vaginal wall swabs (Nugent et al., 1991).

While BV has been implicated in moderate increased risk for HIV-1 infection, several studies reported that women using DMPA have reduced risk of prevalent and recurrent BV (Eschenbach et al., 2000; Hayes et al., 2010; Low et al., 2011; Riggs et al., 2007; Shoubnikova et al., 1997; van de Wijgert et al., 2013). Reduced prevelence of BV in women using DMPA could be because these women often do not menstruate and the presence of haemoglobin in the genital tract has been associated with development of BV (Eschenbach et al., 2000; Pettifor et al., 2009; van de Wijgert et al., 2013). Further studies are needed to understand the mechanism by which DMPA use reduce the risk of BV (Jarosik et al., 1998).

2.3.5 Injectable HCs and susceptibility to sexually transmitted infections (STIs)

Studies have suggested that injectable HC users may be at increased risk of infection with other common STIs (including *C. trachmatis* and HSV-2) but not others (including *N. gonnorhoea*, human papillomavirus (HPV), *T. vaginalis*, and syphilis) (Figure 2.3, number 7). Increased cervical ectopy following injectable HC use and the resultant hypo-oestrogenic state that exists in women using high dose progesterone HCs result in thinning of the mucosal epithelium that result in increased risk for bacterial and viral sexually-transmitted infections that favour infection at the cervix, as well as HIV-1 (Baeten et al., 2001; Critchlow et al., 1995; Harrison et al., 1985; Lavreys et al., 2004; Mohllajee et al., 2006; Morrison et al., 2004; Plourde et al., 1994; van de Wijgert et al., 2013). Table 2.1 summarizes the recent studies that have investigated the relationship between injectable HCs use and risk of STIs. Several of these studies found that use of injectable HCs was associated with a significant increased risk of infection with Chlamydia (Baeten et al., 2001; Lavreys et al., 2004; Morrison et al., 2004), while others did not (Jacobson et al., 2000; Noguchi et al., 2014;

Overton et al., 2008; Pettifor et al., 2009). Similar controversy exists for HSV-2 infection risk in women using injectable HCs, with (Noguchi et al., 2014) reporting enhanced risk for HSV-2, while (Lavreys et al., 2004) did not. For other common STIs, including syphilis, gonorrhea, and HPV, no association with DMPA use was found (Baeten et al., 2001; Guiuliano et al., 2001; Harris et al., 2009; Marks et al., 2011; Moscicki et al., 2001). In contrast to the increased susceptibility noted for infections like chlamydia and HSV-2, albeit controversial, DMPA use was reported to decrease risk for *Trichomonas* infection (Brahmbhatt et al., 2014; Pettifor et al., 2009), although several other groups did not find the same effect (Baeten et al., 2001; Overton et al., 2008; Torok et al., 2009).

It is difficult to assess whether the observed results may reflect systematic behavioural or other differences in exposure to STIs among women using injectable HCs compared to those not selecting this option. This is compounded by the fact that most studies were performed in cross-sectional studies that could not differentiate whether STIs occurred before DMPA use. In addition, other potential confounding factors and heterogeneity in study designs also make it difficult to directly assess the link between injectable HC use and risk for STIs, in the absence of more detailed mechanistic studies.

Table 2.1 Summary of recent studies exploring the relationship between injectable HC use and risk for acquiring STIs

STI	Study	n, population	Adjusted for	DMPA	Risk
C. trachmatis	(Baeten et al., 2001)	948, Kenyan sex workers	Age, education, years of sex work, parity, place of work, number of sex partners per week, number of sex acts per week, condom use	HR 1·6, 95% CI 1·1–2·4, comparing DMPA users with women who were sterilized or non-HC users	Increased
	(Lavreys et al., 2004)	242, HIV-1 positive Kenyan sex workers	Age, education, duration of sex work, parity, number of sex partners per week, condom use	HR 3·1, 95% CI 1·0–9·4, comparing DMPA users with women who were sterilized or non-HC users	Increased
	(Morrison et al., 2004)	819, American patients seeking reproductive health care	Age, race, site, sexual and reproductive health characteristics	HR 4·3, 95% CI 1·7–11·1, comparing DMPA users with non-HC users	Increased
	(Jacobson et al., 2000)	97, HIV-1 negative Baltimore adolescent females	Age, lifetime number of partner	OR 5·44, 95% CI 1·25 – 23·6 comparing DMPA users with non-HC users	No risk
	(Pettifor et al., 2009)	567 HIV-1 negative women	Age, education, and condom use consistency in the past 3 months	IRR 1·2, 95% CI 0·8–1·9, comparing DMPA users with non-HC users	No risk
	(Noguchi et al., 2014)	3,163 women were enrolled in The MTN-003 (VOICE) trial was a Phase 2B, multi-site, randomized, placebo controlled trial	Age, married, cohabiting, education, BL HSV-2, time-varying partners, partner has other partners, and unprotected sex, stratified by site, with censoring at pregnancy	HR 1·09, 95% CI 0·92-1·29, comparing DMPA users with NET-EN users	No risk
	(Overton et al., 2008)	304 HIV-1 negative women	No adjustment	HR 1·19, 95% CI 0·7-1·9, comparing DMPA users with no-DMPA users	No risk
N. gonnorhoea	(Baeten et al., 2001)	948, Kenyan sex workers	Age, education, years of sex work, parity, place of work, number of sex partners per week, number of sex acts per week, condom use	HR 1·1, 95% CI 0·8–1·6, comparing DMPA users with women who were sterilized or non-HC users	No risk
	(Lavreys et al., 2004)	242, HIV-1 positive Kenyan sex workers	Age, education, duration of sex work, parity, number of sex partners per week, condom use	HR 1·0, 95% CI 0·6–1·7, comparing DMPA users with women who were sterilized or non-HC users	No risk

	(Morrison et al., 2004)	819, American patients seeking reproductive health care	Age, race, site, sexual and reproductive health characteristics	Insufficient cases to evaluate risk of gonorrhoea alone; HR for either chlamydia or gonorrhoea 3·6, 95% CI 1·6–8·5, comparing DMPA users with non-HC users	No risk
	(Pettifor et al., 2009)	567 HIV-1 negative women	Age, education, and condom use consistency in the past 3 months	IRR 1·3, 95% CI 0·6–3·0, comparing DMPA users with non-HC users	No risk
	(Noguchi et al., 2014)	3,163 women were enrolled in The MTN-003 (VOICE) trial was a Phase 2B, multi-site, randomized, placebo controlled trial	Age, married, cohabiting, education, BL HSV-2, time-varying partners, partner has other partners, and unprotected sex, stratified by site, with censoring at pregnancy	HR 0·92, 95% CI 0·64 –1·3, comparing DMPA users with NET-EN users	No risk
	(Overton et al., 2008)	304 HIV-1 negative women	No adjustment	HR 1·04, 95% CI 0·6-1·8, comparing DMPA users DMPA users	No risk
HPV	(Marks et al., 2011)	1000 HIV-1 negative women	Age, lifetime number of partners, number of partners L6M, smoking and ever GC/CT/SYP infection.	Risk hazard (RH) 1·24, 95% CI 0·8-1·91 comparing DMPA users with non-HC users	No risk
	(Moscicki et al., 2001)	897 women in HPV and LSIL incidence cohort	No adjustment	HR 0·79, 95% CI 0·2-3·25 comparing DMPA users with non-HC users	No risk
	(Harris et al., 2009)	284 HPV DNA positive women	Age, lifetime number of male partners and parity	OR 4·7, 95% CI, 1·4-15·8 comparing DMPA users with non-HC users	No risk
	(Guiuliano et al., 2001)	2319 women	marital status, employment status,age, number of male partners	OR 2·3, 95% CI, 1·5 – 3·53 comparing injectable HC users with non-HC users	No risk
HSV-2	(Noguchi et al., 2014)	3,163 women were enrolled in The MTN-003 (VOICE) trial was a Phase 2B, multi-site, randomized, placebo controlled trial	Age, married, cohabiting, education, BL HSV-2, time-varying partners, partner has other partners, and unprotected sex, stratified by site, with censoring at pregnancy	HR 2·06 95% CI 1·12 –3·79, comparing DMPA users with NET-EN users	Increased
	(Lavreys et al., 2004)	242, HIV-1 positive Kenyan sex workers	Age, education, duration of sex work, parity, number of sex partners per week, condom use	HR 0·6, 95% CI 0·4–1·0, comparing DMPA users with women who were sterilized or non-HC users	No risk

T. vaginalis	(Baeten et al., 2001)	948, Kenyan sex workers	Age, education, years of sex work, parity, place of work, number of sex partners per week, number of sex acts per week, condom use	HR 0·6, 95% CI 0·4–1·0, comparing DMPA users with women who were sterilized or non-HC users	No risk
	(Torok et al., 2009)			OR 1·4, 95% CI, 0·6 – 3·4 comparing injectable HC users with non-HC users	No risk
	(Pettifor et al., 2009)	567 HIV-1 negative women	Age, education, and condom use consistency in the past 3 months	IRR 0·4, 95% CI 0·1–1·0, comparing DMPA users with non-HC users	Decreased
	(Brahmbhatt et al., 2014)	2374 HIV-1 negative women	sociodemographic, behavioral risk factors, syphilis infection, and BV status	IRR 2·8, 95% CI 1·0–8·0, in DMPA users after 12 months of treatment	Decreased
	(Overton et al., 2008)	304 HIV-1 negative women	No adjustment	HR 1·32, 95% CI 1·0-1·8, comparing DMPA users DMPA users	No risk
Treponema pallidum	(Baeten et al., 2001)	948, Kenyan sex workers	Age, education, years of sex work, parity, place of work, number of sex partners per week, number of sex acts per week, condom use	HR 0·5, 95% CI 0·2–1·4, comparing DMPA users with women who were sterilized or non-HC users	No risk

DMPA=depot medroxyprogesterone acetate; NET-EN=norethisterone enanthate; HC=hormonal contraceptives; CI=Confidence interval; OR=odds ratio; hazard ratio=HR; IRR= incidence rate ratio; RH=risk hazard

2.4 Injectable HCs and HIV-1 shedding from HIV-1 infected women

Injectable HC use in women already infected with HIV-1 may also influence their risk of transmitting HIV-1 to sexual partner/s, by increasing local HIV-1 viral loads in genital secretions (Heffron et al., 2012) (Table 2.2). Heffron et al. (2012) showed that chronically HIV-1 infected women using injectable HCs, in HIV-1 serodiscordant relationships with HIV-1 negative male partners, were twice as likely to transmit HIV-1 to their partners than women not using HCs; and this was associated with significantly elevated concentrations of HIV-1 RNA in genital secretions from in injectable HC users (Heffron et al., 2012). Relatedly, HIV-1 RNA concentrations were shown to be higher in genital secretions taken from naturally cycling HIV-1 infected women during the luteal phase of menstrual cycle, when progesterone predominates, adding further biological plausibility to this association (Benki et al., 2004; Reichelderfer et al., 2000). Earlier studies on the impact of injectable HC use on HIV-1 genital tract viral loads have been conflicting, with several reporting no difference in HIV-1 shedding in female genital secretions in women using injectable HCs compared to those not using HCs (Graham et al., 2010; Lutalo et al., 2013; Morrison et al., 2010).

Table 2.2 Summary of studies evaluating the relationship between injectable HC use in HIV-1 infected women and risk of transmission to their male partners

Study	n, population	Adjusted for	Injectable HC used	Discription of results
(Heffron et al., 2012)	3790 heterosexual HIV-1 serodiscordant couples	Baseline age, baseline plasma HIV-1 viral load, time-varying unprotected sex in last month, time-varying pregnancy	Not differentiated	Cox HR 1·95, 95%CI 0·99 – 3·22; MSM HR 3·01, 95%CI 1·47 – 6·16, comparing injectable HC users with non-HC users
(Mostad et al., 1997)	318 women in STI clinic	CD4, other factors unclear	DMPA	Cervical, OR 2·9, 95% CI 1·5–5·7 and vaginal, OR 1·0, 95% CI 0·3–2·7, comparing DMPA users non-HC users
(Graham et al., 2010)	102 HIV-1 infected non-pregnant women	Plasma viral load, other factors unclear	Not differentiated	HR 1·70, 95% CI 1·7–5·66, comparing DMPA users with non-HC users
(Lutalo et al., 2013)	159 heterosexual HIV-1 serodiscordant couples	HIV-1 viral load	DMPA	OR 1·40, 95% CI 0·30 – 6·49 comparing DMPA users with non-HC users
(Morrison et al., 2010)	188 women seeking family planning	Age at seroconversion, HIV-1 subtype, pregnancy, breastfeeding, STI symptoms, participant behavioural risk, recent unprotected sex, partner recently spending >1night away from home, nonviral STIs, genital ulcer disease, time since infection	DMPA	IRR 1·2, 95% CI 0·8–1·9, comparing DMPA users with non-HC users

However, other unidentified confounding factors are fundamental concerns in all-observational studies such as these (Gray, 2012; Hubacher, 2012; Shelton, 2012). It is possible that women using injectable HCs may use condoms differently to non-HC users (Cushman et al., 1998; Gray, 2012; Sangi-Haghpeykar et al., 2005). The WHO Expert group assessing these factors emphasized the need for studies on HIV-1–discordant couples to quantify the effects of HC use on HIV-1 acquisition, HIV-1

progression in HIV-1 infected women and onward transmission of HIV-1 to men (WHO, 2012).

2.5 Injectable HCs and rate of disease progression in HIV-1 infected women

A randomized control trial evaluating the safety of various HC methods for use by HIV-1 infected women showed that HIV-1 infected women using DMPA experienced moderately more rapid HIV-1 rates of disease progression, measure by CD4 decline, than the control arm (HR 1.39; 95% CI 0.63–3.06), although this was not significant (Stringer et al., 2007). In contrast, observational studies have reported slower rates of disease progression in HC users than non-HC users (Heffron et al., 2013). To further confuse this issue, six additional observational studies found no increased risk for accelerated progression to death, onset of clinical AIDS, decrease of CD4 count <200 or <350 cells/mm^3, initiation of a highly active anti-retroviral therapy (HAART) and death not due to trauma with injectable HC when compared to non-use of HC methods (Allen et al., 2007; Heffron et al., 2013; Heikinheimo et al., 2011; Kilmarx et al., 2000; Morrison et al., 2011; Polis et al., 2010).

A meta-analysis of available clinical data in HIV-1 infected women is needed to resolve this uncertainty. In 2012 and 2015, the WHO published a statement acknowledging that the currently available data does not establish a clear causal association between injectable HCs and HIV-1 shedding nor disease progression. The WHO expert panel further recommended that women using HCs should also be counselled to use condoms to prevent HIV-1 shedding and accelerated disease

progression (WHO, 2012, 2015). These recommendations also stressed the need for further research on longitudinal studies of injectable HCs impact during chronic HIV-1 infection on viral load, CD4 cell count and disease progression, including impacts of initiation, or termination and total duration of injectable HC use (WHO, 2012, 2015).

2.6 Injectable HC and anti-retroviral therapy during HIV infection

Preventing unplanned pregnancies in HIV-1 infected women can significantly decrease HIV-1 transmission from infected mothers to their children and improve a woman's general health. Injectable HCs are widely used to prevent unplanned pregnancies in HIV-1 infected women, but there are concerns that simultaneous use of injectable HCs and highly active anti-retroviral therapy (HAART) may alter the effectiveness of both (Robinson et al., 2012). There are no published data on interactions between NET-EN and the drugs that comprise HAART, but studies have suggested that DMPA can negatively interact with ritonavir-boosted protease inhibitors and efavirenz (Cohn et al., 2007; Robinson et al., 2012). The revised WHO medical eligibility criteria for contraceptive use has not recommended restriction on the use of hormonal contraceptive methods among HIV-1 infected women (WHO, 2015).

2.7 Conclusions

This Chapter summarizes the literature around the relationship between injectable HCs and increased risk of HIV-1 infection in HIV-1 negative women and the impact

of injectable HC use on the infectiousness of HIV-1 infected women to their male sexual partners. Investigations of the possible biological mechanisms driving this risk used *in vitro* laboratory approaches, non-human primates models using SIV vaginal challenges, and *in vivo* clinical studies in women using injectable HCs, each suggesting a positive relationship between long-acting progestin containing injectable HCs and HIV-1 risk. The majority of evidence suggests that injectable HCs have limited effect on HIV-1 disease progression in chronically HIV-1 infected women and that injectable HCs largely does not interact with most anti-retroviral drugs comprising HAART. Many of the clinical studies described were not originally designed to assess this association and, like many observational studies, had many potential confounders. Of these, methodological considerations, such as semen contamination in the cervico-vaginal secretions, inconsistent condom use, age, parity, education, marital status, behavioural risk, pregnancy status, poor follow-up, statistical power and precision were properly controlled for in these studies (Polis and Curtis, 2013). Unless the effects of hormonal contraception on HIV-1 risk are appropriately addressed, the likelihood is that DMPA would indirectly contribute to increased maternal and infant mortality, increase risk of HIV-1 transmission from mother-to-child and from HIV-1 infected women to their male partners.

CHAPTER 3

Lower concentrations of chemotactic cytokines and soluble innate factors in the lower female genital tract associated with use of injectable hormonal contraceptives

CHAPTER 3

Lower concentrations of chemotactic cytokines and soluble innate factors in the lower female genital tract associated with use of injectable hormonal contraceptives

3.1 Abstract

Progesterone-based injectable HCs potentially modulate genital barrier integrity and regulate the innate immune environment in the female genital tract, thereby enhancing risk for STIs or HIV-1 infection. The effects of injectable HC use on concentrations of inflammatory cytokines and other soluble factors associated with genital epithelial repair and integrity was investigated in this Chapter. The concentrations of 42 inflammatory, regulatory, adaptive, growth factors and hematopoetic cytokines, five matrix metalloproteinases (MMPs), and four tissue inhibitors of metalloproteinases (TIMPs) were measured in CVLs from 64 HIV-1 negative women using injectable HCs and 64 control women not using any HCs, in a matched case-control study. There were no differences between groups in the prevalence of BV Nugent score ≥7, or common STIs. In multivariate analyses adjusting for condom use, sex work status, marital status, BV and STIs, median concentrations of the chemokines eotaxin, MCP-1, MDC, the adaptive cytokine IL-15, the growth factor PDGF-AA, and an inhibitor of metalloproteinases TIMP-2 were significantly lower in CVLs from women using injectable HCs than controls. In addition, the pro-inflammatory cytokine IL-12p40 and the chemokine fractalkine were less likely to have detectable levels in women using injectable HCs compared to those not using HCs. Findings from this Chapter suggest that injectable HC use was associated with an immunosuppressive female genital tract innate immune profile. While the relationship between injectable HC use

and STI or HIV-1 risk is yet to be resolved, this data suggest that injectable HCs effects were similar between STI positive and STI negative participants.

3.2 Introduction

Internationally, HCs are widely used by women to prevent unplanned pregnancies. In South Africa, more than half of women aged 15-49 years old use DMPA or NET-EN, with more than 3 times the number of women using DMPA than NET-EN (Department of Health et al., 2007; Sibeko et al., 2011). DMPA primarily provides contraceptive protection by suppressing natural cyclic fluctuations of female sex hormones resulting in a hypoestrogenic state (Jeppsson et al., 1982). The mechanism of action of NET-EN is more complex than that of DMPA. An initial suppression of ovulation overlaps with very high initial plasma progestogen levels, which may decrease after 60 days(Fotherby et al., 1978; Goebelsmann et al., 1979).

As discussed in Chapter 2, high-dose DMPA use is common in the SIV vaginal challenge models because it results in thinning of the vaginal epithelium, which enhances genital SIV infection (Abel et al., 2004; Marx et al., 1996; Trunova et al., 2006; Wieser et al., 2001). The role of DMPA in increasing risk of HIV-1 infection is contentious, with some studies reporting increased risks (Baeten et al., 2007a; Heffron et al., 2012; Hel et al., 2010; Kumwenda et al., 2008; Morrison et al., 2010; Ungchusak et al., 1996), and others finding no such association (Kiddugavu et al., 2003; Kleinschmidt et al., 2007; Myer et al., 2007; Reid et al., 2010; Stringer et al., 2009). The impact of DMPA on genital epitheial barrier intergrity is similary contentiuos (Kiddugavu et al., 2003; Myer et al., 2007). In addition to HIV-1, DMPA has also been associated with an increased risk of *C. trachomatis* infection and decreased risk of acquiring BV and *T. vaginalis* infections (Baeten et al., 2001; van de Wijgert et al., 2013).

It has been hypothesised that DMPA might increase HIV-1 aquisition risk by changing the inflammatory or chemotactic environment of the genital mucosa so as to increase the recruitment of HIV-1 susceptible immune cells to the mucosa (Ildgruben et al., 2003; Miller et al., 2000; Wieser et al., 2001; Wira et al., 2011b; Wira and Veronese, 2011). However, treatment of PBMCs with DMPA has been shown to cause reduced production of several inflammatory and adaptive cytokines (Huijbregts et al., 2013). At the female genital mucosa, suppression of innate immune responses may influence susceptibility to infections. Moreover, MMPs, which are required during normal reproductive processes such as menstruation (Birkedal-Hansen, 1995; Cawston, 1995; Lockwood and Schatz, 1996; Rodgers et al., 1994; Rodgers et al., 1993), may influence epithelial barrier repair in the lower genital tract. MMPs are regulated by specific TIMPs (Fernandez-Catalan et al., 1998; Gomis-Ruth et al., 1997), which may similarly be involved in maintainence of the lower reproductive tract barrier.

Defining the impact of injectable HCs on female genital tract innate immunity in relation to susceptibility to STIs or BV, will provide important insights into biological co-factors influencing HIV-1 risk in women. The aim of this study was to compare concentrations of genital tract soluble immune mediators (including cytokines, MMPs and TIMPs) between women using long-acting injectable HCs and women not using HCs, while accounting for BV and common STIs.

3.3 Materials and Methods

3.3.1 Study design, participants and sample collection

This study included 64 HIV-1 uninfected women using injectable HCs (DMPA or NET-EN) and 64 women not using HCs, enrolled into the prospective CAPRISA 002 observational cohort study of acute HIV-1 infection conducted at the Centre for the AIDS Programme of Research in South Africa (CAPRISA), in Durban, KwaZulu-Natal Province, South Africa, as previously described (Mlisana et al., 2012; van Loggerenberg et al., 2012). Non-HC users were matched to injectable HC users based on age (within 5 years of age) at a 1:1 ratio. Demographic and clinical data were collected at enrolment using a structured questionnaire administered by a trained counsellor. Although data on type of contraception (injectable HCs, COCs, IUDs, condoms, diaphragms, foam and jelly, or were sterilised) was collected no information was collected on whether the injectable contraceptive being used was DMPA or NET-EN. Because of this, injectable HCs in this Chapter included a combination of DMPA and NET-EN users. Women using COC or any other form of HC were excluded from the study, with an exception of IUD users. Laboratory samples, including CVLs were collected from each participant at enrolment by gently flushing the cervix and the lateral vaginal walls with 10ml sterile normal saline, as previously described by Mlisana et al. (2012). Volume of saline recovered after the lavage were not typically recorded. CVLs were transported within 4 hours on ice from the site to the laboratory. In the laboratory, CVLs were centrifuged, the supernatant collected and stored at -80°C. The protocol for this study was approved by the Ethical Review Committees of the University of KwaZulu-Natal and University of Cape Town.

3.3.2 Laboratory testing for STIs and BV

At enrollment, vulvovaginal swabs collected from the posterior fornices and lateral vaginal walls from each woman were tested for *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*, HSV-2 reactivation and *T. vaginalis* using PCR. Gram stain was performed to diagnose BV using Nugent score ≥7 (Mlisana et al., 2012).

3.3.3 Measurement of cytokines and soluble factors in CVL

Concentrations of cytokines, MMPs and TIMPS were measured in CVLs collected at enrollment. Concentrations of 42 cytokines [including IL-1α, IL-3, IL-9, IL-12p40, IL-15, IL-17, epidermal growth factor (EGF), platelet-derived growth factor (PDGF)-AA, transforming growth factor (TGF)-α, vascular endothelial growth factor (VEGF), eotaxin/CCL11, FGF-2, FLT3 Ligand (FLT3L), fractalkine/CX3CL1, G-CSF, growth related oncogene (GRO) family (CXCL1-CXCL3), IFN-α, IFN-γ-induced protein (IP)-10/CXCL10, monocyte chemoattractant protein (MCP)-1/CCL2, MCP-3/CCL7, macrophage-derived chemokine (MDC)/CCL22, MIP-1α/CCL3, MIP-1β/CCL4, PDGF-AB/BB, RANTES/CCL5, soluble CD40 ligand (sCD40L), soluble IL-2 receptor α (sIL-2Rα), TNF-β, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-10, IL-12p70, IL-13, GM-CSF, IFN-γ and TNF-α]; 4 TIMPs [TIMP-1, -2, -3, -4]; and 5 MMPs [MMP-1, -2, -7, -9 and -10]. The Human Cytokine and High Sensitivity LINCOplex Premixed kits (LINCO Research, MO, U.S.A.) were used to measure cytokines; and the TIMP Panel 2 and MMP Panel-2 kits were used to measure TIMPs and MMPs, respectively (Merck-Millipore, Missouri, U.S.A.). CVLs were thawed overnight on ice and filtered by centrifugation using 0.2 μm cellulose acetate filters (Sigma, U.S.A.). All markers were measured in undiluted CVL, except for MMP-9 (which was measured at 50-fold dilution), on a Bio-Plex 100 system (Bio-Rad

Laboratories Inc®, Hercules, California). Bio-Plex manager software (version 5.0; Bio-Rad Laboratories Inc®) was used to analyse the data and all analyte concentrations were extrapolated from the standard curves using a 5 parameter logistic (PL) regression equation. Analyte concentrations that were below the lower limit of detection of the assay were reported as the mid-point between zero and the lowest concentration measured for each analyte. For IL-8, only two samples had readings above the upper limit of detection. For these two samples, IL-8 concentrations were reported as halfway between the highest concentration and the upper limit of the standard curve.

3.3.4 Statistical analyses

Fisher's exact test was used to compare proportions, while a t-test was used to compare ages between groups. To assess the effect of injectable HCs on cytokine levels, linear regression analysis was used. Cytokines that were undetectable in at least a third of women (IL-2, IL-2Rα, IL-3, IL-4, IL-10, IL-12p40, IL-12p70, IL-13, Fractalkine, MIP-1α, IFN-α, EGF, TFG-α, FGF-2, PDGF-AB/BB and MMP-2) were dichotomised (i.e. being rated as either present or absent in each woman) and logistic regression was used to estimate the effect of injectable HCs on the detectability of these cytokines. Linear and logistic regression analyses were adjusted for sex worker status, age, condom use at last sex act, BV and STIs. In addition, interaction terms were tested to assess whether injectable HCs modified the effect of STIs on cytokine concentrations. Because of the small number of women testing positive for cervical STIs, *C. trachomatis*, *N. gonorrhoeae* or *M. genitalium,* these STIs were grouped together [Gonorrhea-Chlamydia-Mycoplasma (GCM)] for the purposes of this

analysis. Statistical analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary).

3.4 Results

This study included a total of 128 women, 64 of whom were non-HC users (including 1 woman using an IUD) and 64 of whom were injectable HC users (either DMPA or NET-EN) (Table 3.1). Of these 128 women, 79.7% were self-reported sex workers and this did not differ between groups. Women using injectable HCs were less likely to be single than those not using injectable HCs (0.0% compared to 4.7%; p=0.057), and less likely to report having multiple partners (52.4% versus 65.6%), although this was not significant. Condom use at last sex act was similar among women using injectable HC and of women not using HCs (53.1% vs 65.6%, p=0.208).

Table 3.1 Baseline demographic and clinical characteristics

Variables	Overall	Injectable-HCs users	Non-HCs users	*P*-value
N	128	64	64	
Age (years)	28 (23-36)	28 (23-36)	29 (23-37)	0.653
Marital status [% (n)]				
Single	2.4% (3)	0.0% (0)	4.7% (3)	0.057
Stable partner	34.7% (44)	41.3% (26)	28.1% (18)	
Married	3.9% (5)	6.4% (4)	1.6% (1)	
Many partners	59.1% (75)	52.4% (33)	65.6% (42)	
Sex work [% (n)]	79.7% (102)	79.7% (51)	79.7% (51)	1.000
Education [% (n)]				
<Grade 8	21.9% (28)	21.9% (14)	21.9% (14)	0.972
Grade 8-10	29.7% (38)	28.1% (18)	31.3% (20)	
Grade 11-12	48.4% (62)	50.0% (32)	46.9% (30)	
Condom use [% (n)]				
Always use condom with steady partner(s)	25.0% (32)	20.3% (13)	29.7% (19)	0.308
Always use condom with casual partner(s)	53.9% (69)	46.9% (30)	60.9% (39)	0.156
Condom use at last sex act	59.4% (76)	53.1% (34)	65.6% (42)	0.208
Positive test or culture result [% (n)]				
Genital discharge	17.2% (22)	12.5% (8)	21.9% (14)	0.241
Bacterial vaginosis (Nugent >7)	51.2% (65)	50.8% (32)	51.6% (33)	1.000
Trichomonas vaginalis	18.9% (24)	19.1% (12)	18.8% (12)	1.000
Chlamydia trachomatis	6.3% (8)	4.8% (3)	7.8% (5)	0.718
Neisseria gonorrhoeae	6.3% (8)	1.6% (1)	10.9% (7)	0.062
Mycoplasma genitalium	1.6% (2)	3.2% (2)	0.0% (0)	0.244
HSV-2 PCR	2.4% (3)	3.2% (2)	1.6% (1)	1.000

*Descriptive statistics are reported as percentages (categorical data) or medians and IQRs (continuous data).

3.4.1 Prevalence of BV and STIs in injectable HC versus non-HC users

To investigate the association between injectable HC use and STIs, the prevalence of *C. trachomatis, T. vaginalis* and BV in women using injectable HCs were compared to those who were not using HCs. At baseline (Table 3.1), more than half of the women in this cohort had BV (nugent score ≥7), and this did not differ significantly between women not using HCs and those using injectable HCs (51.6% and 50.8%, respectively; p=1.000). Nineteen percent of women were infected with *T. vaginalis* and this was similar between groups (19.1% for injectable HC users and 18.8% for non-HC users; p=1.000). The prevalence of *C. trachomatis* (6.3%), *N. gonorrhoeae* (6.3%), *M. genitalium* (1.6%), and HSV-2 (2.4%) were relatively low in this cohort and were also similar between groups (Table 3.1).

3.4.2 Impact of injectable HCs on innate factors in the female genital tract

To better understand how injectable HC use influenced the genital tract innate environment, the concentrations of cytokines, chemokines, growth factors and markers of tissue repair or remodelling (MMPs and TIMPs) were compared in CVL from women using injectable HCs and those not using HCs (Table 3.2). Of the 42 chemokines, growth factors and inflammatory cytokines that were measured, 5/42 (11.9%) had significantly decreased median concentrations in the CVL of women using injectable HCs than those not using HCs, after adjusting for age, condom use, sex worker status, marital status, STIs, and BV. This included several chemokines, including eotaxin [beta-coefficient (β)=-0.334, p=0.004], MCP-1 (β=-0.359, *p*=0.015), MDC (β=-0.364, p=0.003); the growth factor PDGF-AA (β=-0.506, p=0.001); the adaptive cytokine IL-15 (β=-0.240, p=0.038). In addition, pro-inflammatory cytokine IL-12p40 (β=-1.059, p=0.009) and chemokine fractalkine (β=-

0.910, p=0.028) were less likely to be detectable in women using injectable HCs compared to those not using HCs. While linear regression was used to estimate the effect of injectable HCs on concentrations of eotaxin, MCP-1, and MDC, a logistic regression model was fitted to estimate the effect of injectable HC use on the detectability of fractalkine and IL-12p40 because the concentrations of these cytokines were undetectable in at least a third of the women in this study. Furthermore, the median concentration of TIMP-2 was significantly lower in women using injectable HCs than those not using HCs (β=-0.207, p=0.027). In contrast, no differences were observed in MMP concentrations. None of the cytokines, chemokines, growth factors and markers of tissue repair concentrations were significantly different between groups.

Table 3.2 Influence of injectable hormonal contraceptive use on cytokine, MMP and TIMP concentrations in genital secretions

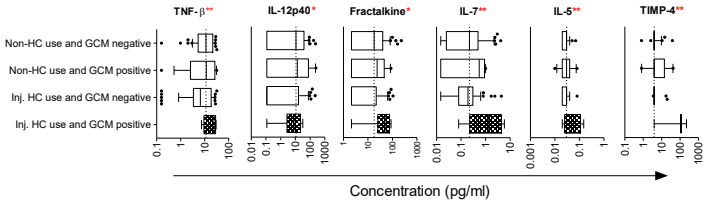
Functional groups	Cytokine	Injectable HC users (n=64)		Non-HC users (n=64)		Multivariate#	
		Median (pg/ml)	IQR	Median (pg/ml)	IQR	Beta coefficient (SE)	*P* value
Pro-inflammatory	TNF-β	7.60	3.36 - 19.05	11.53	5.31 - 21.83	-0.197 (0.104)	0.061
	IL-12p40†	0.12	0.12 - 14.39	12.20	0.12 - 38.24	-1.059 (0.404)	**0.009**
	IL-12p70†	0.02	0.01 - 0.08	0.01	0.01 - 0.09	0.363 (0.414)	0.381
	IL1α	38.02	14.26 - 172.71	87.37	30.40 - 259.69	-0.128 (0.126)	0.311
	IL-6	2.57	0.84 - 11.79	2.56	0.36 - 16.41	0.140 (0.198)	0.481
	TNF-α	0.04	0.03 - 0.07	0.04	0.02 - 0.05	0.223 (0.114)	0.053
	IL-1β	0.79	0.14 - 5.96	0.76	0.19 - 5.81	-0.005 (0.169)	0.979
Chemokine	Eotaxin	0.31	0.26 - 3.09	2.32	0.31 - 5.27	-0.334 (0.114)	**0.004**
	MCP-1	6.33	2.80 - 19.39	13.97	4.85 - 72.18	-0.359 (0.146)	**0.015**
	MDC	10.97	4.60 - 33.28	35.52	12.15 - 63.15	-0.364 (0.119)	**0.003**
	Fractalkine†	2.13	2.13 - 23.60	18.01	2.13 - 40.10	-0.910 (0.414)	**0.028**
	MIP1α†	0.66	0.66 - 5.73	0.66	0.66 - 19.83	-0.422 (0.472)	0.370
	MCP-3	9.35	1.95 - 12.41	12.34	8.47 - 15.42	-0.106 (0.063)	0.097
	IP-10	22.95	3.78 - 88.25	45.96	13.30 - 182.40	-0.393 (0.211)	0.066
	GRO	370.82	134.00 - 1263.72	598.60	236.09 - 1974.89	-0.178 (0.128)	0.167
	MIP-1β	3.18	0.26 - 5.27	4.31	0.70 - 7.93	-0.028 (0.252)	0.911
	IL-8	157.89	69.74 - 510.09	131.05	54.80 - 1029.17	0.112 (0.129)	0.389
	RANTES	3.65	2.24 - 9.62	3.21	0.93 - 9.98	0.202 (0.136)	0.138
Innate	IFNα†	0.68	0.68 - 0.68	0.68	0.68 - 8.91	-0.874 (0.446)	0.050
Hematopoietic	IL-9	0.68	0.01 - 1.24	1.19	0.23 - 1.76	-0.319 (0.188)	0.093
	Flt3L	4.27	0.45 - 9.48	6.56	2.94 - 10.56	-0.174 (0.110)	0.116
	G-CSF	17.17	2.76 - 73.44	26.80	4.71 - 117.07	-0.256 (0.208)	0.222
	GM-CSF	0.14	0.02 - 0.38	0.13	0.01 - 0.47	0.077 (0.141)	0.588
	IL-7	0.25	0.08 - 0.41	0.25	0.02 - 0.61	-0.033 (0.123)	0.791

	IL-3	3.08	0.01 - 14.86	8.35	0.01 - 28.18	-0.229 (0.415)	0.580
Growth Factor	PDGF-AA	4.55	2.54 - 19.81	15.77	5.85 - 118.91	-0.506 (0.146)	**0.001**
	TGF-α	2.35	0.99 - 4.19	3.37	2.14 - 5.79	-0.173 (0.101)	0.089
	VEGF	73.86	17.27 - 151.91	77.09	12.95 - 120.64	-0.033 (0.100)	0.742
	PDGF-AB/BB†	0.09	0.09 - 17.43	0.09	0.09 - 38.66	-0.185 (0.417)	0.658
	EGF†	0.56	0.56 - 2.30	0.56	0.56 - 8.61	-0.632 (0.427)	0.139
	FGF-2†	0.30	0.30 - 8.39	5.32	0.30 - 13.15	-0.539 (0.394)	0.171
Adaptive	IL-15	1.54	0.30 - 1.90	2.08	1.39 - 2.52	-0.240 (0.114)	**0.038**
	IL-5	0.03	0.02 - 0.03	0.03	0.02 - 0.04	0.011 (0.028)	0.690
	IL-17	0.82	0.03 - 1.17	1.07	0.67 - 1.73	-0.158 (0.137)	0.251
	sCD40L	22.84	8.41 - 30.89	22.84	12.64 - 33.53	-0.007 (0.113)	0.954
	IFN-γ	1.31	0.13 - 4.51	0.67	0.01 - 3.09	0.336 (0.215)	0.121
	IL-2†	0.08	0.01 - 0.20	0.08	0.01 - 0.19	-0.444 (0.417)	0.287
	IL-4†	0.02	0.02 - 0.36	0.05	0.02 - 0.38	-0.300 (0.406)	0.460
	IL-13†	0.01	0.01 - 0.01	0.01	0.01 - 0.01	0.217 (0.428)	0.612
	sIL-2Rα†	5.88	0.57 - 10.13	6.10	0.57 - 10.70	0.143 (0.395)	0.717
Anti-inflammatory	IL-1Ra	36313.59	26674.06 - 45012.00	40793.38	27693.84 - 45012.00	-0.038 (0.044)	0.381
	IL-10†	0.02	0.01 - 0.19	0.02	0.01 - 0.24	0.813 (0.429)	0.058
MMPs	MMP-1	5.30	2.40 - 42.71	3.92	2.40 - 53.10	0.135 (0.179)	0.454
	MMP-7	5038.05	379.42 - 23761.51	2247.97	162.27 - 26476.16	0.293 (0.358)	0.416
	MMP-9	13336.27	4963.64 - 73603.95	15753.28	4774.88 - 46728.84	0.203 (0.202)	0.318
	MMP-10	11.70	2.20 - 126.18	5.52	2.20 - 166.11	0.318 (0.235)	0.180
	MMP-2†	118.10	118.10 - 118.10	118.10	118.10 - 118.10	0.808 (0.832)	0.332
TIMPs	TIMP-1	1867.65	854.01 - 6087.20	4356.19	1316.85 - 6634.26	-0.243 (0.189)	0.203
	TIMP-2	11759.57	6497.84 - 16406.50	14878.22	8023.87 - 19246.30	-0.207 (0.091)	**0.027**
	TIMP-3	25.45	25.45 - 32.54	25.45	25.45 - 32.54	0.029 (0.040)	0.472
	TIMP-4	3.92	3.92 - 3.92	3.92	3.92 - 3.92	0.013 (0.068)	0.849

SE=standard error, CI = confidence interval. #Multivariate analysis adjusted for age, marital status, condom use, sex work, STIs and BV as co-variates. †Variables with at least a third of concentrations were undetectable were dichotomised and a logistic regression model was fitted to estimate the effect of injectable contraception on detectability of these cytokines.

3.4.3 Impact of BV and STIs on cytokines in injectable HC versus non-HC users

Because injectable HC use resulted in lower median concentration of genital tract cytokines from several different functional classes (Table 3.2), interactions between injectable HCs and cytokine concentrations in responses to BV or STIs in the female genital tract were assessed. No significant interactions were observed between injectable HC use and cytokine responses to BV (Table 3.3). *T. vaginalis* also had a limited interaction with injectable HC, with only Flt3L demonstrating a significant result (β-estimate of -0.194 in the injectable group vs 0.338 in the non-injectable group, p=0.048, Table 3.4). However, significant interactions were found between gonorrhea, chlamydia or mycoplasma (GCM) infections and injectable HC use for certain cytokines, with TNF-β (p=0.022), IL-5 (p=0.015), IL-7 (p=0.036) and TIMP-4 (p=0.003) being significantly higher in CVLs in response to GCM infections in HC users compared to non-HC users (Figure 3.1, Table 3.5). Furthermore, IL-12p40 (p=0.061) and Fractalkine (p=0.069) showed a trend towards being elevated in the GCM positive women using injectable HCs compared to non-HC users. However, none of these associations remained significant after adjusting for multiple comparisons.


TNF-β**
IL-12p40*
Fractalkine*
IL-7**
IL-5**
TIMP-4**
Non-HC use and GCM negative
Non-HC use and GCM positive
Inj. HC use and GCM negative
Inj. HC use and GCM positive
Concentration (pg/ml)

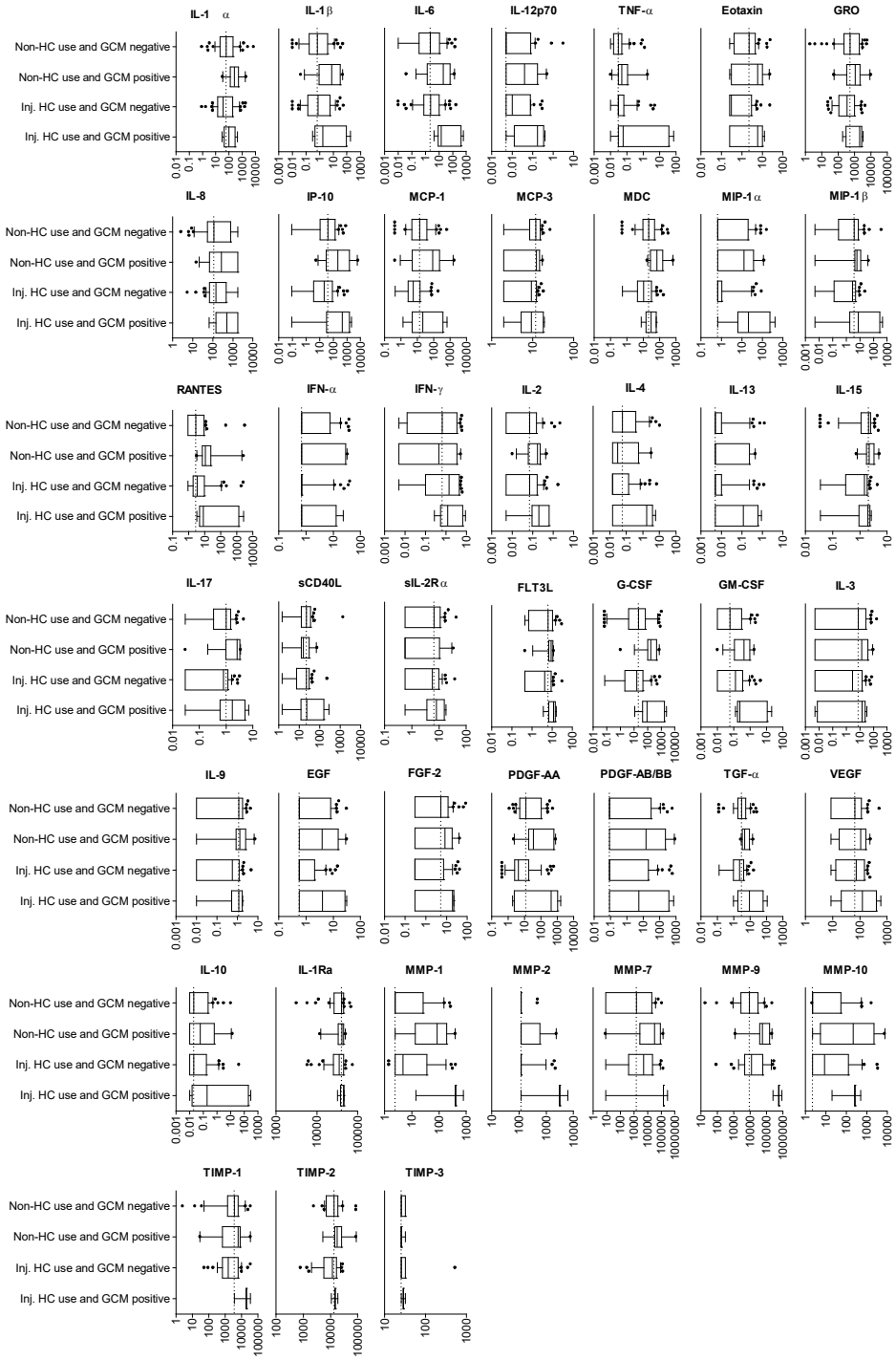

IL-1 α
IL-1β
IL-6
IL-12p70
TNF-α
Eotaxin
GRO
IL-8
IP-10
MCP-1
MCP-3
MDC
MIP-1α
MIP-1β
RANTES
IFN-α
IFN-γ
IL-2
IL-4
IL-13
IL-15
IL-17
sCD40L
sIL-2Rα
FLT3L
G-CSF
GM-CSF
IL-3
IL-9
EGF
FGF-2
PDGF-AA
PDGF-AB/BB
TGF-α
VEGF
IL-10
IL-1Ra
MMP-1
MMP-2
MMP-7
MMP-9
MMP-10
TIMP-1
TIMP-2
TIMP-3
Non-HC use and GCM negative
Non-HC use and GCM positive
Inj. HC use and GCM negative
Inj. HC use and GCM positive
Concentration (pg/ml)

Figure 3.1 Cytokine concentrations in CVLs from women using injectable HCs (DMPA or NET-EN) compared to those not using HCs, stratified according to presence of discharge causing STIs (*C. trachomatis, N. gonnorhoea, M. genitalium*; GCM). Dotted lines indicate the median concentration of each cytokine, MMP or TIMP in women not using HCs and who were GCM negative, as the reference group. ** indicates significant interactions between injectable HC use and GCM with $p<0.05$, while * indicates interactions between injectable HC use and GCM with $p<0.10$. $p<0.10$ received an * because the sample size was too small and due to this, we treated $p<0.010$ as a cutoff instead of of a widlely used $p<0.05$. This was specific for this chapter only.

Table 3.3 Relationship between injectable HC use and cytokine responses to BV

Cytokine	Non-injectable HC group (n=64)		Injectable HC group (n=64)		Interaction p-value
	Est (SE) or OR (95% CI)	p-value	Est (SE) or OR (95% CI)	p-value	
Log TNFB	0.140 (0.118)	0,2409	-0.069 (0.157)	0,6610	0,3318
IL12p40*	0.42 (0.13-1.32)	0,1390	1.53 (0.46-5.11)	0,4866	0,1260
Log IL1a	0.723 (0.171)	<.0001	0.703 (0.183)	0,0003	0,6390
IL12p70*	0.82 (0.26-2.57)	0,7372	0.49 (0.14-1.77)	0,2785	0,4571
Log IL6	0.499 (0.304)	0,1069	0.253 (0.255)	0,3252	0,3663
Log TNFa	0.118 (0.138)	0,3949	-0.019 (0.199)	0,9234	0,3694
Log IL1B	0.918 (0.235)	0,0003	0.692 (0.260)	0,0102	0,3381
Eotaxin	-0.043 (0.161)	0,7890	-0.253 (0.154)	0,1065	0,2558
MCP-1	-0.216 (0.219)	0,3266	-0.222 (0.190)	0,2499	0,7228
MDC	-0.436 (0.169)	0,0128	-0.284 (0.169)	0,0996	0,9605
Fractalkine	1.39 (0.41-4.75)	0,6008	0.44 (0.12-1.56)	0,2028	0,2115
MIP-1a	0.26 (0.07-0.94)	0,0401	0.10 (0.02-0.55)	0,0080	0,3005
MCP-3	0.017 (0.091)	0,8528	-0.061 (0.093)	0,5127	0,5979
IP-10	-0.384 (0.276)	0,1695	-0.890 (0.325)	0,0083	0,1024
GRO	-0.269 (0.201)	0,1851	-0.393 (0.148)	0,0105	0,4073
MIP-1b	0.352 (0.346)	0,3136	-0.397 (0.356)	0,2704	0,1238
IL-8	0.127 (0.207)	0,5439	0.151 (0.158)	0,3435	0,9376
RANTES	0.044 (0.176)	0,8027	0.126 (0.221)	0,5719	0,8289
IFN-a	1.31 (0.44-3.95)	0,6310	0.61 (0.15-2.48)	0,4918	0,5961
IL-9	0.018 (0.251)	0,9427	-0.145 (0.281)	0,6078	0,5482
Flt3L	0.072 (0.143)	0,6174	0.253 (0.149)	0,0969	0,4998
G-CSF	0.230 (0.288)	0,4286	0.336 (0.303)	0,2723	0,8567
GM-CSF	-0.279 (0.187)	0,1419	-0.334 (0.210)	0,1171	0,4021
IL-7	-0.472 (0.191)	0,0166	-0.435 (0.152)	0,0059	0,8176
IL-3	1.65 (0.52-5.19)	0,3945	0.50 (0.15-1.66)	0,2569	0,2198
PDGF-AA	0.056 (0.195)	0,7751	0.201 (0.227)	0,3784	0,8077
EGF	0.73 (0.24-2.23)	0,5752	1.03 (0.31-3.44)	0,9581	0,7923
TFG-a	-0.110 (0.115)	0,3434	-0.161 (0.162)	0,3240	0,6214
FGF-2	0.78 (0.25-2.44)	0,6739	0.35 (0.10-1.18)	0,0897	0,2902
VEGF	-0.072 (0.135)	0,5967	0.024 (0.154)	0,8778	0,6203
PDGF-AB/BB	0.35 (0.10-1.25)	0,1057	0.94 (0.29-3.09)	0,9244	0,5365
IL-15	0.088 (0.133)	0,5108	-0.075 (0.183)	0,6846	0,3678
IL-5	-0.047 (0.041)	0,2627	-0.095 (0.039)	0,0175	0,2774
IL-17	**0.270 (0.173)**	**0,1236**	**-0.126 (0.203)**	**0,5396**	**0,0756***
IL-2	1.10 (0.32-3.79)	0,8788	1.49 (0.43-5.19)	0,5291	
IL-4	0.32 (0.10-1.01)	0,0515	0.45 (0.14-1.45)	0,1800	0,8555
IL-13	0.67 (0.21-2.21)	0,5149	0.95 (0.28-3.20)	0,9278	0,9781
sCD40L	0.010 (0.162)	0,9530	0.237 (0.158)	0,1387	0,2853
sIL-2Ra	0.65 (0.21-2.02)	0,4558	1.60 (0.49-5.17)	0,4345	0,2773
IFN-g	0.024 (0.325)	0,9418	-0.417 (0.286)	0,1505	0,2853
IL-10	0.74 (0.24-2.28)	0,6001	0.60 (0.17-2.08)	0,4208	0,5990
IL-1Ra	0.048 (0.059)	0,4232	-0.022 (0.061)	0,7234	0,4788
MMP-1	0.447 (0.235)	0,0660	0.340 (0.296)	0,2581	0,1338
MMP-2	**8.68 (0.31-244.34)**	**0,2045**	**0.76 (0.07-7.76)**	**0,8188**	**0,0696***
MMP-7	0.971 (0.484)	0,0538	0.473 (0.594)	0,4316	0,1969

MMP-9	0.317 (0.288)	0,2781	0.439 (0.291)	0,1411	0,5754
MMP-10	**0.885 (0.327)**	**0,0110**	**0.520 (0.373)**	**0,1732**	**0,0767***
TIMP-1	0.663 (0.336)	0,0571	0.387 (0.226)	0,0971	0,4700
TIMP-2	-0.170 (0.114)	0,1464	0.059 (0.151)	0,7009	0,4793
TIMP-3	-0.027 (0.016)	0,1022	-0.130 (0.079)	0,1085	0,2085
TIMP-4	-0.003 (0.122)	0,9808	0.031 (0.101)	0,7576	0,6814

Table 3.4 Relationship between injectable HC use and cytokine responses to *T. vaginalis*

Cytokine	Non-injectable group (n=64) Est (SE) or OR (95% CI)	p-value	Injectable group (n=64) Est (SE) or OR (95% CI)	p-value	Interaction p-value
Log TNFB	0.253 (0.172)	0,1460	0.264 (0.194)	0,1777	0,6397
IL12p40*	1.11 (0.21-5.78)	0,9054	0.44 (0.10-1.99)	0,2893	0,4350
Log IL1a	0.156 (0.249)	0,5345	0.299 (0.225)	0,1892	0,8272
IL12p70*	3.84 (0.67-21.97)	0,1305	1.25 (0.30-5.25)	0,7618	0,3970
Log IL6	-0.069 (0.444)	0,8764	0.094 (0.313)	0,7643	0,4874
Log TNFa	-0.112 (0.201)	0,5790	-0.013 (0.244)	0,9583	0,8530
Log IL1B	0.238 (0.342)	0,4897	0.490 (0.319)	0,1307	0,6916
Eotaxin	-0.093 (0.234)	0,6936	-0.279 (0.190)	0,1465	0,4333
MCP-1	-0.372 (0.319)	0,2475	0.401 (0.234)	0,0929	0,2527
MDC	0.028 (0.247)	0,9105	0.032 (0.208)	0,8773	0,2474
Fractalkine	**4.10 (0.56-30.14)**	**0,1659**	**0.37 (0.08-1.76)**	**0,2135**	**0,0879***
MIP-1a	0.91 (0.16-5.13)	0,9128	0.38 (0.06-2.38)	0,3034	0,2131
MCP-3	-0.134 (0.133)	0,3181	-0.017 (0.114)	0,8804	0,6453
IP-10	-0.618 (0.402)	0,1303	0.462 (0.399)	0,2517	0,5036
GRO	-0.001 (0.293)	0,9960	0.633 (0.182)	0,0010	0,1320
MIP-1b	0.605 (0.505)	0,2363	-0.604 (0.438)	0,1735	0,085*
IL-8	0.221 (0.302)	0,4685	0.502 (0.195)	0,0127	0,5838
RANTES	0.076 (0.256)	0,7670	0.059 (0.272)	0,8289	0,3189
IFN-a	0.70 (0.13-3.76)	0,6788	0.25 (0.02-2.51)	0,2381	0,3441
IL-9	0.433 (0.366)	0,2423	-0.043 (0.345)	0,9015	0,3415
Flt3L	**0.338 (0.209)**	**0,1114**	**-0.194 (0.184)**	**0,2968**	**0,0481****
G-CSF	-0.181 (0.420)	0,6679	0.411 (0.372)	0,2744	0,8167
GM-CSF	**0.415 (0.273)**	**0,1344**	**-0.077 (0.258)**	**0,7659**	**0,0816***
IL-7	0.053 (0.279)	0,8510	-0.047 (0.186)	0,8034	0,6187
IL-3	1.09 (0.22-5.48)	0,9157	0.24 (0.06-1.01)	0,0515	0,2587
PDGF-AA	-0.244 (0.285)	0,3957	0.027 (0.279)	0,9230	0,9843
EGF	1.39 (0.29-6.67)	0,6796	0.50 (0.09-2.78)	0,4270	0,1818
TFG-a	-0.141 (0.168)	0.137 (0.199)	0.135 (0.199)	0,5021	0,7017
FGF-2	1.76 (0.31-9.91)	0,5227	0.33 (0.07-1.53)	0,1579	0,1138
VEGF	0.064 (0.197)	0,7462	0.063 (0.189)	0,7404	0,9398
PDGF-AB/BB	0.50 (0.08-3.16)	0,4582	1.45 (0.35-5.92)	0,6087	0,8745
IL-15	0.108 (0.194)	0,5782	0.022 (0.225)	0,9211	0,8090
IL-5	-0.044 (0.060)	0,4662	0.019 (0.047)	0,6940	0,7254
IL-17	0.065 (0.252)	0,7985	0.382 (0.250)	0,1323	0,6550
IL-2	N/A		0.80 (0.18-3.49)	0,7681	
IL-4	**2.94 (0.51-16.88)**	**0,2261**	**0.31 (0.07-1.39)**	**0,1250**	**0,0811***
IL-13	0.33 (0.04-2.50)	0,2838	2.12 (0.50-8.97)	0,3081	0,4429
sCD40L	-0.023 (0.237)	0,9218	-0.414 (0.194)	0,0370	0,2291
sIL-2Ra	2.90 (0.52-16.09)	0,2244	0.32 (0.08-1.28)	0,1072	0,2134
IFN-g	-0.286 (0.474)	0,5487	-0.186 (0.352)	0,5986	0,7263
IL-10	0.89 (0.18-4.45)	0,8839	1.02 (0.22-4.74)	0,9827	0,8151
IL-1Ra	0.102 (0.087)	0,2427	0.027 (0.076)	0,7260	0,4863
MMP-1	0.275 (0.303)	0,3711	0.391 (0.290)	0,1875	0,3679
MMP-2	N/A		4.54 (0.57-36.24)	0,1537	N/A

MMP-7	0.046 (0.624)	0,9419	0.516 (0.583)	0,3826	0,7647
MMP-9	-0.357 (0.371)		0.413 (0.285)	0,1576	0,8143
MMP-10	-0.012 (0.422)	0,9775	0.302 (0.366)	0,4157	0,4630
TIMP-1	0.373 (0.433)	0,3950	0.081 (0.226)	0,7224	0,4718
TIMP-2	-0.021 (0.147)	0,8852	-0.045 (0.151)	0,7692	0,3319
TIMP-3	-0.001 (0.021)	0,9740	-0.053 (0.079)	0,5065	0,8244
TIMP-4	**0.181 (0.158)**	**0,2597**	**-0.067 (0.101)**	**0,5081**	**0,0973***

Table 3.5 Relationship between injectable HC use and cytokine responses to *C. trachomatis, N. gonnorhoea*, and *M. genitalium*

Cytokine	Non-injectable HC group (n=64)		Injectable HC group (n=64)		Interaction p-value
	Est (SE) or OR (95% CI)	p-value	Est (SE) or OR (95% CI)	p-value	
Log TNFB	**-0.412 (0.182)**	**0,0275**	**0.454 (0.275)**	**0,1051**	**0,0223****
IL12p40*	**0.88 (0.16-4.85)**	**0,8823**	**6.74 (0.66-69.02)**	**0,1077**	**0,061***
Log IL1a	0.294 (0.264)	0,2709	0.339 (0.320)	0,2946	0,6074
IL12p70*	0.78 (0.13-4.56)	0,7818	7.76 (0.54-110.71)	0,1306	0,2106
Log IL6	0.636 (0.470)	0,1818	1.214 (0.446)	0,0087	0,3954
Log TNFa	0.380 (0.213)	0,0801	0.780 (0.347)	0,0287	0,1076
Log IL1B	0.356 (0.363)	0,3312	0.741 (0.454)	0,1088	0,8513
Eotaxin	0.268 (0.248)	0,2850	0.422 (0.270)	0,1231	0,2776
MCP-1	0.721 (0.338)	0,0372	0.737 (0.333)	0,0311	0,6318
MDC	0.736 (0.262)	0,0068	0.467 (0.296)	0,1208	0,6865
Fractalkine	**0.35 (0.05-2.43)**	**0,2911**	**4.81 (0.43-53.45)**	**0,2011**	**0,0685***
MIP-1a	8.58 (1.32-55.79)	0,0245	43.14 (1.13-1650.89)	0,0429	0,2762
MCP-3	0.020 (0.141)	0,8874	0.123 (0.163)	0,4550	0,3921
IP-10	1.389 (0.427)	0,0019	0.825 (0.567)	0,1519	0,8071
GRO	0.313 (0.310)	0,3171	0.601 (0.259)	0,0241	0,5302
MIP-1b	0.013 (0.535)	0,9809	0.763 (0.623)	0,2258	0,3116
IL-8	0.065 (0.320)	0,8390	0.480 (0.277)	0,0885	0,7030
RANTES	0.631 (0.271)	0,0238	0.654 (0.387)	0,0967	0,9032
IFN-a	1.31 (0.24-7.19)	0,7571	1.84 (0.22-15.70)	0,5766	0,5242
IL-9	0.107 (0.389)	0,7844	0.478 (0.491)	0,3344	0,5341
Flt3L	-0.025 (0.222)	0,9119	0.511 (0.261)	0,0557	0,1481
G-CSF	1.025 (0.446)	0,0253	1.419 (0.529)	0,0098	0,6122
GM-CSF	0.420 (0.289)	0,1521	0.771 (0.367)	0,0406	0,4856
IL-7	**0.151 (0.296)**	**0,6119**	**0.813 (0.265)**	**0,0034**	**0,0356****
IL-3	0.65 (0.12-3.56)	0,6204	2.81 (0.24-32.98)	0,4119	0,2258
PDGF-AA	0.453 (0.302)	0,1390	1.080 (0.396)	0,0087	0,1035
EGF	2.08 (0.39-11.15)	0,3916	4.31 (0.59-31.51)	0,1506	0,4594
TFG-a	0.340 (0.178)	0,0611	0.658 (0.283)	0,0240	0,1043
FGF-2	1.94 (0.32-11.70)	0,4690	1.98 (0.23-17.04)	0,5345	0,7728
VEGF	-0.040 (0.208)	0,8477	0.266 (0.269)	0,3285	0,3985
PDGF-AB/BB	12.72 (1.75-92.34)	0,0119	3.67 (0.48-27.98)	0,2099	0,6855
IL-15	0.199 (0.206)	0,3384	0.102 (0.319)	0,7512	0,9304
IL-5	**0.067 (0.064)**	**0,2946**	**0.228 (0.067)**	**0,0013**	**0,0151****
IL-17	0.409 (0.267)	0,1316	0.575 (0.356)	0,1118	0,6629
IL-2	N/A		3.04 (0.25-37.42)	0,3850	
IL-4	1.24 (0.21-7.23)	0,8090	1.80 (0.22-14.49)	0,5826	0,5397
IL-13	5.19 (0.77-34.85)	0,0899	5.00 (0.68-36.61)	0,1129	0,7035
sCD40L	-0.062 (0.251)	0,8046	0.125 (0.275)	0,6511	0,3841
sIL-2Ra	0.41 (0.07-2.59)	0,3467	2.67 (0.25-28.20)	0,4138	0,1435
IFN-g	0.054 (0.502)	0,9140	0.313 (0.500)	0,5340	0,3875
IL-10	1.41 (0.24-8.34)	0,7047	1.87 (0.16-21.32)	0,6146	0,6485
IL-1Ra	-0.103 (0.092)	0,2647	0.109 (0.107)	0,3153	0,2746
MMP-1	0.541 (0.332)	0,1135	1.303 (0.586)	0,0332	0,7424
MMP-2	15.27 (0.54-430.22)	0,1095	13.71 (0.40-472.02)	0,1470	0,5269
MMP-7	0.955 (0.685)	0,1731	0.248 (1.177)	0,8345	0,2877
MMP-9	0.820 (0.407)	0,0527	1.713 (0.576)	0,0055	0,2148
MMP-10	0.799 (0.463)	0,0942	0.824 (0.740)	0,2737	0,5375

TIMP-1	-0.449 (0.475)	0,3518	1.118 (0.459)	0,0204	0,1779
TIMP-2	0.237 (0.161)	0,1523	0.255 (0.307)	0,4130	0,9846
TIMP-3	-0.013 (0.023)	0,5641	-0.052 (0.160)	0,7463	0,7807
TIMP-4	**-0.058 (0.173)**	**0,7394**	**0.822 (0.204)**	**0,0003**	**0,0026****

3.5 Discussion

Concentrations of several cytokines and soluble factors were reduced in CVLs of women using injectable HC compared to women who were not using HCs, after controlling for age, condom use, sex work, STIs and BV. Despite this reduction of innate immune responses in the female genital tracts of women using injectable HCs, no differences in prevalence of BV or STIs were found.

Although animal studies using high dose DMPA, have demonstrated that DMPA has immunosuppressive properties both systemically and in the reproductive tract, these studies found that DMPA exerted on effect on viral load and pathogenesis (Abel et al., 2004; Bamberger et al., 1999; Genesca et al., 2007; Gillgrass et al., 2003; Hel et al., 2010; Hughes et al., 2008; Huijbregts et al., 2013; Kleynhans et al., 2013; Kleynhans et al., 2011; Koubovec et al., 2004; Trunova et al., 2006). Fewer studies have been conducted in humans and these have predominantly been performed using PBMCs (Hughes et al., 2008, Kleynhans et al., 2011), with the exception of Huijbregts et al. (2013) who reported reduced cervicovaginal production of IFN-α in women using injectable HCs, the other human studies suggested that DMPA use may actually increase inflammation within the female genital tract (Baeten et al., 2001; Ghanem et al., 2005). In this study, decreased chemotactic cytokine (including eotaxin, fractalkine, MCP-1 and MDC in the multivariate analyses) concentrations observed in women using HCs might influence the trafficking of immune cells to the female genital tract. Fractalkine has been reported to play a role in the recruitment of immune cells to the endometrium, which may be influenced by the presence of DMPA (Hannan et al., 2004). Although these associations potentially suggest HC

interference with chemotaxis, mechanistic studies have not been done to test the relationship between HCs and trafficking of cells within genital tissues.

In the interaction term analysis, it was hypothesized that HC use had a limited impact on cytokine responses to trichomoniasis and gonorrhea, chlamydia and mycoplasma infections, but did not modify the relationship between cytokine concentrations and BV. This analysis suggested that women with gonorrhea, chlamydia and mycoplasma (GCM) infections who were using HCs had increased concentrations of TNF-β, IL-5, IL-7, TIMP-4, IL-12p40 and Fractalkine relative to women with GCM infections who were not using HCs. In addition, the effect size of the relationship between *T. vaginalis* and Flt3L concentrations was larger in women using injectable HCs compared to non-HC users. However, these associations should be interpreted conservatively as none of these associations was significant after adjusting for multiple comparisons and sample sizes for these analyses were relatively small.

High dose DMPA administration to macaques is associated with thinning of the vaginal epithelium (Abel et al., 2004; Genesca et al., 2007; Trunova et al., 2006; Wieser et al., 2001). In this study, significantly reduced concentrations of PDGF-AA were found in CVL from women using injectable HC compared to women not using injectable HCs. This growth factor has been reported to play an important role in restoring the barrier function of the female genital tract following injury, and reduced expression may influence the ability of the epithelial barrier to be regenerated and repaired (Werner and Grose, 2003). Growth factors enhance epithelial repair by stimulating mitosis, spreading and migration of epithelial cells in mouse airways

(Werner and Grose, 2003), and as such, a decrease in these factors could lead to weakened epithelial barriers and reduced epithelial healing in women using injectable HCs.

MMPs and TIMPs play an important role in the degradation and remodeling of the extracellular matrix in the upper reproductive tract during normal reproductive processes (Birkedal-Hansen, 1995; Cawston, 1995; Lockwood and Schatz, 1996; Rodgers et al., 1994; Rodgers et al., 1993). Previous studies have shown that progesterone suppresses the epithelial cell-specific MMPs, working cooperatively with TGF-β to regulate epithelial-specific MMP-7 expression (Bruner et al., 1995; Osteen et al., 1994). In contrast, in the lower reproductive tract, this study found no difference between MMP concentrations in women using injectable HCs compared to those who were not using HC. Previously, Vincent et al. (2002) found that women using DMPA had decreased TIMP-1 and -2 concentrations in their endometrial epithelium and an altered local MMP:TIMP balance in their upper reproductive tracts compared to women not using HCs. Similarly, decreased TIMP-2 concentrations were observed in this study in secretions from the lower reproductive tracts of women using injectable HC compared to those not using HCs. Reduced concentrations of TIMPS may alter the MMP/TIMP ratio which could lead to decreased epithelial barrier integrity.

A limitation of this study is that it was not possible to differentiate DMPA from NET-EN users within the injectable HC user group although these progestin-based HCs may have distinct biological effects. DMPA or NET-EN users may differ behaviorally

or systematically from women who do not use hormonal contraceptives. Another limitation of this study was that stage of the menstrual cycle was not standardized in this cohort although many women using DMPA and Net-EN were not menstruating. In addition, data on prior DMPA or NET-EN use before the time point at which CVL was performed was not available and the length of time on injectable HCs may have an impact of the effect that was measured.

In conclusion, this Chapter shows reductions in CVL concentrations of several chemokines (directing cellular movement within tissue), in addition to an array of proinflammatory and adaptive cytokines, growth factors, and TIMPs in the lower female genital tract of women using injectable HCs. While this study was underpowered to demonstrate the relationship between injectable HC use and STI or HIV-1 risk, the data suggest that injectable HCs effects were similar between STI positive and STI negative participants. Large-scale randomized clinical trials assessing the impact of progestin-derivatives (DMPA or NET-EN) on local and systemic innate and adaptive immune environment, as well as STIs, are needed to further investigate the mechanism(s) by which DMPA or NET-EN might increase risk of HIV-1 infection.

CHAPTER 4

Influence of semen on inflammatory and innate cytokine responses in the female genital tract

CHAPTER 4

Influence of semen on inflammatory and innate cytokine responses in the female genital tract

4.1 Abstract

Semen contains cytokines and activated immune cells that may influence the immune environment of the female genital tract. Inflammatory cytokine concentrations in female genital secretions may influence HIV-1 risk, although the effect of recent sexual intercourse on the cytokine milieu of cervicovaginal secretions has rarely been measured in previous studies. The influence of semen exposure on CVL cytokine concentrations was investigated in this Chapter from 46 HIV-1 negative women, who had no evidence of BV or an STI, by assessing the presence of Y-chromosome by real-time PCR (evidence of unprotected intercourse >48 hours prior to sampling), and PSA by ELISA (evidence of unprotected intercourse ≤48 hours prior to sampling). As in the previous Chapter, concentrations of 42 cytokines, 5 MMPs and 4 TIMPs were measured by luminex. Y-chromosome was detected in 50% (23/46) of CVLs, while PSA was detected in 57% (13/23) of Y-chromosome positive women. More than one third of women (39%, 5/13) determined to have recent sex (PSA+) reported condom use at their last sex act. CVLs collected from PSA+ women had lower concentrations of IL-12p70, sIL-2Rα and IL-2, after adjusting for confounders. Similarly, fractalkine, FGF-2, GM-CSF, IP-10, and TNF-α were decreased CVLs from PSA+ women, although not significantly after adjusting for multiple comparisons. Concentrations of TIMP-1 and MMP-9, both associated with tissue remodelling in the female reproductive tract, were higher in CVLs from PSA+ women. These findings suggest that the presence of semen influenced the cytokine profile in CVL and should be

taken into consideration when investigating biological markers in the female genital tract.

4.2 Introduction

Despite improvements in the formatting and framing of sexual behaviour questionnaires, over-reporting of adherence and safe sexual behaviours have been identified as shortcomings in several biomedical prevention studies (Turner and Miller, 1997; Zenilman et al., 1995). Over-reporting of condom use may lead to inaccurate estimates of the influence of interventions such as vaginal microbicides on risk for HIV-1 and sexual transmitted infections, as sub-analyses are often based on the frequency of unprotected sex.

The presence of semen in the vagina during unprotected sex has been associated with short-term activation of mucosal immunity (Robertson, 2005; Robertson et al., 2009; Sharkey et al., 2012). In addition to spermatozoa, seminal fluid contains potent anti-inflammatory cytokines (TGF-β, IL-10, and PGE2), and pro-inflammatory cytokines [IL-8, secretory leukocyte protease (SLP)-1], all with the capacity to alter the immune environment of the vaginal mucosa (Denison et al., 1999; Sharkey et al., 2007). Seminal fluid also contains signaling molecules that induce expression of IL-1β, IL-6 and leukemia inhibitory factor (LIF) by endometrial epithelial cells *in vitro* (Gutsche et al., 2003; Sharkey et al., 2012). Expression of these cytokines is known to trigger the recruitment and activation of macrophages, DCs and granulocytes (Kachkache et al., 1991; McMaster et al., 1992; Prakash et al., 2003; Sharkey et al., 2012). Seminal fluids increased ectocervical numbers of $CD14^+$ macrophages, $CD1a^+$ DCs and $CD8^+$ T-cells, and decreased numbers of CD4+ T cells 12 hours after unprotected sexual intercourse (Sharkey et al., 2012). Expression of genes involved in inflammation, including those encoding inflammatory cytokines CSF-2, IL-6, IL-8 and IL-1α, were induced in ectocervical cells following exposure to semen, whereas these changes

were not observed in women who engaged in protected intercourse (Sharkey et al., 2012). Moreover, *in vitro* studies suggested that cervical tissue exposed to seminal fluids were responsible for the increased production of several MMPs by ectocervical epithelial cells (Denison et al., 1999; Sharkey et al., 2007). Therefore, objective assessment of semen exposure is important to aid in accurate interpretation of data in studies of the immunological environment in the female genital tract.

Reliable methods have been developed to measure biomarkers of semen exposure to reduce reliance on self-reporting in studies investigating immunological factors in the female genital tract, risk of infection or probability of pregnancy (Mauck et al., 2007; Walsh et al., 2003). PSA and the Y-chromosome are semen markers that have previously been used as indicators of the presence of semen in female genital fluids (Bahamondes et al., 2008; Chomont et al., 2001a; Chomont et al., 2001b; Graves et al., 1985; Kamenev et al., 1989; Minnis et al., 2009; Roewer, 2009). PSA concentrations in vaginal fluid can be detected at concentrations ≥1ng/ml up to 48 hours after unprotected sexual intercourse (Graves et al., 1985; Kamenev et al., 1989; Lawson et al., 1998; Macaluso et al., 1999). Although PSA is relatively sensitive, the utility of PSA as a surrogate indicator for unprotected sex is limited by its rapid decay curve (within 48 hours) (Macaluso et al., 1999; Negri et al., 2000; Obiezu et al., 2001). In comparison, Y-chromosome PCR is a highly stable, sensitive, and specific method to detect spermatozoa-associated DNA fragments of the sex-determining region (SRY) and testis-specific protein Y-encoded (TSPY) genes of the Y-chromosome that are not present on the X-chromosome (Jacot et al., 2013; Kastelic et al., 2009; Reynolds and Varlaro, 1996; Roewer, 2009; Sullivan et al., 1993). Y-chromosomes can be detected in cervicovaginal fluids up to 15 days after exposure to

seminal fluids (Chomont et al., 2001b; Jadack et al., 2006; Kastelic et al., 2009; Zenilman et al., 2005).

Detecting the presence of semen in genital fluids using these biomarkers provide objective tools to measure exposure to semen, allowing for accurate assessment of unprotected sex during HIV-1 prevention trials, and effects of semen on the immunological environment of the female genital tract. In this study, the presence of PSA and Y-chromosomes in CVL collected from HIV-1 uninfected women at high risk of HIV-1 infection was assessed to investigate the influence of recent sexual activity on the cytokine milieu and soluble factors of cervicovaginal secretions in the FGT. In addition, the presence of PSA and Y-chromosomes were also used to determine concordance between self-reporting of consistent condom use and the presence of these markers.

4.3 Materials and Methods

4.3.1 Study design, participants and sample collection

A total of 242 women were enrolled into the prospective CAPRISA 002 observational cohort study of acute HIV-1 infection, as previously described (van Loggerenberg et al., 2008). Demographic and clinical data were collected at enrolment using a structured questionnaire. CVL samples were collected at enrolment from 227 (94%) of these women and 69 (30%) were from women who had no evidence of STI or BV. Because STIs and BV may independently influence the cytokine environment in CVL (Masson et al., 2014), women with laboratory-diagnosed STIs and BV were not included in this study. At enrollment, vulvovaginal swabs collected from the posterior fornices and lateral vaginal walls were tested for *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*, HSV-2 reactivation and *T. vaginalis* by PCR. Gram stain was performed to diagnose BV using Nugent criteria, and Nugent ≥7 were considered to have BV (Mlisana et al., 2012). Women with any STIs or BV. CVLs from the 69 BV/STI negative women were then screened for PSA and Y-chromosome DNA. Further, because cytokine concentrations are higher in CVLs from younger women compared to older women (Masson et al., 2015), Y-chromosome positive and Y-chromosome negative women were age-matched for subsequent cytokine analyses. All Y-chromosome positive women (n=23) were included in subsequent cytokine analyses and an age-matched control group of women (n=23) was randomly selected from the group of women without evidence of semen exposure at a 1:1 ratio using SAS version 9.3 (SAS Institute Inc., Cary).

CVLs were collected by gently flushing the cervix and the lateral vaginal walls with 10 ml sterile normal saline (Masson et al., 2014; van Loggerenberg et al., 2008). CVLs were centrifuged and the supernatant fraction was collected, aliquoted and stored at -80°C for measurement of PSA, cytokine, MMP and TIMP concentrations. The CVL pellet was stored at -80°C for DNA extraction for the Y-chromosone PCR. The protocol for this study was approved by the Ethical Review Committees of the University of KwaZulu-Natal and University of Cape Town, South Africa.

4.3.2 Measurement of cytokine, MMP and TIMP concentrations

The concentrations of 42 cytokines, 5 MMPs and 4 TIMPS were measured in CVL supernatants from these women, according to the method described in Chapter 2. The cytokine panel included IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-12p40, IL-10, IL-12p70, IL-13, IL-15, IL-17, EGF, PDGF-AA, TGF-α, VEGF, eotaxin/CCL11, FGF-2, FLT3L, fractalkine/CX3CL1, G-CSF, GM-CSF, GRO, IFN-α and IFN-γ, IP-10/CXCL10, MCP-1/CCL2, MCP-3/CCL7, MDC/CCL22, MIP-1α/CCL3, MIP-1β/CCL4, PDGF-AB/BB, RANTES/CCL5, sCD40L, sIL-2Rα, TNF-α, TNF-β (High Sensitivity Human Cytokine LINCOplex Premixed kits, LINCO Research, MO, U.S.A.). MMP and TIMP panels included MMP-1, MMP-2, MMP-7, MMP-9, MMP-10, TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (MILLIPLEX® MAP Human MMP or TIMP Panel 2, Merck-Millipore, Missouri. USA). Assays were performed according to the manufacturer's protocol. CVLs were thawed overnight on ice and filtered by centrifugation using 0.2 μm cellulose acetate filters (Sigma, U.S.A.). All markers were measured in undiluted CVL, except for MMP-9 (which was measured at 50-fold dilution), on a Bio-Plex 100 system (Bio-Rad Laboratories Inc®, California, USA). Bio-Plex manager software (version 5.0;

Bio-Rad Laboratories Inc®) was also used to analyse the data and all analyte concentrations were extrapolated from the standard curves using a 5 PL regression equation. Analyte concentrations that were below the lower limit of detection of the assay were reported as the mid-point between zero and the lowest concentration measured for each analyte.

4.3.3 ELISA to detect PSA

Measurement of PSA (also known as human tissue kallikrein-3) was performed on CVL supernatants to detect the presence of male semen. Briefly, 50µl of CVL supernatant was used to detect PSA, with the upper limit of detection of 60 ng/ml and a threshold of positivity of 0.94 ng/ml, according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). Each sample was assayed in duplicate. Every plate included PSA standards (provided in the kit) and a negative control containing sterile PCR-grade water and reaction mix, as proposed by the manufacturer. The average absorbance values for each set of reference standards, control, and sample were measured at 450 nm wavelength using a VersaMax™ absorbance microplate reader (Molecular Devices, LLC).

4.3.4 Detection of Y-chromosome

DNA was extracted from CVL pellets using the Roche® MagNAPure LC DNA Isolation Kit I (Roche Applied Science, Indianapolis, IN), according to the manufacturer's protocol. To control for the integrity and quantity of the extracted DNA, a 268 base-pair fragment of the human β-globin gene was amplified (Bauer et al., 1991; Coutlee et al., 1997). The following primers were used: PC04 ('CAA CTT

CAT CCA CGT TCA CC), and GH20 (GAA GAG CCA AGG ACA GGT AC) (PrimerDesign Ltd, UK). A region of the TSPY1 gene on the Y-chromosome (not found on the X-chromosome) was amplified using a real time qPCR assay (PrimerDesign Ltd, UK). The assay was performed in triplicate for each sample. qPCR was carried out using the Roche LightCycler® 480 system (Roche Diagnostics, GmbH). The negative control (containing no template) and an extraction control (PrimerDesign Ltd, UK) were included in each run. Detection of the Y-chromosome and analysis were performed according to the manufacturer's protocol (PrimerDesign Ltd, UK).

4.3.5 Statistical analyses

Descriptive statistics were used to describe demographic characteristics. Fisher's exact test and Wilcoxon rank sums tests were used to compare categorical variables and continuous data between groups, respectively. To assess the effect of semen exposure on cytokine concentrations, a linear regression model was fitted to log-transformed cytokine concentrations. As in Chapter 3, cytokines with at least a third of concentrations undetectable (including IL-2, IL-2Rα, IL-3, IL-4, IL-10, IL-12p40, IL-12p70, IL-13, Fractalkine, MIP-1α, IFN-α, EGF, TFG-α, FGF-2, PDGF-AB/BB and MMP-2) were dichotomised and a logistic regression model was fitted to estimate the effect of semen exposure on detectability of these cytokines. These models were adjusted for marital status, number of sexual partners, injectable hormonal contraception and sex worker status. Adjustment for multiple comparisons was conducted using the false discovery rate to reduce false positive results (Columb and Sagadai, 2006). Statistical analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary).

4.4 Results

This study included 46 HIV-1 negative women at high risk for HIV-1 infection, with no evidence of any other STI or BV, to quantify the frequency of CVL samples containing semen, and the influence of semen on cytokine concentrations (Table 4.1). Overall, the median age of the women was 40 years (interquartile range (IQR) 35-43), with about half of them (56.5%) being in stable relationships. Women were considered at high risk for HIV-1 infection as 34/46 (73.9%) self-identified as sex workers, with some women participated in sex work while being in a stable or married relationship (56.5%, 26/46).

Only half of the women reported using condoms at their last sex act (54.4%, 25/46), with condoms being used more often with casual partners than with stable partners (56.5% vs. 24.4%, p=0.0006). Forty percent (18/46) of the women reported never using condoms with their stable partners compared to only 13.0% (6/46) reporting never using condoms with their casual sex partners (p=0.0005). Half of these women (50%, 23/46) tested positive for Y-chromosome in CVL pellets by both SRY PCR and real-time PCR [Y-chromosome being stable up to 15 days post-exposure (Zenilman et al., 2005)], while 13 of these 23 (57%) also tested positive for PSA antigen [PSA only being stable for 48 hours post-exposure (Macaluso et al., 1999)]. All PSA positive CVL samples were also Y-chromosome positive (indicating more recent sexual intercourse) but only 10/23 (44%) of Y-chromosome positive CVLs were PSA negative (indicating sexual intercouse >48 hours previously).

Fifty four percent (25/46) of women reported condoms at their last sex act. Of these, 8/25 (32.0%) tested positive for Y-chromosome and 5/25 (20.0%) also tested positive for PSA, suggesting that either condom use was likely over-reported or that their last sex act was protected, but that women may have engaged in unprotected sex before their last sex act. Women who reported never used a condom with their stable partners were more likely to have positive PSA and Y-chromosome results (p=0.012). PSA and Y-chromosome were detected more frequently in the CVL samples from women not using injectable hormonal contraception than injectable hormonal contraception users, although this was not significant (Table 4.1).

Table 4.1 Cohort characteristics according to presence of PSA or Y-chromosome in genital secretions

Variable	Overall	PSA+	PSA-	p-value	Y-chromosome+	Y-chromosome-	p-value
Percentage (n/N)	46	28.3% (13/46)	71.7% (33/46)		50.0% (23/46)	50.0% (23/46)	
Age; Median (IQR)	40 (35-43)	42 (37-44)	40 (34-43)	0.272	40 (35-44)	40 (34-43)	0.743
Marital status [% (n/N)]:							
Single	10.9% (5/46)	15.4% (2/13)	9.1% (3/33)	0.798	8.7% (2/23)	13.0% (3/23)	0.687
Stable partner/married	56.5% (26/46)	53.9% (7/13)	57.6% (19/33)		52.2% (12/23)	60.9% (14/23)	
Many partners	32.6% (15/46)	30.8% (4/13)	33.3% (11/33)		39.1% (9/23)	26.1% (6/23)	
Highest education [% (n/N)]:							
< Grade 8	32.6% (15/46)	53.9% (7/13)	24.2% (8/33)	0.093	39.1% (9/23)	26.1% (6/23)	0.570
Grade 8-10	41.3% (19/46)	38.5% (5/13)	42.4% (14/33)		34.8% (8/23)	47.8% (11/23)	
> Grade 10	26.1% (12/46)	7.7% (1/13)	33.3% (11/33)		26.1% (6/23)	26.1% (6/23)	
Contraception use [% (n/N)]:							
Injectable	23.9% (11/46)	15.4% (2/13)	27.3% (9/33)	0.473	17.4% (4/23)	30.4% (7/23)	0.491
Non-injectable	76.1% (35/46)	84.6% (11/13)	72.7% (24/33)		82.6% (19/23)	69.6% (16/23)	
Sex worker [% (n/N)]	73.9% (34/46)	76.9% (10/13)	72.7% (24/33)	1.000	73.9% (17/23)	73.9% (17/23)	1.000
Condom use at last sex act [% (n/N)]	54.4% (25/46)	38.5% (5/13)	60.6% (20/33)	0.205	34.8% (8/23)	73.9% (17/23)	**0.017**

Condom use with stable partner [% (n/N)]:							
Never	40.0% (18/45)	61.5% (8/13)	31.3% (10/33)	0.201	60.9% (14/23)	18.2% (4/23)	**0.012**
Less than half the time	35.6% (16/45)	23.1% (3/13)	40.6% (13/33)		26.1% (6/23)	45.5% (10/23)	
More than half the time	24.4% (11/45)	15.4% (2/13)	28.1% (9)		13.0% (3)	36.4% (8)	
Condom use with casual partner [% (n/N)]:							
Never	13.0% (6/46)	23.1% (3/13)	9.1% (3)	0.467	17.4% (4)	8.7% (2)	0.265
Less than half the time	30.4% (14/46)	30.8% (4/13)	30.3% (10)		39.1% (9)	21.7% (5)	
More than half the time	56.5% (26/46)	46.2% (6/13)	60.6% (20)		43.5% (10)	69.6% (16)	

IQR = interquartile range

4.4.1 Presence of semen altered cytokine profiles in CVLs

Women who had recent unprotected sex had decreased concentrations of IL-2 (p=0.043), IL-12p70 (p=0.020), and IL-2Rα (p=0.018), compared to women who tested negative for both PSA and Y-chromosome (Table 4.2). In addition, fractalkine (p=0.053), FGF-2 (p=0.095), GM-CSF (p=0.094), and TNF-α (p=0.08) concentrations tended to be reduced in PSA+ women, although not after adjusting for multiple comparisons. In contrast, concentrations of soluble factors TIMP-1 (p=0.039) and MMP-9 (p=0.095) were higher in women who had recent unprotected sex compared to women who tested negative for both PSA and Y-chromosome.

Table 4.2 Relationship between cytokine concentrations and recent semen exposure (≤48 hrs post-sex) in the female genital tract

Functional groups	Cytokine	Condom use or no sex (n=23)		≤48 hours Post-sex (PSA+ Y-chromo+) (n=13)		Multivariate#	
		Median (pg/ml)	IQR	Median (pg/ml)	IQR	Beta coefficient (SE)	*P* value
Pro-inflammatory	TNF-β	4.56	2.49-7.77	3.77	2.49-6.74	-0.325 (0.203)	0.118
	IL-12p40†	19.57	0.12-67.85	5.28	0.12-23.06	-0.189 (0.967)	0.845
	IL-12p70†	0.01	0.01-0.04	0.01	0.01-0.01	-2.529 (1.086)	**0.020**
	IL1α	25.76	15.35-70.12	30.05	21.16-99.74	0.207 (0.220)	0.354
	IL-6	1.04	0.37-15.33	2.15	0.21-9.24	-0.482 (0.374)	0.206
	TNF-α	0.03	0.02-0.04	0.03	0.01-0.03	-0.280 (0.156)	0.080
	IL-1β	0.24	0.06-0.94	0.77	0.04-2.01	-0.142 (0.286)	0.621
Chemokine	Eotaxin	3.57	0.26-6.48	2.29	0.26-3.31	-0.143 (0.270)	0.598
	MCP-1	13.3	4.20-77.18	9.41	4.56-72.49	-0.222 (0.287)	0.444
	MDC	34.09	11.90-60.59	36.66	11.48-60.77	-0.083 (0.204)	0.686
	Fractalkine†	9.83	2.13-59.55	2.13	2.13-18.01	-1.788 (0.926)	0.053
	MIP1α†	0.66	0.66-36.15	0.66	0.66-14.92	-0.124 (0.853)	0.885
	MCP-3	11.41	7.56-16.31	9.35	7.56-11.81	-0.154 (0.125)	0.224
	IP-10	88.54	37.94-211.19	49.73	11.97-186.60	-0.486 (0.294)	0.106
	GRO	400.86	276.49-1554.91	456.21	251.64-618.42	-0.214 (0.227)	0.352
	MIP-1β	2.89	0.01-8.02	2.28	0.01-6.26	-0.687 (0.599)	0.259
	IL-8	147.34	52.26-350.76	428.19	43.64-905.69	0.194 (0.271)	0.478
	RANTES	3.65	2.75-6.93	3.21	2.50-9.11	-0.391 (0.290)	0.185
Innate	IFNα†	1.36	0.68-22.17	0.68	0.68-5.41	-0.968 (0.851)	0.256
Hematopoietic	IL-9	0.38	0.01-1.94	0.01	0.01-1.79	-0.293 (0.412)	0.481
	Flt3L	3.66	0.45-9.78	3.66	0.45-4.27	-0.288 (0.239)	0.235
	G-CSF	29.96	6.02-104.06	5.54	3.76-50.87	-0.667 (0.438)	0.136
	GM-CSF	0.19	0.01-0.54	0.05	0.01-0.16	-0.517 (0.300)	0.094
	IL-7	0.28	0.08-0.56	0.28	0.08-0.43	-0.163 (0.223)	0.468

	IL-3	5.64	0.01-28.00	0.01	0.01-10.42	-0.340 (0.818)	0.678
Growth Factor	PDGF-AA	8.1	4.78-61.91	103.19	4.95-217.97	0.248 (0.258)	0.344
	TGF-α	2.77	1.16-7.72	2.5	0.13-6.65	-0.284 (0.237)	0.239
	VEGF	37.89	8.64-99.70	60.25	17.27-77.93	-0.067 (0.179)	0.709
	PDGF-AB/BB†	10.57	0.09-109.37	0.09	0.09-35.49	-0.996 (0.838)	0.235
	EGF†	0.56	0.56-6.02	0.56	0.56-11.42	0.532 (0.888)	0.549
	FGF-2†	9.29	0.30-17.52	0.3	0.30-5.32	-1.428 (0.855)	0.095
Adaptive	IL-15	1.42	0.07-2.34	0.97	0.04-2.08	-0.289 (0.284)	0.315
	IL-5	0.03	0.02-0.03	0.03	0.03-0.04	-0.043 (0.043)	0.325
	IL-17	0.6	0.03-1.55	0.34	0.03-1.42	-0.250 (0.298)	0.408
	sCD40L	18.39	9.45-30.05	14.74	12.64-22.84	-0.278 (0.203)	0.179
	IFN-γ	3.48	0.27-4.89	4.01	0.07-4.89	-0.006 (0.459)	0.990
	IL-2†	0.05	0.01-0.20	0.01	0.01-0.08	-1.961 (0.969)	**0.043**
	IL-4†	0.02	0.02-0.47	0.02	0.02-0.20	-0.417 (0.864)	0.630
	IL-13†	0.01	0.01-0.01	0.01	0.01-0.01	-1.105 (0.876)	0.207
	sIL-2Rα†	6.1	1.74-12.34	0.57	0.57-6.10	-2.490 (1.052)	**0.018**
Anti-inflammatory	IL-1Ra	34235.15	23157.59-45012.00	25248.88	22666.51-31845.50	-0.077 (0.075)	0.312
	IL-10†	0.02	0.01-0.16	0.02	0.02-0.24	-0.286 (0.968)	0.767
MMPs	MMP-1	2.4	2.40-3.11	2.4	2.40-22.57	0.304 (0.331)	0.366
	MMP-7	623.26	8.10-3546.13	925.5	8.10-4130.87	0.002 (0.718)	0.998
	MMP-9	6269.25	2925.21-23888.75	22178.59	3600.58-62381.61	0.578 (0.334)	0.095
	MMP-10	2.2	2.20-5.52	2.2	2.20-44.45	0.295 (0.411)	0.479
	MMP-2†	118.1	118.10-118.10	118.1	118.10-118.10	-	-
TIMPs	TIMP-1	901.93	130.04-5526.97	4012.86	2262.04-8631.68	0.997 (0.460)	**0.039**
	TIMP-2	17281.32	8597.14-21926.34	14827.82	13307.34-21156.46	0.124 (0.199)	0.538
	TIMP-3	25.45	25.45-32.54	25.45	25.45-32.54	0.020 (0.031)	0.527
	TIMP-4	3.92	3.92-3.92	3.92	3.92-14.80	0.179 (0.135)	0.194

SE=standard error, CI = confidence interval, IQR = interquartile range. #Multivariate analysis adjusted for age, marital status, number of partners, injectable hormonal contraception and sex work as co-variates. †Variables with at least a third of concentrations were undetectable were dichotomised and a logistic regression model was fitted to estimate the effect of semen exposure on detectability of these cytokines and other soluble factors associated with genital epithelial repair and integrity. Bold indicates $p<0.05$ that were significant before adjusting for multiple comparisons. None of the comparisons remained significant after adjusting for multiple comparisons (FDR).

Women who were Y-chromosome positive but PSA negative (suggesting unprotected sex >48 hours but <15 days) had lower concentrations of IL-5 but higher concentrations of PDGF-AA than women negative for both PSA and Y-chromosome ($p=0.029$ and $p=0.041$, respectively, Table 4.3). In addition, concentrations of TNF-α ($p=0.06$) and IL-1β ($p=0.06$) tended to be decreased in women who tested positive for Y-chromosome and negative for PSA compared to women who tested negative for both PSA and Y-chromosome, although not significantly after adjustment for multiple comparisons.

Table 4.3 Relationship between cytokine concentrations and semen exposure (>48 hrs post-sex) in the female genital tract

Functional groups	Cytokine	Condom use or no sex (n=23)		>48 hours Post-sex (PSA negative and Y-chromo positive)(n=10)		Multivariate#	
		Median (pg/ml)	IQR	Median (pg/ml)	IQR	Beta coefficient (SE)	*P* value
Pro-inflammatory	TNF-β	4.56	2.49-7.77	8.42	6.04-15.56	0.311 (0.218)	0.163
	IL-12p40†	19.57	0.12-67.85	9.61	0.12-49.65	-0.329 (0.992)	0.740
	IL-12p70†	0.01	0.01-0.04	0.01	0.01-0.04	-0.855 (0.993)	0.389
	IL1α	25.76	15.35-70.12	46	20.53-87.56	0.117 (0.236)	0.623
	IL-6	1.04	0.37-15.33	1.13	0.35-4.04	-0.543 (0.402)	0.185
	TNF-α	0.03	0.02-0.04	0.03	0.02-0.04	-0.323 (0.167)	0.060
	IL-1β	0.24	0.06-0.94	0.11	0.05-0.17	-0.597 (0.307)	0.060
Chemokine	Eotaxin	3.57	0.26-6.48	1	0.26-5.38	-0.190 (0.289)	0.516
	MCP-1	13.3	4.20-77.18	8.72	4.56-26.15	-0.247 (0.308)	0.429
	MDC	34.09	11.90-60.59	44.06	41.26-77.17	0.213 (0.219)	0.335
	Fractalkine†	9.83	2.13-59.55	5.98	2.13-61.22	-1.078 (0.997)	0.280
	MIP1α†	0.66	0.66-36.15	0.66	0.66-20.24	-0.601 (0.916)	0.512
	MCP-3	11.41	7.56-16.31	10.58	4.24-14.77	-0.112 (0.134)	0.406
	IP-10	88.54	37.94-211.19	70.55	44.48-171.58	0.210 (0.315)	0.511
	GRO	400.86	276.49-1554.91	988.34	523.20-2761.13	0.291 (0.244)	0.241
	MIP-1β	2.89	0.01-8.02	1.28	0.01-4.60	-0.189 (0.643)	0.771
	IL-8	147.34	52.26-350.76	80.93	19.83-596.50	-0.257 (0.291)	0.384
	RANTES	3.65	2.75-6.93	2.98	1.85-7.25	-0.341 (0.311)	0.281
Innate	IFNα†	1.36	0.68-22.17	0.68	0.68-5.65	-1.123 (0.956)	0.241
Hematopoietic	IL-9	0.38	0.01-1.94	1.1	0.01-2.92	0.528 (0.442)	0.240
	Flt3L	3.66	0.45-9.78	5.3	0.45-8.16	-0.011 (0.256)	0.965
	G-CSF	29.96	6.02-104.06	19.42	3.07-60.40	-0.365 (0.470)	0.442
	GM-CSF	0.19	0.01-0.54	0.18	0.01-0.87	-0.243 (0.322)	0.455
	IL-7	0.28	0.08-0.56	0.39	0.12-0.56	0.071 (0.239)	0.769

	IL-3	5.64	0.01-28.00	8.35	0.01-54.16	0.981 (0.956)	0.305
Growth Factor	PDGF-AA	8.1	4.78-61.91	44.23	22.66-170.18	0.587 (0.277)	**0.041**
	TGF-α	2.77	1.16-7.72	2.83	1.59-5.45	0.272 (0.255)	0.292
	VEGF	37.89	8.64-99.70	89.01	49.14-132.04	0.277 (0.192)	0.157
	PDGF-AB/BB†	10.57	0.09-109.37	0.09	0.09-41.33	-1.132 (0.933)	0.225
	EGF†	0.56	0.56-6.02	1.37	0.56-8.54	1.182 (0.987)	0.231
	FGF-2†	9.29	0.30-17.52	0.3	0.30-17.52	-1.404 (0.941)	0.136
Adaptive	IL-15	1.42	0.07-2.34	1.54	0.75-3.17	0.402 (0.305)	0.195
	IL-5	0.03	0.02-0.03	0.03	0.02-0.03	-0.105 (0.046)	**0.029**
	IL-17	0.6	0.03-1.55	0.76	0.03-1.45	0.063 (0.320)	0.845
	sCD40L	18.39	9.45-30.05	28.49	22.84-36.09	0.119 (0.218)	0.589
	IFN-γ	3.48	0.27-4.89	2.19	0.01-4.89	-0.562 (0.492)	0.261
	IL-2†	0.05	0.01-0.20	0.06	0.01-0.08	-0.637 (1.026)	0.535
	IL-4†	0.02	0.02-0.47	0.3	0.02-0.38	1.671 (1.069)	0.118
	IL-13†	0.01	0.01-0.01	0.01	0.01-0.01	-1.137 (0.959)	0.236
	sIL-2Rα†	6.1	1.74-12.34	4.25	0.57-10.92	-1.754 (1.145)	0.126
Anti-inflammatory	IL-1Ra	34235.15	23157.59-45012.00	39327.04	31096.05-45012.00	0.086 (0.080)	0.293
	IL-10†	0.02	0.01-0.16	0.02	0.01-0.06	-0.864 (1.032)	0.403
MMPs	MMP-1	2.4	2.40-3.11	2.4	2.40-2.40	-0.228 (0.312)	0.471
	MMP-7	623.26	8.10-3546.13	162.27	8.10-654.99	-1.023 (0.677)	0.142
	MMP-9	6269.25	2925.21-23888.75	13071.56	3757.91-21198.43	-0.171 (0.315)	0.592
	MMP-10	2.2	2.20-5.52	2.2	2.20-2.20	0.295 (0.411)	0.479
	MMP-2†	118.1	118.10-118.10	118.1	118.10-118.10	-	-
TIMPs	TIMP-1	901.93	130.04-5526.97	3195.76	561.91-4998.14	0.579 (0.424)	0.183
	TIMP-2	17281.32	8597.14-21926.34	15600.36	11444.55-20848.70	0.191 (0.184)	0.307
	TIMP-3	25.45	25.45-32.54	25.45	25.45-32.54	0.003 (0.029)	0.920
	TIMP-4	3.92	3.92-3.92	3.92	3.92-3.92	-0.051 (0.124)	0.683

SE=standard error, CI = confidence interval, IQR = interquartile range. #Multivariate analysis adjusted for age, marital status, number of partners, injectable hormonal contraception and sex work as co-variates. †Variables with at least a third of concentrations were undetectable were dichotomised and a logistic regression model was fitted to estimate the effect of semen exposure on detectability of these cytokines and other soluble factors associated with genital epithelial repair and integrity. Bold indicates $p<0.05$ that were significant before adjusting for multiple comparisons. None of the comparisons remained significant after adjusting for multiple comparisons (FDR).

4.5 Discussion

Despite improvements in the framing of sexual behavior questionnaires, self-reporting does not always yield accurate information; with self-reported condom use biased towards over-reporting (Brener et al., 2003; Durant and Carey, 2000; Mauck et al., 2007; Stuart and Grimes, 2009; Turner and Miller, 1997). Inaccurate self-reported condom use data may lead to imprecise estimates of the biological effects in HIV-1/STIs intervention studies, and knowledge of the potentially confounding presence of semen in genital specimens may be a necessary prerequisite for the accurate study of mucosal immunity. The present study found that about half of the CVL samples collected from sexually active women contained semen markers (PSA and/or Y-chromosome), although nearly a third of women reported condom use at their last sexual act. All PSA positive samples were also positive for Y-chromosome (suggesting sex within 48 hours), while only about half of Y-chromosome positive samples were also positive for PSA (suggesting sex between 48 hours and 15 days prior to sampling).

One fifth of PSA positive and over 30% of Y-chromosome positive women reported that they had used a condom at their last sex act. Women who reported less frequent or no condom use were more likely to test positive for either semen marker. The increased presence of semen markers in CVLs from women in stable relationships may be due to several factors, including marriage and the perception of reduced risk of STIs/HIV-1, desire to conceive, opposition to condom use by male partner, late use or early removal of condoms. Semen markers were detected more frequently in women not using injectable hormonal contraception compared to those using injectable contraception, although this difference was not statistically significant. This may be due to the fact that women who were not using injectable contraception were more likely to be married, have a stable partner, or were trying to conceive.

Previous studies have shown that semen induces an acute inflammatory response and increases recruitment of immune cells to the female genital tract after sex (Robertson, 2005, 2007; Robertson et al., 2009; Sharkey et al., 2012). This study found that CVLs with semen present had reduced concentrations of inflammatory IL-12p70, and adaptive cytokines IL-2, IL-2Rα and IL-5, but increased expression of the growth factor PDGF-AA and soluble factors MMP-9 and TIMP-1, thought to be involved in tissue remodelling and repair in the reproductive tract. IL-12p70 and IL-2 in CVLs may be associated with T-cell activation while IL-5 may be involved in B cell stimulation and differentiation (Olivier et al., 2014; Randall et al., 1993). Recent sex (<48 hours, PSA+) was associated with increased production of TIMP-1 which is known to inhibit gelatinases MMP-2 and -9 involved in remodelling processes of structural proteins in the reproductive tract (Rodgers et al., 1993). Seminal fluids (which can range in volume from 0.1 to 10 ml, Rehan et al., 1975) might dilute vaginal secretions and this might lead to lower concentrations of some cytokines in secretions. Sex which has taken place >48 hours before sample collection (such as those negative for PSA but positive for Y-chromosome) was associated with increased CVL concentrations of the growth factor PDGF-AA, reported to restore barrier function in the female genital tract and enhance epithelial repair by stimulating mitosis, spreading and migration of epithelial cells (Werner and Grose, 2003).

A limitation of this study is that women infected with STIs were excluded (because STIs also drive inflammatory responses in the genital tract), and most of these excluded women were young, hence a relatively small sample size. The influence of semen exposure on genital cytokines was also investigated cross-sectionally instead of longitudinally, where analytes are

assessed in the same women before and after sex. Furthermore, because cytokine concentrations are higher in CVLs from younger women compared to older women, Y-chromosome positive and Y-chromosome negative women were age-matched for subsequent cytokine analyses. All limitations mentioned above are being addressed in further studies.

In conclusion, condom use was not accurately reported in this cohort of women at high risk for HIV-1 infection, confirming the importance of routine objective screening for the presence of semen, especially in the context of HIV-1/STI prevention trials. These findings suggest that the presence of PSA and Y-chromosome may influence the cytokine profile in the female genital tract and should be taken into consideration when investigating immunological factors in this compartment.

CHAPTER 5

In vivo and *in vitro* effects of injectable hormonal contraceptives on innate immune cell activation and cytokine responses

CHAPTER 5

In vivo and *in vitro* effects of injectable hormonal contraceptives on innate immune cell activation and cytokine responses

5.1 Abstract

Suppression of host immune responses in women using injectable HCs has been proposed as a biological mechanism that may contribute to increased risk for HIV-1 acquisition. Monocytes, macrophages and DCs have been shown to be susceptible to immunosuppression following DMPA exposure at physiological concentrations. The aim of this Chapter was (1) to investigate the *in vivo* impact of injectable HCs on blood monocytes and DCs from women using DMPA compared to those using NET-EN, and (2) to compare the *in vitro* effects of DMPA to natural progesterone and cortisol on DC subsets in blood from healthy women not using any form of HC. *In vivo* experiments included PBMCs from 98 HIV-1 uninfected women (40 DMPA users, 40 NET-EN users and 18 non-HC users). For whole blood experiments, blood from women not on HCs was incubated in the presence or absence of increasing concentrations of the exogenous and natural hormones (DMPA, cortisol and progesterone) and TLR4 (LPS) or TLR9 (CpG) agonists were added. Functional (intracellular TNF-a and IL-6 production) and activation phenotype (CD40 and CD86 expression) of mDC (HLA-DR^{+}CD11c^{+}CD123^{-}) subsets were assessed by flow cytometry. Plasma cytokine concentrations were measured by Luminex. Findings in this Chapter suggest that *in vivo* injectable HC use did not affect numbers of circulating monocytes from full blood counts or plasma cytokine levels in HIV-1 uninfected women using DMPA or NET-EN. DMPA did not alter the level of monocyte activation (measured by CD40, CD86 and HLA-DR expression), following *in vitro* stimulation with TLR ligands LPS and CpG. However, DMPA significantly decreased the production of pro-inflammatory TNF-α by mDCs following *in vitro* stimulation with LPS- or CpG ($p=0.0286$). In addition, DMPA-treated mDCs showed

impaired capacity to produce IL-6 after LPS stimulation relative to the control. These *in vitro* experiments suggest that DMPA was capable of down-modulating cytokine production by mDCs, which could potentially alter the broader characteristics of the host innate immune response.

5.2 Introduction

Injectable HCs have been associated with reduced host resistance to pathogens, such as *C. trachomatis*, *N. gonnorhoea*, and HPV by modulating innate mucosal defenses (Bamberger et al., 1999; Koubovec et al., 2005; Koubovec et al., 2004; Morrison et al., 2014). Previous *in vivo* studies and results from Chapter 3 showed that injectable HCs can have an effect on host immunity, by down-regulating production of several proinflammatory cytokines (IL-1α, IL-9, IL-12p40, IL-15, IL-17, MIP-1α, IFNα, Eotaxin, Fractalkine, EGF, FGF2, PDGF-AA, TGF-α, MCP-1, IL-1ra, IL-6, IL-13, TNF-α and GM-CSF) but also increasing cellular susceptibility to HIV-1 infection by up-regulating HIV-1 co-receptors CXCR4 and CCR5 on activated T-cells (Hughes et al., 2008; Huijbregts et al., 2013).

Several studies have demonstrated that injectable HCs inhibit systemic regulators of cellular and humoral immunity (Hughes et al., 2008; Huijbregts et al., 2013). Injacteble HCs also reduce circulating monocyte numbers (Kleynhans et al., 2013; Kleynhans et al., 2011) and suppressing cytokine production by DCs (Hughes et al., 2008; Huijbregts et al., 2013; Huijbregts et al., 2014; Michel et al., 2015). DCs and monocytes are central to host immunity, as they are major antigen presenting cells that recognise and respond early to pathogens in the body, influencing the development of adaptive immunity (Banchereau and Steinman, 1998). DCs are phenotypically and functionally divided into plasmacytoid DCs (pDCs), myeloid DCs (mDCs), and tissue-resident Langerhans cells (LCs) (Muller-Trutwin and Hosmalin, 2005; Shey et al., 2015). Monocytes and myeloid DCs express TLR-2 and TLR-4, pDCs express TLR-7 and TLR-9, and LCs express TRL-2, TLR -4 and TLR -9 (Iijima et al., 2008). HLA class I/II molecules expressed by DCs and co-stimulatory markers

for T and B cells (including CCR7, CD40, CD80 and CD86) were upregulated following antigen capture that make them better antigen-presenting cells (Iwasaki and Medzhitov, 2004; Seder et al., 1993). Following HIV-1 infection, mDCs secrete a series of pro-inflammatory cytokines (including IL-1β, IL-6, IL-10, IL-12 and TNF-α), while pDCs secrete type I interferons, and IL-6 upon TLR-7/8 or TLR-9 stimulation (Palucka et al., 2010). However, the addition of exogenous syntheic progestins (including DMPA) in cultured human pDCs have been shown to downregulate TNF-α and IFN-α production by pDCs exposed to exogenous TLR -7, -8, and 9 ligands (Hughes et al., 2008; Huijbregts et al., 2013; Michel et al., 2015).

Monocytes and DCs are important regulators of T cell differentiation, determining the switch between maturation to Th1, Th2, Th17 or Treg phenotypes (Kaiko et al., 2008; Zhu et al., 2010). Since previous studies have shown that DMPA impacts on monocyte and DC lineage cells (Hughes et al., 2008; Huijbregts et al., 2013; Kleynhans et al., 2013; Kleynhans et al., 2011; Michel et al., 2015), the impact of injectable HC use on systemic cytokines, and numbers of circulating monocytes in women using DMPA or NET-EN was investigated in this Chapter. In addition, the e*x vivo* function of monocyte lineage cells, and the impact of *in vitro* addition of DMPA on mDC and pDC activation and intracellular cytokine production was evaluated.

5.3 Materials and Methods

5.3.1 Study design, samples size and inclusion criteria

For the *in vivo* or *ex vivo* studies, PBMCs from 98 women who had previously participated in the CAPRISA 004 tenofovir 1% gel vaginal microbicide trial, who were either using DMPA (N=40), NET-EN (N=40) or not using HCs (N=18; tubal ligation), were included in this retrospective study (Figure 5.1) (Abdool Karim et al., 2010a). Unlike the CAPRISA002 trial where it was not possible to differentiate the type of injectable HC being used by women, the CAPRISA004 trial questionnaire collected information on whether the women were using DMPA or NET-EN (Sibeko et al., 2011). All women were in the placebo arm of the CAPRISA004 trial (Abdool-Karim et al., 2010). To account for age, half of the women were aged 15-25 years and half were >30 years old in the DMPA and NET-EN groups. Women taking any steroid treatment (including hormonal contraceptives such as IUDs and COCs) were excluded. For the *in vitro* studies, whole blood was obtained from four healthy female volunteers who were not using any HCs. Demographic and clinical data were collected at enrolment using a structured questionnaire administered by a trained counsellor. Data were collected on whether the women were using injectable HCs (DMPA or NET-EN), COCs, IUDs, condoms, diaphragms, foam and jelly, or who were sterilised. Laboratory samples, including vaginal aspirates, CVLs, cytobrushes, vaginal swabs, serum, plasma and PBMCs were collected from each participant throughout the trial. PBMCs were isolated from whole blood by Ficoll treatment using a standard protocol and stored in liquid nitrogen. Full blood counts, including an automated differential count, was performed on each patient sample using Sysmex KX21 hematology analyzer according to the manufacturer's protocol (*Roche* Molecular Systems, Inc, Germany) by the Global Clinical and Viral Laboratory (Amanzimtoti, South Africa). The protocol for this study was approved by the Ethical Review Committee of the University of KwaZulu-Natal. .

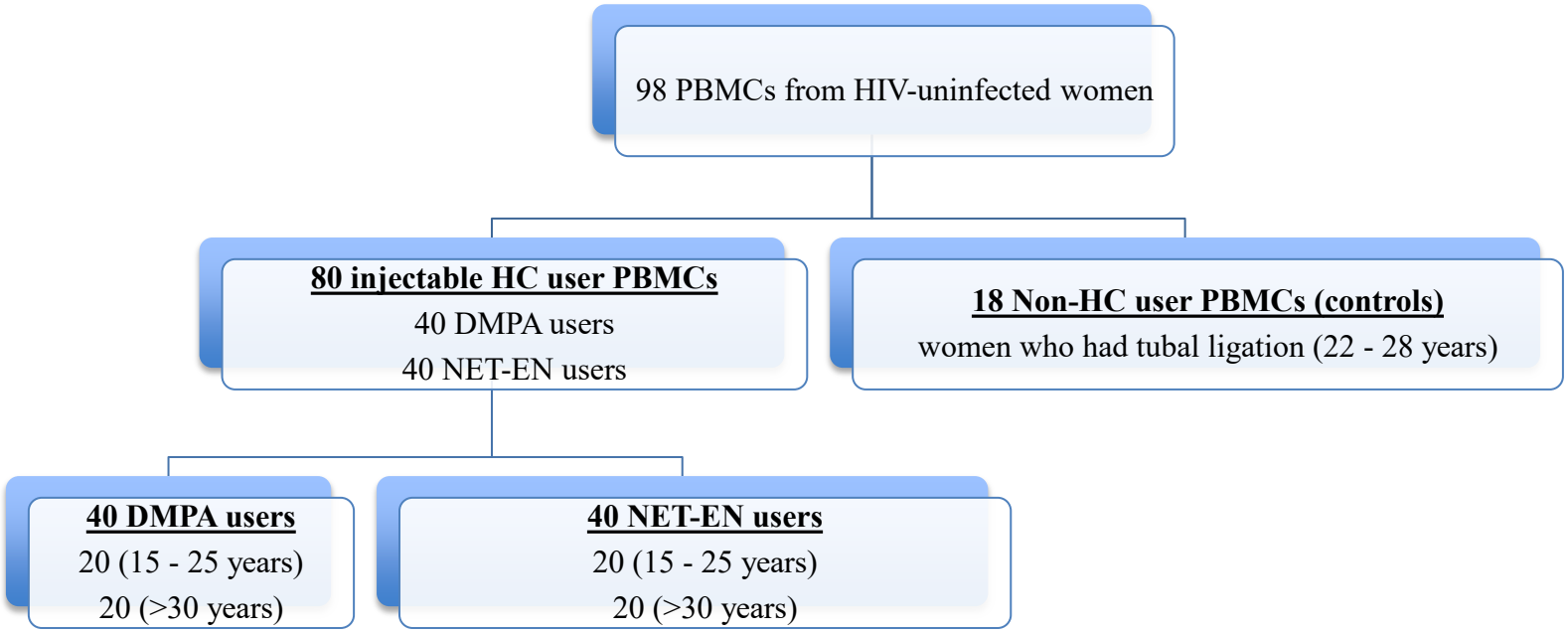


Figure 5.1 Summary of study design for *ex vivo* study. PBMCs from 98 HIV-1 uninfected women (40 DMPA users, 40 NET-EN users and 18 non-HC users as controls) from the CAPRISA 004 were stratified by age.

5.3.2 *Ex vivo* characterization of PBMCs from women using injectable HCs

Cryopreserved PBMCs from HIV-1 negative women who were either users of DMPA, NET-EN or no HCs were rapidly thawed in water bath at 37°C and washed three times with warm R10 culture medium [RPMI culture medium (Lonza, Cologne, Germany) supplemented with 1% penicillin/streptomycin (PenStrep; Gibco, Darmstadt, Germany) and 10% heat-inactivated fetal calf serum (FCS ; Biochrom AG, Berlin, Germany)]. After thawing, PBMCs were rested for 2 hours and viability was determined using trypan blue staining. After resting, thawed cells were incubated with 20µl *LIVE/DEAD*® Fixable Violet Dead Cell Stain for 20 minutes at room temperature, then washed twice with 1X PBS. The cells were then stained with antibodies for monocytes and activation markers for 60 minutes at room temperature in the dark with the following markers for innate cells: Alexa Fluor® 700 anti-HLA-DR, Cyanine 5.5 (Per CP-Cy5.5)-labeled anti-CD123 (pDCs), APC/Cy7 anti-CD11c (mDCs), Qdot 655 anti-CD14 (monocytes; Thermo Fisher Scientific, Waltham, MA), Allophycocyanin (APC)-labeled anti-CD86 (B7-2) and FITC-labeled anti-CD40 as markers of DC activation. At least 50,000 events were captured using a LSRII flow cytometer (BD Immunocytometry Systems). Fluorescence minus one (FMO) staining was used to set gates to differentiate negative and positive populations (Perfetto et al., 2004). FlowJo v9.7 (Tree Star, CA, USA) was used to analyze the data. Results from single-stained and unstained mouse kappa beads were used for colour compensation. Cell doublets were excluded using forward scatter-area versus forward scatter-height parameters. T cells, B cells and NK cells were also excluded from the analyses. The mean fluorescent intensity (MFI) for HLA-DR, CD40 and CD86 were determined in FlowJo. All data have been corrected for background, using the unstimulated condition.

5.3.3 Measurement of plasma cytokines

Concentrations of 12 cytokines in plasma from 100 women (68 DMPA users, 14 NET-EN users and 18 not using any HCs) were measured using Luminex (by Dr Lenine Julie Liebenberg; CAPRISA Lab, Durban, South Africa). These included: IL-1α, IL-1β, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, IL-6, IL-7, IL-8 (CXCL8), IL-10, GM-CSF, IP-10, and TNF-α using the Human Cytokine and High Sensitivity LINCOplex Premixed kits (LINCO Research, MO, USA). Plasma samples were thawed overnight on ice and filtered by centrifugation using 0.2 μm cellulose acetate filters (Sigma, U.S.A.). All markers were measured in undiluted CVL on a Bio-Plex 100 system (Bio-Rad Laboratories Inc®, Hercules, California). Bio-Plex manager software (version 5.0; Bio-Rad Laboratories Inc®) was used to analyse the data and all analyte concentrations were extrapolated from the standard curves using a 5 parameter logistic (PL) regression equation. Analyte concentrations that were below the lower limit of detection of the assay were reported as the mid-point between zero and the lowest concentration measured for each analyte.

5.3.4 Measurement mDC and pDC function and activation

Whole blood from healthy women not on HCs were used for *in vitro* experiments. For measurement of intracellular cytokine production by DC subsets, whole blood (400μl) was incubated with DMPA (Sigma, catalogue number M1629-1G, U.S.A.), cortisol (Sigma, catalogue number H4001-1G, U.S.A.) and progesterone (Sigma, catalogue number P8783-5G, U.S.A.) at varying concentrations (1 mM, 10 mM, 100 mM) and then stimulated for 6 hours with either LPS (final concentration of 2 μg/ml; a TLR4 ligand; Invivogen, San Diego, California, USA) or CpG (final concentration of 1 μg/ml, a TLR9 ligand, Invivogen, San Diego, California, USA). Untreated cells were included as a negative control. For measurement of DC intracellular cytokine production, cells in whole blood were incubated

for 60 minutes at 37°C in a humid incubator with 5% CO_2, after which brefeldin A (10 μg/ml) was added (Sigma, catalogue number B7651, U.S.A.). Incubation was continued for an additional 5 hours at 37°C in a humid incubator with 5% CO_2. For measurement of DC activation, whole blood was stimulated as above but for 18 hours without brefeldin A added. After either a 6 hour (when cytokine production was being measured) or an 18 hour stimulation (when DC activation was being measured), red cells in whole blood were lysed with 1ml BD FACS lysing solution (BD, New Jersey, USA) for 10 minutes at room temperature and stored at -80°C until needed for immunostaining for flow cytometry. In batches, the frozen cells were thawed and stained with antibodies for DC lineage markers and cytokines.

5.3.4.1 Flow cytometry of DC subsets for intracellular cytokine production

For intracellular staining of cytokines to measure DC subset function, hormone-treated cells were washed twice with 3 ml of BD Perm wash buffer, centrifuging each time for 5 minutes at 388 x g. Staining was performed for 60 minutes at room temperature in the dark with the following markers for innate cells: Alexa Fluor® 700 anti- HLA-DR, Cyanine 5.5 (Per CP-Cy5.5)-labeled anti-CD123 (pDCs), APC/Cy7 anti-CD11c (mDCs), Qdot 655 anti-CD14 *(*monocytes; Thermo Fisher Scientific, Waltham, MA), and APC anti-IL-6 and PE Cyanine 7 (PE-Cy7) labeled anti-TNF-α (BioLegend*,* San Diego, California) as functional markers. In addition, cells were also stained (in the same panel) with PE-labeled anti-CD66ace (to exclude neutrophils; BD, New Jersey, USA) and other dump channel markers such as Pacific Blue labeled anti-CD3 (T cells), CD19 (B cells) and CD56 (NK cells) (*BioLegend,* San Diego, California). After staining, cells were resuspended in 1 ml of BD Perm wash buffer and centrifuged for 5 minutes at 388 x g.

For staining cell surface markers to measure DC activation, treated cells were washed once with 3ml of 1% FCS PBS, centrifuging each time for 5 minutes at 388 x g. Staining was performed for 60 minutes at room temperature in the dark with the following markers for innate cells: Alexa Fluor® 700 anti- HLA-DR, Cyanine 5.5 (Per CP-Cy5.5)-labeled anti-CD123 (pDCs), APC/Cy7 anti-CD11c (mDCs), Qdot 655 anti-CD14 *(*monocytes; Thermo Fisher Scientific, Waltham, MA), and Allophycocyanin (APC)-labeled anti-CD86 (B7-2) and FITC-labeled anti-CD40 as markers of DC activation. As with the intracellular staining panel, cells were stained in the same panel with PE-labeled anti-CD66ace and dump channel markers.

At least 500,000 events were acquired using a FACSDiva flow cytometer (BD Immunocytometry Systems). Gates differentiating negative and positive populations were set by FMO staining (Perfetto et al., 2004). Flow cytometry data was analysed using FlowJo v9.7 (Tree Star, CA, USA). Results from single-stained and unstained mouse kappa beads were used to calculate compansations. Cell doublets were excluded using forward scatter-area versus forward scatter-height parameters while cytokine co-expression by DC susbsets was assessed by Boolean gating. T cells, B cells, NK cells and neutrophils were also excluded from the analyses. The MFI for HLA-DR, CD40 and CD86 and frequencies of TNF-α and IL-6 positive cells were determined in FlowJo. All data has been corrected for background using unstimulated data.

5.3.5 Statistical analysis

Descriptive statistics were used to compare demographic characteristics. Mann-Whitney U test was applied for non-parametric independent sample comparisons and Wilcoxon signed rank tests were applied to matched samples for non-parametric comparison. Kruskal-Wallis (Nonparametric One-Way ANOVA) tests were used for non-parametric assessments of variation between groups. *P* values of <0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA, U.S.A.) and SAS version 9.3 (SAS Institute Inc., Cary).

5.4 Results

Table 5.1 summarizes the characteristics of the HIV-1 uninfected women included in the *ex vivo* study. Their median age was 24 years (IQR 21 -31), and almost all (96/98) were in stable relationships. More than 80% of the women reported using condoms at their last sex act (81/98). Age, relationship status and condom use did not differ between groups (DMPA, NET-EN and controls).

Table 5.1 Demographics of women included in the *ex vivo* study

	Overall	Controls	DMPA users	NET-EN users
N	98	18	40	40
Median Age [median (IQR)]	24 (21-31)	33 (29 - 34)	23 (20 - 28)	24 (21 -29)
Relationship status [% (n/N)]				
Casual	2 (2/98)	6 (1/18)	2 (1/40)	0 (0/40)
Stable	98 (96/98)	94 (17/18)	98 (39/40)	100 (40/40)
Condom use at last sex act [% (n/N)]	83 (81/98)	67 (12/18)	88 (35/40)	85 (34/40)

IQR = interquartile range. Control were women not using any form of HC (tubal ligation)

5.4.1 Effects of injectable HC use on monocyte and DC subsets

Since previous studies have suggested that DMPA use may interfere with functional ability of monocyte lineage cells (Kleynhans et al., 2013), the impact of DMPA use on full blood counts and blood monocyte numbers was evaluated in women who were known to be using either DMPA, NET-EN or no/non-injectable HCs (Table 5.2). No differences in the numbers of circulating monocytes in women using injectable HC were noted compared to non-HC users (p=0.512). Additionally, similar numbers of circulating platelets, lymphocytes, basophils, neutrophils and eosinophils were found in both groups. Haemoglobin was slightly lower in women not using any HC (median 13.1 g/dL, IQR 12.25-13.50) compared to injectable HC users (median 14 g/dL, IQR 13.30-14.60; p=0.078), although not significantly.

Table 5.2 Comparison of full blood counts of women using injectable HCs and those not using HCs

Variable	Non-HC users (Tubal Ligation)		DMPA users		NET-EN		p value*
	Median	IQR	Median	IQR	Median	IQR	
Haemoglobin (g/dL)	13.1	12.25-13.50	14	13.30-14.60	13.6	12.40-14.00	0.078
Hematocrit (%)	0.39	0.37-0.40	0.41	0.39-0.43	0.4	0.38-0.42	0.057
Mean corpuscular volume (fL)	86.5	84.80-89.40	88.5	85.40-89.70	87	82.70-92.00	0.713
Mean corpuscular hemoglobin (pg)	29.15	27.90-30.55	30.15	28.70-30.70	29.6	27.20-30.40	0.743
Mean corpuscular hemoglobin conc. (g/dL)	33.5	33.05-34.35	33.85	33.60-34.40	34	33.00-34.40	0.579
Platelets ($x10^3/\mu L$)	310.5	252.50-371.50	266	252.00-290.00	308	246.00-337.00	0.464
White blood cell counts ($x10^3/\mu L$))	7.11	5.57-8.86	6.64	5.87-8.21	6.42	5.52-7.12	0.354
Neutrophils ($x10^3/\mu L$))	3.6	2.72-4.84	4.09	2.30-5.16	3.88	2.98-4.48	0.974
Lymphocytes ($x10^3/\mu L$))	2.32	1.96-2.63	2.11	1.77-3.04	2.09	1.89-2.24	0.217
Monocytes ($x10^3/\mu L$))	0.42	0.37-0.49	0.47	0.31-0.56	0.38	0.32-0.46	0.512
Eosinophils ($x10^3/\mu L$))	0.18	0.08-0.33	0.17	0.06-0.37	0.1	0.07-0.19	0.379
Basophils ($x10^3/\mu L$))	0.02	0.01-0.04	0.02	0.01-0.02	0.02	0.01-0.02	0.564

IQR = interquartile range; p-values were calculated using Kruskal-Wallis ANOVA tests (non-parametric).

To determine whether DMPA use influenced monocyte activation, HLA-DR expression by CD14+ monocytes was measured in PBMCs from women using DMPA, NET-EN or non-injectable HCs. Figure 5.2A summarizes the gating strategy to identify monocytes. The frequency of circulating monocytes was not different in DMPA users compared to non-HC users (median 4.44% of HLA-DR$^+$ cells (IQR, 2.23 – 9.41) versus 2.33% (IQR, 1.66 – 7.07); p=0.220) (Figure 5.2B). Similarly, no difference in the numbers of circulating monocytes in NET-EN users compared to non-HC users [4.69% of HLA-DR$^+$ cells (IQR, 2.24 – 8.12) vs 2.33% (IQR, 1.66 – 7.07); p=0.216] were found. DC activation markers (CD40 and CD86 on CD11c$^+$ mDCs and CD123 pDCs) has not been reported here as these populations were not reliably detected in frozen PBMC samples that had been stored for a long period of time.

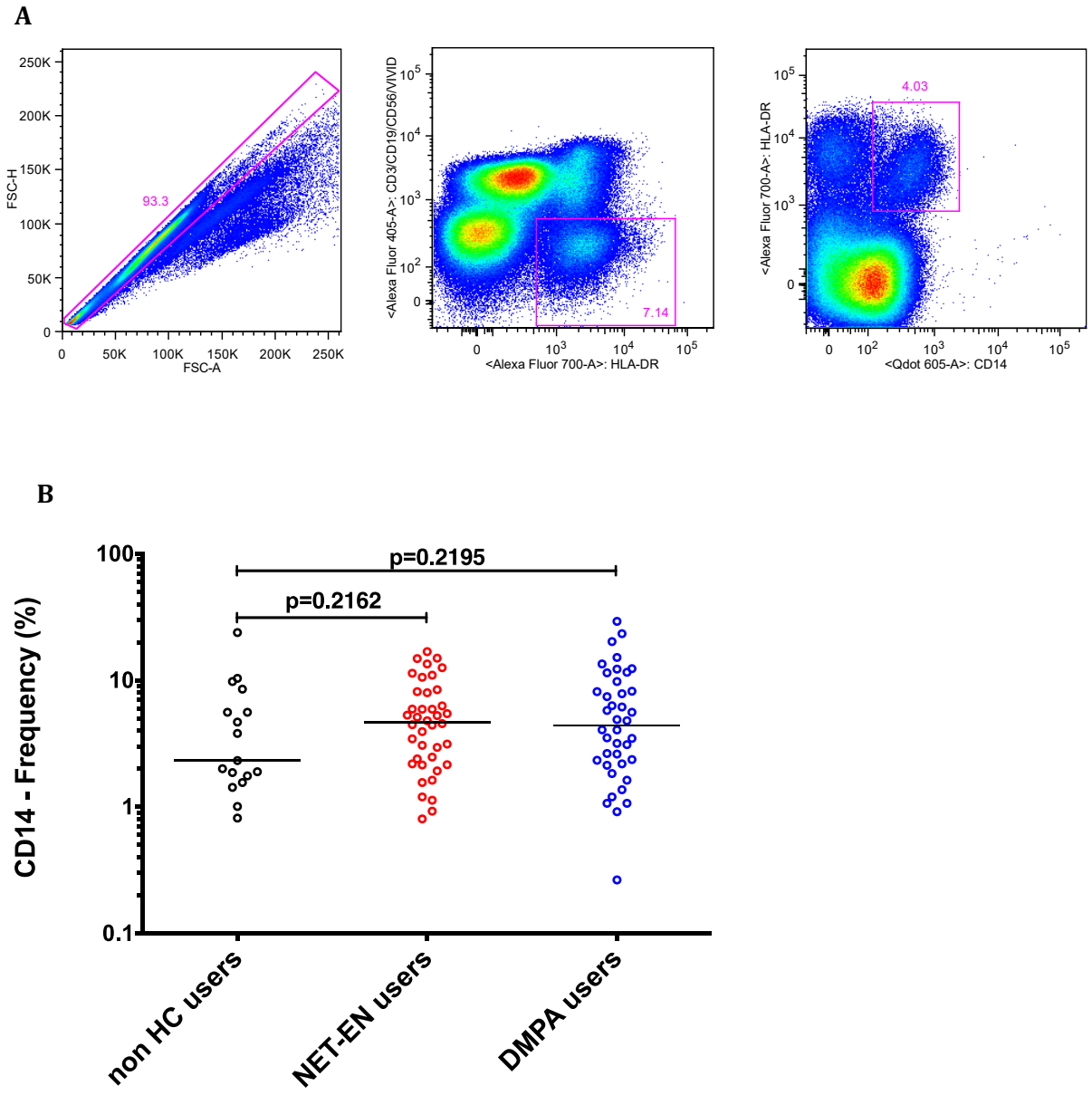


Figure 5.2 Influence of injectable HC use on frequency of monocytes in blood. (A) Gating strategy used to identify CD14+ monocytes in stored PBMCs. CD3/CD56/LIVE/DEAD$^-$ population was used to identify monocytes (CD14$^+$HLA-DR$^+$). (B) Impact of DPMA (blue dots) or NET-EN (red dots) use on frequencies of monocytes in blood from DMPA (n=40) and NET-EN (n=40) users compared to non-HC users (n=18). Bars indicate median values; significance was determined using the Mann-Whitney test. P-values <0.05 were considered significant.

5.4.2 Impact of injectable HCs on innate immune factors in the female genital tract

Previous studies have demostrated that DMPA suppresses production of several cytokines, particularly those related to monocyte lineage function (Hughes et al., 2008; Huijbregts et al., 2013; Kleynhans et al., 2013). To investigate this, the influence of injectable HC on the systemic innate environment was measured by comparing the concentrations of IL-1α, IL-1β, IL-6, IL-7, IL-8, IL-10, GM-CSF, IP-10, and TNF-α in CVL from women using either DMPA, NET-EN, or those not using HCs. None of the cytokines typically produced by DCs and monocytes (including IL-1β, IL-6, IL-10 and TNF-α) were significantly different between groups (Figure 5.3). Concentrations of IL-8, IP-10, and MCP-1 tended to be lower in women using DMPA and NET-EN compared to non-HC controls, although not significantly. This is interesting because MCP-1, IP-10 and IL-8 are all involved in chemoattraction of monocytes or macrophages to sites of infection. The lower concentrations of these specific cytokines in women using injectable HCs suggest that injectable HCs may inhibit migration of monocytes required for immunological surveillance of tissues and response to inflammation.

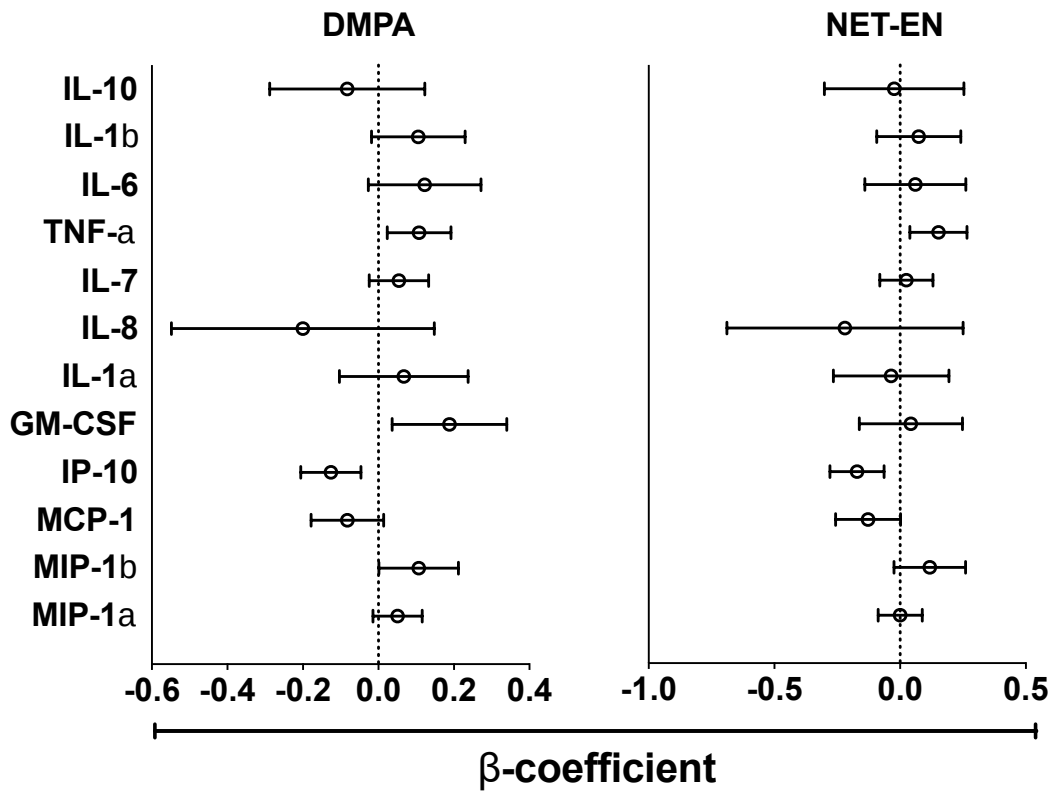


Figure 5.3 Influence of DMPA or Net-EN use on plasma cytokine concentrations compared to those not using HCs. β-coefficients were calculated using logistic regression. A positive β-coefficient indicates that higher concentrations of the cytokine are associated with DMPA or NET-EN use, while a negative β-coefficient indicates that the cytokine concentration is more likely to be lower in plasma from women who were using DMPA or Net-EN compared to non-HC users.

5.4.3 Effect of exogenous hormones on mDC lineage activation

To evaluate whether exogenous addition of DMPA influenced monocyte and DC activation or cytokine production, whole blood from healthy female donors not using HCs were stimulated with LPS (TLR4) or CpG (TLR9) with exogenous addition of natural (progesterone) or synthetic hormones (DMPA) or cortisol at varying concentrations (1, 10

and 100 nM). Figure 5.4 and 5.5 shows the gating strategy used to evaluate cytokine production (IL-6 and TNF-a) and cellular activation (CD40 and CD86 expression), respectively. For this analysis, monocytes were defined as HLA-DR^+ $CD14^+$ cells; mDCs were defined as HLA-$DR^+CD14^-CD11c^+CD123^-$ cells; and pDCs were defined as HLA-$DR^+CD14^-CD11c^-CD123^+$ cells.

While both LPS and CpG increased mDC activation, with higher MFI expression of CD40 on CD11c+ cells, DMPA-, progesterone- and cortisol-treatment did not influence the level of mDC activation induced by these TLR agonists (Figure 5.6). In contrast to CD40, CD86 expression on mDCs was not increased by LPS or CpG stimulation and not affected by exogenously added hormones or cortisol (Figure 5.7). Similar analysis of CD14+ monocytes and CD123+ pDCs yielded the same results and have not been shown.

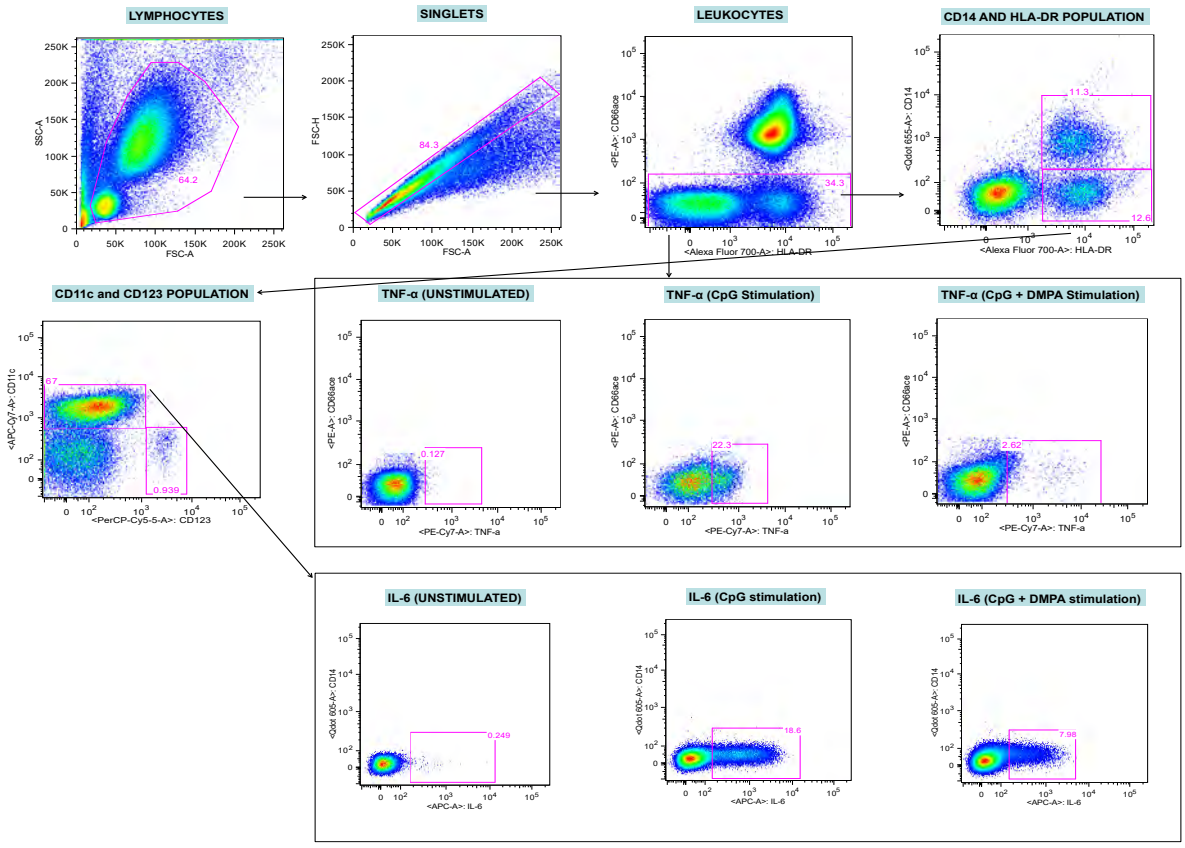


Figure 5.4 Gating strategy used to identify DC subsets and cytokine production of TNF-α and IL-6 that were either unstimulated (control), stimulated with LPS/CpG or a combination of LPS/CpG and hormone (DMPA, cortisol and progesterone). TNF-α production was defined by $CD14^{-}$ $CD11c^{+}$ cells while IL-6 was defined by $CD14^{-}$ $CD11c^{+}$ cells following stimulation of PBMCs with either CpG or LPS for 6 hours.

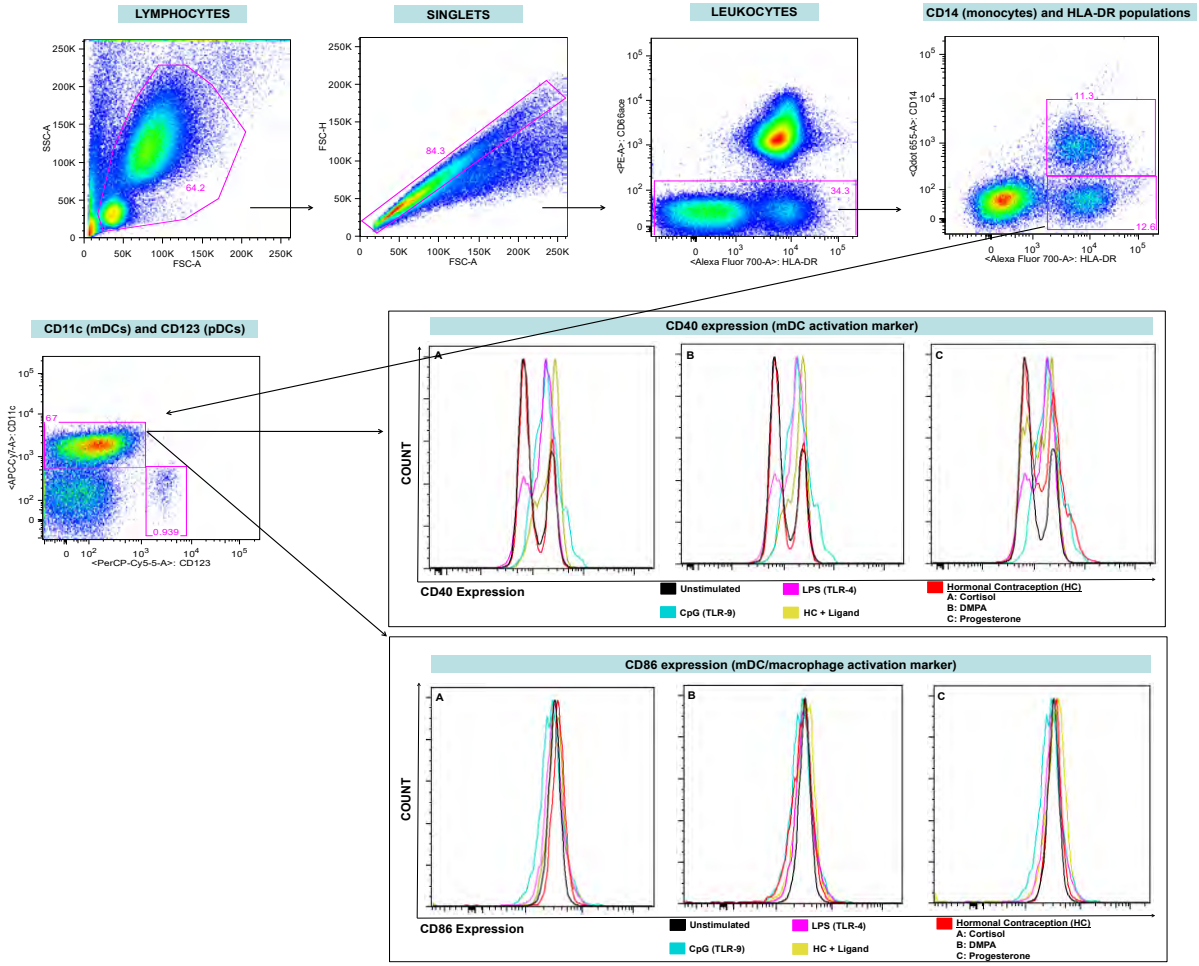


Figure 5.5 Gating strategy for analysis of DC subset activation (CD86 and CD40). Whole blood was either unstimulated (control), or stimulated with LPS/CpG or a combination of LPS/CpG and hormone (DMPA, cortisol and progesterone). Activation markers were defined as CD86 and CD40 following stimulation of PBMCs with either CpG or LPS.

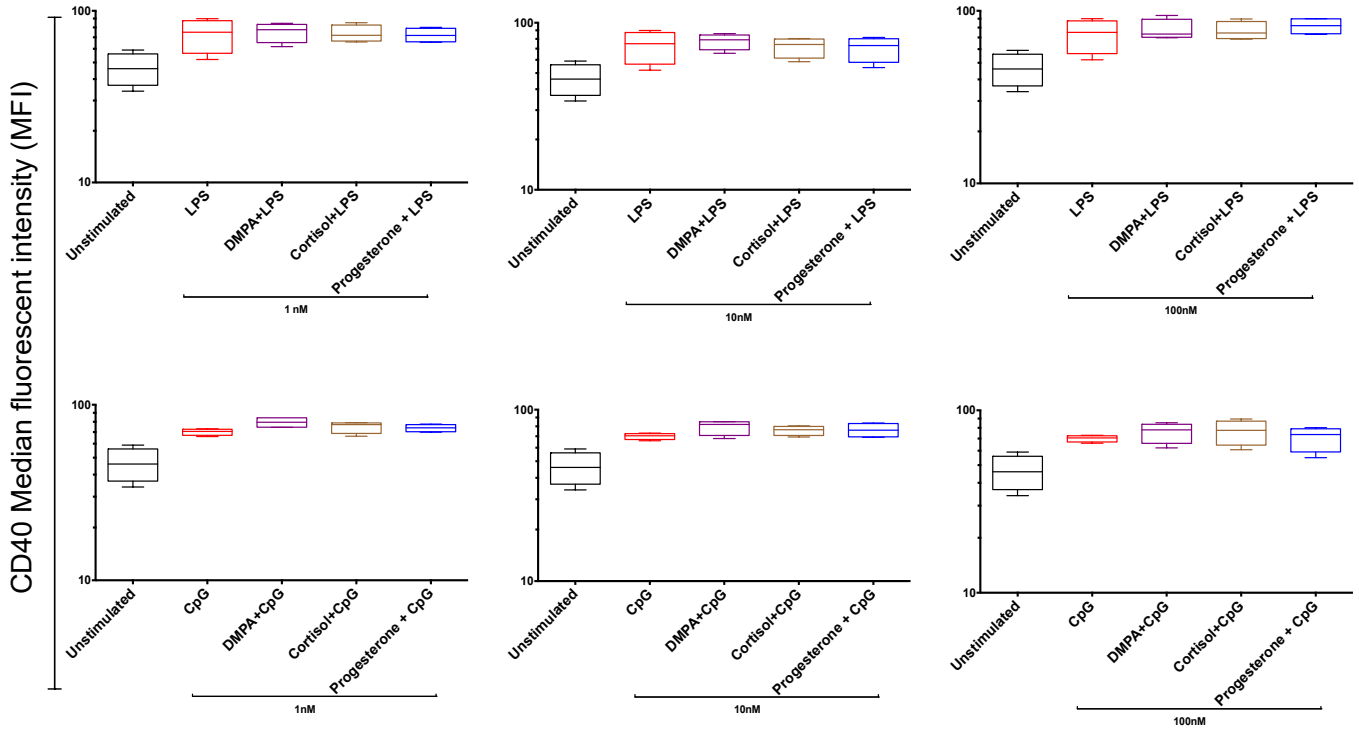


Figure 5.6 Effect of DMPA, cortisol and progesterone on CD40 expression (MFI) by mDCs stimulated with LPS (top panels) or CpG (bottom panels). Whole blood was either not stimulated (black bars), or stimulated with LPS/CpG (red bars), in the presence of DMPA (purple bars), cortisol (brown bars) or progesterone (blue bars). DMPA, progesterone and cortisol were used at varying concentrations (1, 10 and 100 nM). Box plots show the median, 25th and 75th percentile values, while whiskers show the 90 and 10% ranges; significance was determined using the Mann-Whitney test for non-parametric data. P-values <0.05 were considered significant.

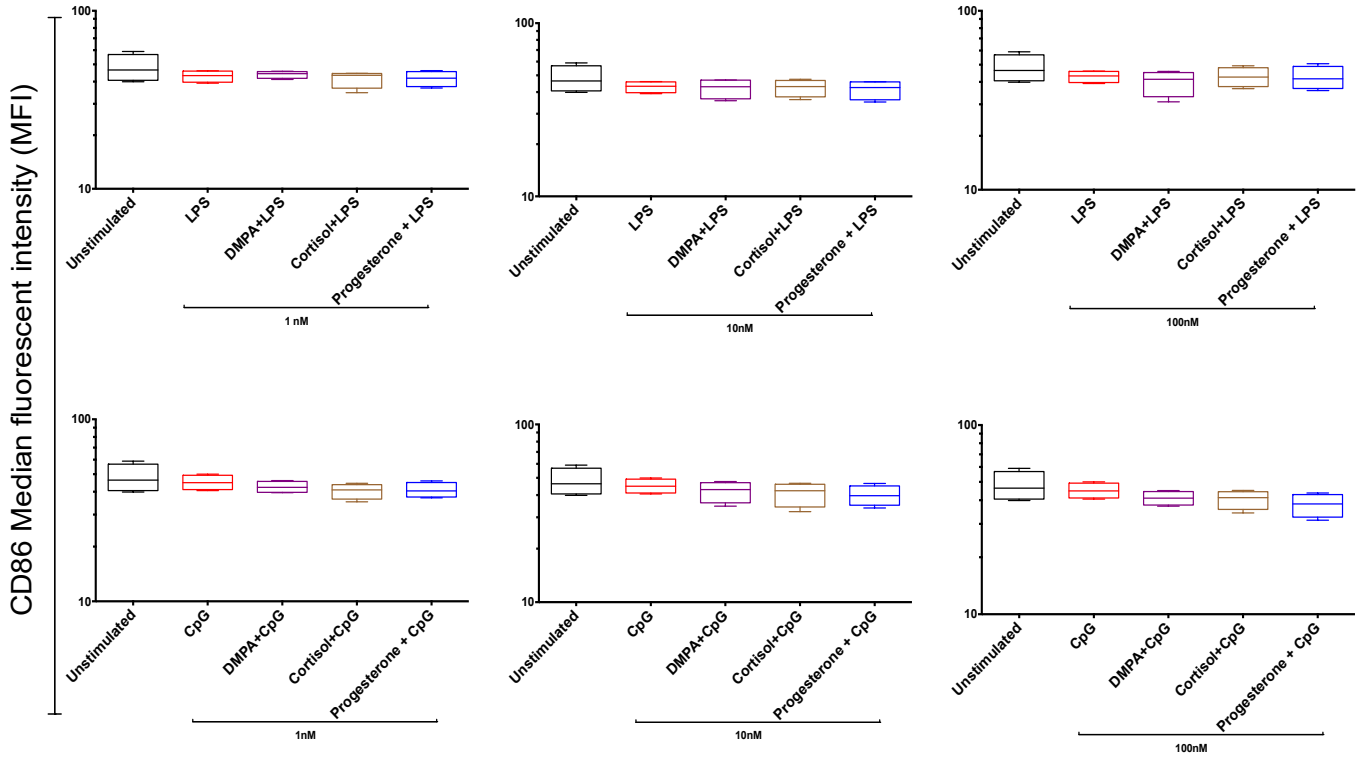


Figure 5.7 Effect of DMPA, cortisol and progesterone on CD86 expression (MFI) by mDCs stimulated with LPS (top panels) or CpG (bottom panels). Whole blood was either not stimulated (black bars), or stimulated with LPS/CpG (red bars), in the presence of DMPA (purple bars), cortisol (brown bars) or progesterone (blue bars). DMPA, progesterone and cortisol were used at varying concentrations (1, 10 and 100 nM). Box plots show the median, 25th and 75th percentile values, while whiskers show the 90 and 10% ranges; significance was determined using the Mann-Whitney test for non-parametric data. P-values <0.05 were considered significant.

5.4.4 Impact of exogenous HCs on mDC function

To investigate whether DMPA use has an impact on DC function *in vitro*, the in vitro effects of DMPA at physiological concentrations were compared to endogenous progesterone. MPA levels in the serum of DMPA users range from 4.5 to 65 nM a few days after administration of the 150 mg dose which then platueaus to about 2.6 nM for three months (Hapgood, 2013; Hiroi et al., 1975; Shrimanker et al., 1978). The levels of endogenous progesterone in the serum of premenopausal women varies from 0.65 nM to about 80 nM between the follicular and luteal phases, respectively (Africander et al., 2011). In this study, IL-6 (Figures 5.8) and TNF-α production (Figures 5.9) by mDCs was assessed following stimulation with LPS/CpG, in the presence of DMPA, cortisol or progesterone at varying concentrations (1, 10 and 100 nM)., as a measure of mDC function. Both DMPA and progesterone significantly reduced mDC TNF-α production following LPS and CpG stimulation, with DMPA having a stronger effect at all concentration measured (Figure 5.8). In contrast, cortisol did not effect TNF-α production by stimulated mDCs.

Similarly, DMPA and progesterone significantly inhibited LPS-induced IL-6 production by mDCs (Figure 5.9). Only varying concentrations of progesterone inhibited CpG-induced IL-6 production by mDCs while DMPA and cortisol did not (Figure 5.9). Due to the fact that only small populations of $CD14^+$ monocytes and $CD123^+$ pDCs were identified in this analysis, LPS/CpG-induced IL-6 or TNF-α production by these subsets was not shown in this Chapter.

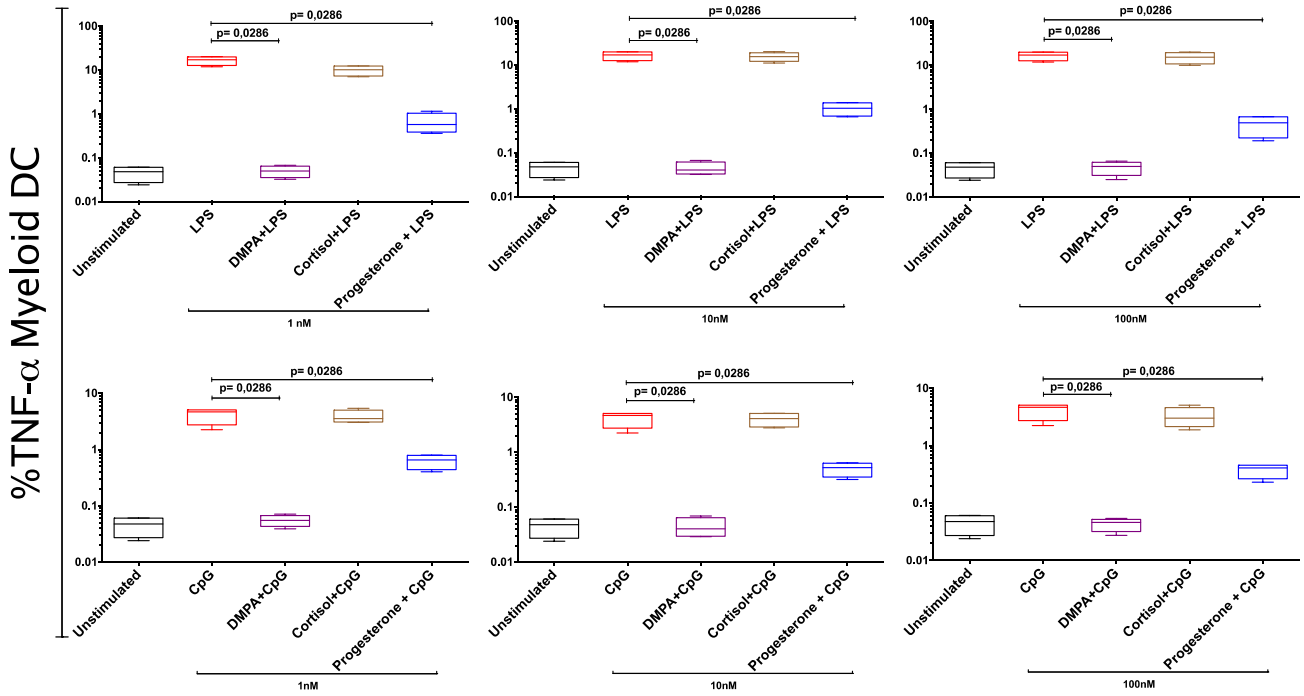


Figure 5.8 Effect of DMPA, cortisol and progesterone on TNF-α production by mDCs stimulated with LPS (top panels) or CpG (bottom panels). Whole blood was either not stimulated (black bars), or stimulated with LPS/CpG (red bars), in the presence of DMPA (purple bars), cortisol (brown bars) or progesterone (blue bars). DMPA, progesterone and cortisol were used at varying concentrations (1, 10 and 100 nM). DMPA and progesterone but not cortisol suppresses TNF-α production by activated mDCs stimulated with LPS or CpG ligands. Box plots show the median, 25th and 75th percentile values, while whiskers show the 90 and 10% ranges; significance was determined using the Mann-Whitney test for non-parametric data. P-values <0.05 were considered significant.

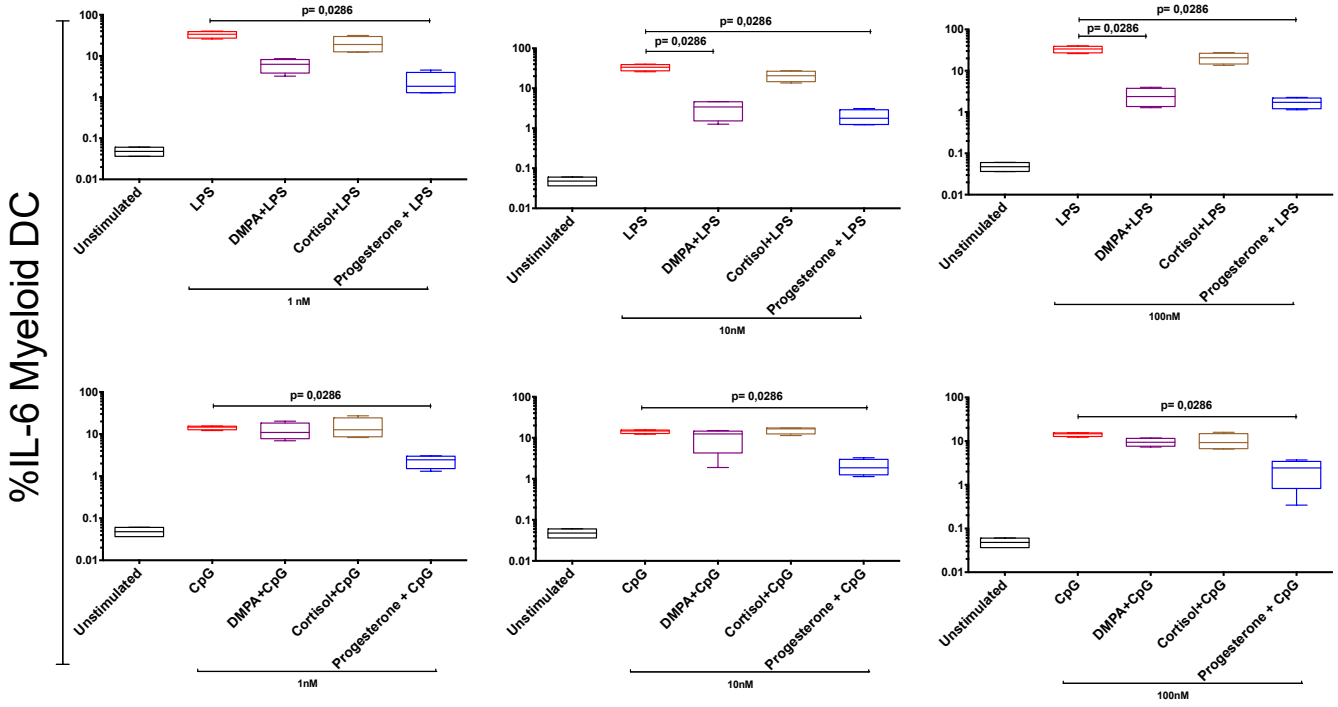


Figure 5.9 Effect of DMPA, cortisol and progesterone on IL-6 production by mDCs stimulated with LPS (top panels) or CpG (bottom panels). Whole blood was either not stimulated (black bars), or stimulated with LPS/CpG (red bars), in the presence of DMPA (purple bars), cortisol (brown bars) or progesterone (blue bars). DMPA, progesterone and cortisol were used at varying concentrations (1, 10 and 100 nM). DMPA and progesterone but not cortisol suppresses IL-6 production by mDCs stimulated with LPS or CpG ligands. Box plots show the median, 25th and 75th percentile values, while whiskers show the 90 and 10% ranges; significance was determined using the Mann-Whitney test. P-values <0.05 were considered significant.

5.5 Discussion

In this Chapter, hormone-mediated effects on monocyte and DC subset function and activation were evaluated: (1) *in vivo* from blood collected from HIV-uninfected women using injectable HCs; and (2) *in vitro* from blood from women using no HCs that was treated with exogenous synthetic and natural hormones (DMPA or progesterone) compared to cortisol. No differences were found in full blood counts, systemic cytokine concentration, or monocyte or DC lineage frequencies between women using DMPA versus those not using HCs, suggesting that HCs did not influence gross monocyte lineage counts. However, DMPA and progesterone decreased the production of TNF-α and IL-6 but not activation of mDCs *in vitro*, suggesting that immunomodulation of DC subsets can occur in the presence of HCs and their natural derivatives.

Kleynhans et al. (2011) perviously reported that women infected with tuberculosis who were concomitantly using DMPA and glucocorticoids had significantly lower numbers of blood monocytes. Furthermore, they also showed that mice treated with DMPA showed suppression in monocyte lineage-specific cytokines (Kleynhans et al., 2013; Kleynhans et al., 2011). In this study, injectable HC use had no effect on numbers of circulating monocytes in women using HCs compared to non-HC users. In addition, DMPA, cortisol and progesterone did not effect the function of monocytes following TLR stimulation with LPS and CpG, in *in vitro* experiments. It is possible that the effect of DMPA on monocytes observed in the previous study was due to the glucocorticoid rather than DMPA treatment. Heimbeck et al. (2010) also showed decreased numbers of circulating monocytes in individuals using glucocorticoid therapy, suggesting that glucocorticoid therapy rather than DMPA may influence monocyte numbers (Heimbeck et al., 2010).

DMPA has previously been shown to inhibit activation of DCs by the TLR3 agonist polyinosinic:polycytidylic acid, measured by increased expression of CD40 and ability to promote allogeneic CD4+ and $CD8^+$ T cell proliferation (Quispe Calla et al., 2015). In mice treated with DMPA or progesterone, decreased expression of CD40 (Vicetti Miguel et al., 2012) and CD86 (Xu et al., 2011) was reported. These studies suggest that the mechanism by which DMPA and/or progesterone inhibit *in vivo* T cell priming and virus-specific T cell expansion was via impaired DC activation and function (Vicetti Miguel et al., 2012; Xu et al., 2011). None of the hormones tested *in vitro* in this study affected CD40 and CD86 expression by mDCs following stimulation with TLR ligands, although CD86 expression under the conditions and concentrations used was not increased by either LPS or CpG stimulation.

Previous i*n vitro* studies have shown that DMPA decreases the ability of pDCs to respond to stimulation by TLR7/8 and TLR9 ligands, measured by production of IFN-α and TNF-α (Huijbregts et al., 2013; Michel et al., 2015). DMPA use has been associated with down-regulation of several cytokines, including IL-6, in mouse serum (Kleynhans et al., 2013) and human cervicovaginal secretions (Hughes et al., 2008). In this study, endogenously added DMPA and progesterone decreased LPS- and CpG-induced TNF-α production by mDCs at varying concentrations (1, 10 and 100 nM). In addition, mDCs treated with DMPA or progesterone displayed impaired capacity to produce IL-6 after LPS stimulation, independent of dose concntration, even at concentration (1 nM) lower than the peak and plateau levels found in the serum of women using DMPA. Only progesterone treatment inhibited production of IL-6 following CpG stimulation. Hartmann et al. (1999) reported kinetic differences in LPS- and CpG-induced cytokine expression by monocytes (4 hours versus 10

hours of stimulation, respectively), and that these ligands use different intracellular pathways for their stimulatory activity. Furthermore, in presence of pathogens, IL-6 production increased activation, proliferation, apoptosis and differentiation of the immune cells (Nakajima et al., 1989), while TNF-α was found to initiate an inflammatory cascade of cytokines, thereby recruiting macrophages and neutrophils to the site of infection (Bahia and Silakari, 2010). IL-6 and TNF-α are also responsible for maturation and migration of dendritic cells to the lymph nodes; thereby influencing the development of adaptive immunity (Banchereau and Steinman, 1998). Therefore, it is possible that the impairment in the ability of DCs to produce these cytokines might lead to reduced immune response against invading pathogens.

In this study, cortisol did not affect mDC cytokine production after LPS stimulation. The failure of cortisol to decrease cytokine expression may be due to its inability to bind to the glucocorticoid receptor complex, as some cytokine genes are regulated by this receptor (Bamberger et al., 1999). DMPA and progesterone are known to bind with high affinity to the glucocorticoid receptor (Bamberger et al., 1999). The results in this Chapter are in contrast with previous *in vitro* studies (Hughes et al., 2008; Kleynhans et al., 2013; Kleynhans et al., 2011) that suggest DMPA mirrors the effect of cortisol instead of its intended analog, progesterone.

Some limitations of this study were that it focused exclusively on mDC lineages, and other HIV-1 target cells, such as monocytes , pDCs and CD4+ T cells, were not evaluated. In addition, PBMCs used for *in vivo* studies were >10 years old, with relatively poor viability that may have influenced the results.

In summary, results from this Chapter suggests that DMPA and its natural analogue progesterone down-regulated certain characteristics of mDC function. These results provide evidence for the biological effects of DMPA on host immunity. More intensive and detailed studies into the biological effect of HCs on immunity is critical to ensure effective, safe HCs, relevant for women at risk of HIV-1 infection.

CHAPTER 6

Influence of injectable HCs on vaginal epithelial thickness, mucosal cytokines, HIV-1 target cell density and depth in the female genital tract during acute HIV-1 infection

CHAPTER 6

Influence of injectable HCs on vaginal epithelial thickness, mucosal cytokines, HIV-1 target cell density and depth in the female genital tract during acute HIV-1 infection

6.1 Abstract

Vaginal epithelial thinning and/or increased density of mucosal HIV-1 target cells are possible mechanisms by which injectable HCs may increase risk for HIV-1 infection in HIV-1 negative women. In women already infected with HIV-1, particularly during acute infection when viral loads peak, alterations in the genital epithelium may increase risk for HIV-1 transmission to sexual partners. Here, the influence of injectable HCs on genital epithelial thickness, mucosal HIV-1 target cell density and depth in recently HIV-1 infected women was investigated. CD4+ T cell and CD68+ macrophage density, both target cells for HIV infection, was measured in vaginal tissue biopsies from acutely-infected women who were either using injectable HCs or not using injectable HCs by immunohistochemical and immunofluorescent staining. Concentrations of 48 cytokines and HIV-1 concentrations were measured in CVL. Blood CD4 counts and plasma viral loads were performed during acute infection and 12 months post-infection. Overall, vaginal epithelial thickness was similar in women using injectable HCs (median 356.9 μm/cc; IQR 302.0-414.2) compared to non-injectable HC users (361.4; 271.0-409.4). The frequency of CD4+ T cells in the vaginal squamous epithelium of injectable HC users was significantly higher than non-injectable HC users [(median 33 cells/mm^2; IQR 23 -74) versus (24; 17-43), p=0.028]. CD68+ target cell density did not differ between women using injectable HCs and those not using injectable HCs [(median 23 cells/mm^2, IQR 17 – 30) versus (21, 15 - 28), p=0.439], although CD68+ macrophages were closer to the vaginal lumenal surface in women using injectable HC than those not using non-injectable HC users (median 97μm, IQR 81.3 – 117. versus 158, 131.8 –

163.8; p=0.021). Furthermore, the frequency of mucosal CD68+ macrophages during the acute infection were positively associated with the concentration of the CCR5 agonist RANTES (beta coefficient (β)=0.779, p=0.024), in addition to macrophage chemotactic cytokine MCP-1 (β=0.453, p=0.041), IP-10 (β=0.568, p=0.042), IL-7 (β=1.332, *p*=0.018), IL-9 (β=0.336, p=0.015), and IL-17 (β=1.058, p=0.007) in CVL, after adjusting for multiple comparisons. Injectable HC use was not associated with higher genital HIV-1 concentrations or more rapid disease progression (measured by CD4 counts or plasma viral loads at 12 months post-infection). It was concluded that women using injectable HC users had increased frequencies of CD4+ T cells in their vaginal stratified epithelium than those not using injectable HCs. CD68+ macrophages correlated with a broad panel of mucosal cytokines and inversely correlated with plasma viral load during acute HIV-1 infection.

6.2 Introduction

The mucosal epithelium of the lower female reproductive tract provide the first line of defence against pathogen entry and mediate the initial host immune response against STIs, including HIV-1 (Kaushic, 2011; Wira et al., 2005a; Wira et al., 2005b). This is also the surface through which HIV-1 is transmitted from an HIV-1 infected women to her sexual partner (Yi et al., 2013). The genital mucosa is also thought to serve as a discrete site for HIV-1 infection, replication and pathogenesis by providing the virus with a steady supply of susceptible target cells (Zhang et al., 1999).

The epithelial barrier of the genital mucosa is also thought to be influenced by hormones, inflammation and infection (Murphy et al., 2014). Studies in naturally cycling non-human primates have demonstrated the effect of hormones on the vaginal epithelium during the menstrual cycle, reporting a thicker epithelial barrier during the estrogen-dominant follicular phase and thinner epithelia during the progesterone-dominant luteal phase (Poonia et al., 2006; Veazey et al., 2012). Non-human primate studies have also suggested dramatic atrophy of the vaginal epithelium following administration of DMPA, although the amounts of DMPA used in these models is generally much higher than typically used in humans per kilogram body weight (Smith et al., 2000; Smith et al., 2004; Trunova et al., 2006). A similar but less pronounced hormonal effect on vaginal epithelial thickness is seen in humans, with decreased number of epithelial layers or reduced barrier thickness being reported during the luteal than follicular phase in naturally cycling women (Mauck et al., 1999; Patton et al., 2000) and in women using DMPA (Mauck et al., 1999). In both humans and non-human primate studies, thinning of the genital tract epithelium during either the luteal phase of the menstrual cycle or in those treated with DMPA, has been associated with increased

susceptibility to certain STIs, including HIV-1, HPV and *C. trachomatis* (Baeten et al., 2005; Marx et al., 1996).

DMPA may alternatively contribute to HIV-1 pathogenesis by facilitating closer contact between target cells in the mucosa and HIV-1 particles because of thinner epithelium (Chandra et al., 2013; Ildgruben et al., 2003; Miller et al., 2000; Wieser et al., 2001; Wira et al., 2011b). DMPA may influence the density of intracellular junction proteins between epithelial cells, potentially allowing better penetration of HIV-1 and facilitating better contact with mucosal target cells (Chandra et al., 2013). Chandra et al. (2013) showed that vaginal biopsies from HIV-1 uninfected women treated with DMPA had higher numbers of CD4+ T cells and macrophages expressing HLA-DR and CCR5 than non-HC counterparts. However, earlier studies reported no link between recruitment or activation of HIV-1 susceptible target cells and injectable HC use, despite resulting in decreased epithelial thickness (Bahamondes et al., 2000; Bahamondes et al., 2014; Kiddugavu et al., 2003; Mauck et al., 1999; Myer et al., 2007).

Acute HIV-1 infection has been associated with increased genital tract inflammatory cytokine responses in women (Roberts et al., 2012), that may result in increased permeability of the mucosal epithelial barrier, allowing HIV-1 easier passage across the mucosal epithelium (Nazli et al., 2010). Inflammatory responses at the genital mucosa have also been shown to enhance HIV-1 pathogenesis, by recruiting highly-activated HIV-1 target cells to the site of initial viral infection and replication (Abel et al., 2005; Li et al., 2009; Meier et al., 2007). In DMPA-treated macaques infected with SIV, cervicovaginal concentrations of MIP-1α, MIP-1β and MIP-3α resulted in recruitment of $CCR5^{+}$ and CD4+ T cells in the endocervical

epithelium (Li et al., 2009). Furthermore, spread of SIV-infected cells from the genital mucosa to ensure a productive systemic infection, correlated with this pro-inflammatory cytokine gradient (Li et al., 2009). In humans, concentrations of IL-1β, IL-6, and IL-8 in CVLs from HIV-1 acutely infected women correlated inversely with CD4+ T cell counts in blood, suggesting the level of genital inflammation present during acute infection predicted adverse CD4 decline during acute infection (Bebell et al., 2008). Increased recruitment of activated HIV-1 target cells to the genital epithelium was shown to facilitate local HIV-1 replication and result in increased local genital HIV-1 shedding from HIV-1 infected women, potentially placing their male partners at higher risk of HIV-1 infection (Gordon et al., 1999; Rottman et al., 1997). In both humans and non-human primates studies, higher levels of T cell activation in blood and plasma viral loads during acute infection predicted faster rates of mucosal and systemic CD4 cell decline and more accelerated disease progression (Brenchley et al., 2004; Douek et al., 2009; Hazenberg et al., 2003; Silvestri et al., 2003).

The aim of this Chapter was to investigate the effect of injectable HC use on vaginal epithelial thickness, availability or depth of HIV-1 targets cells at the genital mucosa and the rate of HIV-1 disease progression (higher viral loads at set-point and CD4 decline to 12 months post-infection) during acute HIV-1 infection. A better understanding of the impact of injectable HC use on density and depth of activated HIV-1 target cells at the mucosa in the presence of genital inflammation during acute infection could lead to better interventions aimed at blocking HIV-1 transmission from an infected women to her partner. In addition, it would also identify therapeutic targets to block male-to-female transmission.

6.3 Materials and Methods

6.3.1 Study participants and sample collection

This retrospective study used 49 cervicovaginal biopsies collected from consenting HIV-1 infected women who had previously participated in the CAPRISA 004 tenofovir 1% gel vaginal microbicide trial, during acute infection (Abdool Karim et al., 2010a). In addition to the cervicovaginal biopsies, matching CVLs, vaginal swabs, and plasma were also collected during this acute infection visit. Vaginal biopsies were snap frozen in liquid nitrogen within 6 hours of collection. CVLs (10ml saline) were collected as described previously in Chapter 3 (section 3.1). CVLs were processed within 6 hours of collection, centrifuged at 388 x g upon receipt in the CAPRISA laboratory, and the supernatant fraction stored at -80°C for measurement of cytokine concentrations and viral loads. Detailed behavioural, structural, and clinical patient information were collected at enrolment into the acute infection study using a structured questionnaire. Data on HC use was collected. Vaginal swabs from each women were also screened for *T. pallidum, C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*, *T. vaginalis,* HSV-2 and BV as previously described in Chapter 3. CD4 counts and plasma viral loads were performed on each women every month for the first 12 months. Plasma RNA concentrations were measured by the ROCHE COBAS Ampliprep-COBAS TaqMan version 2.0 assay (Roche Molecular Systems, Inc, Germany) (Abdool Karim et al., 2010a). Genital tract viral load testing was done using Nuclisens Easyq HIV-1 (version 1.2) according to the manufacturer's protocol (BioMérieux, France) by the the National Health Laboratory Service (NHLS) Clinical Virology laboratory (Groote Schuur Hospital, Cape Town). The detection limit of this assay was ≥50 RNA copies/ml. The protocol for this study was approved by the Ethical Review Committee of the University of KwaZulu-Natal.

6.3.2 Processing of vaginal biopsies

Cervicovaginal biopsies, snap frozen in liquid nitrogen, were embedded in optimum cutting temperature (OCT, Sakura, Torrance, CA, USA) medium in a cryomold to ensure optimum orientation and longitudinal sectioning of the epithelium and then transferred to -20°C. The frozen block was adhered to the cryosectioning chuck at -20°C to ensure the optimal temperature for sectioning, 12 µm sections were cut onto glass slides using the Tissue-Tek® Cryo3® Plus Microtome/Cryostat (Sakura, Torrance, CA, USA), fixed with isopropanol and methanol, and stained with either (i) hematoxylin and eosin (H&E) (to distinguish the stratified squamous epithelium) and (ii) with fluorescently-labelled antibodies against HIV-1 target cells (Cyanine 5 labelled CD4 or DakoCytomation CD68 antibodies; to quantify HIV-1 target cell density within tissue).

For H&E staining, vaginal biopsy sections were transfered into isopropanol for two minutes, then into methanol for 2 minutes and then rinsed in running tap water for 1 minute. Sections were incubated in haematoxylin for 5 minutes and rinsed in running tap water for 5 minutes. The slides were then incubated in eosin for 1 minute. Slides were rehydrated by dipping in absolute ethanol ~20 times. Slides were subsequently washed 3 times with tap water, remaining liquid suctioned and mounted in fluorescent mounting medium (DAKO S3023) and covered with a coverslip. The edges were sealed with clear nail varnish; dried in the dark for 15 minutes and stored in the slide box at 4°C until imaged using a Zeiss AxioScope light microscope interfaced with the AxioVision (4.8.2 version) under 10X magnification.

Immunohistochemical staining of CD4 and CD68 cells was performed to investigate target cell recruitment in vaginal mucosa, in the laboratory of Prof Thomas Hope at Northwestern

University, Chicago (S. Ngcapu, 2014 Fogarty training fellowship). The slides were warmed at room temperature for 5 to 10 minutes and tissue sections were outlined with a PAP pen (Sigma-Aldrich, MO,USA). Slides were fixed at room temperature by placing a freshly made solution of 3.7% formaldehyde in Pipes buffer onto the slide for 10 minutes. Slides were then washed 3 times with PBS and the remaining liquid was suctioned.

For CD4 staining, slides were incubated with 1:200 dilution of the conjugated primary antibody directed against CD4 (OKT4 mouse, Sigma-Aldrich, MO,USA) diluted in donkey serum blocking solution (10% normal donkey serum, 0.1% Triton X-100/0.01%N_aN_3 and 1X PBS) at room temperature for 60 minutes. Slides were washed 3 times with PBS, and the remaining liquid suctioned. Tissue sections were then incubated with hoechst DAPI (staining nuclear material; DAPI used at 1:25000 dilultion; Invitrogen, CA, USA) diluted in donkey serum blocking solution in the dark at room temperature for 10 minutes. The slides were washed 3 times with PBS, remaining liquid suctioned and the tissue sections were finally mounted in fluorescent mounting medium (DAKO S3023, CA, USA) using a 20 mm coverslip (Thermo Fisher Scientific, MA,USA). The edges of the coverslip were sealed with clear nail varnish; dried in the dark for 15 minutes and stored in slide box at 4°C until imaged.

For CD68 staining, slides were initially stained with a 1:200 dilution of unconjugated primary anti-CD68 (DakoCytomation clone EBM11, Dako, CA, USA; diluted in donkey serum blocking solution) at room temperature for 60 minutes. Slides were washed 3 times with PBS and the remaining liquid was suctioned. After washing, the slides were incubated with 1:500 dilution of the secondary antibody (donkey anti-mouse Rhodamine Red X in donkey serum

blocking solution) in the dark at room temperature for 25 minutes. Slides were then washed 3 times with PBS, remaining liquid suctioned. Finally, slides were then incubated with a 1:25000 dilution of hoechst DAPI (to stain nuclear material) diluted in donkey serum blocking solution in the dark at room temperature for 10 minutes. The slides were washed 3 times with PBS, remaining liquid suctioned and mounted in fluorescent mounting medium (DAKO S3023) and a coverslip applied. The edges of the coverslip were sealed with clear nail varnish; dried in the dark for 15 minutes and stored in slide box at 4°C until imaged.

Vaginal biopsy tissue sections, stained with H&E stains, were viewed on the Zeiss AxioScope Light Microscope interfaced with the AxioVision image analysis software programme (4.8.2 version, Zeiss, Oberkochen, Germany). Consecutive lengths (parallel lines) of mucosa were archived from each vaginal biopsy sample, with care taken to avoid oblique and transverse areas. Images were archived-1ed at 10x magnification and stored as .zvi files. Slides were viewed in a blinded fashion by 3 independent assessors, with two assessors using the manual measurements (Sinaye Ngcapu and Dr Desh Archary, CAPRISA Mucosal Laboratory, Durban) and the third using automated measurements (Dr Ann Carias, Northwestern University, Chicago, USA; using specially created algorithms using IDL software, Exelis, VA, USA). Approximately 3 - 4 interactive length measurements of mucosal thickness were manually performed by drawing parallel lines from the epithelium-lamina propria border to the stratum basale (including any lamina propria papillae) and images generated were stored with MS.zvi extension. Information of evident microabrasions or tears in the mucosal epithelium was also noted for each sample. Using the automated system, algorithms in IDL software enabled drawing of two distinct lines, at the luminal surface and where cellular junctions were robust. The distance (thickness) was then calculated from each individual point from line A to the closest distance of another point on

line B, and vice versa. Approximately 2000-7000 interactive length measurements of mucosal thickness were performed per image analysed. Consecutive lengths of mucosal epithelium were archived from each sample with care taken to avoid oblique and transverse areas. This data was extrapolated onto an excel file for unblinding.

For immunofluorescent staining of HIV-1 target cells in vaginal biopsies, images were obtained by deconvolution microscopy on a DeltaVision RT system (Applied Precision, LLC, WA, USA) collected on a digital camera (CoolSNAP HQ; Photometrics, AZ, USA) using a 40x oil objective, and performed at the Hope Laboratory, Northwestern University, Chicago, USA. All images were archived at 40x magnification and stored as z-stacks. SoftWoRx analysis software (MT, USA) was used to measure the shortest distance of intra-epithelial target cells to the surface of tissue epithelium. The cell density was calculated using the following formula:

N_c/A; $A = N_i \times I_1 \times I_2 \times p^2$

Where N_c = number of cells, I_1 = y pixel size, A = area surveyed, I_2 = x pixel size, N_i = number of images, p^2 = pixel size in μm squared

6.3.3 Measurement of cytokine concentrations in CVLs

The concentrations of 48 cytokines were measured in CVL specimens by Luminex multiplexing assays, according to the method described in Chapter 2. These included pro-inflammatory IL-1β, IL-1α, IL-6, IL-12p70, IL-12p40, IL-18, TNF–α, TNF-β, macrophage migration inhibitory factor (MIF), TNF-related apoptosis inducing ligand (TRAIL)], anti-inflammatory (IL-1Rα, IL-10), chemokines (IL-8, IL-16, IP-10, MCP-1, MCP-3, MIP–1α,

MIP-1β, RANTES, cutaneous T-cell attracting chemokine (CTACK), GRO-α, IFN-α2, monokine induced by gamma-Interferon (MIG), eotaxin) adaptive (IFN-γ, IL-2, IL-2RA, IL-4, IL-5, IL-13, IL-15, IL-17), and growth-related cytokines [IL-3, IL-7, IL-9, FGF, G-CSF, GM–CSF, hepatocyte growth factor (HGF), nerve growth factor (NGF)-β, platelet derived growth factor (PDGF)-β, stem cell factor (SCF), stem Cell Growth Factor (SCGF)-β, stromal derived factor (SDF)-1α, and VEGF, leukemia inhibitory factor (LIF), macrophage colony-stimulating factor (M-CSF)] were measured using Bio-Plex Pro Human Cytokine kits and a Bio-Plex MagPix Array Reader (Bio-Rad Laboratories, CA, USA). Prior to assays, CVL supernatants were pre-filtered using 0.2µm Costar Spin-X cellulose acetate filters (Sigma-Aldrich, MO,USA). All assays were performed according to the manufacturer's protocol and samples were assessed in duplicates. Bio-Plex Suspension Array Reader (Bio-Rad Laboratories, CA, USA) was used to collect multiplex data. All analyte concentrations were extrapolated from the standard curves using a 5 PL regression equation. As in Chapter 2, cytokine levels below the lower limit of detection of the assay were reported as the mid-point between the lowest concentration measured for each cytokine and zero.

6.3.4 Statistical analyses

Descriptive statistics were used to describe acutely infected women from which vaginal biopsies were obtained. In order to determine factors associated with CD4 and CD68 cell density, specifically the effect of injectable HCs, a general linear model was fitted to each of these outcomes. Adjusted models included variables age, marital status, condom use, injectable HC use, CAPRISA 004 arm assignment, plasma viral load, presence of sexually transmitted infections and bacterial vaginosis. To determine the effect of individual cytokines on CD4 and CD68 cell density, general linear models were fitted to each of these separately, along with each cytokine, while adjusting for age, marital status, condom use, injectable use,

CAPRISA 004 arm assignment, plasma viral load, presence of sexually transmitted infections and bacterial vaginosis. Spearman's rank correlation was used to explore the relationship between experts measuring epithelial thickness.

Disease progression was defined as having two consecutive CD4 cell counts below 350 cells/µl, after the first 6 months of HIV-1 infection. Time to disease progression was calculated from estimated date of infection, and participants who did not reach this endpoint, were censored at their last visit. Proportional hazards regression models were fitted to time to disease progression, in order to determine the effect of CD4 and CD68 cell density around the time of infection. Data was analysed using SAS version 9.3 (SAS Institute Inc., NC, USA) and graphs were drawn using GraphPad Prism 6 (GraphPad, CA, USA).

6.4 Results

Forty nine vaginal biopsies were collected from acutely HIV-1 infected women (Table 6.1). Of these, 8/49 (16.3%) were using no or non-injectable HCs [3 were using condoms only, 4 were using COCs and 1 was using a non-hormonal IUD] and 41/49 (83.7%) were using injectable HCs (36 were using DMPA and 5 were using NET-EN). The earliest time point post-infection for HIV-1 infected women using injectable HCs was median of 46 days (range 32-66) while non-injectable HC users were enrolled 77 days (46-129) post-infection (p=0.147). Women using no or non-injectable HCs were similar in age to those using injectable HCs [median 23 years (range 22-28) and 25 years (22-28), respectively, p=0.737]. Women reported similar condom use at last sex act [61% (25/41) in injectable HC users compared; 75% (6/8) in no or non-injectable HC users; p=0.693). The majority of women (66%, 27/41) were in the placebo arm of the CAPRISA 004 1% Tenofovir microbicide trial, and this did not differ between injectable HC users and those using no or non-injectable HCs. The median CD4+ T-cell counts and viral load measurements at the acute infection time point (when vaginal biopsies were collected) was 528 cells/µl (range 419-774) and 26 300 RNA copies/ml (3330-105000), respectively, and did not differ if women were using injectable HCs or not (p=0.582 and p=0.335, respectively).

During acute HIV-1 infection, more than half (56%) of the women had BV (Nugent score ≥7), and this did not differ significantly between women not using injectable HCs and those using injectable HCs (p=0.681). Similarly, 21% of women were infected with *C. trachomatis* and this did not differ significantly between groups (p=1.000). The prevalence of *T. vaginalis* (12%), *N. gonorrhoeae* (5%), *M. genitalium* (9%), and HSV-2 (14%) were relatively low in this cohort and were not significantly different in groups (Table 6.1).

Table 6.1 Demographic and clinical characteristics of participants, stratified by injectable HCs use

Characteristic	Overall (N=49) Median (IQR) or % (n)	Injectable HCs users (N=41) Median (IQR) or % (n)	Non-injectable HCs users (N=8) Median (IQR) or % (n)	p-value
Age	24 (22 – 28.0)	25 (22.0 – 28.0)	23 (21.7 – 28.0)	0.737
Stable partner	76 (37/49)	73 (30/41)	88 (7/8)	0.660
Highest level of schooling	12.0 (11.0 – 12.0)	12.0 (11.0 – 12.0)	11.5 (11 – 12.0)	0.281
Treatment arm				
Tenofovir	37 (18/49)	34 (14/41)	50 (4/8)	0.443
Placebo	63 (31/49)	66 (27/41)	50 (4/8)	
Days post infection	49 (32.0 – 89.0)	46 (32.0 – 66.0)	77 (45.8 – 129.0)	0.147
CD4 count (cells/µl)	528 (414.0 – 771.0)	528 (419.0 – 774.0)	578 (328.0 – 762.0)	0.582
Plasma viral load (RNA copies/ml)	26300 (3330.0 – 105000.0)	22000 (3290.0 – 104000.0)	58350 (11890.0 – 342500.0)	0.335
Genital viral load (RNA copies/ml)	7 (2/30*)	585 (399 – 771)	-	-
Condom use at last sex act	63 (31/49)	61 (25/41)	75 (6/8)	0.693
STIs:				
Bacterial vaginosis	56 (24/43)	58 (21/36*)	43 (3/7)	0.681
Trichomonas vaginalis	12 (5/43)	14 (5/36*)	ND	0.572
Neisseria gonorrhoeae	5 (2/43)	6 (2/36*)	ND	1.000
Chlamydia trachomatis	21 (9/43)	22 (8/36*)	14 (1/7)	1.000
Mycoplasma genitalium	9 (4/43)	11 (4/36*)	ND	1.000
Herpes simplex virus type 2	14 (6/43)	17 (6/36*)	ND	0.567

Abbreviations: IQR, interquartile range; ND=no data available; *data was only available for women

6.4.1 Influence of measurement method on vaginal epithelial thickness

To evaluate whether the different epithelial thickness measuring methods and/or inter-individual variability introduced any variance in the measurements taken, all images were measured by 3 independent assessors using two different measurement platforms, one manual and the other fully automated using IDL software (Figure 6.1 and 6.2). No significant difference in epithelial thickness, measured manually between independent assessors, was observed (median 355 μm, 300 – 414 for assessor 1 versus 363, 304 – 417 for assessor 2; p=0.981) (Figure 6.2). Although epithelial thickness measurements correlated positively between assessor 1 and 2, this was not significant (Rho=-0.462, p=0.134). Comparing manual and fully automated measurement, results were significantly different (median 355 μm, 300 – 414 for manual assessor 1 versus 254, 197 – 323 for automated assessor 3, p=<0.0001). Manual measurements from assessor 1 did not correlate with those of automated assessor 3 (Rho=0.107, p=0.464). Because the automated method measurement takes into account the whole of the stratified squamous epithelium, including sinuous patterning of the epithelial rete pegs that extend into the dermal papillae that are probably major contributors to the variability in measurements, automated measurements were used for all the subsequent analyses presented in this Chapter. The manual method was concluded to be outdated allowing only few interactive measurements (3 to 4) compared to 2000-7000 interactive measurements done using automated IDL software. In addition, the automated method was considered to have less bias in measurement of the epithelial thickness.

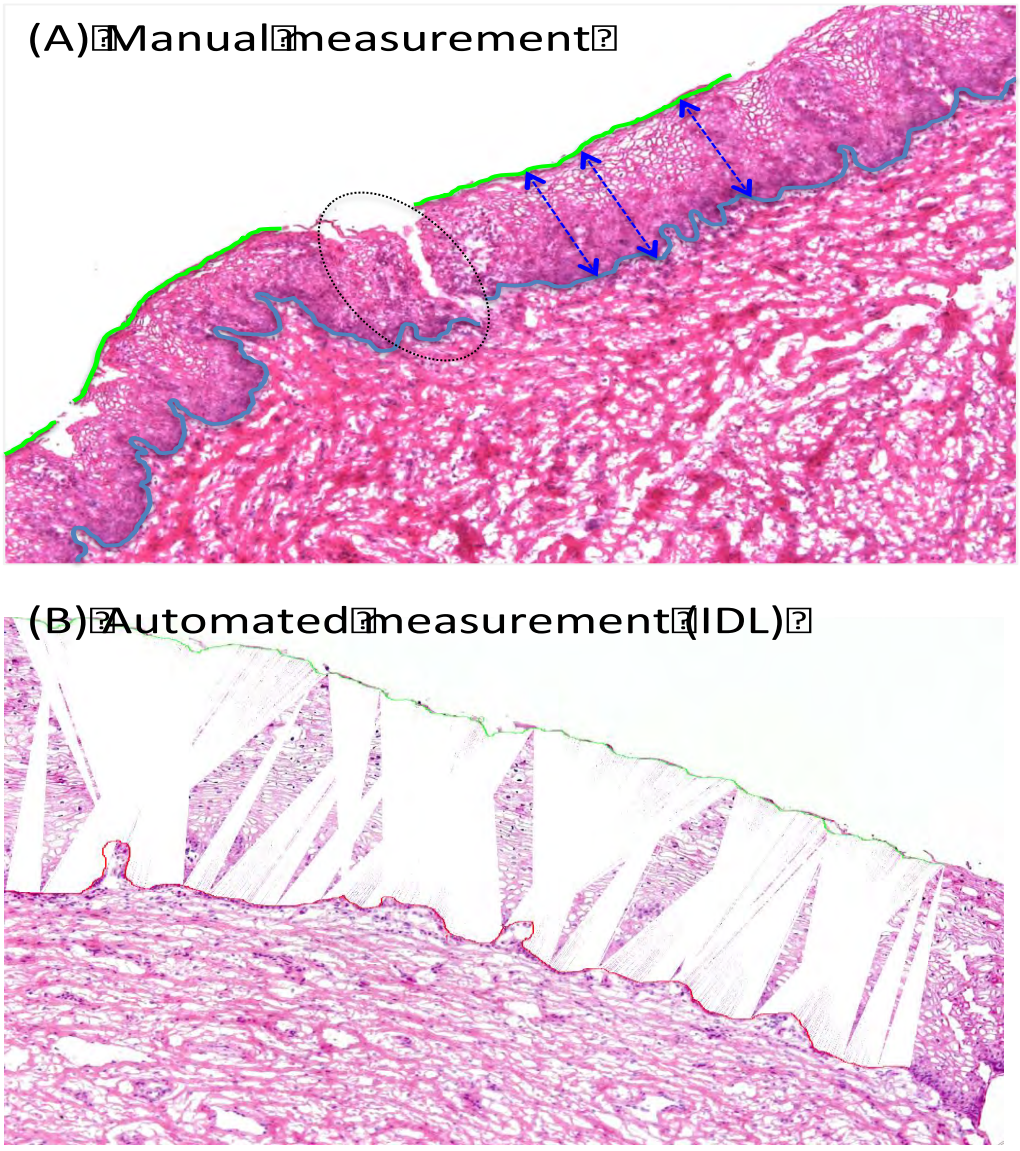


Figure 6.1 Vaginal epithelial thickness measurements in biopsy tissues collected from acutely HIV-1 infected women, who were either using injectable HCs or not. (A) Manual measurements were performed by drawing lines from the epithelium-lamina propria border to the stratum basale (including any lamina propria papillae). The dotted circle represents the microabrasions which may be due to flash freezing were visible in a number of biopsies assessed. (B) Automated random measurements (performed using IDL software, represented by multiple white lines) were done by calculating thickness following 2000-7000 interactive measurements from each individual point between line A (green line) to the closest distance of another point on line B (red line).

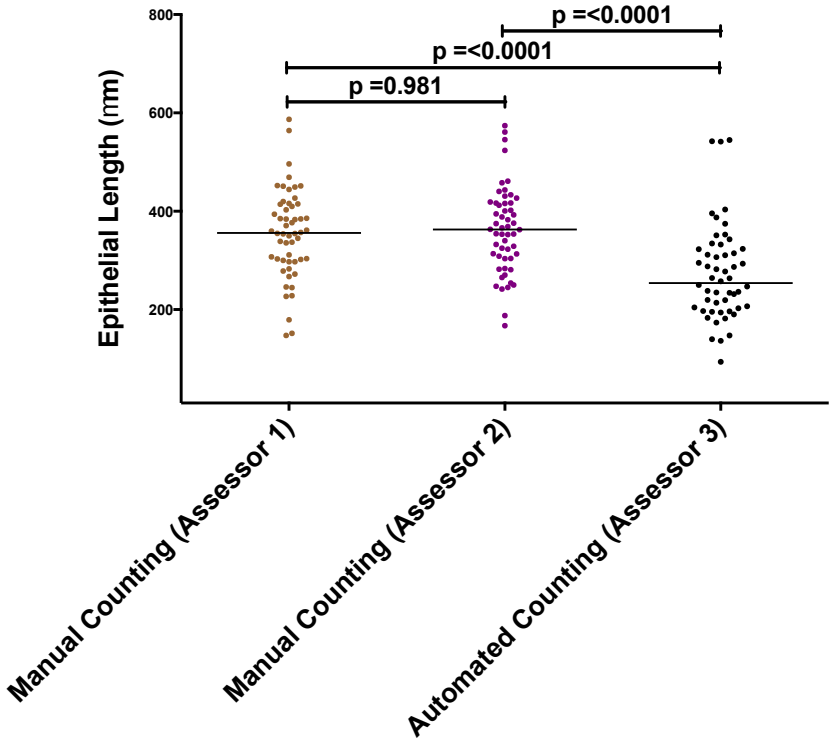


Figure 6.2 Comparison of epithelial thickness measurements from three different experts. Assessor 1 and 2 used the manual counting method while assessor 3 used an automated method in IDL. Manual measurements were performed by drawing approximately 3 to four interactive length measurements lines from the epithelium-lamina propria border to the stratum basale (including any lamina propria papillae) while automated measurements were done by calculating thickness following 2000-7000 interactive measurements that covered the whole stratified squamous epithelium including sinuous patterning of the epithelial rete pegs that extend into the dermal papillae.

6.4.2 Relationship between injectable HC use and vaginal epithelial thickness in HIV-1 infected women

Because previous studies in non-human primates and humans have suggested that DMPA use results in thinning of the vaginal epithelium, the influence of injectable HC use on vaginal epithelial thickness was assessed in vaginal biopsies. No difference in vaginal epithelial

thickness was observed in women using injectable HCs compared to those using no or non-injectable HCs (Table 6.2, p=0.970). Similarly, epithelial thickness was similar if women were stratified according to whether they were using DMPA (p=0.679) or NET-EN (p= 0.583) compared to no or non-injectable HC users.

Table 6.2 Vaginal epithelial thickness in women using injectable HCs

Groups	N	Median thickness (μm) (IQR)	p-value
Non-injectable HCs*	8	361.4 (271.0 – 409.4)	-
Injectable HCs:	41	356.9 (302.0 – 414.2)	0.970
DMPA	36/41	355.9 (280.8 – 414.2)	0.679
NET-EN	5/41	340.8 (244.8 – 410.2)	0.583

Abbreviations: IQR, interquartile range; *Non-injectable HCs include 2/8 no contraception users, 1/8 women using condom only, 4/8 using COCs and 1/8 who used an IUD.

6.4.3 Relationship between injectable HCs and vaginal target cell density and depth in HIV-1 infected women

Thirty of the 49 vaginal biopsies were stained for CD4+ (representing T cells) and CD68+ (representing macrophages) HIV-1 target cell density and depth (two representative images Figure 6.3 and 6.4). Of these 30 vaginal biopsies, 24 were from injectable HC users (22 DMPA and 2 NET-EN) and 6 were from non-injectable HC users (4 COC users, 1 IUD and 1 woman not using any form of HC). The median number of CD4+ T cells within the squamous epithelium was 31 cells/mm^2 (IQR, 20 - 64) (Table 6.3). HIV-1 infected women using injectable HCs had significantly higher numbers of CD4+ T cells within the vaginal squamous epithelium than women using no or non-injectable HCs (p=0.028), after adjusting for age, condom use, study arm, marital status, plasma viral load, any STIs or BV.

CD68+ macrophages were slightly less dense within the stratified squamous epithelium of acute infection vaginal biopsies, with a median number of 23 cells per mm^2 of tissue (IQR, 16 - 30) (Figure 6.4). Unlike CD4+ T cells, no difference in numbers of CD68+ macrophages was found between HC and non-HC groups (p=0.439) or between DMPA and non-HC groups (p=0.439) (Table 6.3). Furthermore, there was no significant correlation between CD4+ and CD68+ target cell densities measured in acutely HIV-1 infected vaginal biopsies (Rho=-0.07, p=0.723).

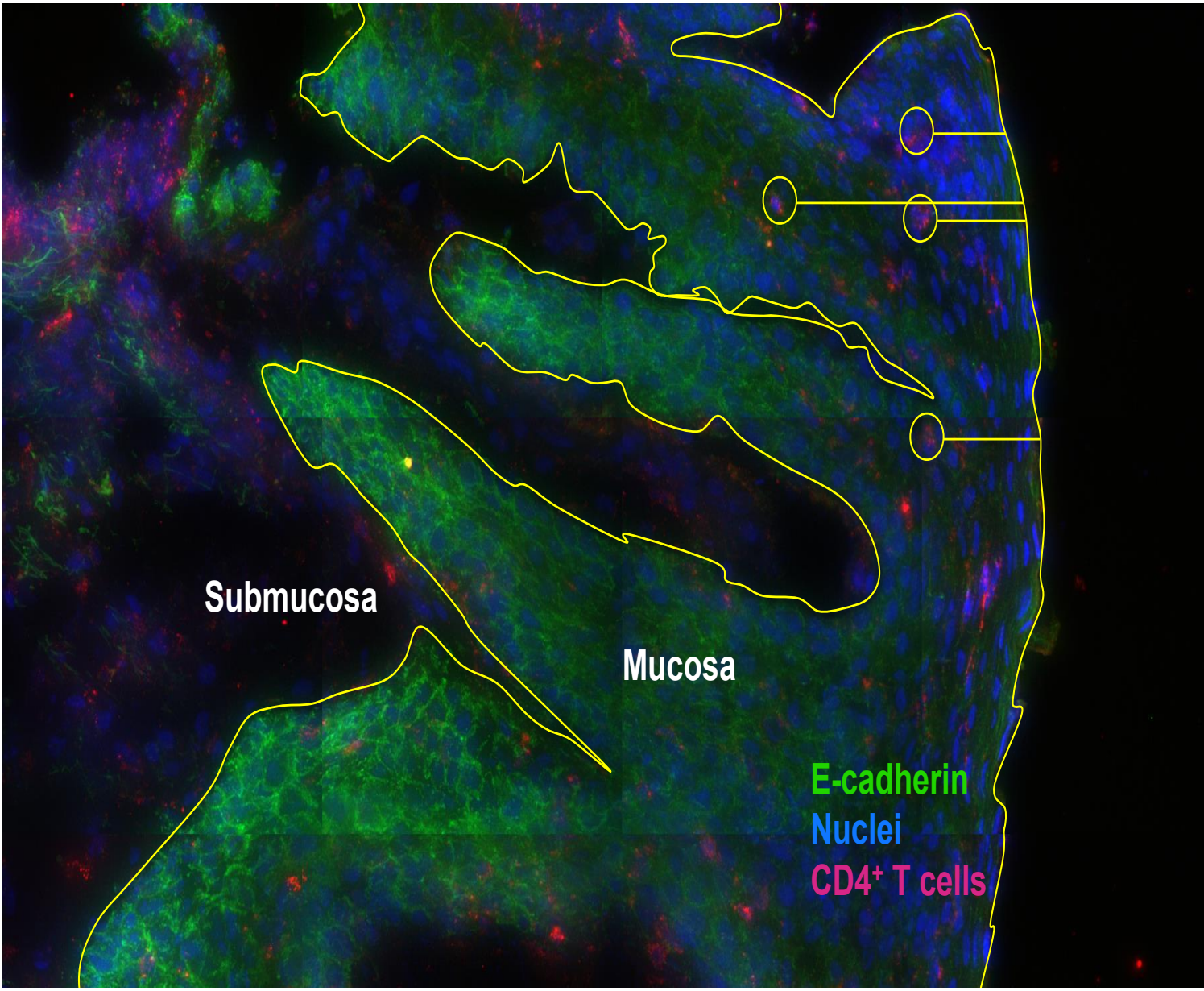


Figure 6.3 CD4+ T cells in vaginal biopsies from acutely HIV-1 infected women. CD4+ cells are shown in red, E-cadherens junctions are shown in green, and DAPI is shown in blue (40X). The shortest distance of intra-epithelial target cells (yellow circle and horizontal line) to the surface of tissue epithelium (yellow vertical line) was also measured.

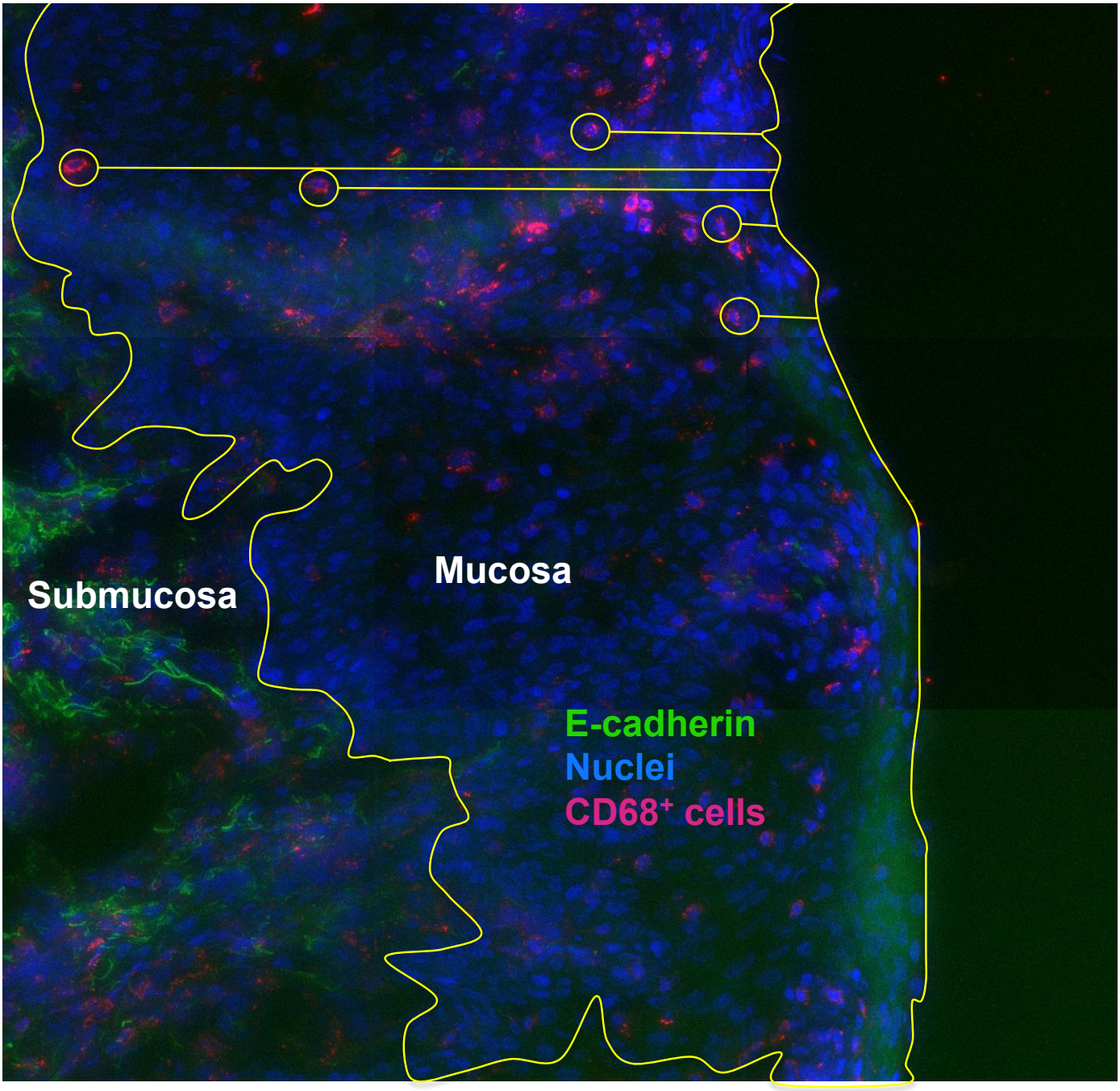


Figure 6.4 CD68+ macrophages in vaginal biopsies from acutely HIV-1 infected women. CD68+ cells are shown in red, E-cadherens junctions are shown in green, and DAPI is shown in blue (40X). The shortest distance of intra-epithelial target cells (yellow circle and horizontal line) to the surface of tissue epithelium (yellow vertical line) was also measured.

*IQR: interquartile range; #Multivariate analysis adjusted for Age, injectables, condom use, study arm, marital status, plasma viral load, any STIs and BV

HIV target cell depth in the tissue, measured by the distance between CD4+ or CD68+ cells and the vaginal lumenal interface in vaginal biopsies was evaluated (Table 6.4, Figures 6.3 and 6.4). CD4+ cells were a median distance of 136 μm from the vaginal lumen, with depth ranging from 59 – 297 μm. According to Carias et al. (2013), HIV-1 can passively diffuse to an average depth of 6.98 ± 0.19μm into undisrupted squamous. However, HIV-1 can breach the 10 - 20 μm thick layer of columnar epithelial and migrate to depths of 50 μm (which was the physical limit of the field microscope used) within the epithelium to reach the underlying target cells (Carias et al., 2013). Taking this as an arbitrary cut-off at which HIV-1 would have easy access to available target cells, ~2 of the 30 CD4+ cells counted in sections evaluated would be within this cut-off. Vaginal CD4+ target cell density did not differ between acutely HIV-1 infected women using injectable HCs compared to those not using or using non-injectable HCs [median 121μm (89 – 204) versus 113 (82 – 187); p=0.951] (Table 6.4).

In contrast to the CD4+ target cells, CD68+ macrophages were closer to the lumen in women using injectable HC compared to non-injectable HC users (median 97, IQR 81 – 117 versus 158, IQR 132 – 164; p=0.021). Similarly, CD68+ macrophages were near the lumen in women using DMPA compared to non-DMPA users (median 98, IQR 71 – 129 versus 158, IQR 132 – 164; p=0.026) (Table 6.4).

Table 6.4 Target cell depth in vaginal biopsies of women using injectable HCs versus non-injectable users

Groups	CD4+ T cells			CD68+ Macrophages	
	N	Median depth in µm (IQR)	P value	Median depth in µm (IQR)	P value
Non-injectable HCs	6/30	113 (82 – 187)	-	158 (132 – 164)	-
Injectable HCs	24/30	121 (89 – 204)	0.951	97 (81 – 117)	**0.021**
DMPA	22/30	121 (87 – 205)	0,905	98 (71 – 129)	**0,026**
NET-EN	2/30	116 (91 – 141)	0,722	104 (92 – 116)	0,167

Abbreviations: IQR, interquartile range; DMPA- depot medroxyprogesterone acetate; NET-EN - norethisterone enanthate; Non-injectble HCs include (condom only (1/6), combined oral contraception (4/6) and intrauterine device (1/6)].

6.4.4 Impact of injectable HCs on genital tract cytokines concentrations

To better understand how injectable HC use influenced the genital tract innate immune environment and HIV-1 target cell recruitment, the concentrations of 42 cytokines, chemokines and growth factors were measured in matching CVLs from these acutely HIV-1 infected women (Table 6.5). CVL concentrations of MIP-1α ([beta-coefficient (β)=0.365, p=0.089], IL-13 (β=0.108, *p*=0.086), IL-4 (β=0.186, p=0.062) and IL-10 (β=0.193, p=0.088) were higher in women using injectable HCs compared to those not using injectable HCs, although these were not significant (Table 6.5). None of the other chemokines, growth factors and inflammatory cytokines were significantly different.

Table 6.5 Influence of injectable hormonal contraceptive use on cytokine concentrations in matching genital secretions

Functional groups	Cytokine	Injectable HC users (n=24)		Non-injectable HC users (n=6)		Multivariate#	
		Median (pg/ml)	IQR	Median (pg/ml)	IQR	Beta coefficient (SE)	*P* value
Pro-inflammatory	TNF-β	0.54	0.29 - 1.01	0.63	0.47 - 0.74	0.206 (0.363)	0.580
	IL-12p40	2.94	2.80 - 3.07	2.86	2.82 - 2.96	0.083 (0.140)	0.564
	IL-12p70	1.24	1.02 - 1.58	1.05	0.72 - 1.24	0.421 (0.306)	0.192
	IL-1α	1.83	1.44 - 2.44	1.51	1.38 – 2.00	0.546 (0.388)	0.183
	IL-18	2.67	1.80 - 3.36	2.79	2.44 - 2.96	-0.023 (0.701)	0.974
	IL-6	0.40	0.26 - 0.76	0.24	0.17 - 0.56	0.218 (0.384)	0.580
	TNF-α	0.65	0.49 - 0.86	0.54	0.23 - 0.65	0.349 (0.223)	0.141
	IL-1β	1.39	0.83 - 1.92	1.00	0.54 - 1.28	0.545 (0.511)	0.305
	TRAIL	1.50	1.01 - 2.24	1.69	0.59 - 1.89	1.470 (1.045)	0.183
	MIF	2.72	1.56 - 3.38	2.88	1.53 - 3.71	0.740 (0.858)	0.404
Chemokine	Eotaxin	0.88	0.85 - 0.96	0.85	0.85 - 0.86	0.102 (0.063)	0.129
	MCP-1	1.29	1.22 - 1.41	1.29	1.18 - 1.44	-0.018 (0.122)	0.886
	MIG	3.00	2.72 - 3.35	2.86	2.32 - 3.16	0.295 (0.430)	0.505
	CTACK	1.66	1.43 - 2.06	1.78	1.56 - 1.85	0.115 (0.297)	0.703
	MIP-1α	0.16	0.16 - 0.24	0.19	0.10 - 0.35	0.365 (0.199)	0.089
	MCP-3	1.64	1.54 - 1.95	1.74	1.49 - 1.82	0.149 (0.232)	0.531
	IP-10	2.52	2.14 - 3.06	2.58	2.02 - 3.02	0.328 (0.478)	0.504
	IL-16	2.04	1.70 - 2.42	2.14	1.68 - 2.19	0.348 (0.360)	0.351
	GRO	2.58	1.57 - 3.01	2.36	1.90 - 2.97	0.252 (0.535)	0.644
	MIP-1β	0.77	0.61 - 1.07	0.59	0.49 - 1.32	0.024 (0.259)	0.927
	IL-8	2.43	1.87 - 3.02	2.14	1.65 - 2.73	0.765 (0.648)	0.258

	RANTES	1.18	1.01 - 1.53	1.08	0.96 - 1.39	0.106 (0.285)	0.716
Innate	IFN-α2	1.52	1.25 - 1.81	1.61	1.42 - 1.678	0.085 (0.245)	0.734
Hematopoietic	IL-9	0.60	0.60 - 0.69	0.60	0.61 - 0.64	0.127 (0.075)	0.114
	G-CSF	1.71	1.42 - 2.58	1.55	1.17 - 2.00	0.082 (0.485)	0.867
	GM-CSF	2.12	2.08 - 2.15	2.14	2.08 - 2.17	0.006 (0.027)	0.837
	M-CSF	2.07	1.71 - 2.20	1.69	1.62 - 2.07	0.372 (0.304)	0.242
	IL-7	0.075	0.02 - 0.18	0.09	0.02 - 0.16	0.143 (0.125)	0.271
	IL-3	2.40	2.16 - 2.74	2.44	2.36 - 2.52	0.080 (0.246)	0.750
Growth Factors	HGF	1.79	1.43 - 2.55	1.80	1.61 - 2.09	0.244 (0.487)	0.624
	LIF	1.35	1.00 - 1.89	1.49	1.20 - 1.59	0.163 (0.384)	0.678
	SCF	1.21	0.84 - 1.51	1.12	0.94 - 1.24	0.314 (0.326)	0.354
	SCGF- β	2.52	2.13 - 3.00	2.60	2.41 - 2.72	0.291 (0.461)	0.538
	SDF-1α	2.27	0.79 - 2.67	2.35	1.77 - 2.48	0.342 (0.720)	0.642
	B-NGF	0.18	0.18 - 0.78	0.30	0.08 - 0.45	0.220 (0.563)	0.702
	VEGF	2.20	2.03 - 2.60	2.08	1.80 - 2.23	0.378 (0.339)	0.284
	PDGF-AB/BB	0.92	0.84 - 1.26	0.64	0.52 - 1.00	0.305 (0.247)	0.238
	Basic FGF	1.29	1.25 - 1.37	1.33	1.21- 1.45	0.016 (0.123)	0.895
Adaptive	IL-15	1.32	1.32 - 1.32	1.32	1.32 - 1.34	0.002 (0.013)	0.889
	IL-5	0.05	0.05 - 0.05	0.12	0.24 - 0.84	0.206 (0.149)	0.190
	IL-17	1.46	1.46 - 1.47	1.46	1.46- 1.46	0.125 (0.089)	0.185
	IFN-γ	1.74	1.46 - 1.91	1.74	1.57 - 1.74	0.177 (0.198)	0.388
	IL-2	1.20	1.20 - 1.20	1.20	1.20 - 1.20	0.018 (0.011)	0.142
	IL-4	0.05	0.05 - 0.05	0.12	0.04 – 0.28	0.186 (0.091)	0.061
	IL-13	0.47	0.47 - 0.57	0.47	0.47 - 0.47	0.108 (0.058)	0.086
	sIL-2Rα	1.95	1.64 - 2.37	2.01	1.77- 2.14	0.159 (0.322)	0.630
Anti-inflammatory	IL-1Ra	3.90	3.62 - 4.24	3.91	3.83 - 4.03	0.254 (0.439)	0.573
	IL-10	0.98	0.89 - 1.14	1.03	0.92- 1.10	0.193 (0.105)	0.085

SE=standard error, CI = confidence interval. #Multivariate analysis adjusted for age, condom use, study arm, marital status, plasma viral load, any STIs and BV

6.4.5 Relationship between genital cytokine concentrations and target cell densities

Of the 42 growth factors and cytokines measured, 6/42 (18.4%) were associated with higher frequencies of CD68+ macrophages, after adjusting for age, marital status, condom use, injectable hormonal contraceptive use, study arm, any STI, BV and plasma viral load (Figure 6.5). These included the chemokines RANTES (β=0.779, p=0.024), MCP-1 (β=0.453, p=0.041), and IP-10 (β=0.568, p=0.042); growth factors IL-7 (β=1.332, *p*=0.018), and IL-9 (β=0.336, p=0.015); and the adaptive cytokine IL-17 (β=1.058, p=0.007). In addition, eotaxin (β=0.049, p=0.05); PDGF-AA/BB (β=0.593, p=0.05) and GRO (β=-0.223, p=0.06) concentrations tended to be associated with increased frequencies of CD68+ macrophages, although weakly. No association was found between any of the CVL cytokines measured and vaginal CD4+ T cell densities (Figure 6.5).

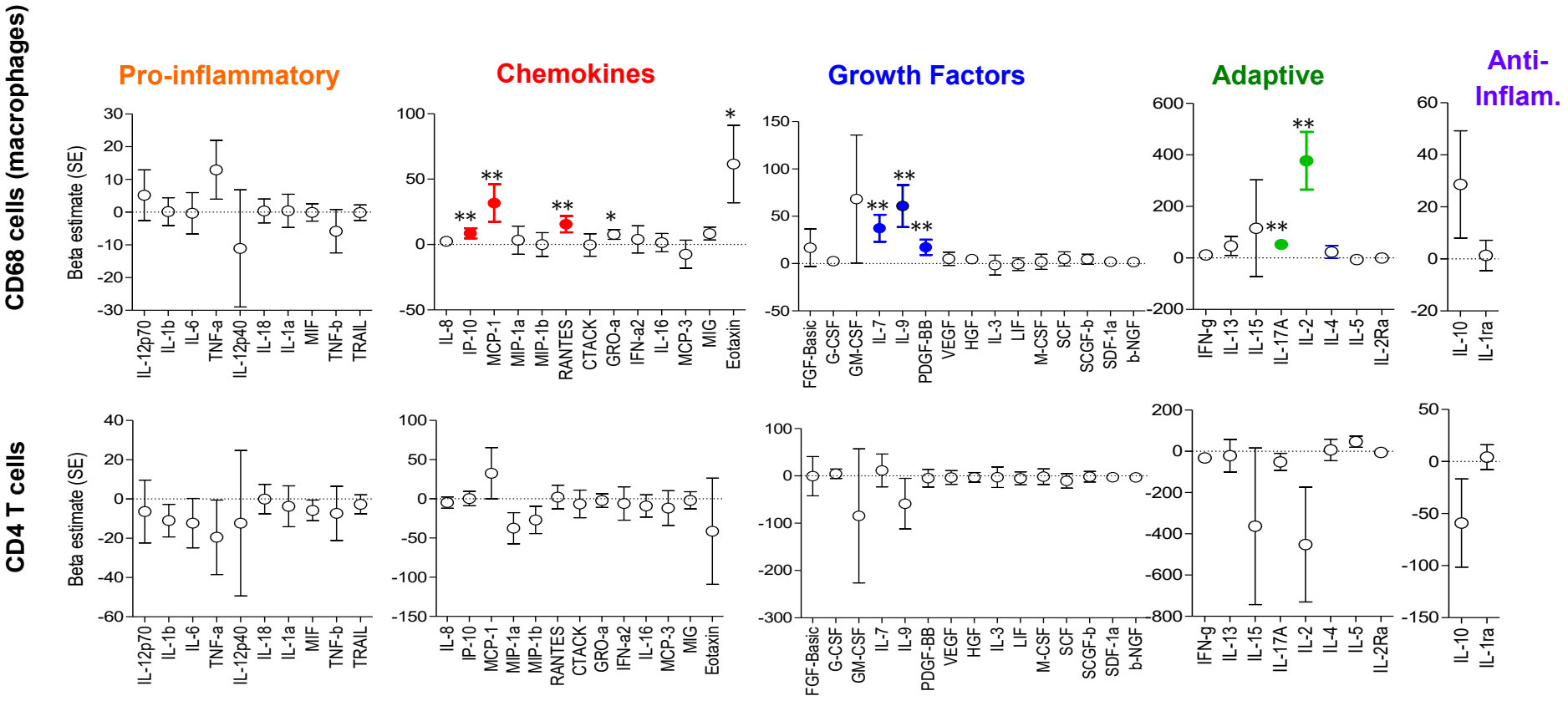


Figure 6.5 Relationship between HIV-1 target cell densties in vaginal biopsies during acute HIV-1 infection and genital cytokine concentrations. Dotted line indicates the effect of cytokine to target cells (for example: for every 1 log increase in RANTES, CD68+ cells in the vaginal biopsy increases by 15 cell/mm^2). Colour and ** indicates significant interaction between target cells and cytokine concentration with $p<0.05$ while colour and * indicates interaction between target cells and cytokine concentration with $p<0.10$.

6.4.6 Genital cytokine concentrations and the depth of target cells

The relationship between cytokines and the depth of target cells to the vaginal lumen was assessed (Figure 6.6). Counterintuitively, an inverse relationship was found between the depth of CD68+ macrophages in biopsy tissues and CVL concentrations of certain cytokines, including growth factors β-NGF (β=-0.809, p=0.043), SCGF-β (β= -0.652, p=0.048), and LIF (β= -0.540, p=0.049); pro-infammatory cytokine IL-18 (β=-1.105, p=0.017); and chemokine IFN-α2 (β=-0.351, p=0.041), after adjusting for age, marital status, condom use, injectable hormonal contraceptive use, study arm, any STI, BV, CD4+ and CD68+ cell density, epithelial thickness and plasma viral load. Furthermore, although not significant, CVL concentrations of IL-1β (β=-0.692, p=0.051); IL-6 (β=-0.453, p=0.069); CTACK (β=-0.394, p=0.068); GRO (β=-0.696, p=0.066); HGF (β=-0.624, p=0.077); IL-2Rα (β=-0.413, p=0.079); SDF-1α (β=-0.908, p=0.085) and TNF-β (β=-0.438, p=0.096) were negatively associated with the depth of CD68+ macrophages. This analysis suggests that CD68+ macrophages are further away from the vaginal lumen in women with the highest lumenal concentrations of cytokines.

In contrast to this finding for CD68+ cells, the depth of CD4+ T cells in the tissues were positively associated with CVL cytokine concentrations: including IL-1β (β=0.712, p=0.047), IL-6 (β=0.635, p=0.017); and MIP-1α (β=0.293, p=0.033), after adjusting for age, marital status, condom use, injectable hormonal contraceptive use, study arm, any STI, BV, CD4+ and CD68+ cell density, epithelial thickness and plasma viral load (Figure 6.6). Similarly, although not significantly, a positive association was found between CVL concentrations of G-CSF (β=0.662, p=0.061); IL-8 (β=0.817, p=0.090) and GRO (β=0.699, p=0.079) and CD4+ T cell depth in biopsies. Unlike CD68+ cells, this finding suggests that CD4+ cells are

closer to the vaginal lumen in women with the highest vaginal lumen concentrations of pro-inflammaorty cytokines.

6.4.7 Factors associated with recruitment of HIV-1 target cells in vaginal biopsies

In order to evaluate other factors that influenced the density of HIV-1 target cells in vaginal biopsies during acute HIV-1 infection, age, marital status, CD4 counts, plasma viral loads, any STIs and BV were considered as drivers of CD4+ and CD68+ target cell density. An inverse relationship was observed between CD68+ cells and acute infection plasma viral loads (Table 6.6). For every 1 Log increase in plasma viral loads, CD68+ cell density in vaginal tissue decreased 5 cells/μm, after adjusting for age, study arm, any STI or BV ($p=0.048$). There was no difference between other factors (age, study arm, any STI or BV) and CD68+ cell density. No associations were found between these factors and CD4+ vaginal cell density.

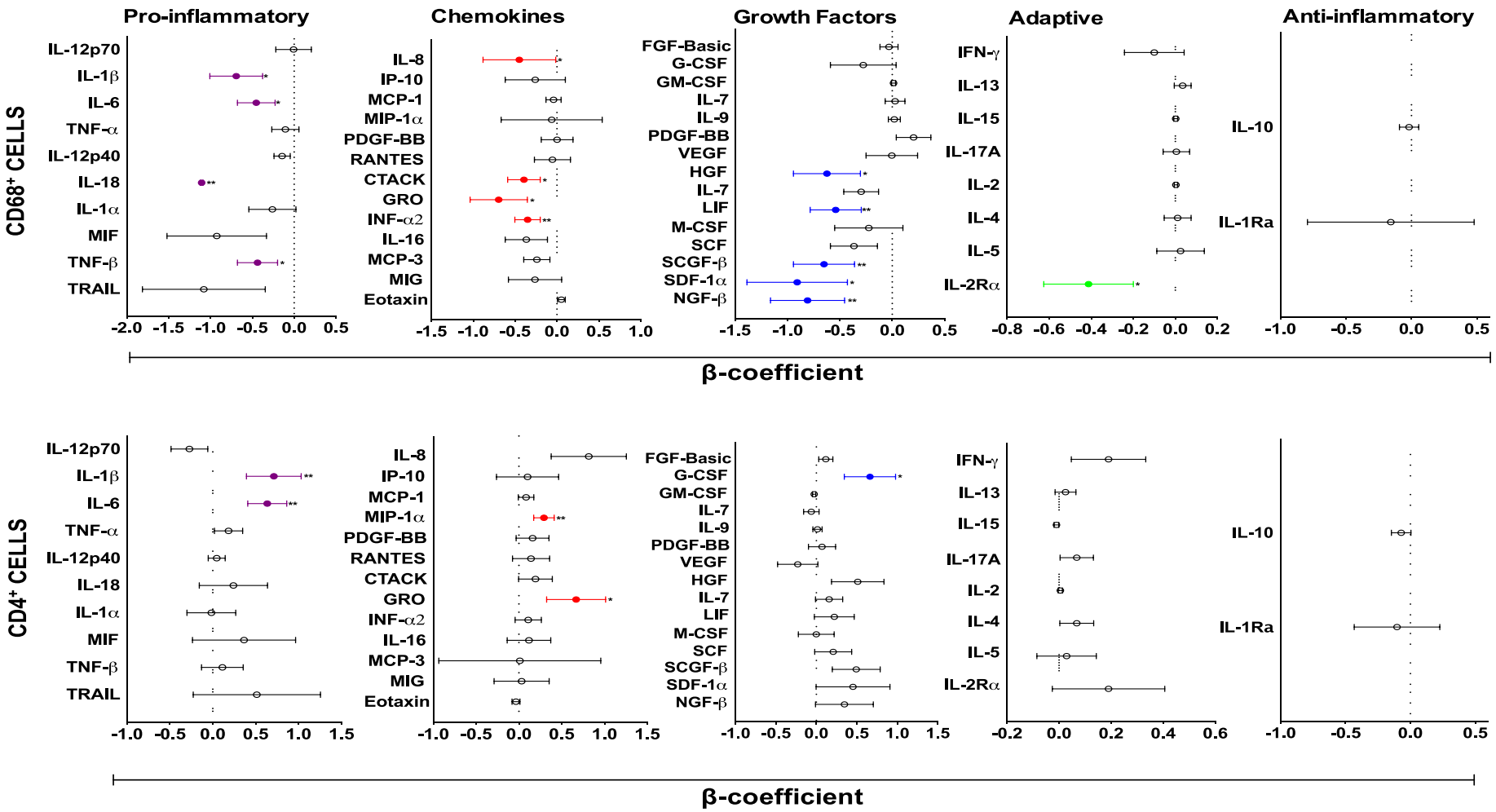


Figure 6.6 Relationship between HIV-1 target cell depth in vaginal biopsies during acute HIV-1 infection and genital cytokine concentrations. Dotted line indicates the effect of cytokine to target cells depth. Colour and ** indicates significant interaction between target cells and cytokine concentration with $p<0.05$ while colour and * indicates interaction between target cells and cytokine concentration with $p<0.10$.

Table 6.6 Factors associated with CD4 and CD68 cell density in vaginal biopsies

Variable	CD4+ T cells				CD68+ macrophages			
	Unadjusted Beta estimate (SE)	p-value	Adjusted Beta estimate (SE)	p-value	Unadjusted Beta estimate (SE)	p-value	Adjusted Beta estimate (SE)	p-value
Age (per 1 year increase)	1.467 (1.067)	0.181	1.542 (1.100)	0.178	-0.287 (0.418)	0.499	-0.570 (0.453)	0.225
Marital status Single vs stable partner	12.891 (12.004)	0.293	7.252 (12.979)	0.583	5.818 (4.545)	0.212	5.423 (5.349)	0.324
Condom use at last sex act No vs Yes	-0.357 (11.577)	0.976	10.452 (12.867)	0.427	3.670 (4.363)	0.408	-1.567 (5.303)	0.771
004 arm Placebo vs Tenofovir	-35.156 (14.850)	0.026	-34.054 (16.855)	0.059	2.640 (6.233)	0.675	-2.369 (6.946)	0.737
Plasma viral load (per 1 log increase)	6.410 (5.504)	0.255	7.437 (6.225)	0.248	-3.202 (2.063)	0.133	-5.434 (2.565)	**0.048**
Any STI	21.342 (11.120)	0.066	10.628 (13.538)	0.443	4.820 (4.337)	0.277	8.576 (5.579)	0.142
BV (Nugent score 7-10)	10.100 (11.672)	0.395	5.332 (11.175)	0.639	0.927 (4.414)	0.835	-0.493 (4.605)	0.916

6.4.8 Influence of injectable HC use on rate of HIV-1 disease progression

Furthermore, the rationale for investigating the influence of vaginal target cell density on rate of progress was that this high influx of target cells in the vaginal epithelium noted in this study would potentially result in higher seeding of an individual during acute infection with HIV and more rapid spread to systemic circulation, potentially also associated with more severe loss of CD4 in women using injectable HCs. In support of this hypothesis, previous work from our group has suggested that acute infection CVL cytokine levels predicted worse CD4 T cell loss during acute infection (Bebell et al., 2008) and more severe CD4 loss over the first year in addition to higher viral loads at set-point (Roberts et al., 2012). These previous studies did not directly assess target cell density in vaginal tissues. In this Chapter, we assessed the influence of injectable HC use on the rate of HIV-1 disease progression, disease progression was measured by plasma viral loads at 12 months post-infection and blood CD4 decline <350 cell/µl after 6 months post infection (at two consecutive visits). Injectable HC users had comparable plasma viral loads at 12 months post-infection to non-injectable HC users [median Log RNA copies/ml 4.34 (IQR 2.72-4.79) and 3.55 (IQR 3.16-4.49), respectively, p=0.7558; adjusted for age, injectables, condom use, study arm, marital status, any STIs or BV. Only 2/30 acutely HIV-1 infected women had detectable HIV-1 in their genital secretions (CVL) so analysis of vaginal HIV-1 target cell density and HIV-1 genital shedding was not possible in this study.

Of the 30 acutely HIV-1 infected women with vaginal biopsies imaged in this study, 10/30 (33.3%) progressed to CD4 < 350 cells/µl within 2 years of HIV-1 infection (considered rapid disease progressors), of which 7/10 were DMPA users 3/10 were non-progressors. No significant association was found between injectable HC use and these markers of disease

progression, after adjusting for age, marital status, condom use, study arm assignment, any STI, BV, plasma viral load.

6.4.9 Influence of vaginal target cell density on rate of HIV-1 disease progression

To assess the influence of vaginal target cell density on the relative rate of HIV-1 disease progression; disease progression was defined as having two consecutive CD4 counts < 350 cells/µl at any time point after 6 months post infection. Of the 30 acutely HIV-1 infected women with vaginal biopsies imaged in this study, 11/30 reached this endpoint, at a median of 10 months post infection (IQR, 6 - 17 months). All those who reached this endpoint did so within 24 months of HIV-1 infection and were classified as rapid disease progressors. However, there was no significant association found between vaginal target cell density during acute HIV-1 infection on rate of HIV-1 disease progression, after adjusting for age, marital status, condom use, injectable hormonal contraception, study arm assignment, any STI, BV, plasma viral load (Table 6.7).

Table 6.7 HIV-1 target cell density and rate of HIV-1 disease progression

	Unadjusted HR (95% CI)	**p-value**	**Adjusted HR (95% CI)**	**p-value**
CD4+ vaginal density	0.98 (0.96-1.01)	0.233	0.97 (0.92-1.03)	0.298
CD68+ vaginal density	1.03 (0.97-1.08)	0.345	1.07 (0.99-1.15)	0.101

Abbreviations: IQR, interquartile range; adjusted for Age, Injectables, condom use, study arm, marital status, plasma viral load, any STIs and BV

6.5 Discussion

Previous studies have suggested that injectable HC use might facilitate contact between cervico-vaginal mucosal target cells and HIV-1 by reducing the integrity and/or thickness of vaginal epithelium and density of tight junction proteins (Chandra et al., 2012; Ildgruben et al., 2003; Miller et al., 2000; Wieser et al., 2001; Wira et al., 2011a). In this study comparing vaginal epithelial thickness in acutely HIV-1 infected women using DMPA, Net-EN or non-HCs, no difference was observed between those using injectable HCs and non-HC users in vaginal epithelial thickness. Injectable HC use may also alter the inflammatory and/or chemotactic environment of the genital mucosa (Ildgruben et al., 2003; Miller et al., 2000; Wieser et al., 2001; Wira et al., 2011b; Wira and Veronese, 2011) and this may indirectly increase the recruitment of HIV-1 susceptible immune cells to the mucosa. Vaginal biopsies from acutely HIV-1 infected women using injectable HCs had increased frequencies of genital CD4+ T cells compared to non-injectable HC users. Unlike HIV-1 negative women discussed in Chapter 3, injectable HC use in acutely HIV-1 infected women was not associated with lower concentrations of cytokines or growth factors in genital secretions. However, several chemokines, hematopoietic and adaptive cytokines in CVLs during acute infection were associated with increased frequencies of vaginal tissue CD68+ macrophages, but not CD4+ cells. Those women with the highest plasma viral loads during acute infection had the lowest numbers of CD68+ cells in vaginal tissue.

Studies have suggested that the thickness of the stratified squamous epithelium is altered by DMPA use (Smith et al., 2000; Smith et al., 2004; Trunova et al., 2006) and hormonal fluctuations during the menstrual cycle (King, 1983; Owen, 1975;

Poonia et al., 2006; Veazey et al., 2012). In this study, epithelial thickness was similar in women using injectable HCs compare to non-injectable HC users. This study did find a difference in epithelial thickness measurement depending on the method used to calculate thickness, with the automated method being adopted for all subsequent analysis because of its ability to make thousands of measurements in an unbiased way. In agreement with this observation about measurement bias, Chandra and colleagues observed up to a 34% inter-observer variability in measurements using different methods of measurement (Chandra et al., 2013). They have suggested that the conflicting results reported in the literature to date on epithelial thickness following HC use may reflect differences in the method of measurement, observer bias, timing of tissue sampling, pharmacokinetics, and inter-individual variability in the biological response to DMPA. Through automated measurements, the whole stratified squamous epithelium including sinuous patterning of the epithelial rete pegs that extend into the dermal papillae was included in all measurements. A recent study suggest that a non-viable stratum corneum from the viable stratum malpighii should be measured instead, to exclude the variance that may occur from the epithelial rete pegs (Tjernlund et al., 2015).

Studies have suggested that DMPA may influence the frequency of immune cells in the vaginal mucosa (Chandra et al. 2012; Ildgruben et al. 2003; Miller et al. 2000). In this Chapter, women using injectable HCs had increased numbers of CD4+ T cells in their vaginal stratified epithelium compared to non-HC users. In HIV negative women, previous studies found no difference in numbers of CD4+ T cells in the stratified epithelium post DMPA treatment (Chandra et al., 2013; Mitchell et al.,

2014). The increased numbers of CD4+ T cells observed in the current study may be due to the fact that these women were already HIV-1 infected at the time of biopsy collection. CD68+ cells in women who use injectable HCs were not significantly different from non-injectable HC users. Density of vaginal immune cells did not predict faster disease progression rates.

Previous studies suggested that blood HIV-1 RNA concentrations positively predicted risk for heterosexual transmision of HIV-1 (Mayaux et al., 1997; Mock et al., 1999; Quinn et al., 2000). Baeten et al. (2007) suggested that DMPA use influenced how infectious an HIV-1 infected women was to her sexual partner as well as the rate at which she progressed during her clinical course of HIV-1 infection (Baeten et al., 2007b). In this study, women using DMPA did not have higher viral load set-points or more severe CD4 decline over 12 months than women using non-HCs, suggesting that the rate of disease progression was similar.

Acutely HIV-1 infected women in this study who had the higher concentrations of pro-inflammatory cytokines RANTES, MCP-1, IP-10, and IL-17; and adaptive/hematopoetic cytokines IL-9 and IL-7 in CVLs also had the highest numbers of CD68+ macrophages in the vaginal stratified epithelium. RANTES is part of the β-chemokine family, upregulated during inflammation, and responsible for the recruitment of lymphocytes (including basophils, eosinophils, natural killer cells and monocytes) to the site of infection (Baggiolini et al., 1997). RANTES, like MCP-1, has variable affinities for its receptors CCR1, CCR2, CCR3, CCR4 and CCR5, which are expressed on the surface of mature macrophages (Baggiolini et al., 1997; Decrion

et al., 2005; Kaufmann et al., 2001). In addition, MCP-1 promotes immune cell activation and recruitment (Decrion et al., 2005). IP-10 is a biomarker for antiviral immune responses (Luster et al., 1985), while IL-17 plays a vital role in pathogen clearance and mediates pro-inflammatory responses by increasing the production of several other cytokines (Freel et al., 2010). This suggests that these cytokines have a potential to increase the risk of HIV-1 acquisition, promoting activation and recruitment of HIV-1 target cells to the genital mucosa.

Although CD68+ macrophage density did not change with HC use, this study also found that CD68+ macrophage density in vaginal biopsies decreased by 5 cell/mm^2 of tissue with every 1-Log increase in plasma viral load. Like CD4+ T cell and DCs, macrophages are the first immune cells to fight the virus at mucosal surface and may also be infected. Macrophages produce cytokines that recruit CD4+ T cell to the site of infection and support viral pathogenesis by increasing the number of primary target cells available for HIV-1 replication during acute HIV-1 infection (Cicala et al., 2011; Koppensteiner et al., 2012). In this study, the density of CD4+ target cells in vaginal tissue did not correlate with the density of CD68+ macrophages. In tissues, macrophages are long-lived reservoirs of HIV-1 and contibute to the inability to achieve complete HIV-1 clearance during acute infection (Galiwango et al., 2012; Wu, 2011). These findings support evidence that vaginal macrophages are available to become productively infected during early HIV-1 infection.

Genital tract cytokine concentrations did not predict vaginal CD4+ T cell densities. Previous studies have shown that pro-inflammatory cytokines in the genital tract

during acute HIV-1 infection were associated with decreased CD4+ T cell counts in blood and increased viral loads during both acute infection (Bebell et al., 2009) and at 12 months post-infection (Roberts et al., 2012). In this study, CD4+ T cells in the stratified squamous epithelium of the vagina from acute infection tended to be negatively associated with pro-inflammatory cytokine concentrations (including IL-12p70, IL-1β, IL-6, TNF-α, IL-12p40, IL-18, IL-1α, MIF, TNF- β and TRAIL). Plasma viral load was also not associated with vaginal CD4+ T cell depletion.

A limitation of this study is that HIV-1 target cells and epithelial thickness were measured in tissue available from women after they seroconverted only, who were predominantly injectable HC users, and no control groups including HIV-1 infected women from other low risk communities or women who remained uninfected from the same CAPRISA 004 communities were available for this study. This study is also relatively small.

In conclusion, injectable HC users had increased frequencies of CD4+ T cells in their vaginal stratified epithelium than in women not using injectables. However, injectable HC use was not associated with thinning of the vaginal epithelial barrier or faster HIV-1 disease progression. Although there was no injectable use effect, the density of CD68+ macrophages in cervicovaginal tissue correlated with a broad panel of mucosal cytokines and inversely correlated with plasma viral loads during acute HIV-1 infection. This study provides valuable insight into possible underlying mechanisms by which genital inflammation may increase HIV-1 risk and subsequent clinical phenotypes during HIV-1 disease course, such as viral set point.

CHAPTER 7

Discussion

CHAPTER 7
Discussion

The impact of injectable HCs on HIV-1 acquisition and transmission is a major public health concern, especially in sub-Saharan Africa, where injectable HCs is the most popular HC choice in women, and HIV-1 prevalence and incidence rates especially in young women remains high (Abdool Karim et al., 2010, Van Damme et al., 2012). This unresolved dilemma has millions of young women having to choose between reliable fertility control methods and the possibility of increased HIV-1 risk and less effective contraception with possible reduced HIV-1 risk. There are several scientific and ethical challenges in resolving this dilemma through randomized controlled trials including that of removing user method choice for fertility control options. Understanding biological and immunological effects of hormonal contraceptive use could provide information to move forward with this dilemma. If biological mechanisms for DMPA-induced HIV-1 risk were clearly demonstrable, more informed policy measures could be implemented in terms of balancing fertility control and HIV-1 risk reduction needs of women in sub-Saharan Africa. The aims of this dissertation were to investigate the biological effects of injectable HC use on the innate environment in the genital tract in HIV-1 negative women at high risk of HIV-1 infection, and HIV-1 infected women during acute HIV-1 infection. Chapter 3 demonstrated that injectable HC use down-modulates expression of specific chemokines (eotaxin, MCP-1, MDC), adaptive cytokines (IL-15), growth factors (PDGF-AA) and an inhibitor of metalloproteinase (TIMP-2), while the pro-inflammatory cytokine IL-12p40 and chemokine fractalkine were less likely to be detected in CVL collected from women at high risk for HIV-1 infection. These findings suggest that the immunosuppressive effects of injectable HCs within the

female genital tract could modulate susceptibility to HIV-1 acquisition. The presence of semen was further found to suppress some of these markers (IL-12p70, sIL-2Rα and IL-2) (Chapter 4), suggesting that exposure to semen in the female genital mucosa may promote an environment conducive to facilitating male-female HIV-1 acquisition.

Chapter 5 studied the effects of exogenous DMPA or progesterone on activation and cytokine production by innate monocyte and DC subsets. This included *ex vivo* experiments in PMBCs from women using DMPA and *in vitro* experiments in whole blood stimulated with exogenous hormones or DMPA. The use of DMPA or NET-EN had no effect on numbers of circulating monocytes. However, DMPA treatment decreased the capacity of mDCs to produce TNF-α and IL-6 in response to TLR stimulation. These findings suggest that the impairment in the ability of DCs to produce these cytokines might lead to reduced immune response against invading pathogens and therefore contribute to the observed increased in HIV-1 acquisition in DMPA users.

During acute and early HIV-1 infection, in studies using vaginal biopsies (Chapter 6), vaginal epithelial thickness was found to be similar in women using injectable HC compared to non-injectable HC users. However, the frequency of CD4+ T cells in the vaginal squamous epithelium of injectable HC users was significantly higher than non-injectable HC users. Injectable HC use was not associated with higher genital HIV-1 concentrations or more rapid disease progression. The findings in Chapter 6 suggest that modulation of cytokine and epithelial barrier factors in the genital tract

rather than epithelial thinning might underly altered HIV-1 acquisition risk in DMPA users.

7.1 Injectable HCs modulate soluble genital immune mediators

Genital tract epithelial and immune cells confer protection against pathogens by secreting a range of antimicrobial peptides, cytokines and chemokines that have inhibitory effects against HIV-1 and other STIs, including recruitment and activation of immune target cells (Murphy et al., 2014). DMPA use may increase a woman's susceptibility to HIV-1 infection by modulating soluble immune mediators that increase recruitment of HIV-1 susceptible immune cells to the mucosa (Ildgruben et al., 2003, Miller et al., 2000, Wieser et al., 2001, Wira et al., 2011, Wira and Veronese, 2011). In non-human primates, the high doses of DMPA that are typically used have immunosuppressive properties both systemically and in the genital tract (Abel et al., 2004, Genesca et al., 2007, Gillgrass et al., 2003). The effects of injectable HC use on concentrations of soluble inflammatory cytokines were investigated in Chapter 3. HIV-1 negative women using injectable HCs had lower concentrations of several chemokines (eotaxin, MCP-1, MDC), the adaptive cytokine IL-15, growth factor PDGF-AA, and TIMP-2 (involved in tissue remodeling) in secretions from the lower female genital tract than women not using HCs, confirming that injectable HCs influence the genital immune environment that may reduce women's genital mucosal barrier function. Decreased chemokine concentrations in women using injectable HCs might influence the trafficking of immune cells to the female genital tract.

In support of decreased immune defenses, previous studies have reported an association between DMPA use and the increased prevalence of cervical candidiasis, and infections with *C. trachomatis*, *N. gonorrhoeae*, and *M. genitalium* (Wand and Ramjee, 2012, Baeten et al., 2001, Morrison et al., 2004). The increased prevalence of common STIs may influence risk for HIV-1 acquisition by altering inflammatory responses or by physically disrupting the mucosal barrier (Baeten et al., 2007a). In Chapter 3, the prevalence of STIs were similar in women using injectable HCs to those not using HCs, although women using injectable HCs with gonorrhea, chlamydia and mycoplasma infections had increased TNF-β, IL-5, IL-7, TIMP-4, IL-12p40 and Fractalkine concentrations compared to women not using HCs with these infections. Similarly, women infected with *T. vaginalis* who were using injectable HCs had higher CVL Flt3L concentrations than women not using HCs. The pro-inflammatory cytokine IL-12p40 promotes production of IFN-γ in $CD8^+$ T cells and DC migration to the site of infection, while TNF-β is a key mediator of epithelial cell inflammatory responses (Svanborg et al., 1999). From these findings, it was assumed that increased IL-12p40 and TNF-β, found normally during mucosal inflammation, are crucial features of mucosal immune defense against infections such as *C. trachomatis.* IL-7, also influenced by DMPA use in women with an STI, regulates survival, proliferation and repertoire diversity of memory T cells and facilitating better CD4 reconstitution in HIV-1 infected patients receiving IL-7 therapy (Mackall et al., 2011). These characteristics of IL-7 potentially place DMPA users, infected with STIs, at a higher risk of HIV-1 infection by acting on CD4 cell survival and proliferation to provide susceptible targets for infection. Despite suppressed genital cytokine responses in injectable HC users, higher inflammatory and adaptive cytokine responses in the genital tracts of women using injectable HCs who have certain STIs

may place them at greater risk of HIV-1 infection compared to women not using HCs. A limitation of Chapter 3 and 4, in which women from CAPRISA002 were included, was that it was not possible to differentiate DMPA from NET-EN users in the injectable HC group as this information was not collected. In addition, we did not monitor the stage of the menstrual cycle in this cohort, nor collect longitudinal data on DMPA or NET-EN use prior sampling. Furthermore, this was a cross-sectional study so the direct effects of contraceptive initiation could not be addressed. Although several important possible confounders were accounted for in each analysis (such as condom use, sex work, marital status, BV and certain STIs), other factors [such as vaginal cleansing (douching), sexual practices (drying, lubricants) and STIs like HPV] were not assessed.

The effects of injectable HC use on concentrations soluble factors associated with genital epithelial repair and integrity were investigated, including growth factors (PDGF-AA, TGF-α, VEGF, PDGF-AB/BB, EGF, FGF-2), MMPs (MMP-1, MMP-2, MMP-7, MMP-9, MMP-10), and TIMPs (TIMP-1, TIMP-2, TIMP-3 and TIMP-4). Lower concentrations of PDGF-AA, reported to be involved in enhancing epithelial repair and restoring barrier function in the female genital tract (Werner and Grose, 2003), was observed in HIV-1 negative women using injectable HCs compared to women not using HCs. Growth factors have been shown to play an important role in restoring genital barrier function by stimulating mitosis, encouraging the spreading and migration of epithelial cells in airways following injury (Werner and Grose, 2003). As such, decreased expression of these factors could lead to a compromised epithelial barrier and reduced epithelial healing in women using injectable HCs. CVLs of women using injectable HCs had lower concentrations of the inhibitor of

MMP, TIMP-2, compared to those not using any HCs, although MMP concentrations were similar between groups (Chapter 3). Furthermore, women using injectable HCs who had gonorrhea, chlamydia and mycoplasma infections had higher TIMP-4 concentrations in CVL compared to those not using HCs who had these infections (Chapter 3). MMPs and their tissue inhibitors (TIMPs) are responsible for genital mucosal epithelial tissue degradation, particularly during menstruation when the endothelium of the uterus undergoes major remodeling (Birkedal-Hansen, 1995, Cawston, 1995, Lockwood and Schatz, 1996, Rodgers et al., 1994, Rodgers et al., 1993). Unlike TIMP-4 that binds to MMP-14 required for monocyte migration (Lockwood and Schatz, 1996), TIMP-2 inhibits activation of MMP-2, which is involved in epithelial tissue remodeling (Rodgers et al., 1993). Thus a decrease in TIMP-2 concentrations will alter the MMP:TIMP ratio. This could lead to enhanced overall activity of MMPs in the reproductive tract and consequently, a weakened epithelial barrier integrity, making the genital tract susceptible to pathogens. Estrogen has been shown to influence vaginal epithelial tight junction proteins and thereby impact on the integrity of the epithelium (Gorodeski, 2007). Modulation of TIMP-2 reported in Chapter 3 suggests that HCs may have an indirect effect on the epithelial barrier integrity in the genital tract, through the modulation of epithelial barrier factors. Future studies should include more epithelial repair markers to elucidate this mechanism.

7.2 Influence of semen on cytokine responses in the lower reproductive tract

Epidemiologic studies that have suggested an association between injectable HC use and increased risk for HIV-1 acquisition were not specifically designed to assess this

association. Some important methodological considerations, such as presence of semen markers which may confound studies of the mechanisms driving this risk, were not evaluated as a result (Polis and Curtis, 2013). Bias reporting of condom use and safe sexual behavior has been identified as shortcomings in several biomedical prevention studies (Turner and Miller, 1997; Zenilman et al., 1995). Previous studies have shown that semen contains high concentrations of anti-inflammatory cytokines (including TGF-β, IL-10, PGE2), inflammatory cytokines (including IL-8, IL-1β, IL-6), and activated immune cells (Olivier et al., 2014), each potentially capable of influencing the immune environment of the lower female genital tract during sexual intercourse (Denison et al., 1999, Robertson, 2005, Robertson et al., 2009, Sharkey et al., 2012a, Sharkey et al., 2007, Sharkey et al., 2012b).

In Chapter 4, the presence of semen in CVL from HIV-1 negative women included in Chapter 3 was investigated, by detection of Y-chromosome (as evidence of sex within 7-14 days) or PSA (as evidence of unprotected intercourse ≤48 hours of sampling). Concentrations of IL-12p70, IL-2 and IL-2Rα were lower in CVLs of women with Y-chromosome and/or PSA present than women negative for these markers. Lower concentrations of cytokines detected in this study may be directly due to immunosuppressive effects of semen; or dilution of vaginal secretions and cytokines by the approximately 3ml volume of semen introduced during ejaculation (ranging from 0.1-10ml; Sharkey et al., 2007). In contrast, concentrations of TIMP-1 and MMP-9 were significantly higher in CVLs from women who tested negative for semen compared to those testing positive. MMPs and TIMPs are responsible for epithelial tissue degradation and remodeling (Birkedal-Hansen, 1995, Cawston, 1995, Lockwood and Schatz, 1996, Rodgers et al., 1994, Rodgers et al., 1993). Results in

Chapter 4 suggest that the presence of semen may influence the cytokine profile in the female genital tract and should be taken into consideration when investigating immunological factors in this compartment.

The presence of semen products PSA and Y-chromosomes were assessed for their usefulness in objectively determining the accuracy of self-reported condom use in Chapter 4. About one fifth of the PSA positive and a third of Y-chromosome positive women, who reported that they had used a condom at their last sex act (Chapter 4), had detectable PSA and/or Y-chromosme in CVLs. This suggests either that condom use was over-reported (for PSA positive individuals) or that unprotected exposure to semen occurred days before the actual use of a condom [where Y chromosome remains stable in the female genital tract for 7-14 days, (Chomont et al., 2001b)]. Inconsistencies between reported condom use and detection of PSA/Y-chromosome in CVL may also be due to participants perceiving some topics as sensitive or the perceived fear of being non-compliant with barrier method use recommended during counselling sessions with study staff.

Semen markers were detected more frequently in women not using injectable HCs compared to those using injectable contraception, although this difference was not statistically significant (Chapter 4). This trend agrees with suggestions that injectable HC users may be behaviourally distinct compared to women not using any HC (Gray, 2012). For example, the presence of semen markers may be due to the fact that women who were not using injectable contraception were more likely to be married, have a stable partner, or were trying to conceive.

7.3 Impact of injectable HCs on innate cell activation and function

Injectable HC may contribute to HIV-1 risk by directly influencing chemotaxis, maturation or activation of mucosal HIV-1 target cells, including genital tract DCs (Huijbregts et al., 2013, Huijbregts et al., 2014, Kleynhans et al., 2013, Kleynhans et al., 2011). DCs are crucial in the early innate response because they recognize viral and bacterial infections through TLR-7/8 and TLR-9, respectively (Kawai and Akira, 2010, Schlaepfer et al., 2006). DMPA has been shown to inhibit TLR-9-induced IFN-α and TNF-α production in pDCs *in vitro* in mice (Hughes et al., 2008) and humans (Huijbregts et al., 2013). Furthermore, *in vitro* studies have also shown that higher concentrations of TNF-α inhibited entry of HIV-1 into primary target cells, CD4 T cells and macrophages and replication in productively infected cells (Herbein et al., 1996, Lane et al., 1999). In Chapter 5, *in vitro* treatment of PBMCs with DMPA impaired the production of TNF-α by mDCs in response to the TLR-4 agonist LPS and the TLR-9 agonist CpG, while exogenously added progesterone and cortisol did not. In addition, DMPA also reduced the production of IL-6 by mDCs in response to LPS (Chapter 5), while cortisol and progesterone did not. IL-6 and TNF-α are responsible for maturation and migration of DCs to the lymph nodes; thereby potentially influencing the development of adaptive immunity more broadly (Banchereau and Steinman, 1998). The suppressive effect of DMPA on TNF-α and IL-6 production by mDCs, in response to LPS and CpG stimulation, may result in a blunted inflammatory response during early stage of HIV-1 infection, thereby favoring the propagation of a founder viral population over immune control. In addition, since DCs are the main antigen-presenting cell and produce cytokines that direct the maturation and differentiation of other parts of the acquired immune response (including B and T cells), this specific impact of DMPA on DC subsets is

likely to have a wide reaching impact on acquired immunity. Due to these non-specific and broad immunosuppressive effects of DMPA, it is plausible that inhibition of TNF-α production by DCs indirectly supports HIV-1 replication. Together, these findings also suggest that cortisol and progesterone do not exert similar immune suppressive effects of innate dendritic cells like DMPA does.

7.4 Effect of injectable HC use on HIV-1 disease progression

In a randomized control trial in HIV-1 infected women in Zambia, faster rates of progression were reported among those using injectable HCs compared to those using IUDs (Stringer et al., 2009). In contrast, several observational studies found that HC use in women did not influence their rate of HIV-1 disease progression in those who become infected (Baeten et al., 2007b, Phillips et al., 2013, Lavreys et al., 2004, Heffron et al., 2013, Polis et al., 2010, Richardson et al., 2007). In the final Chapter of this dissertation (Chapter 6), HIV-1 infected women who were using injectable HCs had a similar disease course as women not using HCs, with similar CD4 counts at 12 months post-infection and similar viral load trajectories during the first year of infection. Although this is a secondary analysis of data from the CAPRISA002 Acute Infection study (van Loggerenberg et al., 2008), no adverse effects of injectable HC use were noted in terms of HIV-1 disease progression. However, the CAPRISA002 cohort as a whole was characterized by high numbers of rapid progressors (Mlisana et al., 2014), so characteristics of this particular cohort may have confounded this analysis.

7.5 Injectable HC use and vaginal squamous epithelial thickness

Several non-human primate and human studies have reported that DMPA use reduces the thickness of the vaginal epithelial lining, and influences the density of intracellular junction proteins between epithelial cells, which has been proposed to facilitate more efficient contact between HIV-1 and potential mucosal HIV-1 target cells (Ildgruben et al., 2003, Miller et al., 2000, Wieser et al., 2001, Wira et al., 2011). In women, however, several studies found no difference in thickness of the vaginal epithelium in those using DMPA compared to those who were not (Chandra et al., 2013, Mitchell et al., 2014, Bahamondes et al., 2000, Mauck et al., 1999). Previous clinical studies reported vaginal epithelial thicknesses ranging from 175-1020 μm, approximately 25-31 epithelial cells layers in thickness (Bahamondes et al., 2000; Chandra et al., 2013; Mauck et al., 1999; Miller et al., 2000; Mitchell et al., 2014). In comparison, DMPA-treated macaques, given higher doses of DMPA per kg than humans (30mg/kg compared to stat dose of 150mg in humans), had only two to three vaginal epithelial cell layers separating lamina propria from the vaginal lumen compared to more than 25 cell layers in non-DMPA treated animals (Marx et al., 1996). In addition, dramatic atrophy of the vaginal epithelium following high-dose DMPA administrations was observed (Marx et al., 1996; Smith et al., 2000; Veazey et al., 2003). In Chapter 6, vaginal epithelial thickness from acutely HIV-1 infected women ranged from 279-413 μm, with a median of 356 μm, and this did not differ between women using injectable HCs and those who were not using injectable HCs.

Two different methods for measuring vaginal epithelial thickness were used in this study (manual and automated), which showed significant variability of the

measurements (Chapter 6). The manual counting method is outdated, subjective and does not take into account unevenness and variability in distances from the basal layer to the luminal surface (including epithelial rete pegs). In contrast, the automated method using IDL software includes measurements from the whole stratified squamous epithelium, including sinuous patterning of the epithelial rete pegs that extend into the dermal papillae, and does a multitude of serial and repeated measurements that scan the entire section (Carias et al., 2013; Tjernlund et al., 2015). This method is more objective and eliminates some bias inherent to manual counting. In addition to methodology used for measurements, other factors may contribute to variation in estimations of epithelial thickness, including timing in the menstrual cycle of tissue sampling (luteal versus follicular phase; which was not taken into account in this study), the pharmacokinetics of endogenous hormones, and biological heterogeneity among individuals in response to DMPA (Chandra et al., 2013). The different methods of measurement also make direct comparison between published studies challenging. Tjernlund et al. (2015) suggested measuring only from the non-viable stratum corneum to the viable stratum malpighii, instead of the whole stratified squamous epithelium, to exclude variance that may occur from the epithelial rete pegs. While Chapter 6 focused on tissue from women after HIV-1 infection, it would be interesting in future studies to include non-infected women.

7.5 Injectable HC use and vaginal HIV-1 target cell density and depth

In Chapter 6, higher numbers of CD4+ T cells were found in the stratified epithelium from vaginal biopsies of acutely HIV-1 infected women using injectable HCs had than in women using no or non-hormonal contraceptive methods (including IUDs). In

HIV-1 negative women, others found no difference in the numbers of CD4+ T cells in the genital stratified epithelium following DMPA treatment (Chandra et al., 2013, Mitchell et al., 2014). If these increased mucosal CD4+ T cell numbers within and just below the surface of the vaginal stratified epithelium that was seen in acute HIV-1 infection reflect pre-infection CD4 T cell density, it is plausible that this may have influenced their risk for HIV-1 acquisition. The median distance of CD4+ cells in the squamous epithelia from the vaginal lumen was 136 μm, ranging from 59 – 297 μm. According to Carias et al. (2013), HIV-1 virions migrate to depths of 50 μm or more within the epithelium to reach the underlying target cells, with 50 μm being the physical limit of depth measured using the field microscope (Carias et al., 2013). Importantly, increased HIV-1 target cell density during acute infection may increase the probability that these women transmit HIV-1 to their sexual partners, since there would be more target cells at the mucosa capable of releasing HIV-1 particles. However, only a few women in this study had detectable HIV-1 RNA in genital secretions, so this could not confirmed.

Previously, pro-inflammatory cytokines in CVL during acute HIV-1 infection were associated with worse disease status and clinical course, including decreased CD4+ T cell counts and increased viral loads during both acute infection (Bebell et al., 2009) and 12 months post-infection (Roberts et al., 2012). In this study, CD4+ T cells in the stratified squamous epithelium of the vagina from acute infection were negatively associated with several pro-inflammatory cytokines (including IL-12p70, IL-1β, IL-6, TNF-α, IL-12p40, IL-18, IL-1α, MIF, TNF- β and TRAIL), although not significantly after adjusting for multiple comparisons. In addition, the depth (proximity of these

cells to the lumen) of CD4+ T cells in vaginal tissues was significantly associated with elevated concentrations of pro-inflammatory cytokine IL-1β and IL-6; and the chemokine MIP-1α. Pro-inflammatory cytokines (IL-1β and IL-6) and chemokines (MIP-1α) play a central role in initiating and sustaining the inflammatory response by recruiting immune cells from circulation and stimulating their differentiation and activation (Charo and Ransohoff, 2006). This suggests that these cytokines have the potential to enhance viral replication by promoting activation and recruitment of CD4+ T cells to the genital mucosa.

Although CD68+ macrophage density was not influenced by HC use, acutely HIV-1 infected women in this study who had the higher concentrations of pro-inflammatory cytokines RANTES, MCP-1, IP-10, and IL-17; and adaptive/hematopoetic cytokines IL-9 and IL-7 in CVLs also had the highest numbers of CD68+ macrophages in the vaginal stratified epithelium (Chapter 6). RANTES is part of the β-chemokine family, up-regulated during inflammation, and responsible for the recruitment of lymphocytes to the site of infection, including basophils, eosinophils, natural killer cells and monocytes (Baggiolini et al., 1997). RANTES and MCP-1 have varying affinities for their cognate receptors, including CCR1, CCR2, CCR3, CCR4 and CCR5, which are expressed on the surface of mature macrophages (Baggiolini et al., 1997; Decrion et al., 2005; Kaufmann et al., 2001). In addition, MCP-1 promotes immune cell activation and recruitment (Decrion et al., 2005). IP-10 is a biomarker for antiviral immune responses (Luster et al., 1985), while IL-17 plays a vital role in pathogen clearance and mediates pro-inflammatory responses by increasing the production of several other cytokines (Freel et al., 2010). This suggests that these cytokines have the

potential to increase the risk of HIV-1 acquisition, through enhancing and stimulating activation and simultaneously recruiting various immune cells that become targets for HIV-1 infection in the genital mucosa.

Although CD68+ macrophage density was positively associated with CVL cytokine concentrations, the depth of CD68+ cells (proximity to the vaginal lumen) was counterintuitively negatively associated with CVL concentrations of several growth factors (including β-NGF, SCGF-β, and LIF), the pro-inflammatory cytokine IL-18, and the chemokine IFN-α2. IFN-α is an important inflammatory cytokine in anti-viral activity during acute HIV-1 infection and during late stage disease. It is expressed by monocytes upon viral infection, however, as monocytes differentiate into macrophages, they selectively lose their ability to produce IFN-α (Francis et al., 1996). IL-18 is an inflammatory cytokine that, in combination with IL-12, causes differentiation of monocytes into macrophages (Yoo et al., 2005). Furthermore, a group of hematopoietic growth factors also regulate differentiation and proliferation of monocytes into macrophages (Hassan et al., 1994). Therefore, it is possible that this association between lower CVL concentrations of inflammatory cytokines and growth factors with depth of CD68+ macrophages could reflect mucosal monocyte differentiation into macrophages, which then migrate out of the tissue towards local lymph nodes to present antigen.

In Chapter 6, CD68+ macrophages were found to be closer to the vaginal lumen in women using injectable HC compared to non-injectable HC users (median depth 97 µm, IQR 81 – 117 µm in injectable HC users; versus 158 µm, IQR 132 – 164 µm in

non-injectable HC users). CD68+ macrophage density in vaginal biopsies also decreased by 5 cell/mm^2 of tissue with every 1-Log increase in plasma viral load, possibly indicative of the impact of high viremia on activation induced macrophage apoptosis. Like CD4+ T cell and DCs, macrophages are the first immune cells to fight the virus at mucosal surface and may also be infected (Cicala et al., 2011; Koppensteiner et al., 2012). Macrophages produce chemotactic cytokines that recruit CD4+ T cell to the site of infection and support viral pathogenesis by increasing the number of these primary target cells available for HIV-1 replication during acute HIV-1 infection (Cicala et al., 2011; Koppensteiner et al., 2012). In tissues, macrophages are long-lived reservoirs of HIV-1, remain as foci of infection that constantly fuel infection and contribute to the inability to achieve complete HIV-1 clearance during acute infection (Galiwango et al., 2012; Wu, 2011).

7.6 Conclusion

In this dissertation, injectable HC use was associated with decreased concentrations of certain pro-inflammatory cytokines, chemokines, growth factors, adaptive cytokines and TIMP-2. *In vitro* evidence further confirmed that DMPA had immunosuppressive effects on DC function, in response to TLR-stimulation. During acute HIV-1 infection, injectable HC use was not associated with thinning of the vaginal epithelial barrier. However, injectable HC was associated with increased numbers of CD4+ T cells in the vaginal epithelium but not with faster HIV-1 disease progression. Despite increased CD4+ T cells in the vagina during acute HIV-1 infection, this dissertation found no evidence that DMPA increased infectiousness of women to their sexual partners, as indicated by genital HIV-1 shedding.

This dissertation provides insight into potential biological mechanisms by which injectable HC use impacts mucosal and systemic immune responses (Hel et al., 2010, Murphy et al., 2014). It is unclear whether the DMPA-mediated changes to systemic and mucosal immune mechanisms described in this dissertation would impact on the susceptibility to HIV-1 infection. However, in light of evidence that injectable HC may modulate HIV-1 risk, large-scale randomized clinical trials assessing the impact of progestin-derivatives (DMPA or NET-EN) on genital and systemic innate and adaptive immune environment, as well as STIs, are needed to further investigate the mechanism(s) by which DMPA or NET-EN might increase risk of HIV-1 infection in women.

7.7 Recommendations for future research

Although various biological mechanisms by which injectable HC modulate host immune responses were investigated in this dissertation, there is still an urgent need to expand this type of biological mechanistic research to inform on decisions around use of fertility control choices in women in high HIV disease burden countries. All observational and laboratory studies that showed DMPA might modulate risk to HIV-1 were secondary analyses of other trials not specifically designed to assess this association. It is imperative to find safe and effective alternatives to DMPA, alternatives that do not suppress the protective capabilities of the immune system at physiological levels. Future studies need to assess in detail potential immunomodulatory effects of other HCs (such as NET-EN, implants, levonorgestrel intra-uterine devices and COCs) on both the systemic and mucosal immune

environment, including inhibition of antiviral activity, epithelial structural integrity and immune cell function. These studies should investigate whether the rate of HIV-1 seroconversion, transmission and disease progression varies in women using these other HCs compared to women not using any HC while concurrently reducing confounding effects as HC choice is modified by age, marital status, and socioeconomic status (Morrison and Nanda, 2012).

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APPENDICES

Appendix 1: Publication derived from the doctoral research

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Lower concentrations of chemotactic cytokines and soluble innate factors in the lower female genital tract associated with the use of injectable hormonal contraceptive

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ABSTRACT

Progesterone-based injectable hormonal contraceptives (HCs) potentially modulate genital barrier integrity and regulate the innate immune environment in the female genital tract, thereby enhancing the risk of STIs or HIV infection. We investigated the effects of injectable HC use on concentrations of inflammatory cytokines and other soluble factors associated with genital epithelial repair and integrity. The concentrations of 42 inflammatory, regulatory, adaptive growth factors and hematopoietic cytokines, five matrix metalloproteinases (MMPs), and four tissue inhibitors of metalloproteinases (TIMPs) were measured in cervicovaginal lavages (CVLs) from 64 HIV-negative women using injectable HCs and 64 control women not using any HCs, in a matched case–control study. There were no differences between groups in the prevalence of bacterial vaginosis (BV; Nugent score ≥7), or common sexually transmitted infections (STIs). In multivariate analyses adjusting for condom use, sex work status, marital status, BV and STIs, median concentrations of chemokines (eotaxin, MCP-1, MDC), adaptive cytokines (IL-15), growth factors (PDGF-AA) and a metalloproteinase (TIMP-2) were significantly lower in CVLs from women using injectable HCs than controls. In addition, the pro-inflammatory cytokine IL-12p40 and the chemokine fractalkine were less likely to have detectable levels in women using injectable HCs compared with those not using HCs. We conclude that injectable HC use was broadly associated with an immunosuppressive female genital tract innate immune profile. While the relationship between injectable HC use and STI or HIV risk is yet to be resolved, our data suggest that the effects of injectable HCs were similar in STI-positive and STI-negative participants.

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1. Introduction

Internationally, hormonal contraceptives (HCs) are widely used by women to prevent unplanned pregnancies. In South Africa, more than half of women aged 15–49 years old use depot medroxyprogesterone acetate (DMPA) or norethisterone enanthate (Net-EN), with more than three times the number of women using DMPA than Net-EN (Sibeko et al., 2011; Department of Health, 2007). DMPA is an aqueous microcrystalline suspension (150 mg/ml) administered by intramuscular depot injection every three months, while NET-EN injection contains 200 mg/ml of norethisterone, which is effective for two months as contraception (Fraser and Weisberg, 1981). DMPA primarily provides contraceptive protection by suppressing natural cyclic fluctuations of female sex hormones, resulting in a hypo-estrogenic state (Jeppsson et al., 1982). In addition, DMPA induces atrophy of the endometrium by decreasing the glycogen content needed to provide energy for the development of the blastocyst after the morula has entered the uterine cavity (Hatcher et al., 2011; Mishell et al., 1968). Less is known about the endogenous effects of Net-EN.

High-dose DMPA use is common in the simian immunodeficiency virus (SIV) vaginal challenge models because it results in thinning of the vaginal epithelium, which enhances genital SIV infection (Abel et al., 2004; Wieser et al., 2001). The role of DMPA in increasing the risk of HIV infection is contentious. While some studies have reported such risks (Morrison et al., 2010; Ungchusak et al., 1996; Baeten et al., 2007; Kumwenda et al., 2008), others have found no association (Kleinschmidt et al., 2007; Reid et al., 2010). The impact of DMPA on genital epithelial barrier integrity is similarly contentious (Kiddugavu et al., 2003; Myer et al., 2007).

In addition to potentially increasing HIV acquisition risk, DMPA has been associated with an increased risk of *Chlamydia trachomatis* infection and a decreased risk of acquiring other sexually transmitted infections (STIs) such as *Trichomonas vaginalis* and bacterial vaginosis (BV) (Baeten et al., 2001; van de Wijgert et al., 2013).

It has been hypothesised that DMPA might increase HIV acquisition risks by changing the inflammatory or chemotactic environment of the genital mucosa so as to increase the recruitment of HIV-susceptible immune cells to the mucosa (Ildgruben et al., 2003; Miller et al., 2000; Wieser et al., 2001; Wira and Veronese, 2011). However, treatment of PBMC with DMPA has been shown to cause reduced production of several inflammatory and adaptive cytokines (Huijbregts et al., 2013). At the female genital mucosa, suppression of innate immune responses may influence susceptibility to infections. Moreover, matrix metalloproteinases (MMPs), which are required during normal reproductive processes (such as menstruation) for extracellular matrix degradation and tissue remodelling in the endometrial compartment of the upper genital tract (Lockwood and Schatz, 1996; Rodgers et al., 1993; Birkedal-Hansen, 1995), may influence epithelial barrier repair in the lower genital tract. MMPs are regulated by specific tissue inhibitors of metalloproteinases (TIMPs) (Fernandez-Catalan et al., 1998; Gomis-Ruth et al., 1997), which may similarly be involved in the maintenance of the lower reproductive tract barrier.

Defining the impact of injectable HCs on female genital tract innate immunity in relation to susceptibility to STIs or BV, will provide important insights into biological co-factors influencing HIV risk in women. The aim of this study was to compare concentrations of genital tract-soluble immune mediators (including cytokines, MMPs and TIMPs) between women using long-acting injectable HCs and women not using HCs, while accounting for BV and common STIs.

2. Materials and methods

2.1. Study design, participants and sample collection

Our study included 64 HIV-uninfected women using injectable HCs (DMPA or Net-EN) and 64 women not using HCs, enrolled into the prospective CAPRISA 002 observational cohort study of acute HIV infection conducted at the Centre for the AIDS Programme of Research in South Africa (CAPRISA), in Durban, KwaZulu-Natal Province, South Africa, as previously described (Mlisana et al., 2012; van Loggerenberg et al., 2012). Non-HC users were matched to injectable HC users based on age (within 5 years of age) at a 1:1 ratio. Demographic and clinical data were collected at enrolment using a structured questionnaire administered by a trained counsellor. Although data on type of contraception (injectable HCs, combined oral contraceptives (COCs), intrauterine devices (IUDs), condoms, diaphragms, foam and jelly, or sterilisation) were collected no information was collected on whether the injectable contraceptive being used was DMPA or Net-EN. We therefore report on injectable HCs in this study with a combination of DMPA and Net-EN users. Women using COC or any other form of hormonal contraception were excluded from the study, with the exception of IUD users. Laboratory samples, including cervicovaginal lavages (CVLs), were collected from each participant at enrolment by gently flushing the cervix and the lateral vaginal walls with 10 ml of sterile normal saline, as previously described by Mlisana et al. (2012). Volume of saline recovered after the lavage was not typically recorded. CVLs were transported within 4 h on ice from the site to the laboratory. In the laboratory, CVLs were centrifuged and the supernatant collected and stored at −80 °C. The protocol for this study was approved by the Ethics Review Committees of the University of KwaZulu-Natal and the University of Cape Town.

2.2. Testing for STIs and BV

At enrolment, vulvovaginal swabs collected from the posterior fornices and lateral vaginal walls from each woman were tested for *C. trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, HSV-2 reactivation and *T. vaginalis* using PCR. Gram staining was performed to diagnose BV using Nugent score ≥7 (Mlisana et al., 2012).

2.3. Cytokine, MMP and TIMP measurements in CVL

Concentrations of cytokines, MMPs and TIMPS were measured in CVLs collected at enrolment. We measured 42 cytokines [including IL-1α, IL-3, IL-9, IL-12p40, IL-15, IL-17, epidermal growth factor (EGF), platelet-derived growth factor (PDGF)-AA, transforming growth factor (TGF)-α, vascular endothelial growth factor (VEGF), eotaxin/CCL11, FGF-2, FLT3 Ligand (FLT3L), fractalkine/CX3CL1, G-CSF, growth related oncogene (GRO) family (CXCL1–CXCL3), IFN-α, IFN-γ-induced protein (IP)-10/CXCL10, monocyte chemoattractant protein (MCP)-1/CCL2, MCP-3/CCL7, macrophage-derived chemokine (MDC)/CCL22, MIP-1α/CCL3, MIP-1β/CCL4, PDGF-AB/BB, RANTES/CCL5, soluble CD40 ligand (sCD40L), soluble IL-2 receptor α (sIL-2Rα), TNF-β, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-10, IL-12p70, IL-13, GM-CSF, IFN-γ and TNF-α]; 4 TIMPs [TIMP-1, -2, -3, -4]; and 5 MMPs [MMP-1, -2, -7, -9 and -10]. The Human Cytokine and High Sensitivity LINCOplex Premixed kits (LINCO Research, MO, USA) were used to measure cytokines; and the TIMP Panel 2 and MMP Panel-2 kits were used to measure TIMPs and MMPs respectively (Merck-Millipore, St. Charles, MO, USA). CVLs were thawed overnight on ice and filtered by centrifugation using 0.2 μm cellulose acetate filters (Sigma, St. Louis, MO, USA). All markers were measured in undiluted CVL, except for MMP-9 (which was measured at 50-fold dilution), on a Bio-Plex 100 system (Bio-Rad Laboratories Inc®, Hercules, CA, USA). Bio-Plex manager software (version 5.0; Bio-Rad Laboratories Inc®) was used to analyse the data and all analyte concentrations were extrapolated from the standard curves using a five-parameter logistic (PL) regression equation. Analyte concentrations that were below the lower limit of detection of the assay were reported as the mid-point between zero and the lowest concentration measured for each analyte. For IL-8, only two samples had readings above the upper limit of detection. For these two samples, IL-8 concentrations were reported as halfway between the highest concentration and the upper limit of the standard curve.

2.4. Statistical analyses

Fisher's exact test was used to compare proportions, while a *t*-test was used to compare the groups with regard to ages. To assess the effect of injectable HCs on cytokine levels, linear regression analysis was used. Cytokines that were undetectable in at least a third of women (IL-2, IL-2Rα, IL-3, IL-4, IL-10, IL-12p40, IL-12p70, IL-13, fractalkine, MIP-1α, IFN-α, EGF, TFG-α, FGF-2, PDGF-AB/BB and MMP-2) were dichotomised (i.e. rated as either present or absent in each woman) and logistic regression was used to estimate the effect of injectable HCs on the detectability of these cytokines. Linear and logistic regression analyses were adjusted for sex worker status, age, condom use at last intercourse, BV and STIs. In addition, interaction terms were tested to assess whether injectable HCs modified the effect of STIs on cytokine concentrations. Because of the small number of women testing positive for cervical STIs, *C. trachomatis*, *N. gonorrhoeae* or *M. genitalium*, these STIs were grouped together [Gonorrhoea–Chlamydia–Mycoplasma (GCM)] for the purposes of this analysis. Statistical analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA).

3. Results

This study included a total of 128 women, 64 of whom were non-HC users (including one woman using an IUD) and 64 of whom were injectable HC users (either DMPA or Net-EN) (Table 1). Of these 128 women, 79.7% were self-reported sex workers and this did not differ between groups. Women using injectable HCs were less likely to be single than those not using injectable HCs (0.0% compared with 4.7%; $p = 0.057$), and less likely to report having multiple partners (52.4% versus 65.6%), although this was not significant. Condom use at last intercourse was similar between the group of women using injectable HC and the group of women not using HCs (53.1% versus 65.6%, $p = 0.208$).

3.1. Prevalence of BV and STIs in injectable HC versus non-HC users

To investigate the association between injectable HC use and STIs, the prevalence of *C. trachomatis*, *T. vaginalis* and BV in women using injectable HCs was compared with those who were not using HCs. At baseline (Table 1), more than half of the women in this cohort had BV (Nugent score ≥7), and this did not differ significantly between women not using HCs and those using injectable HCs (51.6% and 50.8% respectively; $p = 1.000$). Nineteen per cent of women were infected with *T. vaginalis* and this was similar in both groups (19.1% for injectable HC users and 18.8% for non-HC users; $p = 1.000$). The prevalence of *C. trachomatis* (6.3%), *N. gonorrhoeae* (6.3%), *M. genitalium* (1.6%), and HSV-2 (2.4%) was relatively low in this cohort and was also similar in the two groups (Table 1).

3.2. Impact of injectable HCs on innate immune factors in the female genital tract

To better understand how injectable HC use influenced the genital tract innate environment, the concentrations of cytokines, chemokines, growth factors and markers of tissue repair or remodelling (MMPs and TIMPs) were compared in CVL from women using injectable HCs and those not using HCs (Table 2). Of the 42 chemokines, growth factors and inflammatory cytokines that were measured, 5/42 (11.9%) had significantly decreased median concentrations in the CVL of women using injectable HCs than those not using HCs, after adjusting for age, condom use, sex worker status, marital status, STIs, and BV. This included several chemokines, including eotaxin [beta-coefficient (β) = −0.334, $p = 0.004$], MCP-1 ($\beta = -0.359$, $p = 0.015$), MDC ($\beta = -0.364$, $p = 0.003$); the growth factor PDGF-AA ($\beta = -0.506$, $p = 0.001$); the adaptive cytokine IL-15 ($\beta = -0.240$, $p = 0.038$). In addition, pro-inflammatory cytokine IL-12p40 ($\beta = -1.059$, $p = 0.009$) and chemokine fractalkine ($\beta = -0.910$, $p = 0.028$) were less likely to be detectable in women using injectable HCs compared with those not using HCs. While linear regression was used to

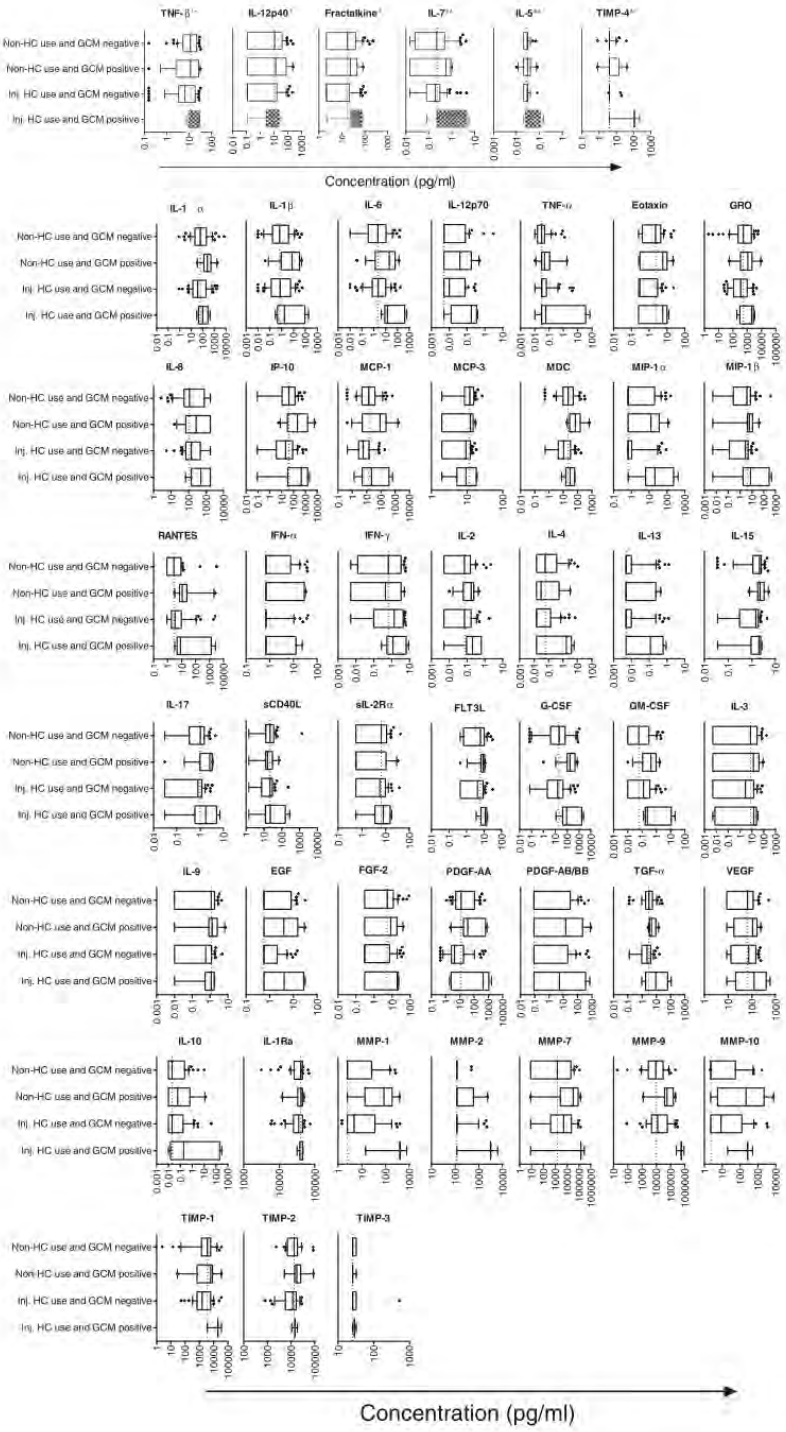
TNF-β
IL-12p40
Fractalkine
IL-7
IL-5
TIMP-4
Non-HC use and GCM negative
Non-HC use and GCM positive
Inj. HC use and GCM negative
Inj. HC use and GCM positive
Concentration (pg/ml)
IL-1 α
IL-1β
IL-6
IL-12p70
TNF-α
Eotaxin
GRO
IL-8
IP-10
MCP-1
MCP-3
MDC
MIP-1α
MIP-1β
RANTES
IFN-α
IFN-γ
IL-2
IL-4
IL-13
IL-15
IL-17
sCD40L
sIL-2Rα
FLT3L
G-CSF
GM-CSF
IL-3
IL-9
EGF
FGF-2
PDGF-AA
PDGF-AB/BB
TGF-α
VEGF
IL-10
IL-1Ra
MMP-1
MMP-2
MMP-7
MMP-9
MMP-10
TIMP-1
TIMP-2
TIMP-3
Concentration (pg/ml)

Table 1
Baseline demographic and clinical characteristics.

Variables	Overall	Injectable HC users	Non-HC users	*P* value
N	128	64	64	
Age (years)	28 (23–36)*	28 (23–36)	29 (23–37)	0.653
Marital status [% (*n*)]				
Single	2.4% (3)*	0.0% (0)	4.7% (3)	0.057
Stable partner	34.7% (44)	41.3% (26)	28.1% (18)	
Married	3.9% (5)	6.4% (4)	1.6% (1)	
Many partners	59.1% (75)	52.4% (33)	65.6% (42)	
Sex work [% (n)]	79.7% (102)	79.7% (51)	79.7% (51)	1.000
Education [% (*n*)]				
<Grade 8	21.9% (28)	21.9% (14)	21.9% (14)	0.972
Grade 8–10	29.7% (38)	28.1% (18)	31.3% (20)	
Grade 11–12	48.4% (62)	50.0% (32)	46.9% (30)	
Condom use [% (*n*)]				
Always uses condom with steady partner(s)	25.0% (32)	20.3% (13)	29.7% (19)	0.308
Always uses condom with casual partner(s)	53.9% (69)	46.9% (30)	60.9% (39)	0.156
Condom use at last intercourse	59.4% (76)	53.1% (34)	65.6% (42)	0.208
Positive test or culture result [% (*n*)]				
Genital discharge	17.2% (22)	12.5% (8)	21.9% (14)	0.241
Bacterial vaginosis (Nugent >7)	51.2% (65)	50.8% (32)	51.6% (33)	1.000
Trichomonas vaginalis	18.9% (24)	19.1% (12)	18.8% (12)	1.000
Chlamydia trachomatis	6.3% (8)	4.8% (3)	7.8% (5)	0.718
Neisseria gonorrhoeae	6.3% (8)	1.6% (1)	10.9% (7)	0.062
Mycoplasma genitalium	1.6% (2)	3.2% (2)	0.0% (0)	0.244
HSV-2 PCR	2.4% (3)	3.2% (2)	1.6% (1)	1.000

* Descriptive statistics are reported as medians and IQRs (continuous data) or percentages (categorical data).

estimate the effect of injectable HCs on concentrations of eotaxin, MCP-1, and MDC, a logistic regression model was fitted to estimate the effect of injectable HC use on the detectability of fractalkine and IL-12p40 because the concentrations of these cytokines were undetectable in at least a third of the women in this study. Furthermore, the median concentration of TIMP-2 was significantly lower in women using injectable HCs than in those not using HCs ($\beta = -0.207$, $p = 0.027$). In contrast, no differences were observed in MMP concentrations. None of the cytokines, chemokines, growth factors and markers of tissue repair concentrations was significantly different in the two groups.

3.3. Impact of BV and STIs on cytokines in injectable HC versus non-HC users

Because we found that injectable HC use resulted in lower median concentration of genital tract cytokines from several different functional classes (Table 2), we assessed whether there were interactions between injectable HCs and cytokine concentrations in responses to BV or STIs in the female genital tract. We did not observe any significant interactions between injectable HC use and cytokine responses to BV (Supplementary Table 1). *T. vaginalis* also had a limited interaction with injectable HC, with only Flt3L demonstrating a significant result (β-estimate of −0.194 in the injectable group versus 0.338 in the non-injectable group, $p = 0.048$, Supplementary Table 2). However, we found significant interactions between gonorrhoea, chlamydia or mycoplasma (GCM) infections and injectable HC use for certain cytokines, with TNF-β ($p = 0.022$), IL-5 ($p = 0.015$), IL-7 ($p = 0.036$) and TIMP-4 ($p = 0.003$). GCM-positive women on injectable HC had increased median concentrations of these cytokines in the CVL, compared with the effect of GCM positivity on cytokine concentrations among the non-HC users (Fig. 1, supplementary Table 3). Furthermore, IL-12p40 ($p = 0.061$) and fractalkine ($p = 0.069$) showed a trend towards being elevated in the GCM-positive women using injectable HCs compared with non-HC users. However, none of these associations remained significant after adjusting for multiple comparisons.

4. Discussion

We found that median concentrations of several cytokines and soluble factors (including several chemokines, pro-inflammatory and adaptive cytokines, growth factors and TIMP were reduced in CVLs of women using injectable HC compared with women who were not using HC, after controlling for age, condom use, sex work, STIs and BV. Despite this reduction of innate immune responses in the female genital tracts of women using injectable HCs, no differences in prevalence of BV, or STIs (including *C. trachomatis*, *N. gonorrhoea*, *T. vaginalis*, *M. genitalium*, or HSV-2) were found.

Fig. 1. Cytokine concentrations in cervicovaginal lavages (CVLs) from women using injectable HCs (DMPA or Net-EN) compared with those not using HCs, stratified according to the presence of discharge-causing STIs (*C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*; GCM). Dotted lines indicate the median concentration of each cytokine, MMP or TIMP in women not using HCs and who were GCM-negative, as the reference group. **Significant interactions between injectable HC use and GCM with $p < 0.05$, while *interactions between injectable HC use and GCM with $p < 0.10$.

Table 2
Influence of injectable hormonal contraceptive use on cytokine, MMP and TIMP concentrations in genital secretions.

Functional groups	Cytokine	Injectable HC users ($n=64$)		Non-HC users ($n=64$)		Multivariate[#]	
		Median (pg/ml)	IQR	Median (pg/ml)	IQR	Beta coefficient (SE)	*P* value
Pro-inflammatory	TNF-β	7.60	3.36–19.05	11.53	5.31–21.83	−0.197 (0.104)	0.061
	IL-12p40[†]	0.12	0.12–14.39	12.20	0.12–38.24	−1.059 (0.404)	**0.009**
	IL-12p70[†]	0.02	0.01–0.08	0.01	0.01–0.09	0.363 (0.414)	0.381
	IL1α	38.02	14.26–172.71	87.37	30.40–259.69	−0.128 (0.126)	0.311
	IL-6	2.57	0.84–11.79	2.56	0.36–16.41	0.140 (0.198)	0.481
	TNF-α	0.04	0.03–0.07	0.04	0.02–0.05	0.223 (0.114)	0.053
	IL-1β	0.79	0.14–5.96	0.76	0.19–5.81	−0.005 (0.169)	0.979
Chemokine	Eotaxin	0.31	0.26–3.09	2.32	0.31–5.27	−0.334 (0.114)	**0.004**
	MCP-1	6.33	2.80–19.39	13.97	4.85–72.18	−0.359 (0.146)	**0.015**
	MDC	10.97	4.60–33.28	35.52	12.15–63.15	−0.364 (0.119)	**0.003**
	Fractalkine[†]	2.13	2.13–23.60	18.01	2.13–40.10	−0.910 (0.414)	**0.028**
	MIP1α[†]	0.66	0.66–5.73	0.66	0.66–19.83	−0.422 (0.472)	0.370
	MCP-3	9.35	1.95–12.41	12.34	8.47–15.42	−0.106 (0.063)	0.097
	IP-10	22.95	3.78–88.25	45.96	13.30–182.40	−0.393 (0.211)	0.066
	GRO	370.82	134.00–1263.72	598.60	236.09–1974.89	−0.178 (0.128)	0.167
	MIP-1β	3.18	0.26–5.27	4.31	0.70–7.93	−0.028 (0.252)	0.911
	IL-8	157.89	69.74–510.09	131.05	54.80–1029.17	0.112 (0.129)	0.389
	RANTES	3.65	2.24–9.62	3.21	0.93–9.98	0.202 (0.136)	0.138
Innate	IFNα[†]	0.68	0.68–0.68	0.68	0.68–8.91	−0.874 (0.446)	0.050
Hematopoietic	IL-9	0.68	0.01–1.24	1.19	0.23–1.76	−0.319 (0.188)	0.093
	Flt3L	4.27	0.45–9.48	6.56	2.94–10.56	−0.174 (0.110)	0.116
	G-CSF	17.17	2.76–73.44	26.80	4.71–117.07	−0.256 (0.208)	0.222
	GM-CSF	0.14	0.02–0.38	0.13	0.01–0.47	0.077 (0.141)	0.588
	IL-7	0.25	0.08–0.41	0.25	0.02–0.61	−0.033 (0.123)	0.791
	IL-3	3.08	0.01–14.86	8.35	0.01–28.18	−0.229 (0.415)	0.580
Growth Factor	PDGF-AA	4.55	2.54–19.81	15.77	5.85–118.91	−0.506 (0.146)	**0.001**
	TGF-α	2.35	0.99–4.19	3.37	2.14–5.79	−0.173 (0.101)	0.089
	VEGF	73.86	17.27–151.91	77.09	12.95–120.64	−0.033 (0.100)	0.742
	PDGF-AB/BB[†]	0.09	0.09–17.43	0.09	0.09–38.66	−0.185 (0.417)	0.658
	EGF[†]	0.56	0.56–2.30	0.56	0.56–8.61	−0.632 (0.427)	0.139
	FGF-2[†]	0.30	0.30–8.39	5.32	0.30–13.15	−0.539 (0.394)	0.171
Adaptive	IL-15	1.54	0.30–1.90	2.08	1.39–2.52	−0.240 (0.114)	**0.038**
	IL-5	0.03	0.02–0.03	0.03	0.02–0.04	0.011 (0.028)	0.690
	IL-17	0.82	0.03–1.17	1.07	0.67–1.73	−0.158 (0.137)	0.251
	sCD40L	22.84	8.41–30.89	22.84	12.64–33.53	−0.007 (0.113)	0.954
	IFN-γ	1.31	0.13–4.51	0.67	0.01–3.09	0.336 (0.215)	0.121
	IL-2[†]	0.08	0.01–0.20	0.08	0.01–0.19	−0.444 (0.417)	0.287
	IL-4[†]	0.02	0.02–0.36	0.05	0.02–0.38	−0.300 (0.406)	0.460
	IL-13[†]	0.01	0.01–0.01	0.01	0.01–0.01	0.217 (0.428)	0.612
	sIL-2Rα[†]	5.88	0.57–10.13	6.10	0.57–10.70	0.143 (0.395)	0.717
Anti-inflammatory	IL-1Ra	36,313.59	26,674.06–45,012.00	40,793.38	27,693.84–45,012.00	−0.038 (0.044)	0.381
	IL-10[†]	0.02	0.01–0.19	0.02	0.01–0.24	0.813 (0.429)	0.058
MMPs	MMP-1	5.30	2.40–42.71	3.92	2.40–53.10	0.135 (0.179)	0.454
	MMP-7	5038.05	379.42–23,761.51	2247.97	162.27–26,476.16	0.293 (0.358)	0.416
	MMP-9	13,336.27	4963.64–73,603.95	15,753.28	4774.88–46,728.84	0.203 (0.202)	0.318
	MMP-10	11.70	2.20–126.18	5.52	2.20–166.11	0.318 (0.235)	0.180
	MMP-2[†]	118.10	118.10–118.10	118.10	118.10–118.10	0.808 (0.832)	0.332
TIMPs	TIMP-1	1867.65	854.01–6087.20	4356.19	1316.85–6634.26	−0.243 (0.189)	0.203
	TIMP-2	11,759.57	6497.84–16,406.50	14,878.22	8023.87–19,246.30	−0.207 (0.091)	**0.027**
	TIMP-3	25.45	25.45–32.54	25.45	25.45–32.54	0.029 (0.040)	0.472
	TIMP-4	3.92	3.92–3.92	3.92	3.92–3.92	0.013 (0.068)	0.849

SE, standard error, CI, confidence interval.
Values shown in bold were those that were significant $p<0.05$.
[#] Multivariate analysis adjusted for age, marital status, condom use, sex work, STIs and BV as co-variates.
[†] Variables with at least a third of concentrations that were undetectable were dichotomised and a logistic regression model was fitted to estimate the effect of injectable contraception on the detectability of these cytokines.

Although some animal studies using high-dose DMPA have demonstrated that DMPA has immunosuppressive properties both systemically and in the reproductive tract (Genesca et al., 2007; Gillgrass et al., 2003; Hughes et al., 2008; Huijbregts et al., 2013; Kleynhans et al., 2013; Trunova et al., 2006), fewer studies have been conducted in humans and these have predominantly been performed using PBMCs (Hughes et al., 2008; Kleynhans et al., 2011). With the exception of Huijbregts et al. (2013), who reported reduced cervicovaginal production of IFN-α in women using injectable HCs, the other human studies suggested that DMPA use might actually increase inflammation

within the female genital tract (Baeten et al., 2001; Ghanem et al., 2005). In this study, decreased chemotactic cytokine (including eotaxin, fractalkine, MCP-1 and MDC in the multivariate analyses) concentrations observed in women using HCs may influence the trafficking of immune cells to the female genital tract. Fractalkine has been reported to play a role in the recruitment of immune cells to the endometrium, which may be influenced by the presence of DMPA (Hannan et al., 2004). Although these associations potentially suggest HC interference with chemotaxis, we have not carried out the mechanistic studies necessary to test the relationship between HCs and trafficking of cells within genital tissues.

In our interaction term analysis, we hypothesised that HC use had a limited impact on cytokine responses to trichomoniasis and gonorrhoea, chlamydia and mycoplasma infections, but did not modify the relationship between cytokine concentrations and BV. This analysis suggested that women with gonorrhoea, chlamydia and mycoplasma (GCM) infections who were using HCs had greater increases in the concentrations of TNF-β, IL-5, IL-7, TIMP-4, IL-12p40 and fractalkine relative to women with GCM infections who were not using HCs. In addition, we found that the effect size of the relationship between *T. vaginalis* and Flt3L concentrations was larger in women using injectable HCs than in non-HC users. However, these associations should be interpreted conservatively as none of these associations was significant after adjusting for multiple comparisons and sample sizes for these analyses were relatively small.

High-dose DMPA administration to macaques is associated with thinning of the vaginal epithelium (Abel et al., 2004; Genesca et al., 2007; Trunova et al., 2006; Wieser et al., 2001). In this study, significantly reduced concentrations of PDGF-AA were found in CVL from women using injectable HC compared with women not using injectable HCs. This growth factor has been reported to play an important role in restoring the barrier function of the female genital tract following injury, and reduced expression may influence the ability of the epithelial barrier to be regenerated and repaired (Werner and Grose, 2003). Growth factors enhance epithelial repair by stimulating mitosis, the spreading and migration of epithelial cells in mouse airways (Werner and Grose, 2003), and as such, a decrease in these factors could lead to weakened epithelial barriers and reduced epithelial healing in women using injectable HCs.

Matrix metalloproteinases and TIMPs play an important role in the degradation and remodelling of the extracellular matrix in the upper reproductive tract during normal reproductive processes (Lockwood and Schatz, 1996; Rodgers et al., 1993; Birkedal-Hansen, 1995). Previous studies have shown that progesterone suppresses the epithelial cell-specific MMPs, working cooperatively with TGF-β to regulate epithelial-specific MMP-7 expression (Bruner et al., 1995; Osteen et al., 1994). In contrast, in the lower reproductive tract, we found no difference between MMP concentrations in women using injectable HCs compared with those who were not using HC. Previously, Vincent et al. (2002) found that women using DMPA had decreased TIMP-1 and -2 concentrations in their endometrial epithelium and an altered local MMP:TIMP balance in their upper reproductive tracts compared with women not using HCs. Similarly, we found decreased TIMP-2 concentrations in secretions from the lower reproductive tracts of women using injectable HC compared with those not using HCs. Reduced concentrations of TIMPS may alter the MMP/TIMP ratio, which could lead to decreased epithelial barrier integrity.

A limitation of our study is that we could not differentiate DMPA from Net-EN users within the injectable HC user group, although these progestin-based HCs may have distinct biological effects. DMPA or NET-EN users may differ behaviourally or systematically from women who do not use any hormonal contraceptives. In addition, we did not monitor the stage of the menstrual cycle in this cohort, nor did we collect longitudinal data on DMPA or NET-EN use prior to sampling. This study did not adjust CVL for dilution; thus, absolute concentrations of cytokines may have been influenced by this factor.

5. Conclusion

We found reductions in CVL concentrations of one proinflammatory cytokine, four chemokines, one growth factor, one adaptive cytokine and one TIMP in the lower female genital tract of women using injectable HCs. While we are underpowered to demonstrate the relationship between injectable HC use and STI or HIV risk, our data suggest that the effects of injectable HC effects were similar in STI-positive and STI-negative participants.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jri.2015.03.007.

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