UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

Frequency and Phenotype of Immune Cell Subsets in different regions of the Human Cervix: Implications for HIV Susceptibility

by

Nashlin Pillay (212523489)

Submitted in partial fulfilment of the requirements for the degree of
Master of Medical Sciences

Department of Medical Microbiology,
CAPRISA Mucosal Immunology Laboratory,
School of Laboratory Medicine and Medical Sciences,
Nelson R. Mandela School of Medicine, College of Health Sciences,
University of Kwa-Zulu Natal

2018

Supervisor: Dr. Sinaye Ngcapu

Preface

The research contained in this dissertation/thesis was completed by the candidate while based in the Discipline of Medical Microbiology, School of Laboratory Medicine and Medical Sciences of the College of Health Sciences University of KwaZulu-Natal, Nelson R Mandela School of Medicine, South Africa under the supervision of Dr Sinaye Ngcapu. The research was financially supported by the DST-NRF Centre of Excellence in HIV Prevention.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

Nashlin Pillay **Signed:** 

Dr. Sinaye Ngcapu **Signed:** 

As the candidate's supervisor I agree to the submission of this thesis

Date: December 6, 2018

Declaration: Plagiarism

I, Nashlin Pillay, declare that:

(i) the research reported in this thesis, except where otherwise indicated or acknowledged, is my original work;

(ii) this thesis has not been submitted in full or in part for any degree or examination to any other university;

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

(iv) this thesis does not contain other persons' writing unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a) their words have been re-written but the general information attributed to them has been referenced;

b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced;

(v) where I have used material for which publications followed, I have indicated in detail my role in the work;

(vi) this thesis is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

(vii) this thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed: Nashlin Pillay

Date: December 6, 2018

Dedication

To My Family and Friends

Acknowledgements

My Family for support

Dr. Sinaye Ngcapu of CAPRISA and UKZN, my supervisor and mentor

CAPRISA mucosal immunology supervisors, for the consist input and knowledge

CAPRISA mucosal laboratory staff, for the help and courtesy

AHRI Leslie labs, for allowing us to use the dissociator

Thandeka and Kewreshnie of HHP, for the assistance in flow cytometry

Wenkosi Qulu, Ph.D. student, for assisting in sample collection

Prince Mshiyeni Memorial Hospital and King Edward Hospital for allowing as to collect samples

CAPRISA Research Fellows for their support and assistance

Ethics Declaration

This study was approved by the Biomedical Research Ethics Committee of the University of Kwa-Zulu Natal (BE218/17). Permission was obtained from the Department of Health to conduct research and both Prince Mshiyeni Memorial Hospital and King Edward Hospital (appendix A-D). HSP and GCP were followed and all participants were consented and willingly participated in this study. Participant identity was kept strictly confidential.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

µl	Microliter
g	Grams
Min	Minute
ml	Millilitre
mm	Millimetre
^{o}C	Degrees Celsius
Rpm	Revolutions per Minute
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cell
ARV	Antiretroviral
BREC	Biomedical Research Ethics Committee
BV	Bacterial Vaginosis
CAPRISA	Centre For the AIDS Programme of Research in South Africa
CCR5	C-C Chemokine Receptor Type 5
CCR6	C-C Chemokine Receptor Type 6
CD1a	Cluster of Differentiation 1a
CD3	Cluster of Differentiation 3
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CD19	Cluster of Differentiation 19
CD14	Cluster of Differentiation 14
CD25	Cluster of Differentiation 25
CD56	Cluster of Differentiation 56
CD127	Cluster of Differentiation 127
CIN	Cervical Intraepithelial Neoplasia
COC	Combined Oral Contraceptive
CST	Community State Type
CVL	Cervicovaginal Lavage
CXCR3	C-X-C Motif Chemokine Receptor 3
CXCR4	C-X-C Motif Chemokine Receptor 4
CXCR5	C-X-C Motif Chemokine Receptor 5
DC SIGN	Dendritic cell specific intercellular adhesion molecule 3 grabbing non-integrin
DC	Dendritic Cell
DMPA	Depot-medroxyprogesterone acetate
FBS	Fetal Bovine Serum
FGT	Female Genital Tract
FMO	Fluorescence Minus One
GP120	Glycoprotein 120
HBSS	Hank's Balanced Salt Solution
HC or H/C	Hormonal Contraceptive
HIV	Human Immunodeficiency Virus
HPV	Human Papillomavirus
HSV-2	Herpes Simplex Virus Type 2
IL	Interleukin
IQR	Interquartile Range
KEH	King Edward VII Hospital
LC	Langerhans Cell
MIP 1α	Macrophage Inflammatory Protein 1 Alpha
MIP-2α	Macrophage Inflammatory Protein 2 Alpha
MIP-1β	Macrophage inflammatory protein 1Beta
NET-EN	Norethisterone enanthate
NK	Natural Killer

PBS	Phosphate Buffered Saline
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed Cell Death Protein Type 1
Pen/Strep	Penicillin/Streptomycin
PMMH	Prince Mshiyeni Memorial Hospital
PrEP	Pre Exposure Prophylaxis
RPMI	Roswell Park Memorial Institute medium
SIV	Simian Immunodeficiency Virus
STIs	Sexually Transmitted Infections
TAH	Total Abdominal Hysterectomy
Th17	T helper cell 17
TFV	Tenofovir
TLR	Toll-like Receptor

Abstract

Background: Although mucosal sites such as the gut have been extensively studied, less is known about the immune cell composition of the human female genital tract, a surface through which HIV is transmitted. This study aimed to determine the frequency of immune cell subsets in endocervical and ectocervical tissue sections of HIV uninfected women.

Methods: Six cervical explant tissue samples were obtained from women undergoing elective hysterectomies in Prince Mshiyeni Memorial Hospital and King Edward VII Hospital, Durban, South Africa. The tissues were dissected into 2mm blocks and mechanically digested and dissociated using the mouse spleen (m_spleen_01) program of the gentle-MACS Dissociator. B and T cell subsets phenotyping were quantified using flow cytometry.

Results: Endocervix and ectocervix tissue was obtained from 4/6 recruited participants and two participants only had endocervix tissue available. Overall, T cell subsets (as marked by CD3+ and CD4+ subsets) were the most abundant cell population compared to B cells (as marked by CD19+ expression) in the cervix (45% vs. 4%, respectively). Although the overall frequency of B cells observed in both endocervical and ectocervical regions was low, the abundance of CD19+ cells was slightly higher in the endocervix (4%) compared to the ectocervix (2%). There was no significant difference in the frequencies of T cell populations (CD3+ and CD4+ cells) in the ectocervix and endocervix. The majority of CD4+ T cell co-receptors found in the cervix were CXCR4 (69%), followed by CD127 (67%), CXCR3 (66%) and CCR6 (63%). CCR5 was detected in 38%, while both CD25 and PD1 were expressed in 34% of CD4+ T cells. None of these CD4+ T cell co-receptors were significantly different in the ectocervix and endocervix, with an exception of CCR6 and PD1 that showed a trend towards being elevated in the endocervix compared to ectocervix, p=0.067. There was no difference in frequencies of both B and T cells amongst women on hormonal contraceptives versus non-hormonal contraceptive users.

Discussion: Although limited by sample size, our data suggest that the T cells (CD3 and CD4+) were more abundant than B cells in the endocervix. Compared to the ectocervix, the endocervix had slightly higher frequencies of CD4+ T cell receptors (CCR6, PD-1, CCR5, CD25) suggesting that endocervix might be a preferential region for HIV infectivity, replication and transmission.

Chapter 1:

Introduction and Literature Review

1.1 Introduction

Sub-Saharan African is home to 14% of the world's population but contributes a disproportionate 70% of all the world's human immunodeficiency virus (HIV) infection, with over 1000 new infections every day (Abdool Karim et al., 2010, Gómez-Olivé et al., 2013, UNAIDS, 2017). The risk for HIV infection in sub-Saharan Africa is skewed towards women, particularly young women between the ages of 15-24 years, with HIV prevalence in young women up to 3 times more compared to their young male counterparts (Figure 1.1) (UNAIDS, 2017, Abdool Karim et al., 2017). It is thought that young women become infected with HIV after fewer acts of unprotected heterosexual intercourse (Pettifor et al., 2005, Glynn et al., 2001) with transmission of HIV across mucosal surfaces in women being the most common route of HIV infection (Abdool Karim et al., 2010).

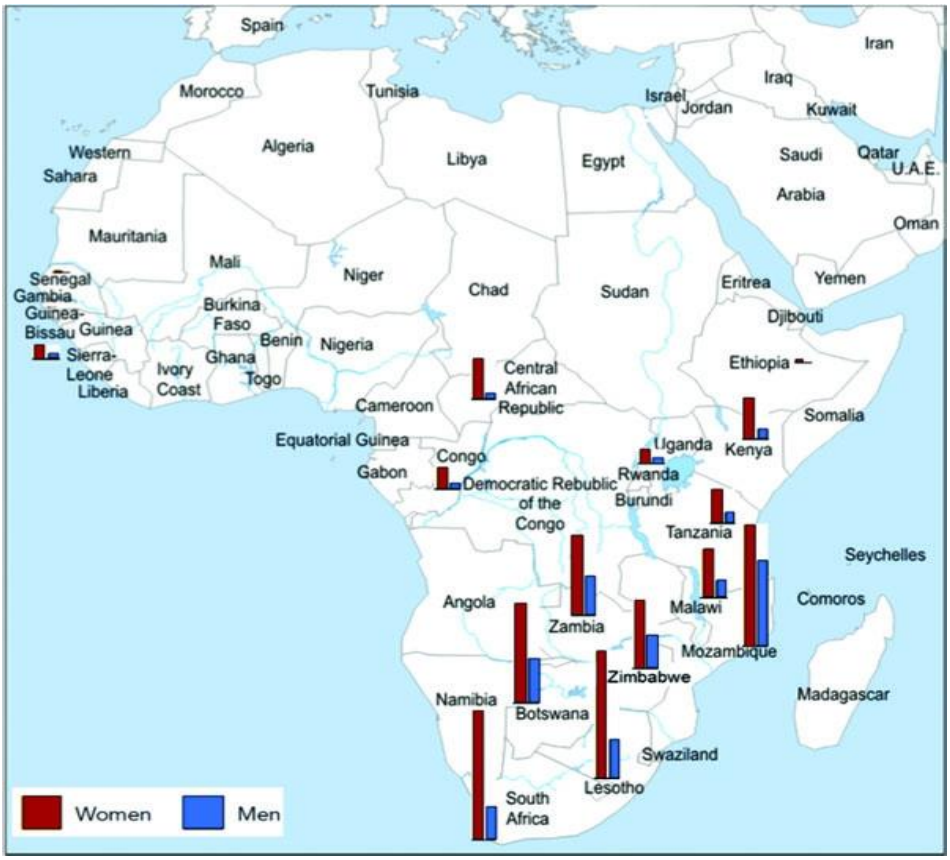

Figure 1. 1. Map showing the disproportionate burden of HIV in young women in sub-Saharan Africa**.** Adolescent girls and young women between the ages of 15-24 years in eastern and southern Africa up to eight times more HIV infection compared to their male peers. Figure adapted from (Abdool Karim et al., 2012).

The mucosal epithelium of the female genital tract (FGT) is the first line of defence against pathogen entry. Despite this, the vagina and cervix remain vulnerable to HIV infection (Hladik and Hope, 2009). Studies have suggested the cervix is the most vulnerable region, with the endocervix being a preferential region for HIV replication compared to the ectocervix (Lee et al., 2015, Trifonova et al., 2014). Also, the endocervix is lined with a more vulnerable single-cell, simple columnar epithelium, while the ectocervix is lined with a stronger multi-layered squamous epithelium (Critchlow et al., 1995, Gray-Swain and Peipert, 2006, Hwang et al., 2011). A few studies have assessed the frequency and distribution of resident immune cells in the human cervix, with T cells being the more dominant population compared to B cells. Several studies showed that CD8+ T cells were the most abundant T cell population than CD4+ T cells in the human cervical mucosa (Givan et al., 1997, Pudney et al., 2005, Saba et al., 2010). In contrast, macrophages (expressed by CD14+ cells and CD103 $CD11b^{+}$ $CX3CR1^{+}$ $DC\text{-}SIGN^{+}$ dendritic cells (DCs), were the most dominant population in the human cervical mucosa (Trifonova et al., 2014). It was further shown that the endocervix has a greater abundance of HIV target cells such as the CD4+ T cells with post-menopausal women illustrated as more vulnerable to HIV infection (Trifonova et al., 2014). This suggests that each region of the FGT differs and one region may be more preferential for HIV due to the abundance of HIV target cells. However age, hormones and contraceptive usage play a vital role in immune cell abundances and the structure of both the ectocervix and endocervix (Trifonova et al., 2014, Reis Machado et al., 2014). Although few studies have defined B cell, T cell and DC population within cervical mucosa, no study has looked at the proportions and distribution of CD4+ co-receptors and their link with HIV infectivity.

For decades, several studies on humanized mice were used to advance understanding of HIV transmission (Olesen et al., 2011, Denton et al., 2008, Wheeler et al., 2011) while others used macaque SIV models to understand SIV (Simian Immunodeficiency Virus) susceptibility (Zhang et al., 1999, Miller et al., 2005, Haase, 2010). Not only did the findings of these studies fail to fully explain the mechanism by which HIV may be transmitted to humans, but several limitations were also noted. These include low immune cell numbers and different cellular response to chemokines secreted by mouse epithelial cells (Hladik and McElrath, 2008, Policicchio et al., 2016). Furthermore, other approaches have also been used to investigate immune responses at the FGT. These methods include the use of cytobrushes and cervicovaginal lavage obtained from cervico-vaginal surfaces, in combination with flow cytometry and Luminex technology (Grivel and Margolis, 2009, Liebenberg et al., 2011, Roberts et al., 2012). As an alternative, human cervical samples have been used to assess women's susceptibility to HIV transmission (Saba et al., 2010, Grivel and Margolis, 2009). Human cervical explants enable the study of HIV infection and cellular or antibody responses in intact tissues,

rather than in single cells, which more closely resembles what happens *in vivo* (Grivel and Margolis, 2009).

Taking all this into consideration, it is clear an extensive understanding of the relationship between abundance of genital immune cell subsets and transmission of HIV is warranted. Specifically, there is a need to better characterise the innate and adaptive immune cells in the endocervix and ectocervix of healthy women. Characterizing the abundance and distribution of the major immune cells within the different regions can help associate a specific region with infectivity and be able to link inflammation of the genital tract to an increased recruitment of HIV target cells.

1.2 Literature Review

1.2.1 The Female Genital Tract

The FGT is a unique system that has an ability to balance defence against invading pathogens with tolerance towards reproductive components such as sperm, embryo and foetus (Nguyen et al., 2014). It is a complex structure composed of keratinocytes linked by different proteins that provide a biological barrier, separating the extrinsic factors from the lymphatic and circulatory systems (Wira et al., 2015). It consists of the stratified squamous epithelial introitus (vaginal opening), the vaginal mucosa and the cervix (Lee et al., 2015, Pudney et al., 2005). The cervix is a cylinder-shaped neck of tissues situated at the lowermost part of the uterus and connects the uterus and vagina (Figure 1.2) (Nguyen et al., 2014).

The cervix consists of the ectocervix, endocervix and the squamocolumnar junction known as the transformation zone (Danforth, 1983). These sites differ structurally, with the vagina and ectocervix characterised by a multi-layered non-keratinised stratified squamous epithelium (Petrova et al., 2013) while the mucosa of the endocervix is made up of a single layer columnar epithelium and within the transformation zone, squamous epithelial cells transform to columnar epithelial cells (Reis Machado et al., 2014, Hladik and Hope, 2009). The transformation zone usually withdraws into the endocervix by the onset of menopause (Singer, 1975). The endocervix is reliant on mucus, tight and adherens junctions in the epithelium while the ectocervix is reliant on the multiple layers of squamous epithelium as a physical barrier of protection against invading pathogens, respectively (Nguyen et al., 2014). Although these layers provide a first line of defence against invading pathogens, the potentially

protective superficial layer is continuously sloughed off, which may result in vulnerability to ascending infections such as HIV (Wira et al., 2005a).

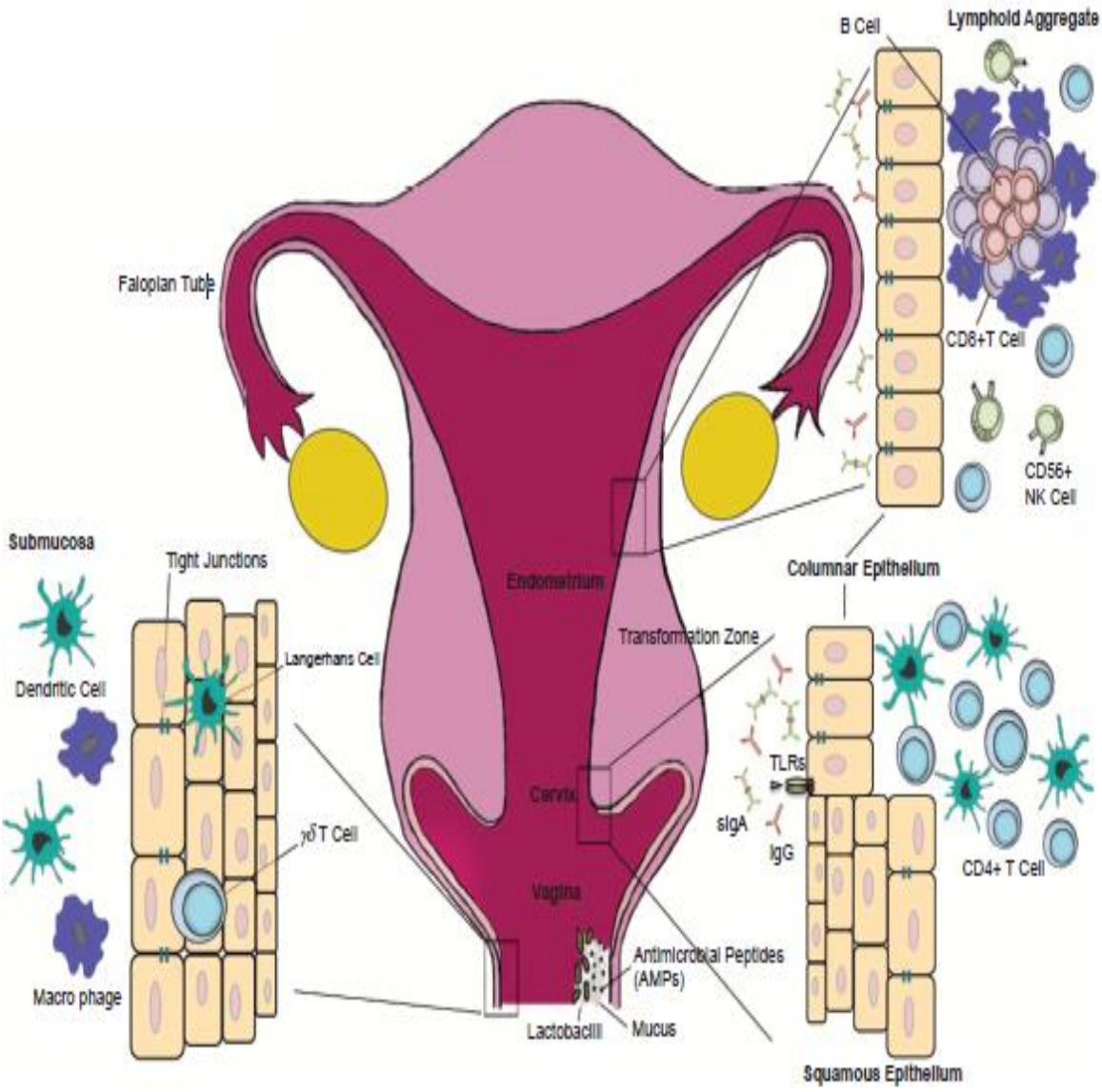

Figure 1. 2. Characterisation of the female genital tract mucosa. The mucosa of the lower FGT, which is made up of the vagina and ectocervix, is defined by a multi-layered squamous epithelium that is held together by tight junctions and houses various immune cells such as dendritic cells, T cells and macrophages. The upper FGT is defined by a single layer of columnar epithelium which houses lymphoid aggregates composed of B and T cells. A healthy FGT is also dominated by antimicrobial peptide producing *lactobacilli.* Figure adapted from (Nguyen et al., 2014).

The epithelium in the FGT secretes a protective moisture, providing a hydrophilic layer of glycoproteins called glycocalyx and a hydrophobic glycoprotein mucus which aids in trapping pathogens, thereby preventing ascending infections (Gipson et al., 1997, Vigil et al., 2009, Reis Machado et al., 2014). Genital epithelial cells also produce various antimicrobial peptides and proteins into the cervicovaginal fluids, express toll-like receptors (TLRs) and secrete inflammatory cytokines in response to antigen binding (Petrova et al., 2013, Wira et al., 2005a). Both the vagina and ectocervix are the primary sites exposed to male seminal fluids and are more prone to invading pathogens compared to the endocervix and transformation zone (Shattock and Moore, 2003, Miller et al., 1992, Pudney et al., 2005). However, the endocervix and transformation zone are thought to be the sites where HIV is most likely to penetrate and access the high number of target cells available (Hladik and Hope, 2009, Lee et al., 2015, Miller et al., 1992, Kell et al., 1992, Pudney et al., 2005, Shattock and Moore, 2003).

1.2.2 Mucosal immune system of the female genital tract

The epidermis of the FGT is home to a small number of different cell types, with keratinocytes being the most abundant (Lee et al., 2015, Wira et al., 2005a). In a healthy FGT, Langerhans cells (LCs) are found within the squamous epithelium and express cluster of differentiation (CD)-1a. LCs have the ability to reach into granulosum and stratum corneum to present foreign antigens and initiate the immune responses (Hickey et al., 2011). Other cells present within the sub-milieu of tissue are DCs, the primary antigen presenting cell (APC) responsible for the induction of host immune response that can be tolerogenic or antigen specific adaptive response (Lee et al., 2015, Amjadi et al., 2014). DCs have been shown to express the HIV binding co-receptors CCR5, as well as a C type lectin DC-SIGN which mediate HIV entry into DCs (Petrova et al., 2013, Wu, 2008).

The epithelial lamina propria of the vaginal mucosa also contains an array of cells, which include different immune cells such as the granulocytes and T cells, while B cells, macrophages and monocytes are present in much smaller numbers (Givan et al., 1997, White et al., 1997). Although relatively few compared to blood, the cervicovaginal mucosa contains leukocytes, with CD8+ T cells being the most abundant, followed by small numbers of CD4+ T cells, CD45+ neutrophils, $CD56^{bright}$ NK cells, CD68+ macrophages and CD1a+ immature DCs (Lee et al., 2015, Pudney et al., 2005). B cells (CD19+ and CD20+ B cells) are also said to be located around these lymphocyte clusters in follicle-like structures of the cervicovaginal mucosa (Lee et al., 2015, Johansson et al., 1999). When comparing immune cell distribution between the ectocervix and endocervix; T and B cells were found

more abundant in the ectocervix while there was no difference in numbers of NK cells, CD14+ macrophages and DCs (Trifonova et al., 2014). With high levels of CD8+ T cells and antigen presenting cells in the endocervix, it is thought to be the site where initial cellular response against pathogens occur (Trifonova et al., 2014, Lee et al., 2015). However, due to the large quantities of CD4+ T cells, macrophages and DCs present in the endocervical mucosa and transformations zone, these regions are also thought to be more vulnerable to HIV infection compared to the ectocervical and vaginal mucosa (Pudney et al., 2005, Trifonova et al., 2014). The FRT also plays residence to a variety of CD4+ T cells subsets which are defined by the co-receptor present (eg. CCR6, CXCR4) on the CD4+ T cell (Lee et al., 2015).

1.2.3 CD4+ T cell co-receptors

One of the most studied co-receptor of the CD4+ T cell CCR5 which is the co-receptor for R5 strains of the HIV virus (HIV-1, HIV-2) (Oppermann, 2004). The gp120 envelope of the HIV virus binds onto the CD4 receptor of the CD4+ T cell and following a conformational change the gp120 protein is able to bind onto the CCR5 co-receptor thus initiating the first step in HIV infection (Alkhatib, 2009). Mutations of the CCR5 co-receptor can lead to resistance against the HIV virus (Quillent et al., 1998). Studies have found that when compared to peripheral blood, CD4+ T cells in the genital significant express more CCR5 (Iyer et al., 2017). Another important CD4+ T cell co-receptor is CXCR4 which also takes part in the binding of X4 tropic HIV virus (Poveda et al., 2007). HIV binding to CXCR4 is for the most similar to R5 strains in which gp120 protein binds first to CD4 and then to CXCR4 and CXCR4 is functionally expressed on cancer cells, playing a role if proliferation and migration (Alkhatib, 2009).

CCR6 expression is commonly a defining marker of Th17 cells which perform both roles inflammation and migration (Yamazaki et al., 2008). Th17 cells additionally play a role in maintaining vaginal epithelial integrity; therefore a reduction may lead to genital inflammation which could further aid viral replication (Stieh et al., 2016). Studies have aslo shown that these Th17 cells are susceptible to HIV and are depleted early within the cervix of HIV infected patients (Sallusto et al., 2012, Weaver et al., 2013). The functionality of CD4+ T cells expressing CXCR3 is trafficking and migration of T cells to lymphoid compartments where they interact with antigen presenting cells which can lead to the production of effector and memory T cells (Groom and Luster, 2011). CD4+ T cells expressing CXCR5 are associated with follicular B helper T cells which aid in B cell communication and the CD127 and CD25 markers are associated with regulatory cells which are differentially depleted during HIV infection (Moser, 2015, Dunham et al., 2008). PD-1 expression on

CD4+ T cells is a marker for cell exhaustion and plays a role in death and regulation of T cells (Riley, 2009).

1.2.4 Microbial environment of the female genital tract and HIV risk

The cervicovaginal mucosa is a non-sterile environment with roughly 10^9 microorganisms per millilitre present (Quayle, 2002, Mirmonsef et al., 2011). The microbial community of healthy women is generally considered to be dominated by *Lactobacillus* species. The *Lactobacillus* spp. are thought to provide protection by lowering the vaginal pH of the lower genital tract through lactic acid production, various bacteriostatic and bactericidal compounds such as hydrogen peroxide (H_2O_2), and through competitive exclusion of other organisms (Figure 1.3) (Ravel et al., 2011, Reis Machado et al., 2014, Klebanoff and Coombs, 1991). A number of different *Lactobacillus* spp. have been described in healthy genital tracts although the most frequent and abundant of these are *L. crispatus, L. gasseri, L. jensenii* and *L. iners,* however, not all *Lactobacillus* species are equally protective, with *L. iners* found in both healthy and bacterial vaginosis (BV) affected vaginal environments (Ravel et al., 2011, Petrova et al., 2013, Gootenberg et al., 2018). These bacteria are also classified into community state types (CST) according to which is most dominant in the microbiota, CST I: *L. crispatus*, CST II: *L. gasseri*, CST III: *L. iners*, CST V: *L. jensenii*, CST V: anaerobic dominance (Gootenberg et al., 2018, Ravel et al., 2011). A genital environment, dominated by *Lactobacillus* species, has been associated with healthy pregnancy outcomes, lack of abnormal vaginal symptoms and urogenital disease and reduced risk for several sexually transmitted infections (STIs), including Human papillomavirus (HPV) and/or HIV (Petrova et al., 2013, Reis Machado et al., 2014, Ness et al., 2005). Replacement of commensal *Lactobacillus* spp. with commensal gram negative bacterial pathogens may lead to BV, a microbial dysbiosis often resulting in vaginitis and discharge and increased HIV risk (Kenyon et al., 2013, Macklaim et al., 2013, Gosmann et al., 2017).

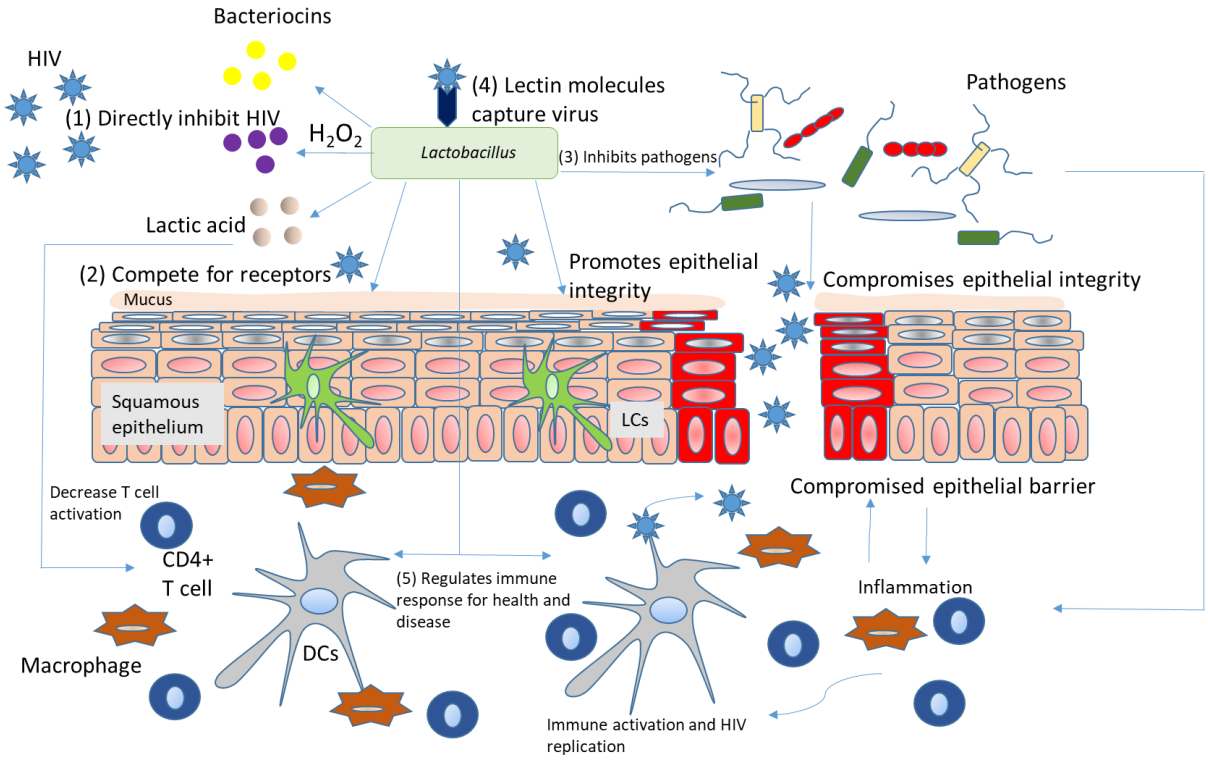

Figure 1. 3. General overview of the mechanism by which *Lactobacilli* species prevent invading pathogens (such as HIV) and overgrowth of strict anaerobic bacteria in the lower female genital tract. A *Lactobacillus* dominated vaginal microbial community has long been associated with health, by inhibiting the colonization of genital pathogens through (1) the production of lactic acid and H_2O_2, (2) competing with other microorganisms for epithelial receptor sites, (3) variety of inhibitory agents such as bacteriocins, (4) Lectin mediated binding and capture of HIV and (5) by regulating the immune response. Reduction in *Lactobacilli spp*. may lead to diverse microbial communities linked with bacterial vaginosis and increased risk of HIV infection, likely via genital inflammation (red colour in epithelium) and recruitment of target cells. Figure adapted from (Petrova et al., 2013).

BV is the most common cause of vaginal discharge in women of reproductive age, although its prevalence varies considerably between ethnic groups. The etiology of BV is not known although it has clearly been associated with sexual activity (Kumar et al., 2011). Reduction in *Lactobacilli* species is generally accompanied by the intense overgrowth of commensal vaginal anaerobic bacteria, which can reach densities 100 to 1000 times higher than found in healthy vaginas, and display significantly increased bacterial diversity (Macklaim et al., 2013). Cultures of organisms from the genital tract of women with BV typically yields a spectrum of facultative anaerobic commensals, including *Gardnerella vaginalis*, *Atopobium vaginae*, *Eggerthella* spp., *Prevotella* spp., BVAB2 (Clostridia-like bacteria), *Megasphaera* type 1, *Mobiluncus* spp, *Ureaplasma urealyticum* and *Mycoplasma hominis* (Shipitsyna et al., 2013). An absence of *Lactobacillus* species, combined with

the presence of *G. vaginalis*, *A. vaginae*, and/or *Prevotella* spp. (Shipitsyna et al., 2013) have been suggested to be highly accurate indicators of BV.

BV has been associated with increased genital inflammation (Anahtar et al., 2015), and an increased risk for HIV infection (Figure 1.3) (Cohen et al., 2012). A recent study by Anahtar et al. (2015) demonstrated that high BV-associated bacterial communities were associated with increased proinflammatory cytokine levels due to APCs sensing bacterial products via TLR6 which leads the activation of (NF)-κβ pathway that ultimately results in inflammation and target cell recruitment (Anahtar et al., 2015). Due to the complexity of BV, which is often different syndromically, some studies have shown BV to be associated with the upregulation of proinflammatory cytokines while others have found BV to be associated with the downregulation of certain cytokines (Passmore et al., 2016, Masson et al., 2014, Kyongo et al., 2012, Deese et al., 2015). Proteomics shows that along with BV being associated with increases in cytokines and cytokine receptors, BV is also associated with changes in proteins that are linked to the breakdown of the mucosal barrier and mucus alterations (Passmore et al., 2016, Borgdorff et al., 2016). Due to its inflammatory nature, BV has been associated with an increased risk of acquiring an STIs, including *Chlamydia trachomatis*, *Trichomonas vaginalis*, herpes simplex virus type 2 (HSV-2) and *Neisseria gonorrhoea* (Al-Harthi et al., 1999, Abbai et al., 2016).

1.2.5 Impact of hormonal changes in the female genital tract

The FGT experiences progressive anatomic changes that are controlled by oestrogen and progesterone from birth through menopause (Beagley and Gockel, 2003). Immune cells in the FGT, including epithelial cells, macrophages, dendritic cells and T cells, are regulated by hormones such as oestrogen and progesterone which vary during the menstrual cycle (Polis et al., 2014). A study investigating the changes in immune cell numbers during different stages of the menstrual cycle found that levels of CD4+, CD8+ T cells and B cells were higher in the ectocervix compared to the endocervix in pre-menopausal women (Trifonova et al., 2014). Only CD4+ T cells remained abundant in post-menopausal women (Trifonova et al., 2014). There were no significant differences in densities of CD14+ macrophages, DCs and LCs within the vagina and cervix when comparing the luteal and follicular phase of the menstrual cycle (Lee et al., 2015, Pudney et al., 2005). Chandra et al. (2013) showed that women with high levels of progesterone [particularly those using depo-medroxyprogesterone acetate, (DMPA)] had significantly higher numbers of activated T cells and macrophages expressing CCR5 compared to their non-progesterone users. In contrast, Mitchell et al.,

(2014) found no difference in density or numbers of T cells or $CCR5^+$ HIV-1 target cells in the lower reproductive tract of women using DMPA compared to non-HC users (Mitchell et al., 2014).

Oestrogen and progesterone have been shown to regulate the thickness of the genital mucosal barrier during the reproductive cycle in women (Beagley and Gockel, 2003, Fahey et al., 2008). Increased levels of oestrogen have been shown to contribute to protection against invading pathogens by increasing the thickness of the vaginal epithelium and production of cervical mucous, as well as being able to aid in the growth of beneficial commensal bacteria such as *Lactobacilli* (Morrison et al., 2015, Wira et al., 2015, Petrova et al., 2013). Although oestrogen may have potential protective effects, data from several studies suggest that high levels of progesterone may have adverse effects on the FGT, resulting in increased risk for HIV infection (Wieser et al., 2001, Wira et al., 2011, Miller et al., 2000, Ildgruben et al., 2003). Studies have shown that the mucosal barrier is compromised during the luteal phase, a phase characterized by high progesterone levels (Wira et al., 2015). It has been shown that high-dose progesterone decreases the integrity and thickness of the genital mucosal barrier in women and the density of intracellular junction proteins in the stratified epithelial layer (Ildgruben et al., 2003, Chandra et al., 2013, Miller et al., 2000, Wira et al., 2011). In contrast, other studies have found no association between progesterone and genital epithelial barrier integrity (Chandra et al., 2013, Mitchell et al., 2014). In non-human primates, high-dose progesterone is commonly used in the simian immunodeficiency virus (SIV) vaginal challenge model, specifically because it thins the vaginal epithelium and significantly increases the efficiency of vaginal transmission (Abel et al., 2004, Trunova et al., 2006, Wieser et al., 2001, Marx et al., 1996). Decreased genital barrier function might facilitate more efficient contact between HIV target cells within the cervicovaginal mucosa and HIV particles entering the vaginal lumen.

1.2.6 Impact of inflammation on the female genital tract

Exploring mucosal risk factors in the FGT is critical for understanding STI/HIV susceptibility. Genital inflammation is the best biological correlate of HIV risk that has been described to date and if elevated prior to infection, has been associated with an approximately three-fold higher risk of acquiring HIV (Masson et al., 2015). Although the mechanisms to explain why some people have comparatively high levels of genital inflammation while others do not, are not fully understood, the likely drivers of genital inflammation are BV and STIs (Masson et al., 2015). In the CAPRISA 004 1% tenofovir microbicide trial, women who acquired HIV during the trial had significantly elevated genital tract inflammatory markers (such as MIP-1α, MIP-1β, IL-8, and IP-10) prior to HIV infection, independent of study arm and age (Masson et al., 2015). It was further demonstrated that genital

inflammation reduced the effectiveness of 1% tenofovir in women, even in those with high adherence to the drug (McKinnon et al., 2018). Inflammation is needed for the clearance of infection, however, it can also lead to the disruption of infected epithelial barriers, which then facilitates access to target cells deep in the tissue (Passmore et al., 2016, Mcgee et al., 1999, Svanborg et al., 1999). Several studies showed decreased functionality of the epithelial barrier in the FGT of women was associated with elevated levels of proinflammatory cytokines (Passmore et al., 2016, Arnold et al., 2016). Furthermore, the detection of either MIP-1α or MIP-1β in CVL samples was associated with >3 fold increased odds of HIV infection (Masson et al., 2015). An e*x vivo* study using postmenopausal ectocervical tissue showed a strong association between genital inflammation and HIV replication (Reis Machado et al., 2014, Rollenhagen and Asin, 2011). Taken together, these data shows that an inflammatory environment favours HIV acquisition in women, regardless of the cause (Passmore et al., 2016).

1.2.7 Female genital tract and sexually transmitted co-infections

STIs, such as human papillomavirus (HPV), *Neisseria gonorrhoea, Mycoplasma genitalium* and *Chlamydia trachomatis*, can lead to reproductive complications and increased HIV susceptibility (Passmore et al., 2016, Laga et al., 1993, Mlisana et al., 2012). The most common impact that STIs have on the FGT is inflammation due to upregulation of pro-inflammatory cytokines and immune cell recruitment (Passmore et al., 2016, Fichorova et al., 2001). The presence of *Mycoplasma genitalium, Chlamydia trachomatis* and *Neisseria gonorrhoea* and the resultant increase in levels of IL-1β, IL-6, IL-8 and soluble CD40L was associated with increased risk of acquiring HIV (Passmore et al., 2016, Masson et al., 2014). HSV-2 has also been associated with elevated levels of DC-SIGN+ DCs and CCR5+ T cells and ulcerative lesions that affect the epithelial barrier and increases HIV susceptibility of the FGT. Furthermore, STIs can also affect vaginal health by displacing the healthy *Lactobacillus* population and altering the vaginal pH, resulting in overgrowth of BV associated organisms and increased HIV susceptibility (Petrova et al., 2013).

1.2.8 HIV prevention strategies in the female genital tract

The development of an effective HIV prevention tool for women is listed as a high research priority to achieve the goals set out by UNAIDS in its Fast-Track strategy to end the epidemic by 2030 (UNAIDS, 2017). Currently, the most common form of prevention used is male and female condoms,

which act as effective barriers against HIV and other STIs (Arkell, 2016). The reasons condoms are the preferred method is that unlike contraceptives and other drugs, there are no adverse side effects other than rare latex allergies, and are effective against pregnancy and STIs (Cohen et al., 2011, Arkell, 2016). Female condoms, although still debatable with a partner, may offer women more control and power in terms of usage as compared to having to negotiate the use of a male condom (Arkell, 2016, Cohen et al., 2011). However, not many women use the female condom due to the lack of awareness of the product, poor basic knowledge and skills to insert and remove it properly, poor acceptance by both females and male partners, and relatively higher cost of this method (Arkell, 2016, Cohen et al., 2011).

Over the past decade, three major trials (Abdool Karim et al., 2010, Baeten et al., 2012, Thigpen et al., 2012) have demonstrated the prevention benefit of the prophylactic use of antiretroviral (ARV) drugs in women, with the CAPRISA 004 trial demonstrating moderate effectiveness in preventing HIV infection in women who used the intravaginal tenofovir (TFV) gel consistently (Abdool Karim et al., 2010). Although product adherence was a crucial factor for effective HIV pre-exposure prophylaxis (PrEP), even with high adherence, protection was modest, underscoring a role for other biological risk factors (Marrazzo et al., 2015, Van Damme et al., 2012, Baeten et al., 2016). Tenofovir, commonly used as a component of ARVs, has been most extensively studied in efficacy trials for PrEP. Regardless of mode, the efficacy of tenofovir depends on cellular transportation and the enzymatic conversions, while tissue permeability and the dynamics of the vaginal environment are also crucial in the FGT (Taneva et al., 2015, Nixon et al., 2014). Intracellularly, TFV undergoes phosphorylation to the active metabolite, tenofovir diphosphate (TFV-DP), that compete with intracellular adenosine triphosphate (dATP, which is elevated in activated cells) to block HIV replication (Andrei et al., 2011, Suo and Johnson, 1998, Nixon et al., 2014). More recently, non-*Lactobacillus* spp. dominance of the vaginal microbiota, more specifically anaerobic BV-associated bacteria such as *G.vaginalis,* has been proposed as a potential contributor to the inability of topical TFV to protect women from HIV infection (Klatt et al., 2017, Hillier et al., 2017), an effect not found with oral TFV-based PrEP (Heffron et al., 2017).

Although both studies showed a negative correlation between tenofovir levels and BV-linked bacteria, several limitations were also noted. For example, these studies used data collected at single random time points, with bacterial proteins (CAPRISA 004 study) and Nugent score (the FAME programme) used to identify vaginal bacterial community groups. In addition, the biodegradation of tenofovir was only shown *in vitro*, but this may not replicate in *in vivo* conditions. Taking all this into consideration, it is clear an extensive understanding of the relationship between vaginal microbiota, genital

inflammation, host and PrEP efficacy are warranted. It is also important that the mucosal micro-environment must be understood to complement research on PrEP. Development of drugs that are not affected by environmental and host factors may lead to more consistent efficacy of PrEP in setting with imperfect drug adherence and high HIV incidence rate.

1.3 Aim and Objectives

Rationale

HIV transmission mostly occur across the mucosal surface of the female genital tract The vaginal and cervical mucosa is thought to serve as a discrete site for HIV infection, replication and pathogenesis by providing the virus with a steady supply of susceptible target cells. It was found that T lymphocytes are more prolific than B cells in the human cervix and that $CD8^+$ T cells outnumber $CD4^+$ T cells in these sites. However, very few studies have looked at the frequencies of local T and B cells in different regions within the human cervix.

Aim:

- To determine the frequency and anatomical distribution of immune cell subsets in endocervical and ectocervical tissue sections of HIV uninfected women.

***Objectives*:**

- To characterize the distribution and phenotype of T cell and B cell population in endocervical and ectocervical tissue sections of HIV uninfected women.
- To compare the availability of immune cell subsets in women using hormonal contraceptives to non-hormonal contraceptive users.

Hypothesis:

The ectocervix will have a greater abundance of immune cells compared to the endocervical region. In addition, women using hormonal contraceptives will have higher numbers of immune cells in their cervix compared to non-hormonal contraceptive users.

Chapter 2:

Frequency and Phenotype of Immune Cell Subsets in different regions of the Human Cervix: Implications for HIV Susceptibility

2.1 Introduction

The mucosal epithelium of the lower FGT provides the first line of defense against pathogen entry and mediates the initial host immune response against STIs, including HIV (Kaushic, 2011, Wira et al., 2005a, Wira et al., 2005b). In addition, this is the surface through which HIV is transmitted from an HIV infected women to her sexual partner (Yi et al., 2013). Furthermore, the vaginal and cervical mucosa is thought to serve as a discrete site for HIV replication and pathogenesis by providing the virus with a steady supply of susceptible target cells (Nguyen et al., 2014). Although several studies have defined the composition of mucosal immune cells in the gut and lungs (Givan et al., 1997, Pudney et al., 2005, Saba et al., 2010), very little is known about immunity in the FGT.

Several approaches have been used to investigate immune responses at the FGT. These methods include the use of humanized mice, macaques, cytobrushes and CVL obtained from cervico-vaginal surfaces, in combination with flow cytometry and Luminex technology (McKinnon et al., 2014, Policicchio et al., 2016, Hladik and McElrath, 2008, Grivel and Margolis, 2009, Liebenberg et al., 2011, Roberts et al., 2012). However, these studies have limitations such as the use of single cells for flow cytometry analysis, low immune cell numbers, different cellular responses to chemokines secreted by mouse epithelial cells and poor translation of non-human primate discoveries into human studies. As an alternative, human cervical samples have been used to assess women's susceptibility to HIV transmission (Givan et al., 1997, Trifonova et al., 2014). Cervical explants enable the study of HIV infection and cellular or antibody responses in intact tissues, rather than in single cells, which more closely resembles what happens *in vivo* (Grivel and Margolis, 2009). In addition, the epithelial lamina propria of the vaginal mucosa also contains an array of cells including T cells, B cells, resident phagocytes, macrophages and monocytes to provide both humoral and cell-mediated immunity (Givan et al., 1997, White et al., 1997).

T lymphocytes have been shown to be more abundant than B cells in the human cervix and that $CD8^+$ T cells outnumber $CD4^+$ T cells in these sites (McKinnon *et al.* 2014). Another study found that CD14+ macrophages, which is comprised of CD103 $CD11b^+$ $CX3CR1^+$ $DC\text{-}SIGN^+$ DCs, were the

most abundant cells in the human cervical mucosa (Trifonova et al., 2014). Although these studies show proportions of cells in the cervical mucosa, very few studies have looked at the abundance of local T and B cells in different regions within the human cervix. This study determined the frequency of immune cell subsets with a particular focus on CD4 T cell co-receptors, in endocervical and ectocervical tissue sections of HIV-uninfected women. Characterizing the abundance of major lymphoid cells and CD4 co-receptor subsets within the cervix and their localization in different anatomical regions can help associate the abundance of vulnerable cells with HIV infectivity and recruitment of HIV target cells based on the functionality of the immune cell.

2.2 Methods and Materials

2.2.1 Study Design

This study included HIV-uninfected women undergoing elective hysterectomies at Prince Mshiyeni Memorial Hospital and King Edward VII Hospital, Durban, South Africa. Patients attended hospitals for various reasons, including early stage cancer, fibroids and abnormal bleeding. All laboratory manipulation of the cervical tissue was performed in the CAPRISA Mucosal Immunology Laboratory, Durban, South Africa. Written informed consent was obtained a day before surgery from women who were deemed mentally fit by study gynaecologist and obstetrician. Patients demographic and clinical data were collected a day before surgery using a structured questionnaire. Consenting women aged 18 years and older, including post-menopausal, as well as hormonal contraceptive users, were enrolled in the study. Women with severely damaged cervical tissues, due to surgery or late stage cancer and high grade cervical intraepithelial neoplasia (CIN) patients, were excluded from the study. Ethical approvals were obtained from Prince Mshiyeni Memorial Hospital, King Edward Hospital, Department of Health (HRKM Ref: 214/18, NHRD Ref: KZ_201805_041) and the University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC: Ref: BE218/17), respectively.

2.2.2 Peripheral blood mononuclear cells for gating strategies

Peripheral blood mononuclear cells *(*PBMCs) were used to compensate for voltages, spill-over of the antibody flourochromes and to establish gating strategies for all subsequent samples. Briefly, PBMCs were isolated from blood acquired from healthy donors using BD Vacutainer® acid citrate dextrose (ACD) tubes (Becton Dickinson (BD), NJ, USA). The ACD tubes were centrifuged at 1600 revolutions per minute (rpm) for 10 minutes (min) at room temperature without brakes to ensure

adequate layer separation. Once centrifuged, the plasma layer was removed and replaced with 2% FBS/PBS (2% Fetal bovine serum (Thermo Fisher Scientific, MA, USA) in phosphate buffered saline (Lonza Group, Basel, Switzerland)) also known as FACS wash buffer, to dilute blood sample. The diluted blood sample was carefully layered over 12 ml of Ficoll-Paque (Sigma-Aldrich, MO USA) in a 50 ml conical tube. The tube was centrifuged at 1400 rpm for 30min without brakes at 4^{o}C. After centrifugation, the plasma layer was removed and the mononuclear cell layer was carefully transferred to a new 50ml conical tube with 40ml of 2% FBS/PBS. The tube was centrifuged at 1600 rpm for 10 min at 4^{o}C. After spinning, the supernatant was decanted and the pellet was resuspended in 10ml of R10 (RPMI 1640 (Lonza Group, Basel, Switzerland) media supplemented with FBS and penicillin/streptomycin (Thermo Fisher Scientific, MA USA) media. Fifty microliters of mononuclear cells in R10 media and 50µl of trypan blue were used to obtain a viable cell count. After counting, mononuclear cells in R10 media were centrifuged at 2000rpm for 6 min at 4^{o}C and the supernatant was discarded. The pellet was resuspended in 2% FBS/PBS at 1ml per million cells, centrifuged and stained at 4^{o}C for 60 minutes in the dark with the following markers: CXCR5-APC; PD-1-BV421; CXCR4-PE-Cy5; CXCR3-PE-Cy7; CD4-BV605; CD25-FITC; CD127-PE; CD19-Alexa Flour 700 (BioLegend, CA, USA); Live/Dead aqua for Amcyan (Thermo Fisher Scientific, MA, USA); CD3-APC-H7; CCR5-PerCp-Cy5.5; CCR6-BV650 (BD, NJ, USA). Compensation for voltages and spillover of the antibody flourochromes were performed and at least 1000,000 events were acquired using a FACSDiva flow cytometer (BD Immunocytometry Systems, NJ, USA). Gates differentiating negative and positive populations were set by fluorescence minus one (FMO) staining, which stains cells with all fluorochromes used in the experiment except one (Perfetto et al., 2004). Cell doublets were excluded using forward scatter-area versus forward scatter-height parameters and dead cells were excluded from the analyses.

2.2.3 Human Cervical Tissue Collection and Preparation

Hysterectomies were performed at Prince Mshiyeni Memorial Hospital and King Edward VII Hospital. After surgery, cervical tissue was cut from the uterus using a scalpel blade and forceps and placed into R15 transport media (containing RPMI 1640, FBS, Pen/Strep, Amphotericin B) and transported to the laboratory on ice within 3 hours from collection. In the petri dish, tissue was rinsed with new transport media (R15) and kept in solution at all times to prevent tissue from drying out. Excessive muscular tissue was trimmed with scalpels and forceps, being sure to limit handling of the epithelial layer. The endocervix and ectocervix were separated and the tissue epithelial layer was carefully dissected into 2mm blocks using biopsy punch.

2.2.4 Tissue block digestion and dissociation

To prepare a single cell suspension, the dissected 2mm tissue blocks were subjected to mechanical digestion and dissociation. Briefly, the tissue blocks were placed into a C-tube (MACS Miltenyi Biotec, CA, USA) containing 40µl of collagenase solution (0.2g of collagenase IV powder (Thermo Fisher Scientific, MA, USA), 2ml of HBSS (Lonza Group, Basel, Switzerland), 8µl of DNase 1 (Thermo Fisher Scientific, MA, USA) and 8ml of R10 media. The solution with tissue blocks was then mechanically digested and dissociated for 60 seconds using mouse spleen (m_spleen_01) program of the gentle-MACS Dissociator (MACS Miltenyi Biotec, CA, USA), as previously described (Trifonova et al., 2014). After dissociation, the cell suspension was incubated for 60 minutes at 37°C. Cells were collected by filtering through a 100µm cell strainer (MACS Miltenyi Biotec, CA, USA), into a new 50ml conical tube. Any material left after the filtering was discarded. During the optimization steps, C-tubes with suspended tissue blocks were allowed to digest and dissociate without using the gentle-MACS Dissociator and compared to those dissociated using gentle-MACS Dissociator. The frequency of cells was too low in those digested without gentle-MACS Dissociator and the rest of the study was carried with mechanical digestion and dissociation.

2.2.5 Tissue Staining and flow cytometry

During subsequent rounds of digestion, the cell suspension was centrifuged at 1800 rpm for 5 minutes at 4^{o}C and cell pellets were resuspended in labelled (endocervix and ectocervix, respectively) BD FACS tubes (BD, NJ, USA) containing 2% FBS/PBS. The cells were centrifuged at 1800 rpm for 3 minutes and the supernatant was discarded. The cells were stained with antibodies for T cell lineage markers and their receptors. Briefly, 50ul of antibody cocktail was added into FACS tubes with resuspended cells and incubated at room temperature for 20 minutes in the dark with the following markers: CXCR5-APC; PD-1-BV421; CXCR4-PE-Cy5; CXCR3-PE-Cy7; CD4-BV605; CD25-FITC; CD127-PE; CD19-Alexa Flour 700 (BioLegend, CA, USA); Live/Dead aqua for Amcyan (Thermo Fisher Scientific, USA); CD3-APC-H7; CCR5-PerCp-Cy5.5; CCR6-BV650 (BD, NJ, USA). Cells were then washed with 150µl 2%FBS/PBS and centrifuged at 1800 rpm for 3 minutes after which supernatant was discarded. After final wash, an additional 150µl of 2% FBS/PBS was added to cells and 200µl of cell fix (BD, NJ, USA) was added to the cell suspension and stored at 4^{o}C until flow cytometry.

Compensation for voltages and spillover of the antibody flourochromes were performed initially using PBMCs and subsequently, BD rainbow beads were used before acquiring samples to maintain and ensure accuracy. At least 100,000 events were acquired using a FACSDiva flow cytometer (BD Immunocytometry Systems). Gates differentiating negative and positive populations were set by FMO staining (Perfetto et al., 2004). Cell doublets were excluded using forward scatter-area versus forward scatter-height parameters and dead cells were excluded from the analyses. Flow cytometry data was analysed, and gating strategies and frequencies were established using the FlowJo v10 software (Tree Star, CA, USA).

2.2.6 Statistical Analysis

Descriptive statistics were used to compare demographic characteristics. Mann-Whitney U test was applied for non-parametric independent sample comparisons and Kruskal-Wallis (Nonparametric One-Way ANOVA) tests were used for non-parametric assessments of variation between groups. Spearman's rank correlation (nonparametric correlation) was used to explore the relationship between immune cell subsets within individual cervical regions and across cervical regions. *P* values of <0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism version 7.03 (GraphPad Software, San Diego, CA, U.S.A.) and SAS version 9.3 (SAS Institute Inc., Cary).

2.3. Results

2.3.1 Study demographics

This study included 6 HIV-uninfected women undergoing elective hysterectomies (Table 2.1). All participants were black South African women, with a median age of 52.5 (47.3 – 58 years). Three of the women self-reported use of hormonal contraception to prevent unintended pregnancy or to control abnormal bleeding, while three did not use any form of hormonal contraceptives. Clinical reasons for hysterectomy varied, with half (3/6) of women with abnormal bleeding and the other half with multiple fibroids. Due to lack of vaginal swabs, this study could not screen for STIs and bacterial vaginosis.

Table 2. 1. Study characteristics of the women undergoing elective hysterectomies

Characteristics	**HIV Negative Women**
	n (%)
N	6
Age (years), median (IQR)	52.5 (47.3 -58)
Hormonal Contraceptives	
DMPA	1 (16.7)
Other (COC or NET-EN)	2 (33.3)
Non-contraceptive users	3 (50)
Reasons for hysterectomies	
Abnormal bleeding	3 (50)
Multiple Fibroid uterus	3 (50)

2.3.2. Gating strategy

To define the abundance of local T and B cells in different regions within the human cervical tissue, this study optimized the gating strategy using both PBMCs and cervical tissues. Figure 2.1 summarizes the gating strategy to identify activated T cell populations by flow cytometry. Cell doublets or aggregates were excluded by gating of singlets while live cells were distinguished into CD19+ B cell and CD3+ T cell populations. Live CD3+ T cell populations were further distinguished into CD4+ T cell populations. General expression of HIV co-receptors (such as CCR5 and CXCR4), inflammatory and regulatory (CCR6, CXCR3, CD25 CXCR5 and CD127) and markers of exhaustion (PD-1) were distinguished in CD4+ T cell population. Gates distinguishing negative and positive populations were defined by fluorescence minus one (FMOs) staining (Figure 2.2), where one

antibody of interest is left out of the panel in order to see which region would and would not have cells when the antibody is present or absent.

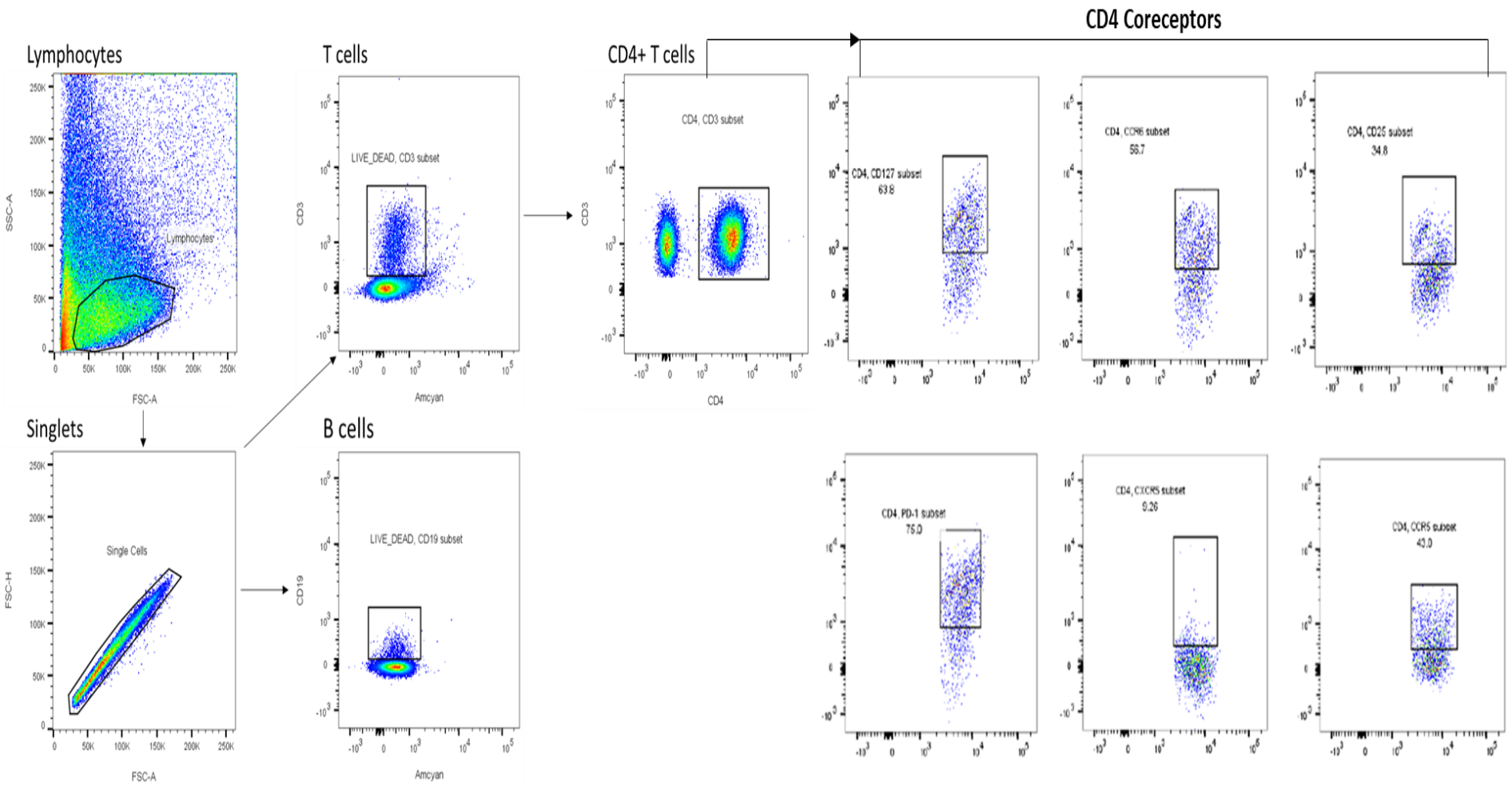

Figure 2. 1. The gating strategy used to identify activated T cell populations by flow cytometry**.** PBMC and genital tract MMCs were stained with fluorochromes-labelled monoclonal antibodies to define CXCR5-APC; PD-1-BV421; CXCR4-PE-Cy5; CXCR3-PE-Cy7; CD4-BV605; CD25-FITC; CD127-PE; CD19-Alexa Flour 700; Live/Dead aqua for Amcyan; CD3-APC-H7; CCR5-PerCp-Cy5.5; CCR6-BV650–expressing populations.

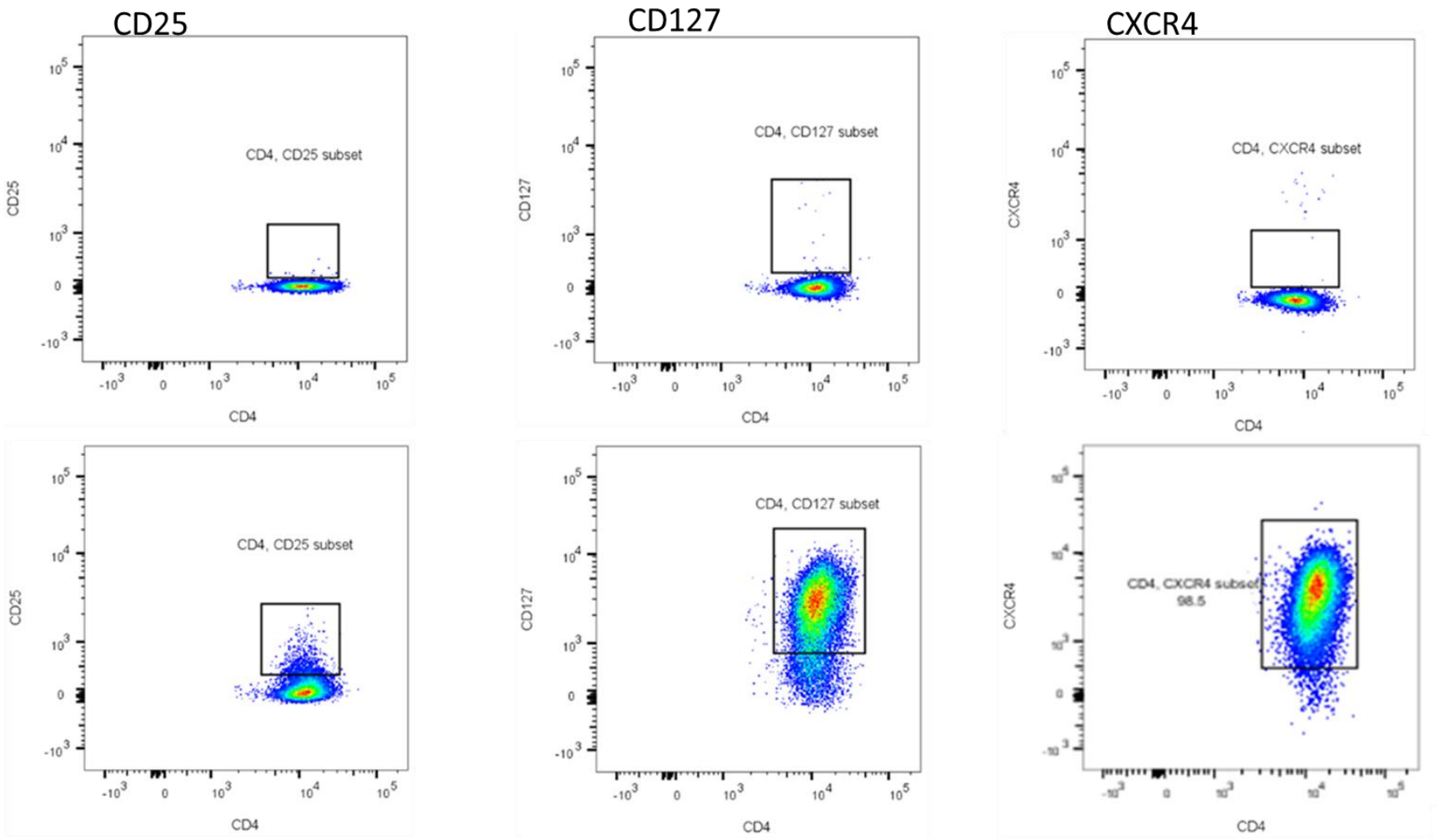

Figure 2. 2. The fluorescence minus one (FMO) gating strategy. Gates distinguishing negative and positive populations were set by fluorescence minus one (FMO) staining, where samples were stained with all antibodies except the one of interest

2.3.3 Comparison of immune subsets in PBMCs and cervical tissue

The frequency of B and T cells, including CD4+ T cell co-receptors in cervical tissues were compared to matching cells found in PBMC samples from HIV-uninfected women. Lymphocytes population (86% vs 27%, p = <0.0001) and the CD3+ cell expression (81% vs 11%, p = 0.001) were more abundant in the PBMCs compared to cervical tissues, respectively (Figure 2.3a). There was no significant difference in the proportion of CD19+ B cells in cervical tissues and PBMCs (4% vs 3%, p = 0.314). Focusing on the CD4+ T cell compartment (Figure 2.3b), the frequency of CD4+ T cells was significantly higher in PBMCs (65%) compared to cervical tissues (45%), p = 0.0009. Frequencies of CCR5 expression (38% vs 29%, p = <0.0001), CCR6 expression (63% vs 27%, p = <0.0001), CXCR3 expression (66% vs 20%, p = <0.0001), CXCR5 expression (19% vs 16%, p = <0.0001) by CD4+ T cells were significantly elevated in the cervical tissues compared to the PBMCs. CXCR4 expression by CD4+ T cells was more abundant in PBMCs than cervical tissues (97% vs 69%, p = 0.001). Compared to PBMCs, cervical CD4+ T cell population had elevated markers of T-regulatory cells CD25 (34% vs 5%, p = <0.0001) and exhaustion marker, PD-1(68% vs 34%, p = <0.0001) but decreased secretion of CD127 (68% vs 89%, p = 0.001).

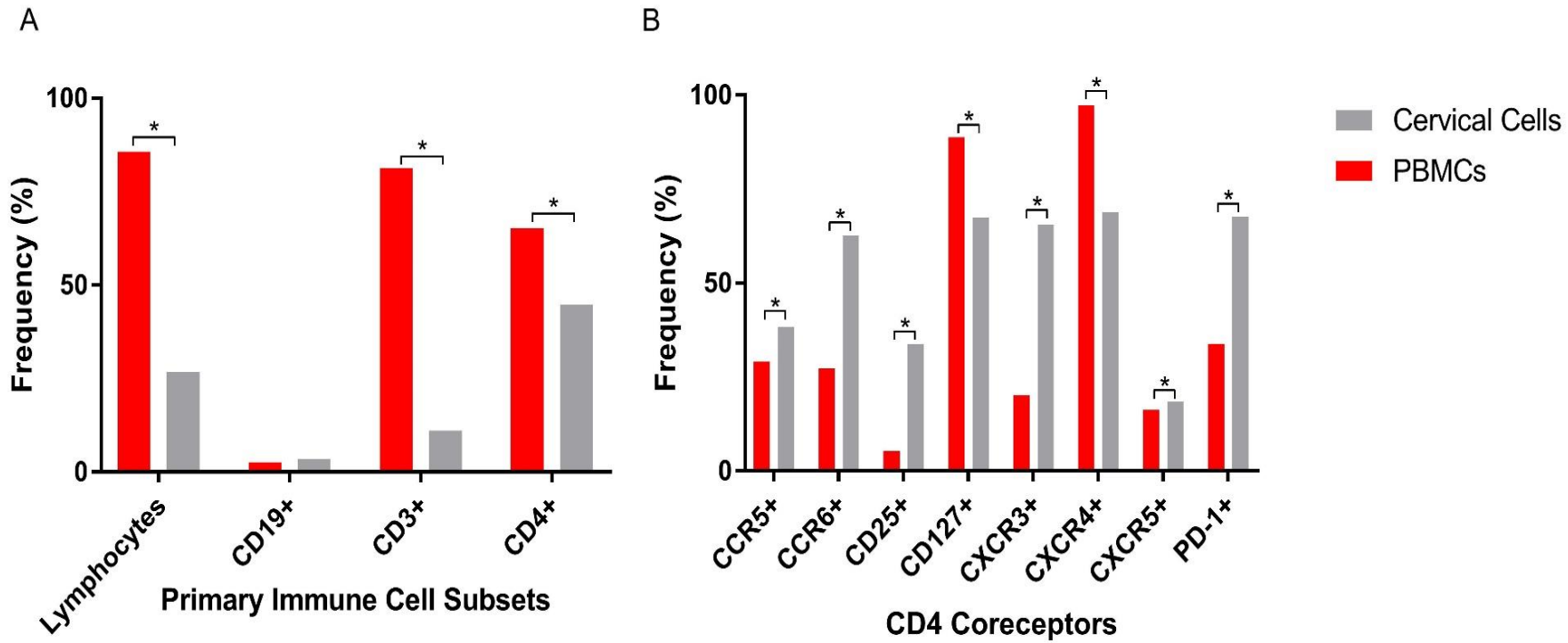

Figure 2. 3. Distribution of immune cell subsets in PMBCs and cervical tissues. The frequency of cervical tissue immune cell subsets (n=6) was compared with PBMCs of normal donors (n=2). A) shows primary immune cell subsets and B) shows CD4 cell subsets. Statistical significance represented as * ($p < 0.05$).

2.3.4 Comparison of immune subsets in cervical tissue compartments

This study defined the abundance and distribution of the major immune cells within the different regions of the cervix (Figure 2.4). Of the 6 participants recruited, only 4 had cervical tissue samples containing both endocervix and ectocervix while endocervix could not be determined in two participants.

In the cervix, 11% of lymphocytes were CD3+ T cells and 3% were CD19+ B cells. Of the CD3+ T cell population, 45% were CD4+ T cells. This study did not measure CD8+ T cell populations but our assumption is that the remaining 55% belongs to CD8 T cell subset. Trifonova et al., (2014) showed that CD8 were the most abundant T cell population compared to CD4 cells in the cervix (Trifonova et al., 2014). No significant differences in frequency between immune cell subsets measured in the ectocervix versus the endocervix were found. The frequency of lymphocytes was similar in the ectocervix compared to the endocervix (31% vs 29%, $p = 0.610$). Similar numbers of CD3 cells were detected in ectocervix (10%) and endocervix (6%). When analysing the CD3+ T cell subsets, 48% of the CD4+ T cells were detected in the endocervix while 46% was found in the ectocervix. The overall frequency of B cells observed in both regions was very low, with 4% of CD19+ cells found in the endocervix and 2% in the ectocervix.

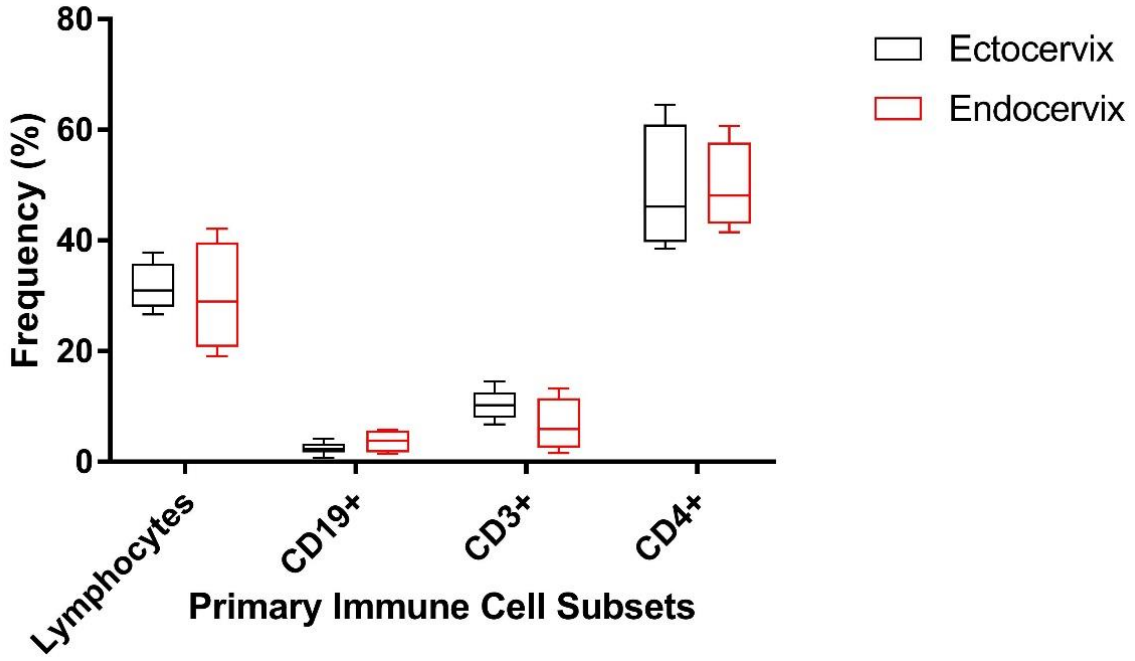

Figure 2. 4. Primary immune cell subset frequencies in the Ectocervix and Endocervix. No significant differences were seen in frequencies of Lymphocytes, B cells and T cells between the two regions, however, observational trends were seen.

2.3.5 Comparison of CD4+ T cell co-receptors in cervical tissue compartments

To analyse the abundance of CD4+ T cell co-receptors in the cervix, we co-stained for CXCR4, CD127, CXCR3 and CCR6, CCR5, CD25 and PD1 (Figure 2.5). Overall, the majority of the CD4+ T cells the co-receptor expressed were CXCR4 (69%), followed by CD127 (67%), CXCR3 (66%) and CCR6 (63%). CCR5 was detected in 38%, while CD25 and PD1 were expressed in 34% of CD4+ T cells, respectively. None of these CD4+ T cells co-receptors were significantly different in the ectocervix and endocervix, with an exception of CCR6 and PD1 that showed a trended towards being elevated in the endocervix compared to ectocervix, p=0.067, respectively. Briefly, the median frequencies of CCR5 (50% vs 31%), CXCR4 (84% vs 78%), CCR6 (65% vs 54%, p = 0.067), CXCR3 (79% vs 61%), were slightly more abundant in the endocervix compared to ectocervix. The other abundant cells in the endocervix compared to ectocervix were regulatory T cells such as CD25

(48% vs 26%) and CD127 (75% vs 71%). Furthermore, frequency of exhausted CD4+ T cells, PD1, was more abundant in the endocervix (77%) than ectocervix (64%).

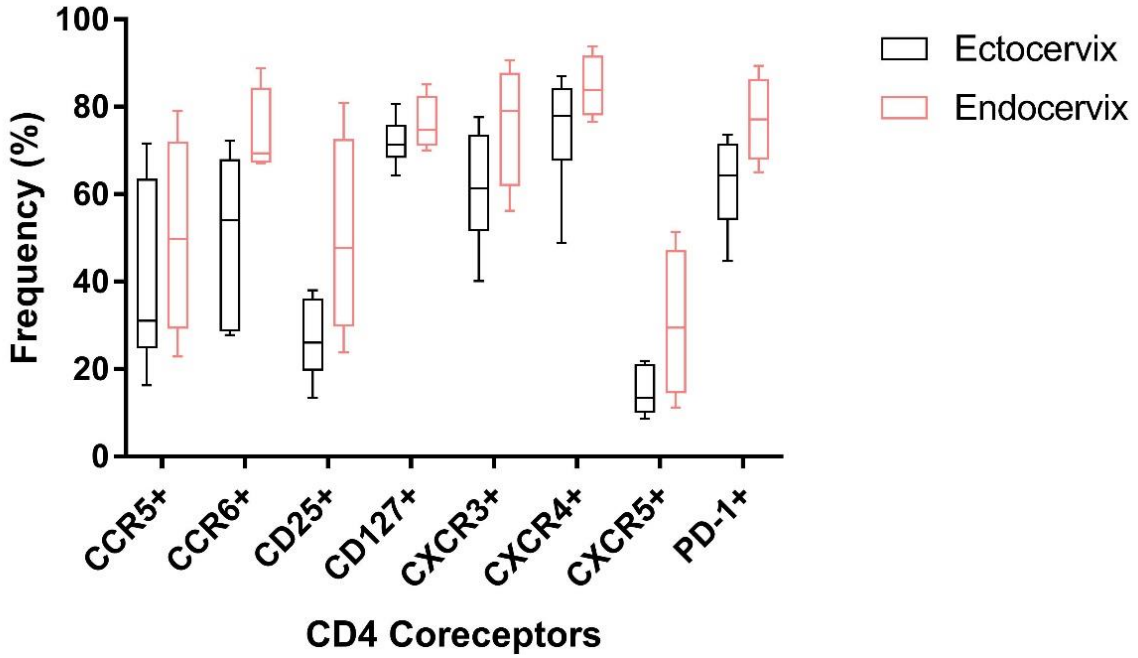

Figure 2. 5. CD4+ cell in the ectocervix and endocervix. No significant difference was seen when comparing immune cell frequencies of CD4 receptor subsets between the two cervical regions.

2.3.6 Hormonal Contraceptive did not influence frequencies of immune cell subsets in the cervical tissues

The impact of hormonal contraceptives on the frequency of immune cells was determined by comparing immune cell subsets in women on HC versus those who were not. There was no difference in immune cell subsets when stratified according to HC use (HC users versus non-HC users) (Figure2.6a). Similarly, CD4+ co-receptors did not differ between the HC groups (Figure2.6b).

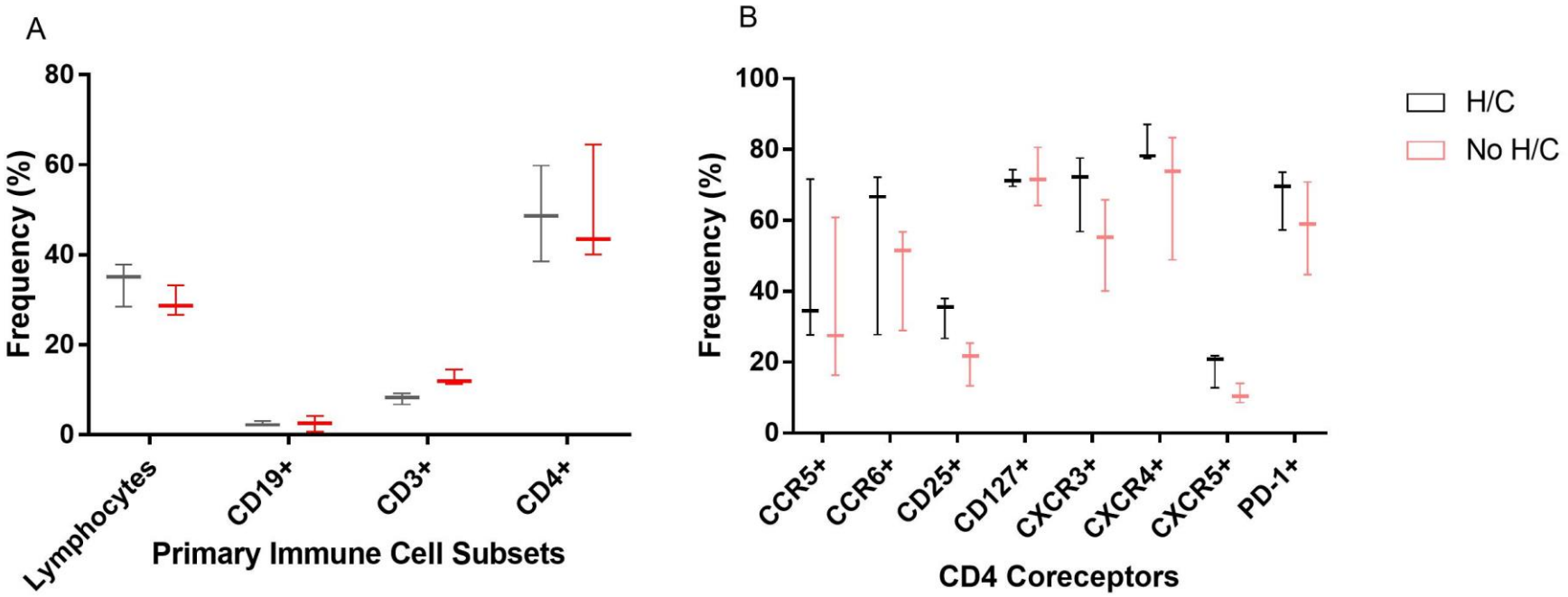

Figure 2. 6. Frequencies of immune cell subsets in the human cervix. (A) Distribution of immune cells amongst HC users and non-HC users. (B) Distribution of CD4+ T cell co-receptors amongst HC users and non-HC user

2.3.7 Relationship between CD4 T cell co-receptors in the ectocervix and endocervix

A Spearman's rank correlation test was used to assess an association between CD4 T cell co-receptors in both the ectocervical and endocervical region. Table 2.2 represents the R and p values for immune cell subsets in both cervical regions. CD4+ T cells expressing CD25 were positively associated with CXCR4 chemokine receptor expression on CD4+ T cells (r = 0.817, p = 0.047) while CCR5 showed a trend towards a negative correlation with CD127 in the ectocervix (r = –0.789, p = 0.062). Within the endocervix, CCR6 cells were positively associated with CD127 CD4+ T cells (r = 0.954, p = 0.046). Similarly, CD127 and CD4+ T cells were also positively associated with PD1 (r = 0.967, p = 0.033). Furthermore, CD25 showed a trend towards a negative correlation with CXCR3 CD4+ cells in the endocervix (r = 0.942, p = 0.058). There was no significant correlation between cervical B and T cell subsets, data not shown.

Table 2. 2. R and P values for the correlation of ectocervical and endocervical CD4+ T cell co-receptor subsets. Significant associations shown in red.

	CCR5	CCR6	CD25	CD127	CXCR3	CXCR4	CXCR5	PD-1
CCR5		R = -0.091 P = 0.846	R = -0.004 P = 0.994	R = -0.789 P = 0.062	R = 0.138 P = 0.795	R = -0.486 P = 0.328	R = 0.509 P = 0.303	R = 0.452 P = 0.368
CCR6	R = 0.194 P = 0.806		R = 0.781 P = 0.067	R = 0.400 P = 0.433	R = 0.500 P = 0.313	R = 0.667 P = 0.148	R = 0.549 P = 0.259	R = 0.548 P = 0.260
CD25	R = -0.407 P = 0.593	R = 0.777 P = 0.223		R = 0.266 P = 0.610	R = 0.598 P = 0.210	R = 0.817 P = 0.047	R = 0.732 P = 0.098	R = 0.611 P = 0.198
CD127	R = 0.302 P = 0.698	R = 0.954 P = 0.046	R = 0.747 P = 0.253		R = 0.299 P = 0.565	R = 0.569 P = 0.239	R = -0.285 P = 0.584	R = -0.460 P = 0.359
CXCR3	R = -0.642 P = 0.358	R = 0.522 P = 0.478	R = 0.942 P = 0.058	R = 0.515 P = 0.485		R = 0.200 P = 0.704	R = 0.706 P = 0.117	R = 0.029 P = 0.956
CXCR4	R = 0.210 P = 0.790	R = 0.909 P = 0.091	R = 0.574 P = 0.426	R = 0.757 P = 0.243	R = 0.285 P = 0.715		R = 0.229 P = 0.662	R = 0.375 P = 0.464
CXCR5	R = -0.613 P = 0.387	R = -0.386 P = 0.614	R = 0.212 P = 0.788	R = -0.231 P = 0.769	R = 0.504 P = 0.496	R = -0.679 P = 0.321		R = 0.682 P = 0.136
PD-1	R = 0.510 P = 0.490	R = 0.868 P = 0.132	R = 0.571 P = 0.429	R = 0.967 P = 0.033	R = 0.327 P = 0.673	R = 0.653 P = 0.347	R = -0.264 P = 0.736	

Ectocervical cells

Endocervical cells

2.4. Discussion

Little is known about the abundance of local T and B cells in different regions within the human cervix. The current study determined the frequency and anatomical distribution of immune cell subsets in endocervical and ectocervical tissue sections of HIV uninfected women undergoing elective hysterectomies. This study found more T cell (CD3+ and CD4+ cells) populations in PBMCs compare to the cervical tissues. Frequencies of CD19+ B cells were similar in the PBMCs and cervical tissues. Consistent with previous studies (McKinnon et al., 2011, Trifonova et al., 2014), this study showed that the expression of CD4+ T cell co-receptors were more pronounced in the cervical tissues compared to the PBMCs. Although there is no precise reason for the difference in immune cell population between blood and cervical mucosa, previous studies have suggested that target cells reside deeper within the sub-basal milieu and only migrate to the cervix when dendritic cells present exogenous antigen on MHC class I molecules, resulting in activation, proliferation and migration to the site of infection (Miller and Shattock, 2003).

Comparing immune cell subsets in cervix, T cells (CD3+ cells) frequencies were higher compared to the B cells (CD19+ cells). When comparing the immune cell subsets in the endocervix and ectocervix, this study found differences in the distribution of B and T cells, with T cells being the most abundant. There were no significant differences seen in immune cells between the compartments and the lack of observed difference may be attributed to small sample size. Although the overall frequency of B cells was very low, CD19+ cells were twice as abundant in the ectocervix compared to the endocervix, consistent with previous reports (Trifonova et al., 2014). The lack of B cells in the cervical tissue may be due to the absence of infection as B cells are known to expand over the course of a primary infection (Hickey et al., 2011). Forty-five percent of CD3+ T cells in the cervical tissues were CD4+ T cells. No significant differences in frequency of CD4+ T cells measured in ectocervix versus endocervix were found. We did not measure CD8+ T cell populations, but it plausible to assume that the remaining unidentified population belongs to CD8 T cell subset. Although there is no significant difference in CD4+ T cells in the cervix, their presence in this site may lead to infection and pathogenesis in presence of a virus (Miller et al., 2005, Zhang et al., 1999). HIV infects the infiltrating CD4+ T cells in the cervical tissue and uses these to disseminate from the site of initial infection to other sites (Zhang et al., 1999, Miller et al., 2005).

As much as CD4 is the main cellular receptor for HIV entry, alone, it is not sufficient for HIV infection. Several chemokine receptors are needed to serve as co-factors that allow HIV entry when co-expressed with CD4 on a cell surface. These may include CXCR4, CXCR3, CCR6, and CCR5. With an exception of CCR6, this study did not find any difference in the proportions of the co-receptors in the ectocervix and endocervix. CCR6 showed a trend towards being elevated in the endocervix compared to ectocervix. CD4+ T cells expressing CCR6 are identified as Th17 cells (Acosta-Rodriguez et al., 2007, Annunziato et al., 2012). Several studies have shown that Th17 cells are highly susceptible to HIV-infection and are depleted early from the cervix of HIV-infected patients (Sallusto et al., 2012, Weaver et al., 2013, Stieh et al., 2016). CCR6+ CD4+ T cells (Th17) play an important role in maintaining vaginal epithelial integrity, and their depletion may prompt genital inflammation that may further drive viral replication and facilitating HIV pathogenesis (Weaver et al., 2013, Stieh et al., 2016). This study cannot confirm whether CCR6+ CD4+ T cells found are Th17 due to the lack of transcriptional regulator RORγt that provides further evidence that these cells are specifically of the Th17 lineage. In contrast to other studies, this study observed a positive correlation amongst CD4+ T cell co-receptors and this may be attributed to small sample size.

Another CD4+ T cell subsets measured in this study were T regulatory cells, such as CD25 and CD127, essential for the maintenance of self-tolerance (Sakaguchi et al., 1995). There was no significant difference in either CD25 or CD127 comparing the ectocervix and endocervix. Co-expression of CD4+CD25+ cells may lead to increased HIV susceptibility in HIV uninfected individuals as these cells may be infected by HIV because they express HIV's primary receptor (CD4) and co-receptors (CXCR4/CCR5). However, it is not known whether the infected CD4+CD25+ Treg cells die due to virus replication or remain effective as regulators (Nilsson et al., 2006, Epple et al., 2006, Oswald-Richter et al., 2004). Increased CD127 levels are strongly associated with non-redundant mechanism of maintaining CD4 T cell survival and proliferation while decreased levels are linked to increased rate of disease progression, increased T-cell death resulting in CD4+ T-cell loss, and impairment of protective functional immunity (Schluns et al., 2000, Chetoui et al., 2010, Fry and Mackall, 2005). Furthermore, this study found a slightly higher frequency of exhausted CD4+ T cells, PD1, in the endocervix than ectocervix. Increased levels of PD-1 are known to inhibit HIV-specific T cells in humans. Taken together, this suggests that

CD4+CD25+ and CD4+PD1+ T cells can mediate an environment that supports HIV survival and persistence by suppressing HIV-specific CD4+ T cells.

Women using the long-acting injectable depot medroxyprogesterone acetate (DMPA) are reportedly at 2-fold increased risk for HIV infection from epidemiology studies, and at an increased risk of transmitting HIV to their sexual partners should they become infected (Baeten et al., 2007, Heffron et al., 2012, Martin et al., 1998, Morrison et al., 2010). The mechanisms by which injectable HCs might contribute to an increased risk of HIV-1 acquisition and transmission is through modulating recruitment of CD4+ T cells (Chandra et al., 2013). Thus we compared the availability of immune cell subsets in women using hormonal contraceptives to non-hormonal contraceptive users. There were no differences in frequencies of immune subsets (both B and T cells) amongst women on hormonal contraceptives versus non-hormonal contraceptive users.

This study had several limitations including small sample size and lack of cervical tissues from younger women. To circumvent this in the future, we have extended the network of hospitals to improve the numbers of young women recruited to the study. Another limitation of this study was that we were unable to differentiate CD8 from CD3 T cell populations. We also focused only on the frequency and distribution of B and T cells and no other HIV target cells such as antigen presenting cells. This will be addressed in future studies, which will also include assessing the abundance of major lymphoid cells within the cervix and their localization in different anatomical regions. The possibility that the cervical tissue may have been in an inflamed state as a result of surgery or during laboratory processing could have influenced the frequency and distribution of immune cell subsets.. Furthermore, all women were of an advanced age (possibly post-menopausal), HIV uninfected, with unknown immune, BV and STI status, which could have altered the frequency and distribution of immune cell subsets. Future sample collection will include cervicovaginal swabs for cytokine, BV and STI screening and matching PBMCs for immunity. We will also collect information on clinical factors which might influence immune cell composition in the genital tract. These include sexual activity, vaginal insertion practices (such as the use of tobacco, snuff, savlon, which can alter the immunological environment of the genital tract) and use of intra-uterine devices.

In conclusion, we characterized the anatomical distribution of T and B cells, comparing different regions within the cervical tissue (endocervix and ectocervix). Endocervical tissue had slightly more immune CD4 T cell co-receptors compared ectocervical tissues. This suggests that the endocervical region might be associated with infectivity through an abundance of HIV target cells. Understanding the clinical links of the HIV susceptible immune cells in different regions of the cervix, as well as its influence in both HIV acquisition and in host defense against invading pathogens, may lead to novel strategies for HIV prevention and contribute to better women's health.

References

ABBAI, N. S., REDDY, T. & RAMJEE, G. 2016. Prevalent Bacterial Vaginosis Infection - a Risk Factor for Incident Sexually Transmitted Infections in Women in Durban, South Africa. *INTERNATIONAL JOURNAL OF STD AND AIDS,* 27**,** 1283-1288.

ABDOOL KARIM, Q., ABDOOL KARIM, S. S., FROHLICH, J. A., GROBLER, A. C., BAXTER, C., MANSOOR, L. E., KHARSANY, A. B., SIBEKO, S., MLISANA, K. P., OMAR, Z., GENGIAH, T. N., MAARSCHALK, S., ARULAPPAN, N., MLOTSHWA, M., MORRIS, L., TAYLOR, D. & GROUP, C. T. 2010. Effectiveness and Safety of Tenofovir Gel, an Antiretroviral Microbicide, for the Prevention of Hiv Infection in Women. *SCIENCE,* 329**,** 1168-1174.

ABDOOL KARIM, Q., BAXTER, C. & BIRX, D. 2017. Prevention of Hiv in Adolescent Girls and Young Women: Key to an Aids-Free Generation. *JOURNAL OF ACQUIRED IMMUNE DEFICIENCY SYNDROME,* 75 Suppl 1**,** S17-S26.

ABDOOL KARIM, Q., KHARSANY, A. B., FROHLICH, J. A., WERNER, L., MLOTSHWA, M., MADLALA, B. T. & ABDOOL KARIM, S. S. 2012. Hiv Incidence in Young Girls in Kwazulu-Natal, South Africa--Public Health Imperative for Their Inclusion in Hiv Biomedical Intervention Trials. *AIDS AND BEHAVIOR,* 16**,** 1870-1876.

ABEL, K., ROURKE, T., LU, D., BOST, K., MCCHESNEY, M. B. & MILLER, C. J. 2004. Abrogation of Attenuated Lentivirus-Induced Protection in Rhesus Macaques by Administration of Depo-Provera before Intravaginal Challenge with Simian Immunodeficiency Virus Mac239. *THE JOURNAL OF INFECTIOUS DISEASES,* 190**,** 1697-1705.

ACOSTA-RODRIGUEZ, E. V., RIVINO, L., GEGINAT, J., JARROSSAY, D., GATTORNO, M., LANZAVECCHIA, A., SALLUSTO, F. & NAPOLITANI, G. 2007. Surface Phenotype and Antigenic Specificity of Human Interleukin 17–Producing T Helper Memory Cells. *NATURE IMMUNOLOGY,* 8**,** 639-646.

AL-HARTHI, L., ROEBUCK, K. A., OLINGER, G. G., LANDAY, A., SHA, B. E., HASHEMI, F. B. & SPEAR, G. T. 1999. Bacterial Vaginosis-Associated Microflora Isolated from the Female Genital Tract Activates Hiv-1 Expression. *JOURNAL OF ACQUIRED IMMUNE DEFICIENCY SYNDROMES,* 21**,** 194-202.

ALKHATIB, G. 2009. The Biology of Ccr5 and Cxcr4. *CURRENT OPINION IN HIV AND AIDS,* 4**,** 96-103.

AMJADI, F., SALEHI, E., MEHDIZADEH, M. & AFLATOONIAN, R. 2014. Role of the Innate Immunity in Female Reproductive Tract. *ADVANCED BIOMEDICAL RESEARCH,* 10.4103/2277-9175.124626.

ANAHTAR, M. N., BYRNE, E. H., DOHERTY, K. E., BOWMAN, B. A., YAMAMOTO, H. S., SOUMILLON, M., PADAVATTAN, N., ISMAIL, N., MOODLEY, A., SABATINI, M. E., GHEBREMICHAEL, M. S., NUSBAUM, C., HUTTENHOWER, C., VIRGIN, H. W., NDUNG'U, T., DONG, K. L., WALKER, B. D., FICHOROVA, R. N. & KWON, D. S. 2015. Cervicovaginal Bacteria Are a Major Modulator of Host Inflammatory Responses in the Female Genital Tract. *IMMUNITY,* 42**,** 965-976.

ANDREI, G., LISCO, A., VANPOUILLE, C., INTROINI, A., BALESTRA, E., VAN DEN OORD, J., CIHLAR, T., PERNO, C. F., SNOECK, R., MARGOLIS, L. & BALZARINI, J. 2011. Topical Tenofovir, a Microbicide Effective against Hiv, Inhibits Herpes Simplex Virus-2 Replication. *CELL HOST MICROBE,* 10**,** 379-389.

ANNUNZIATO, F., COSMI, L., LIOTTA, F., MAGGI, E. & ROMAGNANI, S. 2012. Defining the Human T Helper 17 Cell Phenotype. *TRENDS IN IMMUNOLOGY,* 33**,** 505-512.

ARKELL, C. 2016. *Hiv and the Female Genital Tract–What Does It Mean for Hiv Prevention?* [Online]. CATIE. Available: https://www.catie.ca/en/pif/fall-2016/hiv-and-female-genital-tract-what-does-it-mean-hiv-prevention [Accessed 28 November 2018].

ARNOLD, K. B., BURGENER, A., BIRSE, K., ROMAS, L., DUNPHY, L. J., SHAHABI, K., ABOU, M., WESTMACOTT, G. R., MCCORRISTER, S. & KWATAMPORA, J. 2016. Increased Levels of Inflammatory Cytokines in the Female Reproductive Tract Are Associated with Altered Expression of Proteases, Mucosal Barrier Proteins, and an Influx of Hiv-Susceptible Target Cells. *MUCOSAL IMMUNOLOGY,* 9**,** 194-205.

BAETEN, J. M., DONNELL, D., NDASE, P., MUGO, N. R., CAMPBELL, J. D., WANGISI, J., TAPPERO, J. W., BUKUSI, E. A., COHEN, C. R., KATABIRA, E., RONALD, A., TUMWESIGYE, E., WERE, E., FIFE, K. H., KIARIE, J., FARQUHAR, C., JOHN-STEWART, G., KAKIA, A., ODOYO, J., MUCUNGUZI, A., NAKKU-JOLOBA, E., TWESIGYE, R., NGURE, K., APAKA, C., TAMOOH, H., GABONA, F., MUJUGIRA, A., PANTELEEFF, D., THOMAS, K. K., KIDOGUCHI, L., KROWS, M., REVALL, J., MORRISON, S., HAUGEN, H., EMMANUEL-OGIER, M., ONDREJCEK, L., COOMBS, R. W., FRENKEL, L., HENDRIX, C., BUMPUS, N. N., BANGSBERG, D., HABERER, J. E., STEVENS, W. S., LINGAPPA, J. R., CELUM, C. & PARTNERS PR, E. P. S. T. 2012.

Antiretroviral Prophylaxis for Hiv Prevention in Heterosexual Men and Women. *THE NEW ENGLAND JOURNAL OF MEDICINE,* 367**,** 399-410.

BAETEN, J. M., LAVREYS, L. & OVERBAUGH, J. 2007. The Influence of Hormonal Contraceptive Use on Hiv-1 Transmission and Disease Progression. *CLINICAL INFECTIOUS DISEASES,* 45**,** 360-369.

BAETEN, J. M., PALANEE-PHILLIPS, T., BROWN, E. R., SCHWARTZ, K., SOTO-TORRES, L. E., GOVENDER, V., MGODI, N. M., MATOVU KIWEEWA, F., NAIR, G., MHLANGA, F., SIVA, S., BEKKER, L. G., JEENARAIN, N., GAFFOOR, Z., MARTINSON, F., MAKANANI, B., PATHER, A., NAIDOO, L., HUSNIK, M., RICHARDSON, B. A., PARIKH, U. M., MELLORS, J. W., MARZINKE, M. A., HENDRIX, C. W., VAN DER STRATEN, A., RAMJEE, G., CHIRENJE, Z. M., NAKABIITO, C., TAHA, T. E., JONES, J., MAYO, A., SCHECKTER, R., BERTHIAUME, J., LIVANT, E., JACOBSON, C., NDASE, P., WHITE, R., PATTERSON, K., GERMUGA, D., GALASKA, B., BUNGE, K., SINGH, D., SZYDLO, D. W., MONTGOMERY, E. T., MENSCH, B. S., TORJESEN, K., GROSSMAN, C. I., CHAKHTOURA, N., NEL, A., ROSENBERG, Z., MCGOWAN, I., HILLIER, S. & TEAM, M.-A. S. 2016. Use of a Vaginal Ring Containing Dapivirine for Hiv-1 Prevention in Women. *THE NEW ENGLAND JOURNAL OF MEDICINE,* 375**,** 2121-2132.

BEAGLEY, K. W. & GOCKEL, C. M. 2003. Regulation of Innate and Adaptive Immunity by the Female Sex Hormones Oestradiol and Progesterone. *FEMS IMMUNOLOGY & MEDICAL MICROBIOLOGY,* 38**,** 13-22.

BORGDORFF, H., GAUTAM, R., ARMSTRONG, S. D., XIA, D., NDAYISABA, G. F., VAN TEIJLINGEN, N. H., GEIJTENBEEK, T. B., WASTLING, J. M. & VAN DE WIJGERT, J. H. 2016. Cervicovaginal Microbiome Dysbiosis Is Associated with Proteome Changes Related to Alterations of the Cervicovaginal Mucosal Barrier. *MUCOSAL IMMUNOLOGY,* 9**,** 621-633.

CHANDRA, N., THURMAN, A. R., ANDERSON, S., CUNNINGHAM, T. D., YOUSEFIEH, N., MAUCK, C. & DONCEL, G. F. 2013. Depot Medroxyprogesterone Acetate Increases Immune Cell Numbers and Activation Markers in Human Vaginal Mucosal Tissues. *AIDS RESEARCH AND HUMAN RETROVIRUSES,* 29**,** 592-601.

CHETOUI, N., BOISVERT, M., GENDRON, S. & AOUDJIT, F. 2010. Interleukin-7 Promotes the Survival of Human Cd4+ Effector/Memory T Cells by up-Regulating Bcl-2 Proteins and Activating the Jak/Stat Signalling Pathway. *IMMUNOLOGY,* 130**,** 418-426.

COHEN, C. R., LINGAPPA, J. R., BAETEN, J. M., NGAYO, M. O., SPIEGEL, C. A., HONG, T., DONNELL, D., CELUM, C., KAPIGA, S., DELANY, S. & BUKUSI, E. A. 2012. Bacterial Vaginosis Associated with Increased Risk of Female-to-Male Hiv-1 Transmission: A Prospective Cohort Analysis among African Couples. *PLoS MEDICINE*, 10.1371/journal.pmed.1001251.

COHEN, M. S., CHEN, Y. Q., MCCAULEY, M., GAMBLE, T., HOSSEINIPOUR, M. C., KUMARASAMY, N., HAKIM, J. G., KUMWENDA, J., GRINSZTEJN, B. & PILOTTO, J. H. 2011. Prevention of Hiv-1 Infection with Early Antiretroviral Therapy. *THE NEW ENGLAND JOURNAL OF MEDICINE,* 365**,** 493-505.

CRITCHLOW, C. W., WOLNER-HANSSEN, P., ESCHENBACH, D. A., KIVIAT, N. B., KOUTSKY, L. A., STEVENS, C. E. & HOLMES, K. K. 1995. Determinants of Cervical Ectopia and of Cervicitis: Age, Oral Contraception, Specific Cervical Infection, Smoking, and Douching. *AMERICAN JOURNAL OF OBSTETRICS AND GYNECOLOGY,* 173**,** 534-543.

DANFORTH, D. N. 1983. The Morphology of the Human Cervix. *CLINICAL OBSTETRICS AND GYNECOLOGY,* 26**,** 7-13.

DEESE, J., MASSON, L., MILLER, W., COHEN, M., MORRISON, C., WANG, M., AHMED, K., AGOT, K., CRUCITTI, T. & ABDELLATI, S. 2015. Injectable Progestin-Only Contraception Is Associated with Increased Levels of Pro-Inflammatory Cytokines in the Female Genital Tract. *AMERICAN JOURNAL OF REPRODUCTIVE IMMUNOLOGY,* 74**,** 357-367.

DENTON, P. W., ESTES, J. D., SUN, Z., OTHIENO, F. A., WEI, B. L., WEGE, A. K., POWELL, D. A., PAYNE, D., HAASE, A. T. & GARCIA, J. V. 2008. Antiretroviral Pre-Exposure Prophylaxis Prevents Vaginal Transmission of Hiv-1 in Humanized Blt Mice. *PLoS MEDICINE,* 10.1371/journal.pmed.0050016.

DUNHAM, R. M., CERVASI, B., BRENCHLEY, J. M., ALBRECHT, H., WEINTROB, A., SUMPTER, B., ENGRAM, J., GORDON, S., KLATT, N. R. & FRANK, I. 2008. Cd127 and Cd25 Expression Defines Cd4+ T Cell Subsets That Are Differentially Depleted During Hiv Infection. *THE JOURNAL OF IMMUNOLOGY,* 180**,** 5582-5592.

EPPLE, H.-J., LODDENKEMPER, C., KUNKEL, D., TRÖGER, H., MAUL, J., MOOS, V., BERG, E., ULLRICH, R., SCHULZKE, J.-D. & STEIN, H. 2006. Mucosal but Not Peripheral Foxp3+ Regulatory T Cells Are Highly Increased in Untreated Hiv Infection and Normalize after Suppressive Haart. *BLOOD,* 108**,** 3072-3078.

FAHEY, J., WRIGHT, J., SHEN, L., SMITH, J., GHOSH, M., ROSSOLL, R. & WIRA, C. 2008. Estradiol Selectively Regulates Innate Immune Function by Polarized Human Uterine Epithelial Cells in Culture. *MUCOSAL IMMUNOLOGY,* 1**,** 317-325.

FICHOROVA, R. N., DESAI, P. J., GIBSON, F. C. & GENCO, C. A. 2001. Distinct Proinflammatory Host Responses to Neisseria Gonorrhoeae Infection in Immortalized Human Cervical and Vaginal Epithelial Cells. *INFECTION AND IMMUNITY,* 69**,** 5840-5848.

FRY, T. J. & MACKALL, C. L. 2005. The Many Faces of Il-7: From Lymphopoiesis to Peripheral T Cell Maintenance. *THE JOURNAL OF IMMUNOLOGY,* 174**,** 6571-6576.

GIPSON, I. K., HO, S. B., SPURR-MICHAUD, S. J., TISDALE, A. S., ZHAN, Q., TORLAKOVIC, E., PUDNEY, J., ANDERSON, D. J., TORIBARA, N. W. & HILL III, J. A. 1997. Mucin Genes Expressed by Human Female Reproductive Tract Epithelia. *BIOLOGY OF REPRODUCTION,* 56**,** 999-1011.

GIVAN, A. L., WHITE, H. D., STERN, J. E., COLBY, E., GUYRE, P. M., WIRA, C. R. & GOSSELIN, E. J. 1997. Flow Cytometric Analysis of Leukocytes in the Human Female Reproductive Tract: Comparison of Fallopian Tube, Uterus, Cervix, and Vagina. *AMERICAN JOURNAL OF REPRODUCTIVE IMMUNOLOGY,* 38**,** 350-359.

GLYNN, J. R., CARAEL, M., AUVERT, B., KAHINDO, M., CHEGE, J., MUSONDA, R., KAONA, F., BUVE, A. & STUDY GROUP ON THE HETEROGENEITY OF, H. I. V. E. I. A. C. 2001. Why Do Young Women Have a Much Higher Prevalence of Hiv Than Young Men? A Study in Kisumu, Kenya and Ndola, Zambia. *AIDS,* 15 S51-60.

GÓMEZ-OLIVÉ, F. X., ANGOTTI, N., HOULE, B., KLIPSTEIN-GROBUSCH, K., KABUDULA, C., MENKEN, J., WILLIAMS, J., TOLLMAN, S. & CLARK, S. J. 2013. Prevalence of Hiv among Those 15 and Older in Rural South Africa. *AIDS CARE,* 25**,** 1122-1128.

GOOTENBERG, D. B., MITCHELL, C. M. & KWON, D. S. 2018. Cervicovaginal Microbiota and Reproductive Health: The Virtue of Simplicity. *CELL HOST & MICROBE,* 23**,** 159-168.

GOSMANN, C., ANAHTAR, M. N., HANDLEY, S. A., FARCASANU, M., ABU-ALI, G., BOWMAN, B. A., PADAVATTAN, N., DESAI, C., DROIT, L. & MOODLEY, A. 2017. Lactobacillus-Deficient Cervicovaginal Bacterial Communities Are Associated with Increased Hiv Acquisition in Young South African Women. *IMMUNITY,* 46**,** 29-37.

GRAY-SWAIN, M. R. & PEIPERT, J. F. 2006. Pelvic Inflammatory Disease in Adolescents. *CURRENT OPINIONS IN OBSTETRICS AND GYNECOLOGY,* 18**,** 503-510.

GRIVEL, J. C. & MARGOLIS, L. 2009. Use of Human Tissue Explants to Study Human Infectious Agents. *NATURE PROTOCOLS,* 4**,** 256-269.

GROOM, J. R. & LUSTER, A. D. 2011. Cxcr3 in T Cell Function. *EXPERIMENTAL CELL RESEARCH,* 317**,** 620-631.

HAASE, A. T. 2010. Targeting Early Infection to Prevent Hiv-1 Mucosal Transmission. *NATURE,* 464**,** 217-223.

HEFFRON, R., DONNELL, D., REES, H., CELUM, C., MUGO, N., WERE, E., DE BRUYN, G., NAKKU-JOLOBA, E., NGURE, K., KIARIE, J., COOMBS, R. W. & BAETEN, J. M. 2012. Use of Hormonal Contraceptives and Risk of Hiv-1 Transmission: A Prospective Cohort Study. *THE LANCET INFECTIOUS DISEASES,* 12**,** 19-26.

HEFFRON, R., MCCLELLAND, R. S., BALKUS, J. E., CELUM, C., COHEN, C. R., MUGO, N., BUKUSI, E., DONNELL, D., LINGAPPA, J., KIARIE, J., FIEDLER, T., MUNCH, M., FREDRICKS, D. N., BAETEN, J. M. & PARTNERS PR, E. P. S. T. 2017. Efficacy of Oral Pre-Exposure Prophylaxis (Prep) for Hiv among Women with Abnormal Vaginal Microbiota: A Post-Hoc Analysis of the Randomised, Placebo-Controlled Partners Prep Study. *LANCET HIV,* 10.1016/S2352-3018(17)30110-8.

HICKEY, D. K., PATEL, M. V., FAHEY, J. V. & WIRA, C. R. 2011. Innate and Adaptive Immunity at Mucosal Surfaces of the Female Reproductive Tract: Stratification and Integration of Immune Protection against the Transmission of Sexually Transmitted Infections. *JOURNAL OF REPRODUCTIVE IMMUNOLOGY,* 88**,** 185-194.

HILLIER, S., MEYN, L., BUNGE, K., AUSTIN, M., MONCLA, B., DEZZUTTI, C. & ROHAN, L. Impact of Vaginal Microbiota on Genital Tissue and Plasma Concentrations of Tenofovir. Conference on Retroviruses and Opportunistic Infections (CROI), 2017.

HLADIK, F. & HOPE, T. J. 2009. Hiv Infection of the Genital Mucosa in Women. *CURRENT HIV/AIDS REPORTS,* 6**,** 20-28.

HLADIK, F. & MCELRATH, M. J. 2008. Setting the Stage: Host Invasion by Hiv. *NATURE REVIEWS IMMUNOLOGY,* 8**,** 447-457.

HWANG, L. Y., SCOTT, M. E., MA, Y. & MOSCICKI, A. B. 2011. Higher Levels of Cervicovaginal Inflammatory and Regulatory Cytokines and Chemokines in Healthy Young Women with Immature Cervical Epithelium. *JOURNAL OF REPRODRODUCTIVE IMMUNOLOGY,* 88**,** 66-71.

ILDGRUBEN, A. K., SJÖBERG, I. M. & HAMMARSTRÖM, M.-L. K. 2003. Influence of Hormonal Contraceptives on the Immune Cells and Thickness of Human Vaginal Epithelium. *OBSTETRICS & GYNECOLOGY,* 102**,** 571-582.

IYER, S. S., SABULA, M. J., MEHTA, C. C., HADDAD, L. B., BROWN, N. L., AMARA, R. R., OFOTOKUN, I. & SHETH, A. N. 2017. Characteristics of Hiv Target Cd4 T Cells Collected Using Different Sampling Methods from the Genital Tract of Hiv Seronegative Women. *PLOS ONE,* 12**,** e0178193.

JOHANSSON, E., RUDIN, A., WASSEN, L. & HOLMGREN, J. 1999. Distribution of Lymphocytes and Adhesion Molecules in Human Cervix and Vagina. *IMMUNOLOGY,* 96**,** 272-277.

KAUSHIC, C. 2011. Hiv-1 Infection in the Female Reproductive Tract: Role of Interactions between Hiv-1 and Genital Epithelial Cells. *AMERICAN JOURNAL OF REPRODUCTIVE IMMUNOLOGY,* 65**,** 253-260.

KELL, P., BARTON, S., EDMONDS, D. & BOAG, F. 1992. Hiv Infection in a Patient with Meyer-Rokitansky-Küster-Hauser Syndrome. *JOURNAL OF THE ROYAL SOCIETY OF MEDICINE,* 85**,** 706-707.

KENYON, C., COLEBUNDERS, R. & CRUCITTI, T. 2013. The Global Epidemiology of Bacterial Vaginosis: A Systematic Review. *AMERICAN JOURNAL OF OBSTETRICS AND GYNECOLOGY,* 209**,** 505-523.

KLATT, N. R., CHEU, R., BIRSE, K., ZEVIN, A. S., PERNER, M., NOEL-ROMAS, L., GROBLER, A., WESTMACOTT, G., XIE, I. Y., BUTLER, J., MANSOOR, L., MCKINNON, L. R., PASSMORE, J. S., ABDOOL KARIM, Q., ABDOOL KARIM, S. S. & BURGENER, A. D. 2017. Vaginal Bacteria Modify Hiv Tenofovir Microbicide Efficacy in African Women. *SCIENCE,* 356**,** 938-945.

KLEBANOFF, S. J. & COOMBS, R. W. 1991. Viricidal Effect of Lactobacillus Acidophilus on Human Immunodeficiency Virus Type 1: Possible Role in Heterosexual Transmission. *JOURNAL OF EXPERIMENTAL MEDICINE,* 174**,** 289-292.

KUMAR, N., BEHERA, B., SAGIRI, S. S., PAL, K., RAY, S. S. & ROY, S. 2011. Bacterial Vaginosis: Etiology and Modalities of Treatment-a Brief Note. *JOURNAL OF PHARMACY AND BIOALLIED SCIENCES,* 3**,** 496-503.

KYONGO, J. K., JESPERS, V., GOOVAERTS, O., MICHIELS, J., MENTEN, J., FICHOROVA, R. N., CRUCITTI, T., VANHAM, G. & ARIEN, K. K. 2012. Searching for Lower Female

Genital Tract Soluble and Cellular Biomarkers: Defining Levels and Predictors in a Cohort of Healthy Caucasian Women. *PLOS ONE,* 10.1371/journal.pone.0043951.

LAGA, M., MANOKA, A., KIVUVU, M., MALELE, B., TULIZA, M., NZILA, N., GOEMAN, J., BEHETS, F., BATTER, V. & ALARY, M. 1993. Non-Ulcerative Sexually Transmitted Diseases as Risk Factors for Hiv-1 Transmission in Women: Results from a Cohort Study. *AIDS,* 7**,** 95-102.

LEE, S. K., KIM, C. J., KIM, D.-J. & KANG, J.-H. 2015. Immune Cells in the Female Reproductive Tract. *IMMUNE NETWORK,* 15**,** 16-26.

LIEBENBERG, L. J., GAMIELDIEN, H., MKHIZE, N. N., JAUMDALLY, S. Z., GUMBI, P. P., DENNY, L. & PASSMORE, J. A. 2011. Stability and Transport of Cervical Cytobrushes for Isolation of Mononuclear Cells from the Female Genital Tract. *JOURNAL OF IMMUNOLOGICAL METHODS,* 367**,** 47-55.

MACKLAIM, J. M., FERNANDES, A. D., DI BELLA, J. M., HAMMOND, J. A., REID, G. & GLOOR, G. B. 2013. Comparative Meta-Rna-Seq of the Vaginal Microbiota and Differential Expression by Lactobacillus Iners in Health and Dysbiosis. *MICROBIOME,* 10.1186/2049-2618-1-12.

MARRAZZO, J. M., RAMJEE, G., RICHARDSON, B. A., GOMEZ, K., MGODI, N., NAIR, G., PALANEE, T., NAKABIITO, C., VAN DER STRATEN, A., NOGUCHI, L., HENDRIX, C. W., DAI, J. Y., GANESH, S., MKHIZE, B., TALJAARD, M., PARIKH, U. M., PIPER, J., MASSE, B., GROSSMAN, C., ROONEY, J., SCHWARTZ, J. L., WATTS, H., MARZINKE, M. A., HILLIER, S. L., MCGOWAN, I. M., CHIRENJE, Z. M. & TEAM, V. S. 2015. Tenofovir-Based Preexposure Prophylaxis for Hiv Infection among African Women. *THE NEW ENGLAND JOURNAL OF MEDICINE,* 372**,** 509-518.

MARTIN, H. L., JR., NYANGE, P. M., RICHARDSON, B. A., LAVREYS, L., MANDALIYA, K., JACKSON, D. J., NDINYA-ACHOLA, J. O. & KREISS, J. 1998. Hormonal Contraception, Sexually Transmitted Diseases, and Risk of Heterosexual Transmission of Human Immunodeficiency Virus Type 1. *THE JOURNAL OF INFECTIOUS DISEASES,* 178**,** 1053-1059.

MARX, P. A., SPIRA, A. I., GETTIE, A., DAILEY, P. J., VEAZEY, R. S., LACKNER, A. A., MAHONEY, C. J., MILLER, C. J., CLAYPOOL, L. E., HO, D. D. & ALEXANDER, N. J. 1996. Progesterone Implants Enhance Siv Vaginal Transmission and Early Virus Load. *NATURE MEDICINE,* 2**,** 1084-1089.

MASSON, L., MLISANA, K., LITTLE, F., WERNER, L., MKHIZE, N. N., RONACHER, K., GAMIELDIEN, H., WILLIAMSON, C., MCKINNON, L. R., WALZL, G., ABDOOL KARIM, Q., ABDOOL KARIM, S. S. & PASSMORE, J. A. 2014. Defining Genital Tract Cytokine Signatures of Sexually Transmitted Infections and Bacterial Vaginosis in Women at High Risk of Hiv Infection: A Cross-Sectional Study. *SEXUALLY TRANSMITTED INFECTIONS*, 90**,** 580-587.

MASSON, L., PASSMORE, J. A., LIEBENBERG, L. J., WERNER, L., BAXTER, C., ARNOLD, K. B., WILLIAMSON, C., LITTLE, F., MANSOOR, L. E., NARANBHAI, V., LAUFFENBURGER, D. A., RONACHER, K., WALZL, G., GARRETT, N. J., WILLIAMS, B. L., COUTO-RODRIGUEZ, M., HORNIG, M., LIPKIN, W. I., GROBLER, A., ABDOOL KARIM, Q. & ABDOOL KARIM, S. S. 2015. Genital Inflammation and the Risk of Hiv Acquisition in Women. *CLINICAL INFECTIOUS DISEASES*, 61(2)**,** 260-269.

MCGEE, Z. A., JENSEN, R. L., CLEMENS, C. M., TAYLOR-ROBINSON, D., JOHNSON, A. P. & GREGG, C. R. 1999. Gonococcal Infection of Human Fallopian Tube Mucosa in Organ Culture: Relationship of Mucosal Tissue Tnf-A Concentration to Sloughing of Ciliated Cells. *SEXUALLY TRANSMITTED DISEASES*, 26**,** 160-165.

MCKINNON, L. R., HUGHES, S. M., DE ROSA, S. C., MARTINSON, J. A., PLANTS, J., BRADY, K. E., GUMBI, P. P., ADAMS, D. J., VOJTECH, L. & GALLOWAY, C. G. 2014. Optimizing Viable Leukocyte Sampling from the Female Genital Tract for Clinical Trials: An International Multi-Site Study. *PLOS ONE*, 10.1371/journal.pone.0085675.

MCKINNON, L. R., LIEBENBERG, L. J., YENDE-ZUMA, N., ARCHARY, D., NGCAPU, S., SIVRO, A., NAGELKERKE, N., LERMA, J. G. G., KASHUBA, A. D. & MASSON, L. 2018. Genital Inflammation Undermines the Effectiveness of Tenofovir Gel in Preventing Hiv Acquisition in Women. *NATURE MEDICINE*, 10.1038/nm.4506.

MCKINNON, L. R., NYANGA, B., CHEGE, D., IZULLA, P., KIMANI, M., HUIBNER, S., GELMON, L., BLOCK, K. E., CICALA, C. & ANZALA, A. O. 2011. Characterization of a Human Cervical Cd4+ T Cell Subset Coexpressing Multiple Markers of Hiv Susceptibility. *THE JOURNAL OF IMMUNOLOGY*, 187(11)**,** 6032-6042.

MILLER, C., ALEXANDER, N., VOGEL, P., ANDERSON, J. & MARX, P. 1992. Mechanism of Genital Transmission of Siv: A Hypothesis Based on Transmission Studies and the Location of Siv in the Genital Tract of Chronically Infected Female Rhesus Macaques. *JOURNAL OF MEDICAL PRIMATOLOGY*, 21**,** 64-68.

MILLER, C. J., LI, Q., ABEL, K., KIM, E.-Y., MA, Z.-M., WIETGREFE, S., LA FRANCO-SCHEUCH, L., COMPTON, L., DUAN, L. & SHORE, M. D. 2005. Propagation and Dissemination of Infection after Vaginal Transmission of Simian Immunodeficiency Virus. *JOURNAL OF VIROLOGY,* 79**,** 9217-9227.

MILLER, C. J. & SHATTOCK, R. J. 2003. Target Cells in Vaginal Hiv Transmission. *MICROBES AND INFECTION,* 5**,** 59-67.

MILLER, L., PATTON, D. L., MEIER, A., THWIN, S. S., HOOTON, T. M. & ESCHENBACH, D. A. 2000. Depomedroxyprogesterone-Induced Hypoestrogenism and Changes in Vaginal Flora and Epithelium. *OBSTETRICS & GYNECOLOGY,* 96**,** 431-439.

MIRMONSEF, P., GILBERT, D., ZARIFFARD, M. R., HAMAKER, B. R., KAUR, A., LANDAY, A. L. & SPEAR, G. T. 2011. The Effects of Commensal Bacteria on Innate Immune Responses in the Female Genital Tract. *AMERICAN JOURNAL OF REPRODUCTIVE IMMUNOLOGY,* 65**,** 190-195.

MITCHELL, C. M., MCLEMORE, L., WESTERBERG, K., ASTRONOMO, R., SMYTHE, K., GARDELLA, C., MACK, M., MAGARET, A., PATTON, D., AGNEW, K., MCELRATH, M. J., HLADIK, F. & ESCHENBACH, D. 2014. Long-Term Effect of Depot Medroxyprogesterone Acetate on Vaginal Microbiota, Epithelial Thickness and Hiv Target Cells. *JOURNAL OF INFECTIOUS DISEASES,* 210**,** 651-655.

MLISANA, K., NAICKER, N., WERNER, L., ROBERTS, L., VAN LOGGERENBERG, F., BAXTER, C., PASSMORE, J. A., GROBLER, A. C., STURM, A. W., WILLIAMSON, C., RONACHER, K., WALZL, G. & ABDOOL KARIM, S. S. 2012. Symptomatic Vaginal Discharge Is a Poor Predictor of Sexually Transmitted Infections and Genital Tract Inflammation in High-Risk Women in South Africa. *JOURNAL OF INFECTIOUS DISEASES,* 206**,** 6-14.

MORRISON, C. S., CHEN, P.-L., KWOK, C., BAETEN, J. M., BROWN, J., CROOK, A. M., VAN DAMME, L., DELANY-MORETLWE, S., FRANCIS, S. C. & FRIEDLAND, B. A. 2015. Hormonal Contraception and the Risk of Hiv Acquisition: An Individual Participant Data Meta-Analysis. *PLOS MEDICINE,* 10.1371/journal.pmed.1001778.

MORRISON, C. S., CHEN, P. L., KWOK, C., RICHARDSON, B. A., CHIPATO, T., MUGERWA, R., BYAMUGISHA, J., PADIAN, N., CELENTANO, D. D. & SALATA, R. A. 2010. Hormonal Contraception and Hiv Acquisition: Reanalysis Using Marginal Structural Modeling. *AIDS,* 24**,** 1778-1781.

MOSER, B. 2015. Cxcr5, the Defining Marker for Follicular B Helper T (Tfh) Cells. *FRONTIERS IN IMMUNOLOGY,* 10.3389/fimmu.2015.00296.

NESS, R. B., KIP, K. E., HILLIER, S. L., SOPER, D. E., STAMM, C. A., SWEET, R. L., RICE, P. & RICHTER, H. E. 2005. A Cluster Analysis of Bacterial Vaginosis–Associated Microflora and Pelvic Inflammatory Disease. *AMERICAN JOURNAL OF EPIDEMIOLOGY,* 162**,** 585-590.

NGUYEN, P. V., KAFKA, J. K., FERREIRA, V. H., ROTH, K. & KAUSHIC, C. 2014. Innate and Adaptive Immune Responses in Male and Female Reproductive Tracts in Homeostasis and Following Hiv Infection. *CELLULAR & MOLECULAR IMMUNOLOGY,* 11**,** 410-427.

NILSSON, J., BOASSO, A., VELILLA, P. A., ZHANG, R., VACCARI, M., FRANCHINI, G., SHEARER, G. M., ANDERSSON, J. & CHOUGNET, C. 2006. Hiv-1–Driven Regulatory T-Cell Accumulation in Lymphoid Tissues Is Associated with Disease Progression in Hiv/Aids. *BLOOD,* 108**,** 3808-3817.

NIXON, B., JANDL, T., TELLER, R. S., TANEVA, E., WANG, Y., NAGARAJA, U., KISER, P. F. & HEROLD, B. C. 2014. Vaginally Delivered Tenofovir Disoproxil Fumarate Provides Greater Protection Than Tenofovir against Genital Herpes in a Murine Model of Efficacy and Safety. *ANTIMICROBIAL AGENTS AND CHEMOTHERAPY,* 58**,** 1153-1160.

OLESEN, R., WAHL, A., DENTON, P. W. & GARCIA, J. V. 2011. Immune Reconstitution of the Female Reproductive Tract of Humanized Blt Mice and Their Susceptibility to Human Immunodeficiency Virus Infection. *JOURNAL OF REPRODUCTIVE IMMUNOLOGY,* 88**,** 195-203.

OPPERMANN, M. 2004. Chemokine Receptor Ccr5: Insights into Structure, Function, and Regulation. *CELL SIGNAL,* 16**,** 1201-1210.

OSWALD-RICHTER, K., GRILL, S. M., SHARIAT, N., LEELAWONG, M., SUNDRUD, M. S., HAAS, D. W. & UNUTMAZ, D. 2004. Hiv Infection of Naturally Occurring and Genetically Reprogrammed Human Regulatory T-Cells. *PLOS BIOLOGY,* 10.1371/journal.pbio.0020198.

PASSMORE, J.-A. S., JASPAN, H. B. & MASSON, L. 2016. Genital Inflammation, Immune Activation and Risk of Sexual Hiv Acquisition. *CURRENT OPINION IN HIV AND AIDS,* 11**,** 156-162.

PERFETTO, S. P., CHATTOPADHYAY, P. K. & ROEDERER, M. 2004. Seventeen-Colour Flow Cytometry: Unravelling the Immune System. *NATURE REVIEWS IMMUNOLOGY,* 4**,** 648-655.

PETROVA, M. I., VAN DEN BROEK, M., BALZARINI, J., VANDERLEYDEN, J. & LEBEER, S. 2013. Vaginal Microbiota and Its Role in Hiv Transmission and Infection. *FEMS MICROBIOLOGY REVIEWS,* 37**,** 762-792.

PETTIFOR, A. E., REES, H. V., KLEINSCHMIDT, I., STEFFENSON, A. E., MACPHAIL, C., HLONGWA-MADIKIZELA, L., VERMAAK, K. & PADIAN, N. S. 2005. Young People's Sexual Health in South Africa: Hiv Prevalence and Sexual Behaviors from a Nationally Representative Household Survey. *AIDS,* 19**,** 1525-1534.

POLICICCHIO, B. B., PANDREA, I. & APETREI, C. 2016. Animal Models for Hiv Cure Research. *FRONTIERS IN IMMUNOLOGY,* 10.3389/fimmu.2016.00012.

POLIS, C. B., PHILLIPS, S. J., CURTIS, K. M., WESTREICH, D. J., STEYN, P. S., RAYMOND, E., HANNAFORD, P. & TURNER, A. N. 2014. Hormonal Contraceptive Methods and Risk of Hiv Acquisition in Women: A Systematic Review of Epidemiological Evidence. *CONTRACEPTION,* 90**,** 360-390.

POVEDA, E., BRIZ, V., DE MENDOZA, C., BENITO, J. M., CORRAL, A., ZAHONERO, N., LOZANO, S., GONZÁLEZ-LAHOZ, J. & SORIANO, V. 2007. Prevalence of X4 Tropic Hiv-1 Variants in Patients with Differences in Disease Stage and Exposure to Antiretroviral Therapy. *JOURNAL OF MEDICAL VIROLOGY,* 79**,** 1040-1046.

PUDNEY, J., QUAYLE, A. J. & ANDERSON, D. J. 2005. Immunological Microenvironments in the Human Vagina and Cervix: Mediators of Cellular Immunity Are Concentrated in the Cervical Transformation Zone. *BIOLOGY OF REPRODUCTION,* 73**,** 1253-1263.

QUAYLE, A. 2002. The Innate and Early Immune Response to Pathogen Challenge in the Female Genital Tract and the Pivotal Role of Epithelial Cells. *JOURNAL OF REPRODUCTIVE IMMUNOLOGY,* 57**,** 61-79.

QUILLENT, C., OBERLIN, E., BRAUN, J., ROUSSET, D., GONZALEZ-CANALI, G., MÉTAIS, P., MONTAGNIER, L., VIRELIZIER, J.-L., ARENZANA-SEISDEDOS, F. & BERETTA, A. 1998. Hiv-1-Resistance Phenotype Conferred by Combination of Two Separate Inherited Mutations of Ccr5 Gene. *THE LANCET,* 351**,** 14-18.

RAVEL, J., GAJER, P., ABDO, Z., SCHNEIDER, G. M., KOENIG, S. S., MCCULLE, S. L., KARLEBACH, S., GORLE, R., RUSSELL, J. & TACKET, C. O. 2011. Vaginal Microbiome of Reproductive-Age Women. *PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES,* 108**,** 4680-4687.

REIS MACHADO, J., DA SILVA, M. V., CAVELLANI, C. L., ANTÔNIA DOS REIS, M., MONTEIRO, M. L. G. D. R., TEIXEIRA, V. D. P. A. & ROSA MIRANDA CORRÊA, R. 2014. Mucosal Immunity in the Female Genital Tract, Hiv/Aids. *BioMed research international*, 10.1155/2014/350195.

RILEY, J. L. 2009. Pd-1 Signaling in Primary T Cells. *Immunological reviews*, 229**,** 114-125.

ROBERTS, L., PASSMORE, J. A., MLISANA, K., WILLIAMSON, C., LITTLE, F., BEBELL, L. M., WALZL, G., ABRAHAMS, M. R., WOODMAN, Z., ABDOOL KARIM, Q. & ABDOOL KARIM, S. S. 2012. Genital Tract Inflammation During Early Hiv-1 Infection Predicts Higher Plasma Viral Load Set Point in Women. *Journal of Infectious Diseases*, 205**,** 194-203.

ROLLENHAGEN, C. & ASIN, S. 2011. Enhanced Hiv-1 Replication in Ex Vivo Ectocervical Tissues from Post-Menopausal Women Correlates with Increased Inflammatory Responses. *Mucosal immunology*, 4**,** 671-681.

SABA, E., GRIVEL, J. C., VANPOUILLE, C., BRICHACEK, B., FITZGERALD, W., MARGOLIS, L. & LISCO, A. 2010. Hiv-1 Sexual Transmission: Early Events of Hiv-1 Infection of Human Cervico-Vaginal Tissue in an Optimized Ex Vivo Model. *Mucosal Immunol*, 3**,** 280-290.

SAKAGUCHI, S., SAKAGUCHI, N., ASANO, M., ITOH, M. & TODA, M. 1995. Immunologic Self-Tolerance Maintained by Activated T Cells Expressing Il-2 Receptor Alpha-Chains (Cd25). Breakdown of a Single Mechanism of Self-Tolerance Causes Various Autoimmune Diseases. *The Journal of Immunology*, 155**,** 1151-1164.

SALLUSTO, F., ZIELINSKI, C. E. & LANZAVECCHIA, A. 2012. Human T H17 Subsets. *European journal of immunology*, 42**,** 2215-2220.

SCHLUNS, K. S., KIEPER, W. C., JAMESON, S. C. & LEFRANÇOIS, L. 2000. Interleukin-7 Mediates the Homeostasis of Naive and Memory Cd8 T Cells in Vivo. *Nature immunology*, 1**,** 426-432.

SHATTOCK, R. J. & MOORE, J. P. 2003. Inhibiting Sexual Transmission of Hiv-1 Infection. *Nature Reviews Microbiology*, 1**,** 25-34.

SHIPITSYNA, E., ROOS, A., DATCU, R., HALLEN, A., FREDLUND, H., JENSEN, J. S., ENGSTRAND, L. & UNEMO, M. 2013. Composition of the Vaginal Microbiota in Women of Reproductive Age--Sensitive and Specific Molecular Diagnosis of Bacterial Vaginosis Is Possible? *PLoS One*, 10.1371/journal.pone.0060670.

SINGER, A. 1975. The Uterine Cervix from Adolescence to the Menopause. *BJOG: An International Journal of Obstetrics & Gynaecology,* 82**,** 81-99.

STIEH, D. J., MATIAS, E., XU, H., FOUGHT, A. J., BLANCHARD, J. L., MARX, P. A., VEAZEY, R. S. & HOPE, T. J. 2016. Th17 Cells Are Preferentially Infected Very Early after Vaginal Transmission of Siv in Macaques. *Cell host & microbe,* 19**,** 529-540.

SUO, Z. & JOHNSON, K. A. 1998. Selective Inhibition of Hiv-1 Reverse Transcriptase by an Antiviral Inhibitor, (R)-9-(2-Phosphonylmethoxypropyl)Adenine. *The Journal of Biological Chemistry,* 273**,** 27250-27258.

SVANBORG, C., GODALY, G. & HEDLUND, M. 1999. Cytokine Responses During Mucosal Infections: Role in Disease Pathogenesis and Host Defence. *Current opinion in microbiology,* 2**,** 99-103.

TANEVA, E., CROOKER, K., PARK, S. H., SU, J. T., OTT, A., CHESHENKO, N., SZLEIFER, I., KISER, P. F., FRANK, B., MESQUITA, P. M. & HEROLD, B. C. 2015. Differential Mechanisms of Tenofovir and Tenofovir Disoproxil Fumarate Cellular Transport and Implications for Topical Preexposure Prophylaxis. *Antimicrobial agents and chemotherapy,* 60**,** 1667-1675.

THIGPEN, M. C., KEBAABETSWE, P. M., PAXTON, L. A., SMITH, D. K., ROSE, C. E., SEGOLODI, T. M., HENDERSON, F. L., PATHAK, S. R., SOUD, F. A., CHILLAG, K. L., MUTANHAURWA, R., CHIRWA, L. I., KASONDE, M., ABEBE, D., BULIVA, E., GVETADZE, R. J., JOHNSON, S., SUKALAC, T., THOMAS, V. T., HART, C., JOHNSON, J. A., MALOTTE, C. K., HENDRIX, C. W., BROOKS, J. T. & GROUP, T. D. F. S. 2012. Antiretroviral Preexposure Prophylaxis for Heterosexual Hiv Transmission in Botswana. *The New England Journal of Medicine,* 367**,** 423-434.

TRIFONOVA, R. T., LIEBERMAN, J. & BAARLE, D. 2014. Distribution of Immune Cells in the Human Cervix and Implications for Hiv Transmission. *American journal of reproductive immunology,* 71**,** 252-264.

TRUNOVA, N., TSAI, L., TUNG, S., SCHNEIDER, E., HAROUSE, J., GETTIE, A., SIMON, V., BLANCHARD, J. & CHENG-MAYER, C. 2006. Progestin-Based Contraceptive Suppresses Cellular Immune Responses in Shiv-Infected Rhesus Macaques. *Virology,* 352**,** 169-177.

UNAIDS. 2017. *Unaids Data 2017* [Online]. UNAIDS. Available: http://www.unaids.org/en/resources/documents/2017/2017_data_book [Accessed 28 November 2018].

VAN DAMME, L., CORNELI, A., AHMED, K., AGOT, K., LOMBAARD, J., KAPIGA, S., MALAHLEHA, M., OWINO, F., MANONGI, R., ONYANGO, J., TEMU, L., MONEDI, M. C., MAK'OKETCH, P., MAKANDA, M., REBLIN, I., MAKATU, S. E., SAYLOR, L., KIERNAN, H., KIRKENDALE, S., WONG, C., GRANT, R., KASHUBA, A., NANDA, K., MANDALA, J., FRANSEN, K., DEESE, J., CRUCITTI, T., MASTRO, T. D., TAYLOR, D. & GROUP, F. E.-P. S. 2012. Preexposure Prophylaxis for Hiv Infection among African Women. *THE NEW ENGLAND JOURNAL OF MEDICINE,* 367**,** 411-422.

VIGIL, P., CORTÉS, M. E., ZÚÑIGA, A., RIQUELME, J. & CERIC, F. 2009. Scanning Electron and Light Microscopy Study of the Cervical Mucus in Women with Polycystic Ovary Syndrome. *JOURNAL OF ELECTRON MICROSCOPY,* 58**,** 21-27.

WEAVER, C. T., ELSON, C. O., FOUSER, L. A. & KOLLS, J. K. 2013. The Th17 Pathway and Inflammatory Diseases of the Intestines, Lungs, and Skin. *ANNUAL REVIEW OF PATHOLOGY: MECHANISMS OF DISEASE,* 8**,** 477-512.

WHEELER, L. A., TRIFONOVA, R., VRBANAC, V., BASAR, E., MCKERNAN, S., XU, Z., SEUNG, E., DERUAZ, M., DUDEK, T., EINARSSON, J. I., YANG, L., ALLEN, T. M., LUSTER, A. D., TAGER, A. M., DYKXHOORN, D. M. & LIEBERMAN, J. 2011. Inhibition of Hiv Transmission in Human Cervicovaginal Explants and Humanized Mice Using Cd4 Aptamer-Sirna Chimeras. *JOURNAL OF CLINICAL INVESTIGATION,* 121**,** 2401-2412.

WHITE, H. D., YEAMAN, G. R., GIVAN, A. L. & WIRA, C. R. 1997. Mucosal Immunity in the Human Female Reproductive Tract: Cytotoxic T Lymphocyte Function in the Cervix and Vagina of Premenopausal and Postmenopausal Women. *AMERICAN JOURNAL OF REPRODUCTIVE IMMUNOLOGY,* 37**,** 30-38.

WIESER, F., HOSMANN, J., TSCHUGGUEL, W., CZERWENKA, K., SEDIVY, R. & HUBER, J. C. 2001. Progesterone Increases the Number of Langerhans Cells in Human Vaginal Epithelium. *FERTILITY AND STERILITY,* 75**,** 1234-1235.

WIRA, C., GHOSH, M., SMITH, J., SHEN, L., CONNOR, R., SUNDSTROM, P., FRECHETTE, G. M., HILL, E. & FAHEY, J. 2011. Epithelial Cell Secretions from the Human Female Reproductive Tract Inhibit Sexually Transmitted Pathogens and Candida Albicans but Not Lactobacillus. *MUCOSAL IMMUNOLOGY,* 4**,** 335-342.

WIRA, C. R., FAHEY, J. V., SENTMAN, C. L., PIOLI, P. A. & SHEN, L. 2005a. Innate and Adaptive Immunity in Female Genital Tract: Cellular Responses and Interactions. *IMMUNOLOGICAL REVIEWS,* 206**,** 306-335.

WIRA, C. R., GRANT-TSCHUDY, K. S. & CRANE-GODREAU, M. A. 2005b. Epithelial Cells in the Female Reproductive Tract: A Central Role as Sentinels of Immune Protection. *AMERICAN JOURNAL OF REPRODUCTIVE IMMUNOLOGY,* 53**,** 65-76.

WIRA, C. R., RODRIGUEZ-GARCIA, M. & PATEL, M. V. 2015. The Role of Sex Hormones in Immune Protection of the Female Reproductive Tract. *NATURE REVIEWS IMMUNOLOGY,* 15**,** 217-230.

WU, L. 2008. Biology of Hiv Mucosal Transmission. *CURRENT OPINION IN HIV AND AIDS,* 3**,** 534.

YAMAZAKI, T., YANG, X. O., CHUNG, Y., FUKUNAGA, A., NURIEVA, R., PAPPU, B., MARTIN-OROZCO, N., KANG, H. S., MA, L. & PANOPOULOS, A. D. 2008. Ccr6 Regulates the Migration of Inflammatory and Regulatory T Cells. *THE JOURNAL OF IMMUNOLOGY,* 181**,** 8391-8401.

YI, T. J., SHANNON, B., PRODGER, J., MCKINNON, L. & KAUL, R. 2013. Genital Immunology and Hiv Susceptibility in Young Women. *AMERICAN JOURNAL OF REPRODUCTIVE IMMUNOLOGY,* 69**,** 74-79.

ZHANG, Z., SCHULER, T., ZUPANCIC, M., WIETGREFE, S., STASKUS, K. A., REIMANN, K. A., REINHART, T. A., ROGAN, M., CAVERT, W., MILLER, C. J., VEAZEY, R. S., NOTERMANS, D., LITTLE, S., DANNER, S. A., RICHMAN, D. D., HAVLIR, D., WONG, J., JORDAN, H. L., SCHACKER, T. W., RACZ, P., TENNER-RACZ, K., LETVIN, N. L., WOLINSKY, S. & HAASE, A. T. 1999. Sexual Transmission and Propagation of Siv and Hiv in Resting and Activated Cd4+ T Cells. *SCIENCE,* 286**,** 1353-1357.

Appendices

Appendix A: Ethical Approval

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI
RESEARCH OFFICE
Biomedical Research Ethics Administration
Westville Campus, Govan Mbeki Building
Private Bag X 54001
Durban
4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za
Website http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

21 May 2018

Mr N Pillay (212523489)
Discipline of Medical Microbiology
School of Laboratory Medicine and Medical Sciences
nashpillay15@gmail.com

Dear Mr Pillay

Protocol: Frequency and phenotype of immune cell subsets in different regions of human cervix: implications for HIV susceptibility.
Degree: MMedSc
BREC ref: BE218/17

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	23 May 2018
Expiration of Ethical Approval:	22 May 2019

I wish to advise you that your application for Recertification received on 03 May 2018 for the above protocol has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

This approval will be ratified by a full Committee at its meeting taking place on 12 June 2018.

Yours sincerely

Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics

cc supervisor: ncapu@ukzn.ac.za
cc postgraduate administrator: dudhrajhp@ukzn.ac.za

Appendix B: Department of Health approval for King Edward Hospital

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

DIRECTORATE:
Health Research & Knowledge Management

Physical Address: 330 Langalibalele Street, Pietermaritzburg
Postal Address: Private Bag X9051
Tel: 033 395 2805/ 3188/ 3123 Fax: 033 394 3782
Email:
www.kznhealth.gov.za

HRKM Ref: 214/18
NHRD Ref: KZ_201805_041

Date: 12 June 2018
Dear Mr N. Pillay
UKZN

Approval of research

1. The research proposal titled **'Frequency and phenotype of immune cell subsets in different regions of human cervix: implications for HIV susceptibility'** was reviewed by the KwaZulu-Natal Department of Health.

   The proposal is hereby **approved** for research to be undertaken at King Edward VIII Hospital.

2. You are requested to take note of the following:
   a. Make the necessary arrangement with the identified facility before commencing with your research project.
   b. Provide an interim progress report and final report (electronic and hard copies) when your research is complete.

3. Your final report must be posted to **HEALTH RESEARCH AND KNOWLEDGE MANAGEMENT, 10-102, PRIVATE BAG X9051, PIETERMARITZBURG, 3200** and e-mail an electronic copy to hrkm@kznhealth.gov.za

For any additional information please contact Mr X. Xaba on 033-395 2805.

Yours Sincerely

Dr E Lutge
Chairperson, Health Research Committee
Date: 15/06/18

Fighting Disease, Fighting Poverty, Giving Hope

Appendix C: King Edward Hospital Approval

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

OFFICE OF THE HOSPITAL CEO
KING EDWARD VIII HOSPITAL

Private Bag X02, OVONGELA, 4013
Corner of Rick Turner (Francois Road) & Sydney Road
Tel: 031-3608853, Fax:031-2061457, Email: regine.kluzway@kznhealth.gov.za
www.kznhealth.gov.za

Ref.: KE 2/7/1/(17/2018
Enq.: Mrs. R. Sibiya
Research Programming
26 March 2018

Mr. N. Pillay
Discipline of Medical Microbiology
School of Laboratory Medicine and Medical Sciences
Nelson R. Mandela – School of Medicine
UNIVERSITY OF KWAZULU-NATAL

Dear Mr. Pillay

Protocol: "Frequency and phenotype of immune cell subsets in different regions of human cervix: implications of HIV susceptibility" Degree-MMedSc; BREC REF. NO. BE218/17

Permission to conduct research at King Edward VIII Hospital is provisionally granted, pending approval by the Provincial Health Research Committee, KZN Department of Health.

Kindly note the following:-

- The research will only commence once confirmation from the Provincial Health Research Committee in the KZN Department of Health has been received.
- Signing of an indemnity form at Room 8, CEO Complex before commencement with your study.
- King Edward VIII Hospital received full acknowledgment in the study on all Publications and reports and also kindly present a copy of the publication or report on completion.

The Management of King Edward VIII Hospital reserves the right to terminate the permission for the study should circumstances so dictate.

Yours faithfully

SUPPORTED/NOT SUPPORTED

2018/04/06
DATE

DR. R GREEN-THOMPSON
CLINICAL HEAD – OBSTET&GYNAE.

~~SUPPORTED/NOT SUPPORTED~~

09/04/2018
DATE

DR. SA MOODLEY
ACTING SENIOR MEDICAL MANAGER

Appendix D: PMMH Approval

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

DIRECTORATE: Senior Medical Manager

Mangosuthu Highway, Private Bag X 07
MOBENI
Tel: 031 907 8317/8304 Fax: 031 906 1044 Email:myint.aung@kznhealth.gov.za
www.kznhealth.gov.za

Prince Mshiyeni Memorial
Hospital

Enquiry: Dr M AUNG
Ref No: 15/RESH/2017
Date: 11/09/2017

TO: Sinaye Ngcapu

RE: LETTER OF APPROVAL TO CONDUCT RESEARCH AT PMMH

Dear Researcher;

I have pleasure to inform you that PMMH has granted to conduct research on **"Comparing HIV target cells in genital biopsies from South African women: Understanding the potential susceptibility factors for HIV acquisition"** in our institution.

Please note the following:

1. Please ensure this office is informed before you commence your research.
2. The institution will not provide any resources for this research.
3. You will be expected to provide feedback on you finding to the institution.

With kind regard

DR MYINT AUNG
Senior Medical Manager & specialist in Family Medicine
MBBS, DO(SA), PGDip in HIV (Natal), M.Med.Fam.Med (natal), PhD
Tel: 031 9078317
Fax: 031 906 1044
myint.aung@kznhealth.gov.za

Appendix E: Adult Consent Forms

ADULT CONSENT FORM FOR ENROLMENT TO EXPLANT STUDY:
ADMINISTRATIVE PAGE

Please complete this administrative section prior to completing the consent form for enrolment.

1. Has the participant completed the literacy assessment Yes No
2. Does the participant require and impartial witness Yes No

Staff Note: If the volunteer cannot read, the consent form must be read to the volunteer exactly as written, in the volunteer's language of choice, and a witness must sign this form to confirm that the correct information was given to the volunteer and that the volunteer freely consents to be in this study.

If participant has indicated NO for both question do not proceed

ADULT CONSENT FORM FOR ENROLMENT TO EXPLANT STUDIES:

Version 1.1

April 2014

(Adults)

PRINCIPAL INVESTIGATOR FOR EXPLANT STUDIES

INHLOKO YOCWANINGO LWE EXPLANT

Dr Derseree Archary

CAPRISA, 2 nd Floor Doris Duke Medical Research Institute,

Nelson R Mandela School of Medicine, Durban, South

Africa

E 031 260 4849

Good day and thank you for taking the time to read this consent form or have it read to you!

Sawubona, siyabonga ngokuthi uthathe isikhathi sakho ukufunda noma ukufundelwa incwadi yesivumelwano sokubamba iqhaza ocwaningwenil

Introduction:

Researchers at CAPRISA are trying to understand why some women become infected with HIV during sex while others do not. To help to understand how vaginal tissue protects women from HIV infection, we would like to study cervico-vaginal tissue to be able to show which part of the tissue provide protection. As you are an adult undergoing a planned elective hysterectomy, we would like to invite you to participate in this study by donating healthy tissue removed during your hysterectomy to our study. You do not have to agree to be part of this study and refusing participation will not impact on the quality of your treatment. I will give you information about this CAPRISA study and talk to you today about what your participation would involve and, if you agree to be part of this study, the procedures that you will undergo and what we wilt expect from you and what your rights are,

Isingeniso:

Abacwaningj base CAPRISA bazama ukuqonda ukuthi kunganj abanye abantu besifazane bethola igciwane lesandulela ngculazi ngokuya ocansini kuthi abanye bangalitholi. Ukusiza ekutheni siqonde ukuthi izicubu ezithize zesitho sangasese zibavikela kanjani labantu besifazane ekutholeni igciwane lesandulela ngculazi, sifuna ukucwanjnga ingxenyana yezicubu zesitho sangasese ukubona ukuthi iyiphi ingxenyana okuyiyona eyenza ukuthi bavikeleke. Njengoba ungowesifazane okhulile osethathe isinqumo sokukhipha isibeletho, siceJa ukukumema ukuthi ubambe iqhaza ocwaningweni

lwethu ngokuthi usiphe jzicubu zesibeletho lezi ezizokhishwa kuwena. Awuphoqelekile ukuthi ubambe iqhaza kulolucwaningo kanti ukunqaba angeke kushinsthe indlela ozophathwa ngayo. Uma uvuma ukubamba iqhaza kutolucwaningo Iwase CAPRISA,ngizokunika ulwazi kanye nemigomo okuzomele uyilandele, amalungelo akho kanye nezinto esizozilindela kuwe.

You are asked to read (or have read to you) this consent form in the language of your choice. After you have read this consent form or have had the form read to you, please take your time in deciding if you wish to join this study and sign this form. Feel free to talk to people you trust such as your friends, family, or teachers if you feel you need help to decide to participate or not. This consent form may contain words or terms that you don't understand. Please ask me to explain anything you may not understand. To be sure you understand the contents of this form I will ask you a few questions after you have read this consent form or have had the consent form read to you. If you agree to join this study, we will ask you to sign this form. We wilt give you a copy of this form to keep or if you prefer we can keep this form for you.

Uyacelwa ukuba ufunde (noma ufundelwe) ifomu lokuvuma ukubamba iqhaza ngolimu oluthandwa nguwe. Emva kokufunda lelifomu, sjcela uthathe isikhathi sakho ukucabanga ukuthi uyafuna yini ukubamba iqhaza na bese usayina lelifomu. Ungakhululeka ukukhuluma nabangani bakho obathembayo, umndeni, noma othishela uma udinga usizo ngokuthatha isinqumo. Lelifomu kungenzeka libe namagama ongawaqondisisi kahle. Ngicela ungibuze uma kukhona ongakuqondisisi kahie. Ukuze ngiqiniseke ukuthi uyayiqonda imininingwane yalelifomu ngizokubuza imibuzo embalwa emva kokuba usuljfundile noma walifundelwa. Uma uvuma ukubamba iqhaza sicela usayine lelifomu. Sizokunika elinye ifomu elifana naleli ozoljgcina noma sikugcinile thina.

Background:

Women are at greater risk of getting infected with HIV than men during heterosexual intercourse. For this reason, there is a Jot of research going on to develop strategies to reduce this risk in women. We recently completed a study where we were comparing the risk of HIV transmission in women who used a vaginal gel containing an active substance called Tenofovir at 1%, which is known to inactivate HIV, and women who used the gel but without this active substance, for up to 12 hours before and after sexual intercourse. We showed that women who used the gel containing Tenofovir had a reduced risk of being infected with HIV. We also know that when women had a vaginal inflammation, they were at increased risk of getting infected with HIV.

Esesikwazi:

Abantu besifazane banamathuba amaningi okutheteleka ngegciwane lesandulela ngculazi uma kuqhathaniswa nabesilisa abenza ucansi nabesifazane ngesikhathi kwenziwa ucansi. Jngakho luluningi ucwaningo oluqhubekayo ukuzama izindiela zokwehlisa amathuba okutheleleka ngegciwane lesandutela ngculazi kubantu besifazane. Sisanda kuqeda ukwenza ucwaningo Japho sasibheka khona ukuthi ngabe uketshezi olune Tenofovir luyabavikela yini abesifazane kwigciwane lesanduleta ngculazi uma luqhathaniswa noketshezi olunengenayo iTenofovir uma lushuthekwa esithweni sangasese kuya emahoreni ayishumi nambili ngaphambi kokwenza ucansi kanye nasemuva kokwenza ucansi. Sabe sesikhombisa ukuthi uketshezi olune Tenofovir lwehlisa amathuba abesifazane okutheleleka ngegciwane lesandulela ngculazi. Siyazi nokuthí abesifazane abanesifo socansi banamathuba amaningj okutheleJeka ngegciwane lesandulefa ngculazi.

We still do not fully understand the mechanism why increased inflammation leads to HIV infection and whether this inflammation will eventually impact the efficacy of tenofovir gel if it is licenced as means to prevent HIV infection in women. t will be talking to you about the study that CAPRISA will be undertaking to establish the role of inflammation in the effect of tenofovir gel to prevent HIV infection.

Asikakatholi kahle ukuthj kungani ukuqhubeka kokuvuvuka nokushisa nobuhlungu kuleyondawana esithweni sangasese enegciwane elithile elingasilo elesandulela ngculazi noma leyondawo elimele noma enomuhuzuko ingaholela ekutholeni igciwane lesandulela ngcutazj, futhi ingabe lokukuvuvuka kungaba nomthelela ekusebenzeni koketshezj olune Tenofovir uma isitholakele imvume yokuthi loluketshezi lusetshenziswe. Ngizokhuluma nawe ngocwaningo esifuna ukulwenza eCAPRlSA

ukubheka indima edlawa xxxx ekusebenzeni koketshezi oJune Tenofovir ekuvikeleni igcjwane lesandueJJa ngculazi.

Your participation in this study is voluntary:
Before you learn more about the CAPRISA explant study, it is important that you know the following:

- You do not have to take part in this study if you don't want to.
- If you agree to take part but change your mind later about continued participation, you can do so.
- If you decide not to take part in this study, it will not affect your access to services in your community or hospital.
- If you decide not to take part in this study you will still be able to participate in the other studies in the future.

Ukubamba kwakho iqhaza akuphoqelekile:

Ukubamba kwakho iqhaza kulolucwaningo akuphoqelekile:
Ngaphambi kokuba uzwe kabanzi ngalolucwaningo Iwase CAPRJSA, kubalulekile ukuthi wazi lokhu okulandelayo: . Awuphoqelekile ukubamba iqhaza kulolucwaningo

- Uma uvuma ukubamba iqhaza kodwa ushintsha umqondo ngokuhamba kwesikhathi lokho kuvumelekile . Ukungabambi kwakho iqhaza kutolucwaningo ngeke kukuvimbe ukuthi uthole usizo lwezempilo emtholampilo wangakini noma esibhedieJa
- Uma ungalibambi iqhaza kulolucwaningo uyovumeleka ukubamba iqhaza kolunye ucwaningo esingabanalo ngokuzayo

The rest of this consent form will describe to you the purpose of CAPRISA explant study, when each study procedure wilt take place, procedures for contacting you if necessary, the risks and benefits of participating in this study, and your rights as a study participant.

This study has been reviewed and approved by the University of KwaZulu-Natat's Biomedical Research Ethics Committee (BREC).

Lencwadi yesivumelwano sokubamba iqhaza izokuchazefa kabanzi ngenhloso yocwaningo Iwase CAPRISA, ukuthi sizoluqhuba kanjani ucwaningo kanye nokuthi sizokuthinta kanjani, ubungozi kanye nenzuzo yokubamba iqhaza kanye namalungelo akho.

Lolucwaningo lugunyazwe ikomidi Jezokuphepha kwezocwaningo lase nyuvesi yakwa Zulu-Natal.

Purpose of CAPRISA explant study:

The purpose of this study is to understand how vaginal tissue protects women from HIV infection and how the presence of sexually transmitted infections in this tissue can lower this protection against HIV.

Inhloso yocwaningo lase CAPRISA:

Inhloso yalotucwanjngo ukuqonda ukuthi kungani izicubu ezithize zesitho sangasese zivikela abesifazane ekutholeni igciwane lesandulela ngculazj kanye nokuthi ukuba khona kwezifo zocansi kulezizicubu kwehlisa amathuba okuvikeleka egciwaneni lesandulela ngculazi.

CAPRISA Explant Study Procedures:

After undergoing surgical hysterectomy, a section of your surgically removed tissue Will be taken to the CAPRISA laboratory jn Umbilo in an appropriate solution. In the laboratory, the tissue will be cut into smaller sections and tenofovir gel, other chemicals and HIV will be added to the tissue. We will then determine how your tissue responds to these conditions and blocks HIV infection. We will also store the tissue in order to see what immune cells are present in the tissue. This will give an idea of what happens in the female genital tract.

You will not be able to participate in CAPRJSA explant study until all the consenting procedures relevant to your participation have been completed. You will be notified by study staff when the enrolment survey will take place.

After you have agreed to be part of the CAPRISA explant study, you will sign the consent form.

Imigudu ezolandelwa ukwenza ucwaningo le CAPRISA Explant

Emva kokukhishwa kwesibeteletho, ingxenye yaso izothathwa ifakwe kwingxube ewuketshezi ihanjiswe esikhungweni sokucwaningo Iwamagazi esise CAPRISA. Esjkhungweni sokucwaninga igazi, izicubu zizosikwa zibencane bese zifakwa uketshezi lwe Tenofovir negciwane Jesandulela ngcuJaza kanye neminye imithi. Sizobe sesibheka ukuthi izicubu zirnelana kanjani nemithi esizifake yona. Izicubu eziyosala lapho ziyobe sezigcinwa kwenzela ukuthi sibuye siqhubeke nocwaningo. Lokhu kuzosinika umqondo wokuthi kwenzakalani esithweni sangasese somuntu wesifazane

Angeke ukwazi ukubamba iqhaba kucwaningo Iwase CAPRISA anduba incwadi yesivumelwano sokubamba iqhaza isiqediwe. Uyokwaziswa uma sekufanele ucwaningo luqale Emva kokuba usuvumile ukubamba jqhaza ocwanjngweni Iwase CAPRISA uzosayjna incwadi yesivumelwano.

Enrolment

If you agree (consent) to participate in CAPRISA explant study and, if you are eligible to participate in this study: (1) you will be asked to provide a vaginal swab; (2) you will be asked to donate about 2 tablespoons of blood (the trained nurse will take your blood). The swab and blood collected from

you will allow us to undertake some tests relating to your health. Your swab and blood specimens will be tested in the laboratory for sexually transmitted infections (STD. You will be tested for HIV by rapid antibody test. You will not be excluded from the study based on your HIV or STI results. We will then wait until the operation to collect the tissue samples.

Ukungena ocwaningweni:
Uma uvuma ukubamba iqhaza ocwaningweni Iwase CAPRISA, futhi uvumelekile:

(1) Uyocelwa ukuba usiphe uketshezi lwesitho sakho sangasese
(2) Uyocelwa ukuba usiphe izipuni ezimbiti zegazi (unesi uyothatha igazi). uketshezi negazi okuthathwe kuwe kuyosiza ukuthi senze ucwaningo mayelana nempilo yakho kuphinde kuyoxilongwa ukuthi akunazo yini izifo ezithathelana ngocansi negciwane lesandulela ngculazi. Awuzukhishwa ocwaningweni ngenxa yemiphumela yesandulela ngculazi kanye neye zifo ezithathelana ngocansi. Siyolinda ukuthi uhlinzwe kukhishwe izicubu.

At enrolment when you are admitted to hospital in preparation for surgery, we will allocate to you a unique study number that we call a participant identification number (PID). This number will be our main reference to identify all specimens which we collect from you, including the hysterectomy sample. We will not link your name to your HIV test result or any of your other results and, therefore, when the study is carried out In the laboratory, we will onlyknow your PID number and not your name. We will not share your results with anyone and we will keep your results and number confidential.

Uma sekuqala ucwaningo ususesibhedlela utindele ukuhlinzwa, sizokunikeza inombolo yakho ehlukile. Lenombolo iyona ezosetshenziswa kuwena kuphela ukuhlukanisa izicubu namagazi athathwe kuwe angahlangani nawabanye nanokuthi iziphi izicubu zesibeletho namagazi ezithathe kuwe. Angeke sihlanganise igama lakho kanye nemiphumeta yakho yegciwane lesandulela ngculazi kumbe nemjnye imiphumela yocwaningo, esikhungweni socwaningo Iwamagazi ngeke balibone igama lakho kodwa bayobona lenombolo esizokunika yona. Ngeke sikhombise muntu imiphumela yakhe kanye nenombolo yakho siyokugcina kuyimfihJo.

Contact Procedures:
After you have consented to participate in the study and samples have been collected, we will not have any followup contact with you again. However, you are allowed to contact us at any time after your operation to ask that we discard your tissue sample and not include you in our results. In this case, you will need to tell us the PID number that was allocated to you at enrolment.

Ukuxhumana.

Emva kokuba usuvumile ukubamba iqhaza kulolucwaningo igazi nezicubu zakho ,sezithathiwe ngeke sisaphinde sixhumane nawe. Kodwa uvumelekile ukusithinta ernva kokuhlinzwa uma ufuna silahle zicubu zakho singabe sisazisebenzisa. Uma kunjalo kuyodingeka usitshele inombolo Jena esakunika yona

Risks and/or discomforts:

The study clinical procedures are similar to those experienced by women in routine gynecological examinations or during pre-assessment for elective hysterectomy. You may experience discomfort when having pelvic examinations and/or undergoing phlebotomy for this study. During phlebotomy, you may feel dizzy or faint, and/or develop a bruise or swelling where the needle is inserted. You may become embarrassed, worried, or anxious when completing the HIV-related interviews and/or receiving HIV/STI counseling. You may also become worried or anxious while waiting for the HIV test results or after receiving HIV-positive test results. Trained study staff will be available to help you deal with these feelings.

Ubungozi kanye nobuhlungwana:

Ukuxilongwa okuzokwenziwa kufolucwaningo kuyafana nalokho okwenziwa kubantu besifazane uma beya kudokotela wesifazane ukuyoxilongwa nokulungiselelwa ukukhishwa kwesibeletho. Kungenzeka uzwe ubuhlungwana uma kuxilongwa ingaphakathi lesitho sangasese sakho kanye noma uthathwa igazi - Ngesikhathi uthathwa igazi kungenzeka ube nesiyezi uhuzuke noma kuvuvukale lapho kade kufakwe khona inaliti. Kungenzeka uzenyeze, ukhathazeke noma ube nexhala ngesikhathi ubuzwa imibuzo mayelana negciwane lesandulela ngculazi kanye nezifo zocansi. Kungenzeka ube nexhala ngesikhathi usalinde imiphumela yakho yegciwane lesandulela ngculazi noma emva kokuba uthole imiphumela ekhombisa ukuthi unegciwane lesandulela ngculazi. Abasebenzi abawufundele umsebenzi bazobe bekhona ukukusiza ukuthi ubhekane nemizwa yakho

Benefits:

You and others may benefit in the future from information learned in this study. You will have access to free counselling and testing for HIV and sexually transmitted infections and other HIV risk reduction- If you are infected with HIV or have an STI, you witl be referred for medical care, counselling, and other services and research studies available to you. If you are HIV positive you will have access to care and anti-retroviral treatment if indicated. You may speak with a study counsellor who may help with questions about the study and your health at any time or as needed.

All care provided to you in the context of this study will be in accordance with Department of Health Policies and Guidelines.

lnzuzo:

Wena kanye nabanye abantu nizozuza ngolwazi esizolufunda kulolucwaningo. Nizothola ukwelulekwa nokuhlolelwa igciwane lesandutela ngculazi kanye nokuhlolelwa izifo zocansi ezithathelanayo kanye nokuthi unganciphisa kanjani amathuba okutheleleka ngegcjwane lesanduJela ngculazi mahhala. Uma ngabe usunegciwane lesandulela ngculazi noma isifo socansi uyodluliselwa emtholampiJo lapho uzothola khona usizo, tokwelulekwa. Uma unegciwane lesandulela ngculazi uyosizwa ngokuthi uthole imishanguzo nokunakekelwa kwezempilo. Ungakhuluma nomeluleki ozobe eyingxenye yocwaningo ongakusiza urna unemibuzo mayelana nocwaningo kanye nangempilo yakho. Lonke unakekelo lwezempilo oyoluthola kulolucwaningo luyobe luhambisana nemigomo yomnyango wezempilo.

New findings:

The findings from this study will be published in journals that you or other members of the public can access. Your information will not be revealed in such publications

Imiphumel emisha:

Imiphumela yalolucwaningo iyokhishwa emaphepheni umphakathj ozokwazi ukufinyelela kuwo. Imininingwane yakho ngeke ivezwe kulawo maphepha.

Reasons why you may be withdrawn from the study without your consent:

You may be removed from the study without your consent for the following reasons:

- The study is cancelled by the University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC). . Other administrative reasons.

Kungenzeka ukhishwe kucwaningo ngaphandle kokuthi waziswe ngaiezizizathu elizandelayo•

- Uma ucwaningo luvalwa yiNyuvesi yaKwaZutu-Natali . Kanye nezinye izizathu zokuphatha

Costs to you:

There is no cost to you for taking part in this study. At the enrolment you will receive R50 for participating in this study,

Ukukhokha:

Awuzukhokha lutho ngokubamba kwakho iqhaza kulolucwaningo. Uma sekuqala ucwaningo uyonikweza R50 ngokubamba kwakho iqhaza.

Confidentiality:

All information you share with us will be maintained securely with limited access by CAPRISA study staff, and you will only be identified using a PID. Personal information from your records will not be released without your written permission. You will not be personally identified in any publication or information shared publicly about this study. Your records may be reviewed by the BREC or study sponsors.

Secondly, information that the study staff get from you, your tissue samples will be confidential in most situations. This means that study staff will NOT tell your parent or guardian about results or procedures that you have the right to decide on your own about.

Imfihlo:

Yonke imininingwane osinikeze yona iyobekwa abasabenzi base CAPRISA endaweni evikelekile lapho kungangeni khona noma ubani, kuyobe sekusetshenziswa inombolo yakho esizokunika yona. Imininingwane yakho ngeke idalulwe ngaphandåe kwemvume yakho ebhatwe phansi. Ngeke udalulwe uma sikhipha ulwazi locwaningo sitshela umphakathi. Imininingwane yakho kungenzeka icutshungulwe abaxhasi bocwaningo.

Okwesibili, imininingwane yakho kanye nezicubu eziyothathwa kuwe abasebenza ocwaningweni ziyoba imfihlo ngasosonke isikhathi. Lokho kusho ukuthi ngeke kutshelwe urnzali noma umbheki wakho ngemiphumela uwena kuphela ongabatshela.

Research-Related Injury:

Based on the procedures we propose to undertake in this study and described above it is unlikely that you will be injured as a result of being in this study.

Ukulimala okuhambisana nocwaningo:

Ngenxa yemigudu esizoyilandela kulofucwaningo nesesikuchaze ngenhla mancane amathuba okuthi ungalimala ngenxa yalolucwaningo.

Problems or Questions about participating in this research study:

If you ever have any questions about your participation in this study you should contact Dr Derseree Archary at 031-260 4849 or 0739797212, CAPRISA, Second Floor Doris Duke Medical Research Institute, Durban, 359 King George V Avenue, Glenwood, Durban, South Africa. If you have questions about your rights as a research participant, you should contact the Chairperson of the Biomedical Research Ethics Committee of the University of KwaZulu-Natal at in Durban using the address below:

Research Office
Biomedical Research Ethics Administration
Westville Campus, Govan Mbeki Building
Private Bag X 54001
Durban
4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za

Website: https: //research. ukzn. ac-za/ResearchEthics/Biomedica Research Ethics. aspx

tzinkinga kanye
Uma unemibuzo ngokubamba kwakho iqhaza kulolucwaningo ungathintha u dokotela Derseree Archary kulenamba 031 260 4849 noma 0739797212 CAPRISA Second Floor Doris Duke Medical Research Institute, Durban, 359 King George V Avenue, Glenwood, Durban, South Africa.Uma unemibuzo ngamalungelo akho njengombambiqhaza, ungathinta usihlabo we Biomedical Research Ethics Committee of the University of KwaZuJu-Natal eThekwini kulelikhe'i elingenzansi:

Research Office
Biomedical Research Ethics Administration
Westville Campus, Govan Mbeki Building
Private Bag X 54001
Durban
4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za
Website: https//research.ukzn.ac.za/ResearchEthics/BiomedicalReseaarchEthics

SIGNATURE PAGE:

If you have read the informed consent (or if you have had it read to you) and understand the information, and you voluntarily agree to join this study, please complete the comprehension assessment with the staff member. If you have a good understanding of what you are agreeing to participate in, you will be asked to please sign your name below.

Volunteer's name (print name as it appears in ID k]Birth certificate)	Volunteer's signature	Date
Name of staff member who administered consent (print)	Staff members signature	Date
Witness' name (print)	Witness' signature	Date

Was a copy of the signed copy given to the volunteer: Yes No If no, why not:

Appendix F: CAPRISA PID data capture form

CAPRISA

CENTRE FOR THE AIDS PROGRAMME OF RESEARCH IN SOUTH AFRICA

UNAIDS

CAPRISA IS A UNAIDS COLLABORATING CENTRE FOR HIV PREVENTION RESEARCH

Institution	University of KwaZulu-Natal
Department	CAPRISA
Study name	Explant study: CAP 105
Site name	Prince Mshiyeni Memorial Hospital (PMMH), Umlazi
Participant identification number (PID)	105-MS-/ / / / /
Date of birth	Day: Month: Year:
Ethnicity	Black Indian White Coloured Other
HIV status	Positive
	Negative
	Indeterminate (reason):
	Unknown
History of hormonal contraception	Injectable
	Oral
	Other (Specify):
	None
Reason(s) for Hysterectomy	
Sample(s) collected	Cervical swab
	Blood
	Cervical tissue

Name of study nurse.....

Signature.....

Date.....

Appendix G: Explant protocol

Purpose

This protocol describes the method used to quantify data for all immune cells including lymphocytes and antigen presenting subtypes and for setting up tissue explant cultures.

Background

The immune cells present in the human genital tract are important for protection from sexually transmitted infections and can be a target of viral infections, such as HIV. Therefore, knowledge about the distribution and variability of the different immune cell types is important for understanding the susceptibility to infections and the development of prevention strategies against infection. Thus, human cervical explant tissue can be used effectively to quantify data for all immune cells including lymphocytes and antigen presenting subtypes. In addition, tissue explant cultures offer the best bridge between in vitro testing and in vivo experimentation. Several explant culture set-ups have currently been published. This protocol describes the polarized cervical explant cultures. These cultures can be used to evaluate microbicide product toxicity, effectiveness, and product application times. This can be used to investigate HIV-1 pathogenesis and include but are not limited to testing innate antimicrobial activity of human secretions, the influence of cytokines, semen and hormones on HIV-1 infection, the cell types that are infected after exposure to HIV-1, and the migration of infected cells from the tissues.

Preparation

Biological hood should be decontaminated using 70% ethanol at least 15 minutes prior to use.

Be sure to follow all Good Clinical Laboratory Practice (GCLP) guidelines.

Surgical tools should be sterile autoclaved.

Waste container with disinfectant should be ready for any disposable materials. Be sure to prevent overflow.

Prepare all media according to appropriate instructions.

Storage Requirements

All media should be stored at 2-8°C and brought to room temperature before use.

Procedure

1) **Day 1**:
   - Do checklist (see- appendix 1A)
   - Prepare transport media (R15 and can be stored at 4^0C for 2weeks maximum):
     - ✓ RPMI 225ml
     - ✓ 10% FBS 25ml
     - ✓ 100x Pen/Strep/L-glutamine 2.5ml
     - ✓ 2.5µg/ml Amphotericin B (Fungizone) 2.5ml

Before you leave for the day: Check if the temperature of the of the incubator is set at 37^0C and CO_2 is at 5%

2) Day 2:

1. Check if the temperature of the of the incubator is set at 37^0C and CO_2 is at 5%
2. Make FRESH 13mL Collagenase Digestion Media per patient
   a. Remove Collagenase IV vial from -20°C.
   b. Carefully measure 0.2g of collagenase powder per subject.
   c. Reseal vial, store in desiccators at -20 °C.
   d. In 15mL conical tube add collagenase powder, then add 2ml of HBSS
   e. Vortex well to mix, until powder has completely dissolved, making sure no clumps get stuck on the walls or cap.
   f. Filter solution with 0.22 µm Millipore filter.
   g. Collagenase Digestion Media expires one day from preparation. ****Always make up fresh Collagenase Digestion Media for use****

3) Cervical Explant set-up

1. Tissue processing

   1) Tissue should be put in transport medium (R15) immediately after surgery and transported to the laboratory within 3 hours from collection.
   2) In the Petri dish, rinse tissue with new transport media (R15) or PBS and keep the tissue in solution at all times to tissue drying out.
   3) Trim excessive muscular tissue with surgical tools (Scalpels, forceps), being sure to limit handling of the epithelial layer.
   4) Separate the different sections of the tissue (endocervix and ectocervix) carefully. To do this, use a scalpel, a pair of forceps that can easily grab the tissue, and a blunt pair of forceps to hold the tissue in place while cutting (you may want to start with another person holding the tissue until you have become accustomed to this procedure). When using the forceps, make sure not to puncture the epithelial layer. Afterward, discard the connective tissue into a biohazard bin.
      a. Punch 3mm biopsies of epithelial layer. Using a scalpel, slice excess muscular tissue behind the epithelium so that each explant is about 2mm thick.
      b. Experiment conditions should be performed in duplicate (if possible), therefore attempt to extract an even number of explants.

For Distribution of cells:

1. Transfer 10 x $3mm^3$ pieces (further cut into small pieces for effective digestion) of endocervical or ectocervical explant tissue into the gentleMACS C tube containing Digestion Media (40µl Collagenase solution + 8ml R10 *(appendix 1B)* + 8µl DNAse)
2. Tightly close the C tube and attach it upside down onto the sleeve of the gentleMACS Dissociator
3. Run gentleMACS Program m_spleen_01.
4. After termination of the program, detach C Tube from the gentle MACS Dissociator.
5. Incubate the sample for 30 minutes at 37 °C on a shaker platform at low speed (approximately 120 rpm).
6. Attach C Tube upside down onto the sleeve of the gentle MACS Dissociator
7. Run gentleMACS Program m_spleen_01.
8. (Optional) Centrifuge sample at 1800rpm for 1 minute.
9. Apply the cell suspension to a cell strainer (100 µm mesh size) placed on a 50 mL tube.
10. Rinse strainer with 30 mL R10 medium and collect the flow-through.
11. Centrifuge at 1800rpm and 4 °C for 5 minutes.
12. Discard supernatant

Staining

13. Re-suspend the pellet (with whatever left in the well) and transfer the liquid with cells (~200ul) to a V bottom plate
14. Count cells (optional)
15. Prepare the surface stain Master Mix (see - Appendix 1C)
16. Spin the plate at 1800rpm for 3 min and discard supernatant
17. Add 50µl of surface stain master mix per well
18. Incubate surface stain for 20 mins at room temp in the dark (Cover with foil)
    a. *While waiting, prepare compensation beads (see –Appendix 1D)*
19. Wash cells with 150µl of *PBS-1 (99µl PBS + 1µl FBS, multiply this by number of wells)*
20. Centrifuge at 1800 rpm for 3 mins and discard supernatant
21. Wash cells with 200µl of PBS-1 into each well
22. Centrifuge at 1800 rpm for 3 mins and discard supernatant
23. Resuspend cells in 200ul of 1x cell fix and transfer to Facs tubes
24. Optional, add additional 150µl of PBS-1 into each well
25. Cover with foil and store at 4^oC, if not acquiring the same day
26. Acquire cells for Flow Cytometry (cover your other tubes with foil while acquiring)

Checklist

<u>Equipment and Materials</u>

1. 2-8°C Refrigerator
2. -80°C freezer
3. 5% CO_2 incubator at 37°C
4. Plate Reader
5. Vacuum
6. Pipette Aid
7. Pipettes – range 20, 200, 1000µl

8. Waste container
9. Sharps container
10. brass cork bore
11. Sterile surgical tools
    a. Forceps
    b. Blunt nose scissors

Disposables

1. Pipette tips – range 1, 10, 20, 200, 1000µl
2. 10ml serological pipettes
3. Petri dish
4. Scalpel
5. Biopsy Punch tool – 2mm
6. gentleMACS C tube
7. MACS Dissociator
8. 0.22 µm Millipore filter

Reagents and Consumables

1. Transport media per explant (R15)
    a. RPMI 22.5ml
    b. 10% FBS 2.5ml
    c. 100x Pen/Strep/L-glutamine 125ul
    d. 2.5µg/ml Amphotericin B (Fungizone) 250ul

2. Collagenase Solution per patient
    a. Collagenase powder 0.2g
    b. HBSS 2ml

3. Collagenase Digestion Media
    a. R10 media 8ml
    b. Collagenase Solution 40µl
    c. DNase 8µl

Appendix H: R10 Medium

R10 Medium

1. Thaw a falcon tube containing 55ml of heat inactivated cell-culture tested serum in a 37°C water bath with occasional mixing/swirling. (If serum is not heat inactivated, inactivate by placing in a 56°C water bath for an hour and cool to room temperature)
2. Get a 500ml bottle of RPMI from the cold room
3. Thaw one tube each of Pen/Strep (5ml) and L-glutamine (5ml)
4. Add Pen/Strep and L-glutamine
5. Add 5ml of 1M HEPES
6. Add 55ml of FBS
7. Mix thoroughly

Reagents

Heat-inactivated fetal calf serum (FCS) (Sigma-Aldrich Co., cat. no.F4135)
L-glutamine (200 mM solution) (Cellgro, Mediatech Inc., cat. no. 25-005-CI) Sigma G7513-100ML)
Penicillin/streptomycin (10,000 U ml_1 and 10 mg ml_1) (Cellgro, Mediatech Inc., cat. no. 30-002-CI) (Sigma P4333-100ML)
1 M HEPES buffer (Cellgro, cat. no. 5-060-CI; Mediatech Inc.) (Sigma H3537-100ML)
RPMI 1640 medium (Sigma-Aldrich Co., cat. no. R0885)

Notes:

a. To make RPMI-PLGH (serum free media) skip the addition of FCS.
b. Name and initial bottle.
c. NEVER pipette directly out of the bottle for any experiments. Instead pour out the required amount of media into a falcon tube and work with that. The bottle of R10 is always kept at 4°C.

Appendix I: Surface marker flow panel and PBMC staining

Marker	Flourochromes	VOLUME/ REACTION(50UL TOTAL)
CXCR5	APC	1
Live/Dead	Amcyan	3
PD-1	BV421	1
CXCR4	PE-Cy5	1
CXCR3	PE-Cy7	1
CD3	APC-H7	1
CD4	BV605	1
CD25	FITC	1
CD127	PE	1
CD19	Alexa Flour 700	1
CCR5	PerCP-Cy5.5	1
CCR6	BV650	1

Antibodies purchased from BD Science (Scientific group) and Biolegend

Compensation Tubes

1. Set up during one of the incubation/wash steps during staining procedure
2. Compensation tubes should never be prepared more than 1hr before flow analysis

Label compensation tubes as follows:

a) Add 2ml of PBS-1 to a FACS tube and label it as Unstained
b) Add 6 drops of anti-mouse IgG compensation beads (both positive and negative).
c) Mix well and transfer 120ul of this mixture to all individual tubes
d) Add 2ul of the appropriate antibody to each tube. Mix
e) Incubate at RT for 5min
f) Top up to 300ul of PBS-1 to each tube
g) Store at 4^{o}C no more than 1 hour prior to flow analysis

Appendix J: PMBC isolation and staining protocol

One day before lab work prepare and gather all reagents:

Ficoll

R10

2% FBS PBS

FACS tubes

Falcon 50ml tubes

Serological pipettes

Tube racks

Centrifuge with correct buckets

Pipette tips

Antibodies

PBMC isolation in the morning

1) Spin acd tubes containing blood for 10min at 1600rpm (no brakes)

2) While spinning add 12ml of Ficoll to 50ml tube

3) Once tubes have spun mark the plasma layer at top and bottom making sure to leave a little bit of plasma

4) Using a serological pipette remove the plasma layer and replace with 2% FBS-PBS

5) Mix by pipetting up and down with the serological pipette

6) Layer the blood onto the Ficoll at an angle using the serological pipette drop by drop

7) Once the blood is layered on the Ficoll (make sure there is no blood in the Ficoll layer) Spin the 50ml tube for 30 min at 1400rpm (no brakes)

8) While the tube is spinning prepare the Antibody cocktail
9) After spinning remove the plasma layer (leave a bit in case PBMCs are in) with a serological pipette and discard.

10) Take PMBCs with a little bit of plasma and Ficoll (if the pmbcs are in the Ficoll take the Ficoll but avoid RBCs)

11) Place PBMCs in a new 50ml conical tube and add 40ml of 2%FBS-PBS and spin at 1600rpm for 10min (PBS washes away the Ficoll and plasma)

12) After spin decant the S/N and flick out pellet into remaining media.

13) Add 10ml of R10 media

14) Counting cells: 50ul cells and 50ul trypan blue and mix in a tube and count cells in 2 grids

    #cells (cells/ml) = cells counted/2 x df (1) x 10^4: answer must be in millions

15) Go back to 50ml containing cells and R10 and spin at 2000rpm for 6min and decant S/N then add R10 at 1ml per million cells (eg 2million cells = 2ml R10)

16) Check how many tubes needed for the experiment

PBMC staining

17) Divide the cells equally amongst tubes and calculate the number of cells in each tube (cells must be in million units)

18) Spin tubes for 6 min at 2000 rpm and decant S/N

19) Add 2%FBS-PBS at 1ml per million cells

20) Spin tubes at 2000rpm for 6min and decant S/N

21) Add 50ul Ab cocktail to each tube

22) Incubate tubes with foil for 20 min at $4^{o}C$

23) After incubation wash cells with 2%FBS-PBS at 1ml per mil cells

24) Spin tubes at 2000rpm for 6min

25) If acquiring same day, decant and add PBS + 1 before acquiring cell

26) If acquiring next day, decant add cell fix at 1ml per million cells and store at $4^{o}C$ in the dark.