The Effect of Hormonal Contraception on Mucosal and Peripheral Immunity: Implications for HIV Susceptibility among Women in Nairobi, Kenya

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H80/51272/2016

A thesis submitted to the University of Nairobi in fulfillment of the requirements for the award of the degree of Doctor of Philosophy in Medical Microbiology

DECLARATION

I hereby declare that this thesis is my original work and has not been presented for the award of a degree or any other award in any university.

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Funding:

This study was funded by a grant from the Canadian Institutes of Health Research (CIHR) jointly held by Prof. Keith Fowke and Prof. Charu Kaushic (McMaster University). Fellowship awards from the Queen Elizabeth II Scholars Program and the CIHR International Infectious Diseases and Global Health Training Program to Kenneth Omollo enabled travel for trainings, courses, and laboratory work in Winnipeg, Canada.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to all those who have contributed to the completion of this thesis. Without their support, guidance, and encouragement, this work would not have been possible.

First and foremost, I am profoundly grateful to my PhD advisors Prof. Julius Oyugi, Prof. Keith Fowke, and Dr. Dufton Mwaengo for their unwavering support, expertise, and mentorship throughout the entire research process. Their dedication to my academic and personal growth has been invaluable. Special thanks to Dr. Julie Lajoie for her patience, critical appraisal and scientific stewardship of this study and many others we have done together over the years.

My gratitude also goes to my lab mates Monika Kowatsch, Collin Graydon, Lucy Mwangi, Allison Balasko, Toby Le, Natasha Hollett and Steve Wayne for consistently fostering a warm, humorous and conducive atmosphere within the Fowke Lab. Their insightful feedback, constructive criticism, and valuable suggestions have greatly enriched this study.

I want to express my appreciation to all the research participants who generously contributed their time, data and specimens to this study. I also acknowledge and thank Dr. Joshua Kimai, Julianna Cheruiyot, Maureen Akolo, and John Mungai for assistance in clinical coordination, participant recruitment and specimen collection.

Lastly, I acknowledge my family's steadfast moral support in my academic endeavors.

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ABBREVIATIONS

AIDS	- Acquired Immune Deficiency Syndrome
ANOVA	- Analysis of Variance
BV	- Bacterial Vaginosis
CCL	- C-C Motif Ligand
CCR5	- C-C Chemokine Receptor type 5
CD	- Cluster of Differentiation
CMC	- Cervico Mononuclear Cells
COC	- Combined Oral Contraceptives
CVL	- Cervico Vaginal Lavage
DC	- Dendritic Cells
DMPA	- Depot Medroxyprogesterone Acetate
DNA	- Deoxyribonucleic Acid
FGT	- Female Genital tract
FSW	- Female sex worker
GR	- Glucocorticoid Receptor
HC	- Hormonal Contraception
HIV	- Human Immunodeficiency Virus
IL	- Interleukin
IUCD	- Intra-Uterine Contraceptive Device
MIP	- Macrophage Inflammatory Protein,
MPA	- Medroxyprogesterone acetate
NHP	- Non-human primates
OE_2	- Estradiol
P4	- Progesterone
PBMC	- Peripheral blood mononuclear cells
PBS	- Phosphate buffered saline
PCR	- Polymerase Chain Reaction
PR	- Progesterone Receptor
RNA	- Ribonucleic acid
STI	- Sexually Transmitted Infection
SWOP	- Sex Workers Outreach Project
UNAIDS	- Joint United Nations Program on HIV/AIDS
UNITID	- University of Nairobi Institute of Tropical and Infectious Disease

ABSTRACT

Background

Safe and effective contraception is a critical component of basic healthcare for women. Unfortunately, a number of observational studies have associated depot-medroxyprogesterone acetate (DMPA) - a low-cost, progestin-based contraceptive - with a higher risk of HIV-1 acquisition. DMPA is the most widely used reversible contraceptive among young women living in sub-Saharan Africa (SSA), a region that bears the highest burden of HIV worldwide. An association between DMPA use and HIV acquisition in this region is of public health concern and presents potential challenges to HIV control efforts on the African continent. It is imperative to assess the biological mechanisms that DMPA may exploit to influence HIV acquisition at the genital tract including elevated numbers of $CD4^+$ HIV target cells and increased concentrations of inflammatory cytokines at the endocervical mucosa. Alteration of the bloodstream levels of immune-modulating hormones may also be another mechanism. Defining the impact of DMPA on the immune response is important in understanding the biological factors influencing the risk of HIV acquisition in women, especially sex workers who are already at increased risk compared to women from the general population. Therefore, the aim of this study was to characterize the effect of DMPA on mucosal and peripheral immune responses of sex worker and non-sex worker groups of women.

Objective

To compare levels of T cell activation, concentrations of proinflammatory cytokines/chemokines in blood and the genital mucosa, and plasma levels of cortisol, free triiodothyronine (T3), and free thyroxine (T4) of female sex workers (FSW) and non-sex workers (non-SW) using DMPA and those not using any hormonal contraceptive.

Methods

In this cross-sectional study, 66 FSW and 61 non-SW living in the same community were recruited. Venous blood was collected and peripheral blood mononuclear cells (PBMC) and plasma freshly isolated using the Ficoll-Hypaque technique. Cervical mononuclear cells (CMC) were extracted from cytobrush specimens and processed. Cervicovaginal lavages (CVL) were also obtained. For each participant, paired PBMCs-CMC specimens were stained for *ex vivo* immunophenotyping

with a panel of fluorochrome-conjugated monoclonal antibodies including CD3, CD4, CD8, HLA-DR, CD38, CD69, CCR5, CCR6 and CD161 and Brilliant Violet Stain Buffer. Data were acquired on the LSRII flow cytometer and analysed using FlowJo Software. Cytokine, chemokine and hormone concentrations were determined using multiplex bead arrays. Analyses were performed using GraphPad Prism and R with statistical significance set at $p \leq 0.05$.

Findings

A differential impact of DMPA on the immune response was noted between FSW and non-SW. In non-SW, DMPA elevated genital and peripheral T cell activation and CCR5 expression; they had significantly increased numbers of $CD4^+CD38^+$ and $CD4^+HLADR^+$ T cells along with increased intensity of CCR5 on $CD4^+$ T cells. Similarly, the proportions of endocervical $CD3^+$ cells, and activated $CD4^+CD69^+$ and $CD4^+38^+$ T cells were significantly higher in DMPA users. DMPA using non-sex workers also had higher systemic expression of IFNγ, IL-10, MIG and sCD40L. On the other hand, FSW using no HC had lower T cell activation levels and CCR5 expression compared to non-sex workers. In the systemic compartment, FSWs had significantly lower proportion of $CD4^+CCR5^+$ cells and expression of $CD69^+$ and $CD38^+$ on $CD4^+$ T cells, while in the genital tract, lower proportion of $CD4^+CCR5^+$ cells and expression of the activation markers CD38 and CD69 was observed. Lower systemic concentrations of proinflammatory cytokines (IL-17, TNFα) and of chemotactic proteins (MCP-1) in the plasma as well as lower level of IFNγ in CVL of FSWs on no HC was also observed. But remarkably, similar levels of immune activation and inflammation was observed between FSW and non-SW using DMPA.

By multivariate analysis, plasma MIG was the best marker for differentiating DMPA users from no-HC users. Additionally, Cortisol, T3 and T4 levels were elevated in DMPA users and this correlated with markers of T cell activation.

Conclusion:

DMPA raised genital and peripheral immune activation, CCR5 expression, cortisol, and thyroid hormone levels. While sex workers had decreased levels of immune activation at the genital tract, DMPA counteracted this effect. These findings collectively demonstrate that DMPA alters the immune response and provide a biological mechanism underlying increased susceptibility to HIV in women using DMPA. Therefore, women using DMPA should be counseled to evaluate their individual HIV risk and use appropriate HIV prevention methods alongside the contraceptive.

CHAPTER 1

1 INTRODUCTION

Over 39 million people in the world are presently living with the Human Immunodeficiency Virus and Acquired Immune Deficiency Syndrome (HIV/AIDS), with 85.6 million having been infected with the virus since the beginning of the pandemic in the early 1980s(UNAIDS, 2023). One of the major goals of public health is to combat the spread of the pandemic. Many gains have been made in this regard: the number of HIV-infected people has stabilized and even declined in some regions while long-term antiretroviral therapy has increased life expectancy for infected people. However, Sub-Saharan Africa (SSA) remains the region most heavily affected, accounting for 70% of the 1.3 million new infections worldwide in 2022(UNAIDS, 2023). Kenya has had modest declines in adult HIV prevalence in the last decade. With a prevalence of 4.3%(NACC, 2022), Kenya currently has the third-largest epidemic in the world.

In SSA, women and young girls continue to be disproportionally affected by HIV, accounting for 60% of all HIV infections(UNAIDS, 2023). AIDS-related mortality is also higher in women. In Kenya, young women are twice as likely to become infected than their male counterparts(NACC, 2022). Kenya's epidemic is largely generalized but key populations like sex workers are at greater risk of acquiring the infection. The HIV prevalence estimates in Kenyan female sex workers (FSW) varies from 29%(NACC, 2022) to 45%(Kerrigan et al., 2013). In the capital, Nairobi, a 2015 study estimated that one third of sex workers were living with HIV(Musyoki et al., 2015). Prevention of HIV infections in women, especially those among key populations, is therefore a public health priority.

Efforts to develop more effective biomedical approaches to prevent HIV infections are ongoing and bring optimism among the scientific community. Pre-Exposure Prophylaxis (PrEP) using antiretroviral drugs has been proven to be effective in preventing new infections and is being rolled out in HIV prevention programs around the globe. However, the effectiveness of oral and topical PrEP varies widely, from minus 49% to >90%(Thomson et al., 2016) depending on adherence and biological factors. Genital inflammation is a biological predictor of PrEP and microbicide efficacy in SSA(Hope, 2018). In an analysis of the CAPRISA 004 trial, McKinnon et al reported that women without inflammation who adhered to the 1% Tenofovir gel had protection of

75%(McKinnon et al., 2018). On the other hand, women with genital inflammation who also adhered to the drug had protection of minus 10%(McKinnon et al., 2018). Clearly, inflammation can precipitate the failure of HIV prevention strategies. Inflammation can result from secretion of cytokines/chemokines leading to recruitment of activated CD4+ T cells to the mucosa increasing per cell HIV susceptibility. While the mechanisms that underlie inflammation in Sub-Saharan women are not fully understood, certain factors are potential causes. Sexual and intravaginal practices, dysbiosis of the vaginal microbiome, untreated genital-ulcerative infections and use of injectable hormonal contraceptives have been implicated(Kaul et al., 2015a).

The majority of the women infected with or at-risk of HIV are in their childbearing years (18-49 years) and therefore, need access to safe and effective contraception for birth control. This need for safe, effective contraception is adequately met by long-acting injectable hormonal contraceptives. Unfortunately, various observational studies have shown an association between the use of some hormonal contraceptives and an increased risk of HIV infection. In particular, Depot Medroxyprogesterone Acetate (DMPA), a progesterone-based contraceptive used by a vast majority of women in SSA(Hubacher et al., 2012; Sibeko et al., 2011), has been linked to an increase in the risk of acquiring or transmitting HIV and other sexually transmitted infections (STIs)(Heffron et al., 2012; Morrison et al., 2007; Polis et al., 2014). This is a critical issue that may impede ongoing efforts to control and reverse the HIV pandemic. On the other hand, other epidemiological studies have failed to observe an effect of DMPA or other forms of contraception on HIV infection(Kiddugavu et al., 2003; Myer et al., 2007). There is, therefore, growing debate on the use of DMPA by women who may be at-risk of HIV infection. In a bid to answer this question through a randomized clinical trial, the Evidence for Contraceptive Options and HIV Outcomes (ECHO) trial was conducted in three SSA countries. The trial found no significant difference in HIV incidence among women using DMPA or levonorgestrel (LNG) implant, or the copper-intrauterine device(Evidence for Contraceptive Options and HIV Outcomes (ECHO) Trial Consortium., 2019). In response, the World Health Organization recently reverted the medical eligibility criteria for DMPA from category 2 (advantages generally outweigh risks) to category 1 (no restriction for use) for women at high risk of HIV. However, the ECHO trial itself had several statistical and design challenges, a detailed discussion of which is in section 2.6, making it difficult to rule out an increase or no change in the HIV risk for the three contraceptives compared to one another. Thus, the question of the role of DMPA in HIV susceptibility remains unresolved.

A majority of HIV infections start at mucosal sites. Over 40% of HIV transmissions occur in the female genital tract (FGT) during heterosexual intercourse(Hladik & McElrath, 2008). The immune response at the FGT is important to fight against infectious agents and control the commensal microbiota. The mucosal immune system in the FGT is controlled by the sex hormones, estradiol and progesterone(Kaushic et al., 2011). Notably, sex hormones modulate almost all aspects of immune function in the FGT including antigen presentation, T-cell cytotoxicity and cytokine production, cellular trafficking, secretion of antimicrobial peptides and antibody secretion(Wira et al., 2015; Wira & Veronese, 2014). In hormonal contraception (HC), exogenous sex hormones (synthetic analogues of progesterone and estradiol) are administered to prevent ovulation and endometrial receptivity. These compounds may have an impact on the immune response at the FGT. However, little is known about this aspect. Since they mimic the function of endogenous sex hormones, it is possible that they also play a role in FGT immunity and thus impact a woman's susceptibility to infections. Therefore, some hormonal contraceptives could exert a negative impact on the mucosal immunity and increase the risk of HIV infection.

Identification of the biological mechanisms by which DMPA might increase the risk of HIV infection would provide an understanding of this complex issue. Mechanisms that have been proposed include thinning and/or disruption of the cervicovaginal epithelium, dampening of the innate and adaptive mucosal cellular responses, alteration of the vaginal microbiota, increased proportions of HIV target cells and aberrant inflammation (reviewed by Murphy et al(Murphy et al., 2014)). However, there is still a paucity of *in vitro* and clinical data to support these mechanisms.

In order to address these gaps in knowledge, this thesis will address the question of whether exogenous hormones impact the immune response among active sex workers using DMPA, and no hormonal contraceptives (No HC) recruited at the Sex Workers Outreach Programme (SWOP) clinics in Majengo, Nairobi. Similarly, non-sex workers (considered lower risk and drawn from the general population) residing in the Majengo vicinity and using DMPA and No HC were recruited as a control group. The overall goal of this study was to investigate the impact of hormonal contraception on HIV target T cells, and inflammatory cytokines in the blood and mucosa of female sex workers and non-sex workers in Nairobi.

1.1 JUSTIFICATION AND RATIONALE

Women continue to be disproportionately affected by HIV/AIDS, with most HIV infections occurring at the female genital tract during heterosexual intercourse. Globally, an increasing number of women use oral or injectable hormonal contraceptives, especially in areas of high HIV prevalence like Sub-Saharan Africa. Epidemiological evidence shows that the widely used injectable contraceptive, Depot medroxyprogesterone acetate, increases the risk of HIV acquisition. This is an important, yet under-investigated, public health issue with potentially large implications for health policy. However, inadequate information from clinical and in vitro studies is available to aid in weighing the potential risks of hormonal contraceptive use in individuals at high risk of HIV infection.

Potential mechanisms by which DMPA can increase a woman's risk of HIV acquisition include thinning of the vaginal epithelial barrier, induction of inflammation at the mucosa, interference with innate soluble immune mediators and adaptive cellular responses, alteration of the vaginal microbiota and increase in HIV target cell numbers in the FGT. These mechanisms are incompletely understood. It is, therefore, imperative to study how DMPA and other hormonal contraceptives affect the mucosal and systemic immune system in women who are at high risk of HIV infection. Among the users of DMPA are female sex workers who are at higher risk of HIV exposure compared to non-sex workers. Studies have shown that sex work strongly modulates the systemic and mucosal immune system, and consequently, has an impact on HIV susceptibility. However, it remains unknown whether there is a differential impact of DMPA on the immune response of FSW and women from the general population. Understanding the effect of DMPA on the immune response of a high-risk population compared to a general population is important in aiding women at higher risk of HIV acquisition access better counseling on family planning as well as HIV prevention.

1.2 HYPOTHESIS

This study hypothesizes that DMPA use results in increased levels of inflammatory markers (cytokines/chemokines) and higher frequency of activated CD4+ target T cells in the cervix and peripheral blood of women.

1.3 OBJECTIVES

1.3.1 Broad Objective

To characterize the effect of DMPA on the peripheral and mucosal immunity, and consequently on HIV susceptibility, among women in Nairobi, Kenya

1.3.2 Specific Objectives

1. To compare the effect of DMPA on levels of inflammation-associated cytokines and chemokines in the cervical mucosa and peripheral blood of sex workers and non-sex workers.
2. To compare the effect of DMPA on T cell activation and HIV target cells in the cervical mucosa and peripheral blood of sex workers and non-sex workers.
3. To determine the immune profiles that distinguish the use of DMPA and no hormonal contraception.
4. To determine the effect of DMPA on the endocrine system in relation to inflammation and HIV target cells in sex workers and non-sex workers.

CHAPTER 2

2 LITERATURE REVIEW

2.1 The Human Immunodeficiency Virus

The Human Immunodeficiency Virus (HIV), an enveloped retrovirus of the genus Lentivirus, is the causative agent of Acquired Immune Deficiency Syndrome (AIDS). It was discovered in 1981 in a group of homosexual men presenting with clinical symptoms of immune deficiency in the United States. However, through testing of stored serum and plasma samples, it is now known that the earliest case was in 1959 in a male individual in the Democratic Republic of Congo(T. Zhu et al., 1998). There are two strains of the virus: HIV-1 and HIV-2. HIV-2 is predominant in West Africa, less pathogenic and hasn't caused severe epidemics(McCutchan, 2006). HIV-1 (hereafter simply referred to as HIV) is more transmissible and is responsible for the global epidemic. HIV is further divided into groups which are thought to have arisen from independent transmission events. These are: major (M), outlier (O), non-major and non-outlier (N) and putative (P). Group M is responsible for the majority of HIV/AIDS cases and consists of different subtypes (also called clades) denoted as A to L which are differentially distributed worldwide(Osmanov et al., 2002). Clades A, B and C are the most prevalent(McCutchan, 2006) with subtype C being responsible for almost half of global HIV infections. In Kenya, where this study was conducted, subtype A is dominant (Dowling et al., 2002).

2.1.1 Virion Structure

Unlike most retroviruses, HIV has a cone-shaped capsid core containing two copies of single-stranded RNA (ssRNA) and enzymes (reverse transcriptase and integrase) necessary for infection and viral replication. The genome is 9kb in length with long terminal repeats at either end. Encoded in the genome are structural proteins (Gag, Pol, Env), regulatory proteins (Tat, Rev) and accessory proteins (Nef, Vpu, Vpr, Vif). Surrounding the capsid is a matrix of p17 proteins which in turn is surrounded by the envelope. The envelope is composed of a lipid-bilayer membrane derived from the host cell as well as viral glycoproteins, gp41 (transmembrane) and gp120 (outer surface) embedded as spikes. A single virion can have an estimated average of 14 gp41-gp120 trimers on the surface(P. Zhu et al., 2006). It is the envelope protein complex that determines HIV cellular tropism and mediates fusion with the host cell membrane permitting viral entry.

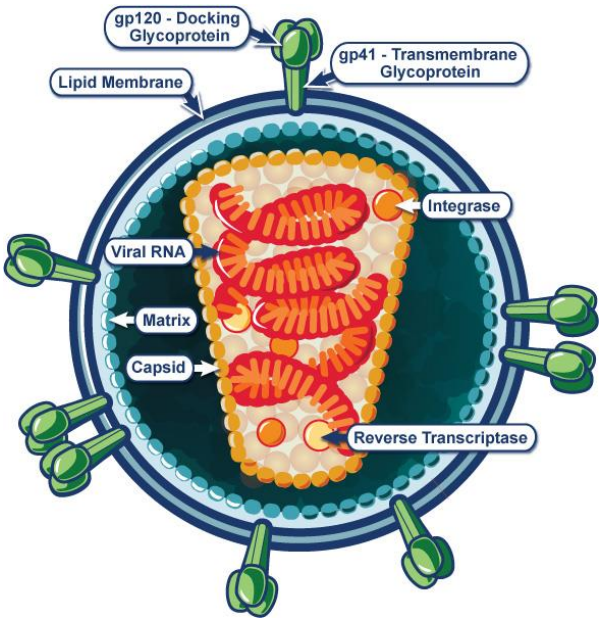

Figure 1.Schematic Illustration of the HIV Virion

[Source: (National Institute of Allergy and Infectious Diseases, 2009)]

2.1.2 HIV Entry into Host Cells

To enter into a host cell, lentiviruses must first bind to a main receptor and after to a co-receptor located at the surface of the cell. HIV infects cells by the binding its gp120 protein to the CD4 receptor on host cell. This receptor is found primarily on CD4+ T lymphocytes, which are the main target cells for HIV. However, CD4 receptors can also be found on dendritic cells, Langerhans cells, macrophages and brain microglia(Haase, 2011). Next, the binding of CD4 and gp120 leads to a conformational change in the Env protein. This exposes the gp120 binding site for co-receptors, allowing the binding to CCR5 (R5 strains) and CXCR4 (X4 strains). R5 strains of the virus are transmitted via mucosal membranes and are seen in early infections whereas X4 strains emerge later in chronic infections and are associated with the progression to AIDS(Schuitemaker et al., 1992).

The fusion of gp120 to CD4 and CCR5/CXCR4 leads to further conformational changes in the gp41 protein. This leads to formation of “the fusion peptide” that inserts into the target cell membrane and facilitates fusion of viral and host membranes(Wilen et al., 2012). Following the fusion, the capsid and genetic material are released into the cytoplasm where the viral reverse transcriptase begins to transcribe the ssRNA into complementary deoxyribonucleic acid (cDNA). For a productive infection to occur, the viral integrase enzyme mediates the cDNA integration into

the host genome where it is transcribed by host RNA polymerase II and new virions are formed. Figure 2-2 outlines this process.

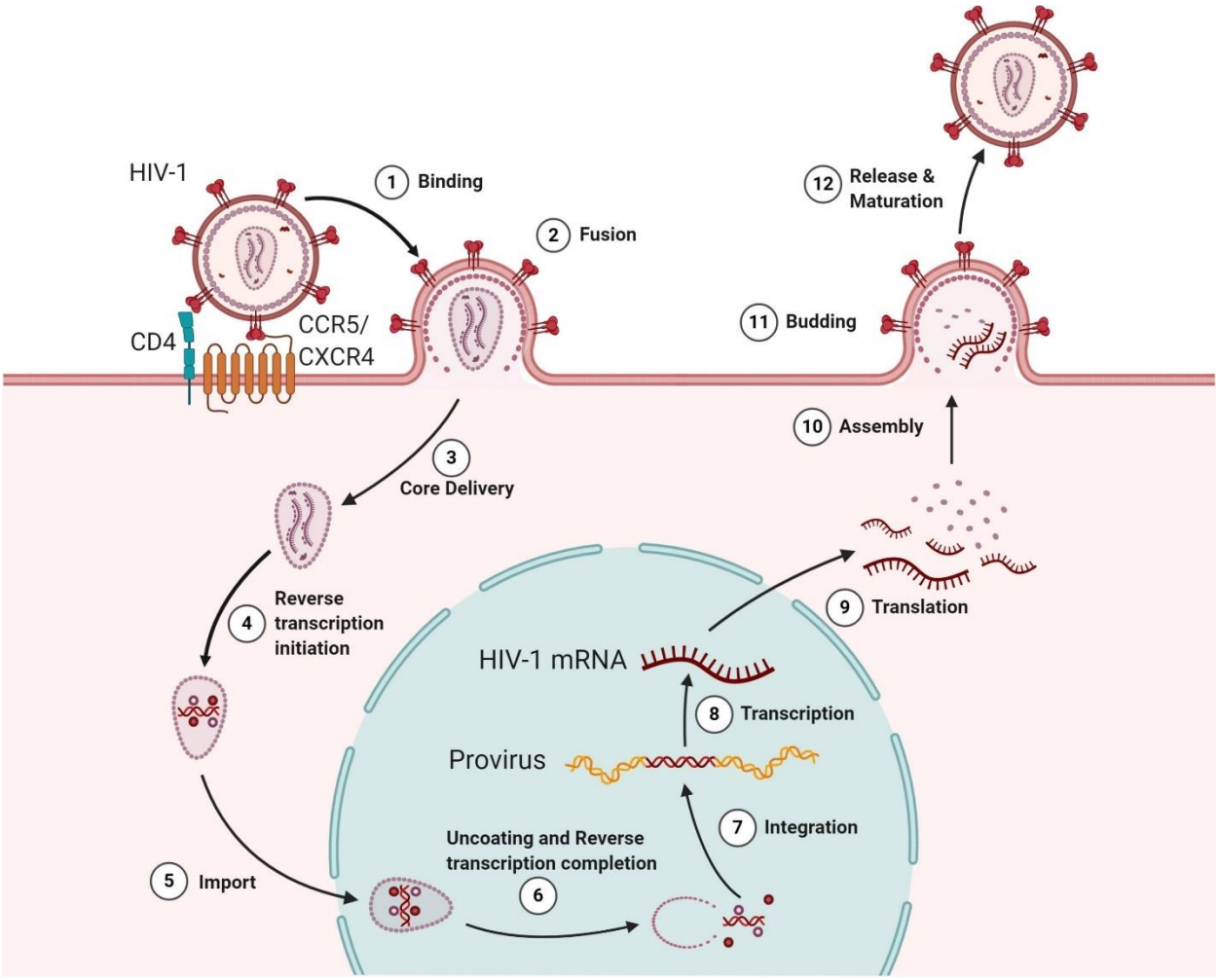

Figure 2. The HIV Replication Cycle.

[Source: (Ramdas et al., 2020)]

2.2 Initial Events During HIV Infection

The majority of HIV infections start at the FGT during heterosexual coitus. Our understanding of the mechanisms of mucosal HIV transmission is still limited because of the technical and ethical difficulties in studying the initial events of HIV transmission in humans. Therefore, many studies have exploited Simian Immunodeficiency Virus (SIV) infected animal models, particularly macaques, to understand the cellular and molecular events following intravaginal or rectal SIV inoculation. In sexual transmission, the genital mucosa is the primary site where infectious HIV

virions (or HIV-infected cells) are shed by the infected partner and come into contact with the epithelial barrier of the non-infected partner. The virus must then cross the epithelial barrier to reach its target cells and establish an infection. As already discussed, transmission is enhanced when the mucosal barrier of the genital tract is perturbed by inflammation, breached by physical trauma or the presence of genital-ulcerative sexually transmitted diseases like syphilis and gonorrhea(Kaul et al., 2015a). However, even in the absence of mucosal trauma, HIV can still bypass the natural barriers and innate defense mechanisms to cause a productive infection. Cell-free or cell-associated virus can cross the epithelium through transcytosis, endocytosis or simply through transmigration across the epithelial cell junctions(Boggiano & Littman, 2007).

The relative contribution of the various anatomical parts of the female genital tract in establishing an infection is still debatable. Although often referred to as "vaginal transmission," studies suggest that the entire FRT may be susceptible to SIV/HIV. The multilayered squamous epithelium of the vagina is thought to reduce penetration of virions while the single-layered transformation zone between the ectocervix and endocervix has been shown to permit penetration of more virions after SIV challenge(Zhang et al., 1999) and is thus believed by many in the field to be more susceptible to HIV. However, the larger surface area of the vaginal wall and the ectocervix compared to the endocervix (>15 times) potentially provides more access points for the virus especially if there are epithelial breaches. It is plausible that infections can occur in the uterus, fallopian tubes and ovaries but there's a paucity of data to demonstrate the contribution of these sites to initial infection. A recent study in macaques demonstrated that SIV rapidly disseminates across the entire lower and upper FRT to the local draining lymph nodes, within 48 hours of exposure(Stieh et al., 2014). Therefore, although most transmission studies have focused on cervicovaginal tissues, it is apparent that the entire FRT mucosa is susceptible to HIV infection.

2.2.1 Target Cells

Various cell types are thought to be involved in the initial cascade of HIV acquisition. Studies in animal models suggest that after crossing the epithelial barrier, HIV may initially infect the Macrophages, Dendritic cells (DCs) and Langerhans cells (LCs) which are present in high density at the genital mucosa. SIV-infected DCs have been shown to be abundant in the vaginal lamina propria of macaques a few minutes after SIV challenge(Hu et al., 2000). Similarly, HIV-infected DCs have been visualized in vaginal biopsies of asymptomatic HIV-infected women(Bhoopat et

al., 2001). DCs express CD4, CCR5 and DC-SIGN which facilitate capture and infection by HIV which is then rapidly transmitted to nearby $CD4^+$ T-cells. DC infection can either be productive or non-productive which has implications on how DCs amplify the infection to $CD4^+$ T cells. In non-productive DC infection, the virions are transmitted through formation of an 'infectious synapse'(Arrighi et al., 2004; McDonald et al., 2003) – a DC-T cell contact zone where the virus and its cellular receptors are highly concentrated – while in productive infections, a 'virological synapse' is formed between the infected DC and a T cell(Jolly & Sattentau, 2004). In carrying out their function of antigen presentation in the draining lymph nodes, infected DCs disseminate the virus to even more circulating T cells.

Similarly, Langerhans cells (LCs) are potent antigen presenting cells found in the vaginal mucosa that play a role in HIV invasion. LCs have protruding dendrites that extend widely across the epithelium towards the lumen where HIV can directly bind to them resulting in trans-infection of other cells (Fahrbach et al., 2007). Hladik *et al*, have observed that intact virions are internalized into the LC cytoplasmic compartments(Hladik et al., 2007). Subsequently, LCs move out of the epithelium on the basal side, thus spreading the infection beyond the point of viral entry. Indeed, intact virions were observed in LCs 60 hours post vaginal challenge(Hladik et al., 2007).

Although DCs and LCs may play an important role in presenting infectious virions to susceptible T cells, studies in humans and non-human primates strongly suggest that $CD4^+$ T cells are the primary HIV target cells during sexual transmission(Joag et al., 2016; McKinnon et al., 2011a; C. J. Miller et al., 2005; Veazey et al., 2003) and can be infected independently of LCs and DCs(Hladik et al., 2007). $CD4^+$ T cells are dispersed throughout the epithelium and lamina propria (often in aggregates) of the endocervix, ectocervix and vaginal mucosa. The bulk of these cells are of the central memory phenotype which have higher CCR5 expression than peripheral T cells(Poonia et al., 2006). HIV virions can penetrate the epithelium into the basal layers where $CD4^+$ T cells reside and infect some founder cells. Furthermore, the early innate immune responses produced by epithelial cells to seminal fluid and to HIV itself may result in inflammation and to the recruitment of more $CD4^+CCR5^+$ T cells increasing chances for the virus to find a target cell and establish an infection. By visualizing fluorescently tagged HIV virions in an organ culture system, Hladik and McElrath have shown that R5-tropic HIV efficiently binds to intraepithelial $CD4^+$ T cells within 2 hours of exposure followed by fusion and productive infection(Hladik &

McElrath, 2008). Infected cells then rapidly leave the epithelium and can be seen confined to the stroma one day after infection(Hladik & McElrath, 2008). In macaque models, mucosal SIV infection directly correlated with the frequency of $CD4^+CCR5^+$ T cells in rectal(Carnathan et al., 2015) and vaginal(Carnathan et al., 2015; Pandrea et al., 2012) tissue prior to SIV challenge. Furthermore, even if there is only a small population of $CD4^+$ T cells and DCs in the epithelium and submucosa of the FGT prior to SIV challenge(Q. Li et al., 2009), within 4 days post-challenge, a significant increase of these cells is observed. The majority of these cells become infected by the virus(Stieh et al., 2016; Zhang et al., 1999). This increase in cells contribute to the expansion of the founder population and to the activation and recruitment of more target cells to the site of infection thereby amplifying the infection. Vaginal $CD4^+$ T cells are also rapidly depleted following intravenous SIV inoculation in macaques(Veazey et al., 2003). Taken together, these data suggest that $CD4^+CCR5^+$ T cells are highly susceptible targets of SIV/HIV infection and that inflammation and cell recruitment fuels the local expansion of HIV in the FGT.

In summary, multiple cell types are implicated in the early events during HIV transmission at the genital tract. In the vast majority of cases, mucosal HIV and SIV infections are initiated by a single founder R5-tropic virus. To establish an infection, the virus may follow two pathways: 1) internalization into dendritic and Langerhans cells and subsequent presentation to CD4+ T cells at the genital mucosa or in draining lymph nodes, or 2) direct infection of a small cluster of CD4+ T cells which amplify the infection locally before moving to the draining lymph nodes. In either case, upon entry into the lymph node, the virus continues to multiply in the numerous follicular CD4+ T cells (Tfh). The infection can then rapidly spread to other organs like the gastrointestinal tract. In this way, the lymph nodes draining mucosal sites provide a reservoir for continued virus replication, amplification and persistence of the infection.

2.3 The Female Reproductive Tract (FRT)

Most HIV infections occur through the mucosa with over 40% of transmissions occurring in women during heterosexual intercourse(Hladik & Hope, 2009). It is therefore important to consider the anatomy of the female reproductive tract when studying HIV transmission. The FRT is anatomically divided into two sections: (1) a lower part (vagina, ectocervix and endocervix) and (2) an upper part (uterus, fallopian tubes and ovaries) (Figure 2-3). Each part plays distinct roles in female reproduction(Wira et al., 2015). In this thesis, the lower FRT is the main focus and is

simply referred to as the female genital tract (FGT). The vagina and ectocervix are covered by a multilayered non-keratinized stratified squamous epithelium whereas the endocervix is lined by a single-layer columnar epithelium. These epithelial cells are joined together by tight junction proteins which regulate the movement of molecules across the epithelium(Wira et al., 2010). Within and beneath the epithelial linings are dendritic cells, Langerhans cells, macrophages and T cells which provide a biological barrier. The 'transformation zone' where the stratified squamous epithelium of the ectocervix transitions into the single layer of the endocervix is enriched with immune cell populations and is believed to be particularly susceptible to HIV infection(Hladik & McElrath, 2008). However, the main site for HIV infection within the FGT remains unknown.

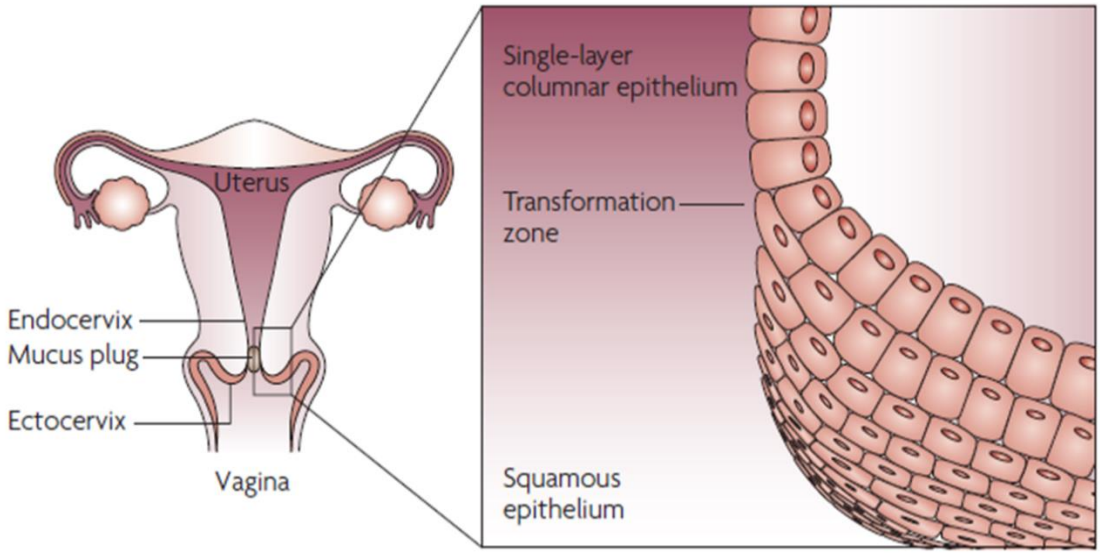

Figure 3. Basic Illustration of The Female Reproductive Tract.

[Source: (Hladik & McElrath, 2008)]

2.3.1 Immunity at the FGT

Being the first line of defense against a broad range of pathogens, the mucosal immune system in the FGT has evolved to meet the unique challenges present in the FGT. These include dealing with the resident vaginal microflora, exposure to allogeneic spermatozoa, protection against sexually transmitted infectious agents and accepting an immunologically semi-distinct foetus during pregnancy. Being an immunologically active site, the microenvironment of the FGT mucosa is a

critical determinant of HIV susceptibility. FGT immunity consists not only on mechanical barriers but also of innate and adaptive immune responses.

2.3.1.1 Mechanical and chemical barriers

The epithelial cells (ECs) lining the FGT play a key role as primary barriers to pathogen entry through desmosomes, adherens, and tight junctions between them. When intact, the epithelium provides a good degree of mechanical protection against pathogens like HIV. These cells also express Toll-like receptors (TLRs) that recognize pathogenic motifs and respond directly to pathogens while relaying signals to other immune cells during invasion(Pivarcsi et al., 2005; Triantafilou et al., 2014). Furthermore, ECs secrete a hydrophobic mucin glycoprotein - a major component of cervical mucus – which serves as a physical barrier by trapping pathogens in its matrix and preventing access to the epithelium. thereby decreasing the rate of HIV sexual transmission. The mucus barrier is therefore important in protecting the upper tract from ascending infections.

The cervicovaginal epithelium is also colonized by commensal flora which is regulated to allow growth of beneficial bacteria while preventing that of pathogens. The FGT microbiome can be classified into microbial communities dominated by species belonging to the genus *Lactobacillus*, and another characterized by overgrowth of strict anaerobes such as *Prevotella* and *Gardnerella*. Microbial communities with diverse anaerobes and low *Lactobacillus* abundance (a clinical condition known as Bacterial Vaginosis (BV)) have been associated with an increased risk of STIs including HIV(Anahtar et al., 2015; Low et al., 2011). *Lactobacilli* produce lactic acid thereby an acidic creating an environment in the FGT that limits growth of and colonization by other microorganisms. Lactic acid has anti-inflammatory properties and has been shown to inhibit HIV *in vitro*(Aldunate et al., 2013). Other studies have also demonstrated that cervicovaginal mucus from individuals with a *Lactobacillus*-dominant flora efficiently traps HIV leading to a slower diffusion of the virus(Lai et al., 2009; Nunn et al., 2015). This indicates that the microbial flora and cervicovaginal mucus work in concert to protect the FGT from infections.

Cervicovaginal mucus also contains immune mediators, including antibodies, cytokines and antimicrobial peptides secreted constitutively and upon infection by ECs and infiltrating leukocytes. Antibodies present at the FGT are primarily IgA and IgG, being both plasma-derived

and locally produced and having a pathogen neutralization and opsonization function(Alexander & Mestecky, 2007; Russell & Mestecky, 2002). A wide array of cytokines including interferons (IFNs), Interleukin (IL)-6, IL-8 play various immunomodulatory and antiviral effects. Genital IL-1β, IL-6, and IL-10 have important roles in the maturation of B lymphocytes to antibody producing plasma cells. In addition, chemokines Macrophage Inflammatory Protein (MIP)-1a, MIP-1b and RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted) not only act as chemoattractant for cells to the FGT but are also natural ligands for the CCR5 receptor and thus play a role in blocking R5-tropic strains of HIV(Cocchi et al., 1995). Antimicrobial peptides secreted at the FGT include secretory leukocyte protease inhibitor (SLPI), lactoferrin, β-defensins and elafin which have all been shown to be constitutively expressed in the FGT and have potent inhibitory activity against HIV(Ghosh et al., 2010; Klotman & Chang, 2006; Novak et al., 2007; Quiñones-Mateu et al., 2003).

2.3.1.2 Immune responses

Should the physical and chemical barriers be ineffective in containing a pathogenic invasion, additional protective immune responses are rapidly switched on. The FGT is populated by immune cells that participate in its protection. Those cells constitute between 6% to 20% of all cells(Monin et al., 2020) present at the FGT and their function is similar to those of leukocytes present in other mucosal tissues and peripheral blood. Among these are antigen presenting cells (APCs) such as LCs, DCs and macrophages which play a role in pathogen surveillance as well as priming of naïve T cells in the mucosa and in draining lymph nodes(Monin et al., 2020). The protective role of other innate immune cells of the FGT has also emerged over time. Genital neutrophils have been shown to form neutrophil extracellular traps (NETs) in co-culture with HIV viral-like particles, contributing to viral inactivation(Barr et al., 2018). Innate-like lymphocyte populations including mucosal-associated invariant T-(MAIT) cells and Natural Killer (NK) cells offer local protection against STIs and participate in immune surveillance. NK cells are activated upon recognition of HIV-infected cells to perform a cytotoxic function thereby limiting viral spread(Quillay et al., 2016).

Similarly, antigen-specific T cells participate in the containment of infections at the FGT. In fact, the majority of the leukocytes found at the FGT are T cells, representing around 50% of all leukocytes present in the FGT(Givan et al., 1997). CD4+ T helper (Th) cells and CD8+ cytotoxic

T lymphocytes (CTL) are distributed throughout the mucosa with CD8+ T-cells often outnumbering CD4+ T-cells(Givan et al., 1997; Lee et al., 2015). CD4+ mediated immunity involves Th1 (cell-mediated), Th2 (humoral), T-regulatory (Treg) and Th17 responses(Hickey et al., 2011). Th1 cell-mediated immunity involves the destruction of intracellular pathogens and induction of CD8+ CTL response which destroy pathogen-infected cells by inducing apoptosis through perforin and granzymes, while a Th2 response activates B cells to secrete immunoglobulins(Reis Machado et al., 2014). A coordinated Th1 and CTL response is critical for containment of *Chlamydia trachomatis* infection(O'Connell & Ferone, 2016) – one of the bacterial infections that increase the risk of HIV acquisition. Antiviral CTL responses in the FGT have been reported. CD8+ T-cells specific for HIV antigens in the cervicovaginal mucosa and are associated with protection from HIV(Gonzalez et al., 2017; Kuebler et al., 2016). T helper 17 (Th17) cells are also involved in host defense against intracellular bacteria and fungi. Mucosal T-cells are also polyfunctional, secreting multiple effector cytokines and chemokines that are associated with control of HIV replication(Gonzalez et al., 2017).

Notably, the immune response at the FGT is modulated through hormone-driven alterations over the course of the menstrual cycle which have profound effects on nearly all aspects of local immunity. The sex hormones, Estradiol and Progesterone, play a key role in regulating the immune responses at the FGT (reviewed in more detail at section 2.5) including immune cell distribution, cytokine and antimicrobial peptide levels and T cell responses. This regulatory effect exerted by the cyclic nature of sex hormones confers the FGT with a unique milieu that is adapted for protection of the host from pathogen invasion while ensuring optimal reproductive outcomes. The combination of these protective mechanisms and physiological functions likely determines the net outcome of HIV exposure. In fact, the low probability of transmission following a single episode of vaginal sex (1:200 to 1:2000)(Shaw & Hunter, 2012) underpins the important role of the mucosal innate immune factors and physical barriers in preventing infection.

2.4 Genital Inflammation and Risk of HIV Transmission

While the aforementioned immune defenses at the FGT provide a good degree of protection against HIV, elevated genital inflammation may heighten the risk of HIV transmission. Inflammation is a normal physiological response to pathogen invasion, tumors or other environmental stimuli, which activates both the innate and adaptive components of the immune

system in a coordinated fashion(Medzhitov, 2007). The inflammatory response results in clearance of the noxious stimuli and initiates a cascade of wound healing processes. A classical pro-inflammatory response is characterized by upregulation of cytokines and chemokines expressed by various cell types including epithelial cells, dendritic cells, macrophages and T cells. Pro-inflammatory cytokines include IL-1, IL-6, TNF-α, and IFN-γ while chemokines include MIP-1α, MIP-1β and IP-10(Dinarello, 2000). In the genital tract, expression of these cytokines and chemokines leads to activation and recruitment of immune cells from the blood to the mucosa to perform effector functions(Nkwanyana et al., 2009). Genital inflammation in women may be driven by the presence of sexually transmitted infections, bacterial vaginosis, intravaginal practices such as douching and use of hormonal contraceptives(Kaul et al., 2015a; Passmore et al., 2016). Regardless of the cause, inflammation and immune activation at the FGT acts a two-edged sword as it provides the host with immune protection but may also create an environment that favors HIV replication and establishment of a productive infection. A chronic or uncontrolled inflammatory state at the genital mucosa may increase the risk of HIV transmission through various ways.

Pro-inflammatory cytokines can cause disruption of the tight junction proteins between epithelial cells, impairing the integrity of this mucosal barrier. Increased levels of TNF-α correlates with disruption of occludin and ZO-1 junction proteins, and enhances translocation of HIV across the epithelial barrier to the submucosa(Nazli et al., 2010). Women with elevated genital pro-inflammatory cytokine concentrations have also been shown to have proteomic markers including the neutrophil-derived matrix metalloprotease (MMP)-8 and MMP9, fibrinogen β-chain and coronin-1 A; all of which are indicative of reduced epithelial barrier function(Arnold et al., 2016). Increased permeability of the genital epithelia would allow HIV and other pathogens to have greater access to target cells beneath the epithelium and establish an infection. Indeed, persistently raised levels of MIP-1α, MIP-1β, and IP-10 was associated with HIV seroconversion in a cohort of South African women(Grobler et al., 2015).

The chemoattractant properties of cytokines and chemokines are also responsible for the recruitment of HIV target cells to the genital mucosa. Inflammation may, therefore, increase HIV infection in this way since viral replication is dependent on the availability of the target cells. In HIV uninfected women, pro-inflammatory cytokines correlate with increased frequencies of activated CD4+ T cells at the endocervix(Kaul et al., 2008). MIP-1α is known to recruit $CCR5^{+}$ T

cells needed to establish infection to sites of inflammation(Stanford & Issekutz, 2003). In addition to their cell-recruiting property, pro-inflammatory cytokines can upregulate intracellular HIV replication by inducing the expression of the transcription factor nuclear factor-kappa B (NF-κB) on lymphocytes(Chêne et al., 1999; Osborn et al., 1989; Weissman et al., 1995). At a population level, baseline genital inflammation facilitated transmission of less infectious HIV virions(Selhorst et al., 2017) and predicted higher viral load and CD4+ T cell depletion in a South African cohort(Roberts et al., 2012). Further, elevated genital cytokine expression predicted non-effectiveness of a 1% tenofovir intravaginal gel despite high adherence(McKinnon et al., 2018) thereby increasing the risk of HIV seroconversion.

On the other hand, reduced inflammation is known to reduce the risk of HIV transmission. Prospective studies in cohorts of women who are HIV-exposed but seronegative (HESN) show a reduction in secretion of pro-inflammatory cytokines and chemokines compared to lower-risk HIV-uninfected controls(Chege et al., 2012; Lajoie et al., 2012a). Reduced systemic and mucosal activation of CD4+ T cells(Card et al., 2009a) along with low level gene transcription(Songok et al., 2012) has also been reported in HESN female sex workers implying that a quiescent immune phenotype at the FGT is protective against HIV acquisition by limiting the proportion of HIV target cells available for infection. These findings further add to our understanding of the role of inflammation in increasing the risk of HIV transmission.

2.5 Sex Hormones and Immunity in the Female Genital Tract

As already noted, multiple cells are involved in the HIV transmission cascade and the proportion and activation level of primary targets (CD4+ T cells) is important for HIV to establish a productive infection. A number of biological and environmental factors can modulate immune functions at the FGT and thereby influence cellular susceptibility to infection. Among these factors are the sex hormones.

Sex hormones estradiol (OE_2) and progesterone (P4) are secreted by the ovaries in women of reproductive age during the menstrual cycle. In response to these hormones, changes occur in the FGT in preparation for ovum production, fertilization, and implantation of the embryo in the uterus(Torrealday et al., 2000). The 28-day menstrual cycle is divided into four stages: the menstrual, proliferative, mid-cycle and secretory phases. The menstrual phase, in which

endometrial shedding occurs, lasts 3-5 days in most women. The proliferative/follicular phase during which the endometrial lining is reconstituted is marked by increasing estradiol levels up to the mid-cycle phase (ovulation) when the levels drastically drop and progesterone levels start rising. After ovulation, the concentration of P4, and to a lesser extent OE_2, steadily increase and peak at the mid-secretory phase. In the absence of fertilization, OE_2 and P4 levels drop, leading to endometrial shedding and onset of menses(Wira et al., 2015). The length of the follicular phase depends on the length of the menstrual cycle. This varies from woman to woman. Actually, only about 25% of women fits within the 28-day cycle and have their ovulation around day 14 or 15(Symul et al., 2019).

Apart from reproduction, sex hormones also play an important modulatory role in the immunological functions, offering conducive conditions for maternal protection and fetal survival(Rodriguez-Garcia, Patel, et al., 2013; Wira et al., 2015). This regulation is site specific between the upper and lower FRT. For example, cell-mediated immunity is decreased in the upper tract during the secretory phase (high progesterone levels) to promote successful implantation(White et al., 1997). In this regard, fluctuation of immune responses at the FGT during the menstrual cycle has informed the identification of a "window of vulnerability" to HIV infection in the progesterone-high phase during which innate, humoral and cell-mediated immunity are suppressed by sex hormones and therefore infection is likely to occur in case of exposure(Wira & Fahey, 2008).

The FGT epithelium is also responsive to hormonal stimuli. OE_2 increases the proliferation of epithelial cells and thickening of the epithelium in both the uterus and the vagina. This has been observed both in animal models and women(Hadzic et al., 2014; Hel et al., 2010). Similarly, low estradiol production is associated with thinning and atrophy of the vaginal epithelium which is observed in post-menopausal women(Santoro & Komi, 2009). In contrast to estradiol, high levels of P4 have been associated with thinning of the vaginal epithelium in animal models(Hadzic et al., 2014; Marx et al., 1996a), although this has not been observed in humans. The integrity of the epithelial barrier is important in preventing HIV from crossing the epithelium to reach submucosal T cells. Notably, post-menopausal women are 4- to 8-fold more susceptible to HIV transmission(Sheffield et al., 2009).

The secretion of cervicovaginal mucus is also tightly regulated by sex hormones. During the proliferative phase, mucus is thin and watery with a low viscosity, facilitating sperm movement into the upper FRT. On the other hand, progestational mucus is thick, viscous and is usually present following ovulation and during the secretory phase, when it functions to impede the movement of pathogens from the lower part into the upper part of the FRT(Hansen et al., 2014). The mucosal epithelium also expresses innate antimicrobial proteins such as secretory leukocyte protease inhibitor (SLPI), α- and β-defensins and antiproteases such as elafin in a hormone cycle-dependent manner(Wira et al., 2015). For instance, OE_2 but not P4 suppresses the secretion of human β-defensin 2 (HBD2) and elafin in the vagina(Patel et al., 2013, 2014). Interestingly, OE_2 also increases uterine secretion of SLPI and HBD2 towards the vaginal lumen from where incoming pathogens would encounter the mucosal surface(Fahey et al., 2008). These findings suggest that the antimicrobial activity of cervicovaginal fluid (CVL) is high in the follicular phase and decreases in the luteal phase.

Sex hormones also regulate the number, tissue distribution and trafficking of leukocytes in the FGT. This is to achieve a balance between fighting infections and immune regulation required for successful fetal implantation and pregnancy. Multiple studies have shown that leukocyte numbers increase in the endometrium in late secretory face and during menses(Givan et al., 1997; King & Critchley, 2010; Yeaman et al., 2001). However, in the lower tract, leukocyte numbers tend to remain constant throughout the menstrual cycle(Pudney et al., 2005). A peak in the recruitment of neutrophils, macrophages and NK cells has been observed during ovulation(Pudney et al., 2005). The recruitment of these immune cells to the genital mucosa is mediated by chemokines secreted by epithelial cells. The differential expression of these chemokines and their corresponding receptors on immune cell subsets across the menstrual cycle suggests a modulating impact of sex hormones. For instance, the endometrial expression of MIG and IP-10 was found to be varied across the menstrual cycle, peaking at the luteal stage following ovulation when NK cell numbers correlated with the concentration of the two chemotactic proteins(Kitaya et al., 2004). The mRNA expression of chemokine receptors CCR5 and CXCR1 is also upregulated during the luteal phase, suggesting a possible role of these receptors in immune cell trafficking to the genital mucosa(Dominguez et al., 2003).

Yeaman et al, have observed that T cells are usually scattered and in small aggregates in the uterus, during the proliferative phase but those lymphoid aggregates are much bigger during the secretory phase(Yeaman et al., 1997). These aggregates are mainly made of memory CD8+ T cells and B cells. Their formation is thought to be a mechanism to prevent the loss of memory T cell populations during menstruation. As mentioned before, the hormonal regulation of the FGT immune function is site-specific. CD8+ T cells predominate in the upper FGT while CD8+ and CD4+ T cells are equally distributed in the lower FGT supporting the notion of greater susceptibility to HIV in the vagina and ectocervix(Rodriguez-Garcia, Biswas, et al., 2013).

The distribution and activation of CD4+ T cells is also hormonally regulated. The follicular phase of the cycle has been associated with elevated proportions of CD4+ T cells expressing the early activation and tissue retention marker, CD69(Boily-Larouche et al., 2018). Histological evidence also suggests that the expression of CCR5 on CD4+ T cells is higher during the follicular phase(Yeaman et al., 2004). In a comparison of the distribution of Th17 cells in the endometrium, endocervix and ectocervix, Garcia *et al* have shown an increased presence of these cells and a higher susceptibility to HIV infection in the endocervix and the ectocervix compared with the endometrium(Rodriguez-Garcia et al., 2014). Although this study did not address the variation in Th17 during the menstrual cycle, pre-menopausal women had decreased numbers of Th17 cells in the endometrium compared to post-menopausal women(Rodriguez-Garcia et al., 2014). This decreased frequency of Th17 cells in the endometrium of pre-menopausal women may be necessary for the successful fertilization and implantation. Their increased presence in the vagina and ectocervix may be critical for protection against fungi and bacteria but also makes the lower FGT particularly more susceptible to HIV infection since Th17 cells are preferential HIV target cells(Brenchley et al., 2008).

In summary, the mucosal immune system at the FGT plays an important role in host protection from pathogens and in reproduction. Estradiol and progesterone regulate the immune response to ensure a balance between successful fertilization, implantation and pregnancy on one hand and immune protection from infectious agents, on the other hand. However, the cyclical pattern in which immune factors are either suppressed or enhanced during the menstrual cycle may create a "window of vulnerability" during the secretory phase for HIV to establish an infection. More work is therefore needed to further elucidate the interface between sex hormones and mucosal immunity.

This may be crucial for the development of a vaccine and/or microbicide against HIV that will be effective in women.

2.6 Hormonal Contraception and Risk of HIV Infection

As discussed above, endogenous sex hormones play a critical role in modulating women's fertility throughout the menstrual cycle. To control pregnancy, some women use exogenous steroid hormones in contraception. Safe and effective contraception is important in preventing unintended pregnancies. Contraception reduces maternal and infant morbidity and mortality, especially in women living in resource-limited settings like Sub-Saharan Africa(Darroch et al., 2008). Use of contraception by HIV-infected women who wish to avoid pregnancy is one of the most cost-effective strategies to prevent mother-to-child HIV transmission(WHO, 2012a). However, the effectiveness of contraceptive methods in preventing pregnancies varies and no method except for condoms can prevent sexual transmission of HIV. Furthermore, little is known about the impact of hormonal contraception on the immune response.

Worldwide, over 100 million women use some form of hormonal contraception(UNDP, 2019). Unfortunately, there is emerging evidence and growing concern that some of them may increase a woman's risk of acquiring HIV. As early as 1991, Frank Plummer and his team at the University of Manitoba published data suggesting that long-term use of oral contraceptives was associated with increased risk of HIV susceptibility in a cohort of FSWs in Kenya(Plummer et al., 1991). Several follow-up studies done in SSA expanded this hypothesis and focused on the injectable contraceptive Depot medroxyprogesterone (DMPA, also known as Depo-Provera). DMPA is a low-cost progestogen-based contraceptive administered as a 3-monthly intramuscular injection and is highly effective (Darroch et al., 2008). About 30% of women who use contraceptives in Kenya choose DMPA(UNDP, 2019). Unfortunately, DMPA has been linked to increased risk of HIV acquisition(Polis et al., 2014, 2016). This potentially increased risk of HIV acquisition has not been consistently observed(Heffron et al., 2012; Kapiga et al., 1998; Kiddugavu et al., 2003; Morrison et al., 2007, 2012; Myer et al., 2007) and therefore there is still ongoing debate on the topic. Furthermore, some of the early evidence comes from epidemiological studies whose results may be complex to interpret due to variations in study design, sample size, population, contraceptive methods studied and adherence to them, plus other residual confounders (reviewed

by Polis et al(Polis et al., 2013)). These challenges limit the conclusions that can be drawn from these studies.

Two well-designed observational studies that explored this issue came from Kenya. In a 10-year prospective cohort study in Mombasa, female sex workers who used DMPA were twice as likely to acquire HIV as their counterparts who used no contraception. In a secondary analysis of data from the Partners in Prevention (PIP) study, women who used DMPA had increased risk of acquiring and transmitting HIV compared with women not using hormonal contraception(Heffron et al., 2012). Whereas the PIP study was not primarily designed to investigate the impact of HC on HIV risk, these findings may have important public health implications. An updated meta-analysis of the observational data suggests a 40% increased risk of HIV associated with DMPA.

Ex vivo laboratory studies have also shown a link between DMPA and HIV. Wang et al observed increased cervical shedding of HIV-1 DNA in HIV seropositive Kenyan women upon start of DMPA(Wang et al., 2004). Studies in non-human primates also add to this controversy. No difference was observed in the plasma viral load of macaques treated with 30mg DMPA 30 days prior to intravenous SIV challenge compared with controls(Sanders-Beer et al., 2010). In contrast, a significant increase in SIV acquisition was observed in macaques that received progesterone implants compared to those which received placebo(Marx et al., 1996b). The progesterone treated animals also progressed faster to AIDS(Marx et al., 1996a). A caveat to these data is that non-human primate studies have limitations like sample size and DMPA dosage (30mg versus 150mg in humans) and their findings cannot be directly extrapolated to human biology.

Given the inconclusive nature of data from observational studies, it was thought that a randomized clinical trial would give clear answers to this question(Consortium., 2014). It was against this backdrop that the ECHO trial was conducted. ECHO was a randomized, open-label, multicenter trial done in Kenya, eSwatini, South Africa, and Zambia. It was aimed at detecting a difference in HIV risk between three methods of contraception. A total of 7829 women aged 16-35 seeking effective contraception were assigned to DMPA-IM every 3 months, a copper IUD, or LNG implant and followed for 18 months with HIV incidence as the primary endpoint. The results of the study showed that by intention-to-treat analysis, the hazard ratios were 1.04 (96% CI 0.82–1.33, p=0.72) for DMPA-IM vs. copper-IUD, 1.23 (0.95–1.59, p=0.097) for DMPA-IM vs. LNG

implant and 1.18 (0.91–1.53, *p*=0.19) for copper-IUD vs. LNG implant(Evidence for Contraceptive Options and HIV Outcomes (ECHO) Trial Consortium., 2019). The study therefore found no statistically significant difference in HIV risk among the methods evaluated.

Prior to the ECHO trial, the WHO had, in response to observational data, upheld the medical eligibility criteria (MEC) at Category 2 for DMPA clarifying that women using progestogen-only injectable contraceptives should be advised to also use condoms and other measures to prevent HIV acquisition(WHO, 2012b). However, in consideration of the ECHO data, WHO updated the MEC to Category 1, encouraging the use of DMPA by all women in any circumstance(WHO, 2019). Despite this, the ECHO trial had limitations that affect interpretation of the results. While the trial found no statistically significant differences in HIV incidence among women in the 3 study arms, HIV incidence was "alarmingly high" in all 3 groups(Evidence for Contraceptive Options and HIV Outcomes (ECHO) Trial Consortium., 2019) suggesting the possibility that the three methods increased HIV risk almost equally. Indeed, ECHO was powered to detect a 50% difference in HIV risk between any of the three methods and thus if the methods increased HIV risk equally, no difference would be detected. It is also uncertain whether the LNG implant was associated with a lower HIV risk compared to DMPA or the copper IUD. Lack of a no-hormonal contraception group means that there's no data on the absolute HIV risk of each individual method. As well, being an open-label trial done at a time when WHO had urged counselling for DMPA users, it is possible that women in the 3 arms received unequal HIV prevention counseling(Hapgood, 2020) and thus, there's a possibility of women changing their sexual behavior after randomization. The study has also been criticized for being unethical for exposing women to known contraceptives with an aim of determining a risky or harmful endpoint – HIV infection(Sathyamala, 2019). It is therefore difficult to conclude that the ECHO trial definitively answered the question of whether hormonal contraceptives have an impact on HIV susceptibility(Hapgood, 2020). Under these circumstances, biological and clinical studies are warranted to provide better understanding and guide policy decisions on this important issue.

2.6.1 Biological mechanisms for increased risk of HIV acquisition associated with DMPA

As already discussed, HIV infection in the FGT is a multifactorial process that involves interplay between the virus, environmental factors like the use of HC and host factors. The virus must cross the epithelial barrier and mucus layer to reach target cells and establish infection. An intact vaginal

epithelium provides a good degree of protection from HIV as evidenced by a transmission risk of 1:200 to 1:2000 heterosexual coital acts. Apart from host factors, other factors like presence of STIs also play a role in facilitating HIV infection. It is plausible then that DMPA may modify these factors in a way to facilitate HIV infection. In the following section, some of the mechanisms that have been explored and could explain how DMPA might increase risk of HIV acquisition will be discussed.

2.6.1.1 Epithelial barrier integrity

DMPA may increase the risk of HIV by thinning the epithelial barrier and disrupting the tight junctions(Murphy et al., 2014). Much of the evidence supporting this comes from non-human primate models. Pigtail macaques are more susceptible to SIV in the progesterone dominated phase of the menstrual cycle or following treatment with MPA; this increase in susceptibility is attributable to marked thinning of the vaginal epithelium(Vishwanathan et al., 2011). Whether such a change is observed in humans is still unclear. No significant increase in vaginal epithelial thickness was observed in biopsies obtained 1- and 3-month post DMPA administration in women(Mauck et al., 1999). Miller et al reported small reductions in epithelial cell layers and thickness in the genital tract of women following 6 months of DMPA use(L. Miller et al., 2000a). More recently, our research group observed a thinner epithelial layer and greater distribution on CD4+CCR5+ T cells towards the vaginal lumen(Edfeldt et al., 2020). How these changes biologically impact HIV acquisition is also not certain.

2.6.1.2 Alterations in Vaginal Microbiome

Lactobacilli protect against infections by maintaining an acidic vaginal pH, producing hydrogen peroxide and other antimicrobial products. Data from human studies suggest that progesterone treatment reduces the population of Lactobacilli in the vaginal microbiota(L. Miller et al., 2000a; Mitchell et al., 2014). Absence of vaginal *Lactobacilli* has been associated with an increased risk of acquiring HIV and other STIs(H. L. Martin et al., 1999; Wiesenfeld et al., 2003). Bacterial Vaginosis (BV) – which is characterized by loss of *Lactobacilli* and overgrowth of commensal anaerobes – also increases HIV acquisition(Atashili et al., 2008). A review by Achilles and Hillier(Achilles & Hillier, 2013) suggests that women who use oral contraceptives have reduced risk of BV, unlike those using DMPA. A negative impact of DMPA on vaginal microbiota may thus provide a mechanism by which it increases HIV risk. A study done almost two decades ago

showed decrease in H_2O_2-producing lactobacilli in women using DMPA, but did not find conclusive evidence of dysbiosis in microbiota(L. Miller et al., 2000a). Roxby *et al* observed a 100-fold reduction in *Gardnerella vaginalis* – a BV associated bacteria - in Kenyan women following initiation of DMPA. The microbiome arm of our study has demonstrated that DMPA-using women had greater diversity in the vaginal flora(Wessels et al., 2019), having controlled for potential confounders by excluding women having STIs and BV. Reduced levels of vaginal glycogen, an important determinant of Lactobacilli colonization of vaginal epithelium, was also observed in these women(Wessels et al., 2019). These results were also seen in a humanized mouse model in which MPA administration increased HIV susceptibility(Wessels et al., 2019). Microbiome changes induced by DMPA may lead to the expression of inflammatory mediators and activation of T cells which directly influence HIV susceptibility.

2.6.1.3 Changes in soluble antimicrobial proteins and immune mediators

Studies have examined the impact of hormonal contraceptives on the expression of cytokines, chemokines and antimicrobial proteins which have inhibitory activity against HIV. A decreased expression of SLPI was observed in endometrial biopsies, following DMPA treatment compared with biopsies collected at baseline(A. Li et al., 2007). In a second study, DMPA users were also found to have higher levels of HNP-1 and lactoferrin than non-users of hormonal contraception whereas SLPI levels were similar(Guthrie et al., 2015). The conflicting nature of these findings shows the need for more studies to investigate the impact of DMPA on expression and levels of innate antimicrobial peptides. As discussed in section 2.4, cytokines and chemokines in the FGT are also under hormonal regulation. These molecules play a role in recruitment of immune cells and mucosal defense. *In vitro*, an increase in Interleukin-8 and decrease in Regulated on Activation, Normal T Expressed and Secreted (RANTES) was observed in cervical cells treated with MPA(Africander et al., 2011). RANTES inhibits HIV by competitively binding to the CCR5 co-receptor on T cells and reduced levels in the FGT could dampen mucosal defense. However, worth noting is that concentrations of exogenous hormones used in *in vitro* studies may exceed the levels that cells get exposed to clinically. Clinical studies are, therefore, needed to unravel this issue.

2.6.1.4 Effect on HIV target cells

DMPA use may facilitate HIV infection by increasing the number of available target cells, especially activated T cells and macrophages, or by dampening cellular immune responses at the FGT. DMPA suppresses the function of human dendritic cells in vitro(Quispe Calla et al., 2015), suggesting impaired antigen presentation. Increased expression of CCR5 has also been suggested by some studies(Chandra et al., 2013; Lajoie et al., 2019). Increases in the proportions of endocervical CD4+ T cells expressing CCR5 have been observed with DMPA compared with non-users(Lajoie et al., 2019). DMPA also increased the numbers of cells bearing surface markers of the T cell lineage (CD45, CD3), T cell activation (HLA-DR) and CCR5 in vaginal tissues(Chandra et al., 2013). These findings suggest that increased expression of CCR5, and increased numbers of activated T cells may contribute to the epidemiological link between DMPA and HIV. However, larger studies are needed to address the impact of different forms of hormonal contraceptives on the number, immune function and activation of mucosal and systemic T cells. Further, there is still a dearth of data on T cell memory phenotypes and Th17 cell frequencies at the ectocervix in the context of DMPA use.

2.6.1.5 Effect on other immune-regulating hormones

Few studies have explored the effect of DMPA on other hormones that play a role in immune regulation. Progestins like DMPA act similar to progesterone by binding to the progesterone receptor (PR) with high affinity to control gene expression. The concentrations of the progestin, steroid receptor and other competing steroids or signaling molecules therefore, determines the biological response within the cell. Contrary to endogenous progesterone, DMPA binds with a high affinity to the glucocorticoid receptor (GR) and is thus capable of exerting a wider array of biological effects. Glucocorticoids such as cortisol are the natural ligands of GR and play a role in regulation of immune responses, including inflammation(Jefferies, 1991). Cortisol is produced as a physiologic response to stress. Stress has profound effects on different components of the immune system such as inflammation, lymphocyte proliferation, and immune cell distribution in lymphoid organs(Glaser & Kiecolt-Glaser, 2005). A study by Virutamasen *et al* found that DMPA users had significantly higher levels of morning cortisol than non-users(Virutamasen et al., 1986). The thyroid hormones, T3 and T4 also modulate the immune response(De Vito et al., 2011). A recent study observed that DMPA users have higher levels of T4 post-initiation of

contraception(Quintino-Moro et al., 2019). To date, there are no data linking DMPA use with alterations in cortisol, T3 and T4 in relation to cellular susceptibility to HIV.

CHAPTER 3

3 MATERIALS AND METHODS

3.1 Study Site

This study was conducted at the Sex Workers Outreach Program (SWOP) Clinics in Nairobi and at the Babadogo City County Clinic. The SWOP clinics are run by Partners for Health and Development in Africa (PHDA) – a collaborative University of Nairobi/University of Manitoba's Research Program - and provide free HIV care and treatment for sex workers. Some of the services offered at the clinics include: HIV testing, counselling for prevention and STI/HIV, provision of free condoms, laboratory testing, and reproductive health services. The Babadogo Clinic is run by the Nairobi City County Clinic and provides maternity and reproductive health services to the general community. Nested within the clinic is a HIV Treatment, Care and Research program operated by PHDA.

3.2 Study Design

This was a cross-sectional comparative study.

3.3 Study Cohort

Female sex workers attending the SWOP clinics and low-risk women from the same geographic area in Nairobi, Kenya were recruited into the study

3.4 Inclusion and Exclusion Criteria

Participants were considered eligible for enrolment in the study if they fulfilled all the inclusion criteria and none of the exclusion criteria as defined below.

3.4.1 Inclusion Criteria

1. For the FSWs, be active in sex work for ≤ 3 years, thus placed in the epidemiological category of heightened susceptibility to HIV.
2. For the non-sex worker group, not being involved in transactional or commercial sex work.

3. For the FSWs, enrolled in any SWOP clinic in Nairobi or for the non-sex workers, attending the Baba Dogo or Pumwani facilities.
4. Uterus and cervix present to allow collection of cytobrush samples.
5. Aged 18 to 50 years old, thus able to give consent and not menopausal.
6. Willing to stop douching for one-day prior to sampling.
7. Willing to undergo a pelvic exam as part of the study procedures.
8. In good health and HIV uninfected by rapid antibody tests.
9. Further criteria specific for each study group were:
    a) DMPA group: Being on DMPA for at least 6 months prior to enrolment
    b) COC group: Having taken oral hormonal contraception that contains both progesterone and estrogen for at least 6 months prior
    c) No contraception group: Should not have taken any oral, injectable or hormonal implant contraception for the past 6 months

3.4.2 Exclusion

1. Being pregnant or having been pregnant in the preceding 12 months as pregnancy alters endocrine parameters and immunity.
2. Breast feeding
3. Having an active classical STIs, yeast infection or bacterial vaginosis as these are factors known to modulate immunity at the genital tract.
4. Being in self-reported menopause
5. Prior hysterectomy
6. No longer involved in sex work for the FSWs group
7. Taken oral contraception that is only progesterone based
8. Using hormonal implant or IUCD as contraceptive methods.

3.5 Sample Size

To detect a 10% difference in chemokine levels and immune activation marker frequency between study groups and assuming an alpha of 5% and a beta of 20% this study required 21 individuals in each study group. Sample size calculation was done using G*Power v3.1 (Heinrich Heine University, Düsseldorf, Germany). However, performing analysis on mucosal samples is

challenging with sufficient lymphocyte yields from about 1:2 cytobrush samples. Therefore, to overcome this challenge, this study aimed to recruit 30 women in each group to get the requisite 21 individuals as outlined below:

- 30 new negative FSWs (less than 3 years of self-declared sex work) using DMPA as hormonal contraceptive
- 30 new negative FSWs (less than 3 years of self-declared sex work) using Combined Oral Contraceptive (COC) as contraceptive method
- 30 new negative FSWs (less than 3 years of self-declared sex work) not using any hormonal contraception method
- 30 HIV negative non-FSWs women using DMPA
- 30 HIV negative non-FSW women using COC
- 30 HIV negative non-FSW women using no hormonal contraceptive

3.6 Definition of subjects and control group

The subjects in this study were FSW using DMPA, COC and those not using any hormonal contraception. The control group were the low-risk women using contraception DMPA, COC and those not using any hormonal contraception.

3.7 Consenting and Recruitment Procedure

Recruitment of participants was done at the SWOP Clinic and Babadogo Clinic by study nurses trained in counseling. Prospective participants were informed about the purpose of the study, the specimens to be collected and how they will be handled, and any benefits associated with being in the study. Two informed consent forms were obtained at separate times: the pre-screening form used to determine participants' eligibility into the study and The Patient Information and Consent Form signed by the participant if they met the eligibility criteria and were willing to participate in the study. During the informed consenting, the study nurses read the consent form to the participants and answered participant's question. Willing participants were given the opportunity to go home with the form to discuss with their family or peer-leader as they wish, and return with the form to the clinic if interested in participating.

3.8 Ethical Approval

This study was approved by the University of Manitoba's Institutional Review Board and the Kenyatta National Hospital/University of Nairobi Ethics Review Committee (P566/08/2015, Appendix IV).

3.9 Study Design: Schematic Outline

This study was nested in the Assessment of the Impact of Exogenous Hormones on the Immune System and Susceptibility to HIV Infection study, approved under KNH-ERC #P132/03/2015. The overall study was investigating the impact of exogenous hormones on the innate immunity-vaginal microbiome axis. As schematically shown below, at the prescreening visit, specimens were collected for HIV and STI testing. HIV testing was done according to national guidelines. Urine samples were tested for *Neisseria gonorrhae* and *Chlamydia trachomatis.* Antibodies to *Treponema pallidum* were tested in plasma using the Rapid Plasma Reagin Test and positive cases confirmed using the Treponema Pallidum Hemagglutination Assay (TPHA). Bacterial Vaginosis (BV) was determined through Nugent scoring of Gram-stained slides. Vaginal swabs were taken to test for *Trichomonas vaginalis* through light microscopy of wet mounts. HIV, BV and STI test results were considered valid for 48 hours from the prescreening visit. During this time, participants were asked to use a condom for the next 36 hours to prevent any semen-induced modification of the vaginal microbiota, or introduction of penile flora into the vagina. They were further required to abstain from sexual activity 12 hours prior to sampling since sexual activity can modify immune factors in the FGT mucosa. Abstinence was confirmed using a rapid test for Prostate Specific Antigen at the enrolment visit.

For the participants using DMPA, sampling was done between week 3-4 post DMPA injection.
For the participants using COC and those not using hormonal contraceptives, sampling was done 5-10 days following menses (one week after starting new cycle) to ensure synchronicity in the menstrual phase when samples are collected.

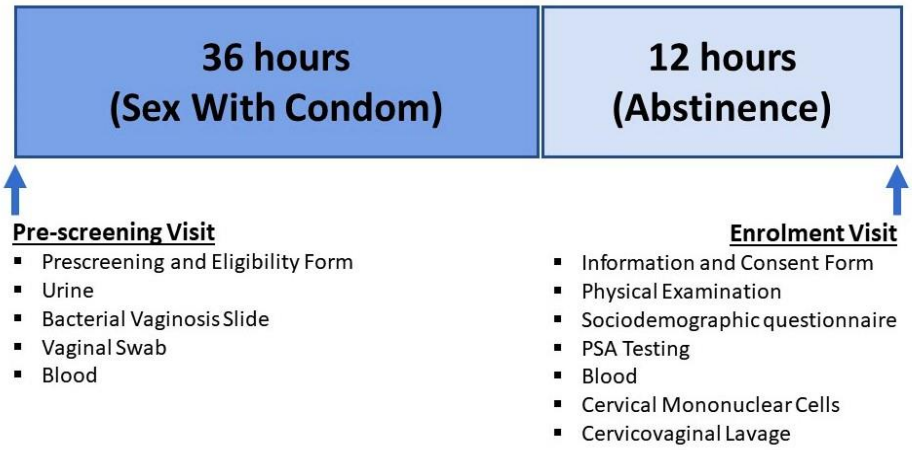

Figure 4. Schematic Outline of the Study Design

3.10 Sociodemographics and clinical examination

Demographic details, reproductive and sexual history, sexual practices, and past medical history were gathered through the use of a standardized questionnaire. The questionnaire covered reproductive health aspects, including information on pregnancy, contraceptive methods, date of last menstruation, duration of hormonal contraception use, douching practices, and other genital hygiene practices. In addition, sex workers provided information related to their sexual practices, such as condom usage, duration of engagement in sex work, and the number of clients and regular partners.

The research nurse/clinical officer conducted an external genital examination to assess the vulva and vaginal opening for indications of redness, irritation, discharge, cysts, genital warts, or any other clinical abnormalities. Subsequently, an internal cervicovaginal examination was performed under a speculum in the lithotomy position. This involved examining the vaginal walls and cervix for evidence of ulceration, inflammation, or any other clinical abnormalities.

3.11 Specimen Collection and Handling

3.11.1 Cervicovaginal Lavage

Cervico-vaginal lavages (CVL) were collected by washing the endocervix with 2 ml of sterile 1x PBS. The fluid was collected from the posterior fornix into a conical tube. The specimen was then

transported on ice to the laboratory where the CVL was centrifuged to remove cellular debris and the supernatant stored at -70°C. Specimens were then shipped to the University of Manitoba for multiplex cytokine and chemokine assays.

3.11.2 Cytobrush samples

Cervico-mononuclear cells (CMCs) were collected using a cytobrush and a plastic spatula, then transferred into a 15ml conical tube containing 10 ml of PBS. To isolate the CMCs from the cell suspension, the tube was vortexed vigorously for 1 minute to dislodge the cells from the cytobrush. In a biosafety cabinet, the mucus on the scraper and cytobrush were squeezed down into the fluid suspension. The suspension was then passed through a 100µm cell strainer into a new conical tube to remove epithelial cells and large mucus particles. The cells were then washed by adding 5ml RPMI media (supplemented with 10% Fetal Bovine Serum (FBS)) through the filter and centrifuging the filtrate at 1600rpm for 10 minutes. The supernatant then decanted and the cell pellet was resuspended by gently flicking the tube followed by another wash with 5ml PBS and centrifugation at 1600rpm for 10 min. The final pellet was resuspended in 100µl of 2% FBS-1x PBS (FACS buffer) in readiness for antibody staining.

3.11.3 Blood

Forty milliliters of venous blood were also collected and transferred into Sodium Heparin tubes. Peripheral blood mononuclear cells were extracted using the Ficoll-Hypaque technique and $1x10^6$ cells used for immunophenotyping. Plasma was cryopreserved at -70°C and shipped to the University of Manitoba for chemokine, cytokine and hormone detection.

3.12 Laboratory Assays

3.12.1 Immunophenotyping Assays

Following isolation, $1x10^6$ PBMCs and the whole CMC pellet were immunophenotyped. Both sets of cells were washed with FACS Buffer and first stained for 30 minutes at 4°C with 1µl ECD-Live-Dead discriminant dye (Invitrogen, Carlsbad, USA) followed by two washes with 1ml FACS Buffer. Cells were then suspended in blocking solution (0.2µg/µl mouse IgG, FACS Buffer, 10% FBS) for 10 minutes at 4°C and thereafter washed once with 500µl FACS Buffer. A mastermix of 10µl PECy5-CD3, 2µl Alexa700-CD4, 5µl APC-H7-CD8, 0.62µl BB515-CCR6, 5µl BV510-

HLA-DR, 1µl PECy7-CD69, 2.5µl BV421-CCR5, 10µl APC-CD161, 20µl PE-CD38 and 50µl Brilliant Violet Stain Buffer (BD Biosciences, San Jose, USA) was then used to stain the cells for 30 minutes at 4°C. Cells were then washed and fixed in 1% paraformaldehyde solution. The stained cells were acquired on an LSRII flow cytometer (BD System, San Jose, USA) and analyzed using FlowJo v10.0.8r1 software (TreeStar, Ashland, USA). The gating strategy employed in analysis is shown in Appendix I. 8-peak sphero beads were ran for cytometer calibration weekly, while Fluorescence Minus One (FMO) controls were also done on a weekly basis to control for specimen gating during analysis.

3.12.2 Cytokine and Chemokine Assays

Cytokine and chemokine concentration in plasma and CVL was determined using Multiplexed Cytokine Bead Arrays on the Milliplex Bead Panel kits (Millipore, Billerica, Massachusetts). This technology allows simultaneous measurement of multiple analytes in one reaction. The proteins analyzed were: IL-1α, IL-1RA, IL-1β, IL-8, IL-10, IL-17, soluble CD40L, IFNγ, Tumor Necrosis Factor (TNF)α, MIP-1α, MIP-1β, Monokine Induced by gamma (MIG)-3, Interferon gamma-induced Protein (IP)-10 and MCP-1.

Prior to use in the assay, samples were completely thawed at 4°C, vortexed to mix and centrifuged to remove particulates. The assay was done according to the manufacturer's instructions. First, 200µl of Wash Buffer was added to each well on the assay plate, left on a plate shaker at room temperature for 10 minutes then decanted. 25µl of standards and controls were to respective wells followed by 25µl of assay buffer to the blank and sample wells. For plasma samples, 25µl of the serum matrix solution was then added to the controls, standard and sample wells. No matrix was used for CVL samples. 25µl of each plasma or CVL specimen was added to the sample wells in duplicate followed by addition to all wells of 25µl of a mastermix of all beads specific for each cytokine to be detected.

Samples were incubated with agitation on a plate shaker for 2 hours at room temperature (plasma) or overnight at 4°C (CVL) based on manufacturer instruction. The plates were then washed twice using 200µl of wash buffer on an automated plate washer with a magnetic plate holder. After this, 25µl of Detection Antibodies were added per well and incubated with agitation for 1 hour followed

by 25µl of Streptavidin-Phycoerythrin per well and another incubation for 30minutes. The plate was then washed twice with 200µl of wash buffer. The beads were resuspended in 150µl sheath fluid before acquisition on the BioPlex-200 (Bio-Rad, Mississauga, Ontario, Canada). The minimum detectable concentration (MinDC) of each cytokine is shown in Table 1 below. Samples with undetectable concentration were assigned half the MinDC.

Table 1. Minimum Detectable Concentrations of Cytokines

Cytokine	MinDC (pg/ml)	Cytokine	MinDC (pg/ml)
IFNγ	0.8	IP-10	8.6
IL-10	1.1	MCP-1	1.9
sCD40L	5.1	MIP-1α	2.0
IL-17	0.7	MIP-1β	3.0
IL-1RA	8.3	TNFα	0.7
IL-1α	9.4	MIG	10.3
IL-1β	0.8	MIP-3	1.6
IL-8	0.4		

3.12.3 Hormone Level Detection

The concentrations of Cortisol and Thyroid Hormones (T3 and T4) were determined in plasma using the Milliplex Magnetic Bead Panel kit. First, the hormones were extracted from plasma using the acetonitrile precipitation method. 250µl of plasma and 375µl acetonitrile was added into a microfuge tube vortexed for 5seconds, and left to sit for 10 minutes at room temperature. The mixture was vortexed again for 5seconds, then centrifuged at 17000 x g for 5 minutes. 500µl of the supernatant was transferred to a new microfuge tube and dried using a Speed Vac at the highest vacuum setting. Dried samples were reconstituted with 200 µl of Assay Buffer, shaken for 10 minutes and then assayed for hormone concentrations using the same protocol as described for cytokines. The minimum detectable concentrations for each hormone are shown in Table 2 below. Samples with undetectable concentrations were assigned half the minDC.

Table 2. Minimum Detectable Concentrations of Hormones

Hormone	MinDC (ng/ml)
Cortisol	0.17
T3	0.08
T4	0.24

3.13 Data Management and Analysis

All study related data were entered into a Microsoft Excel database. Cases were the FSW while the non-sex workers were the controls. Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA) and R 4.2.2. Gaussian/Normality distribution was tested by the D'agostino and Pearson's omnibus normality test and Shapiro–Wilk normality test. Thereafter, the Mann – Whitney U- test was used to compare variables that were not normally distributed between the two groups, while the Student's t-test was used for comparison of normally distributed variables. The Chi-square and Fisher's Exact tests were used to assess the differences in categorical demographic and clinical variables between groups. Spearman correlation was used to perform all correlation tests.

Partial least squares discriminant analysis (PLS-DA) was used to identify combinations of cytokines/chemokines that best predict the use of either DMPA or No-HC. PLS-DA is a linear, multivariate model similar to principal component analysis (PCA) that considers both the covariance between independent predictors and their connection to an outcome. The model generates combinations of independent variables (principal components) which differentiate individual samples by grouping them. Every sample is assigned a score/weight within the component, then automatically plotted to display the group membership. In this way, the cytokine profiles associated with either use of either DMPA or no-HC can be identified. The sparse-PLSDA (sPLSDA) is a variant of the analysis that generates Variable Importance Scores which highlight the most significant cytokines for predicting or distinguishing the contraceptive group. To evaluate the predictive performance of the PLS-DA model, the area under the curve (AUC) was calculated by performing leave-out-one cross-validation; this involved iteratively fitting the model 10-100 times while excluding one observation at a time for prediction purposes. AUC above 0.5 are statistically significant. PLS-DA and sPLS-DA were done using the MixOmics package in R, available at http://mixomics.org/methods/spls-da/.

For all statistical tests, statistical significance was accepted if two-tailed $p \leq 0.05$.

CHAPTER 4

4 RESULTS

4.1 Enrolment

A total of 130 participants who met the criteria were recruited into the study. Sixty-six (66) were FSW: 27 using DMPA, 15 using COC and 24 using no HC, while sixty-four (64) were non-sex workers: 30 on DMPA, 4 on COC and 30 using no HC (Figure 6). Due to the low sample size of women using COC in the both FSW non-SW groups, the COC group was excluded from statistical analysis so the study focused analyses on DMPA and no HC users for robust comparisons.

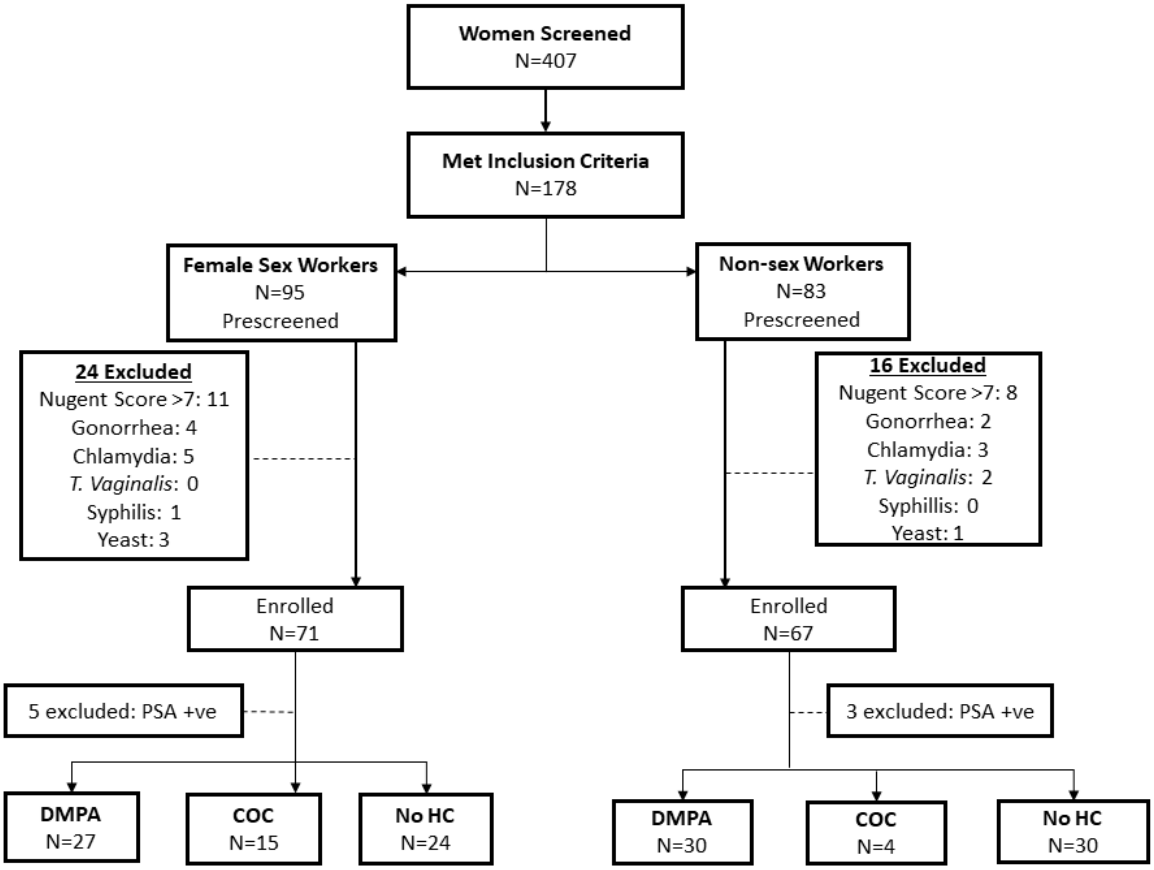

Figure 5. Study Recruitment. Flow chart depicting the screening and enrolment process for study participants.

4.2 Participants' characteristics

Table 3 summarizes the clinical and sociodemographic characteristics of the study participants. No statistically significant differences were observed within FSWs and non-SW in age, duration of contraceptive use and number of regular partners, sexual practices and alcohol use. Because of the enrolment criteria that excluded women having BV, the groups were similar in BV profiles. Some statistical differences were observed in education indices. Non-SW using no HC were likely to have more years of education than their counterparts using DMPA ($p<0.001$) with the majority having high school and college level education. In this group, DMPA use was predominantly (>75%) in women who were married while < 25% in non-committed relationships reported use of DMPA.

Table 3. Sociodemographic and clinical characteristics of female sex workers (FSW) and non-sex workers (Non-SW) enrolled in the study.

	FSW			**Non-SW**		
	DMPA n=27	**No HC n=24**	***p-value***	**DMPA n=30**	**No HC n=30**	***p-value***
Age (years) [#]	27(18-39)	30(19-39)	0.118	29(20-43)	28(18-46)	0.672
Education						
Years of school[#]	10(3-17)	10(6-14)	0.141	9(6-14)	12(6-15)	**0.001**
College[$]	2(7.4%)	2(8.3%)	0.990	1(3.3%)	12(40.0%)	**<0.0001**
Primary[$]	10(37.1%)	9(37.5%)		20(66.7%)	3(10%)	
Secondary[$]	15(55.5%)	13(54.2%)		9(30.0%)	15(50.0%)	
Marital status[$]						
Not married, not living with a man	17(63.0%)	16(66.7%)	0.503	1(3.3%)	23(76.6%)	0.631
Not married, living with a man	2(7.4%)	4(16.6%)				
Married, not living with a man	7(25.9%)	4(16.7%)		6(20%)	2(6.7%)	
Married, living with a man	1(3.7%)	0(0.0%)		21(70.0%)	5(16.7%)	
Widowed, not living with a man				2(6.7%)		
BV diagnosis[$]						
Normal	17(63.0%)	18(37.5%)	0.646	24(51.1%)	23(48.9%)	0.999
Intermediate	7(25.9%)	4(36.4%)		6(46.2%)	7(53.8%)	

Undetermined	3(11.1%)	2(33.3%)				
Alcohol use						
No	13(38.2%)	13(38.2%)	0.781	26(57.8%)	19(42.2%)	0.221
Yes	14(43.8%)	11(34.4%)		5(35.7%)	9(64.3%)	
Contraception and Parity#						
Number of times pregnant	2(0-4)	2(0-5)	0.722	3(1-8)	2(1-5)	0.420
No. of children	2(0-4)	2(0-4)	0.992	2(1-6)	2(0-5)	0.317
Days since last DMPA Injection	34(15-62)	N/A		45 (14-72)	N/A	
Months on contraception	24(14-36)	N/A		36(18-54)	N/A	
Sex Work$						
Years of Sex Work	2(1-4)	2(1-5)	0.402	N/A	N/A	
Number of regular clients	4(1-8)	5(1-12)	0.342	N/A	N/A	
Casual clients in last 5 Days	1(0-4)	2(0-6)	0.509	N/A	N/A	
Sexual Behaviour/Practices$						
Have a regular partner	20(74%)	16(67%)	0.759	24(80%)	18(60%)	0.158
Unprotected sex in last 5 days	12(44%)	8(33.3%)	0.811	11(36.7%)	18(60%)	0.121
Unprotected sex in last 24 hours	0 (0%)	0 (0%)		0 (0%)	0 (0%)	
Oral sex	3(11.1%)	1(4.1%)	0.612	2(6.7%)	2(6.7%)	0.999
Anal sex	0(0%)	2(8.3%)	0.216	2(6.7%)	1(3.3%)	0.999
Practice douching	6(22.2%)	9(37.5%)	0.374	22(73.3%)	24(80%)	0.064

Mean (range), $ n (%)

4.3 Comparison of CD4$^+$ T cell activation phenotypes

T cell activation was evaluated using multiparameter flow cytometry. Activated CD4$^+$ T cells were defined by the single expression of CD69, CD38, and HLA-DR, plus the HIV coreceptor, CCR5. The proportion of cells expressing these markers and the Median Fluorescence Intensity (MFI) of these markers, which is a measure of the per cell intensity of each marker in paired CMC and PBMC specimens, were determined and compared between study groups.

4.3.1 Impact of sex work on T cell activation

Mucosal activation

At the genital tract, FSWs had significantly lower $CD4^+CCR5^+$ T cells (50% vs. 59.9%, p=0.032) and MFI of CD69 and CD38 on $CD4^+$ T cells (1600 vs. 2622, p<0.001; 518.1 vs. 967.5, p=0.004 respectively), Figure 6A.

Peripheral activation

While there were no significant differences in the proportion of activated T cells in peripheral circulation, a trend towards lower proportion of $CD4^+CCR5^+$ T cells in FSWs not using HC (9.21% vs. 13.6%, p=0.087) was observed. In addition, significantly lower MFI of CD38 (1093 vs. 1261, p=0.002) and CD69 (1049 vs. 2056, p<0.001) on $CD4^+$ T cells was observed in FSW (Figure 6B).

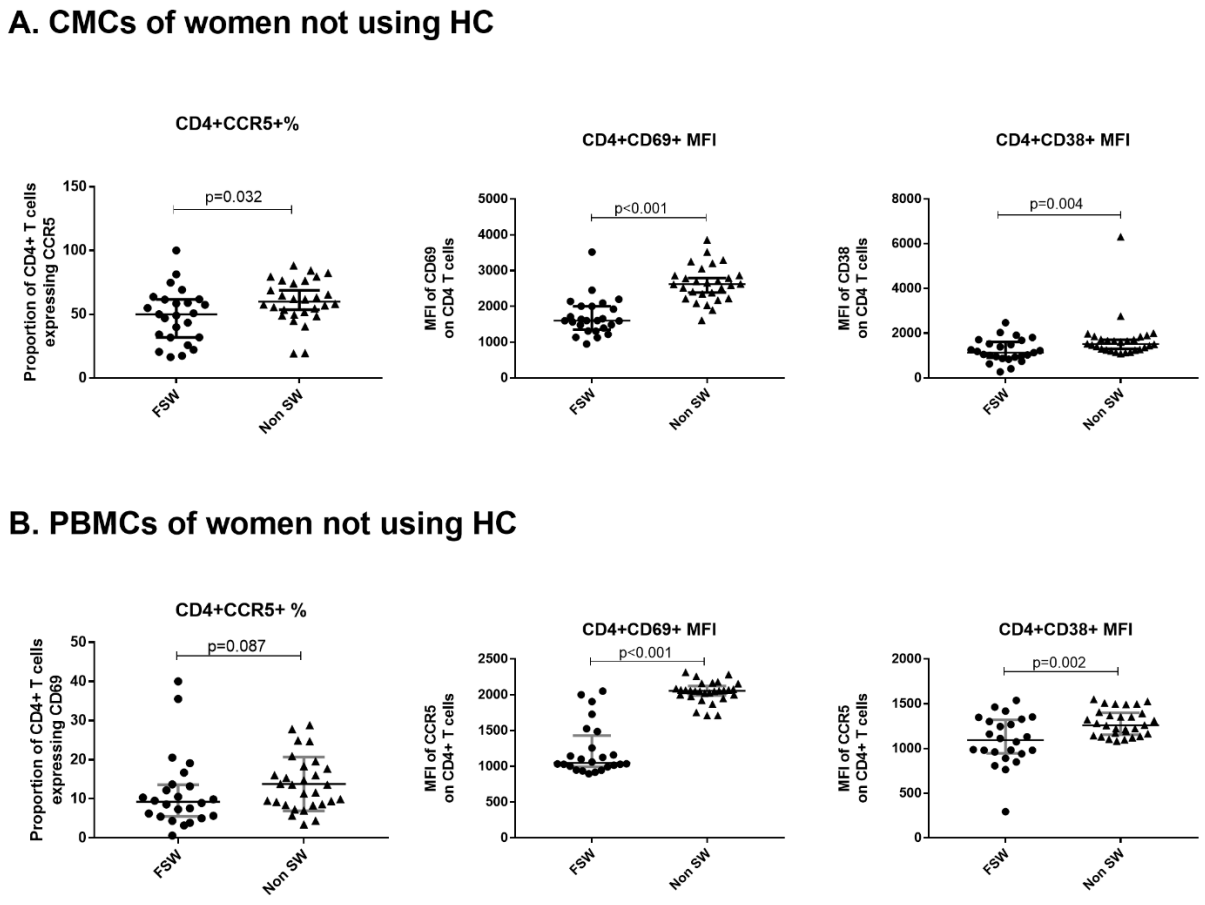

Figure 6. The effect of sex work on T cell activation and CCR5 expression among women not using hormonal contraceptives.

4.3.2 Impact of DMPA on T cell activation

Mucosal activation

In the CMCs of FSWs, DMPA users had a significantly lower proportion of $CD3^+$ T cells than FSWs not using HC (0.9% vs. 5.9%, p=0.029) but no differences were observed in $CD4^+$ T cell frequency, $CD4^+$ T cell activation markers and CCR5 expression (Figure 8A). In non-SW, DMPA users trended towards having a higher proportion of $CD3^+$ cells (4.21% vs. 2.47%, p=0.067), and had higher proportions of $CD4^+CD69^+$ (26.5% vs. 12.6%, p=0.003) and $CD4^+CD38^+$ (39.2% vs. 25.2%, p=0.001) cells, Figure 8B. No differences were observed in $CD4^+$ T cell frequency or other markers of activation.

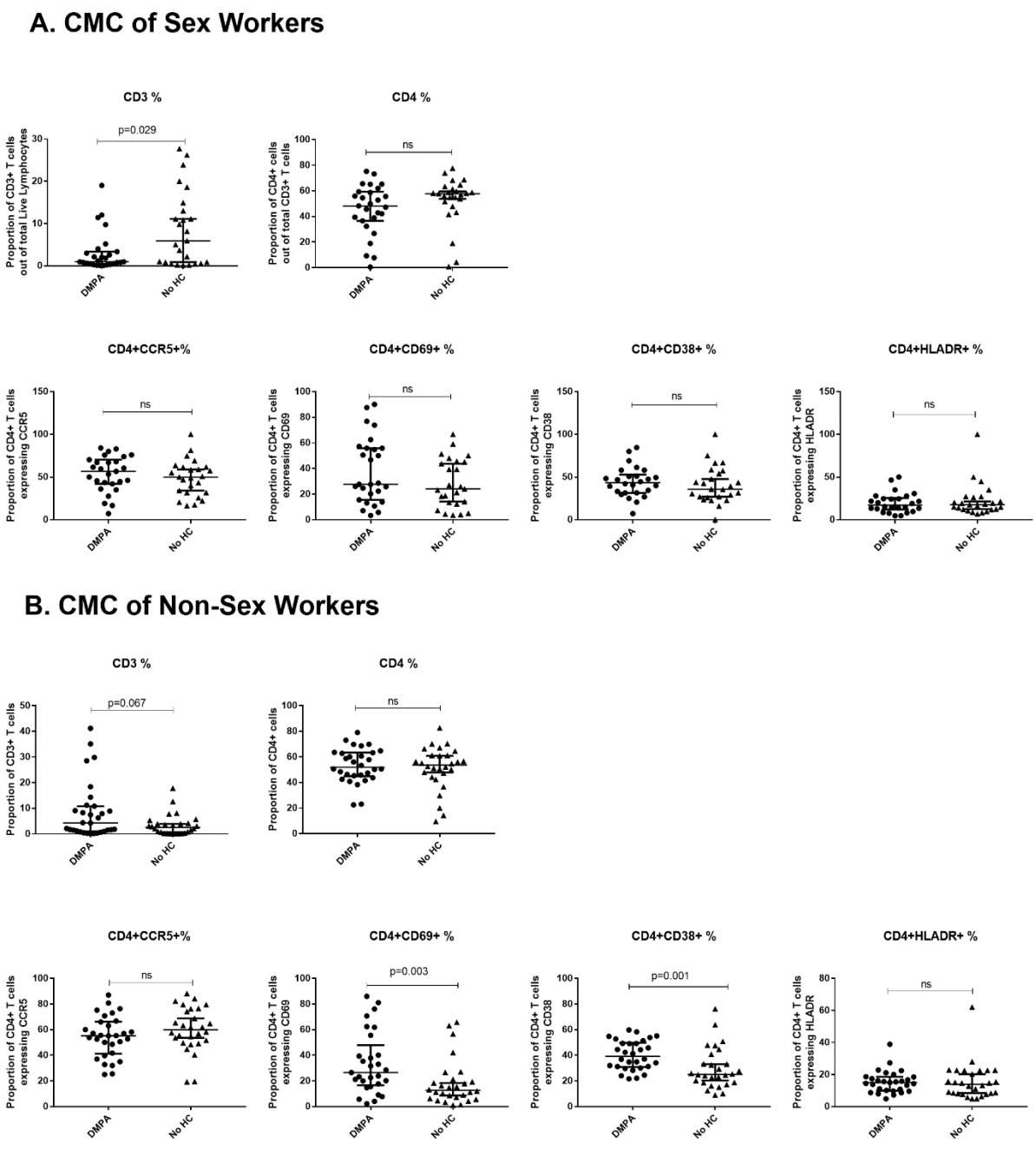

Figure 7. Comparison of cervical mononuclear cells (CMC) Immunophenotypes. ns: Not Significant

Peripheral activation

Among FSW groups, there were no significant differences in expression of activation markers or the expression of CCR5 on blood-derived T cells between DMPA and No HC users (Figure 9A). On the other hand, in non-SW groups, DMPA users had significantly higher expression of CCR5

on a per cell basis (MFI: 1738 vs. 1256, p<0.001). They also had higher proportions of activated $CD4^+$ T cells ($CD4^+CD69^+$ T cells: 3.6 vs. 0.5, p<0.001; $CD4^+CD38^+$ T cells: 50.5% vs 30.3%, p<0.001, $CD4^+HLADR^+$ T cells (4.0% vs. 2.8%, p=0.011), (Figure 9B).

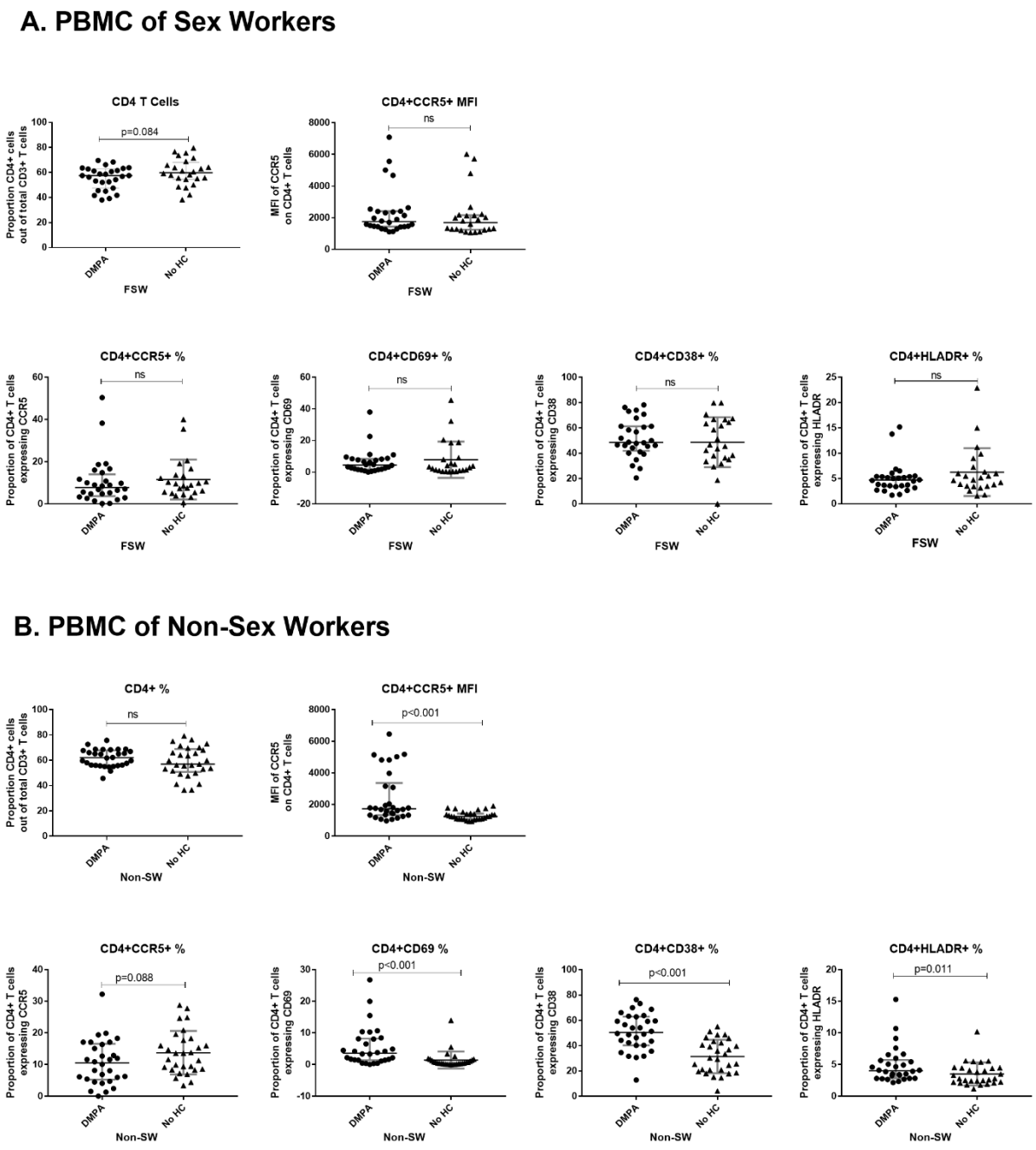

Figure 8. Comparison of peripheral blood mononuclear cells (PBMC) immunophenotypes

4.3.3 Impact of sex work on the immune response during DMPA use

Mucosal activation

Comparison of FSWs and non-SW using DMPA revealed that they had similar levels of endocervical T cell activation (Figure 10A), although FSWs had a diminished proportion of $CD3^+$ cells (0.9% vs. 4.2%, p=0.037, Supplementary Table 1).

Peripheral activation

Analysis of blood-derived $CD4^+$ T cells closely mirrored the observations made at the FGT; FSWs on DMPA had activation levels similar to non-sex workers on DMPA except for MFI of CD38 where sex workers had lower levels (MFI 1133 vs. 1310, p=0.015), (Figure 10B, Supplementary Table 1).

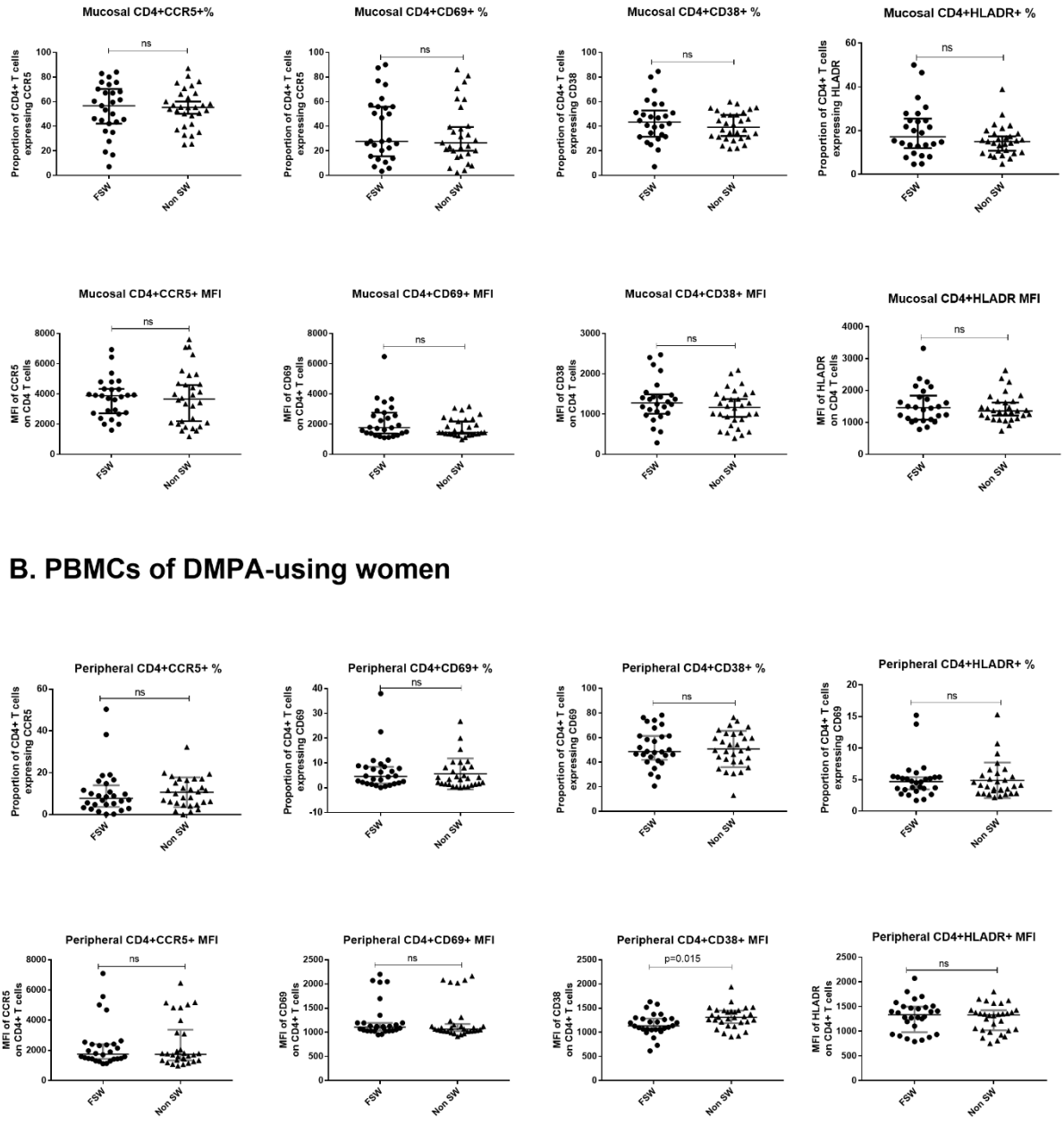

Figure 9. Comparison of $CD4^+$ T cell activation and CCR5 expression in DMPA users

4.3.4 Th17 cells

Th17 cells are a subset of $CD4^+$ T cells that regulate host defense against extracellular bacteria and fungi. Early infection and subsequent depletion of these cells at the genital and gut mucosa suggests that they are a particularly susceptible target population for HIV. The impact of sex work and DMPA use on the frequency of these cells at the genital mucosa and in peripheral circulation was compared. Th17 cells were defined by their co-expression of CD161 and CCR6. The frequencies of Th17 cells expressing the HIV coreceptor CCR5 was also compared.

Impact of sex work on Th17 cells

No differences were observed in the frequencies of Th17 cells, but a trend towards higher frequencies of $CCR5^+$Th17 cells (at the genital tract of FSW compared to non-SW was observed. In peripheral blood, FSW had a significantly higher median proportion of Th17 cells (2.74% vs. 1.11%, p=0.019), Figure 11A.

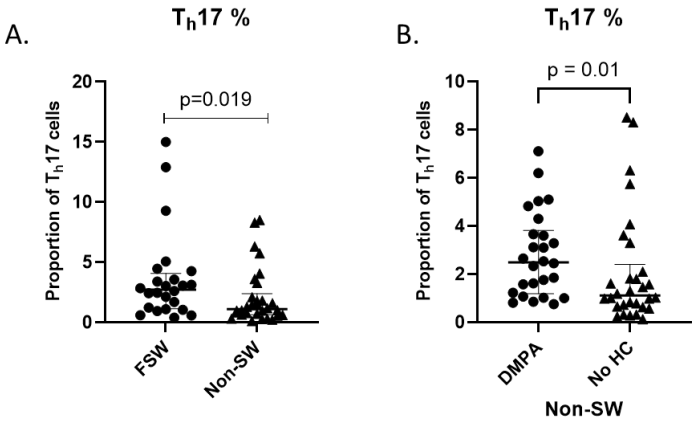

Figure 10. Comparison of Th17 cell frequencies

Impact of DMPA on Th17 cells

No statistically significant differences were observed in the proportions of mucosal or blood-derived Th17 cells or $CCR5^+$Th17 cells in both FSW groups. In non-SW groups, DMPA users had a significantly higher median proportion of Th17 cells in peripheral blood (2.49% vs. 1.11%, p=0.01, Figure 11B) but not at the FGT (Supplementary Table 1).

Impact of sex work on Th17 cells in the context of DMPA use

Comparison of FSWs and non-SW using DMPA revealed that they had similar proportions of both endocervical and peripheral Th17 cells and $CCR5^{+}$Th17 cells (Supplementary Table 1).

4.4 $CD8^{+}$ T cell activation and T_c17 cells

While $CD8^{+}$ T cells (T cytotoxic cells, T_c) are not HIV target cells, they are sensitive to overall immune activation due to cellular crosstalk between the T_h and T_c compartments following pathogenic, immunogenic or xenobiotic stimulus. Induction of a $CD8^{+}$ T cell response and functional maintenance of $CD8^{+}$ subsets is often dependent on a robust $CD4^{+}$ T cell response(Laidlaw et al., 2016). Therefore, the activation of $CD8^{+}$ T cell phenotypes as a proxy measure of DMPA and sex work-induced immune activation was assessed. A comparable pattern of $CD8^{+}$ T cell activation was noted, akin to what was observed in the $CD4^{+}$ T cell compartment. The entire dataset for this analysis is presented in supplementary table 2.

Impact of sex work

Comparison of FSW and non-SW not using HC revealed that the sex workers had significantly higher proportions of $CD8^{+}CCR5^{+}$ cells (p=0.016), and a trend towards higher proportion of $CD8^{+}CD69^{+}$ T cells (p=0.07), but lower MFI of CD69 (p<0.001) at the FGT.

In peripheral blood, FSW had significantly higher levels of $CD8^{+}CD69^{+}$ cells (p<0.001), $CCR5^{+}$Th17 cells (p=0.011) and MFI of HLADR (p=0.028), but significantly lower MFI of CD69 (p<0.001).

Impact of DMPA

At the FGT, the markers significantly higher in DMPA using FSW were: the MFI of CCR5 (p=0.014), proportion of $CD8^{+}CD38^{+}$ T cells (p=0.007), and MFI of CD38 on $CD8^{+}$ T cells (p=0.012). In non-SW, the MFI of CD69 was significantly higher in no HC users (p<0.001). A trend towards significantly higher proportions of $CD8^{+}CD69^{+}$ cells (p<0.06) and the MFI of HLADR (p=0.057) was also observed.

On the other hand, in circulating $CD8^{+}$ T cells, the MFI of CD38 was significantly higher in DMPA using FSW (p=0.032). In non-SW, the markers that were significantly higher in DMPA users were: CCR5 MFI (p=0.002), proportion of $CD8^{+}CD69^{+}$ cells (p<0.001), $CD8^{+}CD38^{+}$ cells (p=0.001),

CD38 MFI (p=0.005), $CCR5^{+}$Tc17 cells (p=0.05) and the MFI of HLA-DR (p=0.006). The MFI of CD69 was however significantly higher in no-HC using non-SW (p<0.001).

Impact of sex work in the context of DMPA use

The levels of $CD8^{+}$ T cell activation and frequencies of Tc17 cells were comparable between DMPA users among FSW and non-SW, and this observation held true for both FGT and blood-derived T cells.

4.5 Comparison of cytokine expression

The next analysis focused on determining the impact of DMPA use and sex work on the expression of cytokines and chemokines that modulate inflammation.

4.6 Impact of sex work on cytokine expression

FGT cytokines

IFN-γ was the only cytokine with significantly lower concentration in FSWs (0.4pg/ml vs. 3.3pg/ml, p<0.001, figure 12A) in this comparison. No differences were observed for other cytokines.

Plasma cytokines

For the women using no HC, the sex worker group had lower plasma concentration of MCP-1 (224.7pg/ml vs. 365.2pg/ml p=0.003), TNFα (10.03pg/ml vs. 12.13pg/ml, p=0.045), and IL-17 (4.91pg/ml vs.7.96pg/ml, p=0.010), relative to the non-sex worker group (Figure 12B).

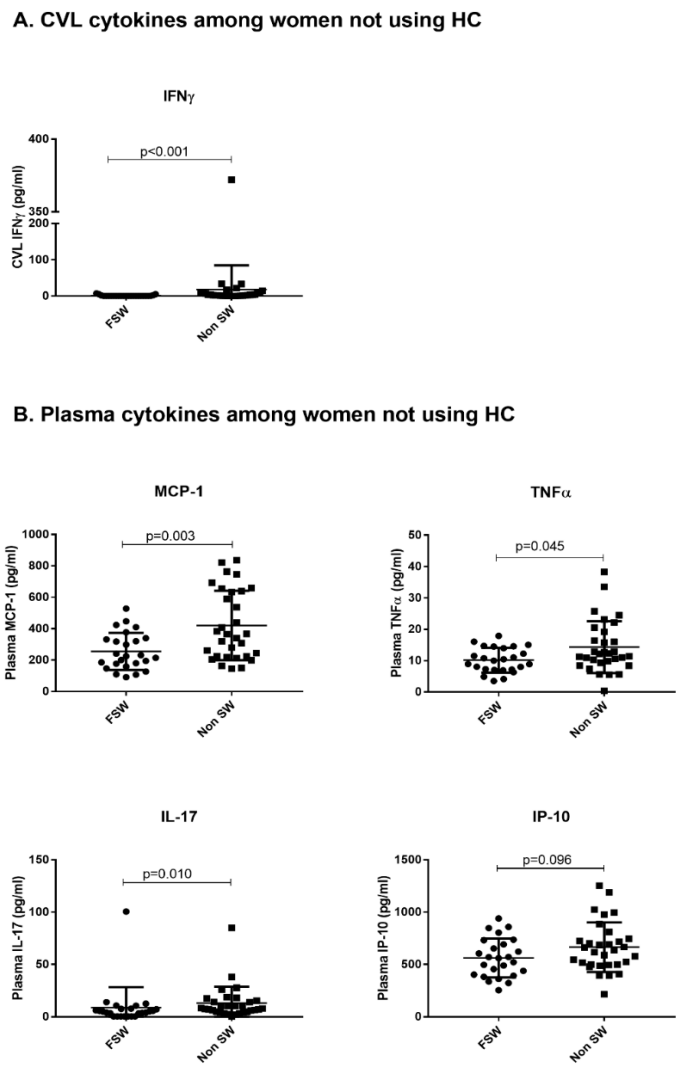

Figure 11. The effect of sex work on cytokine expression among women not using hormonal contraceptives.

4.6.1 Impact of DMPA on cytokine expression

FGT cytokines

Among FSW groups, no significant differences in FGT cytokine expression were observed between DMPA and no HC users, except for MCP-1 which was higher in no HC users (9.9 pg/ml vs. 106.9 pg/ml, p=0.001). Among the non-SW, only IFNγ (0.4 pg/ml vs. 3.3 pg/ml, p=0.001) and MCP-1 (23.7 pg/ml vs.74.8 pg/ml, p=0.011) were significantly higher in the no HC users. No significant differences in expression of other FGT cytokines were observed between DMPA and no HC users.

Plasma cytokines

Analysis of FSW groups revealed that IL-10 (4.8 pg/ml vs. 1.4 pg/ml, p=0.039) was significantly increased in DMPA users compared to those not using any HC (Figure 13A). No differences were seen between the two FSW groups for all other cytokines. Among the non-sex worker groups, DMPA use was characterized by significantly higher concentration of IFNγ (13.3pg/ml vs. 8.2pg/ml, p=0.043), MIG (1733pg/ml vs. 118.3pg/ml, p<0.001) and sCD40L (1457 pg/ml vs. 257.3pg/ml, p<0.001) in the blood. On the other hand, the non-sex working women not using HC had higher levels of IL-17 (3.85pg/ml vs. 7.96pg/ml p<0.001), MCP-1 (184.5pg/ml vs. 365.2pg/ml p<0.001) and IL-1RA (7.8pg/ml vs. 40.3pg/ml p<0.001) than DMPA users (Figure 13B).

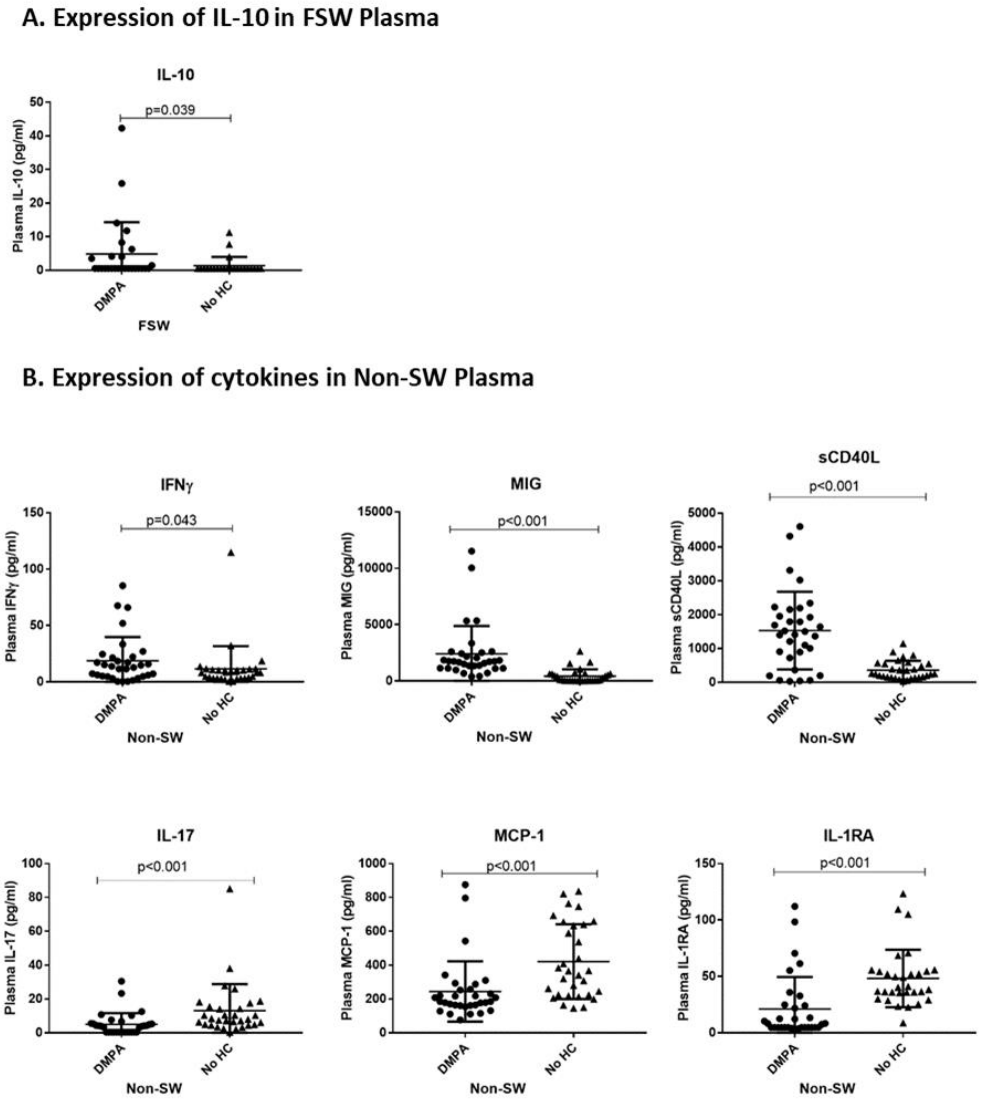

Figure 12. The impact of DMPA on plasma cytokine expression

4.6.2 Impact of sex work on cytokine expression during DMPA use

Analysis of the combined effect of sex work and DMPA use on cytokine expression showed no differences in mucosal cytokine expression in FSWs using DMPA versus non-sex workers on DMPA except for IL-1β.

For plasma cytokines, FSWs had higher levels of IL-17 (5.67pg/ml vs. 3.85pg/ml, p=0.035) and MCP-1 (251.8pg/ml vs. 184.5pg/ml, p=0.050), but lower levels of MIP-3 (10.8 pg/ml vs. 15.3 pg/ml, p=0.008). The levels of IL-1RA also trended towards being higher (15.78pg/ml

vs.7.825pg/ml, p=0.071) in FSWs (Figure 14). All other plasma cytokines were similarly expressed in FSWs and non-sex workers using DMPA.

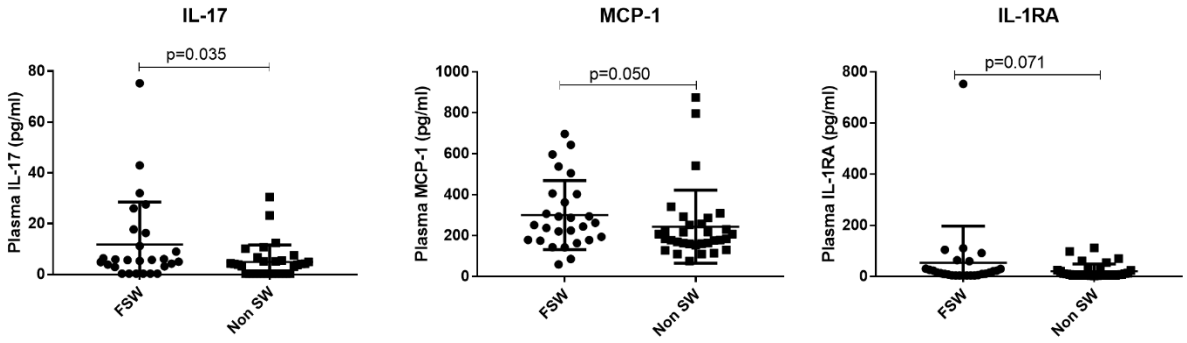

Figure 13. Comparison of plasma cytokine expression among women using DMPA

4.6.3 Expression versus non-expression of cytokines

To determine if DMPA use influenced expression or non-expression of cytokines, participants were classified as either expressors or non-expressors for each cytokine and X2 tests were done to determine the differences in proportions of expressors and non-expressors in each group. Expressors were those that had detectable cytokine concentrations, while non-expressors were those that had concentrations below the limit of detection of each individual cytokine.

Using this classification, it was observed that in non-SW, there was a greater proportion (76.7%) of DMPA users not expressing IFNγ at the genital tract, while in the no HC majority (86.7%) of those were expressors. No difference was observed for plasma IFNγ, or other cytokines overally.

Table 4. Comparison of the proportions of women expressing or not expressing IFNγ

	FSW			**Non-SW**		
	DMPA	**No HC**	***p-value***	**DMPA**	**No HC**	***p-value***
	n (%)	**n (%)**		**n (%)**	**n (%)**	
CVL						
Expressors	11 (40.7%)	6 (24.0%)	*0.245*	7 (23.3%)	26 (86.7%)	***<0.001***
Non-Expressors	16 (59.3%)	19 (76.0%)		23 (76.7%)	4 (13.3%)	
Plasma						
Expressors	27 (100.0%)	22 (88.0%)	*0.104*	27 (90.0%)	28 (93.3%)	*>0.999*
Non-Expressors	0 (0.0%)	3 (12.0%)		3 (10.0%)	2 (6.7%)	

4.7 Cytokine/chemokines profiles that distinguish DMPA and No-HC use

Partial least squares discriminant analysis (PLS-DA) was used to identify combinations of cytokines/chemokines that best predict the use of either DMPA or No-HC. For this analysis, both FSW and non-SW groups were analyzed together as either DMPA or no-HC users.

FGT Cytokines

A less distinct separation of DMPA and no-HC users on either of the PLS components was observed (Figure 15A). The model was predictive with an AUC of 0.66 (p=0.0023, Figure 15B). MCP-1, MIG, IP-10 and IL-1β had the highest variable importance scores in the model (Figure 15C. Univariate analysis of these cytokines confirmed significantly higher expression of MCP-1, IP-10 and IL-1β in no-HC users (Figure 15D).

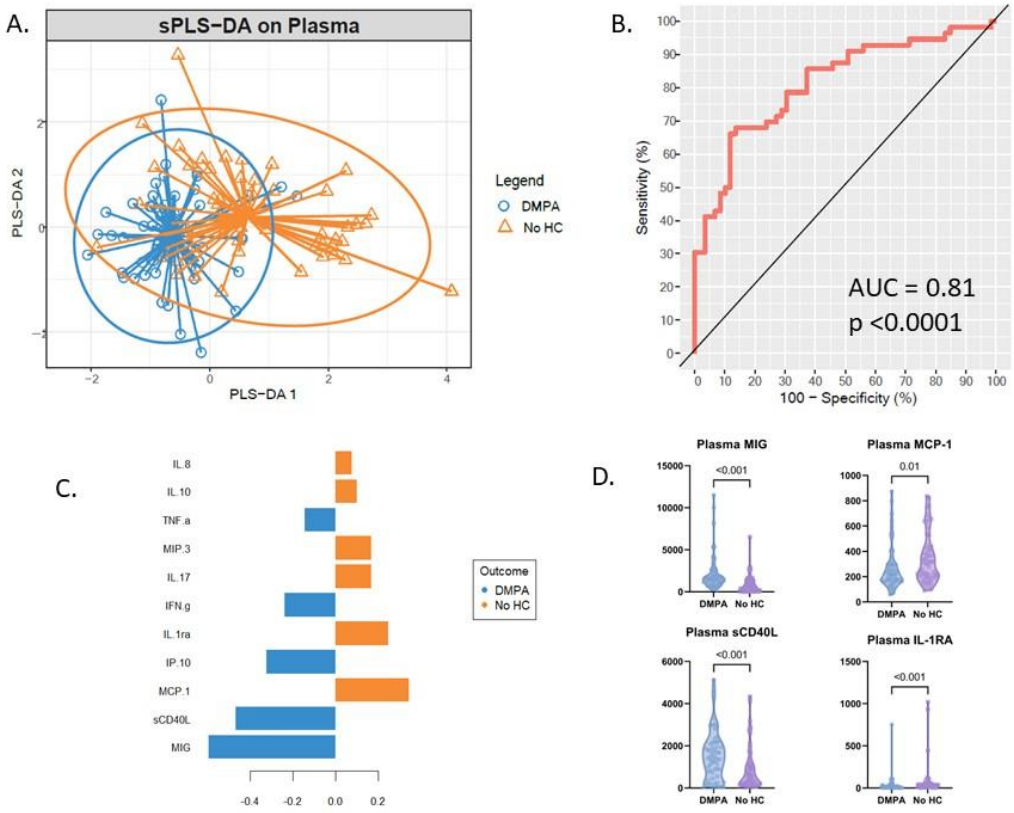

Figure 14. Identification of multivariate mucosal cytokine profiles associated with DMPA and No HC Use

Plasma Cytokines

PLS component 1 of the PLS-DA model of mucosal cytokines best distinguished DMPA users from the no-HC users, with a clear separation of the two groups seen on component 1 (Figure 16A). The model was predictive with an AUC of 0.81 ($p<0.0001$, Figure 16B). Importantly, the cytokine score loadings on component 1 and variable importance scores demonstrated that MIG, sCD40L, MCP-1 and IP-10 were the most important cytokines in classifying DMPA and no-HC users (Figure 16C). Side-by-side univariate comparison of the same cytokines further revealed significantly higher levels of MIG and sCD40L in women using DMPA (Figure 16D).

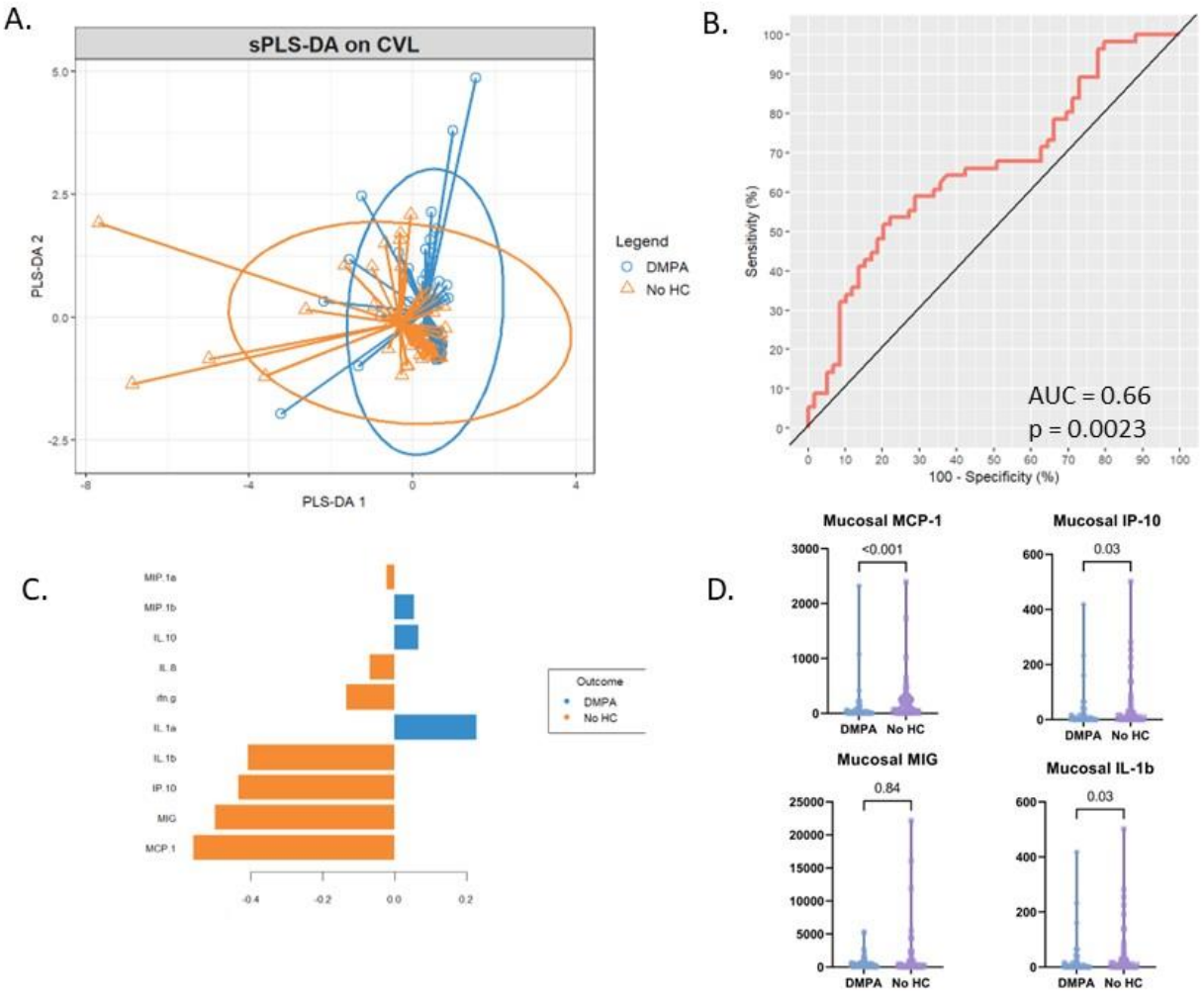

Figure 15. Identification of multivariate plasma cytokine profiles associated with DMPA and no HC Use

4.8 The effect of DMPA on Cortisol and Thyroid Hormones

The levels of cortisol, T3 and T4 in the plasma of both FSW and non-SW were assessed to determine if hormonal contraception affects the expression of these immune-modulating hormones.

In FSW, the median concentrations of cortisol were 76.6ng/ml (log 1.88ng/ml) and 9.0ng/ml (log 0.95ng/ml) for DMPA and no HC users, respectively. The median concentrations of T3 were 1.12ng/ml (log 0.05ng/ml) for DMPA users and 0.37ng/ml (log -0.43ng/ml) for no HC. respectively, while for T4 the values were 42.7ng/ml (log 1.63ng/ml) and 13.6ng/ml (log 1.13ng/ml) for DMPA and no HC respectively. For all the hormones, DMPA users expressed significantly higher levels compared to those not using any hormonal contraceptive (Figure 17A).

In non-SW, the median concentrations of cortisol were 51.8ng/ml (log 1.71ng/ml), and 47.0ng/ml (log 1.67ng/ml) in DMPA and no HC users, respectively. The median concentrations of T3 were 1.28ng/ml (log 0.11ng/ml) and 0.46ng/ml (log -0.87ng/ml) and for T4 they were 41.4ng/ml (log 1.61ng/ml) and 44.3ng/ml (log 1.65pg/ml) for DMPA and no HC users, respectively. No statistical differences were observed in the expression of cortisol and T4 among the groups. However, levels of T3 were significantly higher in the DMPA group compared to no HC users (Figure 17B).

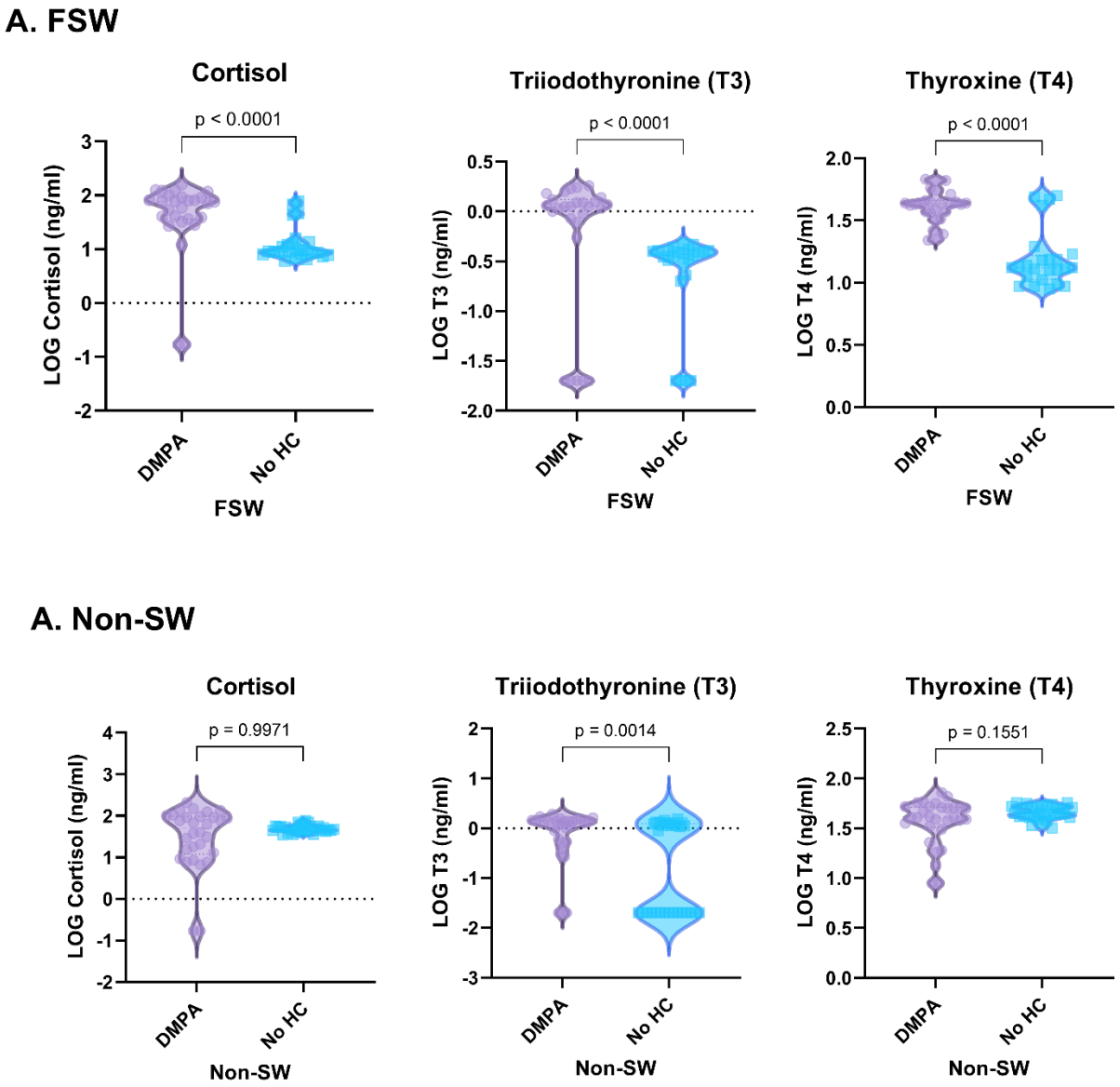

Figure 16.Expression of Cortisol, T3 and T4 in FSW and non-SW

Next, differences in hormone expression between the FSW and non-SW groups were assessed. Non-SW not using any HC had significantly higher levels of cortisol compared to their sex worker counterparts. No differences were observed in T3 expression among the groups. For T4, non-sex workers not using HC had significantly higher levels compared to their FSW counterparts. Table 5 shows the comparisons between the groups using DMPA and no HC.

Table 5. Log10 expression of Cortisol, T3 and T4 in FSW and Non-SW

	DMPA Users			No HC Users		
	FSW	**Non-SW**	*p*	**FSW**	**Non-SW**	*p*
Cortisol	1.88(1.56 - 1.97)	1.7(-1.07 - 1.98)	0.474	0.95(0.90 - 1.12)	1.66(1.64 - 1.74)	**<0.001**
T3	0.05(-0.11 - 0.12)	0.11(-0.27 - 0.16)	0.537	-0.43(-0.61 - 0.39)	-0.87(-1.7 - 0.09)	0.447
T4	1.63(1.56 - 1.66)	1.61(1.51 - 1.71)	0.345	1.12(1.02 - 1.22)	1.65(1.61 - 1.72)	**<0.001**

*Data are Median (Interquartile Range)

4.8.1 Effect of Cortisol, T3 and T4 on $CD4^+$ T cells

Since T cells express glucocorticoid and TH receptors, the next analysis performed was an exploratory association analysis between hormones and markers of T cell activation to evaluate the impact of these hormones on immune activation in the context of hormonal contraception.

Effect on mucosal T cells

Since $CD4^+$ T cells at the genital mucosa are the primary targets of HIV infection, an analysis was performed to determine whether peripheral hormone levels correlated with activation of HIV target cells in the mucosal compartment. DMPA-using sex workers had a negative correlation between cortisol levels and $CD4^+CCR5^+$ T cell proportion (r= -0.4263, p=0.042). T3 concentration negatively correlated with the MFI of activation markers CD38, CD69 and HLADR (r= -0.8085, p<0.001; r=0.4352, p=0.038; r= -0.5971, p=0.002 respectively), while T4 levels were positively correlated with the proportion of $CD4^+CCR5^+$ (r= 0.6363, p=0.001), $CD4^+CD38^+$ (r=0.4251, p=0.043) and $CD4^+HLADR^+$ (r= -0.5971, p=0.002) T cells (Figure 18). In addition, T4 was positively correlated with the MFI of CD69 (r=0.5128, p=0.012) and HLADR (r=0.4551, p=0.029) on $CD4^+$ T cells (Figure 18).

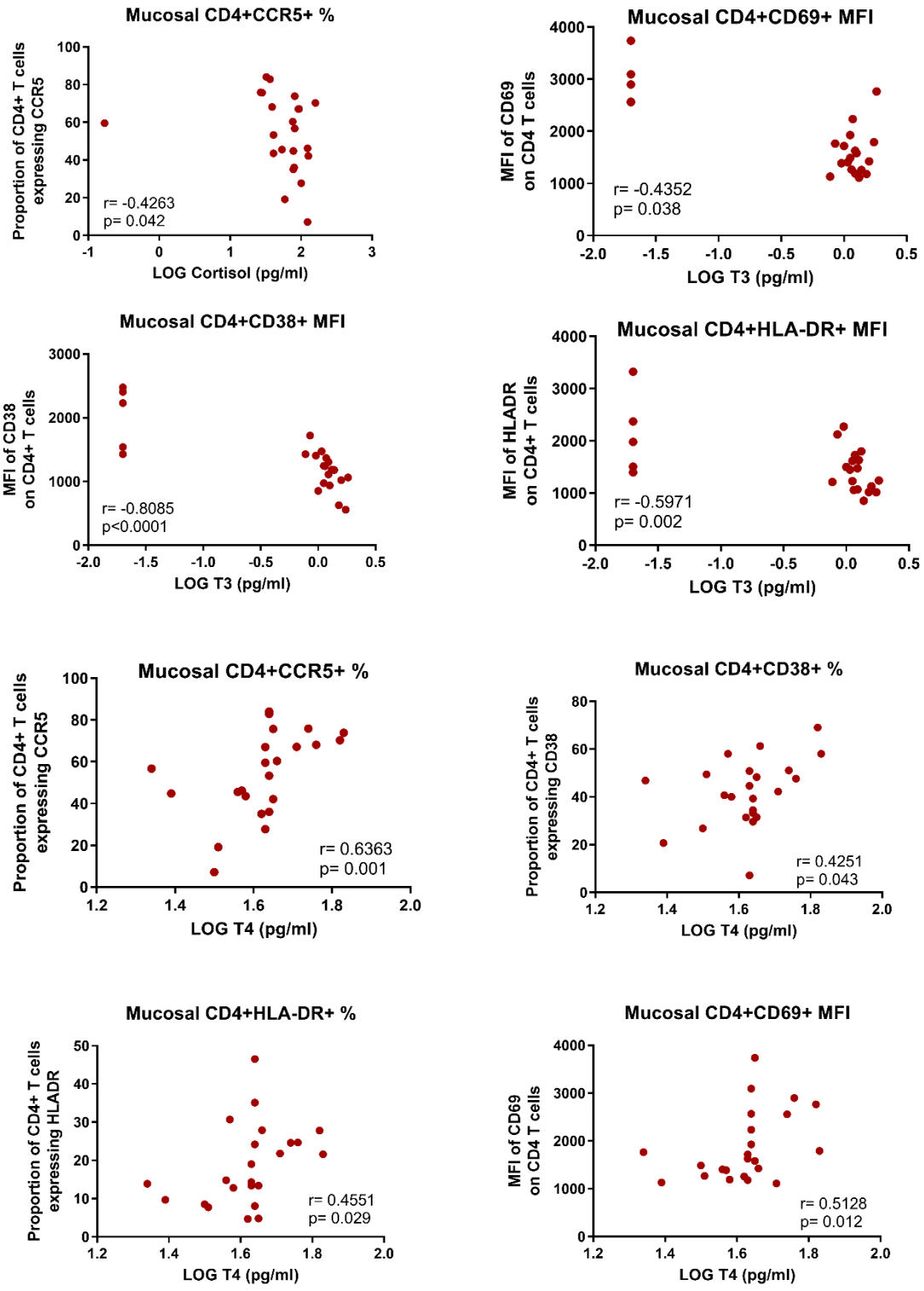

Figure 17. Correlation of hormones with activation markers of mucosal CD4+ T cells among FSW using DMPA

In no HC users, cortisol was positively correlated with the proportion of $CD4^+CCR5^+$ T cells (r=0.4633, p=0.029) and the MFI of CD69 (r=0.5588, p=0.007), while the same markers were negatively correlated with T3 expression. Blood level of T4 positively correlated with the MFI of CD38 (r=0.4809, p=0.023) and CD69 (r=0.4379, p=0.042) on $CD4^+$ T cells (Figure 19).

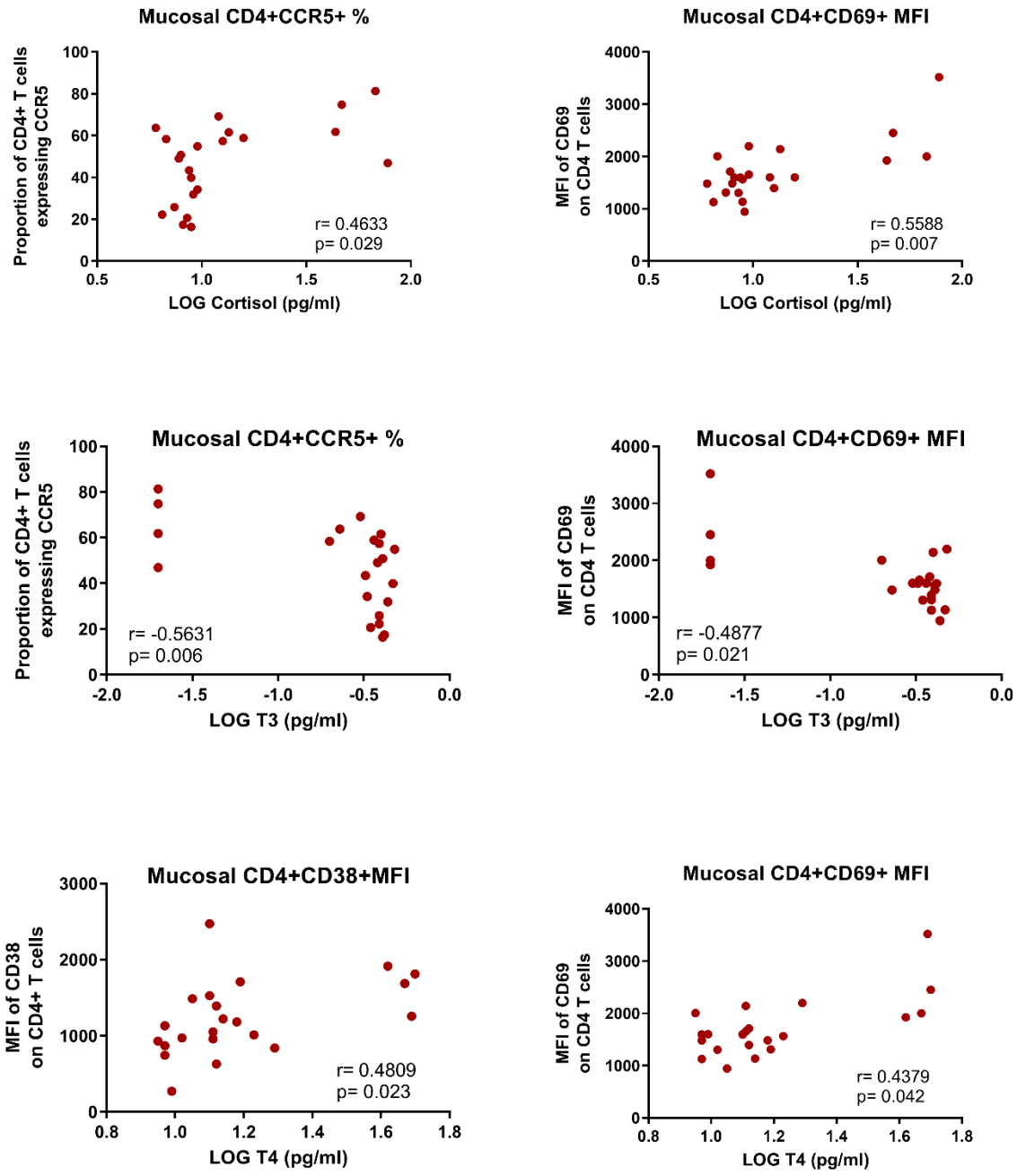

Figure 18. Correlation of hormones with activation markers of mucosal $CD4^+$ T cells among FSW not using HC

In non-sex workers, DMPA users displayed minimal correlations between the blood hormone levels and markers of T cell activation. Cortisol concentration correlated with the MFI of CCR5 (r=0.3682, p=0.049) and T3 inversely correlated with CD69 MFI (r= -0.5014, p=0.006), (Figure 20A). No effect of T4 on T cell activation markers was observed. On the other hand, a number of T cell markers were impacted by the three hormones in non-sex workers not using any HC. Cortisol was positively correlated with the MFI of CCR5 (r=0.4774, p=0.01), but negatively with the number of $CD3^+$ lymphocytes (r= -0.506, p=0.006) and $CD3^+CD4^+$ T cells (r= -0.501, p=0.006). T3 also negatively correlated with the number of $CD3^+$ lymphocytes (r= -0.3807, p=0.045), but positively with the MFI of CCR5 (r=0.4982, p=0.007). Finally, T4 had a positive correlation with the relative proportion of $CD4^+HLADR^+$ T cells (r= -0.4053, p=0.032), (Figure 20B).

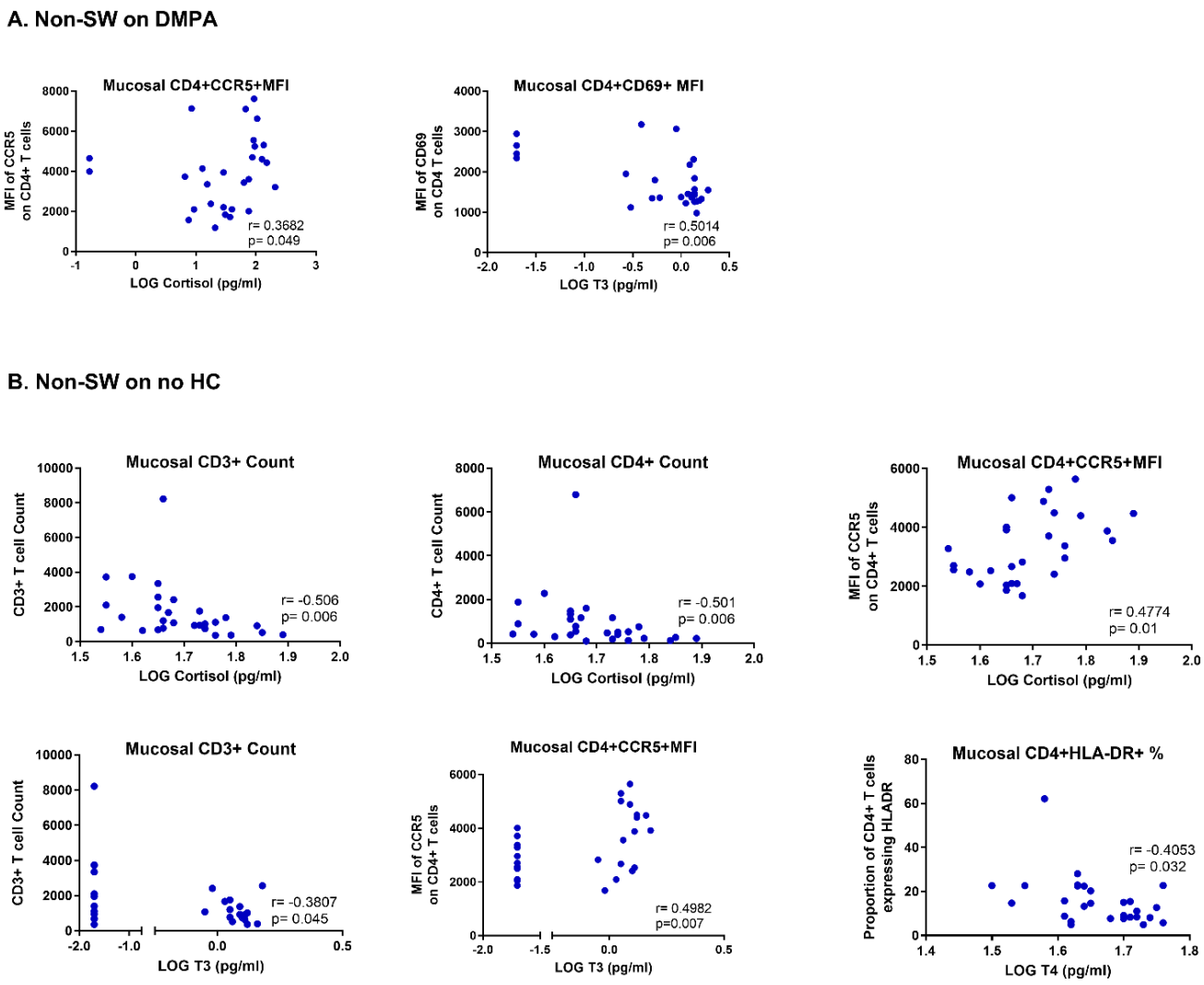

Figure 19.Correlation of hormones with activation markers of mucosal $CD4^+$ T cell activation among non-SW groups

In summary, when assessing the impact of hormones on mucosal T cells, Cortisol and T3 negatively correlated with markers of T cell activation and HIV target cells in DMPA-using FSW while T4 increased the expression of the same markers. These observations were to the opposite direction in no HC users. In addition, in non-SW not using HC, the three hormones had an association not only with the number of T cells at the mucosa but also their activation status.

Effect on peripheral T cells

The next analysis performed was an association analysis between hormones and circulating T cell activation to evaluate the impact of these hormones on systemic immune activation in the context of hormonal contraception. In FSW using DMPA, cortisol was inversely correlated with the median fluorescent intensity (MFI) of HLA-DR on $CD4^+$ T cells (r= -0.4359, p=0.02) on a per-cell basis. T3 levels were inversely correlated with the MFI of CD69 (r= -0.471, p=0.01) and HLA-DR (r= -0.5795, p=0.002). Levels of T4 positively correlated with the proportion of $CD4^+CCR5^+$ T cells (r=0.441, p=0.02) but negatively correlated with the MFI of CCR5 on these cells (r= -0.4251, p=0.03), Figure 21A. In sex workers not using HC, cortisol had an inverse correlation with the proportion of $CD4^+CD69^+$ (r= -0.4447, p=0.03) and $CD4^+HLADR^+$ (r= -0.437, p=0.04) T cells. No correlations with T3 were observed. T4 level was positively correlated with the MFI of CD38 (r=0.4312, p=0.04) and CD69 (r=0.5316, p<0.01) on $CD4^+$ T cells. A negative correlation of T4 levels and the proportion of $CD4^+CCR5^+$ (r= -0.492, p=0.02) and $CD4^+HLADR^+$ (r= -0.5548, p<0.01) T cells was also observed (Figure 21B).

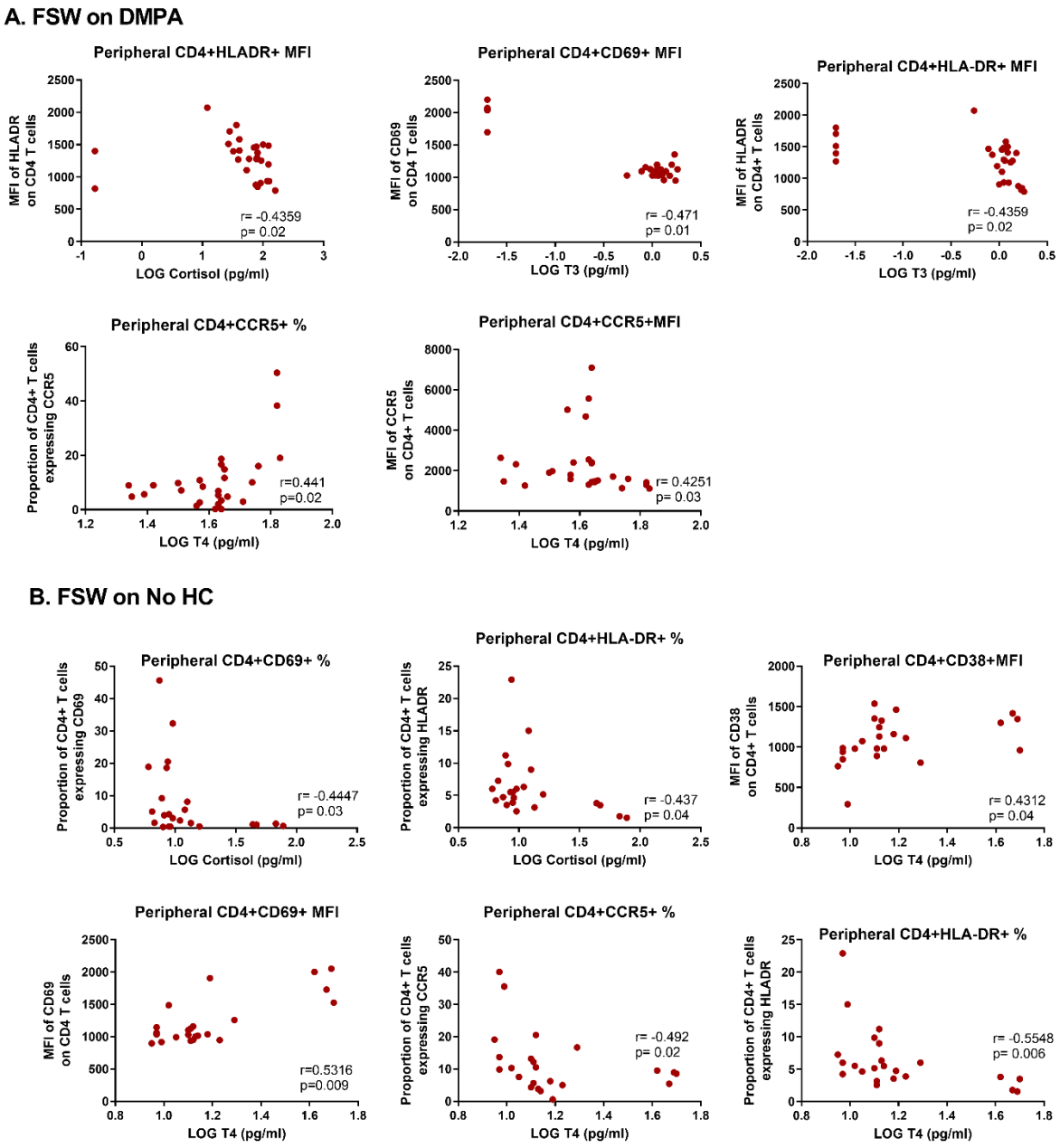

Figure 20. Correlation of hormones with activation markers of peripheral CD4+ T cell activation among FSW

In non sex workers using DMPA, cortisol was positively correlated with the relative number of circulating $CD3^+$ lymphocytes (r=0.3781, p=0.04) and $CD3^+CD4^+$ T cells (r=0.4082, p=0.03). T3 expression positively correlated with the proportion of $CD4^+CD69^+$ T cells (r=0.3883, p=0.03) and the MFI of CCR5 (r=0.4223, p=0.02) on $CD4^+$ T cells. T4 levels correlated positively with the MFI of CD69 (r=0.4670, p<0.01) on $CD4^+$ T cells (Figure 22A). In non-DMPA users, cortisol

showed no correlation with any marker of T cell activation. T3 level had a negative correlation with the proportion of $CD4^{+}CD69^{+}$ T cells (r= -0.4848, p<0.01) and the MFI of CD38 (r= -0.4069, p=0.03). Finally, T4 had an inverse relationship with the MFI of CCR5 (r= -0.4081, p=0.03) on peripheral $CD4^{+}$ T cells. These data are shown in Figure 22B.

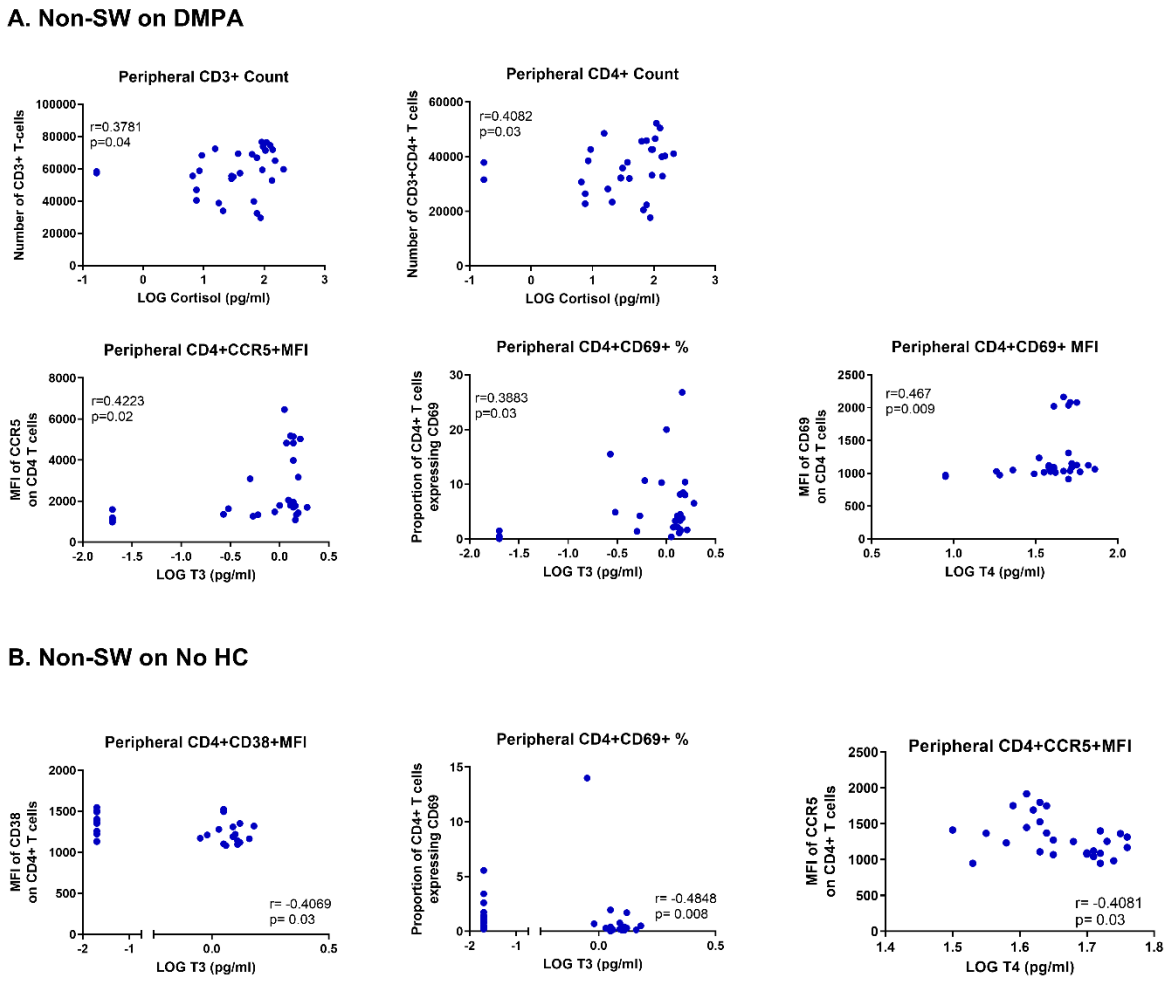

Figure 21. Correlation of hormones with activation markers of peripheral CD4+ T cell activation among non-SW

Overall, these results show that Cortisol and T3 levels in DMPA-using sex workers negatively correlated with markers of T-cell activation in peripheral blood. However, the same markers positively correlated with Cortisol, T3 and T4 positively in DMPA-using non sex workers.

CHAPTER 5

5 DISCUSSION

Understanding the biological mechanisms that underlie the epidemiological connection between DMPA usage and HIV acquisition is of significant importance for public health, especially in Sub-Saharan Africa where high HIV prevalence and DMPA use overlap. This study was designed to investigate the role of DMPA use on mucosal and peripheral inflammation and HIV target cell frequency, and immunomodulatory hormone expression in women from Nairobi, Kenya. These findings show that DMPA use is associated with T cell activation and inflammation at the genital tract of women drawn from the general population and in high-risk FSW, which may in turn contribute to higher risk of HIV acquisition.

The initial observation when describing participant's sociodemographic characteristics was that majority of non-SW who were not using hormonal contraception had more years of education compared to those using DMPA. Education is one of the social factors that greatly influence women's contraceptive choices(Mutumba et al., 2018), as evidenced by the strong correlation between education and contraceptive uptake in SSA (Larsson & Stanfors, 2014a; Shapiro & Tambashe, 1994; Wuni et al., 2018). This could be due to a heightened sexual health awareness in women with more education. Interestingly, Larsson *et al* have reported that unlike other countries within SSA, in Kenya, education matters more in women's decision between use and non-use, rather than choice of a particular contraceptive(Larsson & Stanfors, 2014b). Marital status is another factor that determines contraceptive use. Indeed, 25% of the non-SW using no HC were married, while majority (70%) of the ones on DMPA were married. This finding aligns with prior studies in Kenya which showed that married women tend to favor long-term reversible contraception(Jalang'o et al., 2017; Ndugwa et al., 2011). However, it's important to note that our present study was not specifically designed to investigate the contribution of education and marital status on contraceptive preferences, which limits our ability to interpret this data comprehensively.

The primary aim of this study was to investigate the impact of DMPA on the immune response in a cohort of Kenyan women who were not involved in sex work, as well as those actively engaging in sex work and enrolled in a research and healthcare program in Nairobi.

Among non-SW, DMPA users had significantly increased numbers of blood-derived $CD4^+CD38^+$ and $CD4^+HLADR^+$ T cells along with increased intensity of CCR5 on $CD4^+$ T cells. Both CD38 and HLA-DR are surface markers of activation with CD38 expressed mid-stage of cell activation, whereas HLA-DR typically appears later in the T cell activation cascade(Card et al., 2009b). The MFI of CCR5 on $CD4^+$ T cells was also increased in the DMPA group. CCR5 is a chemokine receptor whose primary role is in chemotactic homing of effector and memory T cells to extravascular sites (Fukada et al., 2002). It is also utilized by HIV as the co-receptor for entry into susceptible target cells. *In-vitro* studies have previously shown that MPA (a longer lasting progestin) not only increases the surface expression of CCR5 on activated T cells, but also inhibits its downregulation(R. P. Huijbregts et al., 2013), and also increases its mRNA expression on tissues. Collectively, our findings indicate that chronic T cell activation and an increased CCR5 density on CD4+ cells in the bloodstream could be a mechanism through which DMPA influences T cell migration and susceptibility to HIV.

Furthermore, in the same non-SW, proportions of endocervical $CD3^+$ cells and activated $CD4^+CD69^+$ and $CD4^+38^+$ T cells were significantly higher in DMPA users. Chandra *et al* have previously reported a significant increase in the numbers of $CD3^+$ lymphocytes in vaginal tissue following short term DMPA use (Chandra et al., 2013) suggesting an influx of T cells to the mucosa upon initiation of DMPA. CD69 is one of the earliest activation markers(Ziegler et al., 1994), in addition to being a tissue retention marker(Kumar et al., 2017; Turner et al., 2014). By inhibiting the expression of sphingosine-1-phosphate receptor-1 (S1PR1), – a promoter of cell egress towards the bloodstream – CD69 impairs egress of T cells from tissues and promotes residency. Genital $CD4^+CD69^+$ T cells are recognized as preferential targets of HIV infection(McKinnon et al., 2011b) given their capacity to create a transcriptionally favorable environment viral replication. Therefore, our findings indicate that DMPA use not only drives activation of T cells at the FGT, but also results in the retention of a pool of the activated cells in the mucosa. This retention potentially exposes the cells to HIV upon sexual exposure.

DMPA also had a noticeable impact on the expression of innate cytokines and chemokines. Among the non-SW groups, individuals using DMPA exhibited higher systemic expression of IFNγ, IL-10, MIG, and sCD40L, which suggests a heightened level of inflammation compared to non-DMPA users. Interestingly, women who did not use hormonal contraception displayed a different

cytokine profile, marked by elevated levels of IL-17 and IL-1RA, an anti-inflammatory cytokine that inhibits the inflammatory actions of other IL-1 cytokines(Palomo et al., 2015). Notably, no differences were observed in the cytokine profiles in the female genital tract between the two groups of non-SW. In summary, these findings indicate that, in non-SW, DMPA promotes inflammation in the bloodstream and immune activation at the FGT, potentially increasing the risk of HIV infection.

A majority of non-SW using DMPA did not express IFNγ at the genital tract. Huijbregts *et al* have shown that DMPA inhibits production of IFNγ by T cells *in vitro*(R. P. H. Huijbregts et al., 2013). The same study reported lower systemic and genital of IFNα in women using DMPA, alongside a reduced capacity of plasmacytoid DCs to respond to activation by Toll-like Receptors (TLR) ligands(R. P. H. Huijbregts et al., 2013). Separately, decreased proportions of IFNγ–producing $CD4^+$ and $CD8^+$ T cells has been associated with prolonged DMPA use(Matubu et al., 2021).While these studies looked at systemic IFNγ expression, our study adds further data showing that at the cervicovaginal mucosa, fewer women using DMPA express IFNγ compared to no HC users. This observation was specifically noticeable in non-sex workers, suggesting a potential variance in the interferon response to DMPA between them and sex workers. In any case, suppression of effector mechanisms such as interferon response at HIV's portal of entry may favor viral replication in founder cell populations by inhibiting early immune cell signaling and response to infection. This could be a potential mechanism contributing to HIV acquisition during DMPA use.

Cytokines and chemokines can be produced by various cell types in response to diverse stimuli, including pathogens, as well as endogenous sex hormones and hormonal contraceptives. Since the simultaneous expression of multiple cytokines/chemokines occurs during a typical immune response, it is important to assess their concentrations not just in isolation but as components of a complex, interrelated system. Using the PLS-DA approach that considers the multivariable combinations of cervicovaginal and plasma cytokines, it was noted that plasma MIG (CXCL9), sCD40L, MCP-1 (CCL2) and IP-10 (CXCL10) were influential pro-inflammatory markers that differentiated DMPA use from no HC, with MIG being the most important. Indeed, even in univariate comparison, MIG levels were significantly higher in DMPA users. MIG is an inflammatory chemokine with a crucial role in the recruitment of activated T cells to sites of

infection, as the other three chemokines. An association of MIG with DMPA use further underscores the role of DMPA in elevating inflammation.

This study had a unique advantage of including both FSW and non-SW, as it was crucial to ascertain whether DMPA induces a comparable immune response pattern in both low-risk and high-risk women. This is particularly significant, given that involvement in sex work has previously been associated with alterations in the immune response. Unprotected heterosexual intercourse leads to exposure of the FGT to immune stimulants such as spermatozoa, leukocytes, cytokines, and even pathogens present in seminal fluid. Sexual activity thus triggers an inflammatory response marked by expression of pro-inflammatory cytokines and the recruitment of activated T cells to the genital mucosa(Sharkey et al., 2012) – a milieu that is favorable for HIV transmission (Haase, 2011). Sex work therefore results in constant exposure of the FGT to immune stimulants from multiple sources, especially with incorrect and inconsistent condom use with clients. This frequent immunostimulatory exposure is thought to possibly induce development of an immune tolerance phenotype.

The early years of a woman's initiation of sex work are associated with a heightened risk of HIV seroconversion. Epidemiological analysis has shown that in the Pumwani cohort, HIV incidence in FSW engaged in sex work for less than two years was 3.5%, while it was 1.5% for those with over two years of experience (McKinnon et al., 2015a). The same study also revealed a 23% decline in the risk of HIV seroconversion for each additional year in sex work (McKinnon et al., 2015a). Although the precise mechanisms behind this declining susceptibility to HIV in FSWs have not been fully clarified, biological factors may play a role. Previous research has suggested that active engagement in sex work is associated with a blunted secretion of proinflammatory cytokines and a decrease in T cell activation at the FGT, with these observations becoming more pronounced with longer durations of sex work(Lajoie et al., 2014). Therefore, it is possible that sex work and hormonal contraception are converging immune stimulants for FSWs, directly impacting their susceptibility to HIV. Thus, the present study aimed to determine the influence of sex work and DMPA on the immune response by comparing FSW to non-SW not using hormonal contraception, as well as FSW to non-SW on DMPA.

Compared to non-SW, FSWs using no HC have lower T cell activation levels and CCR5 expression. In the bloodstream, FSWs had significantly lower proportion of $CD4^+CCR5^+$ cells and expression of $CD69^+$ and $CD38^+$ on $CD4^+$ T cells, while at the genital tract, lower proportion of $CD4^+CCR5^+$ cells and expression of the activation markers CD38 and CD69 was observed. This activation pattern was also observed in the $CD8^+$ fraction. They also had a lower plasma concentration of proinflammatory cytokines (IL-17, TNFα) and of chemotactic proteins (MCP-1) as well as lower level of IFNγ in CVL. These findings are reminiscent of previous reports of an immune quiescent state in FSW (Card et al., 2009a; Lajoie et al., 2012b), which is quite evident even within a year of active sex work and gets more pronounced with cumulative years(Lajoie et al., 2014). It was also demonstrated in our pilot study, that interruption of sex work significantly affects systemic memory T cell phenotypes, underscoring the immune impact of sex work(Omollo et al., 2016). Therefore, once again, these data show that sex work is associated with reduction in immune activation. The factors driving this immunological tolerance-like state remain to be fully elucidated. One potential explanation is the development of tolerance to allo-antigens from sexual partners. Allogeneic immunity and tolerogenic responses to a partner's cells has been documented (Kingsley et al., 2009), pointing to the idea that extended exposure to sex-related antigens from various sources (as encountered in the context of sex work) could result in a decrease in baseline immune activation.

Remarkably, in contrast to the reduced immune activation made in FSW not using hormonal contraception, when the immune responses of FSW and non-SW using DMPA were compared, no differences were found. In fact, they exhibited similar levels of immune activation and inflammation. Thus, while engagement in sex work would typically result in reduced immune activation, the use of DMPA leads to an increase in T cell activation and inflammation in FSWs, reaching a level similar to that observed in non-SW using DMPA. This interesting finding implies that DMPA use among FSW mitigates the potent immunomodulatory effects of sex work, which could have significant implications for HIV susceptibility within this group. Sex workers are already at higher risk of HIV infection, and are considered a key population in HIV prevention programs. Any factor that modifies the immune response in these women to a more activated phenotype would certainly have consequences for HIV susceptibility. A number of observational studies have linked the use of DMPA with an increased risk of HIV acquisition among FSW(Lavreys et al., 2004; H. L. J. Martin et al., 1998; McKinnon et al., 2015b). For example, in

the Mombasa cohort, FSW who used DMPA had a two-fold likelihood of contracting HIV compared to their counterparts who did not use any hormonal contraception(Lavreys et al., 2004; H. L. J. Martin et al., 1998). While the mechanisms for this association may not be clear, our current study suggests that the absence of immune downregulation during sex work among FSW using DMPA may be a contributing factor. It is conceivable that inflammation and the subsequent activation of HIV target cells occur to a greater extent in FSW using DMPA, thereby elevating their risk of infection. Conversely, those who do not use any HC are able to downregulate inflammation, thus reducing their susceptibility to HIV infection. Further studies may be needed to elucidate the role of DMPA in upregulation of immune responses in FSW.

Finally, a mutual relationship exists between the endocrine and immune systems, and haemostatic changes in one system potentially affects the function of the other(Chryssikopoulos, 1997; Klein, 2021). It was noted that use of DMPA resulted in significantly higher levels other immunomodulatory hormones: cortisol and thyroid hormones. In FSW, use of DMPA resulted in significantly higher levels of cortisol. This finding is consistent with a previous report by Virutamasen *et al* of increased level of morning cortisol in DMPA users compared to non-users(Virutamasen et al., 1986). Although a progestin, DMPA can bind to the glucocorticoid receptor (GR), with a binding affinity similar to that of cortisol(Hapgood et al., 2018). Therefore, it is possible that DMPA competitively binds to the GR thus preventing the binding, and uptake, of cortisol into cells resulting in increased levels of cortisol in blood.

T3 expression was observed to be higher in DMPA users independent of sex work status, while T4 expression was only elevated in DMPA-using FSW. An increase in free T4 (but not free T3) levels has been reported in a cohort of Brazilian women 12 months post-initiation of DMPA (Quintino-Moro et al., 2019). Oral progesterone therapy is also associated with increased T4 levels(Sathi et al., 2013), suggesting a role for the progestin in thyroid function. A possible mechanism underlying these observations is the hypoestrogenic effect of DMPA. Oestrogens cause a decrease in the urinary clearance rate of the thyroxine-binding globulin (TBG), which increases its serum concentration and consequently reducing levels of free T3 and T4 (Bartalena & Robbins, 1992; Torre et al., 2020). By suppressing the secretion of oestrogen(L. Miller et al., 2000b), DMPA reduces the levels of TBG, thus, altering the fine regulation of plasma thyroid hormones and elevating fT4 and fT3 concentrations. The long-term physiologic relationship

between DMPA and thyroid hormones remains under-investigated and, therefore, the clinical implication of these observations remains unknown. It is notable that in our study, despite the increase in levels of T3 and T4 in DMPA users, participants were of general good health and none presented with clinical symptoms of thyroid disease. However, it is speculative that this effect of DMPA may be relevant to consider for women intending to initiate use of the contraceptive while already experiencing thyroid dysfunction.

The association of the alterations in hormone levels with markers of $CD4^+$ T cell activation may represent an understudied mechanism by which DMPA modulates the immune system. In FSW, cortisol levels correlated with mucosal CD4+CCR5+ T cells, the primary target for HIV, but this relationship differed in DMPA users who had higher plasma cortisol levels. Correlation between T4 levels and T cell activation markers was also observed in no HC users and was more pronounced in DMPA users. This suggests that cortisol and T4 levels influence CD4+ T cell activation, a significant factor in HIV susceptibility, particularly in the context of DMPA use. Furthermore, a differential response was also observed between FSW and non-SW, suggesting that sex work is a possible intermediate factor in how DMPA affects expression of immune-modulating hormones. Further studies needed to understand the relationship between these hormones and immune responses.

5.1 **Strengths and limitations**

This study has a number of strengths that deserve emphasis. Measures were taken to minimize potential confounders by excluding women with STIs and bacterial vaginosis – factors that have been identified as drivers of inflammatory perturbation at the genital tract. Additionally, given that seminal fluid exposure has been associated with inflammation and recruitment of immune cells at the FGT(Sharkey et al., 2012), the presence of semen was carefully controlled by testing for PSA. None of the women included in the study exhibited detectable PSA in their cervico-vaginal lavage at the time of sampling. Furthermore, only women who had been using contraception for at least 6 months were intentionally selected as a more stringent inclusion criterion. Additionally, the inclusion of FSWs allowed assessment of the impact of DMPA in women who are actually at high risk of HIV infection in a country with high HIV prevalence. Lastly, our wide approach which involved collecting paired genital and blood specimens for immunological assessment of cytokine/chemokines and lymphocytes, allowed us to analyze immune markers in both the

mucosal and peripheral compartments, providing a comprehensive understanding of DMPA's effect on the immune system.

Nevertheless, there are some limitations that are important to consider in the interpretation of the data presented herein. One is the sampling women who were not on any hormonal contraception 5-10 days post-menses. This is the proliferative/follicular phase of the cycle, and was chosen so as to minimize variability of the cycle among women with varying lengths of the menstrual cycle. It is also easier to stage the proliferative phase using verbal recall responses, since women can easily remember the beginning and end of their menses. However, the proliferative phase corresponds to the high-estrogen phase of the menstrual cycle and consequently, the results may not accurately reflect the predominant impact of endogenous progesterone, which characterizes the luteal phase occurring in the latter half of the menstrual cycle. The luteal phase has a variable length that makes it challenging to minimize sampling variability. This limits the interpretation of this study when comparing progestins with endogenous progesterone.

The cross-sectional study design only allows for one-time comparison among women using contraceptives of their choice. This restricts our ability to perform longitudinal evaluation of the immune response before and after initiation of DMPA. Another limitation is that *ex vivo* mechanistic studies to determine how DMPA, cortisol and thyroid hormone interact with glucocorticoid receptors on T cells were not performed and therefore, a causative link between DMPA use and changes in hormone levels and activity cannot be concluded from this study.
An additional possible limitation is the utilization of CVL for the analysis of cytokines and chemokines. Compared to swabs, CVL lacks the precision of site-specific measurements and is inherently diluted with the introduction of buffered saline. Nonetheless, CVL samples serve as valuable tools for assessing the overall state of inflammation in the FGT. Notably, previous studies, including those involving women from the Pumwani Community(Lajoie et al., 2012c, 2018), have successfully employed CVL. To maintain consistency with this prior research and to gain a more comprehensive understanding of genital inflammation, CVL was collected for the evaluation of proinflammatory cytokines.

Lastly, the low use of COC in the cohorts at the stime of the study meant that the study was statistically underpowered for COC comparisons so they were excluded from analyses. This limits

the findings as it was not possible to show the immune-endocrine impact of COC which has been associated with a lower risk of HIV acquisition(Balkus et al., 2016; Morrison et al., 2012).

5.2 **Conclusion**

This study has demonstrated that use of DMPA amplifies both genital and systemic immune activation, along with the elevation of CCR5 expression among non-sex workers. Furthermore, it reiterates that engagement in sex work diminishes immune activation at the genital tract. More importantly, while it is recognized that participation in sex work modulates the immune system, this study reveals, for the first time, that DMPA exerts a more potent immunomodulatory effect since its usage counteracts the impact of sex work on mucosal and peripheral immune responses. Moreover, these data suggest that both DMPA-associated changes in the endocrine system may contribute to the sex-steroid regulation of T cell activation. Collectively, these findings signify that DMPA alters the immune response and offers a biological mechanism for the increased susceptibility to HIV in women, particularly sex workers, using the contraceptive.

5.3 **Recommendations**

Basal genital inflammation is one of the factors contributing to the HIV pandemic in Sub Saharan Africa. Women in this region are reported to have elevated genital inflammation compared to those from other regions of the world(Kaul et al., 2015b). The mediators of this genital inflammation on the continent are not fully elucidated. Our study provides data to support the hypothesis that use of DMPA possibly influences genital inflammation and recruitment of HIV target cells to the mucosa. At a policy and program science level, there is need to enhance medical and HIV prevention counselling for women, especially sex workers, intending to use DMPA as a contraceptive. This counselling should take into account the potential individual HIV risk and offer HIV prevention options that are relevant and accessible to the individual. There is also need for policy bodies such as Ministry of Health and the Pharmacy and Poisons Board to clearly define the medical eligibility criteria for women intending to use DMPA, taking into account the national HIV indices. For basic scientists, there is need for further studies exploring the impact of DMPA on other innate immune cell subsets that are co-players in the HIV transmission cascade, such as dendritic cells and NK cells. These studies should explore the intricate link between DMPA and the hormone response of cells and longitudinal assessment of the immune quiescence phenotype.

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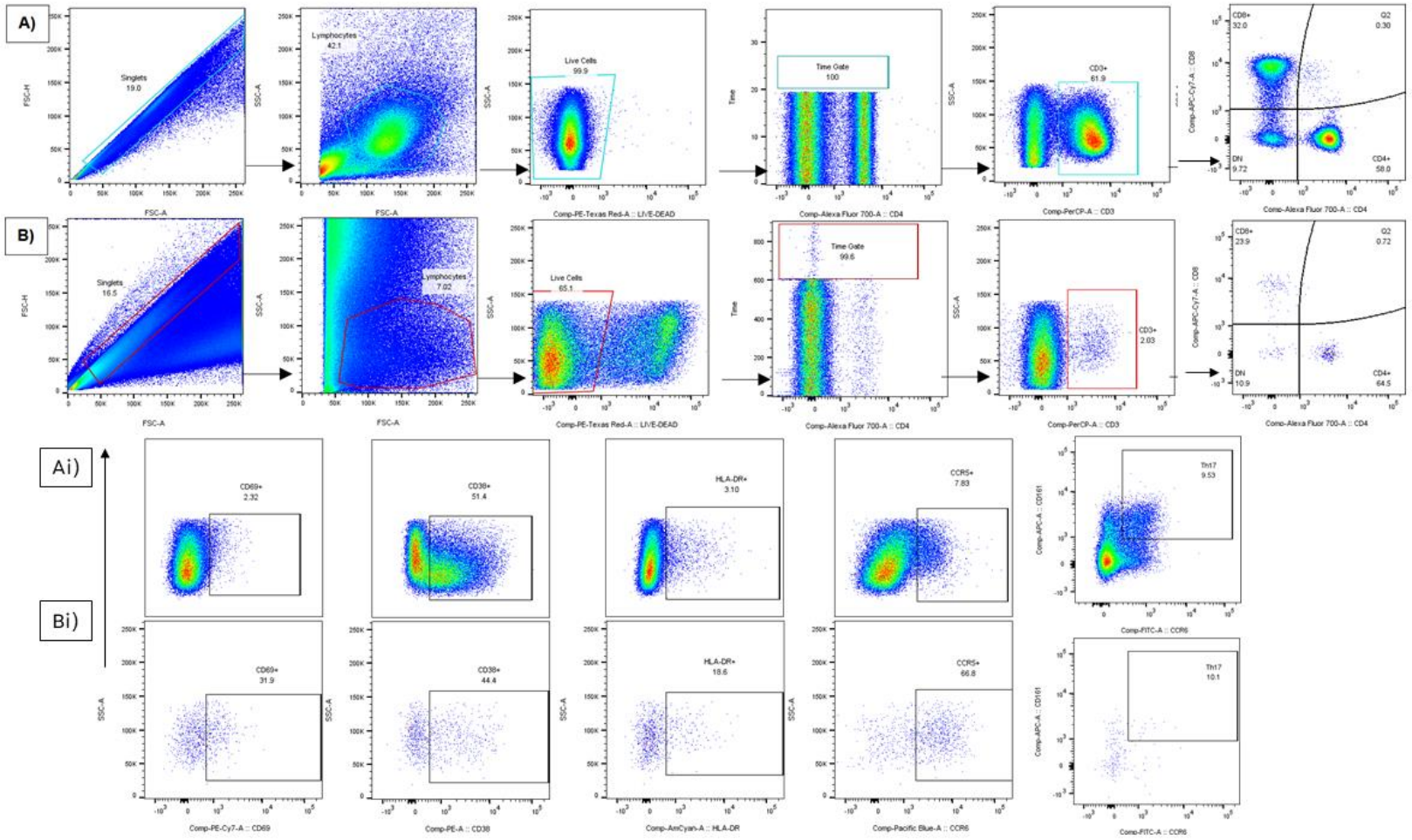
7 APPENDICES

Appendix I. Flow cytometry gating strategy.



Representative plots show gating on singlets, lymphocytes, live cells, internal control consistency in cell flow, CD3+ cells and CD3+CD4+ and CD3+CD8+ cells in PBMC (A) and CMC (B). Further gating for activation markers, CCR5 and Th17 cells was done on the CD4+ T cell fraction of PBMC (Ai) and CMC (Bi).

Appendix II. Supplementary Table 1: CD4+ T cell Immunophenotypes

	Female Sex Workers			Non-Sex Workers			FSW on No HC vz non-SW on No HC	FSW on DMPA vz Non-SW on DMPA
Mucosal	**DMPA**	**No HC**	***p***	**DMPA**	**No HC**	***p***	***p***	***p***
CD3+ %	0.93 (0.4 - 3.33)	5.91 (0.65 - 13.9)	0.029	4.2 (0.98 - 10.8)	2.5 (0.22 - 4.1)	0.067	0.027	0.037
CD4+ %	48.2 (36.5 - 59.3)	57.7 (49.8 - 62.4)	0.100	51.9 (44.9 - 63.4)	53.6 (44.9 - 61.1)	0.587	0.403	0.169
CD4+CCR5+ %	56.7 (42.1 - 70.3)	50 (31.8 - 61.2)	0.314	55.3 (41.2 - 66.2)	59.9 (50.1 - 75.8)	0.197	0.032	0.883
CD4+CCR5+ MFI	3886 (2723 - 4334)	2981 (2311 - 4268)	0.204	3660 (2070 - 4828)	3118 (2425 - 4301)	0.283	0.940	0.784
CD4+CD69+ %	27.7 (15.5 - 55.9)	24.1 (12.9 - 45.4)	0.132	26.5 (16.7 - 47.9)	12.7 (6.5 - 20.7)	0.003	0.032	0.533
CD4+CD69+ MFI	1764 (1389 - 2766)	1600 (1352 - 2004)	0.228	1459 (1340 - 2299)	2622 (2255 - 2864)	<0.001	<0.001	0.283
CD4+CD38+ %	43.4 (31.5 - 52.8)	35.7 (26.4 - 55.7)	0.538	39.2 (30.9 - 49.8)	25.1 (18.7 - 38.9)	0.001	0.025	0.445
CD4+CD38+ MFI	1276 (1009 - 1490)	1130 (898 - 1606)	0.470	1169 (815 - 1491)	1513 (1255 - 1813)	<0.001	0.003	0.234
CD4+HLADR+ %	17.2 (12.03 - 25.5)	17.6 (11.9 - 26.7)	0.936	15.0 (9.9 - 18.5)	13.9 (8.2 - 22.4)	0.554	0.080	0.231
CD4+HLADR+ MFI	1462 (1087 - 1843)	1579 (1098 - 1905)	0.565	1360 (1140 - 1661)	1674 (1471 - 1942)	0.006	0.273	0.811
Th17 %	5.0 (2.44 - 13.1)	3.5 (1.5 - 7.9)	0.196	6.5 (2.5 - 8.9)	3.9 (2.1 - 7.1)	0.135	0.952	0.171
CCR5+ Th17 %	84.0 (68.3 - 95.4)	89.1 (79.8 - 100)	0.270	85.8 (68.4 - 95.9)	81 (68.1 - 90.9)	0.594	0.995	0.328
CD4+CD69+CCR5+	41.2 (13.9 - 50.0)	25.1 (9.5 - 33.8)	0.173	22.9 (13.6 - 36.9)	26 (13.8 - 34.4)	0.712	0.376	0.254
CD4+CD38+HLA-DR+	12.8 (6.19 - 22.2)	10.4 (6.14 - 16.0)	0.682	7.9 (6.1 - 10.5)	8.2 (5.5 - 16.1)	0.467	0.068	0.062
Peripheral								
CD4+CCR5+ %	7.8 (3.7 - 14.0)	9.2 (5.5 - 13.6)	0.369	10.5 (5.3 - 16.6)	13.6 (8.5 - 18)	0.088	0.087	0.392
CD4+CCR5+ MFI	1754 (1443 - 2416)	1704 (1252 - 2173)	0.253	1738 (1337 - 3373)	1256 (1089 - 1431)	0.0004	0.003	0.825
CD4+CD69+ %	4.5 (1.7 - 8.5)	3.2 (1.1 - 8.9)	0.435	3.6 (1.4 - 8.3)	0.5 (0.25 - 1.6)	<0.001	0.0002	0.484
CD4+CD69+ MFI	1106 (1031 - 1195)	1049 (995 - 1430)	0.296	1066 (1022 - 1172)	2056 - 1991 - 2122)	<0.001	<0.001	0.323
CD4+CD38+ %	48.5 (41.8 - 61.3)	46.1 (35.3 - 65.9)	0.539	50.5 (40.3 - 62.9)	30.3 (20.2 - 42.8)	<0.001	0.0004	0.787
CD4+CD38+ MFI	1133 (1058 - 1283)	1093 (945 - 1320)	0.313	1310 (1188 - 1449)	1261 (1154 - 1399)	0.703	0.001	0.015
CD4+HLADR+ %	3.5 (4.7 - 5.4)	4.9 (3.5 - 7.0)	0.409	4.0 (2.8 - 5.7)	2.8 (2.2 - 4.4)	0.010	0.002	0.702
CD4+HLADR+ MFI	1335 (979 - 1497)	1324 (965 - 1470)	0.672	1330 (1018 - 1427)	1615 (1511 - 1805)	<0.001	<0.001	0.541
Th17 %	2.09 (1.26 - 3.12)	2.6 (1.1 - 3.9)	0.5	2.5 (1.2 - 3.8)	1.11 (0.64 - 2.4)	0.01	0.019	0.696
CCR5+ Th17 %	31.2 (22.2 - 39)	30.5 (19.7 - 41.5)	0.912	28.1 (21.5 - 38.2)	25.1 (15.9 - 32.3)	0.24	0.211	0.624
CD4+CD69+CCR5+	1.17 (0.52 - 3.1)	0.62 (0.2 - 3.9)	0.392	0.59 (1.1 - 2.8)	0.7 (0.35 - 1.43)	0.06	0.997	0.99
CD4+CD38+HLA-DR+	2.6 (1.4 - 4.5)	1.9 (1.44 - 2.83)	0.428	3.5 (2.9 - 4.7)	1.6 (1.2 - 2.5)	<0.001	0.503	0.293

Appendix III. Supplementary Table 2: CD8+ T cell Immunophenotypes

	Female Sex Workers			Non-Sex Workers			FSW on No-HC vs. non-SW on No-HC	FSW on DMPA vs. non-SW on DMPA
Mucosal	**DMPA**	**No HC**	***p***	**DMPA**	**No HC**	***p***	***p***	***p***
CD8+ %	28.3(18.18 - 41.9)	26.15(20.03 - 30.23)	*0.428*	27.8(17.2 - 37.4)	27.0(20.1-33.1)	*0.940*	*0.492*	*0.740*
CD8+CCR5+ %	60.6(45.8 - 75.7)	55.55(36.85 - 71.38)	*0.651*	71.1(46.3-83.1)	74.8(53.1-82.4)	*0.625*	***0.016***	*0.265*
CD8+CCR5+ MFI	3988(3199 - 4699)	2710(2324 - 4031)	***0.014***	4219 (2310-5224)	3130(2596-3565)	*0.141*	*0.539*	*0.788*
CD8+CD69+ %	36.9(21.00-48.40)	31(11.09 - 42.35)	*0.425*	30.7(12.60-44.90)	14.85(9.9-20.7)	*0.060*	*0.070*	*0.239*
CD8+CD69+ MFI	1484(1159-2240)	1513(1291 - 2002)	*0.708*	1266(1059-1950)	2090(1927-2348)	***<0.001***	***<0.001***	*0.398*
CD8+CD38+ %	57.2(41.7-73.4)	41.6(31.80 - 47.10)	***0.007***	50.9(41.10-60.80)	40.2(32.1-54.9)	*0.111*	*0.848*	*0.141*
CD8+CD38+ MFI	1507(1187-1823)	1106(755.0 - 1320)	***0.012***	1156(1014-1618)	996(883-1478)	*0.251*	*0.663*	*0.117*
CD8+HLADR+ %	25.7(14.3-37.2)	20.5(14.05 - 33.85)	*0.425*	23.9(14.60-31.10)	28.9(18.1-43.2)	*0.069*	*0.202*	*0.289*
CD8+HLADR+ MFI	1322(1188-1725)	1201(1083 - 1485)	*0.139*	1300(1183-1591)	1209(1065-1448)	*0.057*	*0.774*	*0.893*
T_C17 %	0.95(0.35 - 3.84)	2.7(0.85 - 6.93)	*0.124*	2.15(0.88 - 4.39)	1.5(0.95 - 2.29)	*0.338*	*0.212*	*0.171*
CCR5+ T_C17 %	100(100 - 100)	100(90.65 - 100)	*0.370*	100(87.5 - 100)	100(92.3 - 100)	*0.933*	*0.995*	*0.328*
CD8+CD69+CCR5+ %	35.4(19.3 - 56.7)	28.0(12.0 - 40.8)	*0.217*	21.4(13.05 - 46.9)	14.1(26.1 - 44.4)	*0.632*	*0.638*	*0.254*
CD8+CD38+HLA-DR+ %	20.4(10.7 - 29.3)	14.0(9.44 - 22.4)	*0.210*	13.6(9.25 - 21.25)	13.2(9.72 - 23.2)	*0.770*	*0.924*	*0.062*
Peripheral								
CD8+CCR5+ %	16.1(9.56 - 23.6)	17.2(12.2 - 25.1)	*0.498*	17.35(11.25 - 26.88)	11.11(3.585 - 39.70)	*0.530*	*0.494*	*0.502*
CD8+CCR5+ MFI	1987(1670 - 2346)	1842(1481 - 2517)	*0.475*	2043(1673 - 2978)	1664(1466 - 1954)	***0.002***	*0.196*	*0.363*
CD8+CD69+ %	7.76(2.970 - 12.00)	6.29(1.95 - 10.07)	*0.360*	5.92(4.2 - 8.66)	1.62(0.875 - 3.25)	***<0.001***	***<0.001***	*0.234*
CD8+CD69+ MFI	1046(984 - 1249)	1282(1110 - 1778)	*0.775*	1082(949.5 - 1290)	1791(1646 - 1869)	***<0.001***	***<0.001***	*0.840*
CD8+CD38+ %	26.7(14.90 - 41.60)	21.3(16.7 - 35.7)	*0.621*	32.6(24.33 - 43.83)	20.7(16.25 - 29.45)	***0.001***	*0.315*	*0.222*
CD8+CD38+ MFI	912(743 - 966)	760(610 - 880.5)	***0.032***	882(751 - 965)	775(715 - 803)	***0.005***	*0.425*	*0.972*
CD8+HLADR+ %	8.63(6.12 - 13.8)	7.87(6.18 - 12.5)	*0.803*	9.61(6.92 - 13.93)	11(7.68 - 17.80)	*0.093*	*0.066*	*0.754*
CD8+HLADR+ MFI	1117(1018 - 1232)	1110(958 - 1235)	*0.546*	1102(986 - 1259)	980(907.5 - 1073)	***0.006***	***0.028***	*0.695*
T_C17 %	0.73(0.37 - 1.39)	0.74(0.35 - 1.86)	*0.746*	0.71(0.41 - 1.44)	0.44(0.14 - 1.12)	*0.065*	*0.087*	*0.801*
CCR5+ T_C17 %	68.4(62.1 - 82.7)	77.5(68.3 - 89.8)	*0.161*	77.6(63.3 - 85.8)	68.0(58.0 - 75.5)	***0.050***	***0.011***	*0.203*
CD8+CD69+CCR5+ %	3.2(1.46 - 5.52)	2.64(0.91 - 5.28)	*0.501*	2.45(1.52 - 6.04)	2.10(1.29 - 3.95)	*0.532*	*0.864*	*0.871*
CD8+CD38+HLA-DR+ %	4.21(2.83 - 5.22)	2.74(1.90 - 4.34)	*0.093*	3.46(2.90 - 4.66)	3.39(2.0 - 4.19)	*0.367*	*0.697*	*0.377*

Appendix IV. Ethical Approval

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22nd December, 2015

Kenneth Odiwuor Omollo
Dept. of Medicial Microbioloy
School of Medicine
College of Health Sciences
University of Nairobi

Dear Kenneth,

Revised research proposal: The Impact of Hormonal Contraceptives on Susceptibility to HIV Infection among Women in Nairobi, Kenya (P566/08/2015)

This is to inform you that the KNH- UoN Ethics & Research Committee (KNH-UoN ERC) has reviewed and **approved** your above proposal. The approval periods are 22nd December 2015 –21st December 2016.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH-UoN ERC before implementation.
c) Death and life threatening problems and serious adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH-UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH- UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH- UoN ERC for each batch of shipment.
g) Submission of an *executive summary* report within 90 days upon completion of the study.

This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/ or plagiarism.

For more details consult the KNH- UoN ERC website **http://www.erc.uonbi.ac.ke**

Yours sincerely,

PROF. M.L. CHINDIA
SECRETARY, KNH-UoN ERC

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