In vitro effects of intravaginal insertion products (IVIPs) on biomarkers of inflammation and immune cellular activation in the era of HIV

By

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Of

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PREFACE

The experimental work described in this thesis was conducted at the Centre for the AIDS Programme of Research in South Africa laboratory, Nelson R. Mandela School of Medicine, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa, under the supervision of Dr Pamela Gumbi. The research was financially supported by the DST-NRF centre of Excellence in HIV Prevention.

This work has not been submitted in any form for any degree or diploma to any tertiary institution, where use has been made of the work of others, it is duly acknowledged in the text, the results reported are due to investigations by the candidate.

Rejoice Zanele Hlophe signed: 

Dr Pamela Gumbi signed: 

As the candidate's supervisor, I agree with the submission of this thesis.

Date: November 8, 2019

DECLARATION

I Rejoice Zanele Hlophe, declare that:

The research reported in this thesis, except where otherwise indicated or acknowledged, is my original work.

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

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Signed: 

Date: November 8, 2019

DEDICATION

This work is dedicated to my family and future generations.

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ETHICS DECLARATION

This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BE382/16). HSP and GCP were followed and all participants/ donors were consented and willingly participated in this study. Participant identity was kept strictly confidential.

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ACRONYMS

$	Dollars
%	Percentage
µl	Microlitre
ACD	Acid Citrate Dextrose
AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen presenting cell
APC-H7	Allophycocyanin-H7
BD	Becton Dickson
BV	Bacterial Vaginosis
CAPRISA	Centre for the AIDS Programme of Research in South Africa
CCR5	C-C chemokine receptor type 5
CD	Cluster of differentiation
CO2	Cytochrome Oxidase 2
DAMPs	Damage-associated molecular pattern molecules
DC	Dendritic cell
DC SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DD	Distilled Deionized
DMSO	Dimethyl Sulfoxide
EDTA	Ethylene diamine tetra-acetic acid
FACS	Fluorescence-activated cell sorting
FBS	Foetal Bovine Serum
FGT	Female genital tract
FMO	Fluorescent Minus One
FSC	Forward scatter
FSC-H	Forward scatter-height

G	Gram
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HIV	Human immunodeficiency virus
HIV-1	Human Immunodeficiency type 1
HLA	Human leukocyte antigen
HLA-DR	Human leukocyte antigen
HPV	Human Papilloma Virus
HSRC	Human Sciences Research Council
IFN	Interferon
IL	Interleukin
IP	Interferon-gamma-induced protein
IVIPs	Intravaginal Insertion Products
KZN	KwaZulu-Natal
L. crispatus	*Lactobacillus crispatus*
L. jensenii	*Lactobacillus jensenii*
L. vaginalis	*Lactobacillus vaginalis*
LIF	Luekin Inhibitory factor
MCP-1	Monocyte Chemoattractant Protein 1
MFI	Median fluorescent intensity
Mg	Milligram
Min	minutes
MIP	Macrophage inflammatory protein
ml	Millilitre
NF-KB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
ºC	Degrees Celsius
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffer saline

PerCP-Cy5.5	Peridininchlorophyll protein-Cy5.5
PerCP-Cy7	Peridininchlorophyll protein-Cy7
pH	Potential Hydrogen
PHA	Phytohemagluttin
PMT	Photomultiplier tube
PrEP	Pre-exposure prophylaxis
QC	Quality Control
RANTES	Regulated on Activation, Normal T Expressed and Secreted
RO	Reverse osmosis
RPMI	Roswell Park Memorial Institute
RT	Room temperature
Spp.	Species
SRHR	Sexual reproductive health rights
SSC	Side scatter
STI	Sexually transmitted infection
TCR	T cell receptor
THC	Tetrahydrocannadinol
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor -alpha
UNAIDS	Joint United Nations Programme on HIV/AIDS
Vivid	Violet-fluorescent reactive dye
x g	Relative centrifugal force
X	Times

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ABSTRACT

Rationale

The use of vaginal products is associated with increased HIV acquisition risk, but the mechanism is not fully understood. Vaginal practices entail the use of a wide variety of products which can alter the vaginal environment to achieve a desired state. Strong motivations for vaginal practices include women's desire to maintain stable relationships, manage health, hygiene and sexuality. This adjustment of the vaginal microenvironment may increase HIV acquisition risk. High levels of inflammation and immune activation in the female genital tract are associated with a threefold increase in HIV acquisition risk. We hypothesized that intravaginal insertion products (IVIPs) may be linked to high levels of inflammation and immune activation in the female genital tract which may subsequently lead to an increased risk of HIV acquisition

Objective

The pH of the IVIPs (Kuber, Snuff, Alum, Savlon and Rose water) was measured and the cytotoxicity of the IVIPs was evaluated by determining their effect on cell viability at different dilutions (Neat/stock, 1/5, 1/10, 1/100 and 1/1000). The mechanisms by which potassium aluminium sulfate ("Alum") and smokeless tobacco ("snuff") impact cellular activation and inflammation were investigated using peripheral blood mononuclear cells (PBMCs) *in vitro*.

Methods

The pH of alum, snuff, kuber, savlon and rose water was measured at different dilutions (Neat/Stock, 1/5, 1/10, 1/100 and 1/1000). The effect of the IVIPs on cell viability was determined by exposing PBMCs to the different dilutions of IVIPs mentioned above. PBMCs from 26 HIV-negative healthy donors were unstimulated (negative control) or stimulated for 3 hours at 37°C with 1/1000 dilutions of 450 mg/ml of alum or snuff and 10µg/ml of PHA (positive control). The PBMC supernatants were collected following PBMC stimulation, and eleven cytokines were measured from 12 of the 26 PBMC supernatants. Pro-inflammatory (IL-1β, TNF-α, IL-6), chemokines (IL-8, IP-10, MIP-1α, MIP-1β, MCP-1), hematopoietic (IL-7, GM-CSF) and regulatory (IL-10) cytokines were measured using Bio-Plex multiplex assay. The activation status of T lymphocytes was determined by evaluating $CD38^+$, HLA-DR^+, dual expression of $CD38^+$HLA-DR^+ and

chemokine receptor $CCR5^+$ expression from $CD4^+$ and $CD8^+$ T cells using flow cytometry assay.

Results

Alum stock solution was acidic with a pH of 2.62 whereas the snuff stock solution was basic with a pH of 9.11. Alum and savlon were found to have high cytotoxicity. Snuff exposed cell resulted in a significantly increased CCR5 chemokine expression in $CD4^+$ T cells when compared to the unexposed cells (p=0.0483) and also when compared to alum exposed cells (p=0.0446). However, snuff exposure did not significantly increase any of the activation markers in $CD8^+$ T cells and it did not change the inflammatory cytokine profile. In $CD8^+$ T lymphocytes the $CD38^+$ biomarker was significantly more expressed in unexposed cells compared to the alum exposed cells (p=0.0185). Alum exposed cells significantly increased expression of $HLADR^+$ (P=0.0348) and also the dual expression of $CD38^+HLA\text{-}DR^+$in $CD8^+$ T cells (p=0.0208) when compared to the unexposed cells and was also associated with significantly high levels of cytokines IP-10 (p=0.039), MCP-1 (P=0.0024), MIP-1α (p=0.0005), IL-6 (P=0.0005), TNF-α (P=0.0020), IL-7 (P=0.0005) and GM-SCF (P=0.0005) when compared to the unexposed cells.

Conclusion

This study is the first of its kind to identify a possible link between intravaginal insertion products and inflammation. Alum, in particular, was more inflammatory compared to snuff. These findings may help explain the previous observations of an increased HIV acquisition risk in IVIP users. Future research can extend the current pilot study on an *invitro* human vaginal epithelial cell model. Knowledge from this work and future studies is crucial in developing new female-initiated interventions for preventing HIV acquisition.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW.

1.1 Introduction

1.1.1 Global HIV epidemiology

Human immunodeficiency virus (HIV) and Acquired immunodeficiency syndrome (AIDS) have been the most critical public health pandemic that has threatened human survival and security since its first discovery in 1981(Gottlieb et al., 1981). Approximately 77.3 million people have been infected with HIV since the start of the epidemic, and it has claimed 35.4 million lives through AIDS-related illnesses(UNAIDS, 2018). According to the UNAIDS report, HIV infections are currently estimated to be 36.9 million worldwide, with 1.8 million new HIV infections in 2017 or about 5000 new infections per day (UNAIDS, 2018). Although significant progress has been made since the antiretroviral therapy (ART) rollout; with an estimated 21.7 million on ART and 940,000 people dying of AIDS in 2017, a 47% decrease from 3.4 million in 1996, HIV remains a leading cause of death worldwide and the leading cause of death globally among women of reproductive age (UNAIDS, 2018).

1.1.2 HIV epidemic in Sub-Saharan Africa

Developing countries are profoundly affected by the HIV epidemic with more than 90% HIV infections, a more massive toll (70%) of these infections being from Africa (UNAIDS, 2018). Sub-Saharan Africa (SSA) is the greatly affected region with 19.0 million HIV infections (UNAIDS, 2018) and one of the prime causes of death is HIV/AIDS. There were 960 000 reported new HIV infections with 470 000 people who lost their lives due to AIDS-related causes in 2015, with only 54% of the population on ART. Figure 1.1 shows the adult prevalence of HIV globally, with SSA having the highest HIV prevalence.

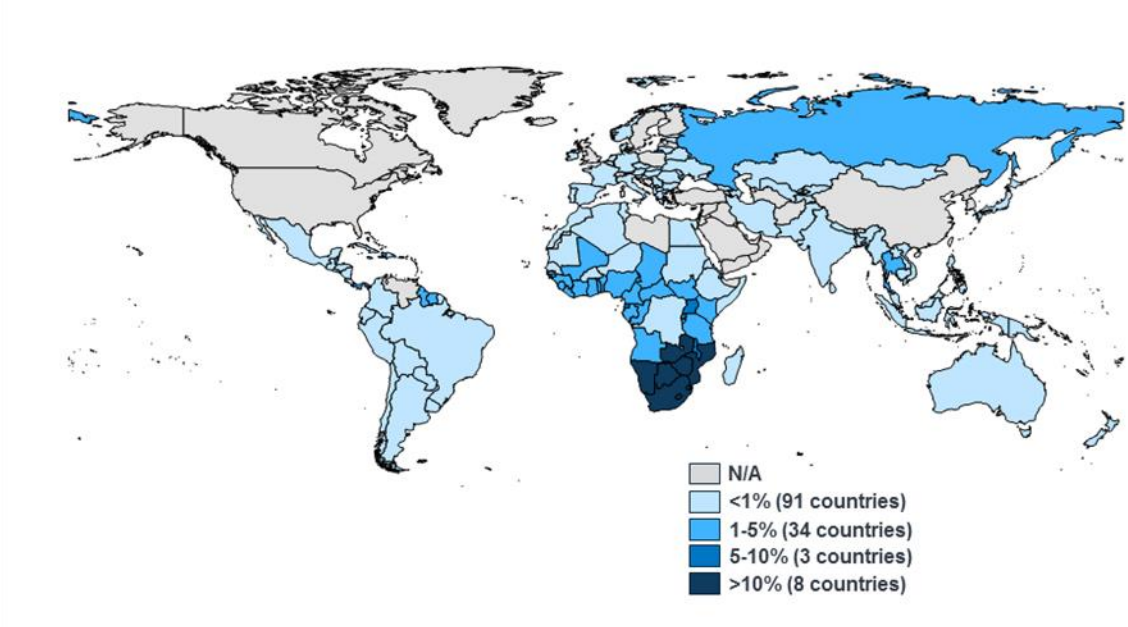

Figure 1. 1. Adult prevalence of HIV infection in Sub-Saharan Africa compared to other geographic regions. Dark blue colour indicates areas with the highest prevalence, whereas the light blue shades indicate areas with the lowest prevalence. Sub-Saharan Africa has more than two-thirds of all people living with HIV globally (figure adapted from UNAIDS, 2017).

1.1.3 HIV Epidemic in South Africa

South Africa remains the epicenter of the HIV epidemic and has the highest number of people living with HIV in the world (7.2 million) (UNAIDS, 2018). Although SA is home to approximately 1% of the world's population, it contributes 14% of the global HIV infection burden, which is about 1000 new infections a day (UNAIDS, 2018). In 2015, 7 million people were living with HIV, with 380 000 new infections and 180 000 people dying from AIDS-related deaths (UNAIDS, 2015). The country invests $1.5 billion yearly towards HIV/AIDS programs, it has the most extensive antiretroviral therapy program globally. However, with all the prevention programs implemented, the HIV prevalence in the general population is still high (19.2%), but it varies from region to region. KwaZulu-Natal (KZN) has the highest HIV prevalence of almost 25.2% compared to other provinces like Northern Cape and Western Cape with 13.9% and 12.6% prevalence, respectively (HSRC, 2018).

1.1.4 HIV epidemic in KwaZulu-Natal

KwaZulu-Natal (KZN) is the second largest province in South Africa and the most vulnerable to HIV/AIDS, with the highest infection rate and therefore referred to as the epicenter of HIV/AIDS (Kiepiela et al., 2014). In South Africa, there are 12 Districts (Harry Gwala, uMgungundlovu, eThekwini, uThukela, iLembe, Zululand, Ehlanzeni, Gert Sibande, Ugu, eHlanzeni, King Cetshwayo and Buffalo city) with the highest HIV prevalence and KZN province having the most affected districts (Woldesenbet et al., 2018).

High levels of migration to urban areas by the rural population in KZN is accompanied by high levels of sexual activity outside of the primary relationship (Collinson et al., 2007). These extra sexual activities often lead to increased risk of acquiring HIV, with resultant progression to AIDS and leading to AIDS-related deaths. Deaths often result in a multitude of orphans, most of whom have no means of surviving, so they depend on transactional relationships with older sexual partners for survival, thus exposing themselves to increased risk of acquiring HIV-1 (Meyer, 2013).

1.1.5 HIV infection in women

One of the significant disparities in HIV infectivity is gender with higher gender imbalance observed in profoundly affected regions such as SSA. A disproportionate impact of the HIV epidemic is found in women; women are four times more likely to be infected with HIV compared to men. Not only do young women aged 15-24 years have HIV rates higher than their male peers, but they also acquire HIV infection five to seven years earlier than their male peers (Karim et al., 2010). According to a UNAIDS report, adolescent girls and young women accounted for 20% of new HIV infections in 2015 globally (UNAIDS, 2016), whereas in SSA 25% of new HIV infections were observed in adolescent girls and young women. That study further showed that among adults, 56% of new HIV infections occurred in women, suggesting that women are at a higher HIV risk compared to men. A recent cross-sectional household survey of randomly selected individuals aged 15-49 years in two neighboring sub-districts with a high burden of HIV infection in KZN reported HIV prevalence of 59.8% in women aged 25-40 years, 40.3% in men aged 25-40 years, 22.3% in

women younger than 25 years, and 7.6% in men younger than 25 years (Figure 1.2, (Kharsany et al., 2018)). They further suggested that sexual partnering between young women below the age of 25 and older men aged 25-40 years, who might have acquired HIV from women of similar age group, is a key feature of the sexual networks driving transmission in young women (Kharsany et al., 2018).

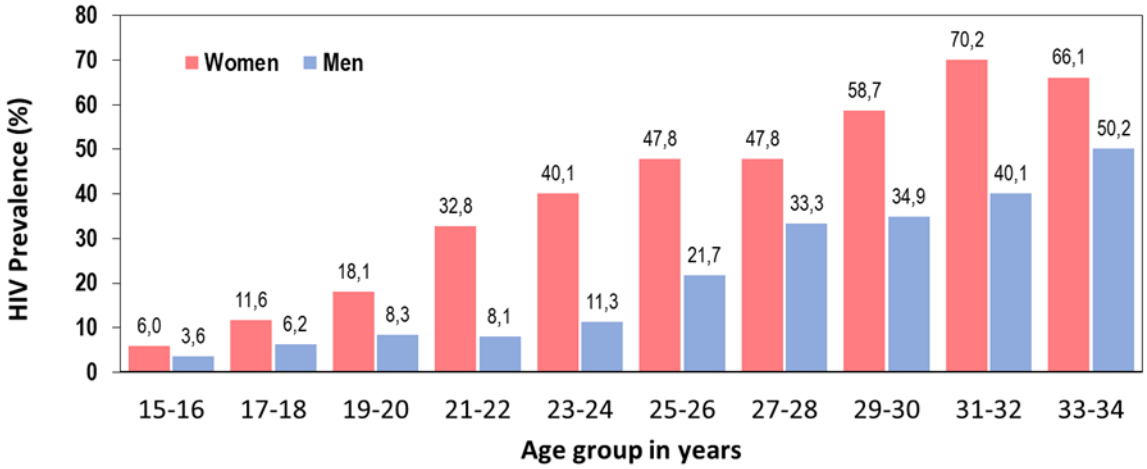

Figure 1. 2. Shows age and gender-specific HIV prevalence in a survey carried out in 2 sub-districts of KZN (Kharsany et al., 2018). HIV prevalence in women aged 15-24 years was 22.3% (20.2-24.4) compared with 7.6% (6.0-9.3; p<0.0001) in men of the same age. This disproportionately high HIV prevalence in females versus male peers is observed from the age of 15 years up to more than 30 years whereas the prevalence in females is more than 70% at age 31-32 years.

1.1.6 Why are young women so vulnerable to HIV infection?

1.1.6.1 Economic status

Young women from poor backgrounds are determined to come out of poverty, and often have little or no access to education (Fetters et al., 1998a). These women exchange sex for money and food and become vulnerable in quest of basic income (Stoebenau et al., 2016). Hence, these relations often expose young women to unsafe sexual behaviors, low condom use and an increased risk of sexually transmitted infections (Glynn et al., 2001). This

unfavorable economic status forces women to depend on their male companions to provide for them and their children (Heise and Elias, 1995).

Poverty has resulted in urbanisation in search of employment. Urban settings have a higher HIV prevalence than rural settings, with sex work common in poverty stricken women (Dodoo et al., 2007). On the other hand, economically empowered girls tend to be involved in safer sexual behaviors and remain in school leading to a reduced HIV prevalence and incidence (Cluver et al., 2016, Taaffe et al., 2016).

1.1.6.2 Gender-Based Violence and Gender inequality

Physical and sexual violence against women and children is a significant crisis facing southern Africa, and it is linked to perceptions of male superiority (Jewkes et al., 2010). South Africa is said to have the highest number of cases of gender-based violence in the world, and these cases include rape and domestic violence (Onyejekwe, 2004). Usually, men exert power over women through sexuality and women who went through physical and sexual partner violence were reported to be 1.5 times more likely to acquire HIV compared to women who had not experienced violence (García-Moreno, 2013). Intimate partner violence has been shown to inflict fear in women and has imposed difficulties in the uptake of HIV testing and counseling, disclosing HIV status, treatment uptake and adherence (Durevall and Lindskog, 2015, Hatcher et al., 2014). Additionally, violence (physical, emotional, verbal, economic) has been linked to low adherence to Pre-exposure prophylaxis (PrEP) medication, thus disrupting HIV prevention strategies (Roberts et al., 2016).

Younger women are four times more likely to be sexually assaulted than older women (Snyder, 2000) and younger girls who have been victims of rape before the age of 18 are more likely to experience subsequent sexual assaults as adults (Gidycz et al., 2001). Rape carries an increased risk of HIV acquisition compared to consensual sex because it is a forced sexual act and may usually cause genital trauma that increases the risk of HIV infection. In the case of virgins and child rape, the risk of HIV acquisition is heightened because this causes severe injuries in the vaginal epithelium which serve as entry points for HIV (Van der Straten et al., 1995). Sexual abuse of children has been linked to increased risk of engagement in high-risk behavior such as having multiple sexual partners, the use of drugs

and alcohol which leads to risky behavior and increases the chances of HIV acquisition (Dembo et al., 1992, Laga et al., 2001, Pettifor et al., 2004, Stöckl et al., 2013). Women from limited resource settings are greatly affected by violence because they have limited means to secure legal rights including women working as sex workers, who are at a particularly increased risk of physical and sexual abuse (Dearing and Hequembourg, 2014).

1.1.6.3 Lack of access to healthcare

In settings where HIV services are available, they are perceived as primarily for married women or women older than 18 women. Health care providers lack youth-friendly services (Mantell et al., 2009). The Lack of access to reproductive health (SRH) services means that women are not able to exercise their sexual and reproductive health rights (SRHR) (Karim et al., 2010). The Adolescent and Youth Friendly Service (AYFS) approach has been promoted in South Africa by the National Department of Health and partners, as a means of standardizing the quality of adolescent health services in the country. Currently, the facilities have the essential components for general service delivery in place, but adolescent-specific service provision is lacking (Lince-Deroche et al., 2015).

1.1.6.4 Early sexual debut

The early sexual debut has been linked to an increased risk of HIV acquisition (Pettifor et al., 2004). Most biologically based studies have found the risk of contracting HIV through heterosexual intercourse to be higher for women compared to men (UNAIDS, 2018). Physiological and immunological immaturity of the female genital tract (FGT) at the time of first sexual debut increases young women's susceptibility to HIV infection and other sexually transmitted infections (STIs). An immature FGT may lack tolerance to semen exposure, therefore eliciting an immune response that results in the recruitment of cells, including HIV target cells, to the site of semen exposure and thus predisposes women to increased HIV infection risk (Kigozi et al., 2009). In addition, an early sexual debut exposes the woman to an increased HIV infection risk due to an extended sexual activity duration because the early sexual exposure would mean a more extended sexual history in one's lifetime and more prolonged HIV infection risk exposure (Pettifor et al., 2004, Stöckl et al., 2013).

1.1.7 Biological factors that increase the risk of HIV susceptibility in women

While the causes of women's vulnerability to HIV has not yet been fully elucidated, the disproportionate impact of the HIV epidemic on women suggest that other factors beyond behavior may be contributing to the heightened vulnerability in young women. The exposed mucosal surface of the vagina may facilitate HIV acquisition. Further, the high levels of activated immune cells in the female genital tract, and the increased expression of HIV co-receptors in cervical cells compared to foreskin cells may also explain why women have a higher per-sexual act risk of HIV acquisition than men (Boily et al., 2009).

1.1.7.1 Cervical ectopy

The extension of the columnar epithelium from the endocervical canal towards the ectocervix is termed cervical ectopy, and some studies have found this condition to be a risk factor for HIV acquisition (Venkatesh and Cu-Uvin, 2013). The endocervical columnar epithelium is thin from having a single layer of glandular cells in close association with underlying vascular cervical stroma whereas the ectocervix is covered by a stratified squamous epithelium (Jacobson et al., 2000). The ectopic tissue allows easy access to the blood and lymphatic systems. Therefore there is a decrease mucosal barrier integrity that allows for easy access to sexually transmitted infections, including HIV (Collier et al., 1995, Critchlow et al., 1995, Ritter et al., 1988). Cervical ectopy is more common in adolescent girls, pregnant women, and in women on hormonal contraceptives. Hence, Adolescent girls are likely to have larger areas of ectopy or immature epithelium that could increase the chances of acquiring HIV and other STIs (Moscicki et al., 2001). A study by Moench et al. (2001) has found that immature cervical epithelium in healthy young women was associated with cervicovaginal inflammatory and regulatory cytokines and chemokines.

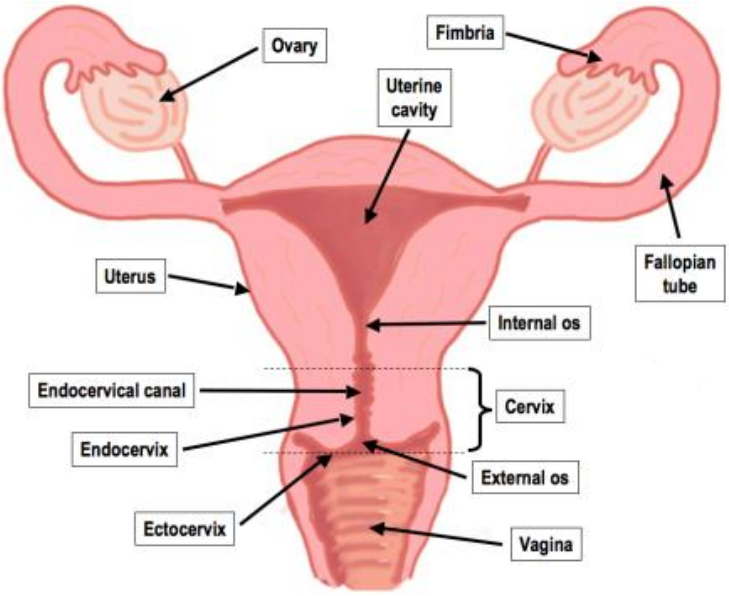

Figure 1. 3. Shows the female reproductive system (Thomas, 2013).

1.1.7.2 Mucosal Surface of the vagina

Although vaginal intercourse carries a lower HIV transmission probability per exposure event (1 in 200 to 1 in 2000) than anal intercourse or parenteral inoculation, it contributes to more HIV incident cases (Hladik and Doncel, 2010). Women mostly engage in heterosexual intercourse which carries a higher risk of HIV infection, because during the intercourse a large inoculum of infected seminal fluid is deposited into the vagina which has a large susceptible surface area and can stay for several hours inside the vagina, thus extending the duration of the HIV infection risk. Heterosexual intercourse can also result in micro-abrasions in the vagina, which can serve as an entry point for the virus to enter the bloodstream (Powers et al., 2008). The female vaginal membrane has higher permeability compared to that of the penis (Baden and Wach, 1998, Yi et al., 2013). Therefore, the risk factors associated with successful heterosexual transmission of HIV infection include: (i) Vaginal epithelial damage as a result of trauma-related abrasions or lesions caused by the presence of STIs (Draughon, 2012); (ii) viral load in plasma and in genital secretions (Morrison et al., 2010, Wilson et al., 2008); (iii) the use of hormonal contraceptives or hormonal changes associated with menstruation which cause the thinning of the genital epithelium (Brawner et al., 2016a, Ngcapu et al., 2015); and (iv) abundance of activated HIV target cells (CD4+ T cells and Langerhans cells expressing CCR5) recruited along an

inflammatory gradient as a result of trauma or sexually transmitted infections (Rancez et al., 2012, Stieh et al., 2014).

1.1.7.3 Inflammation and immune activation in the female genital tract

Female genital tract (FGT) inflammation is the response of FGT tissue to injury or pathogen invasion, this response involves a well-organized cascade of fluid and cellular changes within the tissue. The affected FGT tissue becomes red and swollen due to increased blood flow and due to fluid accumulation, respectively. The changes are a result of vascular response to inflammation, and the response involves changes in vessel capacity and consequently blood flow. Increased blood flow results from the vessel dilation and the hydrostatic pressure increase, which also causes the escape of plasma proteins into the extracellular space due to vascular permeability increase. The retraction of FGT endothelial cells caused by chemical mediators leaves intercellular gaps between the cells. These gaps often lead to unfavorable development of chronic inflammation. Chemotaxis is the process of the movement of leukocytes to the area of inflammation. Monocytes and granulocytes respond to chemotactic factors and move along a concentration gradient. Leukocytes play an important role in the inflammatory response of microbial killing after the immune cell activation. The activation of the immune response due to the presence of pathogens, irritation and tissue damage leads to the release of cytokines, which initiate the acute phase response of inflammation (Haase, 2010). Physical contact between cells and solid particles leads to phagocytosis and the release of antibacterial proteins such as defensins and lysosomes, which affect the bacterial permeability by causing the bacteria to leak to death. The release of the lysosomal products from the cell damages local tissue and can kill microorganisms outside the cell. Inflammation of prolonged duration is termed chronic inflammation and it is characterised by tissue destruction and tissue repair substances found at the same time (Janeway et al., 1996).

1.1.8 Genital inflammation and immune activation's association with increased risk of HIV acquisition.

Vaginal epithelial cell damage or trauma from infections and irritation can cause immune activation in the female genital tract; this reaction can increase the expression of soluble

immune proteins. The presence of immune proteins in the FGT triggers the expression of HIV co-receptors to the cervicovaginal mucosa and thus increasing susceptibility to HIV infection (Fichorova et al., 2001a). Chemokines can be found constitutively or be induced; therefore, they are classified according to their function, i.e., homeostatic and inflammatory, respectively. Inflammatory chemokines MIP-1α, MIP-1β, RANTES, and CCL5 induce the expression of CCR5 receptors within the CD4 and CD8 T cell subsets (Raport et al., 1996).There is little or no expression of CCR5 in the naïve T cell population, but its primarily expressed on memory cells (Sato et al., 2007). Upon T cell activation, the expression of the inflammatory chemokine receptor is upregulated and HIV R5- tropic strains primarily target CCR5 T cells. Therefore, an increased density of target cells in the genital mucosa may contribute to increased HIV susceptibility (Margolis and Shattock, 2006). The presence of dendritic cells in the female genital mucosa can play a role in the HIV acquisition by taking up HIV with lectin-binding receptor DC-SIGN interaction with the HIV gp120 and thus infecting CD4 T cells in trans (McDonald et al., 2003, Turville et al., 2004). Genital inflammation is multi-causal; however, it is still not completely understood, and some of the possible causes are discussed below.

1.1.8.1 Possible causes of immune activation and inflammation in the FGT

I. Sexually transmitted infections (STIs):

Sexually transmitted infections are associated with inflammation, the inflammatory response in the FGT is important for sexual transmitted infection clearance. The genital inflammatory response is detrimental prior to HIV exposure as it increases the likelihood of acquiring HIV infection (Masson et al., 2015, Morrison et al., 2014), through predisposition of HIV co-receptor CCR5. Some studies have established that inflammation is caused by bacterial pathogens such as *Neisseria gonorrhoea*, as well as viral and protozoan infections such as Herpes Simplex Virus (HSV)-2, and *Chlamydia trachomatis* (Chaban et al., 2014, Ghartey et al., 2014). Some STIs are asymptomatic therefore women harbour infections unknowingly resulting in genital inflammation and hence, increased HIV acquisition risk (Mlisana et al., 2012). However, asymptomatic STIs can only account for 20% of the genital inflammation (Serwadda et al., 2003). HIV susceptibility is increased by concurrent sexually transmitted infections (Schellenberg and Plummer, 2012).

Changes in vaginal pH may lead to opportunistic *Staphylococcus aureus* to stimulate the innate immune system in the vaginal epithelial cells through the secretion of exotoxins. A study conducted by Peterson et al. (2005), revealed that *S. aureus* causes a two-fold up-regulation of genes coding chemokines or cytokines. *Candida albicans* is a polymorphic fungus that can be found in the vagina and it forms part of the normal constituents of the microflora and it is the cause of vaginal thrush. *C. albicans* can grow in different physical forms including unicellular yeast, pseaudohyphae, and hyphae. Based on *C. albican's* physical form it can be associated with infection (pseudo, pseaudohyphae, and hyphae) and it can also be associated with harmless colonization (unicellular yeast). Through the interactions between pattern recognition receptors (PRRs) found on the dendritic cell's surface and pathogen-associate molecular patterns (PAMPS) found on the fungal cell wall, the dendritic cells are activated. This interaction results in the activation of different antigen specific immune responses and the recognition of *C. ablicans* is accompanied by the secretion of cytokines that drive the activation and differentiation of the naïve T-lymphocyte into one of the possible T-helper (Th) subsets (Richardson and Moyes, 2015). Schaller et al. (2005) showed that *C. ablican's* strains activated interleukins, tumor necrosis alpha, and gamma interferons that are responsible for tissue damage of vaginal epithelial cells. Some STIs are ulcerative and the disruption of cervico-vaginal epithelial integrity by ulcerative genital diseases such as HSV or *Haemophilusducreyi* may increase HIV-1 acquisition risk by allowing direct lamina propria infection by HIV-1 (Chen, 2012).

II. Changes in vaginal flora

Bacterial Vaginosis (BV) a condition that is characterized by an imbalance in *Lactobacilli* bacteria in the vagina and it is the most common cause of vaginitis and a number of studies have linked BV to increased risk of HIV acquisition by compromising the vaginal epithelial integrity or by triggering the immune activation in the female genital tract through cytokine and chemokine upregulation (Low et al., 2011b, Spear et al., 2007). BV induces an inflammatory response by upregulating the secretion of pro-inflammatory cytokines, namely IL-6, IL-8, TNF-α, and IL-1β and also induces IL-12, IL-23 and p24 secretion and activation markers HLA-DR, CD86, CD83 and CD40 on dendritic cells, therefore, increasing the risk

of acquiring HIV infection (Masson et al., 2015). Hence, BV causes mucosal inflammation by activating the cervico-vaginal environment creating an HIV permissive environment (Thurman et al., 2015).

Aerobic vaginitis (AV) is also caused by an imbalance in the vaginal bacteria i.e. disruption in the lactobacillus- dominated vaginal microbiota. However, AV is characterized by proliferation of enteric aerobic bacterial organisms including *Staphylococcus aureus, Enteroccoci, Escherichia coli,* and group B *streptococcus;* leukocytes and parabasal cell-infiltration, over-inflammation, pH>6, itching and burning, red vaginal mucosal inflammation, dyspareunia, yellowish discharge and by an increased IL-1β and IL-6. AV is present in 2-25% of women and it is associated with STIs such as *N. gonorrhoea, T. vaginalis,* and *C. trachomatis,* which results in ascending genital inflammation and infection (Kaambo et al., 2018).

III. Exposure to seminal proteins

Greater fluctuations in vaginal flora are associated with frequent intercourse (Schwebke et al., 1999b). In the FGT the recruitment and activation of dendritic cells (DC), granulocytes, macrophages, and $CD4^+$ T cells results from the chemokine/cytokine expression observed after sexual intercourse and may predispose women to the elevated risk of HIV acquisition (O'leary et al., 2004, Prakash et al., 2003). Gutsche et al. (2003) showed higher expression of IL-1β, IL-6, and Leukin inhibitory factor (LIF) in epithelial cells when cultured with seminal plasma. A study conducted by Sutherland et al. (2012) has revealed that the presence of seminal plasma in the cervical adenocarcinoma cells induced the expression of inflammatory COX-1 and COX-2, IL-6, IL-11, and chemokines CXCL1 and CXCL8. Seminal plasma interacts with human cervical and vaginal tissues to induce pro-inflammatory cytokine production required for immune tolerance during conception (Sharkey et al., 2007).

IV. Hormones

Hormonal cycling, hormonal contraception use alter cervical immunity in different ways known to increase the risk of HIV acquisition through increased levels of pro-inflammatory

cytokines (Morrison et al., 2014). Reduced epithelial glycogen in the vagina decreases the lactic acid production resulting in the loss of its anti-inflammatory activities, therefore, resulting in a dysbiotic vaginal flora which is associated with inflammation (Wrenn et al., 1968). Progesterone may cause adverse effects on the female genital tract resulting in an increased risk of HIV infection. Wira et al. (2015) showed that the mucosal barrier is compromised during the high progesterone luteal phase. Reduced estrogen levels after menopause can results in inflammation in the vagina. Depot medroxyprogesterone acetate (DMPA) is a risk factor for HIV acquisition through inflammation (Byrne et al., 2016, Murphy et al., 2015).

V. Vaginal trauma and sexual trauma

The vagina experiences both anatomic and physiological changes during sexual intercourse. Mucosal micro-abrasions and laceration have been observed in several women for up to 80 hours after coitus, and this mucosal damage leads to inflammation which increases the risk of acquiring HIV (Astrup et al., 2012). The presence of cervico-vaginal lymphocytes which could be activated in response to injury increases the susceptibility to infection and dysregulation of epithelial tight junctions in the female genital tract, resulting in increased access for pathogens and increased permeability (Brawner et al., 2016b, Porter et al., 2016). Inflammation can result in increased blood flow and elevated capillary pressure which increases frictional tension in the vagina (Berman and Bassuk, 2002). Sexual assaults result in vaginal wounds which are damaged mucosal surfaces of the vagina, which are estimated to occur in 22-90% of all sexual assaults (Draughon, 2012). Wound healing consists of the sequential release of cytokines, chemokines, proteases and growth factors by platelet, macrophages, neutrophils, and fibroblasts (Shah et al., 2012). The process of wound healing starts with inflammation that occurs as a result of sexual assaults and followed by the repair process which releases specific immune factors that that control inflammation and recruit specific cells for repairing the mucosa. Immune mediator's improper/ untimely expression can result in delayed wound healing and increased susceptibility to pathogens such as HIV (Werner and Grose, 2003).

VI. Introduction of foreign matter into the vagina

Introduction of foreign matter into the vagina is done for different reasons with the common reason being an adjustment to the labia, clitoris, vagina, or the vaginal environment. These acts are referred to as vaginal practices, and they include heating of the vulvar area, external washing, douching, external product application, intra-vaginal insertion, physical modifications, and oral ingestion (Scorgie et al., 2009). Some studies have looked at the biological trauma in the natural structure of the vagina and have found that microabrasions, lesions, and inflammation are caused by vaginal products introduced into the vagina (Brotman et al., 2008, Fashemi et al., 2013, Low et al., 2011b).

1.2 Literature review

1.2.1 Vaginal practices

Vaginal practices involves introduction of foreign matter into the vagina and a wide range of products is typically used for this practice, including personal hygiene products, a variety of medicines, commercially available products and other environmentally sourced items such as leaves, herbs and powdered stone (Allen et al., 2010, Hawes et al., 1996). Vaginal products are mainly used for sexual satisfaction of male partners who desire a warm, tight, and dry vagina, for increased sexual pleasure (Gafos et al., 2010). In some cases, intra-vaginal practices may also be used as part of treatment for vaginal discharge, suspected STIs, and for hygiene purposes (Gresenguet et al., 1997). Vaginal practices are alleged to be linked to adverse health outcomes and increased susceptibility to BV and transmission of STIs including HIV, through disruption of genital mucosa, change in vaginal flora and by altering vaginal pH (Hilber et al., 2007, Myer et al., 2006). The association between increased HIV susceptibility and vaginal practices is possible but varying in epidemiologic studies (Low et al., 2011a). With very little knowledge about the impact of vaginal practices on the vaginal immune microenvironment, the understanding biological mechanism by which intra-vaginal products may influence women's susceptibility to HIV is now critical to ensure the effectiveness of new HIV prevention methods.

1.2.1.1 Epidemiology of vaginal practices and different types of vaginal practices

There are many different types of vaginal products, which are used for different reasons. Vaginal practices prevalence is higher in places where male partners have a strong preference for dry sex (Beksinska et al., 1999). Women with multiple sexual partners, including female sex workers, are reported to frequently indulge in these practices (Scorgie et al., 2009). A number of factors cause women to use vaginal practices such as (i) vaginal cleansing, (ii) treatment of sexually transmitted infections (STIs) or vaginal discharge, (iii) prevention of pregnancy or to induce abortion, (iv) enhancement of sexual pleasure and (v) as a 'love potion' to attract or retain partners (Hilber et al., 2007). In SSA, the prevalence of vaginal practices ranges from 6 to 98%, and female sex workers reported the highest prevalence (Allen et al., 2010, Fonck et al., 2001). There are six distinct types of vaginal

practices which have been previously identified: (i) external washing with or without products (ii) external application of products around the vulva, (iii) anatomical modification, (iv) intravaginal cleansing with or without products, (v) intravaginal insertion of products and (vi) oral ingestion of specific product to alter the vaginal environment (Hutchinson et al., 2007, Myer et al., 2006).

1.2.1.2 Commonly used products in vaginal practices

A variety of products are used in vaginal practices, and these products range from pharmaceutical, household, and traditional products. Several studies have documented the use of leaves and herbs (including tobacco) in vaginal practices, whereas other studies have documented detergent and antiseptic use. For this thesis, products previously identified as commonly used in the KZN province will be described. Some of the commonly used products in South Africa were discovered in Vulindlela, KZN, which is one of the highest burden HIV district in South Africa. Eleven group discussions ("boot camps") were conducted between 2013 -2015 with 495 African females aged 13-24, and three targeted focus group discussions (FGDs) with 24 African females aged 16-25 (Humphries et al., 2018).Products include Kuber chewing tobacco which is applied into the vagina for drying purposes; it contains ~20% nicotine, cannabidiol and delta 9-tetra cannabinol (THC); the primary ingredient is Indian hemp/marijuana). Alum (aluminium sulfate) is a colorless astringent compound, which is inserted vaginally for douching; also a common adjuvant used in vaccine preparations, known to stimulate the toll-like receptor (TLR) 7 inflammatory pathway and activate NF-Kb. Rose water (flavored water made from steeping rose petals into the water, used as a vaginal douche).Snuff (chewing tobacco; which is made from ground or pulverised tobacco leaves and contains nicotine, it is inserted vaginally as a sexual stimulant and a tightening agent). Table 1.1 summarises a list of vaginal products commonly used in KwaZulu-Natal, these products include Alum (Gafos et al., 2010), tobacco (Ghys et al., 2001) antiseptic liquids (McClelland et al., 2006, Myer et al., 2006) and rose water (Humphries et al., 2018).

Table 1. 1. Details of the commonly used intravaginal inserted products (IVIPs)

IVIPs	Ingredients	Commercial uses	Vaginal uses	References
Alum	Potassium Aluminium sulfate ($KAl(SO_4)_2$)	treatment of mouth ulcers, water purification, food preservative	douching/ tighten	(Gafos et al., 2010, Humphries et al., 2018)
Snuff	Tobacco, ammonium carbonate, salt, preservatives	smoking and chewing	sexual stimulant/ tightening	(Humphries et al., 2018)
Kuber	Scented Khaini leafy tobacco	air freshener, chewing	libido-enhancing /Drying	(Humphries et al., 2018, Waxman et al., 2016)
Rose water	Distilled water with rose flavouring	cooking, skin toner	treating vaginal odour	(Humphries et al., 2018)
Savlon	Chlorhexidine gluconate, cetrimide, n-propyl alcohol benzyl benzoate	antiseptic liquid	douching	(McClelland et al., 2006, Myer et al., 2006)
Household products	Lemon juice, vinegar, OMO	Consumption, Detergents	cleansing	(Hilber et al., 2010, Low et al., 2011a)

1.2.2 Definition of a healthy female genital tract suitable for prevention of pathogenic microbes

The genital mucosa is protected by multiple barriers such as mechanical barriers (tight junctions and mucosal epithelia) and chemical barriers (mucus, antimicrobial factors, and innate immune cells), together these barriers resist the invasion of the genital mucosa by

pathogens (Langbein et al., 2002, Marchiando et al., 2010). Intercellular spaces between epithelial cells are sealed by intact, tight junctions, therefore preventing paracellular penetration by viruses and pathogens. The multi-layered vaginal mucosal epithelia are stratified and supported by the fibrous connective tissue, and the lamina propria and epithelial cells provide immune functions by producing cytokines and antimicrobial factors that repel and kill microbial antigens (Sotolongo et al., 2012), whereas the endocervical mucosa is a monolayered epithelium. The disruption of tight epithelial junctions leads to the opening of paracellular spaces in between the epithelial cells and therefore leading to paracellular penetration by pathogens and viruses. This disruption is also associated with the upregulation of pro-inflammatory cytokines, thus increasing the acquisition risk of HIV.

A healthy vagina is naturally acidic and has a pH below 4.5 (Ravel et al., 2011a). Vaginal epithelial cells metabolise glycogen to produce glucose under the estrogen influence, and after that, *lactobacilli* convert glucose to lactic acid (Hillier et al., 1992). Some *lactobacilli* species, particularly *L. crispatus* and *L. jensenii* can produce hydrogen peroxide (H_2O_2), which is toxic to many pathogenic microbes. However, it favors the growth of *Lactobacillus* species. Therefore, a healthy female genital environment is also dominated by *Lactobacilli* species. The capacity of lactobacilli to adhere and compete for adhesion sites in the vaginal epithelium and also to produce antimicrobial compounds (hydrogen peroxide, lactic acid, bacteriocin-like substances), is important in the impairment of colonization by pathogens such as *E. coli, Candida* species, *Gardnerella vaginalis* and *Mobiluncus* species (Borges et al., 2014). However, some studies have found that a lactobacilli dominated microbiome is likely found in European women whereas in African women a diverse microbial profile is displayed and therefore a healthy vaginal definition is not straightforward as ethnicity seem to be influencing the composition of vaginal microbiome (Ravel et al., 2011b, Zhou et al., 2010, Zhou et al., 2009).

An association between inadequate vaginal *lactobacilli* and the development of several STIs, including HIV, has been reported (Petrova et al., 2015, Petrova et al., 2013). An unhealthy vagina typically has an alkaline pH, higher numbers of *Gardnerella vaginalis*, *Mycoplasma hominis, Prevotella, Peptostreptococcus, Mobiluncus,* Bacteroides *Atopobium vaginae* and

Megaptera species (Borges et al., 2014). Bacterial Vaginosis is defined by the disequilibrium in the vaginal microbiota, with a decline in the number of lactobacilli species, increased pH, and inflammation. An illustration comparing a healthy vagina versus a vagina with BV is shown in Figure 1.4.

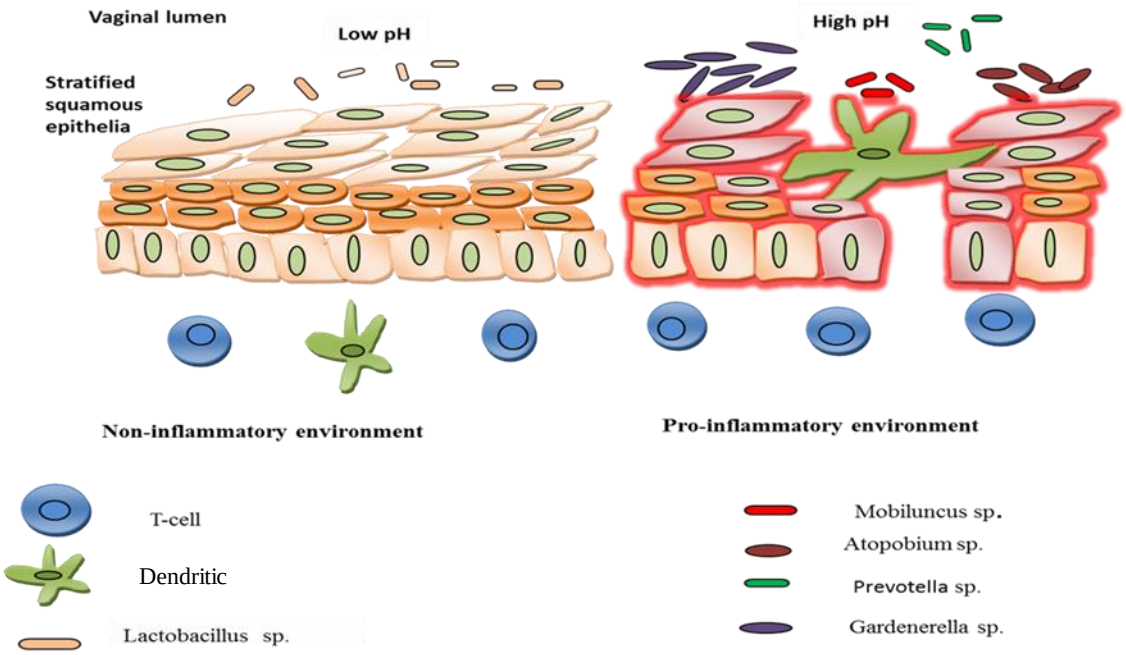

Figure 1. 4. Shows a comparison of a healthy vagina versus a vagina with BV. A healthy vagina is defined by an intact squamous epithelium, low pH and dominance of *Lactobacilli* species which produces lactic acid that inhibits the growth of anaerobic bacteria and helps maintain a low vaginal pH (< 4.5) which generates a non-inflammatory environment resulting into an eubiosis state. An unhealthy vagina can have high vaginal pH (>4.5), an environment that allows the growth of anaerobic bacteria leading to a pro-inflammatory and a dysbiotic environment that favours BV.

1.2.3 Impact of vaginal products on vaginal health

1.2.3.1 The impact of altering vaginal pH

Some vaginal practices such as douching can potentially wash away good bacteria in the vagina as some of the products are antibacterial and therefore harsh for the vaginal environment resulting in the elimination of bacterial species in the vagina even the good

bacteria such as lactobacilli. Several vaginal products contain ingredients that are potentially harmful to the mucosal barrier of the FGT and may potentially influence susceptibility to HIV or STI infection by altering normal vaginal pH, which is typically acidic at pH 3.8- 4.5 (O'Hanlon et al., 2013).

A vaginal pH of less than 3.5 might be considered too acidic for the vagina as this pH might cause vaginal tissue disruption, which can lead to inflammation. Inflammation has been associated with increased risk of HIV infection acquisition, through pro-inflammatory factors such as IL-8 and MIP3-α which are associated with recruitment and activation of HIV target cells into the genital mucosa (Arnold et al., 2016). Whereas an elevated vaginal pH shifts the vaginal environment in favor of pathogenic microbiome such as *Gardnerella vaginalis*, where its overgrowth leads to the formation of an infected biofilm which then accommodates numerous gram-negative anaerobes (Hardy et al., 2015).Hence, BV, which has been associated with more than the three-fold increased risk of acquiring HIV infection (Cohen et al., 2012). The effect of vaginal insertion products on vaginal pH requires further investigation.

1.2.3.2 Disruption of genital mucosa by vaginal products, cellular activation, inflammation and risk to HIV infection

Some vaginal products used for the dry sex effect can disrupt the genital mucosa therefore leading to easy penetration of pathogens to the immune cells, which can lead to an increased risk of HIV acquisition (Hilber et al., 2010, Low et al., 2011b). The act of unlubricated sexual intercourse is termed "dry sex," and women often engage in this type of sexual intercourse for the benefit of their male partners who desire tight and dry vaginas for increased sexual pleasure (Gafos et al., 2010). To achieve this "dry sex state," women use a range of products such as environmental sourced herbs, powdered stone, house cleaning detergents, cloths, and commercially available products. However, the perception of dry sex may cause intense friction which is associated with micro-abrasions in the vaginal epithelium leading to a broken mucosal barrier that allows for pathogen penetration leading to the recruitment of pro-inflammatory cytokines and an inflamed site thus increasing HIV acquisition risk (Masson et al., 2015). Vaginal products used to achieve dry sex state may also decrease or

eliminate protective bacterial species responsible for pathogen eradication in the vagina (Martino and Vermund, 2002).

1.2.3.3 The effect of vaginal products on the vaginal microbiome

The impact of vaginal insertion products on the vaginal microbiome is unknown. However, intravaginal practices are associated with the disturbance of vaginal flora which is termed vaginal dysbiosis (Brotman et al., 2008, McClelland et al., 2006). Some studies have found an association between BV and vaginal practices (Brotman et al., 2008, McClelland et al., 2006). However, Rugpao et al. (2008) found no correlation between cleaning inside the vagina and developing BV. Vaginal practices have not only been linked to vaginal dysbiosis, but also other STIs. A study by Tsai et al. (2009) demonstrated that the chances of acquiring STIs were 1.8 times greater for participants who douched regularly compared to participants who never douched or those who douched occasionally. On the other hand, Ness et al. (2005) found that irregular douching was not associated with gonococcal and chlamydial infections. Intravaginal practices have also been linked to the risk of HIV infection (Ghys et al., 2001, McClelland et al., 2006, Myer et al., 2006, Van de Wijgert et al., 2000). However, Myer et al. (2006) found no correlation between HIV acquisition and vaginal practices. There is a gap in the literature about the mucosal activation following exposure to intravaginal insertion products and sexual activity. Several studies have suggested a link between IVIPs and increased susceptibility to HIV infection (Low et al., 2011b). However, other studies have not found any association. While some cohort studies have demonstrated significant associations between IVIPs and HIV risk (Brotman et al., 2008, Fashemi et al., 2013, Low et al., 2011b), the mechanism by which IVIPs impact vaginal microenvironment have been speculated in a few studies (Brotman et al., 2008, Fashemi et al., 2013, Fichorova et al., 2001b, Francis et al., 2016). However, the actual mechanisms in which IVIPs impact the mucosal microenvironment are not known and the direct causal pathway that links IVIPs with HIV acquisition has not yet been demonstrated. A summary of the postulated effects of vaginal insertion products on the vaginal environment and health is illustrated in the figure below (Figure 1.5)

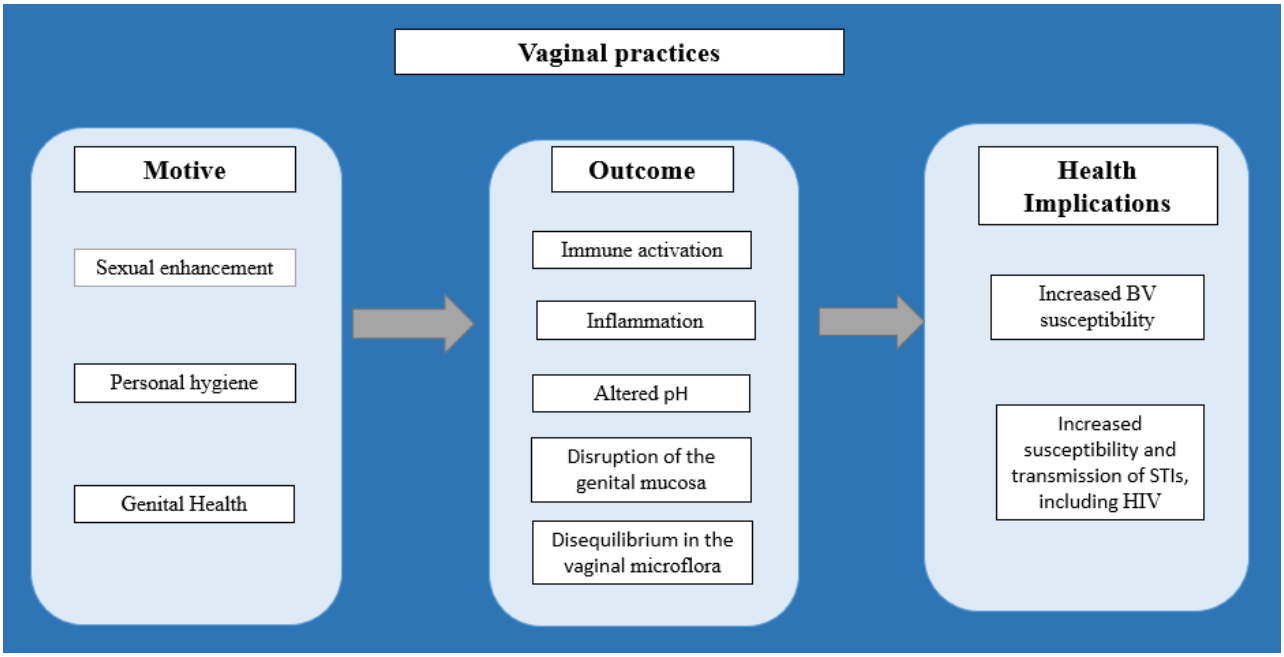

Figure 1. 5. Shows a conceptual diagram of the causal pathway of vaginal practices, BV, STIs and HIV susceptibility. Young women insert products into their vaginas mainly for sexual enhancement, treatment of STIs and personal hygiene; this is suspected of leading to vaginal dysbiosis, vaginal trauma and altered pH which may cause BV and increased risk to HIV and STI infections

1.2.4 Immunologic and microbiological events associated with mucosal trauma

1.2.4.1 Sexual trauma

Some studies have shown that there is a short-term activation of the mucosal immune system that is associated with sexual intercourse where deposition of seminal fluid in the FGT has been linked to pro-inflammatory cytokine and chemokines expression (Denison et al., 1999, Sharkey et al., 2007). The female reproductive tract microbiota is affected by sexual intercourse; greater fluctuation in the vaginal flora is associated with frequent intercourse (Schwebke et al., 1999a).

1.3 Rationale of the study

Globally, adolescent females are the highest risk group for HIV infection, and vaginal trauma potentially increases this risk. The lower reproductive tract of adolescents soon after sexual debut may represent a naive reactive state which lacks tolerance to seminal fluid, combined with delayed response to pathogens. In this naïve age group of young women, early sexual debut and the use of traditional or commercial vaginal inserted products may compound the risk of mucosal trauma which may lead to a higher risk of HIV and other infections. This study takes advantage of the information on vaginal products already provided by young women living in rural KZN where HIV prevalence is high. It seeks to explore immune responsiveness to IVIPs using a PBMC model. Alum and snuff were found to be the products that were most commonly used by young women, and these two products were taken further to investigate how these IVIPs effect on immune cellular activation and inflammation. The ultimate goal is to inform clinical trials and advice adolescent females in developing countries on how they can effectively prevent their risk of HIV infection.

1.4 Study aims and objectives

Aim:

To investigate the *in vitro* effects of IVIPs exposure on the biomarkers of inflammation and cellular activation.

Objectives:

1. To identify the pH of IVIPs (Snuff, kuber, alum, rose water and savlon) and to evaluate the cytotoxicity of the IVIPs by determining their effect on cell viability at different dilutions.
2. To look at the effect of IVIPs (Snuff and alum) on immune cellular activation and inflammation using PBMCs *in vitro*.

Hypothesis:

IVIPs will lead to cellular immune activation, with subsequently increased inflammation, thereby elevating the risk of HIV-1 infection.

CHAPTER 2: MATERIALS AND METHODS

2.1 Laboratory safety conditions and procedures

All experiments were carried out inside the biosafety class II hood with appropriate clothing and precautions. Table 2.1 shows the general laboratory reagents used to carry out the experimentation prepared as recommended. RPMI-1640 media (Invitrogen) was extensively used in this project and it was developed at Roswell Park Memorial Institute; it is suitable for long term culture of peripheral blood lymphocytes. This media has a pH 8 formulation and able to supports a wide variety of cells in suspension if adequately supplemented with serum, 10% heat-inactivated foetal bovine serum (FBS, Sigma Aldrich) was added, heated to 56°C for 30 minutes to inactivate complement proteins. FBS facilitates cell survival and proliferation, and it also increases the viscosity of the media and thus protects the cells from mechanical damages during agitation of suspension culture and 6% penicillin/streptomycin (Sigma Aldrich) was added to prevent bacterial and fungal contamination of cells.

Table 2. 1. General laboratory reagents

Reagent	**Composition**	**Storage condition**	**Supplier**
R10	RPMI 1640 MEDIUM, 10% FBS, 6%penicillin/streptomycin	4ºC	Prepared in the laboratory
Foetal Bovine Serum FBS	Blood from fetal calves	-20 ºC	Invitrogen
Phosphate Buffered Saline	0.138M NaCl,0.0027M KCI (pH 7.2)	RT	Invitrogen
Trypan blue	$C_{34}H_{28}N_6O_{14}S_4$	15-30 ºC	Lonza- Bioscience
Distilled De-ionized water	No impurities and minerals	RT	Supplied by the laboratory

2.2 Ethical approval

The study was conducted at CAPRISA (Centre for the AIDS Programme of Research in South Africa) located in Durban, KwaZulu-Natal province, South Africa. Twenty-six black African female donors from the urban part of Durban, who are aged between 18 to 30 years old and healthy with no known chronic sicknesses donated blood. The eligibility criteria were HIV negative status, not pregnant, did not use IVIPs and their marital status was either married or single. Women with unknown HIV status, HIV infected individuals, pregnant, breastfeeding and taking medication for chronic sicknesses were excluded from the study. All participants agreed to sign an informed consent before the commencement of the study. Ethical approval for the study was granted by the University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC), ref no: BE382/16.

2.3 Selection and preparation of intravaginal insertion products

Information about some commonly used intravaginal insertion products were discovered at Vulindlela Research clinic in Mafakatini, Pietermaritzburg, South Africa through Eleven group discussions ("boot camps"). The group discussions were conducted from 2013-2015 with 495 African females aged 13-24 (Humphries et al., 2019). These products included alum (Potassium aluminium sulfate $KAl(SO_4)_2$), rose water (distilled water with rose flavouring), savlon (chlorhexidine gluconate, cetrimide, n-propyl alcohol benzyl benzoate), kuber (Scented Khaini leafy tobacco), and snuff (tobacco, an ammonium carbonate salt, preservatives). These products were purchased from street vendors and local shops in Durban CBD. Figure 2.1 below shows the format in which selected IVIPs are purchased.

Figure 2. 1. Shows the photographs of all IVIPs selected in the format they are mostly purchased. Kuber, Alum, and Snuff are solids; Savlon and Rosewater are liquids.

2.3.1 Intravaginal insertion products (IVIPs) processing

All products were kept at room temperature before use. Solid products of the same kind from different containers or packets were transferred into sterile zip bags and mixed to ensure uniformity. The concentrations of IVIPs used by women are not known and they vary between individuals. Stock solutions of snuff and kuber products were prepared as described in Harris et al. (2012) at a concentration of 450 mg/ml, by weighing 18g and adding 40ml of phosphate-buffered saline (PBS). Similarly, alum stock solution was also prepared at a concentration of 450 mg/ml to retain dilution ratio of the solid IVIP. In order to completely dissolve alum salt, the mixture had to be heated into a water bath at 65ºC for 10-15 minutes with constant agitation. Aluminium sulfate and water mixture forms aluminium hydroxide. Mixtures were homogenised using a vortex mixer and left at room temperature overnight to allow for maximum extraction and filtered using the 0.22µm corning bottle-top vacuum filter

systems (Sigma Aldrich). Savlon and rose water were used directly for experimentation as they were in liquid form with an unknown concentration of each product; however, sterile distilled water (provided by the laboratory) was used as a control for osmotic pressure changes in rose water. Table2.2 shows how IVIP dilutions were prepared.

Table 2. 2. Shows how IVIPs dilutions were prepared.

Products	**Dilution factor**	**Volume of stock solution (450mg/ml) or Volume of neat liquid product (µl)**	**Volume of R10 media (µl)**	**Product concentration**
Solids: (Snuff, kuber and Alum)	1	5000	0	450mg/ml
	1/5	1000	4000	90 mg/ml
	1/10	500	4500	45 mg/ml
	1/100	50	4950	4.5 mg/ml
	1/1000	5	4995	0.45 mg/ml
Liquids: (Savlon and Rosewater)	1	5000	0	100%
	1/5	1000	4000	20%
	1/10	500	4500	10%
	1/100	50	4950	1%
	1/1000	5	4995	0.1%

2.3.2 pH measurement of IVIPs at different concentrations

pH used to measure the concentration of hydrogen ions H+ in a solution and therefore measure whether the solution is acidic or basic. The pH scale ranges from 0 to14, 7 = neutral, <7is acidic and >7 is basic. The lower female genital tract is a very pH-sensitive part of the body. The healthy vagina is acidic with a pH below 4.5. This acidic pH keeps the vaginal environment free from bacterial pathogens as it is microbicidal. Therefore, it's crucial to measure the pH of the different IVIPs selected, to determine the effect they might impose on the vaginal pH

2.3.2.1 Calibration of a pH meter

The pH meter was calibrated daily before use. It was switched on for at least 30 minutes before use to allow adequate time for the pH meter to warm up. The electrode was taken out of its storage solution and rinsed with reverse osmosis water (RO) under an empty waste beaker. The electrode was gently blotted dry with a paper towel. Three buffers that were pre-warmed to room temperature were used for calibration. The electrode was immersed into the first buffer with a pH of 7; the electrode was left in the buffer until the reading was stable then rinsed with RO water. The electrode was then immersed into the second buffer with a pH of 4 and left in the buffer until the reading was stable and rinsed with RO water. Then the electrode was immersed into the third buffer with a pH of 10 and left in the buffer until the reading was stable, and it was rinsed with the RO water. The pH meter calibration status was checked, and if the calibration passed, then proceeded to sample pH measurement.

2.3.2.2 Measurement of pH in selected vaginal products

Five millilitres of IVIPs (Snuff, Alum, kuber, rose water and savlon) at different dilutions (stock,1/5,1/10,1/100 and 1/1000) were added into labelled falcon 15ml conical centrifuge tubes and pH was measured using a benchtop pH meter (Mettler-Toledo AG). The pH meter's glass electrode was immersed into each dilution for each IVIPs until the reading was stable, and it was recorded. The glass electrode in the pH meter probe was rinsed with RO water in between different dilutions and also in between different IVIPs.

2.4 Peripheral Blood Mononuclear Cell (PBMC) processing

PBMCs are blood cells that can give selective immune system responses, and they are the major cells in the immunity of the body (Pourahmad and Salimi, 2015). Therefore, they are suitable cell types for characterisation of lymphocytes. The most common method for isolating PBMCs from anticoagulant- treated blood specimens is the density gradient centrifugation (Bøyum, 1983); this centrifugation uses a density gradient media such as histopaque medium. This medium can separate different cell populations through the exhibition of unique migration patterns based on cell density, from whole blood each cell

population creates a distinct layer resulting into four distinct layers (erythrocytes, granulocytes, PBMCs and Plasma) then the PBMCs layer can be isolated.

Blood samples were collected from healthy female donors (to rule out the donors' immunological histories that could influence heterogeneous responses) by venepuncture into sterile glass blood collection tubes with acid citrate dextrose (ACD, BD Biosciences). ACD is an anti-coagulant for whole blood. Blood samples were processed within 4 hours of collection. The Ficoll –density gradient centrifugation was used to obtain the PBMC buffy layer. Freshly isolated PBMCs were isolated with reagents described in Table 2.3. To improve cell viability, freshly isolated PBMCs were used.

Table 2. 3. Reagents used for PBMC isolation.

Reagent	**Composition**	**Storage condition**	**Supplier**
Histopaque- 1077	endotoxin tested solution of polysucrose and sodium diatrizoate, adjusted to a density of 1.077g/mL	2-8ºC	Sigma Aldrich
1XPBS with 2% FBS	PBS + 2% of FBS	4ºC	Prepared in the laboratory
FACS wash	PBS + 1% of FBS	4ºC	Prepared in the laboratory

2.4.1 Peripheral Blood Mononuclear Cell (PBMC) isolation

Peripheral Blood Mononuclear Cells (PBMCs) were isolated using Histopaque-1077 medium (Sigma-Aldrich), which enables quick recovery of viable lymphocytes from blood and separates cells by density gradient centrifugation. Two ACD tubes of blood per donor were spun at 2290 x g for 10 min, and the top layer plasma was discarded. The remaining blood was diluted with 1xPBS-2% FBS at RT. Diluted blood was then gently mixed by pipetting up and down, and the cells were layered over 12ml of histopaque in a 50 ml leucosep tubes (Sigma Aldrich). Cells were spun down at 1753 x g for 30 minutes with no

brake. The sample separated after the histopaque spin into four layers the plasma at the top, followed by the PBMC interface layer just below this layer there was a histopaque layer containing granulocytes and the bottom layer were the erythrocytes. Erythrocytes (red blood cells) aggregate together, and these dense cell clusters sediment on the very bottom of the tube. Granulocytes are located in the histopaque medium layer, directly above the erythrocytes. PBMC layer was found in-between the plasma and the histopaque layer; it was white buffy interphase and was collected without disturbing the red blood cells at the bottom of the tube. Cells were transferred into a new 50mlleucosep tube and washed with 40ml 1XPBS-2%FBS at RT. Cells were spun at 2290 x g for 10 minutes, and the supernatant was discarded. Cells were re-suspended and mixed with 10 ml of R10 growth media.

2.4.2 Cell counting using trypan blue exclusion method

To obtain the desired number of cells, it was crucial to first count the total number of live cells from the 10ml PBMC/media suspension. Trypan blue staining technique was used to count the number of viable mononuclear cells. This staining technique was used to distinguish between viable and dead cells selectively. Viable cells have an intact cell membrane, and the dye cannot transverse through the membrane (Strober, 2015), viable cells appeared colourless. In contrast, dead cells do not have an intact cell membrane. Therefore, the stain transverses the membrane, and the cells were stained blue. In each counting chamber, two squares were selected from the grid and were used to count the average number of cells using the formula provided below (the counting formula was adapted from (abcam.com).

A volume of 50µl of the cell suspension was mixed with 50µl of trypan blue (WhiteSci) in a 96 well plate and cells were rested in the dye for 2 minutes to allow for optimal staining. 10µl of the solution was transferred into the counting chambers of Fastread slides (Davis Diagnostic Pty Ltd.) and viewed under a light microscope at 400× magnification. Two squares were counted in the grid and used to count the average number of cells. Each square represents a total volume of 10^4 cm^3, and 1 cm^3 is equivalent to 1 ml. Therefore, the total number of cells was determined using the following calculation: concentration (cells per ml) = average number of cells x 10^4 x 2 (dilution factor).

2.5 PBMC exposure to IVIPs

2.5.1 Viability testing

It was previously reported that 80% of the viability of PBMCs is recommended before starting experimentation (Glisic-Milosavljevic et al., 2005), therefore it was crucial to determine the optimal times of exposure and optimal dilution that would give the optimum viability. When PBMCs were exposed to IVIPs for a longer period >3hours it led to poor cell viability, however, at 3hours of IVIP exposure the cell viability improved, therefore 3hours was chosen as the optimal time for the exposure of IVIPs. One million PBMC suspension was added into round bottom falcon tubes (Becton Dickson) in order to have enough cells to acquire at least 50 000 events from the lymphocyte population and also to compensate for cell loss during fixation. PBMCs were exposed to IVIPs at different dilutions (stock, 1in 5, 1in10, 1in100 and 1in1000) including distilled water which was used as a control for osmotic pressure changes in rose water. Each IVIP exposure were done in triplicates per dilution.

The 1in1000 dilution was the only dilution that had a cell viability of ≥60% from all the products used, and therefore it was found to be the optimal dilution for all IVIPs used. Thereafter the optimal IVIP dilution was used for PBMCs exposure to evaluate the impact of IVIPs on cell activation and inflammation. Snuff and alum were selected to be representative products due to financial constraints. Thereafter PBMCs were exposed to the optimal dilution of snuff and alum. One million cell suspension was added into round bottom falcon tubes (Becton Dickson). Cells were spun at 2290 x g for 10min, the supernatant was decanted, and the tubes flicked to re-suspend the cells,1000µl of the (1 in 1000 dilution of Snuff and Alum) were added to the labelled tubes and incubated for 3hours at 37ºC in the presence of 5% CO2.

2.5.2 Experiment controls

R10 (RPMI 1640, 10% FBS, 6% penicillin/streptomycin) media was used as a negative control, which served as a comparison for IVIP treated cells. PHA (Phytohaemagglutinin, Sigma) was used as a positive control to ensure that the assays worked and that the cells were functional. PHA is extracted from a plant called Phaseolusvulgaris that contains potent cell-agglutinating and mitogenic properties. It binds a variety of glycoproteins on the surface of T cells and binds the T cell antigen receptor. The monoclonal antibodies in the T cell antigen receptor can trigger T cell activation. PHA is a multivalent lectin. Therefore, it does not only bind T cell surface glycoproteins, but it also cross-links these cell antigens with other T cell surface molecules, therefore, generating an activation signal (Karmańska et al., 1996).1000µl of the R10 media and 10 µg/ml of PHA were added to the labelled round bottom falcon tubes (Becton Dickson) containing $1x10^6$ cells suspension. As in IVIP treated cells, cells were stimulated for 3 hours at 37ºC in the presence of 5% CO2.

2.6 Investigation of cellular immune activation using flow cytometry

The aim of this test was to evaluate the activation status of $CD4^+$ and $CD8^+$ T cells by $CD38^+$ and HLA-DR^+ activation markers after exposure to IVIPs, T Cell-mediated immune responses depend directly on the interaction between T-lymphocytes and the cells bearing the antigen. Flow cytometry uses fluorescence to measure the physical and chemical characteristics of cells or other particles such as latex beads etc. Fluorescent dyes can intercalate cellular components such as DNA or RNA. A fluorescently labelled cell in a liquid suspension when passed through a laser becomes excited and moves from its resting state to an excited electronic singlet state, and upon returning to its resting state it emits light at a higher wavelength, and the duration of this process is quite rapid, and it lasts for 1-10 nanoseconds (Brown and Wittwer, 2000). A fluorochrome (fluorescent label) when bound to a cell component, the fluorescent intensity will represent the amount of that cell component. The amount of energy required is different for each fluorophore (fluorescent marker). The use of multiple fluorochromes allows detection of several cell properties to be measured simultaneously and from a population of fluorescently labelled cells a flow

cytometer can sort cells into negative and positive cells of interest and the cells of interest can be further analysed based on the conjugated antibodies (Brown and Wittwer, 2000).

2.6.1 Configuration of the flow cytometer

When designing a flow panel, it is very crucial to have knowledge of the flow cytometer to be used. To know how many markers can be included in the panel, it becomes crucial to know the configuration of the cytometer and how many lasers the instrument has. The BD LSRII Fortessa flow cytometer (BD Bioscience) used has three lasers (blue, violet and red) and 12 channels available as shown in figure 2.2.

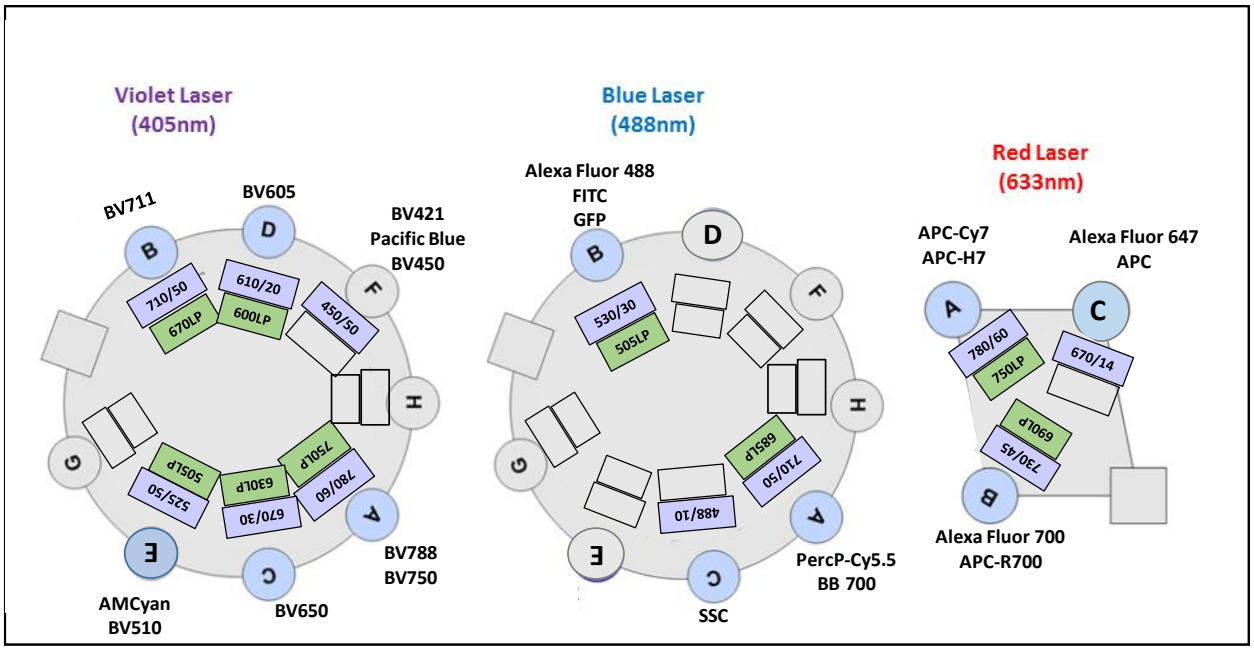

Figure 2. 2. Shows the configuration of LSRII flow cytometer channels and lasers (BD Biosciences)

A 7-colour flow panel was used to identify T lymphocyte activation. The unwanted cells were identified in the dump channel (Pacific blue), a dump channel was essential for placing CD19 because it identifies B cells that are not of interest. A viability dye (live/ dead fixable violet dye) conjugated to Pacific blue was essential for reducing the background noise that could result from the uptake of antibodies by dead cells that bind non-specifically. Therefore, the dump channel Pacific blue served to clean up the analysis by removing contamination in

the downstream analysis. T cell lymphocytes can recognise antigens because they are tolerant of self-molecules and highly sensitive to non-self-molecules. Studying how T cell activates immune responses is essential for understanding immune regulation disorders (Wan, 2010).Live T cells ($CD3^+$) were identified by the expression of T cell membrane receptors CD4 and CD8 antigens with the functions of T helper and cytotoxic T cells, respectively. The activation of these cell subsets was assessed with $CD38^+$, HLA-DR^+ activation markers and also through the dual expression of these markers ($CD38^+$HLA-DR^+). To identify HIV target cells and HIV co-receptor expression $CCR5^+$ was used as a marker.

2.6.2 Selection of markers

To determine the in-vitro effects of IVIPs on immune activation, a number of important markers needed to be included. Incorporating a viability marker (Vivid) was crucial for discrimination of viable cells from non-viable cells as PBMCs are most likely to bind antibodies non-specifically. To exclude B-cells CD19 antibody was included, and that improved specificity and sensitivity of the assay. Since this study was investigating the T-lymphocytes activation, CD3, CD4, and CD8 markers were included because they differentiate T lymphocytes' lineage. At the activation of T lymphocytes was determined by looking at the CD38 and HLA-DR activation markers. Chemokine receptor CCR5 an HIV co-receptor was included for identifying the HIV target cells.

2.6.3 Optimization of the flow cytometry panel

2.6.3.1 Selection of fluorochromes

In polychromatic flow cytometry, there is the variability of fluorochromes that can be chosen; however, not all markers are available commercially on all fluorochromes. Therefore, antibody selection is a very crucial step when constructing a panel. An ideal panel would include bright antibodies which give the best separation, therefore, limiting spectral overlap. Fluorochromes with identical excitation and emission properties but different spillover characteristics were not used simultaneously in this panel. Details of the chosen flow cytometry marker are shown in table 2.4 below, the viability marker fluoresces in the Pacific blue channel. Therefore, CD19 and Dead cells were placed on the same channel

(Pacific blue channel), as they both needed to be excluded. CD3 was placed in the APC-H7 channel, CD4 was placed in the PECy5.5 channel, CD8 was placed in the BV711 channel, HLA-DR was placed in the Alexa-fluor-700 channel, CD38 was placed in the PE-Cy7 channel, and CCR5 was placed in the APC channel.

Table 2. 4. Details of the chosen flow cytometry panel

Reagent	**Channel**	**Storage**	**Supplier**
Live /Dead fixable violet dye	Pacific blue	-20ºC	Molecular probes
CD19	Pacific blue	4ºC	Invitrogen
CD3	APC-H7	4ºC	BD
CD4	PECy5.5	4ºC	Invitrogen
CD8	Bv711	4ºC	Biolegend
CD38	PE-Cy7	4ºC	eBiosciences
HLA-DR	Alexa flour 700	4ºC	Biolegend
CCR5	APC	4ºC	BD

2.6.3.2 Staining non-viable cells using live/dead fixable violet dead cell stain ("Vivid")

The vivid dye is a reactive amine dye, and it is a viability dye. This dye is suitable for identifying dead cells in samples by permeating cell membranes of dead cells and reacting with free amines in the cell's cytoplasm. However, this dye is excluded by live cells because it cannot penetrate their intact cell membranes. When cells are fixed, the dye is only associated with the dead cells, and they can be identified by the fluorescence emitted when the dye is excited by lasers (Perfetto et al., 2010).

The stock concentration of Vivid dye was prepared with 25µg of an amine-reactive dye and DMSO. Lyophilized DMSO that comes with a kit was thawed in a 37ºC water bath until completely thawed, 50µl of DMSO was added into a vial of lyophilised dye.1µL, the optimum volume (after titration) of 500µg/ml stock was added to 39µl of distilled water, and then 1µl of this concentration was added into 49µl of PBS, resulting into a working concentration. This working concentration was used to make a master mix, and 50µl of the

master mix was added to 150µl of PBS containing cells and incubated at RT for 20 min in the dark. Cells were washed twice with PBS and stained with fluorochrome-conjugated antibodies. Table 2.5 below shows the list of the reagents used to stain surface markers.

Table 2. 5. List of reagents used to stain surface markers

Reagents	**Storage conditions**	**Supplier**
Dulbecco's PBS	4ºC	Sigma Aldrich
10X CellFix	4ºC	BD
Anti-mouse compensation beads	4ºC	BD

2.6.4 Antibody staining of IVIP exposed PBMCs

Cells were suspended after the 3-hour incubation by gentle shaking of the FACS tubes and settling for 3 min, spun at 2290 x g for 10min and transferred the supernatants into Eppendorf tubes (Sigma Aldrich). The supernatants were stored at -80 ºC to be used for the measurement of genital tract cytokines. One millilitre of FACS wash was added into the tubes and spun at 2290 x g for 10min, and the supernatant was discarded. The cells were washed with 150µl of PBS and 50µl of the vivid master mix was added to the tubes and suspended with a pipette. The tubes were covered with foil and incubated for 30min in the dark at 37ºC. FACS wash (200µl) was added into each tube and centrifuged at 800 x g for 5min at 4 ºC. Fifty microlitres (50µl) of the chemokine master mix Allophycocyanin labelled anti-CCR5 and FACS wash was added and incubated for 30 minutes in the dark at 37ºC because it stains optimally in this temperature. Cells were washed twice with 200µl of

PBS-1, and 50µl of the surface antibody master mix (Allophycocyanin-H7 labelled anti-CD3, phycoerythrin cyanine 5.5–labelled anti-CD4, Brilliant Violet 711–labelled anti-CD8, phycoerythrin cyanine 7-labeled anti-CD38, Alexa flour 700-labelled anti-HLA-DR, Pacific blue-labelled anti-CD19 and Live/dead was added, re-suspended and incubated in the dark for 20min at RT for 20min. Cells were washed twice with 200µl of FACS wash. 150µl of 1X cellFix (Becton Dickinson) was added, re-suspended, wrapped with foil and acquired in a BD LSRII Fortesa flow cytometer (BD Biosciences). Table 2.6 below shows how the master mix preparation was made for one reaction.

Table 2. 6. Shows master mix preparation for one reaction and the pre-titrated volumes

	X1 reaction
Chemokine mastermix stained at 37ºC	
CCR5 APC	5 µl
PBS-1	45 µl
Surface antibody master mix stained at RT	
CD19 Pac blue	2µl
CD3 APC-H7	1.5µl
CD4 PE-Cy5.5	0.5µl
CD8 BV711	0.5µl
CD38 PE-Cy7	1µl
HLA-DR Alexa flour 700	2µl
FACS WASH	**42.5 µl**

2.6.5 Compensation controls

Each fluorochrome in the selected panel has an excitation and emission spectra when the fluorescence from the spectra overlap more than one fluorochrome can be detected in a single channel. A range of light wavelengths that add energy to a fluorochrome is the excitation spectrum, and it causes the fluorochrome to emit light in another range of wavelengths

referred to as emission spectrum which is shown in the figure. Therefore, compensation is used to correct for spectral overlap by removing the signal spillover from one fluorochrome into any other parameter, ensuring that fluorescence detected from a particular detector is from a specific channel only. Compensation controls are a single colour controls stained with one fluorophore per control using the exact test sample conditions (Bagwell and Adams, 1993).

Compensation beads (BD Bioscience) were stained with individual fluorochrome-conjugated antibodies for use as single-colour compensation controls. Briefly, 7 FACS tubes were labelled with each fluorochrome and one negative tube. The compensation beads were vortexed and one drop placed into each tube, and appropriate volume of each antibody (according to the antibody titrations) was added and centrifuged at 800 x g for 3min at 4ºC. Beads that react with mouse antibodies were used for all antibodies. The tubes were vortexed and re-suspended, incubated for 20 min in the dark at RT. PBS (1ml) was added to each tube and centrifuged at 800 x g for 3 min at 4ºC. The supernatants were decanted, and 150µl of 1X cell fix (1ml of 10X cell fix added to 9ml DD water) was added to each tube and vortexed briefly.

2.6.5.1 Preparation of Rainbow beads

The intensity of the fluorescence is dependent on the functionality of the flow cytometer machine, including laser power, alignment, temperature, and optical density. The first step when setting up the flow experiment is adjusting the sensitivity of the detectors to optimise instrument performance for the run. However, these voltages are not always optimal for the next assay run. Therefore, rainbow beads are acquired under optimised instrument settings to generate a set of target values that would reflect the instrument sensitivity (Holmes K, 2001). Rainbow beads are fluorescent particles that emit as a single peak in each instrument channel, therefore to set-up the instrument these beads are acquired first, and the voltages are adjusted until the bead peak hits the target values (Maecker and Trotter, 2009). Three hundred microliters of PBS-1 were added to the tube labelled "Rainbow", and after that the rainbow beads were vortexed, one drop was added to the tube. The mixture was vortexed and stored on ice.

2.6.5.2 Fluorescence minus one control (FMO)

To properly identify the spread of the fluorochromes into the channel of interest, and FMO control is used. FMOs are important because they help determine where the gates should be placed in a multicolour panel. To accurately discriminate between positive and negative signals, FMO controls are mandatory (Roederer, 2001). FACS tubes were labelled with each fluorochrome (Allophycocyanin-H7 labelled anti-CD3, phycoerythrin cyanine 5.5–labelled anti-CD4, Brilliant Violet 711–labelled anti-CD8, phycoerythrin cyanine 7-labeled anti-CD38, Alexa flour 700-labelled anti-HLA-DR, Pacific blue-labelled - Live/dead). One million PBMCs suspensions were added into round bottom falcon tubes (Becton Dickson). Cells were spun at 2290 x g for 10min, the supernatant was decanted, and the tubes flicked to re-suspend the cells,1000µl of the (1 in 1000dilution of Snuff and Alum) were added to the labelled tubes and incubated for 3hours at 37ºC in the presence of 5% CO2. An appropriate volume of each antibody (according to the antibody titrations) was added minus one labelled in the tube and centrifuged at 800 x g for 3min at 4ºC. The tubes were vortexed and re-suspended, incubated for 20 min in the dark at RT. PBS (1ml) was added to each tube and centrifuged at 800 x g for 3 min at 4ºC. The supernatants were decanted, and 150µl of 1X cell fix (1ml of 10X cell fix added to 9ml DD water) was added to each tube and vortexed briefly.

2.6.6 Data acquisition

After staining, cells were stored at 4ºC in the dark and acquired within 24h. PBMC samples were acquired on a BD LSRII Fortessa flow cytometer (BD Biosciences), Flow cytometry analysis was performed using FlowJo software version 10 (Tree star) with FacsDiva software. The number of events collected was 500000- 1000000.

2.6.7 Data QC and Management

Time gates used to exclude fluorescence shifts that may occur during acquisition were used. N by N plots were conducted per run to generate the correct compensation matrix plot and

adjustments done after plot acquisition. The compensation matrix was applied to compensation controls for the trace of Tandem dye degradation.

2.7 Investigation of biomarkers of inflammation using Bio-Plex

Cell proteins such as Cytokines and chemokines are expressed by all cell types, including epithelial and endothelial cells, and they are also immunologically expressed. These proteins can interact with specific receptors on target cells to facilitate physiological responses such as haematopoiesis, growth, immunity, and inflammation, and their dysregulation is associated with pathological conditions. Detection of the cytokine profile allows determination of the type of preferentially activated immune responses. Using the multiplexed fluorescent bead-based detection assay such as Bio-Plex allows for the detection of multiple cellular proteins simultaneously. A schematic representation of Bio-Plex assay principle is shown in figure 2.3.

The aim of this test was to evaluate the PBMC immune response to IVIP exposure measured by cytokine and chemokine production. Supernatants from PBMCs of n=12 individuals (collected after 3 hours of exposure) stored at -80°C freezer were thawed and concentration of all cytokines and chemokines (interleukin (IL)-1β, IL-6,tumour necrosis factor (TNF)–α and granulocyte-macrophage colony-stimulating factor (GM6, and granulocyte-macrophage colony-stimulating factor (GM–CSF); chemokines (interleukin (IL)-8,interferon gamma-induced protein (IP)-10, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)–1α and macrophage inflammatory protein (MIP)-1β); Hematopoietic cytokines (interleukin (IL)-7) and regulatory cytokines (interleukin (IL)-10) were measured using Bio-Plex Pro Human Cytokine kits (Bio-Rad Laboratories) and a Bio-Plex MagPix Array Reader (Bio-Rad Laboratories) according to the manufacture's protocol. All samples had the same freeze-thaw history at the time of testing. Bio-Plex assay kit reagents were brought to room temperature excluding the detection antibody, detection antibody diluent, streptavidin-phycoerythrin and magnetic beads were kept in a 4ºC fridge. The sensitivity of the Bio-Plex assay kit ranged between 0.2 and 45.2pg/ml for each of the 11 cytokines measured

The Bio-Plex multiplex system uses fluorescently labelled beads each colour coded to discriminate the individual tests in a multiplex suspension following a stepwise fashion as demonstrated in the figure. Capture antibodies directed to the desired biomarker are covalently coupled to the magnetic beads. The biomarker of interest in a sample detects the reaction of the beads in the sample. The unbound proteins are removed by a series of washes after and a biotinylated detection antibody is added to create a sandwich complex. The addition of streptavidin-phycoerythrin (fluorescent indicator) conjugate forms the final detection complex.

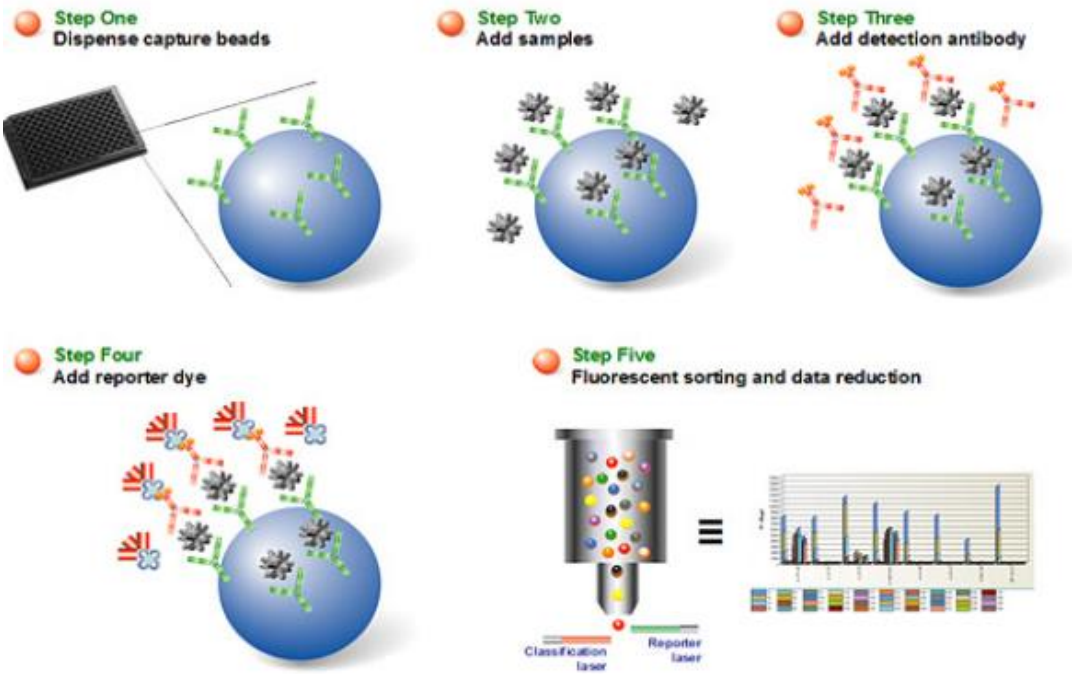

Figure 2. 3. Schematic representation of a bio-Plex assay principle (the figure was adapted from Bio-Rad.com). Step one is the dispensation of capture antibodies, and step two is the addition of the sample, step three is the addition of the detection antibody, step four is the addition of the reporter dye and step five fluorescent sorting and data reduction. This experiment aimed to investigate the inflammatory biomarkers in PBMC culture supernatants after exposure to IVIPs.

2.7.1 Bio-Plex plate preparation

Fifty microliters of thawed supernatant samples were placed in wells of the v-bottom 96 well plates (Costar) master plate, to limit the variability of sample exposure as a result of magnetic beads. Two 27-Plex Pro plates were prepared. Each plate included six duplicated supernatant samples as controls for reproducibility measurements within a plate and thirty supernatant samples each duplicated to investigate the reproducibility of cytokine measurements across plates. A representative plate layout is shown in table 2.7 below.

Table 2. 7. Representative plate layout showing the plate design.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S9	S9	5 Snuff_2018/11/12_R10	5 Snuff_2018/11/12_R10	13 Snuff_2018/11/13_PHA	13 Snuff_2018/11/13_PHA	21 Snuff_2018/11/14_1/1000	21 Snuff_2018/11/14_1/1000	29 Snuff_2018/12/14_R10	29 Snuff_2018/12/14_R10
B	S2	S2	S10	S10	6 Snuff_2018/11/12_1/1000	6 Snuff_2018/11/12_1/1000	14 Snuff_2018/11/13_R10	14 Snuff_2018/11/13_R10	22 Snuff_2018/11/14_PHA	22 Snuff_2018/11/14_PHA	30 Snuff_2018/12/14_1/1000	30 Snuff_2018/12/14_1/1000
C	S3	S3	Blank	Blank	7 Snuff_2018/11/13_PHA	7 Snuff_2018/11/13_PHA	15 Snuff_2018/11/13_1/1000	15 Snuff_2018/11/13_1/1000	23 Snuff_2018/11/14_R10	23 Snuff_2018/11/14_R10	31s Snuff_2018/12/19_PHA	31s Snuff_2018/12/19_PHA
D	S4	S4	Cntrl	Cntrl	8 Snuff_2018/11/13_R10	8 Snuff_2018/11/13_R10	16 Snuff_2018/11/13_PHA	16 Snuff_2018/11/13_PHA	24 Snuff_2018/11/14_1/1000	24 Snuff_2018/11/14_1/1000	32s Snuff_2018/12/19_R10	32s Snuff_2018/12/19_R10
E	S5	S5	1 Snuff_2018/11/12_PHA	1 Snuff_2018/11/12_PHA	9 Snuff_2018/11/13_1/1000	9 Snuff_2018/11/13_1/1000	17 Snuff_2018/11/13_R10	17 Snuff_2018/11/13_R10	25 Snuff_2018/11/14_PHA	25 Snuff_2018/11/14_PHA	33s Snuff_2018/12/19_1/1000	33s Snuff_2018/12/19_1/1000
F	S6	S6	2 Snuff_2018/11/12_R10	2 Snuff_2018/11/12_R10	10 Snuff_2018/11/13_PHA	10 Snuff_2018/11/13_PHA	18 Snuff_2018/11/13_1/1000	18 Snuff_2018/11/13_1/1000	26 Snuff_2018/11/14_R10	26 Snuff_2018/11/14_R10	34s Snuff_2018/12/19_PHA	34s Snuff_2018/12/19_PHA
G	S7	S7	3 Snuff_2018/11/12_1/1000	3 Snuff_2018/11/12_1/1000	11 Snuff_2018/11/13_R10	11 Snuff_2018/11/13_R10	19 Snuff_2018/11/13_PHA	19 Snuff_2018/11/13_PHA	27 Snuff_2018/11/14_1/1000	27 Snuff_2018/11/14_1/1000	35s Snuff_2018/12/19_R10	35s Snuff_2018/12/19_R10
H	S8	S8	4 Snuff_2018/11/12_PHA	4 Snuff_2018/11/12_PHA	12 Snuff_2018/11/13_1/1000	12 Snuff_2018/11/13_1/1000	20 Snuff_2018/11/14_R10	20 Snuff_2018/11/14_R10	28 Snuff_2018/11/14_PHA	28 Snuff_2018/11/14_PHA	36s Snuff_2018/12/19_1/1000	36s Snuff_2018/12/19_1/1000

2.7.1.1 Beads Preparation and Validation

Anti-cytokine beads (10X) stock solution was mixed by vortexed at medium speed and sonicated for the 20s. A 10-fold working dilution was prepared by adding 575 µl of beads into 5175 µl of the Bio-Plex assay Buffer to make a total volume of 5750 µl of the bead working solution and covered with foil for protection from light. Beads working solution was vortexed and 25µl of solution added into each well. Vacuum filter washed (2X) with 100 µl of Bio-Plex Wash Buffer at the Bio-Plex Pro II (BIO-RAD) automated washing station. A 50 µl vortexed standard added into the appropriate well, blanks were also included. Subsequently, 50µl of vortexed samples were added to each corresponding well. Plate covered with adhesive sealer and blotted at the bottom then covered with a foil, incubated for 30 minutes at room temperature shaking at a speed of 1082 x g for 30 seconds and reduced to 80 x g for the remaining incubation time.

2.7.1.2 Preparation of detection antibody

Multiplex antibody detection solution (10X) provided in a kit was gently vortexed, and working stock dilution was prepared with Bio-Plex Detection Antibody diluent. Plate sealer was removed, and filter washed (3X) with 100 µl of Bio-Plex Wash Buffer at the Bio-Plex Pro II (BIO-RAD) automated washing station. Afterwards, 25 µl gently vortexed detection antibody added into each well of 96 well plates. Plate covered with plastic adhesive sealer and blotted at the bottom then covered with a foil, incubated for 30 minutes at room temperature shaking at a speed of 1082 x g for 30 seconds and reduced to 80 x g for the remaining incubation time.

2.7.1.3 Preparation of Streptavidin-PE

Vigorously vortexed Streptavidin stock solution PE (100X) stock solution was used to prepare 100-fold diluted working dilution in the Bio-Plex assay buffer. Plate sealer was removed, and filter washed 3X with 100 ml of Bio-Plex wash buffer. Subsequently, 50 ml of vortexed streptavidin-PE working solution added in each well. The plates were covered with plastic adhesive sealer and blotted at the bottom then covered with a foil, incubated for 30 minutes at room temperature and left in a mechanical shaker at a shaking speed of 1082

x g for 30 seconds and reduced to 80 x g for the remaining incubation time. The washing step with a Bio-Plex wash buffer was repeated three times at the Bio-Plex Pro II (BIO-RAD) automated washing station. Beads were re-suspended in each well with 125 ml of Bio-Plex assay buffer.

2.7.1.3 Standard preparation

Five hundred microliters of the standard diluent were added into lyophilised standard and vortexed for 4s before being incubated on ice for 30min. Polypropylene tubes (BD) were used for the standard's serial dilutions, 72µl was added to the first tube, and 150µl of the standard diluent was added subsequently to the other tubes until the tenth standard tube and incubated. The reconstituted standard was vortexed and 1:4 serial dilutions performed by transferring 50µl of standard one into standard two with 150µl of the standard diluent and 50µl was added subsequently between tubes until tube S10.

2.7.2 Data acquisition

Hundred and twenty-five microlitres of the assay buffer were added into each well for the data acquisition using the Bio-Plex MAGPIX MultiPlex Reader (Bio-Rad). The plates were covered with a plastic adhesive plate sealer, and bottom blotted and shook at a speed of 646 x g for 30 min. The sealer was removed, and the plate read.

2.8 Statistical analysis

Statistical analyses were performed using GraphPad version 7 and Wilcoxon matched-pairs signed-rank test was used for comparing two matched samples. All cytokine concentrations were log10 transformed to achieve normality. The data from different donors were tested for normality using Shapiro-wilk normality test. The percentage coefficient of variation (% CVs) of the inter-plate and intra-plate were analysed using descriptive statistics reported as medians and interquartile range. Statistical significance was referred to as a p-value less than or equal to 0.05, and it was presented by the asterisk rating system ($P < 0.05$ *, $P < 0.01$ ** and $P< 0.001$***). Unsupervised hierarchical clustering was performed using R version 3.6.0. Flow cytometry data were analysed in FlowJo version10 (Tree star) and Excel

(Microsoft) prior to statistical testing. Multiplex data was collected using Bio-Plex Manager software version 6 (Bio-Rad), and a five-parameter logistic (5PL) regression formula was used to calculate sample concentrations from the standard curves.

CHAPTER 3: RESULTS

3.1 IVIP pH measurements at different dilutions

The female genital tract is a pH sensitive part of the body. A normal vaginal pH level is (3.8-4.5) and an introduction of a foreign product in the vagina can alter the normal vaginal pH, therefore we measured the pH of all the IVIPs that were selected at different dilutions and the results are shown in table 3.1

Table 3. 1. Shows the pH profile of intravaginal insertion products (IVIPs) at different dilutions.

Dilutions	Alum	Snuff	Kuber	Rose water	Savlon
Stock/Neat	2.62	9.11	8.21	4.21	6.00
1/5	3.32	9.32	8.19	7.29	7.37
1/10	3.52	9.22	7.85	7.36	7.39
1/100	5.44	8.92	7.66	7.41	7.42
1/1000	7.23	8.20	7.74	7.42	7.44
R10 media	7.43				

Alum stock solution and undiluted rose water solution are strongly acidic (pH 2.62 and 4.21, respectively) and were buffered with the addition of R10 media to be weakly basic. Snuff and kuber stock solutions are basic (pH 9.11 and 8.21, respectively), and the addition of R10 media does not seem to have a distinctive effect on the pH. The undiluted savlon solution is weakly acidic (pH 6.0), and it was buffered with the addition of the R10 to be weakly basic. The R10 medium used for diluting IVIPs has a weak basic pH of 7.43

3.2 Optimal IVIP dilution

A challenging factor with IVIPs exposure was not having a standard concentration for each product because IVIPs preparation varies according to individuals. Therefore, a range of IVIP dilutions (1/5, 1/10, 1/100 and 1/1000) was made from the 450 mg/ml stock (alum, snuff, and kuber) or neat (rose water and savlon) IVIPs. This experiment aimed to determine the product dilution that would give the optimal viability after cell exposure to 3 hours at 37°C, in the presence of 5% CO2. Figure 3.1 shows the percentage of T cells from the peripheral blood identified using the expression of CD3 after exposure to different IVIPs dilutions. Blood was drawn from four different donors who were kept constant between IVIPs.

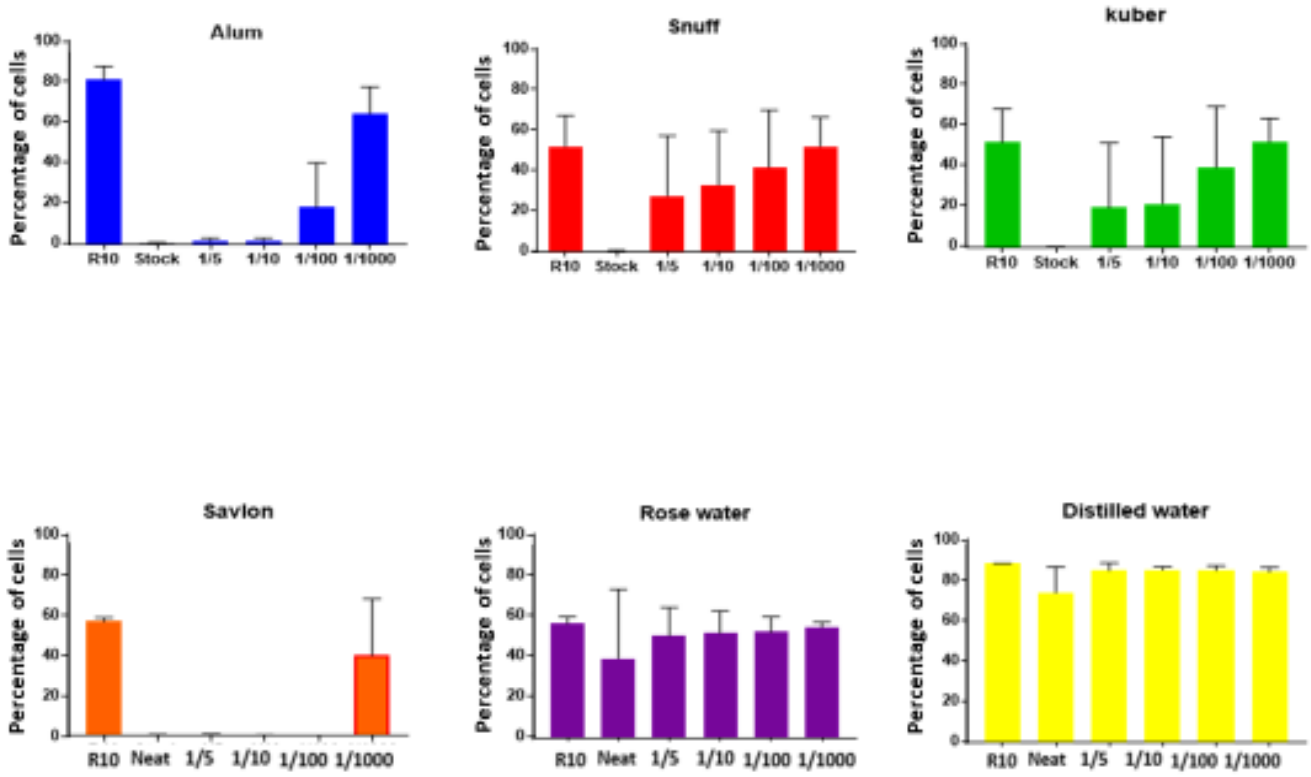

Figure 3. 1. Shows cell viability expression on peripheral T lymphocytes stained with Vivid viability marker after stimulation with R10 media and being exposed to 450 mg/ml stock solution (snuff, kuber and alum), neat (savlon, rose water and distilled water), 1/5, 1/10, 1/100 and 1/1000 dilutions of snuff, kuber, alum, savlon, rose water and distilled water. Mean data of 4 donors is represented and error bars indicate the standard deviation.

$CD3^+$ Lymphocytes isolated from PBMCs exposed to IVIPs (alum, snuff, kuber, savlon, rose water and distilled water) for 3 hours and stained with the optimal volume (1.5µl) of anti-CD3, APC-H7. PBMCs from 4 different donors were used for each condition (R10 media, stock/neat, 1/5, 1/10, 1/100, 1/1000) per IVIP and the same donors were used for each IVIP testing (Figure 3.1). The percentages shown correspond to CD3+ cells (mean ± SD).

Alum exposure results in a high percentage of cell death. The viability of CD3+ T cells after a 3-hour alum exposure ranged from 2 to 70%, for stock, 1/5, 1/10, 1/100 and 1/1000, the mean viability is 2%, 5%, 7%, 22%, and 70%, respectively. Snuff exposure results in a low percentage of cell death. The viability of CD3+ T cells after a 3-hour exposure to snuff ranged from 2 to 50%. For stock, 1/5, 1/10, 1/100 and 1/1000, the mean viability is 2%, 30%, 35%, 40% and 50%, respectively. Cell exposure to kuber results in a low percentage of cell death. The viability of CD3+ T cells after a 3-hour kuber exposure ranged from 2 to 55%. For stock, 1/5, 1/10, 1/100 and 1/1000, the mean viability is 2%, 25%, 30%, 40% and 55%, respectively (Figure 3.1).

Cells exposure to savlon results in a high percentage of cell death. The viability of CD3+ T cells after a 3-hour savlon exposure ranged from 1 to 40%. For neat, 1/5, 1/10, 1/100 and 1/1000, the mean viability is 1%, 1.5%, 2%, 2.5% and 40%, respectively. Cell exposure to rose water results in a low percentage of cell death. The viability of CD3+ T cells after a 3 hour rose water exposure ranged from 40% to 60%. For neat, 1/5, 1/10, 1/100 and 1/1000, the mean viability was 40%, 55%, 56%, 57% and 60%, respectively. Exposure to distilled water results in a very low percentage of cell death; distilled water was used as a control for rosewater stimulation. To check if the results obtained from rose water stimulation were because of water or because of the rose extracts because water diffusion by osmosis across the cell membrane could result in swelling of the cells and bursting causing the cells to die. The viability of CD3+ T cells after a 3 hour distilled water stimulation ranged from 75% to 90%. For neat, 1/5, 1/10, 1/100 and 1/1000, the mean viability is 75%, 85%, 86%, 88% and 90%, respectively (Figure 3.1). Stimulation of cells for 24 hours led to significantly

reduced cell viability (results not shown), therefore, the rest of the stimulations described in this thesis were done for the duration of 3 hours.

Alum and snuff were selected as representative products to be used for further experiments due to the following reasons: (i) they were one of the most commonly used IVIPs compared to others (Humphries et al., 2019) (ii) and also due to budget constraints, only two products could be tested extensively.

3.3 Optimal titer of all antibodies

To ensure that all antibodies are used at a volume that provides the best separation of negative and positive populations, it is important that titrations are performed on all antibodies. Mean fluorescent intensity (MFI) graph is the measure of a cell fluorescence distribution level and it was quantified using the median for the positive and the negative populations, the titre is chosen at the lowest volume where saturation is reached. The signal to noise ratio graph is also used to determine the optimal titre, the ratio is calculated by dividing the positive population (the signal) by the negative population (the noise, and the optimal titre is the highest ratio value. Table 3.2 shows the optimal titre for all antibodies used in this experiment. All titrations were stained with Vivid to avoid an incorrect reading that could result from dead cells accumulating in the antibody.

Table 3. 2. Shows the optimum volume of all the antibodies in the panel.

Marker	Channel	Optimum titre (µl)
Live/dead Vivid	Pacific blue	1
CD19	Pacific blue	2
CD3	APC-H7	1.5
CD4	PE-Cy5.5	0.5
CD8	BV711	0.5
CD38	PE-Cy7	1
HLA-DR	Alexa fluor 700	2
CCR-5	APC	5

3.3.1 Titration of viability dye Vivid

Vivid as the dead cell marker also required titration. Different titration volumes (1µl, 2µl, and 5µl) were used. The ideal concentration should give a clear separation between the negative and positive populations. Figure 3.2 A demonstrates the titration of vivid, the negative population represents the live cells, and the positive population represents dead cells; figure 3.2 B shows how the optimal titre was able to separate between the negative and positive populations in a flow plot of vivid against CD3 and figure 3.2 C shows the positive signal relative to the background in the MFI and signal to noise ratio graphs. The chosen volume of 1 µl was based on the maximum separation of the negative and positive populations and also based on minimal spillover. The volume of 1µl was acceptable because when considering reactive amine dye, the lowest background signal is more important than the absolute positive signal. Vivid staining was performed before surface staining to prevent it from attaching to the antibodies' amine groups.

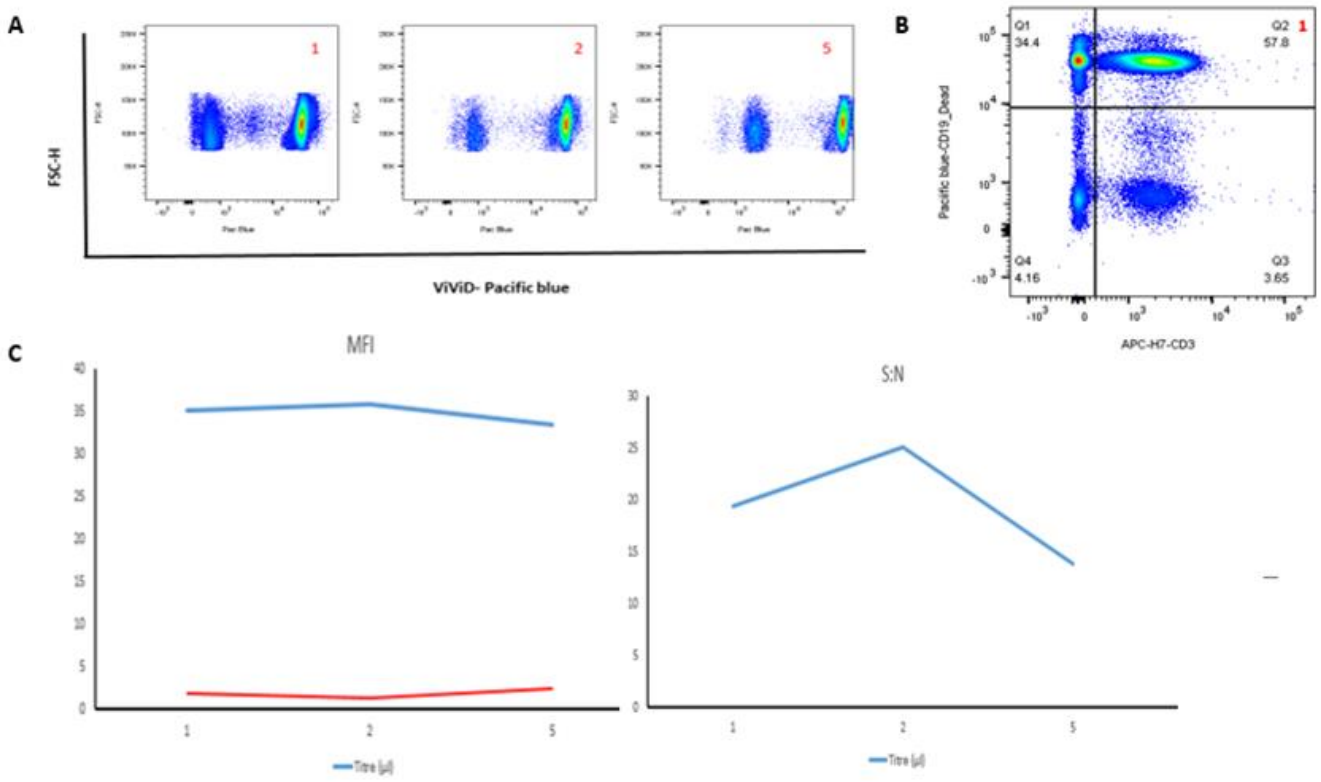

Figure 3.2. Representative titration of a reactive amine dye Vivid- Pacific used for staining dead cells. Unstimulated PBMC cells were stained with dilutions of vivid dye (1, 2 and 5 µl). After vivid staining was complete, cells were stained with anti-CD3 using standard staining procedures. (A) Shows flow plots showing the separation of the positive and negative populations from the three vivid dye dilutions, the value at the top right corner of each flow plots represents the volume of vivid added. (B) Shows a representative flow plot of the working dilution of 1 µl of vivid and anti-CD3. (C) Illustrates the differences in the positive signal relative to the background staining, the MFI graph CD19+(red) CD19- (blue), signal to noise ratio = positive MFI / negative MFI, the separation is acceptable at the lower dilution 1 µl which also gave a lower background.

3.3.2 Titration of surface markers

The next step after viability staining is surface marker staining, PBMCs were stained as described in chapter 2. As an example titration of APC-CCR5 antibody is shown in figure 3.3, at the lower concentration (0.30µl) of APC-CCR5 no distinct positive population could

be identified. The MFI graph revealed that 5µl of this antibody was the optimum to use in the assays

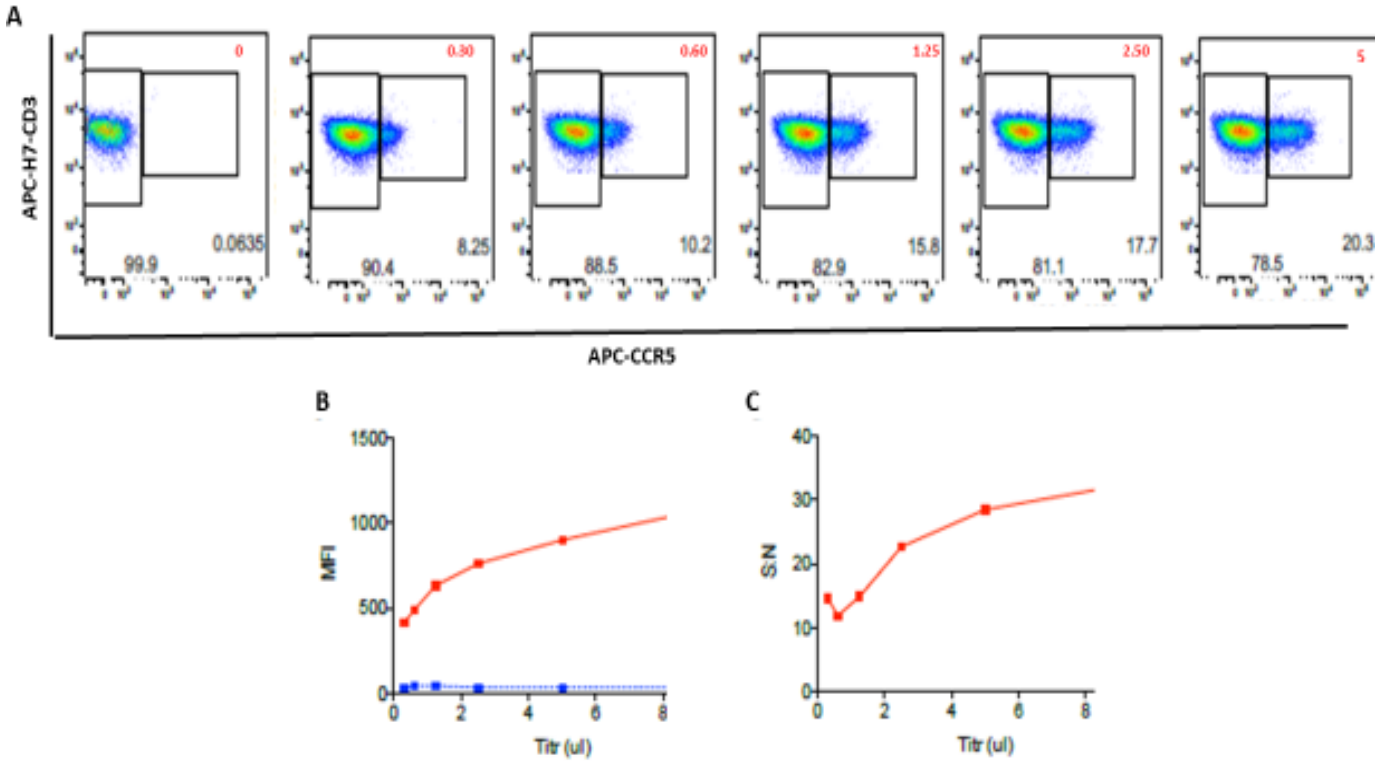

Figure 3.3. Representative titration of APC-CCR5. (A) Flow plots showing the separation of the positive and negative populations from the five APC-CCR5 dilutions 0, 0.30, 0.60, 1.25, 2.50 and 5 µl indicated at the top right corner of each flow plots. (B) Illustrates the differences in the positive signal relative to the background staining, the MFI graph CCR5+ (red) and CCR5- (blue) (C) signal to noise ratio = positive MFI / negative MFI, the separation is acceptable at the dilution 5µl.

3.3 Gating strategy

In flow cytometry analysis gating is one of the fundamental steps because gates are placed upon the cell populations of interest to investigate and quantify these populations. The first step in the gating strategy used included the time plot to remove any inconsistencies that might have occurred during acquisition, by gating where there are no interferences. Gating on forward scatter- height against forward scatter- area ensures that the fluorescence detected is from single cells by excluding the duplets. When distinguishing cell populations, the first step is based on forward and side scatters properties, forward and side scatter give an

estimation of the size, the granularity of the cells, and they help to distinguish cell types. Our cell type of interest was lymphocytes. Plotting the dump channel (pacific blue) against CD3 APC-H7 ensured the exclusion of cell debris, dead cells, and monocytes by gating on the positive CD3 lymphocyte population to further examine T cells. The $CD3^+$ T cells were further identified and gated by the expression of $CD4^+$ and $CD8^+$ T cells subsets, the relative expression of $CD38^+$, HLA-DR^+ activation markers, and $CCR5^+$ the HIV co-receptor (Figure 3.4).

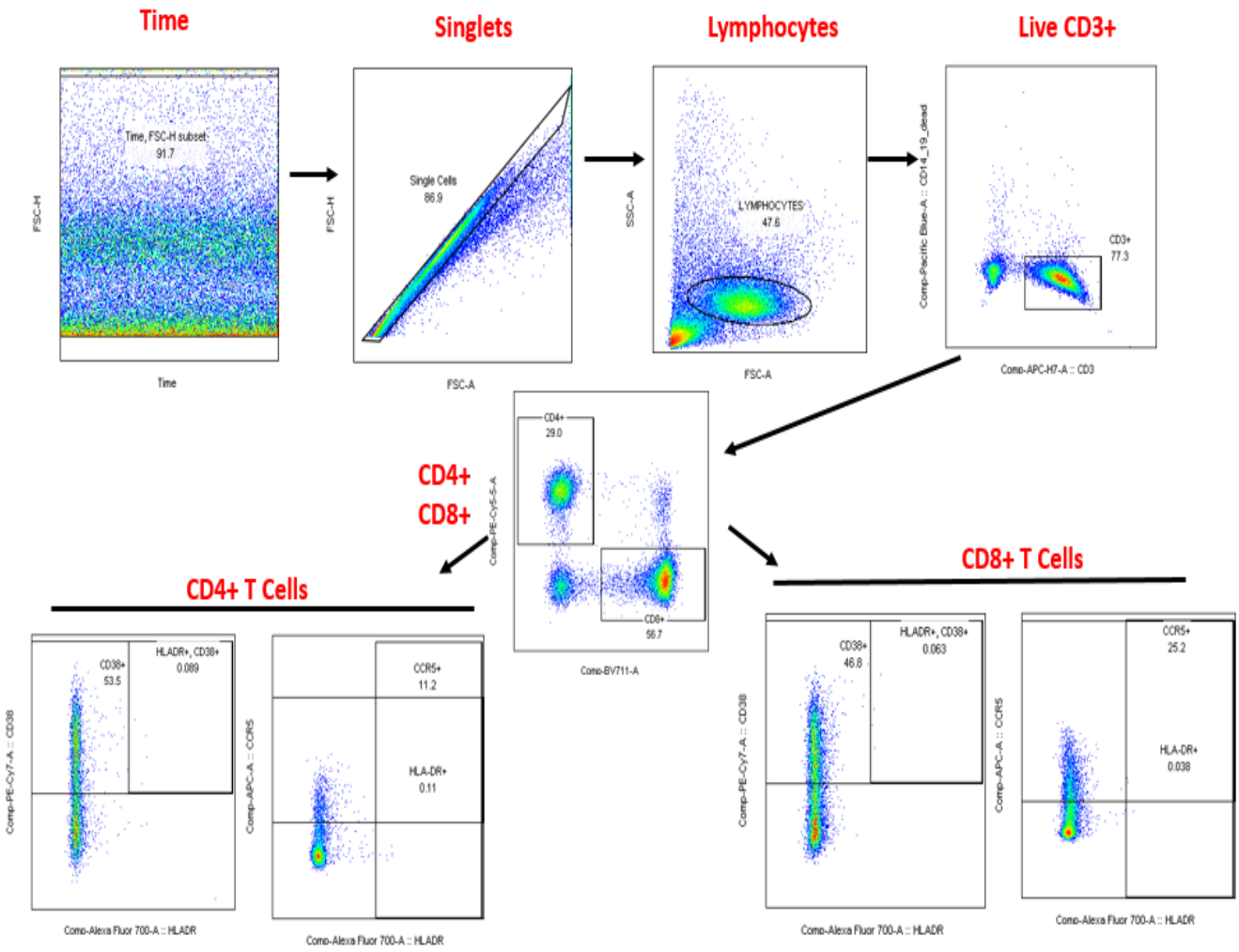

Figure 3.4. Shows the sequential gating strategy applied for this analysis. The first flow plot shows the measurement of forward scatter height and time to remove any inconsistencies in the flow of cells to ensure a higher quality of data. The second plot shows the measurement of forward scatter height and forward scatter area to remove clumps/ duplets and determine the single cells inside a diagonal gate. From the singlets, the analyses were narrowed by plotting side scatter area, and forward area scatter for identifying lymphocytes (inside an oval gate) while excluding the B cells, monocytes, granulocytes, and dead cells. From the lymphocytes gate, viability gate is set up by gating live CD3+ T cells by plotting dump channel using Pacific blue (dead cells, CD14 and CD19) with CD3+ T cells. Live CD3+ T cells were further subdivided into CD4+, and CD8+ T cells and these were subsequently analysed for activation markers of interest HLA-DR+ and CD38+.

3.4 Flow cytometry controls

To be certain that the results were not influenced by the background variation and non-specific effects, the following controls were applied.

3.4.1 Fluorescent minus one (FMO) controls

FMO control ensures proper gate positioning, taking into consideration the fluorescence spread. Ensuring that there were no spillovers, each FMO was plotted against all the other fluorochromes in the panel. Representative plots for CD38-PE-Cy7 FMO is shown in Figure 3.5 All FMO controls for all the fluorochromes in the panel are shown in Figure3.6.

Channel	unstained	FMO control	Fully stained
Pacific blue	.....	Live /Dead fixable violet dye/CD19	Live /Dead fixable violet dye/CD19
APC-H7	.....	CD3	CD3
PECy5.5	.....	CD4	CD4
Bv711	.....	CD8	CD8
PE-Cy7	.....	.....	CD38
Alexa flour 700	.....	HLA-DR	HLA-DR
APC	.....	CCR5	CCR5

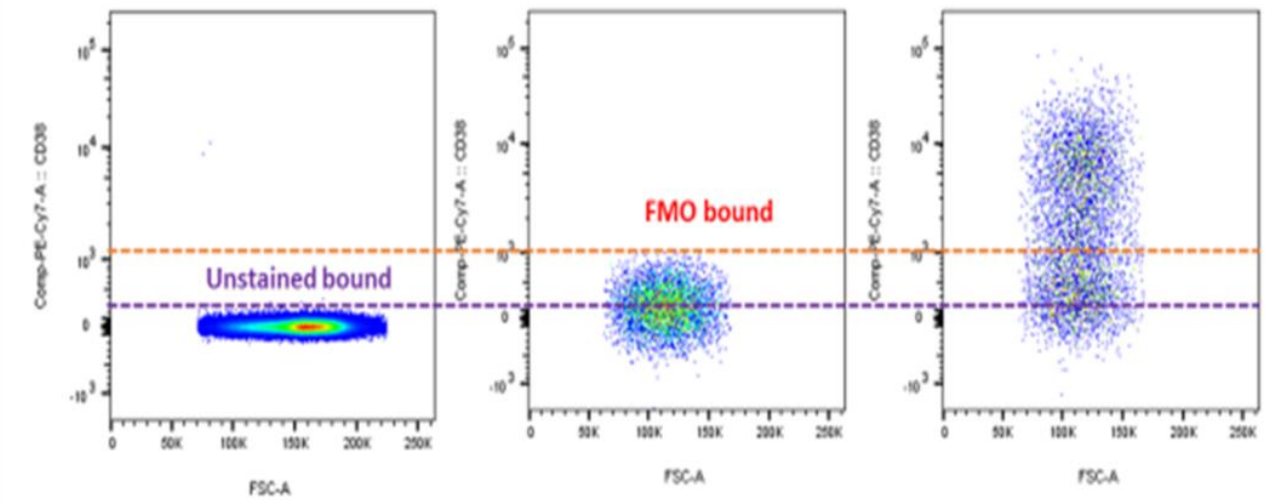

Figure 3. 5. Flow cytometry plots showing an application of an FMO control to determine the fluorescence spread. The FMO control shows the fluorescence spread into the PE-Cy7 channel compared to an unstained control. The red dotted line represents the FMO gating boundary compared to the unstained boundary in purple.

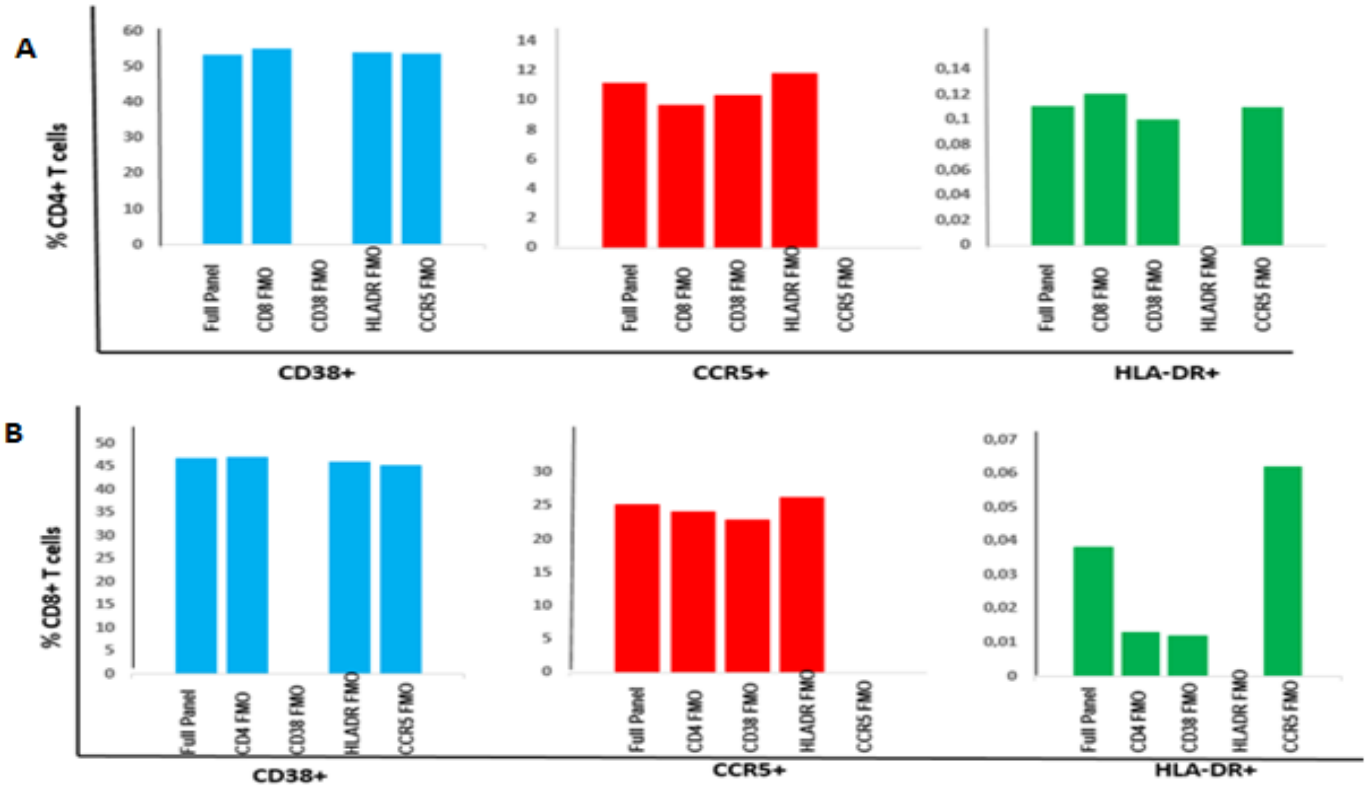

Figure 3. 6. Shows FMO's from $CD4^+$ and $CD8^+$ T cells for all fluorochromes in the panel. (A) shows the percentage of $CD4^+$ T cells for all the fluorochromes in each FMO channel for CD38, CCR5, and HLA-DR. (B) shows the percentage of $CD8^+$ T cells for all the fluorochromes in the Each FMO channel for CD38, CCR5, and HLA-DR. There is no fluorescent spillover into any of the channels in the panel, for both $CD4^+$ and $CD8^+$ T cells. All cells were gated from $CD3^+$T cells, percentages of each cell subset were used.

3.4.2 Unstained control

In order to determine the negative population, the level of background auto-fluorescence and to set voltages correctly, an unstained control was acquired, and from the unstained population, the level of background fluorescence and auto-fluorescence were determined. After that, the voltages and negative gates were set appropriately. Figure 3.7 shows the

negative and positive cell populations, therefore, the position where the negative gate should be placed.

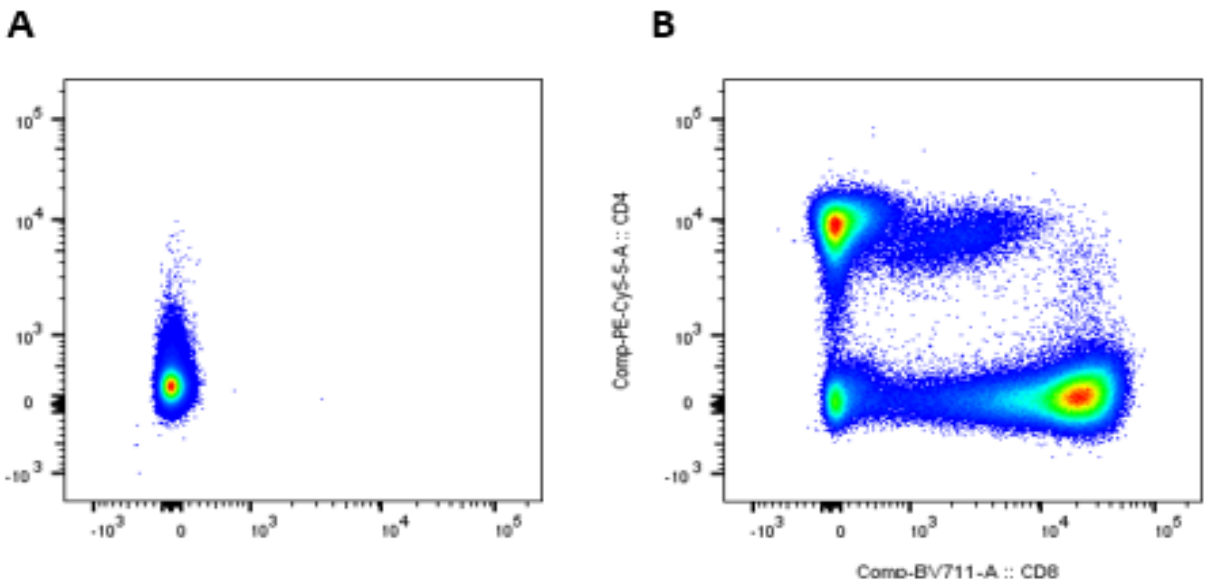

Figure 3. 7. Flow cytometry plots showing (A) unstained lymphocytes are used to set the negative population, allowing (B) the positive population stained with CD4 PE-Cy5.5 and CD8 BV711to be visualised.

3.4.3 Compensation Controls

Compensation controls were included as previously discussed in Section 2.5.3. When using multiple fluorophores, a compensation control is stained with a single antibody, and single stains are essential for determining the levels of compensation because they reveal the level of spectral overlap between different fluorophores, and compensate for this overlap. Determining which colour to match to which antibody in the reagent panel is the most critical factor because some fluorophores are brightly expressed while others are dimly expressed and reagents' signals optically interfere with each other. The emission spectra of the fluorochromes in the panel were viewed using the spectral viewer from (www.thermofisher.com). Pacific blue is brightly expressed in a violet laser. APC is brightly expressed in a red laser. APC-H7 is dimly expressed in a red laser. BV711, PE-Cy5.5 is moderately expressed in a blue laser. BV711 is brightly expressed on the violet laser. Alexa

Fluor 700 is dimly expressed on the red laser. PE-Cy7 is brightly expressed in the yellow green laser. The spectral overlap was compensated for from all the fluorochromes used in the panel, and their overlap was minimal except for BV711 and Alexa Fluor 700; APC-H7 and PE-Cy7 shown in figure 3.8 (www.thermofisher.com).

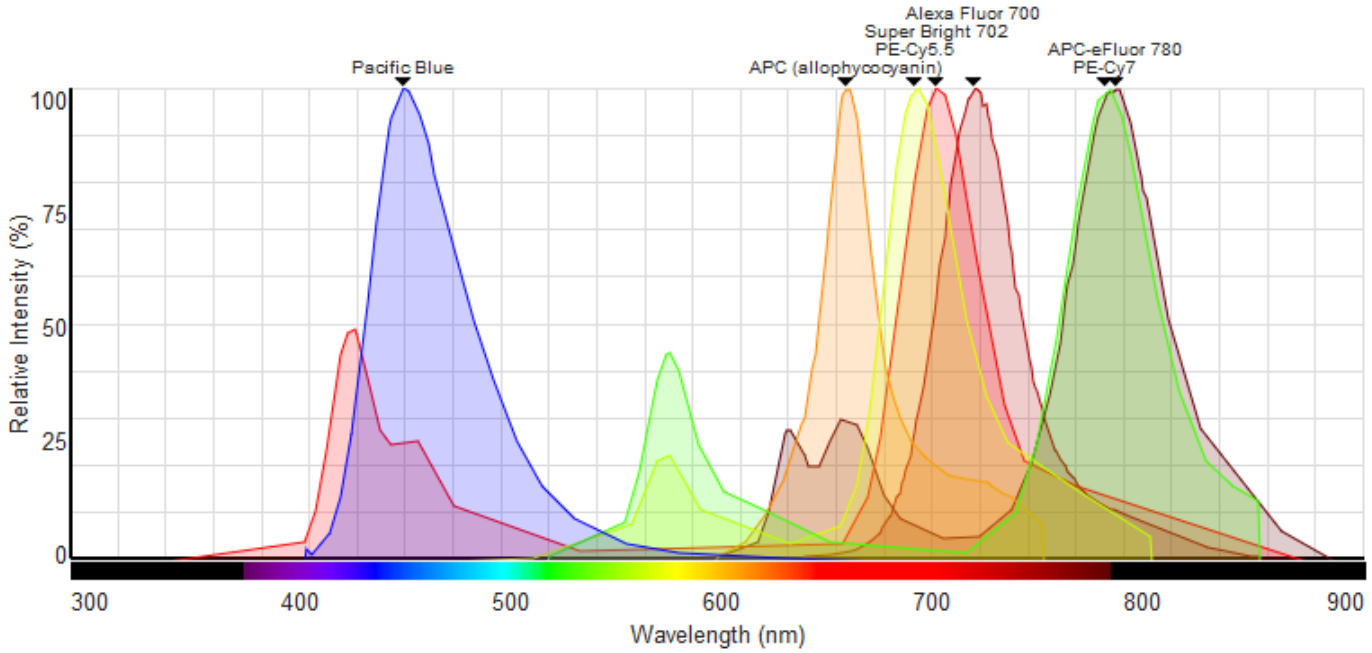

Figure 3.8. Shows emission spectra of fluorochromes using SpectraViewer (www.thermofisher.com). Emission spectra of Alexa Fluor 700 overlap with Brilliant violet 711 they are in the same channel, and PE-Cy7 overlaps with APC-H7 in the spectral viewer. APC-fluor has similar spectral properties to APC-H7, Super bright 702 has similar spectral properties to BV 711 and hence they were compensated.

3.5 The effects of IVIPs on Immune activation of T cells by flow cytometry assay

Immune activation of T cells after exposure to IVIPs was investigated. Here, we used the expression of CD38, HLA-DR, and CCR5 on the surface of CD4+ or CD8+ T cells to represent the level of T cell activation. Representative flow plots from one of the 26 participants for CD4 T cells (Figure 3.9) and CD8 T cells (Figure 3.10) are shown. For each participant, the negative control (R10 media), Snuff, Alum, and PHA (positive control) after 3 hours of exposure are shown.

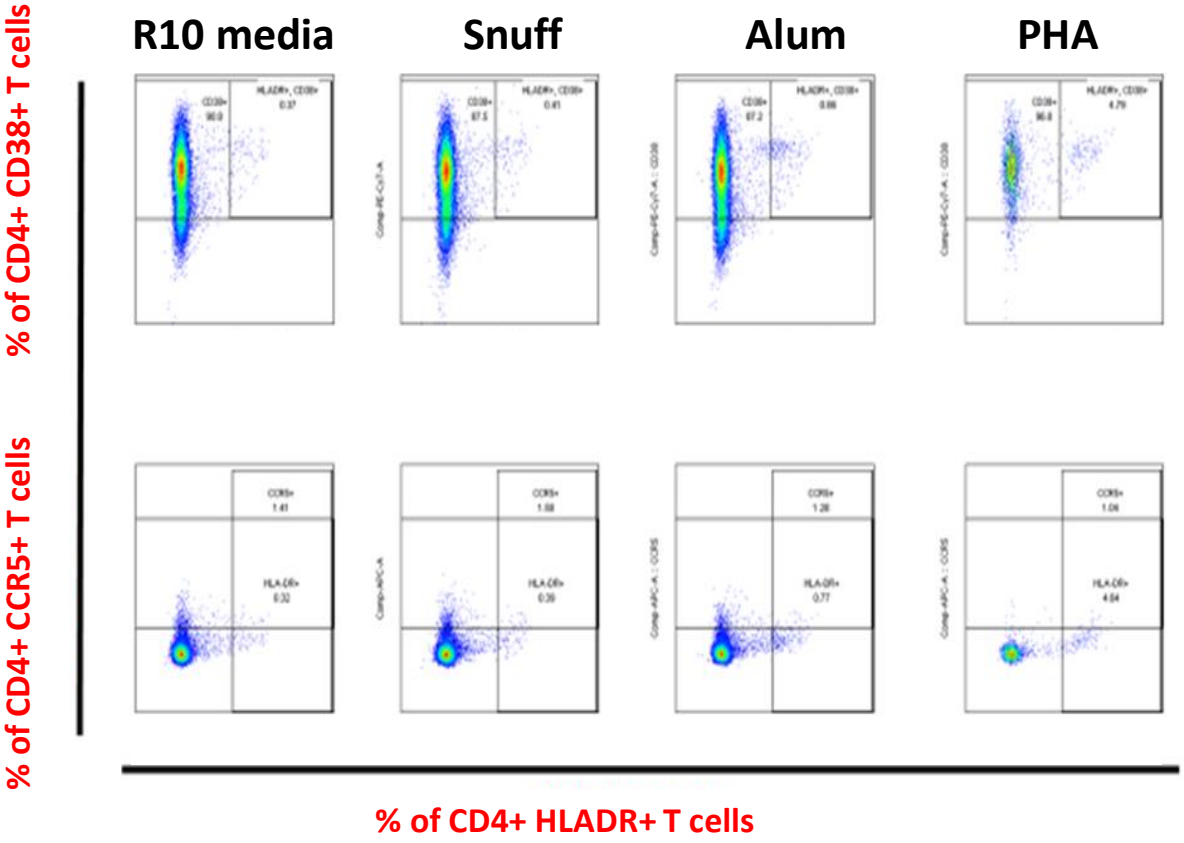

Figure 3.9. Representative flow cytometry dot plots showing impacts of IVIPs and PHA on the expression of cellular activation markers (CD38, HLA-DR) and CCR5 in $CD4^+$ T cells After 3 hours of IVIP exposure at 37 °C.

$CD4^+$ T cells were expressing the $CD38^+$ and HLA-DR^+ similarly irrespective of the condition. For this reason, cells co-expressing HLA-DR, and CD38 were mainly used to mark highly activated cells. CCR5 is less expressed in the negative control, snuff, and alum exposure and it is not expressed in PHA stimulated cells. $CD4^+$ T cells show significant CCR5 down-regulation following PHA stimulation (Figure 3.9).

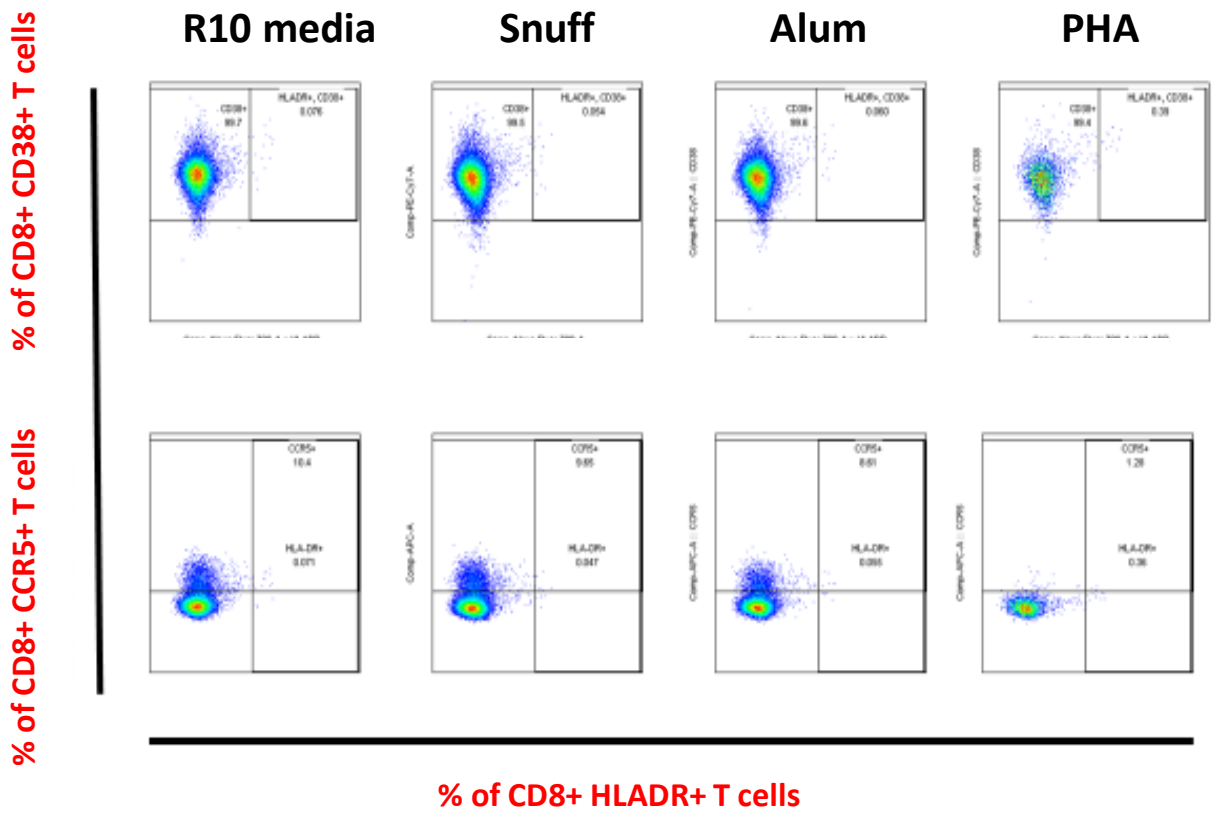

Figure 3.10. Representative flow cytometry dot plots showing the activation status (CD38, HLA-DR and chemokine receptor (CCR5) expression in CD8+ T cells following stimulating with snuff, alum, PHA and R10 media for 3 hours at 37 °C.

$CD8^+$ T cells were expressing the $CD38^+$ activation marker in all the conditions. $CCR5^+$ is slightly expressed in the negative control, snuff, and alum, however, T cells show significant CCR5 down-regulation following PHA stimulation (Figure 3.10). HLA-DR^+ $CD8^+$ T cells were not expressed in all conditions therefore in CD8 T cells were not highly activated (HLA-DR^+ $CD38^+$).

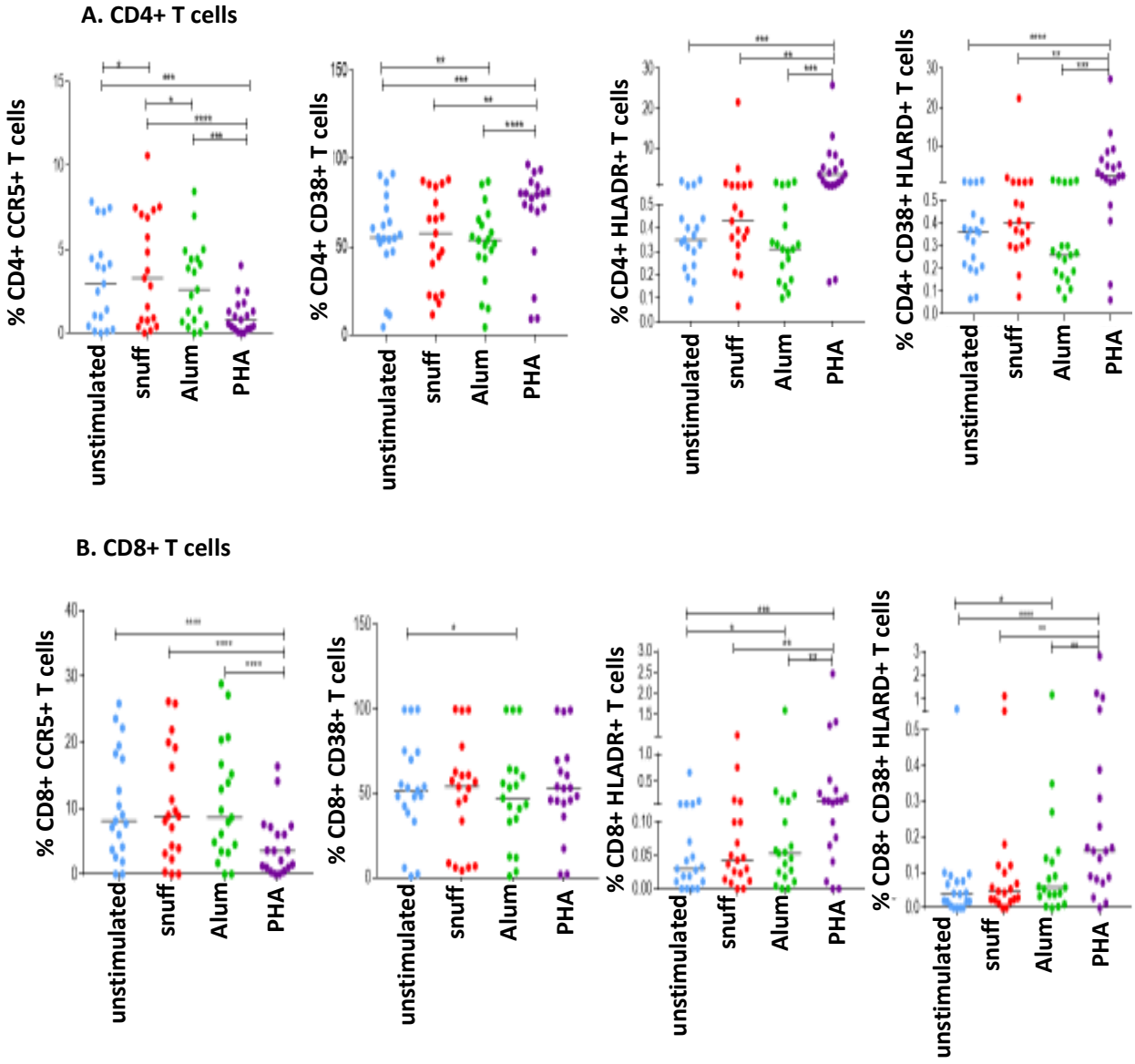

Figure 3.11. Cross-sectional analysis of IVIPs exposure-specific T cell responses in 26 donors. Scatter plots showing the expression of CCR5$^+$, CD38$^+$, HLA-DR$^+$ and CD38$^+$ HLA-DR$^+$ in CD4$^+$ T cells (panel A) and in CD8$^+$ T cells (panel B). PBMC samples were exposed to snuff, alum, and PHA for 3h, followed by staining and acquisition. Horizontal lines represent medians. Statistical comparisons were performed using the Wilcoxon test. Significant differences are shown by an asterisk (*) system (P < 0.05 *, P < 0.01 ** and P< 0.001*).**

In figure 3.11, the expression of CCR5$^+$ is significantly lower in PHA stimulated cells when compared to the unstimulated cells (p=0.0001), snuff (p=0.0001) and alum (p=0.0002)

exposed cells in the $CD4^+$ T cell population. Similarly, in the $CD8^+$ T cell population the expression of the CCR5 biomarker is significantly lower in PHA stimulated cells when compared to the unstimulated cells (p=0.0001) and also when compared to the snuff (p=0.0001) and alum (p=0.0001) exposed cells. In the $CD4^+$ T cells the CCR5 biomarker is significantly higher in the snuff exposed cells compared to the unexposed cells (p=0.0483) and also when compared to the alum exposed cells (p=0.0446).

The expression of $CD38^+$ biomarker in the $CD4^+$ T cell lymphocytes is significantly high in the PHA stimulation compared to the unstimulated cells (p=0.0001) and also when compared to the snuff (p=0.0061) and alum (p=0.0001) exposed cells. The $CD38^+$ activation marker is significantly highly expressed in unexposed $CD4^+$ T cells when compared to alum exposed $CD4^+$ T cells (p=0.0051). In $CD8^+$ T lymphocytes the CD38+ biomarker is significantly highly expressed in unexposed cells compared to the alum exposed cells (p=0.0185).

HLA-DR^+ biomarker in the $CD4^+$ T lymphocytes is significantly highly expressed in the PHA stimulated cells when compared to the unstimulated cells (p=0.0003) and also when compared to snuff (p=0.0071) and alum (p=0.0003) exposed cells. In the $CD8^+$ T lymphocytes the HLA-DR^+ biomarker is also significantly highly expressed in PHA stimulated cells compared to unstimulated cells (p=0.0003) and also when compared to snuff (p=0.0060) and alum p=0.0023) exposed cells. Alum exposed $CD8^+$ T cells also show a significantly higher expression of HLA-DR^+ when compared to the unexposed cells (p=0.0348).

In $CD4^+$ T lymphocytes the dual expression of HLA-DR^+CD38^+ is significantly higher in PHA stimulated cells compared to the unstimulated cells (p=0.0001) and also when compared to the snuff (p=0.0046) and alum (p=0.0002) exposed cells. Similarly, in the CD8+ T lymphocytes the dual expression of HLA-DR^+CD38^+ is significantly higher in PHA stimulated cells compared to the unstimulated cells (p=0.0001) and also when compared to the snuff (p=0.0054) and alum (p=0.0028) exposed cells. Alum exposed $CD8^+$ T cells also show a significantly higher expression of HLA-DR^+CD38^+ when compared to the unexposed cells (p=0.0208).

3.6 The effect of IVIPs exposure on biomarkers of inflammation using Bio-plex immunoassay

Cytokine measurements were evaluated on thawed supernatant specimens collected following exposing cells with snuff alum and PHA for 3 hours at 37 °C. The cytokine concentrations were measured by using the Bio-Plex Pro Human cytokine assay (Bio-Rad) as per manufacturer instruction. Only 12 donors were included for this part of the study in duplicates. Eleven cytokines were evaluated: pro-inflammatory cytokines (interleukin (IL)-1β, IL-6, tumour necrosis factor (TNF)–α and granulocyte-macrophage colony-stimulating factor (GM–CSF)); chemokines (interleukin (IL)-8, interferon gamma-induced protein (IP)-10, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)–1α and macrophage inflammatory protein (MIP)-1β)); Hematopoietic cytokines (interleukin (IL)-7) and regulatory cytokines (interleukin (IL)-10).

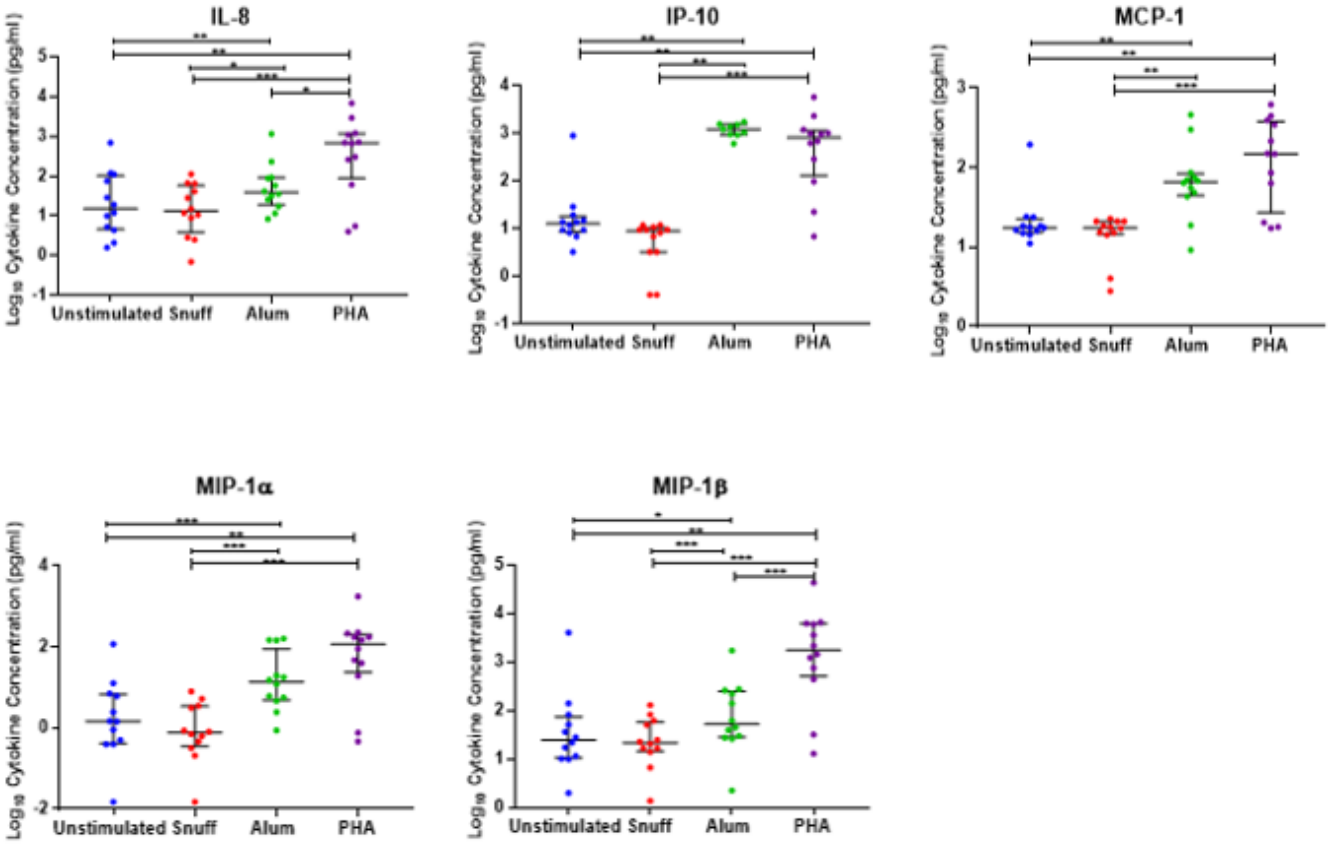

Figure 3.12. Shows Scatter plots comparison of chemokine concentrations in PBMC supernatants samples (n=12) harvested after cells were exposed to snuff, alum, PHA and R10 media for 3 hours at 37 °C. Chemokine concentrations were measured in supernatants from cells left unstimulated (blue circles), snuff exposed cells (red circles), alum exposed (green circles), and PHA stimulated (purple circles). Black solid lines represent the median, upper and lower quartile. P value ≤ 0.05 was considered significant. Abbreviations: IL-8, interleukin-8; IP-10, interferon-γ inducible protein-10; MCP-1, monocyte chemoattractant protein-1; MIP-1α and β, macrophage inflammatory protein-1alpha and beta.

In figure 3.12, PHA stimulation in all chemokines results to a significantly higher chemokine expression when compared to the unstimulated cells. Cells exposed to alum significantly had a higher expression of IL-8 chemokine compared to snuff exposed (p=0.0122). Cell exposure to PHA show a significantly higher expression of IL-8 when compared to both the alum and snuff exposure (p=0.0161) and (p=0.0010) respectively. IP-10 expression is significantly higher in cells exposed to alum compared to unexposed

cells (p=0.0039). Alum exposure results into a significantly higher expression of IP-10 when compared to snuff exposure (p=0.0039). PHA stimulated cells show a significantly increased expression of IP-10 when compared to snuff exposed cells (p=0.0010). The expression of MCP-1 in alum exposed cells is significantly higher when compared to the unexposed cells (p=0.0024) and also when compared to snuff exposed cells (p=0.0015). PHA Stimulated cells results into a significantly higher expression of MCP-1 when compared to snuff exposed cells (p=0.0005). MIP-1α expression in the alum exposed cells is significantly higher compared to the unexposed cells (p=0.0005) and also when compared to snuff exposed cells (p=0.0005). PHA stimulated cells expressed significantly higher MIP-1α when compared to the snuff exposed cells (p=0.0010). MIP-1β expression in alum exposed cells is significantly higher when compared to the unexposed cells (p=0.0161) and also when compared to snuff exposed cells (p=0.0010). MIP-1β expression is significantly higher in PHA stimulation compared to snuff exposed cells (p=0.0010).

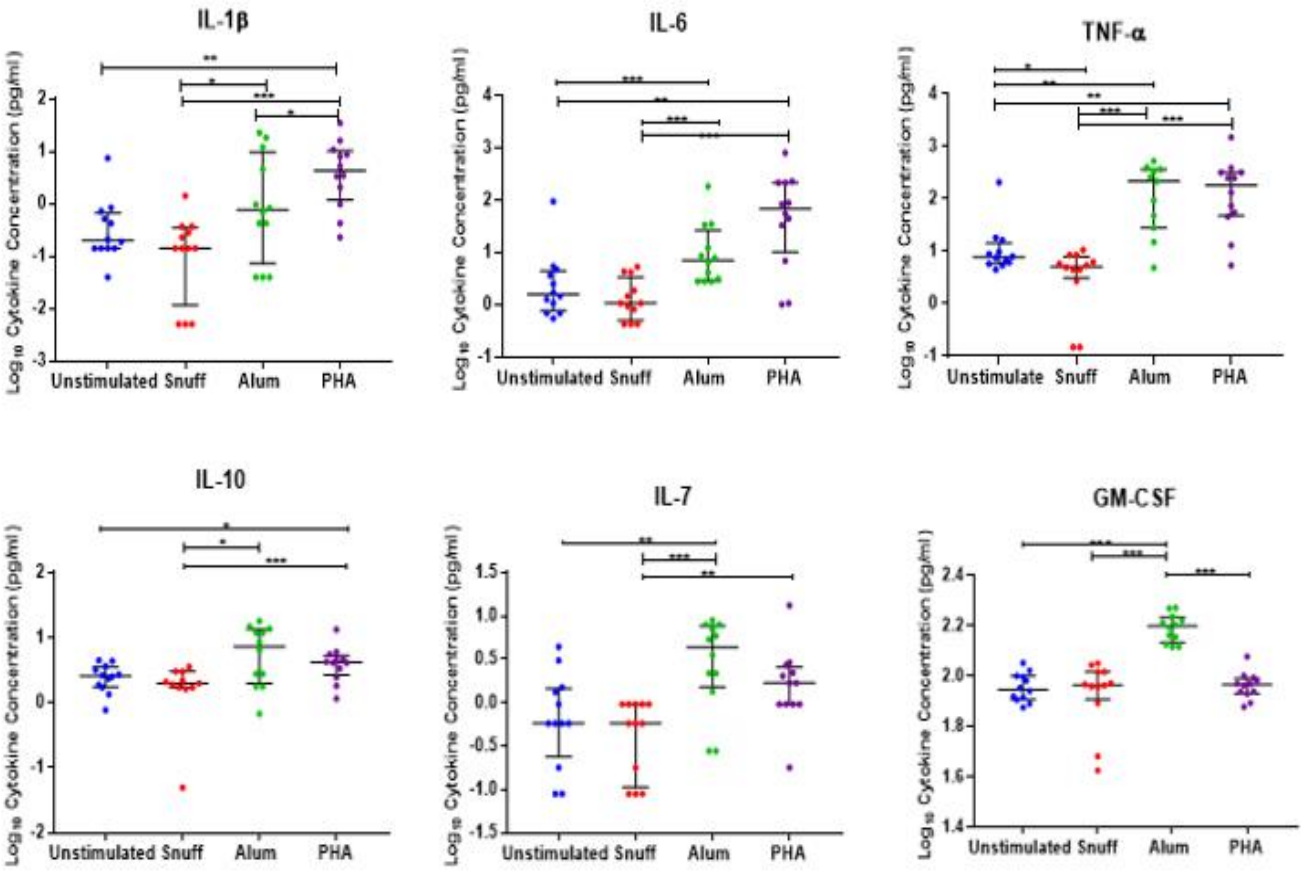

Figure 3.13. Shows Scatter plots comparison of pro-inflammatory (IL-1β, IL-6, TNF-α), hematopoietic (IL-7, GM-CSF) and regulatory (IL-10) cytokine concentrations in PBMC supernatants samples (n=12) harvested after cells were exposed to snuff, alum, PHA and R10 media for 3 hours at 37 °C. Cytokine concentrations were measured in supernatants from cells left unstimulated (blue circles), snuff exposed cells (red circles), alum exposed cells (green circles), and PHA stimulated cells (purple circles). Black solid lines represent the median, upper and lower quartile. P value ≤ 0.05 was considered significant. Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; TNF-α, tumour necrosis factor-alpha.

In figure 3.13, PHA stimulation show significantly higher expression of pro-inflammatory cytokines IL-1β, IL-6, TNF-α (p=0.0029, p=0.0034 and p=0.0024) and regulatory cytokine –IL-10 (p=0.0269) when compared to the unstimulated cells. Alum exposed cells show significantly higher expression of IL-6 and TNF-α (p=0.0005) and (p=0.0020) respectively when compared to the unexposed cells. Alum exposure show significantly higher

expression of pro-inflammatory IL-1β, IL-6, TNF-α (p=0.0322, p=0.0005, p=0.0010), regulatory IL-10 (p=0.0322) and also of hematopoietic IL-7, GM-CSF (p=0.0005, p=0.0005) cytokines when compared to the snuff exposure. PHA stimulation show significantly higher expression of pro-inflammatory IL-1β, IL-6, TNF-α (p=0.0005, p=0.0010, p=0.0005), regulatory cytokine IL-10 (p=0.0010) and also hematopoietic IL-7 (p=0.0088) cytokines when compared to snuff exposed cells.

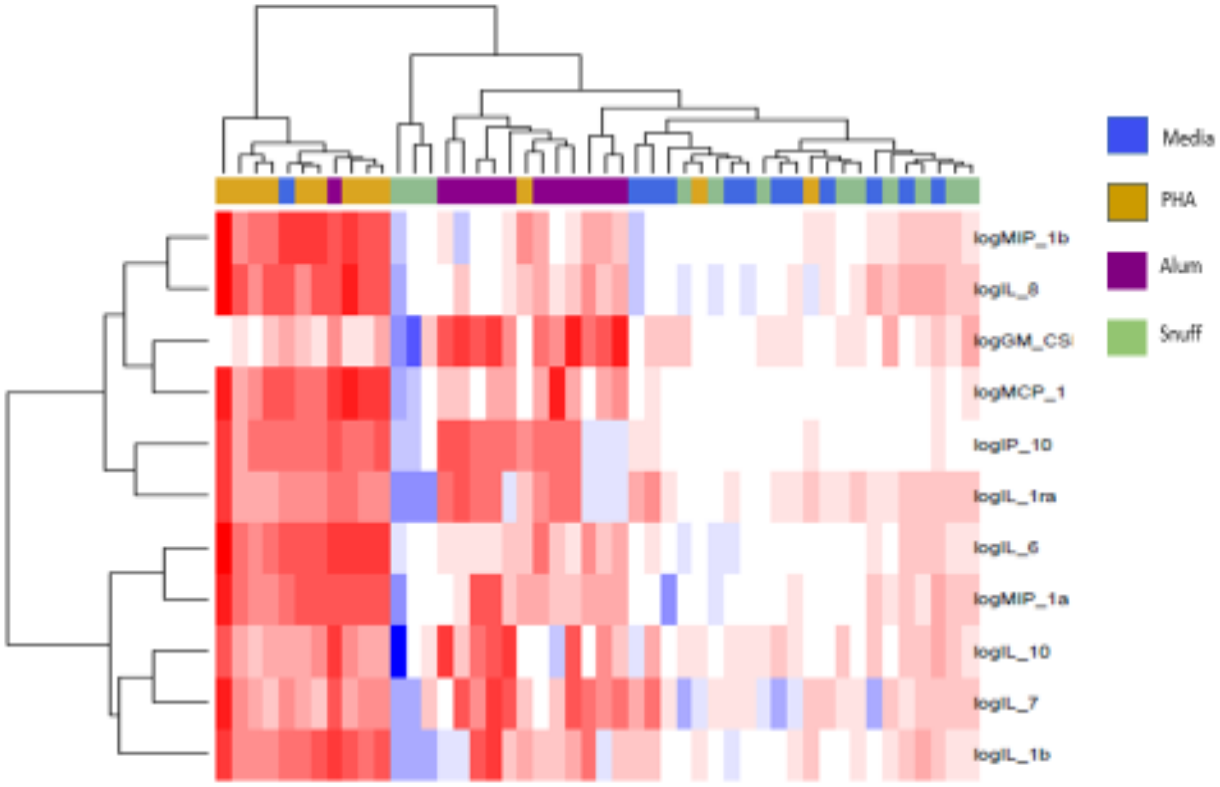

Figure 3.14. Unsupervised hierarchical clustering to show variation in cytokine expression by PBMCs cells following exposure to snuff (green boxes), alum (purple boxes), PHA (positive control; orange boxes) and unstimulated (blue boxes). Cytokine concentrations are indicated using a colour scale that ranges from blue (lowest) through pink to red (highest). The dendrogram above the heat map illustrates degrees of relatedness between cytokine profiles and different conditions. The dendrogram on the left-hand side of the heat map indicates relationships between the expression profiles of the 11 analysed cytokines. Supernatant samples are from 12 donors. Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; IP-10, interferon-γ inducible protein-10; MCP-1,

monocyte chemoattractant protein-1; MIP, macrophage inflammatory protein; TNF-a, tumour necrosis factor-alpha.

In figure 3.14, PHA stimulations show an upregulated cytokine concentration and tended to cluster together however, one PHA sample shows poor cytokine expression. Exposure to alum also show an upregulated cytokine expression and tended to cluster together next to PHA. Snuff exposed cells and media (unstimulated/unexposed) cells show a moderate to low cytokine expression, respectively and tended to cluster together however, one media control sample shows high cytokine expression.

CHAPTER 4: DISCUSSION

Many women use vaginal products for different reasons. However, the use of traditional or commercial vaginal insertion products may upset the normal pH in the vagina, which is important for maintaining the healthy vaginal immune barrier environment. Through the change of pH or direct mucosal trauma triggered by these products, inflammation, and activation of immune cells in response to trauma may lead to a higher risk of HIV and other infections. While IVIPs have been linked to increased risk of acquiring HIV (Allen et al., 2010, Fonck et al., 2001), the exact mechanism by which the use of IVIPs predispose women to elevated levels of acquiring HIV is poorly understood. In this era of a high burden of HIV, it has become crucial to find ways to reduce HIV infections, especially in young women, because they are mostly infected with HIV (UNAIDS, 2018). It is therefore essential to evaluate the exposure effect of vaginal products used by young women on immune cells, to possibly identify the events that may lead to HIV acquisition risk. This study used a PBMC model to determine the exposure effects of intravaginal insertion products on biomarkers of inflammation and cellular immune activation *in vitro*. The vaginal products evaluated in this study were kuber (chewing tobacco which contains ~20% nicotine, cannabidiol and delta 9-tetra cannabinol (THC); the primary ingredients is Indian hemp/marijuana), alum (Potassium aluminium sulfate), rose water (flavoured water made from steeping rose petals into the water, used as a vaginal douche) and snuff (tobacco which is made from ground or pulverized tobacco leaves and contains nicotine). Alum and snuff were selected for thorough assessment in this study because of their common use and also due to budget constraints.

IVIPs have been suspected of causing biochemical changes in the vagina (Van de Wijgert et al.,2000), they increase pH in the vagina, therefore, encouraging the growth of microorganisms associated with bacterial vaginosis which is evidently linked to increased risk of HIV infection acquisition in women (Atashili et al., 2008). Hence, it was crucial to evaluate the pH of intravaginal insertion products. The stock solution of Alum, rose water and savlon were acidic (pH of 2.62, 4.21 and 6.00 respectively). The results from this study confirm the hypothesis that some vaginal products may alter the normal vaginal pH of 4.5.

A study conducted by Cohen (1969), which showed that some Candida species are able to grow at a pH of 2 and lower and they can acidify the environment as a strategy to damage host tissues (Naglik et al., 2003).

Exposure of cells to alum and savlon resulted in high percentage of cell death, indicating the high toxicity effect of these products. The high toxicity of alum and savlon would imply that damage is inflicted by using these products unless they are diluted extensively (which is unlikely in real life). These IVIPs may cause physical damage to the epithelial layer and create an environment that favours the survival and growth of opportunistic organisms by exposing resident cells to damage-associated molecular pattern molecules (DAMPs) therefore leading to enhanced inflammation. One of the motivations behind the use of alum has been the dry sex effect (Humphries et al., 2019) and dry sex causes vaginal tears which are linked to increased risk of HIV acquisition (Dosekun and Fox, 2010). Hence, the change in the vaginal pH caused by alum together with the dry sex effect may predispose women to heightened risk of HIV acquisition, because the vaginal epithelium tissue damage would induce a protective inflammatory response and this response would bring more immune cells to the damaged area to help repair the damaged tissue while providing a doorway or an easy entry of the HIV virus into the female genital tract. Physical damage to vaginal epithelial cells could also mimic abrasions and allow leakage of IVIPs into underlying tissues. Aluminium hydroxide (ALOH) is used as an adjuvant in vaccines despite it toxicity. It has been shown that alum injection induces inflammation (White et al., 1955). However, it is unclear how alum triggers the inflammatory response, some believe that alum causes cytotoxicity leading to the leakage of DAMPs that eventually causes inflammation through TLR. A study by Hanfen et al., (2007) demonstrated that alum is capable of activating caspase-1 by inducing the release of mature IL-1β and IL-18 in cells that are activated by the TLR agonist. However, activation of caspase-1 is believed to require phagocytosis of alum particles. They further demonstrated that alum inhibited IP-10 production while it up regulated IL-10 production. There are many different forms of alum that are used in human studies, in this study we used $KAL(SO_4)_2$, in vaccines they use Aluminium hydroxide (ALOH) (Li et al., 2007), while in water purification they use aluminium hydro(Oxide) (AO) prepared from aluminium sulfate (Mulugeta et al., 2014). We don't know if the mechanism

of action of all the different forms of alum is the same or not, however, in all these different forms alum is acknowledged to be toxic. Future studies can compare the different forms of alum to determine if their mechanism of action is the same or if there are differences in how they work and their uses.

Snuff and kuber on the other hand, were quite basic (less toxic) stock solution pH 9.11 and 8.21 respectively. A basic pH may cause clearing of lactic acid-producing bacteria resulting in an unstable vaginal microflora. The disturbance of the normal vaginal microflora can lead to the up-regulation of immunological responses, causing inflammation in the vagina. Disequilibrium in the vaginal microbiota is termed BV, and it has been associated with more than three-fold increased risk of acquiring HIV infection (Atashili et al., 2008, Cohen et al., 2012). Women use IVIPs usually before a sexual encounter and in this case snuff may buffer the vaginal microenvironment and this alone may be detrimental to women's health, after a sexual encounter the addition of semen into the woman's vagina that was pre-treated with snuff may cause a double negative effect, because semen also neutralizes the vagina (Du Plessis et al., 2013) which may also further increase the risk of infection either by changing immune responses or creating favourable environment for STIs and other infections such as BV.

Following full optimization of a multiple colour flow cytometry panel, the CCR5 expression and the activation status of IVIP exposed T cells was examined by evaluating the expression of CCR5, HLA-DR, CD38 in T cells after no stimulation/IVIP exposure (negative control) and exposure to optimal concentrations of snuff, alum, and PHA (positive control). The cervicovaginal mucosa is lined with epithelial cells that are not susceptible to infection (Dezzutti et al., 2001). The vaginal epithelium has flattened squamous epithelial cells comprising of immune cells upon a breach, HIV can cross the physical barrier through breaks in the epithelium (Miller et al., 2005) and through microtears incurred as a result of sexual intercourse associated with vaginal trauma that may be exacerbated by an IVIP use. HIV penetrates the epithelium rapidly, and thereafter it has access to numerous susceptible target cells that lie within and below the epidermal layer, such as macrophages, dendritic cells, and. CD4 T cells that express CCR5 are the main target of HIV in the mucosa (Li et al., 2015).

Increased susceptibility to HIV infection of activated CD4-T cells has been correlated with increased CCR5 expression (Meditz et al., 2011). Hyperimmune activation of T cells, effected by PHA stimulation, was accompanied by a reduced expression of CCR5 in T cells in this study. Contrary snuff exposed cells results in significantly more CCR5 expression in CD4+ T cells, compared to unstimulated cells, alum and PHA stimulations. Paxton et al. (1998) associated low CCR5 cell surface expression after PHA stimulation with high levels of β-chemokines. Hence, the CCR5 cell surface receptor gets internalized after binding to its natural ligands (Alkhatib et al., 1997), which are upregulated upon activation and high β-chemokine levels may consequently result in reduced CCR5 expression. These previous findings, therefore, suggest that CCR5 expression and levels of β-chemokines should be used together to determine the ultimate levels of CCR5 cell surface expression. The β-chemokines measured in this study were MIP-1α and MIP-1β, and these were both significantly more produced following exposure to alum and PHA, compared to snuff stimulated and unstimulated cells, and this might explain the lower surface expression of CCR5 in PHA and alum stimulated cells. PBMC exposure to snuff, on the other hand, led to significantly higher expression of CCR5 by CD4 T cells, and in addition, MIP-1α and MIP-1β were both significantly less produced following snuff exposure. In this study, it was shown that higher expression of CCR5 expressed by CD4 T cells after exposure to IVIPs was only evident when β-chemokines levels were low, and this was the case with cells exposed to snuff. As shown in Appendix A (Table 1), there was a weak but significant negative association between MIP-1β and CCR5 expression by CD4+ T cells which were unstimulated ($r=-0.165$, $p=0.01$), exposed to snuff ($r=-0.069$, $p=0.016$) and alum ($r=-0.142$, $p=0.015$). This association between MIP-1β and CCR5 expression was not significant in CD4+ T cells stimulated with PHA (APPENDIX A, Table 1). This may be due to the hyper activation effect of PHA or a small sample size, since there were only 12 donors with matched data for CCR5 expression and chemokine concentrations.

To assess the T cell activation effect by IVIPs, the CD38 and HLA-DR activation markers were measured because these cell surface markers are upregulated upon activation, each at a different stage of the activation process.CD38 marker is an early T cell activation marker while HLA-DR is a late T cell activation marker (Bajnok et al., 2017, Tenca et al., 2003).

Dual expression of CD38 and HLA-DR was also assessed because these cells are considered to be highly activated cells and may be more appropriate for evaluating the effect of IVIPs on immune activation. Dual expression of CD38 and HLA-DR by $CD4^+$ T cells and $CD8^+$ T cells was significantly higher in PHA stimulated cells. Similarly, $CD8^+$ T cells exposed to alum, had significantly higher dual expression of CD38 and HLA-DR compared to unstimulated cells, indicating the presence of highly activated $CD8^+$ T cells following alum exposure. CD38 and HLA-DR dual expression by $CD8^+$ T cells did not change significantly in snuff stimulated cells compared to unstimulated cells. The dual expression of CD38 and HLA-DR on T cells determined the hyperactivation status, and alum is therefore associated with hyperactivation of $CD8^+$ T cells because of the co-expression of CD38 and HLA-DR that was significantly increased in alum stimulated $CD8^+$ T cells. Alum seemed to enhance the activity of CD8 T cells in this model, and this may be associated with its adjuvant properties.

In addition to alum inducing higher levels of immune activation (especially in the $CD8^+$ T cell population) compared to unstimulated cells and snuff, alum induced significantly higher levels of inflammation (measured by expression of a broad panel of inflammatory, anti-inflammatory and haematopoietic cytokines). The comparatively elevated levels of inflammation in alum stimulated cells could be due to several factors such as the immunity-boosting effect of alum. The adjuvant effects of alum have been attributed to its ability to prolong antigen exposure to the immune system. However, from the results observed in this study and other studies (Kool et al., 2008, Li et al., 2008, McKee et al., 2009) it is clear that alum can be recognized by the innate immune system leading to upregulation of several pro-inflammatory cytokines and chemokines.

Cytokines and chemokines (except IL-1β and IL-10) were significantly elevated in alum stimulated cells, compared to snuff and unstimulated cells. The cytokine concentrations in alum stimulated cells were in most cases, similar to the concentrations of cytokines in PHA (positive control) stimulated cells. A number of studies have investigated how alum achieves these inflammatory effects. A number of *in vitro* experiments have shown that alum activates the NLRP3 inflammasome in macrophages which, in turn, activates caspase-1 and the

consequent production of cytokines (Eisenbarth et al., 2008, Kool et al., 2008, Li et al., 2008). A study byMcKee et al. (2009), showed that many pro-inflammatory cytokines and chemokines are rapidly produced *in vivo* after exposure to alum. Eosinophils, neutrophils, monocytes, NK cells, NKT cells, and DCs, are rapidly recruited to the site of injection. Similarly, a strong inflammatory state was associated with alum exposure in this study. Cells exposed to snuff, on the other hand, showed a lower cytokine and chemokine expression and its effect was not statistically different from unstimulated cells.

The observations made in this study may explain an increase in inflammatory cytokines previously seen in women who had reported using products other than water for intravaginal practices (Kyongo et al., 2015). The use of IVIPs, particularly alum, could possibly, be associated with vaginal inflammation, which has been associated with a high risk of acquiring HIV infection (Masson et al., 2015). HIV infects and replicates more efficiently in an inflammatory environment (Narimatsu et al., 2005). Recruitment of activated cells after the use of alum may increase susceptibility to HIV infection. MIP-1α and MIP-1β are the CCR5 ligands, and they are upregulated upon activation (Lederman et al., 2006), and they inhibit the entry and fusion of HIV by downregulating the chemokine receptor (Moody et al., 2010). This can be used to explain the result discussed above of CCR5 downregulation, and chemokine elevation in alum stimulated cells. However, these chemotactic chemokines also play a huge role in inflammation, which is known to recruit T cells that are preferentially infected by HIV, especially CD4 T cells that are attracted by MIP-1β (Bhavsar et al., 2014). Pro-inflammatory cytokines play a role in responding to foreign antigens; however, these cytokines in large quantities could potentially induce tissue damage (Czaja, 2014). We noted elevated pro-inflammatory cytokines (TNF-α, IL-8, IL-6, GM-CSF) after exposure to alum thus, the use of alum may exert some damage in the vaginal tissue. The effect of IVIPs on the vaginal tissue would be influenced by the cytokine levels and how long it takes for women to douche with these products and the frequency of use of these products. The PBMC model used in this study does not show any effect of snuff on the immunological markers which were investigated in this study, therefore we are unable to draw conclusions about snuff, although the pH of snuff is a lot higher than the ideal vaginal pH, which may exert a negative effect on the vaginal microenvironment as has been discussed above. Snuff also

seemed to increase the expression of CCR5 receptors on CD4+ T cells, which may likely increase the risk of HIV acquisition.

Given that the experimental work done in this study was on PBMCs and not vaginal cell lines, we are unable to assign the effects of alum douching on vaginal cells conclusively. We acknowledge that PBMCs may not behave in the same manner as vaginal cell lines. PBMCs are one of the best models for assessing immunological responses and their characteristics are very interesting because they are primary cells, they are not immortalized, have the genetic backgrounds of real patients, and they are easy to isolate from whole blood (Burczynski and Dorner, 2006). The limitation is that PBMCs alone cannot completely mimic what would take place *in vivo*. There are several other limitations, such as the amount of the products used by women could not be established as this varies from individuals. The concentrations of IVIPs used and the duration of exposure were solely based on cell viability. The duration of IVIP use by women is not known as it also varies from individuals.

In conclusion, this study is the first of its kind to identify possible mechanisms that link intravaginal insertion products and HIV. Alum, in particular, was more inflammatory compared to snuff. Future research can extend the current pilot study by looking at the effect of intravaginal insertion products on the actual vaginal cells. Knowledge from this work and future studies is crucial in developing new female-initiated interventions for preventing HIV acquisition through sexual mucosal transmission taking into account the use and impacts of IVIPs. Since a lot of women in South Africa, particularly in KZN, engage in intravaginal practices, education on vaginal health is critical. Women need to be educated about IVIPs and their possible impact on the vaginal flora, vaginal PH, epithelial damage and inflammation because women naively engage in intravaginal practices to merely have control over their sexuality and to improve hygiene without realizing the potentially harmful nature of these products and the negative effect these may exert on their vaginas and overall wellbeing.

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APPENDIX A: RELATIONSHIP BETWEEN CD4+ T CELL IMMUNE ACTIVATION AND BIOMARKERS OF INFLAMMATION

Table- 1. Spearman's correlation observed between chemokines and CCR5 expression levels by CD4+ T cells in 12 donor PBMCs exposed to IVIPs.

		CCR5+ expression by CD4+ T cells			
Chemokine		**Unstimulated**	**Snuff**	**Alum**	**PHA**
MIP-1α	r	-0.087	0.027	0.273	0.241
	p-value	**0.003**	**0.005**	**0.006**	0.293
MIP-1β	r	-0.165	-0.069	-0.142	0.273
	p-value	**0.010**	**0.016**	**0.015**	0.445
IL-8	r	-0.060	-0.007	-0.361	0.262
	p-value	**0.008**	**0.013**	**0.014**	0.962
IP-10	r	-0.173	0.321	-0.746	0.352
	p-value	**0.007**	**0.008**	0.095	0.905
MCP-1	r	-0.224	0.223	0.104	0.177
	p-value	**0.008**	**0.013**	**0.012**	0.536

Table- 2. Spearman's correlation observed between cytokines and CCR5 expression levels by $CD4^+$ T cells in 12 donor PBMCs exposed to IVIPs

		CCR5+ expression by CD4+ T cells			
Cytokine		Unstimulated	Snuff	Alum	PHA
IL-7	r	-0.235	0.297	0.040	0.355
	p-value	**0.002**	**0.003**	**0.004**	**0.007**
GM-CSF	r	-0.190	0.024	0.193	-0.317
	p-value	**0.013**	**0.025**	**0.017**	0.461
IL-10	r	-0.029	0.176	-0.092	0.288
	p-value	**0.003**	**0.008**	**0.005**	**0.022**
IL-6	r	-0.214	-0.053	-0.355	0.188
	p-value	**0.004**	**0.006**	**0.007**	0.251
IL-1β	r	-0.521	0.236	0.053	0.162
	p-value	**0.002**	**0.002**	**0.002**	**0.021**
TNF-α	r	-0.305	0.204	-0.020	0.209
	p-value	**0.006**	**0.007**	**0.030**	0.538

Table- 3. Spearman's correlation observed between chemokines and activation marker expression levels by CD4+ T cells in 12 donor PBMCs exposed to IVIPs.

		CD38+ expression by CD4+ T cells				HLA-DR+ expression by CD4+ T cells			
chemokine		Unstimulated	Snuff	Alum	PHA	Unstimulated	Snuff	Alum	PHA
MIP-1α	r	0.625	0.264	0.234	0.087	-0.715	-0.131	-0.080	0.256
	p-value	**0.0003**	**0.0002**	**0.0005**	**0.0003**	0.717	0.260	**0.003**	**0.001**
MIP-1β	r	0.367	0.178	0.224	0.036	-0.269	-0.017	0.211	0.205
	p-value	**0.0004**	**0.0002**	**0.0005**	**0.0004**	**0.0001**	**p<0.0001**	**p<0.0001**	**p<0.0001**
IL-8	r	0.497	0.174	0.346	0.262	-0.340	-0.259	0.046	0.255
	p-value	**0.0004**	**0.0002**	**0.0005**	**0.0004**	**0.0006**	**0.002**	**p<0.0001**	**p<0.0001**
IP-10	r	0.508	-0.337	-0.063	-0.028	-0.364	0.086	-0.063	0.329
	p-value	**0.0004**	**0.0002**	**0.002**	**0.0004**	**0.0002**	**0.012**	**p<0.0001**	**p<0.0001**
MCP-1	r	0.431	-0.199	0.526	-0.079	-0.175	0.125	-0.065	0.323
	p-value	**0.0004**	**0.0003**	**0.0005**	**0.0004**	**p<0.0001**	**p<0.0001**	**p<0.0001**	**p<0.0001**

Table- 4. Spearman's correlation observed between cytokines and activation marker expression levels by CD4+ T cells in 12 donor PBMCs exposed to IVIPs

		CD38+				HLA-DR+			
Cytokine		Unstimulated	Snuff	Alum	PHA	Unstimulated	Snuff	Alum	PHA
IL-7	r	0.044	0.191	0.553	0.261	-0.030	-0.505	-0.665	0.217
	p-value	**0.0003**	**0.0002**	**0.0004**	**0.0003**	0.103	**0.012**	0.375	0.407
GM-CSF	r	0.145	-0.282	0.153	-0.395	0.306	0.278	-0.190	0.706
	p-value	**0.0005**	**0.0003**	**0.0006**	**0.0004**	**p<0.0001**	**p<0.0001**	**p<0.0001**	**p<0.0001**
IL-10	r	0.267	-0.121	0.210	0.122	-0.231	0.141	-0.210	0.140
	p-value	**0.0004**	**0.0006**	**0.0005**	**0.0003**	**0.011**	0.9299	**0.029**	0.306
IL-6	r	0.484	0.220	0.211	0.039	-0.266	-0.354	0.251	0.300
	p-value	**0.0003**	**0.0002**	**0.0005**	**0.0003**	0.202	0.627	**0.002**	**0.0008**
IL-1β	r	0.356	0.236	-0.062	0.162	-0.278	0.087	-0.020	0.307
	p-value	**0.0003**	**0.0002**	**0.0004**	**0.0003**	**0.007**	**0.0005**	0.423	0.443
TNF-α	r	0.339	-0.246	0.207	0.059	-0.280	0.087	-0.020	0.307
	p-value	**0.0004**	**0.0002**	**0.0013**	**0.0004**	**p<0.0001**	0.158	**p<0.0001**	**p<0.0001**

APPENDIX B: ETHICAL APPROVAL

INYUVESI
YAKWAZULU-NATALI

19 August 2016

Ms RZ Hlophe (209508525)
Discipline of Medical Microbiology
School of Laboratory Medicine and Medical Sciences
zhlophe@ymail.com

Title: ***in vitro*** **effect of exogenous vaginal insertion products (VIPs) on biomarkers of genital inflammation and cellular activation.**
Degree: MMedSc
BREC REF NO: BE382/16

EXPEDITED APPLICATION

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 22 June 2016.

The study was provisionally approved pending appropriate responses to queries raised. Your response dated 15 July 2016 to queries raised on 07 July 2016 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval and may begin as from 19 August 2016.

This approval is valid for one year from **19 August 2016.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **RATIFIED** by a full Committee at its next meeting taking place on **13 September 2016.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Professor J Tsoka-Gwegweni
Chair: Biomedical Research Ethics Committee