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INYUVESI YAKWAZULU-NATALI

Type I, II, and III interferon responses in the female genital tract.

By

Slindile Ngubane (217016499)

Supervisor: Dr Aida Sivro

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College of Health Sciences

University of KwaZulu-Natal

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ABSTRACT

Background: Interferon (IFN) responses have the ability to restrict HIV replication at the point of exposure as well as during acute infection, however, they also contribute to persistent immune activation and $CD4^+$ T cell depletion during chronic infection. IFN responses restrict viral replication at the female genital tract, however, their expression and regulation can be affected by other co-infections, bacterial vaginosis as well as sexual and hygienic practices. This could be particularly important for HIV transmission risk in young women. Here, we aimed to determine the causes and consequences of type I, II, and III interferon upregulation at the female genital tract (FGT) in the context of HIV infection.

Methods: Five interferons (IFNα2, IFNβ, IFNγ, IFNλ1, IFNλ2) were measured using the Bio-Plex Pro Human Inflammatory Panel 1 Assay in 238 longitudinal softcup samples from the CAPRISA 083 STI study that investigated point of care testing and treatment for BV/STIs . IFN concentrations in softcup samples of women with STIs/BV (n=170) were compared to samples without STIs/BV (n=68) at baseline. Linear and logistic regression models were utilized to assess the association between mucosal IFN expression and the presence of BV/STIs in women. The association between IFNs and other mucosal cytokine/chemokines was measured using linear regression models. Effects of BV/STI treatment on IFN expression at three-time points (baseline, 6-, and 12-weeks post treatment initiation) was assessed. Additionally, we measured IFN expression in softcup specimens from the CAPRISA 012C study, that investigates passive antibody immunization for HIV prevention. In matching cytobrush samples, we used flow cytometry to characterize $CD4^+$ T cell phenotype. The effect of mucosal IFN expression on $CD4^+$ T cell profiles was assessed using Spearman correlation analysis.

Results: Following the univariable and multivariable analyses adjusting for participant age, contraceptive use, and number of sex partners in the last two months; IFNα2 was significantly associated with presence of BV/STIs (aOR 2.677, 95% CI 1.125-6.366, p=0.026). In the multivariable model, correcting for participant age, contraceptive use and number of sex partners in the last two months we observed significant associations between concentrations of IFNα2 and increased IL-10 (p=0.021), IL-4 (p=0.006), IL12p70 (p=0.030), FGF-basic (p=0.043), PDGF-BB (p=0.005), and decreased IL-3 (p=0.019). Additionally, we observed significant association between

IFNβ and decreased IL-18 (p=0.006); IFNγ and decreased IL-1RA (p=0.045) and GM-CSF (p=0.006); IFNλ1 and decreased IL-10 (p=0.009), TNF-β (p=0.018), Eotaxin (p=0.004), IL-3 (p=0.046), IL-9 (p=0.004) and M-CSF (p=0.050); IFNλ2 and increased IL-2 (p=0.014), decreased TRAIL (p=0.018) and LIF (p=0.033). No significant effect of BV and/or STI treatment on mucosal IFN expression were observed. We observed that IFNα2 was positively correlated with percentage of CD69+ $CD4^+$ T cells (r=0.3007, p=0.0185), CCR5+CCR6+ (r=0.2707, p=0.0436) as well as total CCR6+ activated T cells (r=0.2878, p=0.0315) and %Treg-like $CD4^+$ T cells (r=0.2985, p=0.0195). IFNα2 was also negatively correlated with %HLADR+CD38- CD4+ T cells (r=-0.2711, p=0.0346). There were no significant correlations observed between $CD4^+$ T cell profiles and IFNβ, IFNγ, IFNλ1 and IFNλ2.

Conclusion: The IFNs play an important role in the FGT by contributing to the local immune defence against infections, particularly viral infections. Our study highlights the negative side of chronic IFN upregulation. We identified STIs and BV as important drivers of IFNα2 levels and inflammation at the female genital tract. Importantly, mucosal IFNα2 levels were associated with presence of HIV target cells at the FGT. If IFNs are to be used for treatment and prevention of viral FGT infections, the impact of other variables such as microbiome and other co-infections on IFN signalling and regulation needs to be considered.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
APC	Antigen presenting cell
α4β7	Alpha-4-beta-7
BMI	Body mass index
BV	Bacterial vaginosis
CAPRISA	Centre for the AIDS programme of research in South Africa
CCR5	Chemokine (C-C motif) receptor type 5
CHW	Community health workers
COVID-19	Coronavirus disease 2019
CVL	Cervicovaginal lavage
DC	Dendritic cells
EGF	Epidermal growth factors
FCS	Flow cytometric standard
FGF	Fibroblast growth factor
FGT	Female genital tract
FRT	Female reproductive tract
GALT	Gut-associated lymphoid tissue
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSV-2	Herpes simplex virus 2
IFN	Interferon
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor 1
IL-10	Interleukin 10
IP-10	Interferon gamma induced protein 10
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
JAK-STAT	Janus kinase-signal transducer and activator of transcription
KZN	KwaZulu-Natal
M-CSF	Macrophage colony-stimulating factor

MALT	Mucosa-associated lymphoid tissue
MIP-1α	Macrophage inflammatory protein 1 alpha
MIP-1β	Macrophage inflammatory protein 1 beta
MMP	Matrix metalloproteinases
NALT	Nasopharyngeal-associated lymphoid tissue
NHPs	Non-human primates
NFκB	Nuclear factor kappa B
NK	Natural killer
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
pDCs	Plasmacytoid dendritic cells
PDGF-BB	Platelet-derived growth factor BB monomer
PID	Participants identification
PRR	Pattern recognition receptors
RT	Room temperature
SA	South Africa
SA-PE	Streptavidin-phycoerythrin
$sIgA^+$	Surface immunoglobulin A positive
SIV	Simian immunodeficiency virus
STI	Sexually transmitted infection
TGF-β	Transforming growth factor beta
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
TNF	Tumor necrosis factor
TLR	Toll-like receptors
TRIAL	TNF-related apoptosis-inducing ligand
VEGF	Vascular endothelial growth factor
UN	United Nations
UNAIDS	Joint United Nations Programme on HIV/AIDS

Chapter 1: Introduction

1.1 HIV/AIDS Epidemic

1.1.1 Global HIV statistics

Human Immunodeficiency Virus (HIV) is a virus that targets and weakens the body's immune system resulting in a compromised response against other viral and bacterial infections. HIV and the resulting Acquired Immunodeficiency Syndrome (AIDS) continue to be a major global public health issue, claiming 40.4 million lives since the start of the epidemic. In 2022, there were an estimated 39 million people living with HIV, with 37.5 and 1.5 million being adults and children between the ages of 0-14 years, respectively, and 53% being women and girls (UNAIDS, 2023). There were, also, 1.3 million people who became newly infected with HIV (reduction of 59% since the peak observed in 1995) as well as 630 000 people who died from HIV/AIDS-related illnesses. As of the end of December 2022, 29.8 million people had access to antiretroviral therapy (ART) which is more than three times higher than 7.7 million people who had access to it in 2010 (UNAIDS, 2023).

The 90-90-90 targets proposed by the Joint United Nations Programme on HIV/AIDS (UNAIDS) in 2014 were later adopted by the UN General Assembly and officially proposed as the global target for 2020. These targets set out goals of having 90% of HIV-infected people know their status; have 90% of HIV-positive people accessing treatment; and 90% of those accessing treatment have their viral loads suppressed. These targets were missed at the end of 2020, although not by a wide margin, with 84% of HIV-infected people knowing their HIV status, 87% of HIV-positive people having access to treatment and 90% of people accessing treatment being virally suppressed (Global, 2021, UNAIDS, 2021). Within Africa, 7 countries (including Botswana, Eswatini, Namibia, Rwanda, Uganda, Zambia and Zimbabwe) had reached the set targets. The new 95-95-95 testing and treating targets set to be achieved by the end of 2025 are hoped to significantly reduce new HIV infections, AIDS-related deaths and HIV inequalities while aiding with getting the global HIV response back on track in order to end AIDS as a public threat by 2030. According to UNAIDS (2023), access to HIV treatment has resulted in the prevention of almost 20.8 million AIDS-related death in the past three decades which is an overall reduction of 69% since the

peak in 2004. In 2022, 71% of people living with HIV had reached viral load suppression enabling them to live long healthy lives while having zero risk of sexually transmitting HIV.

The coronavirus disease 2019 (COVID-19) pandemic had a profound impact on HIV treatment and prevention efforts. COVID-19 mitigation measures such as national lockdowns limited people's movement and international travels bans and flight cancellations affected the transportation and supply of medicine (Abdool Karim and Baxter, 2022). This resulted in a decline in the number of patients who attended health care facilities, subsequently affecting HIV testing and monitoring (Doherty, 2020). Furthermore, the Global fund to fight AIDS, Tuberculosis and Malaria reported that there was a 41% and 37% decrease in HIV testing and referrals for diagnosis and treatment, respectively, during the first, most stringent, lockdowns in 2020, compared to the same period in 2019 (UNAIDS, 2021). A rise in annual new infections was recorded in Eastern Europe, central Asia, Middle East, North America and Latin America over the past decade. In 2021, everyday an estimated 4000 people became infected with HIV, with one death due to HIV/AIDS occurring every minute. The delay in progress on HIV has devastating consequences. If the current trends continue, 1.2 million people will become infected with HIV in 2025, which is three times the target of 370,000 new infections (UNAIDS., 2022).

1.1.2 HIV in Sub-Saharan Africa

For the past forty years since the discovery of HIV, sub-Saharan Africa remains the most impacted region by the HIV epidemic. Over two thirds (67%) of the 39 million people infected globally reside in this region despite the fact that it only accounts for 14.78% of the global population. In 2022, sub-Saharan Africa accounted for 63% of the 4000 new HIV infections, daily, amongst adults and children (UNAIDS, 2023). South Africa (SA) continues to carry the highest global burden of HIV with approximately 7.6 million people reported to be living with HIV in 2022 (Stats, 2022). Swaziland carries the highest HIV prevalence rate with 25.9% of adults living with HIV, followed by SA, Mozambique, and Namibia at 17.8%, 11.6% and 11%, respectively. In SA, the KwaZulu-Natal (KZN) province holds the highest HIV prevalence rate

among adults (17.6%) and pregnant women (41.1%) (Simbayi et al., 2019, Woldesenbet et al., 2019).

1.2 Vulnerable groups and key populations at increased risk of HIV

Key populations include people who are at increased risk of acquiring HIV due to their behavior while vulnerable groups include people whose living conditions are placing them at risk of HIV infection, with the two often overlapping.

Key populations as well as their sexual partners fall under the people who are at greater risk of acquiring HIV, having accounted for 70% of the global new HIV infections (UNAIDS, 2021). These include gay men and other men who have sex with men who are at 28 times greater risk of acquiring HIV as opposed to heterosexual men; female sex workers who are at 30 times greater risk as opposed to women in the general population; transgender women who are at 14 times greater risk as opposed to other adults; as well as people who inject drugs who are at 35 time greater risk than the people who do not inject drugs (Global, 2021, UNAIDS, 2021).

Globally, women and girls accounted for more than half (53%) of the people who are living with HIV as well as 46% of all new infections in 2022 (UNAIDS, 2023). Furthermore, around 4000 adolescent girls and young women between the ages of 15-24 years became HIV infected per week, with 3100 of these infections occurring in sub-Saharan Africa. Generally, women acquire HIV through heterosexual transmission and are likely to contract it, at least, five to seven years earlier than men (Karim et al., 2010b), thus resulting in the global prevalence among young girls and women being higher than that observed among males of the same age (United Nations Programme on HIV/AIDS and HIV/AIDS, 2013). In KZN, HIV prevalence in women aged 15-24 years was 22.3% compared to 7.6% in men of similar age (Kharsany et al., 2018).

Additionally, the COVID-19 pandemic has also had a profound effect in heightening pre-existing vulnerability among women who are at high risk of acquiring HIV due to combined negative economic and social effects. Factors such as the epidemic of domestic violence against women was greatly intensified worldwide during the pandemic especially in African countries like Nigeria and SA where it was more common amongst young women (aged 15-24) living with HIV (Lamontagne et al.,

2022). Furthermore, the number of births in young girls/teenagers (aged 10-14) and adolescent girls (aged 15-19) increased by 48.7% and 17.9%, respectively, between 2017 and 2021 in SA (Barron et al., 2022). This highlights the vulnerabilities of young women and demands that the underlying socio-economic issues, such gender power disparity, as well as access to care, be addressed urgently.

1.3 Factors associated with HIV risk

1.3.1 Social and behavioral factors associated with increased risk of infection

HIV acquisition risk varies between different individuals and is dependent on a number of both biological and behavioral factors. Factors such early age of sexual debut, which is defined as having had sexual intercourse at or before the age of 16, have been associated with ensuing sexual behaviors that are risky such as having multiple partners, low condom and contraceptive use (Greenberg et al., 1992, Manning et al., 2000). In Africa, studies have shown that there is a correlation between engaging in sexual acts at a young age and HIV incidence (Boileau et al., 2009, Pettifor et al., 2004). Additionally, young women being sexually partnered with older men, who may have contracted HIV from women of similar age, is a key factor that is driving HIV transmission/prevalence in SA. This has been observed in a study conducted in KZN, suggesting that men between the ages of 25-40 years were the primary source of HIV acquisition in women between the ages of 15-25 years. It was also stated that majority of these men had contracted HIV from women between the ages of 25-40 years (de Oliveira et al., 2017).

A diverse set of vaginal practices such as drying, tightening and intra-vaginal cleaning have placed women at a higher risk of acquiring STIs and HIV (Scorgie et al., 2009). Women in some southern African countries practice “dry sex” which involves inserting drying agents into the vagina such as a dry cloth, herbs or even chemicals in order to produce the required dry, tight and “hot” vagina (Ramjee and Daniels, 2013). This practice is believed to provide sexual pleasure that is heightened for the male partner. However, for women, it causes friction which may lead to tearing of delicate vaginal mucosal lining, thus increasing the risk of acquiring HIV. Furthermore, the use of chemicals cause inflammation, lesions, and alters the vagina’s natural pH level also potentially increasing the risk of contracting HIV and other STIs (Ramjee and Daniels,

2013). In a study conducted by Smit et al. (2011) in KZN, it was reported that most women (90.2%) performed at least one vaginal practice with internal cleaning being the most common one. Although, hygiene was observed as the driving factor, 23.2% of women still practiced intravaginal cleaning around the time of sex.

Studies conducted previously have also shown that there is a relationship between poverty and HIV (Gebreegziabher et al., 2020, Naidoo et al., 2017). This has been observed globally as well as in SA (Gibbs et al., 2020, Poku et al., 2020). In 2020, KZN had more than 11 million people with approximately 3.2 million being extremely impoverished (Statistics South Africa, 2020). Ranking as the third poorest province in SA, this overlaps with the high HIV prevalence. Since community health workers (CHW) first serve communities that are living below the upper-bound poverty line (Stats, 2018) and help improve the community's access to primary health care, the shortage of CHW observed in KZN has led to some communities not being able to access the HIV services offered by them, thus missing the opportunity to be linked to basic health care (Haber et al., 2017, Perriat et al., 2018).

Alcohol abuse in the form of weekend binge drinking also tends to have effects on sexual decision making, condom negotiation skills and condom use. Women who partake in heavy episodic drinking have demonstrated to be more prone to use condoms inconsistently and incorrectly, be subjected to sexual violence and are at a higher risk of acquiring STIs, including HIV (Chersich and Rees, 2008, Scorgie et al., 2012).

1.3.2 Biological factors associated with HIV acquisition

Research studies examining the complex interactions between host, virus, and environment, have identified a number of genetic and immunological contributors to the variation in HIV susceptibility. One of the best characterized genetic factors linked to natural HIV resistance is the delta 32 mutation in the CCR5 gene, present in 10% of Caucasian individuals (Galvani and Novembre, 2005). Several other genetic factors were associated with HIV risk including HLA class 2 particularly *DRB1* and *DQB1* polymorphisms (Tang et al., 2004), CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL5 (Singh and Spector, 2009). HIV acquisition risk has also been associated with a number of immunological factors including increased frequency of pro-inflammatory Th17 cells

(Stieh et al., 2016) and α4β7 integrin expressing $CD4^+$ T cells (Sivro et al., 2018), thought to be the preferential targets for HIV replication.

Women, specifically, were also found to possess greater physiological risk of acquiring HIV as opposed to men. This is mainly due to the fact that women have a greater mucosal surface area and are likely to be exposed to infectious fluids and pathogens for extended periods during coitus as well as increased risk of tissue injury (Ramjee and Daniels, 2013). STIs, bacterial vaginosis (BV) and other biological factors such as genital inflammation have also been associated with increased HIV risk in young women (Passmore et al., 2016).

An inflamed female genital tract creates a favorable environment for HIV to replicate and establish itself as a productive infection (Passmore et al., 2016). Women who had elevated pro-inflammatory cytokines such as IP-10, MIP-1α, and MIP-1β in their genital tracts were observed to be at an increased risk of acquiring HIV (Masson et al., 2015). This is due to the fact that these abovementioned pro-inflammatory cytokines are chemotaxis for HIV target cells such as DCs, T cells, and macrophages (Dieu-Nosjean et al., 1999). Moreover, MIP-1α and MIP-1β are also ligands for the CCR5 HIV co-receptor and particularly recruit CCR5+ HIV target cells into tissues (Mueller and Strange, 2004) which may increase HIV transmission due to the fact that HIV replication is dependent on the presence of these immune target cells (Wira et al., 2005). Additionally, presence of genital inflammation was shown to undermine the effectiveness of tenofovir gel in preventing HIV acquisition in women from the CAPRISA 004 trial (Karim et al., 2010a, McKinnon et al., 2018). This study demonstrated that immunological factors affecting HIV risk can modify the success rates of future HIV prevention methods and that reducing genital inflammation as well as improving overall genital health in young women should be a key strategy in combating the HIV epidemic.

STIs are the most frequent cause of inflammatory cytokine upregulation as well as recruitment of immune cells to the genital mucosa (Levine et al., 1998, Masson et al., 2014). They have been found to promote the transmission of HIV by causing destruction to the protective mucosal barriers and recruiting susceptible immune cells such as macrophages and CD4+ T-helper cells to the site of infection (Kalichman et al., 2011). In a study conducted in SA, it was shown that *Mycoplasma genitalium,*

Chlamydia trachomatis and *Neisseria gonorrhoeae* infections were associated with increased risk of contracting HIV (Mlisana et al., 2012). Additionally, chlamydia followed by gonorrhoea, HSV-2, trichomoniasis, and BV were correlated with highest inflammatory cytokine levels in the genital tract (Masson et al., 2014).

The female genital tract microbiome was also shown to play a key role in vaginal health and HIV susceptibility. A healthy female genital tract has been defined as one that is predominantly comprised of *Lactobacillus* species (particularly *L. jensenii* and *L. crispatus*) which plays an important role in defending the vaginal mucosa against various infections (Ravel et al., 2011). Therefore, the disruption of the *Lactobacillus* species as well as other gram-negative anaerobes that form the commensal microbiome in the genital tract results in the syndrome of BV (Vásquez et al., 2002). Many studies have shown that molecular-BV is common in young African women. Molecular-BV is a term that is generally used for "non-optimal" bacterial communities that are depleted of *Lactobacillus* spp. and are characterized by molecular methods (McKinnon et al., 2019). Furthermore, molecular-BV has been associated with genital inflammation as well as an increased risk of acquiring HIV (Anahtar et al., 2015, McClelland et al., 2018). According to a prospective study that was conducted by Gosmann et al. (2017) in young South African women, it was reported that women colonized with bacterial communities that were highly diverse, had a 4.4-fold increased risk of acquiring HIV as opposed to women with a vaginal microbiota dominated by *Lactobacillus crispatus.*

1.4 Mucosal immune response

1.4.1 Overview of mucosal immune responses

Mucosal surfaces are lined by epithelial cells that form a physical barrier serving to protect the body against pathogens as well as external foreign substances. They are the key link between the host and its environment and represent a major portal of entry for various pathogens (Monin et al., 2020). Mucosal surfaces are also more susceptible to infection due to their structure and permeability that enables them to carry out physiological activities including food absorption (gut), reproduction (uterus and vagina), gas exchange (lungs) and sensory activities (throat, mouth, nose and eyes) (Janeway Jr et al., 2001). Higher mammals have acquired a distinctive mucosal

immune system designed to protect the mucosal surfaces from invading pathogens (McGhee and Fujihashi, 2012).

Various measures serve to provide protection against potential pathogens in the mucosal surfaces. Immune responses are broadly grouped into two categories known as the innate and adaptive immunity. The adaptive immune response includes defense mechanisms that are pathogen (fungal, bacterial, and viral) specific. In order for the immune response to be effective against pathogens, it requires antigen presenting cells (APCs) to process the antigen and present it to T and B cells which induces cell activation. This is followed by the activation of lymphocyte effector functions such as antibody synthesis, cytokine production as well as cytotoxicity. Therefore, protection may be conveyed either through the destruction of specific pathogens directly or indirectly by T cells and/or B-cells that produce neutralizing antibodies (Wira et al., 2005).

The innate immune system, recognized as the first line of defense, differs from the adaptive immune system in the specificity and type of receptors for antigen, cells involved [DCs, natural killer (NK) cells, neutrophils, epithelial cells and macrophages] and the nature of response to antigenic challenge (Janeway Jr and Medzhitov, 2002, Medzhitov and Janeway Jr, 2000). The main function of the innate immune system is to control and prevent invasion of pathogens. It carries this out by recognizing foreign structures in the host and depends on conserved germline encoded receptors that recognize pathogen-associated molecular patterns (PAMPs) located in groups of microorganisms (Medzhitov and Janeway Jr, 2000). The PAMPs are bound by pattern recognition receptors (PRRs) of the host which are expressed on/in the cells of the innate immune system. One of the groups of PRRs are toll-like receptors (TLRs) that are expressed by DCs, neutrophils, macrophages, epithelial cells as well as NK cells (Wira et al., 2005). In response to PAMPs, the TLR signals various adapters and molecules resulting in immune cells being recruited and the production of intracellular and secreted antimicrobial factors that not only link the innate and acquired immunity but can also directly kill invading pathogens (Akira and Takeda, 2004, Yang et al., 1999).

The mucosal immune system is organized into inductive and effector sites depending on their functional and anatomical properties. According to McGhee and Fujihashi

(2012) the mucosal inductive sites are jointly referred to as the mucosa-associated lymphoid tissue (MALT) which include the lymphoid sites, nasopharyngeal-associated tissues (NALT), and gut-associated lymphoid tissues (GALT). The MALT is covered by epithelial cells (excluding goblet cells), underlying lymphoid cells, and a subset of differentiated microfold cells which play a role in initiating a mucosal immune response. It also serves to provide a continuous source of memory B and T cells that migrate to mucosal effector sites (Boyaka and Fujihashi, 2019). Additionally, MALT consists of T cell regions and areas enriched with B cells that have a high frequency of surface IgA-positive ($sIgA^+$) B cells as well as a subepithelial area containing APCs, inclusive of DCs, which play an important role in initiating a specific immune response (McGhee and Fujihashi, 2012).

The mucosal effector sites, on the other hand, include the lamina propria regions of the secretory glandular tissues as well as upper respiratory, gastrointestinal, and female reproductive tracts which contain mucosal effector cells that are antigen specific such as memory B and T cells and plasma cells that produce IgA (Brandtzaeg, 2007). In humans, interleukin-10 (IL-10) and transforming growth factor-β (TBG-β) together with IL-4 have been shown to promote B cell switch to IgA (Asano et al., 2004).

The microbiota found at mucosal surfaces has been associated with restitution post injury, regulating cellular growth, maintaining barrier function as well as inducing, developing, and modulating immune responses. Although the mucosal immune system utilizes different mechanisms to protect the host from invading pathogens, it has coevolved to nurture, monitor, and exploit the commensal microbiota (Neish, 2014). According to Neish and Denning (2010), commensal bacteria may also influence the differentiation of T cells at locations other than the mucosa, induce states that are cytoprotective and modulate epithelial innate signaling pathways. Additionally, distinct taxa within the microbiota have been observed to play a role shaping cell autonomous, innate, and adaptive responses. Therefore, the commensal microbiota has a significant impact on the mucosal immune system, as well as physiology and disease in humans (Neish, 2014).

1.4.2 Mucosal immune response in the female genital tract

The mucosal immune system in the female genital tract is controlled by hormones that facilitate the distribution of various cell populations, transportation of immunoglobins (Igs), level of cytokines as well as antigen presentation in the genital tissues during the reproductive cycle (Wira et al., 2002). In addition to protecting against agents that are infectious, the mucosal immune system encounters a diverse set of challenges in different parts of the female reproductive tract (FRT) thus leading to different responses in the lower (ectocervix and vagina) and upper (endocervix and uterus) regions of the FRT (Monin et al., 2020).

Between these regions, the structural lining of the FRT is significantly different which represents their respective functional characteristics. The upper FRT is lined by a monolayer of columnar epithelial cells that adapt in response to sex hormones in order to facilitate successful implantation and pregnancy whereas the lining of the lower FRT comprises of protective layers of non-keratinized stratified squamous epithelium which is populated by numerous immune cells that serve to protect against invading pathogens as well as support high levels of commensal microbes (Fig 1.) (Givan et al., 1997, Wira et al., 2015). During the menstrual cycle, Patton et al. (2000) observed that the composition of immune cells in the upper FRT were altered in response to the hormonal changes as opposed to the lower FRT which remained relatively unchanged. However, emerging evidence has shown that immune function in the lower FRT may differ across the menstrual cycle (Saba et al., 2013, Wira et al., 2015). According to Monin et al. (2020), the lower FRT has four APC subsets present which include macrophages, intra-epithelial Langerhans cells, lamina propria $CD14^+$ and $CD14^-$ DCs. Since the vaginal mucosa does not consist of the MALT, priming of adaptive responses occur in draining lymph nodes. Therefore, upon infection, the APCs are mobilized to the draining lymph nodes to prime naïve T cells. The Langerhans cells and lamina propria $CD14^-$ DCs are directed towards Th2 activation as well as regulatory functions whereas macrophages and lamina propria $CD14^+$ play a role in the priming of Th1 responses and simulate classical innate cells that respond to molecules that are derived from pathogens via TLRs (Duluc et al., 2013).

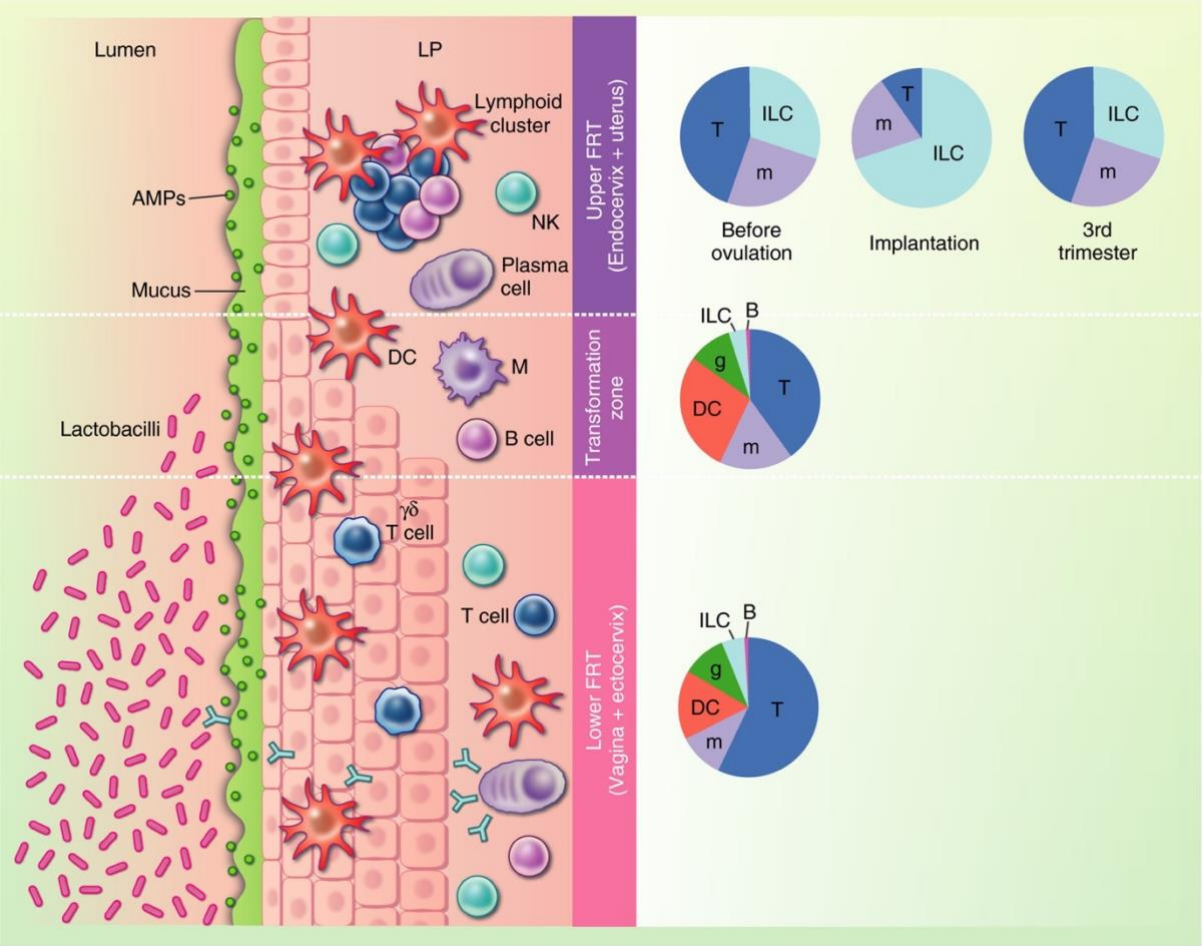

Figure 1: FRT non-immune and immune barriers. The composition of immune cells located in the FRT are indicated in the pie charts. In the upper FRT, this composition differs by stages of the menstrual cycle or pregnancy. AMPs, antimicrobial peptides; g, granulocyte; ILC, innate lymphoid cell; LP, lamina propria; m, monocyte/macrophages; NK, natural killer; T, T cells (Monin et al., 2020). Source: https://onlinelibrary.wiley.com/doi/full/10.1111/imm.13136#

Similar to the gut, the vagina consists of commensal flora whereby the growth of microbes that are beneficial are permitted by the immune system while those that are pathogenic are inhibited. These microbes are prevented from entering the uterus by the cervix which allows the passage of sperm. Once fertilization occurs, resulting in pregnancy, the mucosal immune system in the uterus adapts to allow the fetus, that is immunologically distinct from the mother, to co-exist with her for 9 months while preventing pathogens from entering (Monin et al., 2020). In order to overcome these challenges, the FRT evolved into compartments such as the ovaries, fallopian tubes, uterus, cervix and vagina that are regulated by progesterone and estradiol. These compartments display aspects of the mucosal immune system that contribute to the survival of the fetus and mother (Wira et al., 2005) as well as mediating a fine balance

between maintaining tissue integrity and function and protecting against pathogens (Monin et al., 2020).

1.5 Overview of interferon responses

Interferons (IFNs) are multifunctional, anti-viral cytokines that have been vastly utilized in clinical settings. They belong to a family of autocrine and paracrine cytokines which are normally secreted by host cells in response to pathogens, especially viruses (Wang et al., 2017b). IFNs were previously classified based on their cell origin which resulted in only types α, β, and γ, originating from leucocytes, fibroblast and activated T cells, that were infected by viruses, respectively. However, the discovery of IFN-λ in 2003 resulted in IFNs being classified as types I, II, and III. This is due to the fact that IFN-λ did not fit into the three pre-existing IFN types as it utilized distinct class II cytokine receptor complex (Kotenko et al., 2003, Sheppard et al., 2003). This resulted in the classification being in accordance with the different binding receptors (Fig. 2).

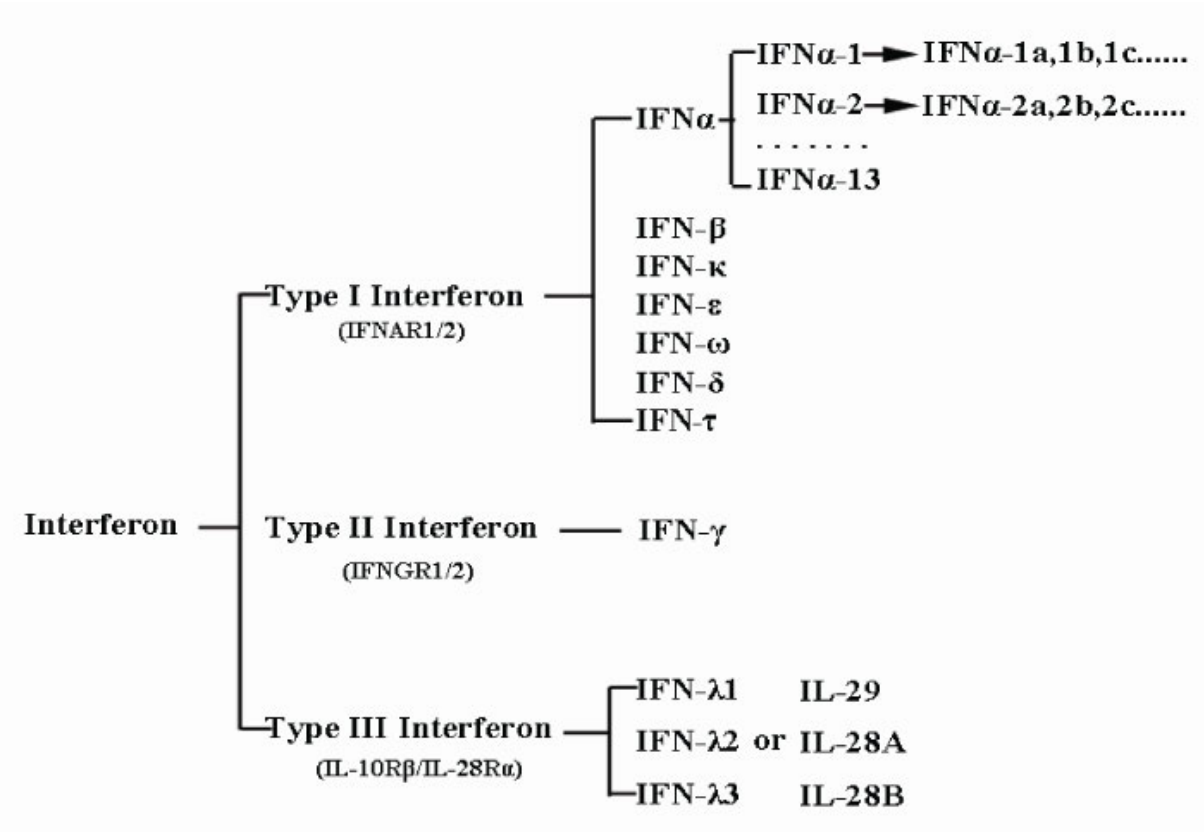

Figure 2: Interferon classification. Type I and III are inclusive of more than two subtypes, except type II (Wang et al., 2017b). Source: https://www.heighpubs.org/hjb/figures/abb-aid1001-g001.png

The IFN response forms part of the initial line of defense against viruses. This is carried out by the viral infection first being recognized by innate immune sensors which in turn activates a response from the different types of IFNs (Park and Iwasaki, 2020). In humans, type I IFNs are inclusive of subtypes α, -β, -ε, -κ, and -ω which ubiquitously bind to the type I IFN receptor that is expressed in an auto- and paracrine manner. IFN regulatory factors (IRF) along with nuclear factor kappa B (NFκB) are normally involved in the induction of IFNs by binding to IFN promoters which ultimately initiate the transcription of interferon stimulated genes (ISGs). Following induction, the IFNs bind to their respective receptor molecule that is expressed on the cell surface (De Weerd and Nguyen, 2012). IFN type I ligand binding to its receptor results in the heterodimerization of IFNAR1 (IFN- α/β receptor α chain) and IFNR2 (IFN- α/β receptor β chain) leading to autophosphorylation of Janus kinase (JAK). This then causes the heterodimerization of signal transducer and activator of transcription 1 (STAT1) and STAT 2 by phosphorylation resulting in a cascade referred to as the JAK-STAT pathway. The IRF9 is then recruited by the STAT1-STAT2 heterodimer which is responsible for the formation of the ISG factor 3 complex. Once this complex has entered the nucleus, it binds to the IFN-stimulated response element that is found on the ISG promotor region, ultimately initiating ISGs transcription (Fig. 3) (Darnell Jr et al., 1994). ISGs have the ability to disrupt every step of the viral replication thus providing both anti- and pro-inflammatory effects (Schoggins and Rice, 2011, Ramasamy and Subbian, 2021).

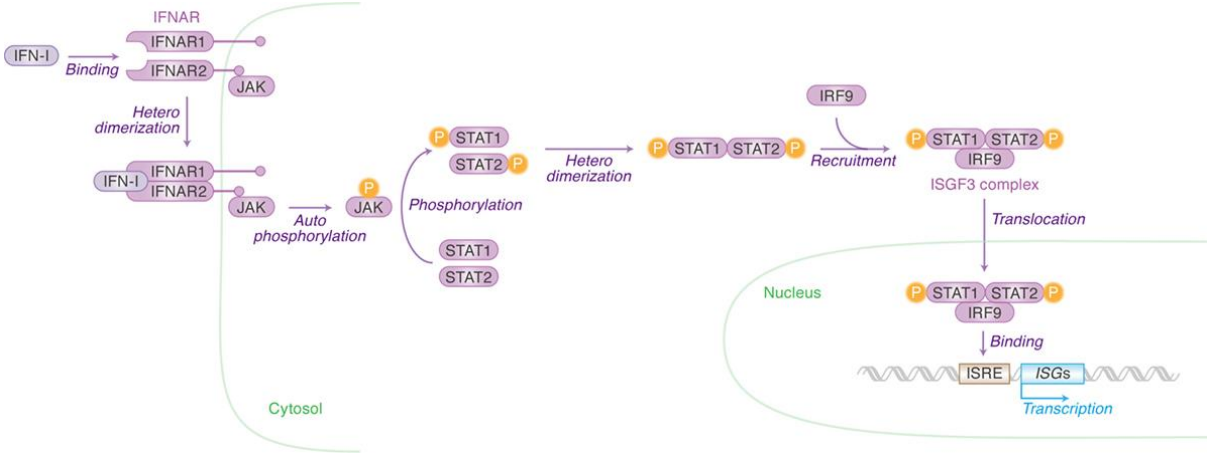

Figure 3: Intracellular signalling pathway initiated by IFN type I resulting in ISG expression. Purple, in italics, indicates cellular actions that are triggered by IFN type I. Cellular molecules that are in relation to the JAK-STAT pathway are indicated in purple whilst cellular organelles are in green. Phosphorylation is indicated with a "P" in a yellow circle (Soper

et al., 2018). Source: https://www.frontiersin.org/files/Articles/309017/fimmu-08-01823-HTML/image_m/fimmu-08-01823-g002.jpg

Since type I IFNs are produced relatively early during a viral infection, they play an important role in activating the antiviral innate immune response such as NK cell effector functions (Lee et al., 2017). They also have the ability to activate the adaptive immune system which promotes the development of immunological memory as well as high-affinity B and T cell responses that are antigen specific (Ivashkiv and Donlin, 2014). IFN type II is mainly produced by NK cells during innate antiviral immune response thus promoting antiviral immunity and has been observed to act as an important link between the innate immune response and adaptive immune response activation (Vivier et al., 2008). In addition to these responses, IFN type II is crucial in various immune responses to pathogens other than viruses, allergic reactions as well as tumor control (De Weerd and Nguyen, 2012). Lastly, IFN type III also plays a pivotal role in antiviral immune activities and binds to the type III IFN receptor that is expressed on certain myeloid and epithelial cells. It commonly functions at anatomical barrier sites and possess effects that are unique to hematopoietic cells such as neutrophils (Kotenko et al., 2019). Although IFN type I and III induce ISG signatures that are similar, the signalling observed from type I IFN results in a rapid induction and decline of ISG expression (Lazear et al., 2019).

1.6 Interferon stimulated genes

Any gene that is induced during an IFN response is defined as an ISG. This includes all three types of IFNs and their subtypes (Schoggins, 2019). The identification of ISGs across many cell line types has been made possible by advances in the RNA sequencing gene profiling by observing changes in the transcriptome in response to IFN stimulation (Yang and Li, 2020). The Interferome database is an online resource that catalogs the results from the gene expression profiling studies (Rusinova et al., 2012).

More than 300 ISGs were identified in early microarray studies (de Veer et al., 2001). However, the meta-analysis of ISG transcriptomes conducted by Schoggins et al. (2011) demonstrated that IFN type I induced approximately 450 genes in various cell backgrounds from different mammals. It has been noted that more genes are induced by cells of hematopoietic linage, with almost 1000 genes observed in mouse or

chimpanzee cells treated with IFN-α (Mostafavi et al., 2016, Lanford et al., 2006). Therefore, suggesting that the number of ISGs might be higher. This was then supported by the study that utilized cross-comparative analysis and RNA-sequencing to profile the transcriptional response that is induced by IFNs in primary fibroblasts from one bird and 9 different mammals (Shaw et al., 2017). Here, it was observed that about 10% of the human genome was regulated by IFNs in human cells.

The well-known function of ISGs is their ability to inhibit viral replication. They achieve this by employing diverse mechanisms that target various stages of the viral life cycle for different viruses.

1.7 Interferon response at the mucosa

The roles played by the different types of IFNs tend to overlap in restricting viral infections at the mucosa (Walker et al., 2021). Generally, once the PAMPs are identified by PRRs, it results in IFNs being induced and secreted by infected epithelial or immune cells (Walker et al., 2021). For type I IFNs, the protective effects frequently include limiting systemic viral infection and dissemination from the initial site of infection. This has been observed in HSV-2 genital tract infection in mice whereby loss of IFN type I signaling led to an increased viral replication locally and systemic dissemination to the central nervous system (Conrady et al., 2011). Additionally, IFN-ε, which is expressed in a hormone regulated manner within the female reproductive tract in mice and human, plays an important role in restricting HSV-2 (Nickodem et al., 2018). Fung et al. (2013) also observed that mice lacking IFN-ε had a decreased ISG expression at baseline and HSV-2 replication was increased within the reproductive tract. This ultimately led to the increased dissemination of the virus into the spinal cord and brain stem, compared to wild type mice. Although type I IFNs are crucial for preventing viral spread, they have also been shown to be the drivers of pathology in murine genital HSV infection (Lebratti et al., 2021).

Type II IFN has been shown to have an impact on the function of innate immune cells such as DCs and macrophages as well as stimulating adaptive immunity that is antigen specific (Nathan et al., 1983, Douvas et al., 1985). According to Billiau and Matthys (2009) type II IFN play an important role at regulating overall inflammatory responses to pathogens and they are also well known as broad spectrum antimicrobial

agents. During a mucosal viral infection, IFN-γ has an influence on APCs resulting in enhanced stimulation of the adaptive antiviral response to help clear the infection as well as generate memory for future infections (Goldszmid et al., 2012). With respect to macrophages, the potent viral replication inhibitor induced by IFN-γ also causes vasodilation thus allowing for an increased extravasation of recruited immune cells to the inflamed and infected site (Schroder et al., 2004). Furthermore, Liu et al. (2001) stated that in HSV-1 and -2 infections, IFN-γ is an important innate immune regulator particularly in controlling an infection that has disseminated beyond the epithelium.

Type III IFNs have a more localized activity at mucosal sites as opposed to type I IFNs. This is due to the fact that there is a restricted expression of their receptor mainly in epithelial cells as well as limited immune cell types in humans and mice (Wells and Coyne, 2018). Therefore, in mucosal infections, type III IFNs are crucial for infection control, particularly in the early stages, without triggering a systemic inflammatory response. In the context of HSV-2 infection, type III IFNs potently restrict the replication of HSV in mucosal epithelial cells (Lopušná et al., 2014). Moreover, IFN-λ has also been shown to control the Zika virus replication in the female reproductive tract due to the fact that high vaginal viral titers were observed in *Ifnlr1*$^{-/-}$ mice while IFN-λ treatment in mice with sufficient *Ifnlr1* limited viral replication. This ultimately supports the notion that this IFN type is likely active against many pathogens at this mucosal site (Caine et al., 2019).

1.8 Role of interferon responses in HIV infection

1.8.1 HIV transmission and acute infection

Acute HIV-1 infection is linked to a significant decrease in mucosal $CD4^+$ T cells of the gastrointestinal and genitorectal tracts (Schneider et al., 1995). The female genital tract represents one of the first points of entry for HIV during sexual intercourse and it is the key to understanding HIV transmission. Experimental and clinical studies have shown that HIV-1 can be transmitted through both the upper and lower FGT, particularly the transformation zone in the cervix which is deemed as a site that is highly susceptible to infection due to the large number of targets cells present in the lamina propria, below the epithelium (Ferreira et al., 2014). Once the HIV has been transmitted in the mucosa, an “eclipse period” occurs. This is when the virus enters

the CD4$^+$ cells and initiate the production of new virions followed by an exponential increase in virus levels with the peak being reached at approximately 21-28 days leading to symptom presentation of acute retroviral syndrome (Haase, 2011).

An increase in inflammatory cytokine expression, such as IFN type I and III, is one of the first indicators of immunological response to HIV infection (Stacey et al., 2009). The link between type I IFN and HIV has been studied extensively in the past 30 years with numerous reports demonstrating elevated plasma levels of IFN type I as well as ISGs in HIV-infected patients (Bosinger and Utay, 2015). In acute infections, pDCs are thought to be major producers of type I IFNs, particularly IFN-α. Their recruitment to the site of infection is normally mediated by virus induced MIP-3α/CCL20 in endocervical epithelium. This, in turn, recruits macrophages and T cells via MIP-1β chemokines, ultimately transforming the local submucosal environment into a CD4$^+$ T cell rich environment, thereby creating conditions that are favourable for the growth of viral founder population (Haase, 2011). However, pDCs also activates NK cells and upregulate antiviral restriction factors (Fig. 4) that have the ability to hinder the entire life cycle of HIV from viral entry (TRIM5α), reverse transcription (APOBEC3G and SAMHD1), nuclear entry (MX2), transcription (Schlafen 11 and GBP5) to budding (tetherin) (Utay and Douek, 2016, Bosinger and Utay, 2015) sparking much debate on whether this early induction of IFN is beneficial or detrimental to the host.

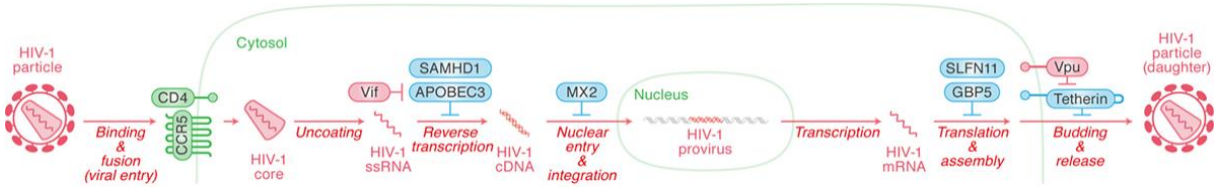

Figure 4: Restriction factors that control HIV-1 replication. Red (in italics) indicates viral replication steps. Cyan indicates restriction factors that inhibit viral replication at respective steps while accessory proteins responsible for counteracting the action of a few restriction factors are indicated in red. Viral components and HIV-1 markers as well as cellular organelles are indicated in pink and green, respectively (Soper et al., 2018). Source: https://www.frontiersin.org/files/Articles/309017/fimmu-08-01823-HTML/image_m/fimmu-08-01823-g003.jpg

According to Herbeuval and Shearer (2007) early production of IFNs and IFN-mediated immune responses may stop or partially restrict the transmission of HIV by activating innate antiviral factors and inducing apoptosis in HIV-1 infected cells. This is supported further by observations made in individuals infected with HIV-1, who have

the ability to maintain a viral load that is undetectable in the absence of ART, called elite controllers. Moreover, pDCs from elite controllers were not only correlated with the production of IFN (Machmach et al., 2012) but also had an increased capacity in reducing HIV production as well as inducing apoptosis of T cells that are infected with HIV compared to viremic patients. Lastly, systemic HIV infection is established by relatively IFN-resistant founder viruses, with IFN-mediated antiviral activity contributing to the HIV transmission bottleneck (Iyer et al., 2017b). Previous studies have also demonstrated similar findings whereby innate immune responses in the mucosa induce potent antiviral properties through the upregulation of ISGs, following infection (Neil et al., 2008, Van Damme et al., 2008).

Although IFN production in acute phase has been linked to the control of viral replication and protection against infection, it also drives immune activation as well as an influx of other immune cells that support the replication and spread of the virus (Bosinger and Utay, 2015, Sivro et al., 2014). It is also believed that the IFN-α responses brought on by acute HIV infection contribute to acute phase $CD4^+$ T-cell apoptosis. Therefore, the increased expression of FAS ligand as well as TNF-related apoptosis-inducing ligand (TRIAL) in plasma, which occurs at the same time as an increase in IFN-α levels, is the first sign that infection-induced apoptosis has occurred (Gasper-Smith et al., 2008). According to Hardy et al. (2007) both non-infectious and infectious HIV-1 induce IFN-α, thereby activating TRIAL expression on pDCs causing selective apoptosis of $CD4^+$ T-cells that are not infected as well as immunopathogenesis induced by IFN-α. The virus then replicates at a rapid rate and disseminates to other lymphoid tissues such as the GALT where up to 80% of $CD4^+$ T cells are lost within the first three weeks post HIV-1 infection (Mehandru et al., 2004, Dandekar, 2007). Therefore, the effects conferred by IFN-α in acute HIV-1 infection can be both protective and damaging (Sivro et al., 2014).

1.8.2 In chronic HIV infection

The dysfunction of T-cells due to cellular exhaustion tends to be apparent during chronic infections. This is normally in response to continuous antigen stimulation, resulting in immunosuppressive factors, such as PD-1, IL-10, and TIM-2, being upregulated (Wherry and Kurachi, 2015). Chronic immune activation and inflammation, in addition to immunosuppressive mechanisms, exacerbate exhaustion

of T cells, thereby, decreasing infection control (Wilson and Brooks, 2013). Sustained IFN upregulation is thought to be the cause of diminished T-cell function (Cheng et al., 2017), and it also exerts detrimental effects on various immune cells in chronic HV-1 infection (Wang et al., 2017a).

As HIV-1 worsens, the pDCs tend to migrate from the blood due to cell death and accumulate in lymph nodes. Here, they acquire an activated but immature phenotype whilst secreting significantly higher IFN-α levels before perishing (Lehmann et al., 2010). Excessive amounts of type I IFNs may potentially lead to the dysregulated activation of pDCs as well as their depletion. Although, the specific mechanism as to how the pDCs interact with type I IFNs have not been clearly elucidated, it has been observed that IFNs derived from HIV-activated pDCs promotes the expression of indoleamine 2,3-dioxygenase which favours the development of Tregs that are immunosuppressive (Manches et al., 2008) whilst resulting in dysfunctional T-cells (Boasso et al., 2007).

Similarly, the production of IFN-γ in individuals infected with HIV-1 can be seen in as early as the acute phase and continues throughout the course of infection. While it is initially produced to clear the primary infection, IFN-γ along with other inflammatory cytokines play a role in establishing chronic immune activation that worsen clinical diseases that are associated with AIDS (Roff et al., 2014).

1.9 Study rationale

IFNs, known as key mediators of viral immunity, trigger the ISGs, thus initiating an antiviral response. IFN responses can restrict viral replication at the point of exposure as well as during acute infection, however they also contribute to persistent immune activation and $CD4^+$ T cell depletion during chronic infection (Sivro et al., 2014). IFN responses can be dysregulated at the FGT, prior to viral infection, due to external influences such as other viral infections, vaginal microbiota composition, and in response to sexual activity as well as various sexual hygiene practices.

IFNα2 was identified as one of the strongest predictors of HIV risk in a previous CAPRISA 004 study looking at the cytokines associated with HIV acquisition. Contrary to that, IFNβ was associated with decreased risk of HIV infection. The results for IFNα2 are at first counter-intuitive; type I IFN-stimulated anti-viral pathways play a critical role in restricting early HIV replication, as supported by the evidence showing that systemic HIV infection is established by relatively IFN-resistant founder viruses with IFN-mediated antiviral activity contributing to the HIV transmission bottleneck (Iyer et al., 2017a). Furthermore, IFNα2 administration in rhesus macaques can prevent or delay the initial viral infection (Sandler et al., 2014). One explanation is that chronic/prolonged stimulation of IFNα2 drive immune activation, thus leading to a decrease in antiviral gene expression, thereby impairing the control of viral replication (Acharya and Liu, 2020, Sandler et al., 2014). Similar was observed in non-human primates (NHP) where IFNα2 exposure eventually led to desensitization and worse clinical outcomes (Sandler et al., 2014). IFNα2 induction in NHP models led to infiltration of immune cells as well as broad inflammation that increased HIV infection and disease progression (Abdulhaqq et al., 2014). With regards to IFNβ, mucosal application of IFNβ protected macaques from repeated intravaginal challenges with a simian human immunodeficiency virus (SHIV) (Veazey et al., 2016). Interestingly, IFNβ has been used for treatment of multiple sclerosis due to its anti-inflammatory properties and has higher affinity for the IFNAR1 compared to IFNα2 (Ramgolam et al., 2009, Severa et al., 2006).

While the interaction between IFN and HIV infection has been studied in several human and animal models, the driving factors of IFN upregulation at the FGT remain

understudied. Additionally, the relationship between mucosal IFN levels and the mucosal cellular environment remains uncharacterized. Here we assessed the drivers of IFN upregulation and the association between IFN expression and cellular immune activation at the FGT.

Hypothesis:

Increased IFNα2 levels at the FGT will be associated with generalized inflammation, presence of dysbiosis and STIs and increase in HIV susceptible $CD4^+$ T cell phenotype. On the contrary, we do not expect to see the same association for IFNβ.

1.10 Aims and objectives

Overall aim: To determine the drivers of type I, II, and III IFN upregulation at the FGT and the associated changes in the immune environment.

Specific objectives:

1. To characterise biological and behavioral drivers of chronic IFN upregulation at the FGT.
2. To assess the impact of IFN upregulation on cellular CD4+ T cell profile in the FGT.

Chapter 2: Materials and Methods

2.1 Ethics

This study was approved by the University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC/00004142/2022). The CAPRISA 083 and 012C studies were both approved by BREC (BFC410/15) and (BREC/00002492/2021), respectively.

2.2 Study cohort

The samples used for this study came from two CAPRISA cohorts, the CAPRISA 083 and 012C studies.

CAPRISA 083: The softcup samples utilized to study mucosal interferon expression with respect to presence and treatment of STIs and BV were obtained from the CAPRISA 083 (Changing the STI care model to reduce genital inflammation and HIV risk in South African women) prospective cohort study. Participants were recruited from Prince Cyril Zulu Communicable Disease Clinic situated in Durban, SA. Women between the ages of 18-40 years who were sexually active, not pregnant or living with HIV were eligible to participate in the study. This was followed by informed consent and subsequent enrollment into the study. Women who had been diagnosed with STIs were offered a single dose of antibiotics: 1g oral azithromycin for *Chlamydia trachomatis*; 250 mg intramuscular ceftriaxone and 1g oral azithromycin for *Neisseria gonorrhoeae* and 2g oral metronidazole for *Trichomonas vaginalis*. BV and intermediate microbiota were diagnosed by Nugent scores 7-10 and 4-6, respectively. Women diagnosed with BV or intermediate microbiota were treated with a single oral dose of 2g metronidazole while those with candidiasis were treated with a 500 mg clotrimazole pessary as well as 1% clotrimazole cream, if vulval symptoms were apparent. Additionally, women with BV/intermediate microbiota or STIs at baseline were followed longitudinally, with genital swab, cytobrush and softcup specimens collected at 6 and 12 (study exit) weeks.

CAPRISA 012C: We additionally used softcup and cytobrush specimens from the CAPRISA 012C cohort, (A double-blinded, randomized, placebo-controlled phase III trial to assess extended safety and tolerability of subcutaneous CAP256V2LS and VRC07-523LS in HIV-negative women). Participants were recruited at two different

sites: the CAPRISA eThekwini (Durban) and Vulindlela (Howick) clinical research site. HIV negative women, between the ages of 18-30 years, as well as with negative pregnancy tests were eligible to participate in the study while those with acute or chronic medical conditions, prior participation in an investigational HIV vaccine trail, or currently receiving immunosuppressive therapy were excluded. Once the informed consent form was signed, the participants were placed into three different groups (1a, -b, and -c) and received a single dose of the study product (CAP256V2LS and VRC07-523LS or placebo) at enrollment, administered into the skin subcutaneously as separate injections, with repeat doses at either 16 (4 monthly) or 24 (6 monthly) weeks dosing intervals. The amount of the study product administered was dependent on the participants weight which was used to calculate the dose. The cytobrush samples were obtained at visit 1000 (enrollment) and 1160 (4 months post enrollment) for the 4 monthly injection group as well as at visit 2000 (enrollment) and 2240 (6 months post enrollment) for the 6 monthly injection group.

2.3 Sample collection and processing

2.3.1 Genital menstrual cup specimens

Menstrual cups (SoftCup; Instead, San Diego, California, USA) were inserted into the vagina of the participant for an hour to collect cervicovaginal fluid for the purpose of identifying cytokines and chemokines in the genital tract. Specimens were collected into 50ml conical tubes and transported to the CAPRISA laboratory, on ice, where they were processed upon arrival. The tube was centrifuged for 10 minutes at 2000 rpm at 22°C in order to remove CVL content followed by the removal of the menstrual cup. The same speed, temperature, and time were used to centrifuge the tube for the second time. In instances where the supernatant separated, 50 µl of it was aspirated into a cryovial, diluted with 200 µl of phosphate-buffered saline (PBS), vortex and cryopreserved at -85 °C until use.

Frozen samples were thawed at 4°C overnight. The following day, samples were diluted 1:5 and 60 µl aliquot was plated out in 96 well plate for IFN measurements.

2.3.2 Vaginal cytobrush specimens

A cytobrush is utilized to sample the endocervical canal for the purpose of assessing the effects of mucosal interferon expression on CD4+ T cell phenotypes. Two brushes were collected for each participant. Briefly, each cytobrush was pushed into the canal, no deeper than the length of the brush which is approximately 1.5 – 2.0 cm. Each brush was then gently rotated 360 degrees twice and pulled straight out and placed immediately into 15 ml conical tubes containing 3 ml of R10 media (RPMI supplemented with 10% Foetal Calf Serum, L-glutamine, streptomycin, and penicillin). Once the excess handle of the brushes had been cut off, the cap of the tube was secured, labeled with the participants identification (PID), visit code, date and placed into a ziplock bag that was sealed, kept on an ice pack, or wet ice and transported to the laboratory within 2 hours of sample collection where it was processed upon arrival. The cells and/or mucus were dislodged from the cytobrush in the 3 ml transport medium (tube 1) by briefly vortexing, double gloving and gently wringing the brush with two fingers in order to maximize cell recovery. The cytobrush was then transferred into a new 15 ml conical tube (tube 2) containing 3ml of R10 media and placed on ice. The original tube (tube 1) was then centrifuged for 10 minutes at 1400 rpm at 4°C. Following the 10 minutes, 500 µl of the supernatant was aliquoted into 5 labelled (with PID, visit code and date) cryovials and the pellet was resuspended by gently flicking the tube with a finger. The tube that contained the cytobrush in 3 ml R10 media (tube 2) was briefly vortexed, to ensure that most cells were recovered, and the media was then transferred using a Pasteur pipette into tube 1 containing the resuspended pellet. This was repeated until tube 1 had approximately 10 ml followed by discarding the cytobrush in tube 2. Tube 1 was then centrifuged again for 10 minutes at 1400 rpm at 4°C. The supernatant was discarded, and the pellet was resuspended by gently flicking the tube. An additional 500 µl of R10 media was added to the resuspended pellet and gently mixed by pipetting up and down about 3-5 times. Prior to cell count, 20 µl of cell suspension was added to 20 µl of 0.4% trypan blue stain (1:2). This was then gently pipette mixed up and down in a well of a 96-well U-bottom plate followed by 20 µl of that mixture being transferred into a counting chamber which was placed under a light microscope for the mononuclear cells to be counted. The remaining cell suspension in tube 1 was transferred into two cryovials and centrifuged for 30 seconds at room temperature (RT). The supernatants were then discarded and the pellet was

resuspended. This was followed by transferring one of the resuspended pellets being lysed using 350 µl RLT lysate with β-mercaptoethanol (9.9 ml of RLT lysate and 0.1 ml β-mercaptoethanol) for downstream RNAseq analysis and stored at -80°C while the other pellet was used immediately for cellular phenotyping using flow cytometry.

2.4 Interferon measurements

Interferons (IFNα2, IFNβ, IFNγ, IFNλ1 and IFNλ2) were measured from stored menstrual cup supernatants using Bio-Plex Pro Human Inflammatory Panel I Assay following the manufacturer's instructions. The coupled magnetic beads were first diluted to 1X, vortexed at medium speed and 50 µl of the bead mixture was added to each well of the flat bottom plate using a multichannel pipette. The plate was then washed two times with 100 µl of Bio-Plex wash buffer. This was followed by vortexing the samples, standards, blank and control and adding 50 µl to the allocated wells. The plate was then covered with sealing tape, protected from light with an aluminium foil and incubated for 1 hour in a shaker set at 250 rpm at RT. When 10 minutes was left of the incubation step, the detection antibodies were vortexed for 15 seconds, centrifuged briefly to collect liquid and diluted to 1X. The plate was then washed three times with 100 µl of wash buffer and 25 µl of the vortexed diluted detection antibodies were added to each well of the plate. Sealing tape and aluminium foil was used to cover the plate which was then incubated in the dark for 30 minutes on a shaker set at 250 rpm at RT. 100x Streptavidin-phycoerythrin (SA-PE) was vortexed for 5 seconds, briefly centrifuged to collect liquid, diluted to 1X and protected from light in the 10 minutes that remained of the incubation step. This was followed by the plate being washed three times with 100 µl of wash buffer and 50 µl of the vortexed diluted SA-PE was added to each well. Aluminium foil and sealing tape was used to cover the plate which was incubated at 250 rpm in the dark for 10 minutes at RT. The plate was washed three times again with 100 µl of wash buffer and the magnetic beads were then resuspended in 125 µl assay buffer, covered in sealing tape and placed in a shaker for 30 seconds set at 250 rpm. The tape was then removed and the plate was analysed using the Bio-Plex 200 system. Data was exported to excel for further analysis. Samples with values below the limit of detection were assigned a value of half the limit of limit of detection (LOD/2).

2.5 Cytokine/chemokine and MMP measurements in CAPRISA083

This data was generously donated by Prof. Lenine Liebenberg, Dr. Andile Mtshali (cytokine/chemokines) and Nonsikelelo Ndlela (MMPs). Pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-12p40, IL-12p70, IL-18, MIF, TNF-α, TNF-β, TRAIL), adaptive response cytokines (IL-2, IL-4, IL-5, IL-13, IL-15, IL-17, IL-2RA), regulatory cytokines (IL-10, IL-1RA), chemotactic cytokines (CTACK, Eotaxin, Gro-α, IL-8, IL-16, IP-10, MCP-1, MCP-3, MIG, MIP-1α, MIP-1β, RANTES) and growth factors (β-NGF, FGF-basic, G-CSF, GM-CSF, HGF, IL-3, IL-7, IL-9, LIF, M-CSF, PDGF-BB, SCF, SCGF-β, SDF-1α, VEGF) were measured using Bio-Plex Pro-Human Cytokine Group I and II panels (Bio-Rad Laboratories, USA). The collagenase MMP-1, gelatinase MMP-2 and MMP-9, metrilysin MMP-7 and stromelysin MMP-10 were measured using MILLIPLEX MAP Human MMP Magnetic Bead Panel 2 (MILLIPORE, Billerica, MA). All assays were performed as per manufacturer's instructions. This data was available for baseline, 6- and 12 weeks post-treatment.

2.6 Flow cytometry

Cell suspension was transferred into a well of a V-bottom 96-well plate and centrifuged at 2100 rpm for 3 minutes. The supernatant was discarded and the cells were stained using an antibody master mix (**Table 1**) and covered with foil for 20 minutes at RT. Cells were stained for the following markers; CD3, CD4, HLA-DR, CD38, CD69, CD25, CD127, CCR6, CCR5; staining also included pre-titrated amounts of Vivid (Invitrogen) to discriminate dead from live cells. Following incubation, cells were washed twice using PBS-1 (PBS supplemented with 10% Foetal Calf Serum). Following the second wash, the pellet was resuspended in 200 µl 1X BD cell-fix (340181) and transferred into a labelled 5 ml polystyrene round-bottom tube. An additional 200 µl cell-fix was used to rinse the well and added into the tube. Samples were then acquired on BD Fortessa instrument. Entire sample was acquired. The data was then exported as flow cytometric standard (FCS) files and were analysed by hierarchal gating using FlowJo v10.8.1 software and exported to Microsoft excel.

Table 1. Flow cytometry panel

Cell marker	Fluorochrome	Ab clone	Company	Catalogue number	Volume (µl) / 50 µl reaction
CD3	APCCy7	SK7	BD Biosciences	560176	1
CD4	PE-TR	RPA-T4	BioLegend	300548	1
HLA-DR	AF700	L243	BioLegend	307626	1
CD38	FITC/AF488	HIT2	BD Biosciences	555459	1
CD69	APC/AF647	L78	BD Biosciences	340560	1
CD25	PE	M-A251	BioLegend	355104	1
CD127	BV711	A019D5	BioLegend	351328	1
CCR6	BV786	G034E3	BioLegend	353422	1
CCR5	BV421	2D7/CCR5	BD Biosciences	562576	1
Live/dead	Amcyan	-	Thermo Fisher Scientific	L34957	3

2.7 Flow cytometry gating

Lymphocytes were identified by gating forward scatter-area (FSC-A) versus side scatter-area (SSC-A). Single cells were identified by gating FSC-A versus forward scatter-height (FSC-H). Following that, live cells were defined as cells not staining for live/dead aqua stain (Invitrogen). $CD4^+$ T cells were identified by gating phycoerythrin-Texas red (PE-TR)-CD4 versus allophycocyanin-cyanine7 (APC-Cy7)-CD3. This was followed by observing the expression of the different markers on $CD4^+$ T cells. We then assessed the expression of CCR5 and CCR6, followed by T cell activation measured by CD38 and HLA-DR on $CD4^+$ T cells. Regulatory T cells (Tregs) were defined as $CD127^{low}$ and $CD25^+$ cells while acute activation and tissue resident cells were measured by the expression of CD69. Additionally, we analyzed the expression

of CCR5, CCR6, and Tregs (CD127^{low} and CD25^{+}) on activated T cells (CD38^{+} and HLA-DR^{+}) (**Figure 5**.).

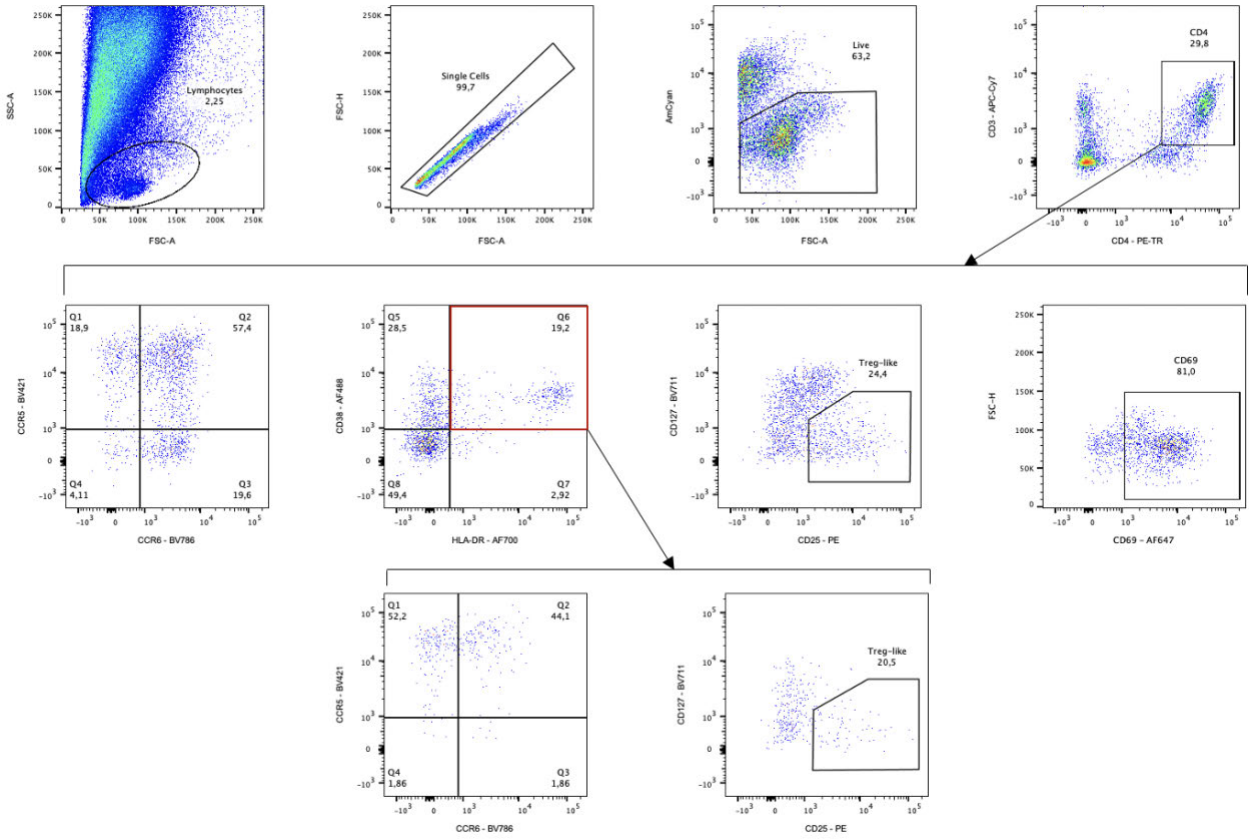

Figure 5: Flow cytometry gating from a representative CAP012C cytobrush sample.

2.8 Data analysis

Data was analyzed and graphed using SPSS v28 and GraphPad Prism v9.5.0, respectively. Spearman correlation was used to correlate IFN levels to mucosal $CD4^+$ T cell profiles. Differences in IFN levels between various categories were analyzed using Mann-Whitney tests when comparing two groups and Kruskal-Wallis tests with a Dunn's multiple comparison post-test when analyzing more than two groups. Friedman matched-pairs rank sum test was utilized to compare IFN concentrations at baseline, 6- and 12 weeks post-treatment. When analyzed as continuous variables, cytokine data were $\log_{10}$ transformed to normalize distributions, if detection was in >60% of samples. Samples with less than 60% detectability were analyzed as binary variables. A logistic univariable regression model was used to examine the effects of demographic, clinical and behavioral variables on mucosal IFNα2, -β, -λ1 and -λ2 while a linear univariable regression model was used for IFNγ. A logistic univariable and multivariable regression model examining the effects of BV/STIs on mucosal IFNα2, -β, -λ1 and -λ2 while a linear univariable and multivariable regression model was used for IFNγ. Linear mixed models, correcting for participant age, contraceptive use, and number of partners in the last two months, were fitted in order to assess the relationship between IFNs and genital cytokine concentration at baseline.

Chapter 3: Results

3.1 Study cohort characteristics

CAP083 cohort characteristics at baseline: The original study enrolled 267 HIV uninfected women, however due to sample availability only 238 were included in the main analysis of this study. Women were between the ages of 18-40 with the median of 23 (IQR 21-27). Of these, 234 (98.3%) reported that they only engaged in coitus with males, utilized condoms sometimes (65.1%) and had one sexual partner in the last two months (86.1%). Most women (71.4%) tested positive for at least one STI [*Chlamydia trachomatis (n=34), Neisseria gonorrhoeae (n=11), Mycoplasma genitalium (n=11), or Trichomonas vaginalis (n=10)*] and/or BV. Eighty-four women (35.2%) reported that they were currently on some form of contraception, with progesterone injections (20.6%) being the most commonly used. Most women tested negative for viral STIs such as HSV-1 (91.2%) and HSV-2 (94.1%) while just more than half tested negative for HPV (66.4%). Vaginal candidiasis was diagnosed in only about a quarter (19.3%) of these women. The baseline characteristics of women who had STI and/or BV detected and those that did not were compared in **Table 2**.

Table 2. Demographic characteristics of women enrolled into the CAPRISA 083 study at baseline.

Variables	Overall (N=238)	STI and/or BV detected (N=170)	STI and BV negative (N=68)	p-value
Age (median, IQR)	23 (21 - 27)	23 (21 - 26)	23 (21 - 27)	0.989
No. of sexual partners in the last 2 months (median, IQR)	1 (1 - 1)	1 (1 - 1)	1 (1 - 1)	
Gender of sex partners (N, %):				
Male and female	4 (1.7)	3 (1.8)	1 (1.5)	
Male	234 (98.3)	167 (98.2)	67 (98.5)	
Condom use (N, %):				0.928
Always	10 (4.2)	7 (4.1)	3 (4.4)	
Sometimes	155 (65.1)	112 (65.9)	43 (63.2)	
Never	73 (30.7)	51 (30.0)	22 (32.4)	
Contraceptive use*(N, %)	84 (35.2)	63 (37.1)	21 (30.9)	0.453
BV category (N, %):				
Normal (0-3)	75 (31.5)	7 (4.1)	68 (100)	
Intermediate/subclinical (4-6)	82 (34.5)	82 (48.2)	0	
BV (7-10)	81 (34.0)	81 (47.6)	0	
Nugent score (median, IQR)	5 (3, 7)	6 (5, 8)	2 (2, 3)	
Candida infection (N, %)				0.586
Positive	46 (19.3)	31 (18.2)	15 (22.1)	
Negative	192 (80.7)	139 (81.8)	53 (77.9)	
Any STI infection**(CT/NG/TV/MG)	56 (23.5)	56 (32.9)	0	
Chlamydia trachomatis	34 (14.3)	34 (20.0)	0	
Neisseria gonorrhoeae	11 (4.6)	11 (6.5)	0	
Trichomonas vaginalis	10 (4.2)	10 (5.9)	0	
Mycoplasma genitalium	11 (4.6)	11 (6.5)	0	
HSV-1 (N, %):				0.136
Positive	21 (8.8)	12 (7.1)	9 (13.2)	
Negative	217 (91.2)	158 (92.9)	59 (86.8)	
HSV-2 (N, %):				0.550
Positive	14 (5.9)	9 (5.3)	5 (7.4)	
Negative	224 (94.1)	161 (94.7)	63 (92.6)	
HPV status (N, %):				0.755
Positive	73 (30.7)	51 (30.0)	22 (32.4)	
Negative	158 (66.4)	114 (67.1)	44 (64.7)	
Missing	7 (2.9)	5 (2.9)	2 (2.9)	

*BV, bacterial vaginosis; HPV, human papilloma virus; HSV, herpes simplex virus; STI, sexually transmitted infection. *People who use contraceptives. **Any STI included any of CT, NG, TV, or MG*

CAP012C cohort characteristics at enrollment: We obtained cytobrushes from a total of 66 women, median age 24 (IQR 21-27). The median body mass index (BMI) was 27.2 kg/m^2 (IQR 23.7-31.2) rendering them overweight. Most women had stable partners (93.9%) and used a condom sometimes (75.8%) during sexual intercourse. Most women were practicing some form of birth control with nuristerate (administered subcutaneously every 8 weeks) accounting for 30.3% followed by depo-provera (administered subcutaneously every 12 weeks, 24.2%), subdermal implant and other forms of contraception (21.2%), lastly IUCD and oral contraceptives (1.5%). The median age of the spouses/regular partner was 28 years (IQR 25-30) with most of them circumcised (89.4%, **Table 3**).

Table 3. Demographic characteristics of CAPRISA 012C participants at enrollment

Variables	Total (N=66)
Age (median, IQR)	24 (21 - 27)
BMI (median, IQR)	27.2 (23.7 - 31.2)
No. of sexual partners in the last 30 days (N, %):	1 (1 - 1)
None	1 (1.5)
1 partner	62 (93.9)
2 – 4 partners	3 (4.5)
Relationship status (N, %):	
Casual partner	3 (4.5)
Stable partner	62 (93.9)
No partner	1 (1.5)
Vaginal sex in the last 30 days *(N, %)	55 (83.3)
Condom use (N, %):	
Always	7 (10.6)
Sometimes	50 (75.8)
Never	9 (13.6)
Contraceptive method (N, %):	
Depo-Provera	16 (24.2)
IUCD	1 (1.5)
Nuristerate	20 (30.3)
Oral contraceptive	1 (1.5)
Subdermal implant	14 (21.2)
Other	14 (21.2)
Insert anything in the vagina in the last 30 days (N, %):	
Yes	3 (4.5)
No	63 (95.5)
Diagnosed or treated for an STI **(N, %)	1 (1.5)
Worried getting infected with HIV (N, %):	
Very worried	30 (45.5)
Somewhat worried	25 (37.9)
Not worried at all	11 (16.7)
Age of spouse/regular partner (median, IQR)	28 (25 - 30)
Circumcised partner (N, %):	
Yes	59 (89.4)
No	6 (9.1)
Study arm (N, %):	
CAP2562LS + VRC07-523LS4 at 4 monthly dosing intervals	29 (43.9)
CAP2562LS + VRC07-523LS4 at 6 monthly dosing intervals	37 (56.1)

*IUCD, intrauterine contraceptive device. *People who had vaginal sex in the last 30 days*
***People who were diagnosed or treated for an STI*

3.2 Detectability of IFNs in softcup samples

First, we wanted to assess the detectability of IFN expression in the softcup specimens from the two parent studies. As observed in **Figure 6 and 7**, most of the IFNs had a low level of detectability except for IFNγ which was >60% detectable. IFNβ had the lowest detectability at only 6.1% and 8.89% in CAPRISA 083 and 012C study samples, respectively.

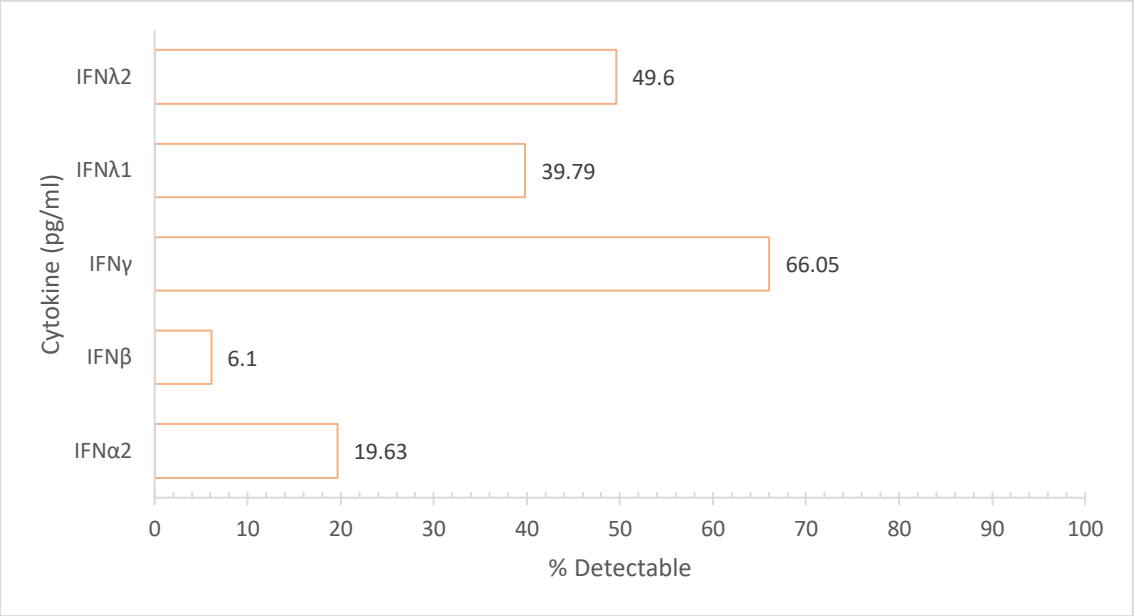

Figure 6: Detectability of IFNs in softcup samples from the CAPRISA 083 cohort (N=377)

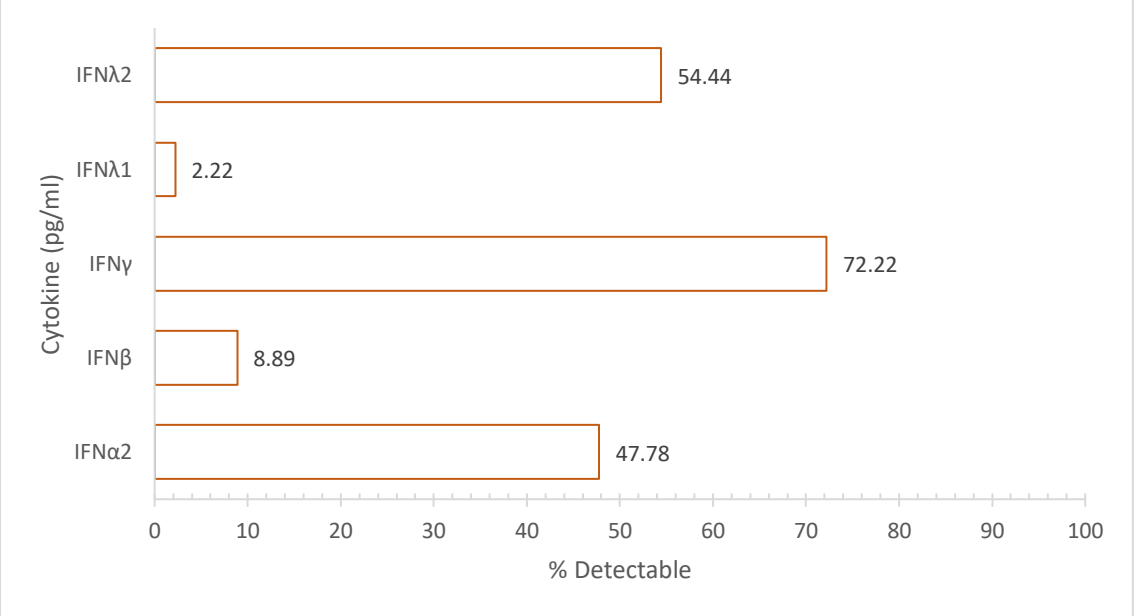

Figure 7: Detectability of IFNs in softcup samples from the CAPRISA 012C study (N=90)

3.3 Effect of clinical and behavioral variables on mucosal IFN expression in CAPRISA 083 participants

In order to determine which behavioural and clinical variables had a significant impact on mucosal IFN expression we ran a binary logistic regression with binarized IFNs (IFNα2, IFNβ, IFNλ1 and IFNλ2) as the outcome and a linear regression with IFNγ as an outcome. We observed that participant age was associated with increased IFNλ1 (OR 1.073, 95% CI 1.013-1.137, p = 0.016) and IFNλ2 expression (OR 1.067, 95% CI 1.008-1.130, p = 0.026). Contraceptive use was associated with increased IFNβ (OR 2.960, 95% CI 1.016-8.627, p = 0.047) while number of sexual partners in the last 2 months was associated with increased IFNλ1 (OR 2.126, 95% CI 1.015-4.454, p = 0.046). Presence of any STIs (CT/NG/TV/MG) was associated with increased IFNλ2 (OR 2.031, 95% CI 1.108-3.723, p = 0.022); and presence of BV was significantly associated with increased IFNα2 (OR 2.546, 95% CI 1.123-5.770, p = 0.025, **Table 4**). Based on these results, we corrected for participant age, contraceptive use, and number of sexual partners in the last 2 months in the future models since any STIs/BV were points of interest.

Table 4. Univariable logistic regression model examining the effects of demographic, clinical and behavioral variables on mucosal IFN expression.

Variable	IFNα2		IFNβ		IFNλ1		IFNλ2	
	OR (95% CI)	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**
Age	0.984 (0.915 – 1.057)	0.651	1.002 (0.894 - 1.122)	0.980	1.073 (1.013 – 1.137)	**0.016**	1.067 (1.008 – 1.130)	**0.026**
Contraceptive use	0.671 (0.332 – 1.359)	0.268	2.960 (1.016 – 8.627)	**0.047**	0.323 (0.767 – 2.283)	0.315	0.667 (0.386 – 1.153)	0.147
Candidiasis	1.640 (0.771 – 3.490)	0.199	1.567 (0.476 – 5.165)	0.460	0.869 (0.444 – 1.704)	0.684	0.964 (0.502 – 1.853)	0.913
No. of sexual partners in the last 2 months	1.546 (0.658 – 3.633)	0.317	0.436 (0.106 – 1.798)	0.251	2.126 (1.015 – 4.454)	**0.046**	1.165 (0.581 – 2.336)	0.666
Any STI	1.187 (0.567 – 2.486)	0.649	0.218 (0.028 – 1.697)	0.146	1.487 (0.809 – 2.732)	0.201	2.031 (1.108 – 3.723)	**0.022**
BV (0-3 normal; 4-10 BV)	2.546 (1.123 – 5.770)	**0.025**	1.285 (0.395 – 4.174)	0.677	0.924 (0.526 – 1.623)	0.783	1.442 (0.821 – 2.533)	0.203
HSV2	1.733 (0.518 – 5.796)	0.372	1.154 (0.141 – 9.467)	0.894	0.263 (0.057 – 1.201)	0.085	0.533 (0.162 – 1.752)	0.300
HSV1 and 2	0.843 (0.328 – 2.168)	0.723	0.397 (0.051 – 3.120)	0.380	1.138 (0.546 – 2.370)	0.730	0.788 (0.376 – 1.651)	0.528
HPV	0.972 (0.481 – 1.963)	0.937	1.088 (0.358 – 3.306)	0.881	0.694 (0.385 – 1.248)	0.222	0.850 (0.483 – 1.495)	0.573

Table 5. Univariable linear regression model examining the effects of demographic, clinical and behavioral variables on mucosal IFNγ expression.

Variables	IFNγ	
	β-coefficient (95% CI)	**p-value**
Age	0.040 (-0.064 – 0.145)	0.446
Contraceptive use	-0.227 (-1.228 – 0.775)	0.656
Candidiasis	0.521 (-0.690 – 1.731)	0.398
No. of sexual partners in the last 2 months	-0.422 (-1.714 – 0.869)	0.520
Any STI (CT/NG/TV/MG)	0.969 (-0.153 – 2.091)	0.090
BV (0-3 normal; 4-10 BV)	0.189 (-0.841 – 1.219)	0.718
HSV2	-1.350 (-3.377 – 0.677)	0.191
HSV1and2	-0.388 (-1.739 – 0.963)	0.572
HPV	-0.241 (-1.284 – 0.803)	0.650

3.4 Association between STIs/BV and mucosal IFN expression

Linear and logistic regression models were utilized to assess the association between mucosal IFN expression and presence of BV/STIs in women. Following the univariable and multivariable analyses adjusting for participant age, contraceptive use, and number of sex partners in the last two months; IFNα2 was significantly associated with presence of STIs/BV (aOR 2.677, 95% CI 1.125-6.366, p = 0.026, Table 6, Figure 8). No significant changes were observed for IFNβ, IFNλ1, IFNλ2 (Table 6) and IFNγ (Table 7) (Figure 8).

Table 6. Logistic regression model examining the effect of STI/BV on mucosal IFNs.

Variables	Univariable				Multivariable			
	OR	95% CI		p-value	aOR	95% CI		p-value
		Lower	Upper			Lower	Upper	
IFNα2	2.594	1.098	6.131	**0.030**	2.677	1.125	6.366	**0.026**
IFNβ	1.107	0.340	3.604	0.866	1.104	0.332	3.672	0.872
IFNλ1	1.038	0.580	1.859	0.899	0.987	0.544	1.791	0.965
IFNλ2	1.620	0.901	2.911	0.107	1.672	0.919	3.041	0.092

Table 7. Linear regression model examining the effect of STI/BV on mucosal IFNγ.

Variables	Univariable				Multivariable			
	β-coefficient	95% CI		p-value	aβ-coefficient	95% CI		p-value
		Lower	Upper			Lower	Upper	
IFNγ	0.157	-0.903	1.217	0.770	0.179	-0.887	1.245	0.741

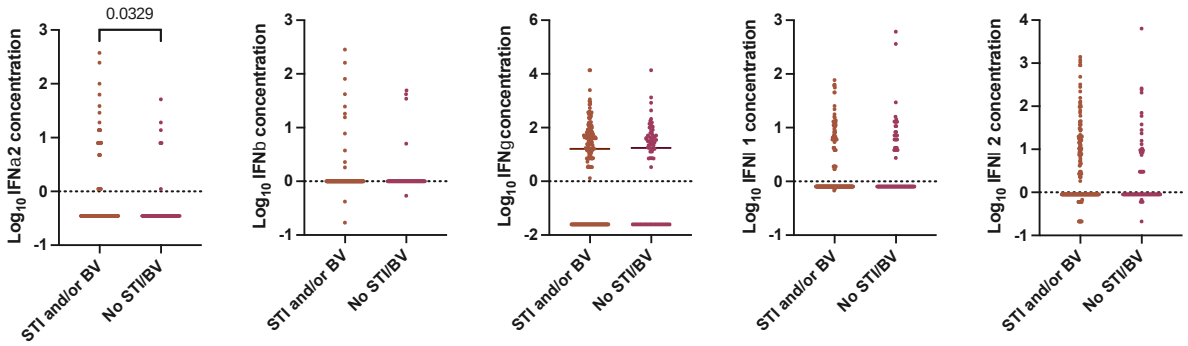

Figure 8: IFN concentrations in softcup samples of CAPRISA 083 women with STIs/BV (n=170) and without STIs/BV (n=68) at baseline. Mann-Whitney U-test was utilized in order to compare the IFN concentration medians between the two groups. IFN concentrations were log transformed and significant p-values are displayed on the graph(s).

3.5 Association between mucosal IFNs and other mucosal cytokines/chemokines

The genital tract cytokine, chemokine, and matrix metalloproteinase (MMP) profiles of the women at baseline were associated with IFNs. In the univariable model, the following cytokines/chemokines were significantly associated with IFNα2 (**Table 8**): IL-10 (p=0.016), IL-15 (p=0.044), IL-4 (p=0.002), IL-12p70 (p=0.025), RANTES (p=0.040), IL-3 (p=0.023), IL-9 (0.047), PDGF-BB (p=0.002). Cytokines associated with IFNβ (**Table 9**) included IL-18 (p=0.019) and IL-1β (p=0.045) while those associated with IFNγ (**Table 10**) included IL-1RA (p=0.045) and GM-CSF (p=0.005). IFNλ1 (**Table 11**) was associated with IL-10 (p=0.005), IL-2RA (p=0.040), IL-12p40 (p=0.025), TNF-β (p=0.007), Eotaxin (p=0.002), RANTES (p=0.043), IL-3 (p=0.024), IL-9 (p=0.001), LIF (p=0.044) and M-CSF (p=0.031) while IFNλ2 (**Table 12**) was associated with IL-2 (p=0.013), IL-12p40 (p=0.025) and TRAIL (p=0.040). In the multivariable model correcting for participant age, contraceptive use, and number of sex partners in the last two months, we observed significant associations between concentrations of IFNα2 and increased IL-10 (aβ 0.153, 95%CI 0.063-0.760, p=0.021), IL-4 (aβ 0.177, 95%CI 0.150-0.871, p=0.006), IL12p70 (aβ 0.144, 95%CI 0.045-0.844, p=0.030), FGF-basic (aβ 0.132, 95%CI 0.014-0.886, p=0.043), PDGF-BB (aβ 0.182, 95%CI 0.192-1.071, p=0.005), and decreased IL-3 (aβ -0.156, 95%CI -0.978 – -0.090, p=0.019). For IFNβ we observed an inverse association with IL-18 (aβ -0.174, 95%CI -1.321 – -0.227, p=0.006). For IFNγ we observed an inverse association with IL-1RA (aβ -0.131, 95%CI -0.224 – -0.003, p=0.045) and GM-CSF (aβ -0.175, 95%CI -0.207 – -0.035, p=0.006). IFNλ1 was associated with decreased IL-10 (aβ -0.173, 95%CI -0.665 – -0.095, p=0.009), TNF-β (aβ -0.155, 95%CI -0.669 – -0.062, p=0.018), Eotaxin (aβ -0.189, 95%CI -1.031 – -0.203, p=0.004), IL-3 (aβ -0.133, 95%CI -0.737 – -0.006, p=0.046), IL-9 (aβ -0.190, 95%CI -0.603 – -0.118, p=0.004) and M-CSF (aβ -0.129, 95%CI -0.447 - 0.000, p=0.050). Lastly, IFNλ2 levels were associated with increased IL-2 (aβ 0.163, 95%CI 0.104-0.914, p=0.014), decreased TRAIL (aβ -0.153, 95%CI -0.923 – -0.086, p=0.018) and LIF (aβ -0.135, 95%CI -0.504 – -0.022, p=0.033, **Figure 9**).

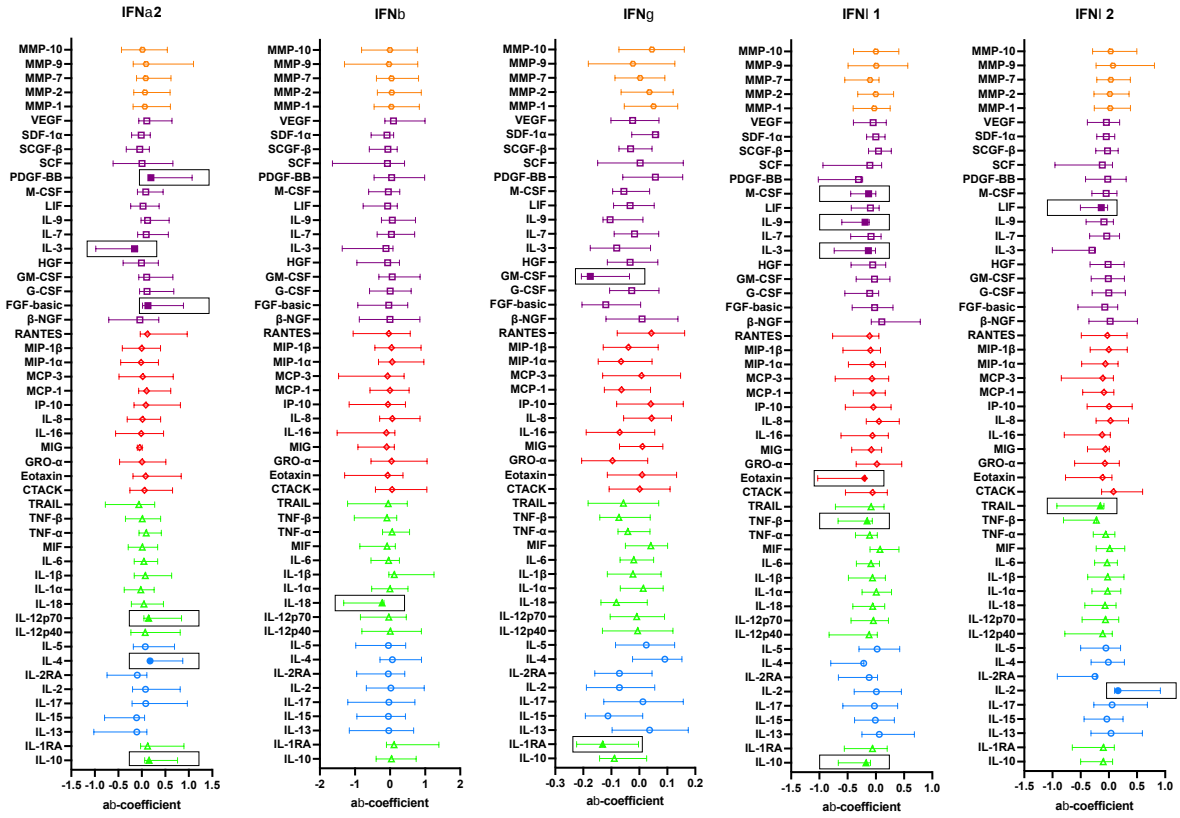

Figure 9: Associations between mucosal IFNs and cervicovaginal cytokines/chemokines at baseline. Multivariable linear mixed models were used to assess the relationship between IFNs and genital cytokine/chemokine concentrations. Inflammation, adaptive response, chemokines, growth factors and MMPs were indicated by green triangles, blue circles, red diamonds, purple squares and orange hexagons, respectively. Significant p-values ($p<0.05$) are indicated by filled shapes and boarders.

Table 8. Linear regression model examining the association between mucosal IFNα2 and mucosal cytokine/chemokines

Variables	Univariable				Multivariable			
	β-coefficient	95% CI		p-value	aβ-coefficient	95% CI		p-value
		Lower	Upper			Lower	Upper	
IL-10	0.156	0.080	0.763	**0.016**	0.153	0.063	0.760	**0.021**
IL-1RA	0.105	-0.081	0.823	0.107	0.123	-0.025	0.897	0.064
IL-13	-0.101	-0.986	0.116	0.121	-0.106	-1.021	0.106	0.111
IL-15	-0.131	-0.851	-0.012	**0.044**	-0.111	-0.793	0.060	0.092
IL-17	0.100	-0.128	1.041	0.125	0.083	-0.209	0.968	0.205
IL-2	0.069	-0.229	0.772	0.286	0.079	-0.197	0.818	0.230
IL-2RA	-0.084	-0.692	0.143	0.197	-0.097	-0.740	0.107	0.142
IL-4	0.200	0.213	0.936	**0.002**	0.177	0.150	0.871	**0.006**
IL-5	0.100	-0.095	0.790	0.123	0.074	-0.181	0.697	0.248
IL-12p40	0.040	-0.363	0.687	0.544	0.072	-0.230	0.817	0.270
IL-12p70	0.146	0.058	0.838	**0.025**	0.144	0.045	0.844	**0.030**
IL-18	0.068	-0.165	0.534	0.299	0.044	-0.224	0.462	0.495
IL-1α	0.014	-0.283	0.354	0.826	-0.023	-0.375	0.262	0.726
IL-1β	0.101	-0.084	0.726	0.119	0.075	-0.161	0.635	0.242
IL-6	0.066	-0.122	0.384	0.309	0.044	-0.162	0.337	0.490
MIF	0.044	-0.212	0.438	0.496	0.009	-0.290	0.337	0.883
TNF-α	0.109	-0.035	0.439	0.095	0.095	-0.060	0.414	0.142
TNF-β	0.026	-0.295	0.445	0.690	0.010	-0.347	0.402	0.884
TRAIL	-0.039	-0.686	0.367	0.552	-0.061	-0.775	0.274	0.347
CTACK	0.029	-0.349	0.553	0.657	0.057	-0.252	0.653	0.384
Eotaxin	0.104	-0.093	0.925	0.109	0.081	-0.188	0.837	0.213
GRO-α	-0.004	-0.497	0.470	0.959	0.005	-0.473	0.514	0.935
MIG	0.022	-0.263	0.375	0.730	-0.096	-0.048	0.007	0.144
IL-16	-0.011	-0.551	0.465	0.867	-0.012	-0.558	0.464	0.856
IL-8	0.037	-0.250	0.456	0.566	0.016	-0.312	0.401	0.805
IP-10	0.069	-0.225	0.749	0.290	0.086	-0.165	0.823	0.191
MCP-1	0.078	-0.134	0.559	0.229	0.101	-0.068	0.618	0.116
MCP-3	0.019	-0.485	0.654	0.770	0.021	-0.485	0.670	0.752
MIP-1α	0.022	-0.341	0.481	0.738	-0.015	-0.449	0.354	0.815
MIP-1β	0.013	-0.370	0.456	0.837	-0.002	-0.415	0.399	0.969
RANTES	0.133	0.025	1.015	**0.040**	0.119	-0.033	0.965	0.067
b-NGF	-0.030	-0.647	0.401	0.644	-0.042	-0.703	0.362	0.529
FGF	0.123	-0.016	0.854	0.059	0.132	0.014	0.886	**0.043**
G-CSF	0.120	-0.020	0.698	0.064	0.112	-0.047	0.681	0.087
GM-CSF	0.086	-0.118	0.608	0.184	0.105	-0.065	0.661	0.107
HGF	0.019	-0.322	0.431	0.775	-0.008	-0.399	0.352	0.903
IL-3	-0.147	-0.940	-0.070	**0.023**	-0.156	-0.978	-0.090	**0.019**
IL-7	0.116	-0.029	0.620	0.074	0.093	-0.090	0.566	0.154
IL-9	0.129	0.005	0.594	**0.047**	0.123	-0.014	0.583	0.061
LIF	0.065	-0.154	0.467	0.321	0.027	-0.236	0.368	0.666
M-CSF	0.104	-0.051	0.491	0.111	0.085	-0.094	0.454	0.196

PDGF-BB	0.205	0.275	1.149	**0.002**	0.182	0.192	1.071	**0.005**
SCF	0.007	-0.590	0.661	0.911	0.005	-0.613	0.661	0.941
SCGF-β	-0.030	-0.301	0.186	0.644	-0.044	-0.329	0.162	0.505
SDF-1α	-0.012	-0.214	0.178	0.858	-0.012	-0.218	0.182	0.861
VEGF	0.112	-0.041	0.652	0.083	0.106	-0.065	0.642	0.110
MMP-1	0.109	-0.062	0.780	0.095	0.064	-0.186	0.609	0.296
MMP-2	0.120	-0.022	0.792	0.064	0.067	-0.171	0.601	0.274
MMP-7	0.119	-0.024	0.741	0.066	0.085	-0.114	0.623	0.175
MMP-9	0.107	-0.104	1.165	0.101	0.092	-0.183	1.100	0.160
MMP-10	0.063	-0.263	0.765	0.337	0.014	-0.434	0.543	0.826

Table 9. Linear regression model examining the association between mucosal IFNβ and mucosal cytokine/chemokines

Variables	**Univariable**				**Multivariable**			
	β-coefficient	**95% CI**		**p-value**	**aβ-coefficient**	**95% CI**		**p-value**
		Lower	**Upper**			**Lower**	**Upper**	
IL-10	0.029	-0.434	0.689	0.655	0.039	-0.398	0.743	0.551
IL-1RA	0.115	-0.070	1.398	0.076	0.112	-0.102	1.392	0.090
IL-13	-0.039	-1.175	0.624	0.547	-0.035	-1.160	0.673	0.602
IL-15	-0.037	-0.889	0.487	0.565	-0.047	-0.946	0.441	0.474
IL-17	-0.047	-1.307	0.600	0.466	-0.033	-1.203	0.709	0.611
IL-2	0.014	-0.723	0.907	0.825	0.023	-0.676	0.973	0.723
IL-2RA	-0.040	-0.892	0.468	0.540	-0.050	-0.954	0.421	0.446
IL-4	0.055	-0.341	0.857	0.397	0.065	-0.289	0.895	0.315
IL-5	-0.037	-0.929	0.516	0.574	-0.047	-0.977	0.445	0.462
IL-12p40	0.020	-0.724	0.984	0.764	0.007	-0.805	0.894	0.918
IL-12p70	-0.043	-0.854	0.426	0.511	-0.038	-0.845	0.461	0.563
IL-18	-0.152	-1.234	-0.110	**0.019**	-0.174	-1.321	-0.227	**0.006**
IL-1α	-0.007	-0.545	0.490	0.918	-0.002	-0.523	0.509	0.979
IL-1β	0.130	0.014	1.325	**0.045**	0.118	-0.034	1.249	0.063
IL-6	-0.037	-0.532	0.292	0.568	-0.043	-0.541	0.267	0.506
MIF	-0.069	-0.811	0.244	0.291	-0.086	-0.861	0.150	0.168
TNF-α	0.067	-0.184	0.591	0.302	0.056	-0.214	0.556	0.383
TNF-β	-0.089	-1.017	0.181	0.171	-0.089	-1.020	0.188	0.176
TRAIL	-0.035	-1.089	0.622	0.591	-0.054	-1.210	0.489	0.404
CTACK	0.065	-0.358	1.106	0.315	0.055	-0.417	1.049	0.396
Eotaxin	-0.083	-1.367	0.291	0.202	-0.071	-1.293	0.367	0.273
GRO-α	0.047	-0.500	1.070	0.475	0.042	-0.543	1.054	0.529
MIG	-0.091	-0.884	0.148	0.162	-0.098	-0.918	0.124	0.134
IL-16	-0.085	-1.367	0.277	0.193	-0.107	-1.508	0.137	0.102
IL-8	0.063	-0.289	0.858	0.330	0.062	-0.297	0.855	0.341
IP-10	-0.055	-1.133	0.451	0.397	-0.058	-1.163	0.440	0.376
MCP-1	-0.011	-0.613	0.518	0.868	-0.003	-0.573	0.544	0.960
MCP-3	-0.061	-1.367	0.481	0.346	-0.074	-1.465	0.400	0.262

MIP-1α	0.064	-0.333	0.999	0.326	0.061	-0.332	0.964	0.338
MIP-1β	0.061	-0.352	0.988	0.351	0.043	-0.433	0.884	0.501
RANTES	-0.045	-1.098	0.525	0.487	-0.038	-1.051	0.575	0.564
β-NGF	0.001	-0.843	0.859	0.985	-0.002	-0.877	0.849	0.975
FGF	-0.057	-1.026	0.395	0.383	-0.038	-0.921	0.502	0.562
G-CSF	0.000	-0.590	0.586	0.995	0.002	-0.584	0.602	0.976
GM-CSF	0.039	-0.411	0.772	0.548	0.058	-0.322	0.856	0.373
HGF	-0.066	-0.925	0.295	0.310	-0.071	-0.945	0.267	0.271
IL-3	-0.111	-1.330	0.092	0.087	-0.115	-1.363	0.082	0.082
IL-7	0.037	-0.375	0.685	0.565	0.040	-0.366	0.700	0.538
IL-9	0.063	-0.247	0.718	0.337	0.063	-0.246	0.725	0.333
LIF	-0.064	-0.758	0.250	0.322	-0.071	-0.769	0.206	0.257
M-CSF	-0.046	-0.601	0.283	0.478	-0.048	-0.612	0.278	0.460
PDGF-BB	0.029	-0.560	0.889	0.655	0.047	-0.458	0.987	0.471
SCF	-0.074	-1.599	0.428	0.428	-0.077	-1.640	0.415	0.241
SCGF-β	-0.058	-0.574	0.216	0.374	-0.064	-0.593	0.201	0.332
SDF-1α	-0.094	-0.551	0.083	0.148	-0.088	-0.540	0.104	0.183
VEGF	0.095	-0.145	0.982	0.145	0.096	-0.150	0.996	0.148
MMP-1	0.042	-0.464	0.912	0.522	0.035	-0.455	0.834	0.563
MMP-2	0.042	-0.449	0.882	0.523	0.051	-0.359	0.891	0.403
MMP-7	0.054	-0.360	0.891	0.404	0.043	-0.388	0.809	0.489
MMP-9	-0.019	-1.192	0.882	0.768	-0.031	-1.296	0.788	0.632
MMP-10	0.001	-0.831	0.842	0.989	-0.003	-0.809	0.773	0.964

Table 10. Linear regression model examining the association between mucosal IFNγ and mucosal cytokine/chemokines

Variables	Univariable				Multivariable			
	β-coefficient	95% CI		p-value	aβ-coefficient	95% CI		p-value
		Lower	Upper			Lower	Upper	
IL-10	-0.087	-0.141	0.027	0.182	-0.089	-0.142	0.026	0.176
IL-1RA	-0.130	-0.222	-0.003	**0.045**	-0.131	-0.224	-0.003	**0.045**
IL-13	0.042	-0.090	0.179	0.518	0.037	-0.097	0.175	0.569
IL-15	-0.112	-0.193	0.012	0.084	-0.112	-0.193	0.012	0.084
IL-17	0.025	-0.115	0.171	0.699	0.013	-0.127	0.157	0.838
IL-2	-0.064	-0.183	0.061	0.324	-0.071	-0.189	0.055	0.278
IL-2RA	-0.071	-0.159	0.045	0.273	-0.071	-0.159	0.045	0.275
IL-4	0.105	-0.016	0.163	0.105	0.091	-0.024	0.152	0.151
IL-5	0.034	-0.080	0.137	0.602	0.025	-0.085	0.126	0.700
IL-12p40	-0.011	-0.139	0.117	0.865	-0.006	-0.132	0.120	0.927
IL-12p70	-0.009	-0.103	0.089	0.892	-0.009	-0.104	0.090	0.894
IL-18	-0.072	-0.133	0.037	0.266	-0.082	-0.137	0.028	0.194
IL-1α	0.018	-0.067	0.088	0.788	0.014	-0.068	0.085	0.824
IL-1β	-0.013	-0.109	0.089	0.838	-0.023	-0.114	0.078	0.710
IL-6	-0.010	-0.067	0.057	0.873	-0.020	-0.069	0.051	0.755
MIF	0.053	-0.047	0.112	0.419	0.041	-0.050	0.101	0.509

TNF-α	-0.031	-0.072	0.044	0.638	-0.041	-0.076	0.038	0.518
TNF-β	-0.076	-0.143	0.037	0.244	-0.073	-0.141	0.039	0.263
TRAIL	-0.053	-0.181	0.075	0.418	-0.057	-0.183	0.069	0.372
CTACK	-0.010	-0.119	0.101	0.874	0.001	-0.108	0.110	0.991
Eotaxin	0.020	-0.106	0.144	0.765	0.010	-0.114	0.133	0.879
GRO-α	-0.099	-0.208	0.027	0.129	-0.096	-0.206	0.030	0.143
MIG	0.012	-0.071	0.085	0.857	0.011	-0.071	0.084	0.867
IL-16	-0.065	-0.186	0.061	0.321	-0.070	-0.190	0.055	0.277
IL-8	0.052	-0.051	0.121	0.423	0.044	-0.056	0.115	0.495
IP-10	0.042	-0.080	0.158	0.519	0.041	-0.081	0.157	0.526
MCP-1	-0.059	-0.124	0.046	0.364	-0.064	-0.125	0.040	0.314
MCP-3	0.003	-0.135	0.143	0.957	0.008	-0.131	0.147	0.907
MIP-1α	-0.053	-0.142	0.058	0.411	-0.065	-0.147	0.045	0.299
MIP-1β	-0.027	-0.121	0.080	0.683	-0.039	-0.129	0.067	0.535
RANTES	0.052	-0.072	0.171	0.421	0.043	-0.079	0.162	0.502
β-NGF	0.014	-0.113	0.142	0.827	0.010	-0.119	0.138	0.884
FGF	-0.114	-0.200	0.012	0.080	-0.120	-0.205	0.005	0.062
G-CSF	-0.019	-0.101	0.075	0.776	-0.027	-0.106	0.070	0.683
GM-CSF	-0.180	-0.212	-0.037	**0.005**	-0.175	-0.207	-0.035	**0.006**
HGF	-0.024	-0.109	0.074	0.712	-0.033	-0.114	0.066	0.605
IL-3	-0.082	-0.176	0.038	0.206	-0.081	-0.175	0.040	0.217
IL-7	-0.017	-0.090	0.069	0.798	-0.017	-0.090	0.069	0.797
IL-9	-0.098	-0.127	0.017	0.133	-0.104	-0.130	0.013	0.110
LIF	-0.031	-0.094	0.057	0.630	-0.033	-0.092	0.053	0.594
M-CSF	-0.050	-0.092	0.040	0.441	-0.055	-0.095	0.037	0.395
PDGF-BB	0.064	-0.054	0.163	0.327	0.057	-0.059	0.155	0.379
SCF	0.008	-0.142	0.162	0.898	0.003	-0.149	0.157	0.959
SCGF-β	-0.024	-0.071	0.048	0.708	-0.031	-0.073	0.045	0.636
SDF-1α	0.059	-0.026	0.070	0.363	0.057	-0.027	0.069	0.383
VEGF	-0.024	-0.101	0.069	0.712	-0.024	-0.101	0.070	0.714
MMP-1	0.059	-0.055	0.151	0.364	0.051	-0.054	0.137	0.397
MMP-2	0.046	-0.064	0.135	0.485	0.036	-0.065	0.121	0.556
MMP-7	0.016	-0.082	0.105	0.810	0.003	-0.087	0.091	0.962
MMP-9	-0.020	-0.179	0.132	0.764	-0.023	-0.182	0.127	0.727
MMP-10	0.054	-0.072	0.178	0.405	0.045	-0.073	0.161	0.461

Table 11. Linear regression model examining the association between mucosal IFNλ1 and mucosal cytokine/chemokines

Variables	Univariable				Multivariable			
	β-coefficient	95% CI		p-value	aβ-coefficient	95% CI		p-value
		Lower	Upper			Lower	Upper	
IL-10	-0.180	-0.675	-0.120	**0.005**	-0.173	-0.665	-0.095	**0.009**
IL-1RA	-0.064	-0.556	0.184	0.324	-0.061	-0.558	0.203	0.359
IL-13	0.057	-0.250	0.653	0.380	0.062	-0.245	0.683	0.354

IL-15	-0.024	-0.411	0.280	0.708	-0.009	-0.375	0.329	0.896
IL-17	-0.033	-0.602	0.357	0.615	-0.026	-0.584	0.385	0.687
IL-2	-0.001	-0.413	0.406	0.987	0.011	-0.383	0.453	0.870
IL-2RA	-0.133	-0.694	-0.016	**0.040**	-0.118	-0.662	0.031	0.074
IL-4	-0.217	-0.803	-0.215	<0.001	-0.215	-0.798	-0.211	<0.001
IL-5	0.033	-0.269	0.456	0.612	0.022	-0.297	0.424	0.729
IL-12p40	-0.146	-0.911	-0.063	**0.025**	-0.120	-0.828	0.027	0.066
IL-12p70	-0.050	-0.446	0.197	0.447	-0.043	-0.439	0.223	0.522
IL-18	-0.067	-0.435	0.135	0.302	-0.056	-0.406	0.157	0.383
IL-1α	0.006	-0.247	0.273	0.921	0.009	-0.244	0.279	0.896
IL-1β	-0.064	-0.496	0.166	0.328	-0.061	-0.485	0.169	0.343
IL-6	-0.123	-0.404	0.007	0.058	-0.087	-0.344	0.064	0.178
MIF	0.058	-0.144	0.386	0.369	0.074	-0.104	0.409	0.243
TNF-α	-0.122	-0.379	0.007	0.059	-0.110	-0.361	0.027	0.092
TNF-β	-0.174	-0.706	-0.112	**0.007**	-0.155	-0.669	-0.062	**0.018**
TRAIL	-0.080	-0.697	0.160	0.218	-0.085	-0.715	0.144	0.192
CTACK	-0.047	-0.504	0.232	0.466	-0.058	-0.538	0.205	0.378
Eotaxin	-0.197	-1.050	-0.231	**0.002**	-0.189	-1.031	-0.203	**0.004**
GRO-α	0.003	-0.387	0.402	0.969	0.018	-0.349	0.461	0.785
MIG	-0.106	-0.475	0.043	0.102	-0.080	-0.427	0.102	0.226
IL-16	-0.081	-0.676	0.150	0.210	-0.061	-0.617	0.221	0.353
IL-8	0.055	-0.165	0.411	0.401	0.055	-0.169	0.415	0.408
IP-10	-0.061	-0.588	0.207	0.346	-0.043	-0.542	0.271	0.513
MCP-1	-0.084	-0.469	0.097	0.197	-0.052	-0.398	0.168	0.423
MCP-3	-0.092	-0.796	0.130	0.158	-0.068	-0.719	0.227	0.306
MIP-1α	-0.078	-0.539	0.130	0.229	-0.060	-0.486	0.172	0.348
MIP-1β	-0.109	-0.621	0.049	0.094	-0.095	-0.583	0.082	0.139
RANTES	-0.131	-0.821	-0.012	**0.043**	-0.112	-0.767	0.053	0.087
β-NGF	0.086	-0.138	0.714	0.184	0.106	-0.081	0.789	0.110
FGF	-0.035	-0.454	0.260	0.593	-0.021	-0.419	0.303	0.753
G-CSF	-0.123	-0.576	0.010	0.058	-0.108	-0.547	0.050	0.103
GM-CSF	-0.022	-0.348	0.246	0.736	-0.022	-0.349	0.249	0.743
HGF	-0.072	-0.479	0.134	0.268	-0.055	-0.439	0.176	0.401
IL-3	-0.146	-0.764	-0.053	**0.024**	-0.133	-0.737	-0.006	**0.046**
IL-7	-0.099	-0.470	0.060	0.129	-0.085	-0.446	0.093	0.198
IL-9	-0.211	-0.636	-0.161	**0.001**	-0.190	-0.603	-0.118	**0.004**
LIF	-0.131	-0.510	-0.007	**0.044**	-0.095	-0.435	0.058	0.133
M-CSF	-0.140	-0.462	-0.022	**0.031**	-0.129	-0.447	0.000	**0.050**
PDGF-BB	-0.245	-1.048	-0.342	<0.001	-0.234	-1.021	-0.308	<0.001
SCF	-0.120	-0.984	0.029	0.065	-0.105	-0.937	0.103	0.115
SCGF-β	0.019	-0.168	0.229	0.765	0.047	-0.129	0.274	0.480
SDF-1α	-0.016	-0.180	0.140	0.809	0.000	-0.164	0.163	0.995
VEGF	-0.060	-0.416	0.152	0.361	-0.048	-0.397	0.186	0.476
MMP-1	-0.070	-0.533	0.157	0.283	-0.027	-0.401	0.253	0.657
MMP-2	-0.038	-0.435	0.234	0.556	-0.001	-0.320	0.314	0.985
MMP-7	-0.119	-0.603	0.021	0.068	-0.101	-0.549	0.055	0.109
MMP-9	0.013	-0.469	0.572	0.845	0.009	-0.492	0.565	0.893
MMP-10	-0.030	-0.518	0.322	0.645	0.002	-0.394	0.407	0.975

Table 12. Linear regression model examining the association between mucosal IFNλ2 and mucosal cytokine/chemokines

Variables	Univariable				Multivariable			
	β-coefficient	95% CI		p-value	aβ-coefficient	95% CI		p-value
		Lower	Upper			Lower	Upper	
IL-10	-0.094	-0.477	0.073	0.150	-0.100	-0.499	0.066	0.133
IL-1RA	-0.103	-0.652	0.071	0.115	-0.097	-0.648	0.097	0.147
IL-13	0.049	-0.274	0.611	0.454	0.040	-0.317	0.595	0.549
IL-15	-0.056	-0.486	0.191	0.392	-0.036	-0.440	0.251	0.590
IL-17	0.087	-0.150	0.786	0.182	0.057	-0.266	0.684	0.387
IL-2	0.160	0.105	0.898	**0.013**	0.163	0.104	0.914	**0.014**
IL-2RA	-0.219	-0.899	-0.245	<0.001	-0.222	-0.914	-0.245	<0.001
IL-4	0.030	-0.277	0.364	0.648	-0.008	-0.315	0.276	0.897
IL-5	-0.016	-0.400	0.312	0.809	-0.052	-0.499	0.209	0.422
IL-12p40	-0.145	-0.893	-0.061	**0.025**	-0.110	-0.780	0.060	0.093
IL-12p70	-0.058	-0.457	0.173	0.375	-0.061	-0.474	0.175	0.365
IL-18	-0.047	-0.382	0.178	0.473	-0.068	-0.425	0.128	0.290
IL-1α	0.003	-0.249	0.260	0.967	-0.022	-0.301	0.212	0.735
IL-1β	0.006	-0.310	0.341	0.925	-0.021	-0.375	0.268	0.744
IL-6	-0.021	-0.235	0.170	0.752	-0.032	-0.252	0.150	0.620
MIF	0.044	-0.171	0.349	0.499	0.015	-0.223	0.282	0.817
TNF-α	-0.037	-0.246	0.136	0.571	-0.056	-0.276	0.108	0.388
TNF-β	-0.220	-0.797	-0.219	<0.001	-0.222	-0.806	-0.218	<0.001
TRAIL	-0.133	-0.855	-0.020	**0.040**	-0.153	-0.923	-0.086	**0.018**
CTACK	0.047	-0.228	0.493	0.469	0.083	-0.129	0.599	0.204
Eotaxin	-0.080	-0.633	0.153	0.220	-0.112	-0.769	0.054	0.088
GRO-α	-0.085	-0.641	0.129	0.192	-0.070	-0.607	0.186	0.297
MIG	-0.068	-0.389	0.120	0.299	-0.060	-0.380	0.009	0.366
IL-16	-0.120	-0.783	0.024	0.065	-0.120	-0.791	0.027	0.067
IL-8	0.052	-0.167	0.398	0.422	0.028	-0.226	0.348	0.676
IP-10	-0.010	-0.421	0.360	0.876	0.004	-0.386	0.413	0.947
MCP-1	-0.096	-0.486	0.068	0.138	-0.087	-0.465	0.089	0.181
MCP-3	-0.125	-0.896	0.008	0.050	-0.108	-0.845	0.080	0.105
MIP-1α	-0.032	-0.409	0.248	0.628	-0.063	-0.483	0.162	0.328
MIP-1β	0.018	-0.285	0.376	0.786	-0.001	-0.331	0.326	0.988
RANTES	-0.007	-0.423	0.377	0.909	-0.027	-0.488	0.322	0.687
β-NGF	0.029	-0.325	0.513	0.659	0.023	-0.353	0.506	0.725
FGF	-0.061	-0.517	0.182	0.346	-0.072	-0.550	0.157	0.274
G-CSF	0.011	-0.264	0.314	0.866	-0.001	-0.298	0.292	0.983
GM-CSF	-0.022	-0.432	0.240	0.732	-0.008	-0.312	0.276	0.904
HGF	0.007	-0.284	0.318	0.910	-0.013	-0.333	0.272	0.843
IL-3	-0.237	-0.992	-0.308	<0.001	-0.238	-1.004	-0.300	<0.001
IL-7	-0.030	-0.323	0.200	0.644	-0.037	-0.340	0.190	0.578
IL-9	-0.080	-0.386	0.089	0.219	-0.086	-0.402	0.081	0.191
LIF	-0.126	-0.492	0.002	0.052	-0.135	-0.504	-0.022	**0.033**
M-CSF	-0.033	-0.274	0.162	0.612	-0.047	-0.301	0.142	0.479
PDGF-BB	0.005	-0.343	0.371	0.938	-0.019	-0.413	0.307	0.772

SCF	-0.108	-0.917	0.078	0.098	-0.115	-0.957	0.063	0.085
SCGF-β	-0.015	-0.218	0.171	0.812	-0.022	-0.231	0.165	0.741
SDF-1α	-0.043	-0.209	0.105	0513	-0.045	-0.215	0.106	0.505
VEGF	-0.044	-0.374	0.183	0.498	-0.044	-0.382	0.191	0.511
MMP-1	0.040	-0.234	0.444	0.542	0.024	-0.258	0.384	0.699
MMP-2	0.048	-0.204	0.451	0.458	0.018	-0.265	0.358	0.771
MMP-7	0.062	-0.159	0.456	0.342	0.035	-0.215	0.381	0.584
MMP-9	0.083	-0.179	0.839	0.203	0.074	-0.225	0.810	0.266
MMP-10	0.057	-0.227	0.596	0.378	0.032	-0.289	0.498	0.603

3.6 Effect of BV/STI treatment on mucosal IFN expression

Next, we assessed the impact of BV/STI treatment on mucosal IFNs expression. Here, women diagnosed and treated for BV/STIs at baseline were retested after 6- and 12 weeks. Of the 170 women with BV/STIs at baseline, 77 (45.29%) were treated for their respective diagnosis and only 60 (35.29%) were retested at 6- and 12 weeks post treatment. The 60 women were then grouped based on their BV/STIs status including BV only (n=25), STIs only (n=3), BV detected (n=57, irrespective of STI detection), STIs detected (n=35, irrespective of BV detection), BV and STIs (n=32), and BV/STIs (n=60) in order to assess the effect of BV and/or STI treatment on mucosal IFN concentrations. Most women were able to clear their STIs (88.57%, 31/35) while only 24.56% (14/57) those had their BV resolved by week 12. We observed no significant effect of BV and/or STI treatment on mucosal IFN expression (**Figure 10**).

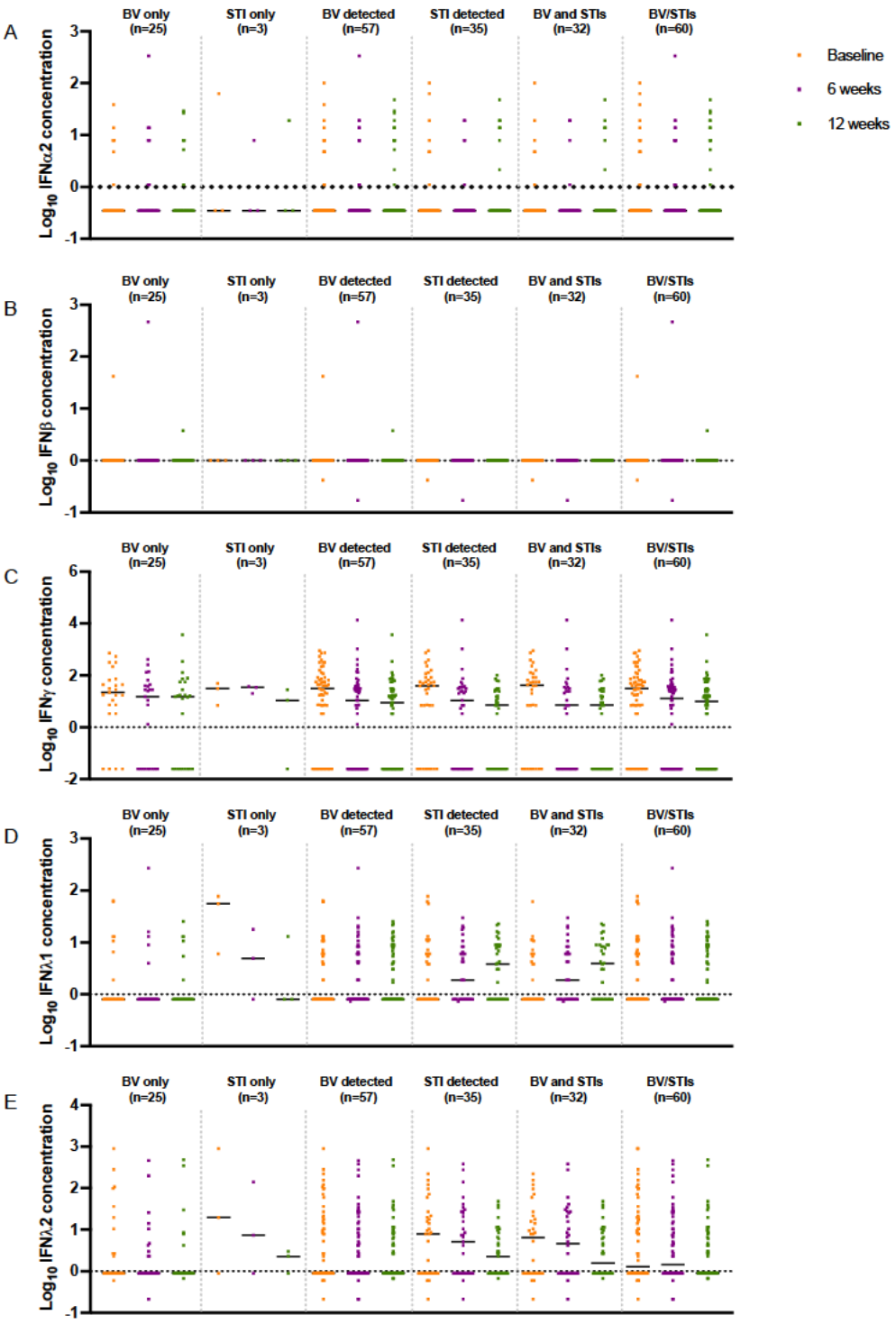

Figure 10: IFN concentrations in softcup samples of women with BV/STIs at baseline and 6- and 12 weeks post treatment. The independent effects of BV and STI treatment in women with BV/STIs at baseline was assessed only in those that returned for the 6- and 12 week visits. Women were further classified as having BV only, STI only, BV detected, STI detected, BV and STIs as well as BV/STIs. Baseline, 6- and 12 week visits are presented by orange, purple and green dots, respectively. The line indicates the median. Friedman matched-pairs rank sum test was used in order to compare the IFN concentrations between the matched softcup samples across all visits. IFN concentrations were log transformed.

3.7 Correlation between mucosal IFNs and mucosal CD4$^+$ T cell profile

The median percentage of CD4$^+$ T cells across all samples was 9.2 (IQR 3.4-14.58). The percentage of other populations of interest are specified in **Table 13.** Mucosal IFN concentrations in matching CAPRISA 012C softcup specimens were correlated with measured CD4$^+$ T cell profiles in the cytobrush specimens. We observed that IFNα2 was positively correlated with percentage of CD69 on CD4$^+$ T cells (r=0.3007, p=0.0185), CCR5+CCR6+ (r=0.2707, p=0.0436) as well as total CCR6+ on activated T cells (r=0.2878, p=0.0315) and %Treg-like on CD4$^+$ T cells (r=0.2985, p=0.0195). IFNα2 was also negatively correlated with %HLADR+CD38- on CD4+ T cells (r=-0.2711, p=0.0346, **Table 14, Figure 11**). There were no significant correlations observed between CD4$^+$ T cell profiles and IFNβ, IFNγ, IFNλ1 and IFNλ2.

Table 13. Percentage of target populations in the FGT cytobrush

Target populations	Median	Interquartile range (IQR)
%CD4+ T cells	9.2	3.4-14.58
%CCR5 on CD4+ T cells	86.80	77.23-91.48
%CCR6 on CD4+ T cells	55.34	43.99-65.93
%HLADR on CD4+ T cells	22.85	14.23-36.65
%CD38 on CD4+ T cells	43.30	31.98-52.40
%HLADR+CD38+ T cells	17.70	10.70-26.13
%Treg-like CD4+ T cells	18.20	11.53-24.60
%CD69 on CD4+ T cells	80.00	68.60-87.53
%CCR5 on HLADR+CD38+ T cells	98.60	94.40-100.0
%CCR6 ob HLADR+CD38+ T cells	34.54	20.15-50.00

Table 14. Significant Spearman correlation between IFNs and CD4⁺ T cell profiles in total cytobrushes (n=62)

IFNα2 correlation	Spearman r	p-value
%CD69 on CD4+ T cells	0.3007	0.0185
%CCR5+CCR6+ on HLADR+CD38+ CD4+ T cells	0.2707	0.0436
%CCR6 on HLADR+CD38+ CD4+ T cells	0.2878	0.0315
%HLADR+CD38- CD4+ T cells	-0.2711	0.0346
%Treg-like CD4+ T cells	0.2985	0.0195

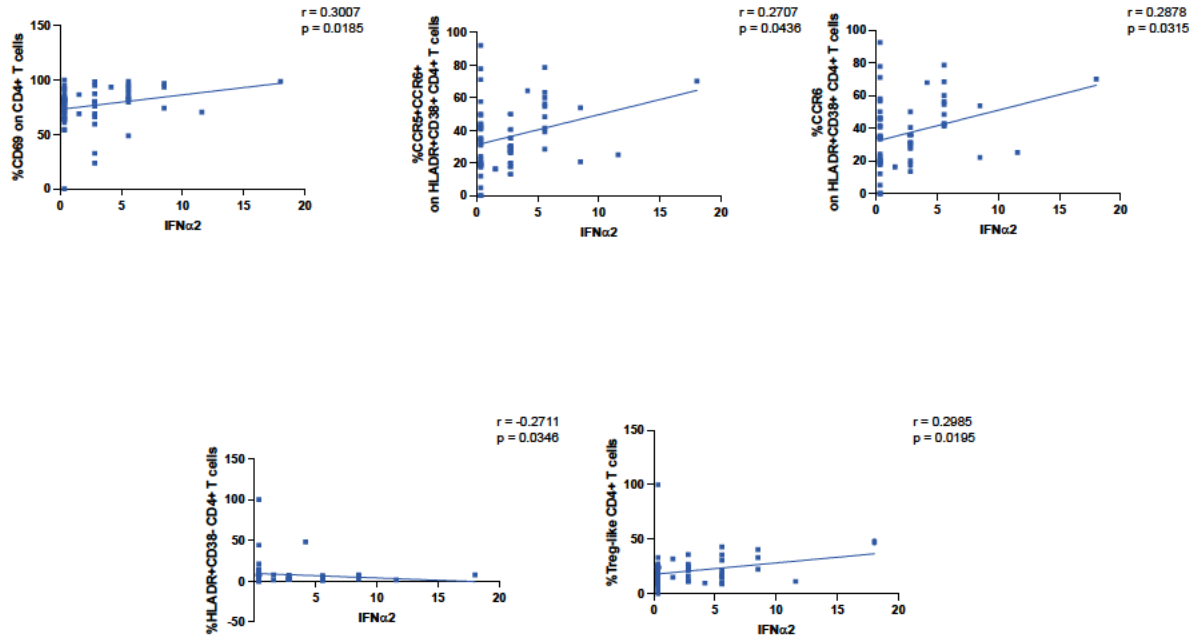

Figure 11: Significant spearman correlation between IFNα2 and CD4⁺ T cell profiles in total cytobrushes (n=62). Linear regression line was added for visualization purposes only.

Chapter 4: Discussion

One of the first immune responses that are activated during a viral infection are IFNs. They play an important role in controlling the replication and dissemination of the virus, particularly at mucosal surfaces, which are important sites for host exposure to infections (Walker et al., 2021). It is crucial to inhibit the establishment and spread of the virus from these mucosal sites in order to prevent severe infection as well as reduce the negative effects of inflammation. IFNs have been unsuccessfully utilized to try and prevent SIV infection, highlighting the gaps in knowledge on their regulation and induction of antiviral response at the FGT, especially in the context of HIV transmission. IFN responses can be impacted by various factors including presence of other co-infections and suboptimal microbiome. This study examined the drivers of type I, II, and III IFN upregulation at the FGT and the associated changes in the mucosal immune environment.

Here we measured the expression of 5 different IFNs (IFNα2, IFNβ, IFNγ, IFNλ1 and IFNλ2) in two CAPRISA cohorts and associated their expression with various clinical, demographic, and immunological parameters. Overall, IFNs had low detectability in mucosal specimens with some variation between the two cohorts likely due to different clinical and demographic characteristics, including presence of BV/STIs at baseline.

When examining the association between mucosal IFN expression and presence of BV/STIs, we found that IFNα2 was significantly elevated in the presence of BV/STIs. With more than 50 different pathogens that can be sexually transmitted; *Chlamydia trachomatis*, HIV, HSV, HPV, hepatis B, *Neisseria gonorrhoea*, *Treponema pallidum* (syphilis) and *Trichomonas vaginalis* are known as the most common STIs. These have also been classified as having detrimental effects on the female genital mucosa (Mwatelah et al., 2019). This is due to the STIs ability to induce a large inflammatory response which not only causes damage to the epithelial barrier but also reduces the natural innate defence of the host by recruiting HIV target cells thereby increasing the risk of acquiring HIV (Arnold et al., 2016). Although the STI induced inflammatory response is aimed to protect the host, it often favours the pathogen. A similar trend has also been observed in IFNs whereby increased immune activation and an influx of other immune cells perpetuate the replication as well as the dissemination of the virus (Bosinger and Utay, 2015). Presence of BV in the FGT is also associated with

increased risk of HIV acquisition (van De Wijgert et al., 2008, Lennard et al., 2018). Previous studies conducted in South African women with STI infections highlighted that inflammatory cytokine levels were in fact increased (Passmore et al., 2016) and women who possessed a vaginal microbiome that was *Lactobacillus* spp. deficient were more susceptible to acquiring both STIs and HIV (Gosmann et al., 2017). According to Masson et al. (2016), BV and STIs were associated with elevated inflammatory cytokine levels such as IFNα2, IFNγ, IL-1α, IL-1β, IL-8 and TNF-α. This is consistent with what we observed in our study whereby IFNα2 was significantly elevated in women with BV/STIs, with no significant associations observed for the other IFN types. Interestingly, we observed no changes in mucosal IFN levels following BV/STI treatment. While this could be due to the sample size and detectability limitations, it could also indicate chronic IFNα2 upregulation that persists after treatment. Previous study looking at the effect of treatment on cytokine/chemokine expression in the same cohort found a significant reduction in GRO-α, MIG, G-CSF, HGH, β-NGF, SCF, and SCGF-β post-treatment of STIs, with no changes in IFNα (Garrett et al., 2021). The management of BV continues to be a global challenge due to the frequent recurrences, particularly among women of African descent (Van De Wijgert et al., 2014), and inadequate treatment options. In a study conducted by Mtshali et al. (2021), it was observed that the complete clearance of BV was suboptimal with recurrence rates being high post-treatment. The study also stated that despite treatment, most women still experienced persistent BV. This was due to the fact that the metronidazole treatment temporarily reduced the relative abundance of anaerobes (*G. vaginalis* and *A. vaginae*) associated with BV and induced *L. iners* dominant state that had a high chance of transitioning back to BV (Mtshali et al., 2021). Therefore, this could be another plausible explanation as to why we do not observe any changes in IFNs post-treatment.

Results from this study have shown associations between the IFNs and other genital cytokines/chemokines. Cytokines are polypeptides/glycoproteins that act on signaling molecules and cells that stimulate them towards sites of infection, trauma, and inflammation. They play an important role in the development as well as regulation of immune system cells (Zhang and An, 2007). In this study, IL-10 which is classified as a cytokine with potent anti-inflammatory properties that inhibit innate and acquired immune response that are undesirable (Couper et al., 2008) was associated with two

IFNs (-α2 and -λ1). While IFNs generally have pro-inflammatory effects, the balance between IL-10 and IFNs is crucial for immune homeostasis since IL-10 is responsible for the termination of inflammatory responses, thereby reducing the damage of self-tissues in the process of eradication of pathogens (Zhang and An, 2007). Notably, we observed that IFNα2 was associated with increased IL-4. IFNα is generally associated with antiviral response and induces Th1 immune response, while IL-4 induces Th2 immune response and is associated with allergy and defense against certain parasites (Kim and Lee, 2011). IFNα and IL-4 often display actions that are antagonistic against each other in the context of IgE production, expression of MHC class II receptors and differentiation of Th1 versus Th2 cells (Huber et al., 2010, Sriram et al., 2007). This has also been observed between IFNγ and IL-4 (Paludan, 1998). Overall, the antagonistic effects contribute to the regulation of immune responses creating a balance that is crucial for the avoidance of immune related disorders (Romagnani, 1997, Paludan, 1998). Additionally, IFNα and IL-12 are innate cytokines that play an important role in defense against viruses (Biron, 1999). IL-12 is known to exist in two isoforms: IL-12p40 and IL-12p70. The biologically active form, IL-12p70, is a heterodimer composed of a subunit that is inducible (IL-12p40) and a subunit (IL-12p35) that is constitutively expressed in some cells while they are regulated in others (Hochrein et al., 2000). IL-12p70 stimulates T cells and NK cells to proliferate, increases cytotoxicity and promote the production of IFNγ. However, previous studies conducted by Nguyen et al. (2000) and Byrnes et al. (2001) observed that IL-12 production is inhibited by high concentrations of IFNα which is contradictory to what was observed in this study whereby IFNα2 was associated with increased IL-12p70. This may have been due to the fact that they both play a role of enhancing the immune defense against viral infections by promoting the activation of various immune cells.

We observed an association between IFNβ and decreased levels of IL-18 which is a potent pro-inflammatory cytokine belonging to the IL-1 family that is involved in host defense against infection and stimulation of both the innate and acquired immune responses (Sims and Smith, 2010). The inverse association of it to IFNβ could contribute to anti-inflammatory nature of IFNβ. Additionally, IFNλ2 levels were associated with increased IL-2 which is commonly produced by $CD4^+$ and $CD8^+$ T cells and plays a role in the development of T_{reg} cells, stimulates the synthesis of antibodies

as well as promoting differentiation and proliferation of NK and Th cells (Ferreira et al., 2018). We also observed that IFNλ1 and IFNλ2 was associated with decreased TNF-β and TRAIL, respectively. TNF-β, a Th1 cytokine, is produced primarily by activated B and T lymphocytes (Aggarwal et al., 2012). In addition to being a potent mediator of immune and inflammatory responses, it is associated with regulating biological processes such as apoptosis, differentiation, and proliferation of cells (Vassalli, 1992). TRAIL is a member of the TNF superfamily commonly referred to as a death ligand cytokine that is mostly utilized by effector immune cells to kill malignant cells (Alizadeh Zeinabad and Szegezdi, 2022). Additionally, various immune cells including DCs, monocytes, macrophages, eosinophil granulocytes, NK cells, B and T cells express TRAIL (Zahn et al., 2011, Mariani and Krammer, 1998).

In this study, IFNα2 was associated with increased FGF-basic and PDGF-BB while inversely associated with IL-3. There was also an inverse association between IL-3, IL-9, and M-CSF with IFN-λ1. Additionally, IFNγ and IFNλ2 was inversely associated with GM-CSF and LIF, respectively. These cytokines were classified as growth factors which are natural signaling molecules that control how cells migrate, proliferate and differentiate during the wound healing process (Macri and Clark, 2009). There are three phases to the wound healing process: the inflammatory, proliferative and remodeling phase which happens consecutively in a continuous manner while overlapping each other occasionally (Braund et al., 2007). Growth factors play an important role in regulating inflammatory responses, promoting the formation of granulation tissue, and initiation of angiogenesis (Park et al., 2017). During the inflammatory phase that is characterized by hemostasis and inflammation, activated platelets release growth factors like epidermal growth factors (EGF), platelet-derived growth factor (PDGF), transforming growth factor alpha and beta (TGF-α and -β), and insulin-like growth factor 1 (IGF-1) which normally diffuse into surrounding tissues and draw monocytes and neutrophils to the wound by chemotactic attraction (Braund et al., 2007, Kiritsy and Lynch, 1993). This is followed by monocyte differentiation into macrophages thereby facilitating the proliferation phase that is characterized by granulation tissue formation and angiogenesis induction (Martin, 1997). The macrophages release vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF-basic) which are primarily responsible for angiogenesis (Martin, 1997). The positive association between IFNα2 and FGF-basic and PDGF-BB may

have been a result of inflammation and facilitating wound healing of possible lesions in the FGT since the women forming the cohort were sexually active.

Activated CD4+ T cells are known as the main target of HIV infection and their depletion ultimately leads to AIDS (Alvarez et al., 2013). In recent years, there have been few studies that examined specific T helper (Th) cell subsets impacted by HIV infection. Th17 cells which secrete cytokines, including IL-17 and IL-22, that are essential for controlling bacterial and fungal infections as well as preserving mucosal integrity (Korn et al., 2009). According to Alvarez et al. (2013), Th17 cells loss has been associated with HIV disease progression in both animal and human models. This is due to the fact that there is an increase in the bacterial products being translocated across the mucosal barrier, increased viral load and hyperactivation of the immune system (Cecchinato et al., 2008, Favre et al., 2009). Additionally, depletion of Th17 cells was evident in the early stages of pathogenic SIV infection in rhesus macaques (Brenchley et al., 2008) while McKinnon et al. (2011) also observed a severe depletion in a subset of Th17 cells in human cervical tissues, identifying these cells as primary HIV target cells at the mucosa. In this study, we observed that IFNα2 was associated with cellular markers of HIV susceptible cells, particularly activated cells and cells expressing CCR6 (Th17), at the FGT. These results show that IFNα2 is associated with immune markers of increased risk of HIV infection in young South African women and could explain the observation made in the CAPRISA 004 study where IFNα2 was the main cytokine associated with increased HIV risk.

Study strengths and limitations

The samples utilized for this study came from the area with the highest HIV incidence and prevalence in the world. Another important strength of the study was the access to fresh mucosal specimens and ability to correlate soluble markers to cellular profiles at the site of viral exposure and transmission.

One of the limitation of this study was that the cohorts were complex with a number of measured and unmeasured variables and coinfections that could confound the data. Secondly, the detectability of most IFNs was low in the both the study cohorts utilized. Therefore, other assays could be explored for more sensitive IFN detection (example:

meso scale discovery assays or standard ELISAs). The sample size is relatively small considering the clinical complexity of the cohort studied. This particularly impacted the assessment of the independent effects of BV or STI treatment due to the low number of participants with BV or STIs only. Lastly, our study is correlative in nature and other models need to be utilized to determine causality.

Future directions

The reported results should be validated in other relevant cohorts and different geographical areas. Future studies should look at IFNα and IFNβ responsiveness in different cell types at the FGT. This could be achieved by stimulating the cytobrush or biopsy samples with different types of IFNs and performing single cell RNAseq analysis. Tissue explant models could be utilized to study causality. Impact and regulation of other IFNs such as type III IFNs and IFNε (that has been found to block HIV infection) (Tasker et al., 2016), should be examined at the FGT.

Overall conclusion

The IFNs play an important role in the FGT by contributing to the local immune defence against infections, particularly viral infections. They achieve this by upregulating antiviral proteins which help with preventing the establishment and dissemination of viral infection as well as regulating immune responses. Our study highlights the negative side of chronic IFN upregulation. Here, we identified that IFNα2 was associated with presence of BV and STIs and increased cellular markers on HIV susceptibility potentially explaining its association with increased HIV risk in CAPRISA 004. If IFNs are to be considered for HIV prevention and treatment, the location and timing of the administration has to be considered carefully. Additionally, the impact of other variables such as microbiome and other co-infections on IFN signalling and regulation has to be considered.

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Appendices

Appendix A: Poster presentations

During my degree, I had the opportunity to present my research in the form of a poster presentation at two national conferences:

1. **Slindile Ngubane**, Thando Maseko, Nikkishia Singh, Farzana Osman, Sinaye Ngcapu, Andile Mtshali, Lenine Liebenberg, Nonsikelelo Ndlela, Desh Archary, Nigel Garrett, Sharana Mahomed, Lyle Mckinnon, Aida Sivro. **Type I, II, and III interferon responses at the female genital tract.** 20 – 23 June 2023. Track 2: Basic and Clinical Sciences. 11th SA AIDS Conference. (Poster presentation)

2. **Slindile Ngubane**, Thando Maseko, Nikkishia Singh, Farzana Osman, Sinaye Ngcapu, Andile Mtshali, Lenine Liebenberg, Nonsikelelo Ndlela, Desh Archary, Nigel Garrett, Sharana Mahomed, Lyle Mckinnon, Aida Sivro. **Type I, II, and III interferon responses at the female genital tract.** 27 November – 2 December 2023. 18th International Congress of Immunology – IUIS. (Poster presentation)

Type I, II, and III interferon responses at the female genital tract

Slindile Ngubane[1], Thando Maseko[1], Nikkishia Singh[1], Farzana Osman[1], Sinaye Ngcapu[1,2], Andile Mtshali[1,2], Lenine Liebenberg[1,2], Nonsikelelo Ndlela[1], Desh Archary[1,2], Nigel Garrett[1,3], Sharana Mahomed[1], Lyle Mckinnon[1,4], Aida Sivro[1,2,4,5]

[1]Centre for the AIDS Programme of Research in South Africa (CAPRISA), Durban, South Africa
[2]University of KwaZulu-Natal, Medical Microbiology, Durban, South Africa
[3]University of KwaZulu-Natal, Public Health Medicine, Durban, South Africa
[4]University of Manitoba, Medical Microbiology and Infectious Diseases, Winnipeg, Canada
[5]JC Wilt Infectious Disease Research Centre, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Canada

Introduction

- Interferon (IFN) responses have the ability to restrict HIV replication at the point of exposure and during acute infection. However, they also contribute to persistent immune activation and CD4$^+$ T cell depletion during chronic infection.
- At what point, and by what mechanisms IFN response turns from protective to pathogenic remains unknown.
- Here, we aimed to characterize mucosal IFN expression and identify drivers of IFN upregulation in the female genital tract (FGT).

Methods

- The samples used for this study were from two CAPRISA cohorts, the CAPRISA 083 and 012C studies.
- Five cytokines (IFNα2, IFN-β, IFNγ, IFN-λ1, and IFN-λ2) were measured using the Bio-Plex Pro Human Inflammatory Panel 1 Assay in 238 and 90 softcup samples from the CAPRISA 083 and 012C study, respectively.
- As IFNs were shown to upregulate CCR5 expression, mucosal IFN levels were correlated to CD4$^+$ T cell phenotypes characterized by flow cytometry in matching cytobrush samples.

Results

CD4$^+$ T cell phenotypic characterization using cytobrush samples

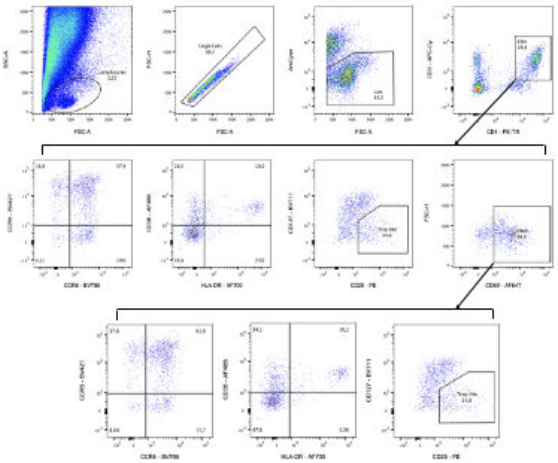

Figure 1. Flow cytometry gating from a representative CAP012C cytobrush sample

Mucosal IFNα2 levels associated with CD4$^+$ T cell phenotypes

Table 1: Spearman correlation between IFNs and T cell phenotypes

	IFNα2	IFNβ	IFNγ	IFNλ2
%CCR6 on CD4+T cells	r= -0.211 (p= 0.070)	r= 0.249 (p= 0.031)	r= 0.0284 (p= 0.809)	r= -0.272 (p= 0.016)
% Treg like cells	r= 0.171 (p= 0.142)	r= -0.022 (p= 0.852)	r= 0.2707 (p= 0.019)	r= 0.098 (p= 0.402)

- There was a negative correlation between IFNα2 and IFN-λ2 with the percentage of CCR6$^+$ CD4$^+$ T cells at the FGT, while IFN-β correlated positively with the percentage CCR6$^+$ CD4$^+$ T cells at the mucosa.
- IFNγ was positively correlated with the percent of T regulatory-like cells at the FGT.

Women with STIs and/or BV had significantly higher levels of IFNα2

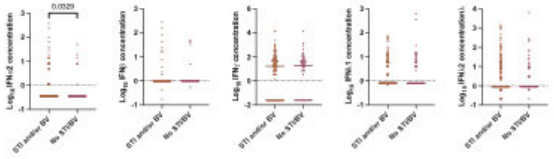

Figure 2. IFN concentrations in softcup samples of women with STIs/BV (n=170) and without STIs/BV (n=68) at baseline. Mann-Whitney U-test was utilized in order to compare the IFN concentration medians between the two groups. IFN concentrations were log transformed and significant p-values are displayed on the graph(s).

Mucosal IFNα2 levels associated with other mucosal inflammatory markers

Table 2: Spearman correlation between IFNs and genital tract cytokines/chemokine profiles of women

	IFNα2	IFNβ	IFNγ	IFNλ1	IFNλ2
IL-10	r= 0.213 (p< 0.001)	**r= 0.138 (p= 0.033)**	r= -0.067 (p= 0.301)	**r= -0.175 (p= 0.007)**	r= -0.093 (p= 0.152)
IL-1Ra	r= 0.090 (p= 0.166)	**r= 0.138 (p= 0.033)**	**r= -0.131 (p= 0.043)**	r= -0.074 (p= 0.257)	r= -0.118 (p= 0.070)
MMP7	**r= 0.149 (p= 0.022)**	r= 0.095 (p= 0.140)	r= -0.013 (p= 0.842)	r= -0.096 (p= 0.141)	r= 0.055 (p= 0.401)
IL-15	**r= -0.135 (p= 0.038)**	r= -0.001 (p= 0.988)	r= -0.086 (p= 0.187)	r= -0.030 (p= 0.643)	r= -0.035 (p= 0.589)
IL-17	**r= 0.130 (p= 0.045)**	r= 0.004 (p= 0.942)	r= 0.020 (p= 0.757)	r= -0.067 (p= 0.305)	r= 0.051 (p= 0.437)
IL-2	r= 0.063 (p= 0.336)	r= 0.029 (p= 0.659)	r= -0.063 (p= 0.337)	r= -0.003 (p= 0.965)	**r= 0.150 (p= 0.021)**
IL-2Rα	r= -0.054 (p= 0.404)	r= -0.087 (p= 0.179)	r= 0.056 (p= 0.389)	r= -0.091 (p= 0.162)	**r= -0.131 (p= 0.044)**
IL-4	**r= 0.210 (p= 0.001)**	r= 0.063 (p= 0.331)	r= 0.071 (p= 0.275)	**r= -0.151 (p= 0.020)**	r= 0.039 (p= 0.545)
IL-12p70	**r= 0.212 (p= 0.001)**	r= 0.100 (p= 0.125)	r= -0.047 (p= 0.471)	**r= -0.135 (p= 0.037)**	r= -0.075 (p= 0.251)
IL-6	**r= 0.146 (p= 0.024)**	r= -0.017 (p= 0.790)	r= 0.019 (p= 0.772)	r= -0.126 (p= 0.053)	r= 0.021 (p= 0.747)
Eotaxin	r= 0.112 (p= 0.084)	r= -0.005 (p= 0.939)	r= 0.037 (p= 0.573)	**r= -0.162 (p= 0.012)**	r= -0.029 (p= 0.662)
FGF-basic	**r= 0.149 (p= 0.021)**	r= -0.034 (p= 0.602)	r= -0.088 (p= 0.174)	r= -0.085 (p= 0.191)	r= -0.041 (p= 0.534)
GM-CSF	r= 0.130 (p= 0.045)	r= 0.046 (p= 0.480)	**r= -0.211 (p= 0.001)**	**r= -0.149 (p= 0.021)**	**r= -0.147 (p= 0.023)**
IL-3	**r= -0.138 (p= 0.034)**	r= -0.097 (p= 0.134)	r= 0.087 (p= 0.181)	r= -0.084 (p= 0.195)	**r= -0.147 (p= 0.024)**
IL-7	**r= 0.168 (p= 0.009)**	r= 0.094 (p= 0.148)	r= 0.049 (p= 0.452)	r= -0.054 (p= 0.411)	r= 0.026 (p= 0.689)
IL-9	**r= 0.173 (p= 0.007)**	r= 0.088 (p= 0.174)	r= -0.016 (p= 0.810)	**r= -0.131 (p= 0.043)**	r= -0.035 (p= 0.590)
M-CSF	**r= 0.176 (p= 0.006)**	r= 0.059 (p= 0.363)	r= 0.023 (p= 0.719)	**r= -0.131 (p= 0.044)**	r= -0.002 (p= 0.971)
PDGF-BB	**r= 0.267 (p= 0.001)**	r= 0.122 (p= 0.059)	r= 0.008 (p= 0.900)	r= -0.205 (p= 0.001)	r= -0.010 (p= 0.878)
VEGF	**r= 0.150 (p= 0.021)**	r= 0.109 (p= 0.092)	r= 0.042 (p= 0.514)	r= -0.034 (p= 0.597)	r= 0.019 (p= 0.768)

Discussion and conclusion

- Mucosal IFNα2 expression was significantly correlated with the presence of STIs/BV and with other pro-inflammatory markers at the mucosal surfaces.
- Presence of BV/STIs and associated inflammation likely impact IFN signaling and the antiviral pathways. This could have negative impact on HIV susceptibility due to impaired IFN response at the site of exposure.

The authors would like to thank all the study participants for their participation in the original study. Additionally, they would like to thank the laboratory team from CAPRISA and University of KwaZulu-Natal for their support during the completion of this project.

Type I, II, and III interferon responses at the female genital tract

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- As IFNs were shown to upregulate CCR5 expression, mucosal IFN levels were correlated to CD4+ T cell phenotypes characterized by flow cytometry in matching cytobrush samples.

Results

Women with STIs and/or BV had significantly higher levels of IFNα2

Table 1: Logistic regression model examining the effect of BV/STIs on mucosal IFNs

	Univariable				Multivariable			
Variables	OR	95% CI		p-value	aOR	95% CI		p-value
		Lower	Upper			Lower	Upper	
IFNα2	2.594	1.098	6.131	**0.030**	2.677	1.125	6.366	**0.026**
IFNβ	1.107	0.340	3.604	0.868	1.104	0.332	3.672	0.872
IFNλ1	1.038	0.580	1.859	0.899	0.987	0.544	1.791	0.965
IFNλ2	1.620	0.901	2.911	0.107	1.672	0.919	3.041	0.092

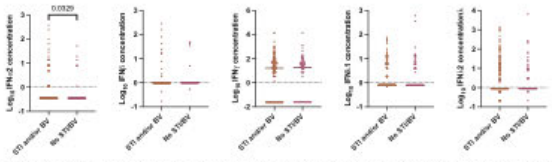


Figure 1. IFN concentrations in softcup samples of women with STIs/BV (n=170) and without STIs/BV (n=68) at baseline. Mann-Whitney U-test was utilized in order to compare the IFN concentration medians between the two groups. IFN concentrations were log transformed and significant p-values are displayed on the graph(s).

BV/STI treatment does not alter IFN concentrations at 6- and 12 weeks

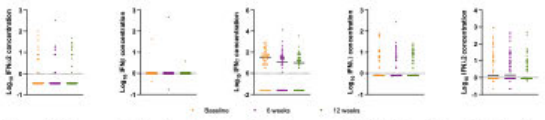


Figure 2. IFN concentration in softcup samples of women with BV/STIs and treated at baseline compared to 6- and 12 weeks post treatment. Friedman matched-pairs rank sum test was used in order to compare the IFN concentration medians between the matched softcup samples across all visits. IFN concentrations were log transformed and no significant p-values were observed.

Association between mucosal IFNs and cytokines/chemokines

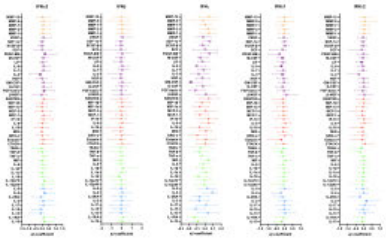


Figure 3. Associations between mucosal IFNs and cervicovaginal cytokines/chemokines at baseline. Multivariable linear mixed models was used to assess the relationship between IFNs and genital cytokines/chemokine concentrations. Inflammation, adaptive response, chemokines, growth factors and MMPs were indicated by green triangles, blue circles, red diamonds, purple squares and orange hexagons, respectively. Filled shapes indicated significant p-values (p<0.05)

CD4+ T cell phenotypic characterization using cytobrush samples

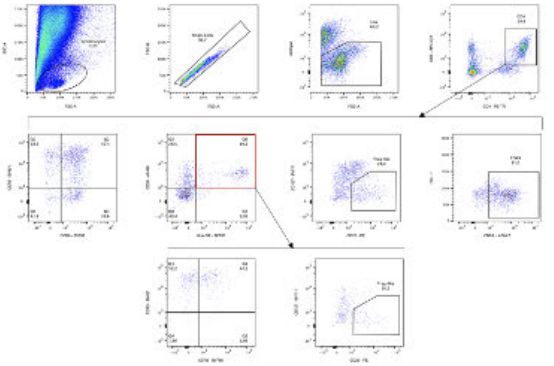
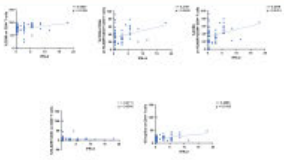


Figure 4. Flow cytometry gating from a representative CAP012C cytobrush sample

Association between mucosal IFNα2 levels and CD4+ T cell phenotypes

Table 2: Spearman correlation between IFNs and T cell phenotypes

Correlation	Spearman r	p-value
IFNα2 vs %CD69 on CD4+ T cells	0.3007	0.0185
IFNα2 vs %CCR5+CCR6+ on HLADR+CD38+ CD4+ T cells	0.2707	0.0436
IFNα2 vs %CCR6 on HLADR+CD38+ CD4+ T cells	0.2878	0.0315
IFNα2 vs %HLADR+CD38- on CD4+ T cells	-0.2711	0.0346
IFNα2 vs %Treg-like on CD4+ T cells	0.2985	0.0195



- IFNα2 was positively correlated with the percentage of CD69 on CD4+ T cells, CCR5+CCR6+ as well as total CCR6+ on activated T cells and Treg-like CD4+ T cells

Discussion and conclusion

- Mucosal IFNα2 expression was significantly correlated with the presence of STIs/BV and activated Th17 profile at the mucosa.
- Presence of BV/STIs and associated inflammation likely impact IFN signaling and the antiviral pathways. This could have negative impact on HIV susceptibility due to impaired IFN response at the site of exposure.

The authors would like to thank all the study participants for their participation in the original study. Additionally, they would like to thank the laboratory team from CAPRISA and University of KwaZulu-Natal for their support during the completion of this project.

Appendix B: Co-authored manuscripts

ADDITIONAL MANUSCRIPTS AND CONTRIBUTION TO OTHER RESEARCH PROJECTS

Throughout my degree I assisted with several other projects which resulted in three co-author manuscripts. One manuscript was a result of my participation in the rapid antigen test evaluations for identification of SARS-CoV-2 infections. Two additional manuscript resulted from providing assistance to a fellow student with sample processing, flow cytometry staining and reading.

1. Natasha Samsunder, Gila Lustig, **Slindile Ngubane**, Thando Glory Maseko, Santhuri Rambaran, Sinaye Ngcapu, Stanley Nzuzo Magini, Lara Lewis, Cherie Cawood, Ayesha BM Kharsany, Quarraisha Abdool Karim, Salim Abdool Karim, Kogieleum Naidoo, Aida Sivro. **Field evaluations of four SARS-CoV-2 rapid antigen tests during SARS-CoV-2 Delta variant wave in South Africa**. *BMC Prognostic and Diagnostic Research*. July 25, 2023. PMID: 37491317

2. Thando Glory Maseko, Santhuri Rambaran, **Slindile Ngubane**, Lara Lewis, Sinaye Ngcapu, Razia-Hassan-Moosa, Derseree Archary, Rubeshan Perumal, Nesri Padayatchi, Kogieleum Naidoo, Aida Sivro. **NK cell phenotypic profile during active TB in people living with HIV-evolution during TB treatment and implication for bacterial clearance and disease severity**. *Scientific Reports*. July 20, 2023. PMID: 37474556

3. Thando Glory Maseko, **Slindile Ngubane**, Marothi Letsoalo, Santhuri Rambaran, Derseree Archary, Natasha Samsunder, Rubeshan Perumal, Surie Chinappa, Nesri Padayatchi, Kogieleum Naidoo and Aida Sivro. **Higher plasma interleukin -6 levels are associated with lung cavitation in drug-resistant tuberculosis**. *BMC Immunology*. August 31, 2023. PMID: 37503696

Manuscript 1:

Samsunder *et al.*
Diagnostic and Prognostic Research (2023) 7:14
https://doi.org/10.1186/s41512-023-00151-3

Diagnostic and Prognostic Research

RESEARCH Open Access

Field evaluations of four SARS-CoV-2 rapid antigen tests during SARS-CoV-2 Delta variant wave in South Africa

Natasha Samsunder[1], Gila Lustig[1], Slindile Ngubane[1], Thando Glory Maseko[1], Santhuri Rambaran[1], Sinaye Ngcapu[1,2], Stanley Nzuzo Magini[1], Lara Lewis[1], Cherie Cawood[3], Ayesha B. M. Kharsany[1,2], Quarraisha Abdool Karim[1,4], Salim Abdool Karim[1,4], Kogieleum Naidoo[1,5] and Aida Sivro[1,2,5,6,7*]

Abstract

Background Rapid antigen tests detecting SARS-CoV-2 were shown to be a useful tool in managing the COVID-19 pandemic. Here, we report on the results of a prospective diagnostic accuracy study of four SARS-CoV-2 rapid antigen tests in a South African setting.

Methods Rapid antigen test evaluations were performed through drive-through testing centres in Durban, South Africa, from July to December 2021. Two evaluation studies were performed: nasal Panbio COVID-19 Ag Rapid Test Device (Abbott) was evaluated in parallel with the nasopharyngeal Espline SARS-CoV-2 Ag test (Fujirebio), followed by the evaluation of nasal RightSign COVID-19 Antigen Rapid test Cassette (Hangzhou Biotest Biotech) in parallel with the nasopharyngeal STANDARD Q COVID-19 Ag test (SD Biosensor). The Abbott RealTime SARS-CoV-2 assay was used as a reference test.

Results Evaluation of Panbio and Espline Ag tests was performed on 494 samples (31% positivity), while the evaluation of Standard Q and RightTest Ag tests was performed on 539 samples (13.17% positivity). The overall sensitivity for all four tests ranged between 60 and 72% with excellent specificity values (> 98%). Sensitivity increased to > 80% in all tests in samples with cycle number value < 20. All four tests performed best in samples from patients presenting within the first week of symptom onset.

Conclusions All four evaluated tests detected a majority of the cases within the first week of symptom onset with high viral load.

Keywords COVID-19, SARS-CoV-2, Antigen rapid diagnostic test, Performance evaluation

*Correspondence:
Aida Sivro
sivroaida@gmail.com
Full list of author information is available at the end of the article

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NK cell phenotypic profile during active TB in people living with HIV-evolution during TB treatment and implications for bacterial clearance and disease severity

Thando Glory Maseko[1,3], Santhuri Rambaran[1], Slindile Ngubane[1], Lara Lewis[1], Sinaye Ngcapu[1,2], Razia Hassan-Moosa[1,3], Derseree Archary[1,2], Rubeshan Perumal[1,3], Nesri Padayatchi[1,3], Kogieleum Naidoo[1,3] & Aida Sivro[1,2,3,4,5]✉

Natural killer (NK) cells, key effector cells of the innate immune system, play an important role in the clearance and control of *Mycobacterium tuberculosis* and HIV infections. Here, we utilized peripheral blood specimens from the Improving Retreatment Success CAPRISA 011 study to characterize NK cell phenotypes during active TB in individuals with or without HIV co-infection. We further assessed the effects of TB treatment on NK cell phenotype, and characterized the effects of NK cell phenotypes during active TB on mycobacterial clearance and TB disease severity measured by the presence of lung cavitation. TB/HIV co-infection led to the expansion of functionally impaired $CD56^{neg}$ NK cell subset. TB treatment completion resulted in restoration of total NK cells, NK cell subset redistribution and downregulation of several NK cell activating and inhibitory receptors. Higher percentage of peripheral $CD56^{bright}$ cells was associated with longer time to culture conversion, while higher expression of NKp46 on $CD56^{dim}$ NK cells was associated with lower odds of lung cavitation in the overall cohort and the TB/HIV co-infected participants. Together these results provide a detailed description of peripheral NK cells in TB and TB/HIV co-infection and yield insights into their role in TB disease pathology.

Manuscript 3:

Maseko *et al. BMC Immunology* (2023) 24:26
https://doi.org/10.1186/s12865-023-00563-2

BMC Immunology

RESEARCH Open Access

Higher plasma interleukin – 6 levels are associated with lung cavitation in drug-resistant tuberculosis

Thando Glory Maseko[1,2], Slindile Ngubane[1], Marothi Letsoalo[1], Santhuri Rambaran[1], Derseree Archary[1,3], Natasha Samsunder[1], Rubeshan Perumal[1,2], Surie Chinappa[1], Nesri Padayatchi[1,2], Kogieleum Naidoo[1,2] and Aida Sivro[1,2,3,4,5*]

Abstract

Background Lung cavitation is associated with heightened TB transmission and poor treatment outcomes. This study aimed to determine the relationship between systemic inflammation and lung cavitation in drug-resistant TB patients with and without HIV co-infection.

Methods Plasma samples were obtained from 128 participants from the CAPRISA 020 Individualized M(X)drug-resistant TB Treatment Strategy Study (InDEX) prior to treatment initiation. Lung cavitation was present in 61 of the 128 drug-resistant TB patients with 93 being co-infected with HIV. The plasma cytokine and chemokine levels were measured using the 27-Plex Human Cytokine immunoassay. Modified Poisson regression models were used to determine the association between plasma cytokine/chemokine expression and lung cavitation in individuals with drug-resistant TB.

Results Higher Interleukin-6 plasma levels (adjusted risk ratio [aRR] 1.405, 95% confidence interval [CI] 1.079–1.829, p = 0.011) were associated with a higher risk of lung cavitation in the multivariable model adjusting for age, sex, body mass index, HIV status, smoking and previous history of TB. Smoking was associated with an increased risk of lung cavitation (aRR 1.784, 95% CI 1.167–2.729, p = 0.008). An HIV positive status and a higher body mass index, were associated with reduced risk of lung cavitation (aRR 0.537, 95% CI 0.371–0.775, p = 0.001 and aRR 0.927, 95% CI 0.874–0.983, p = 0.012 respectively).

Conclusion High plasma interleukin-6 levels are associated with an increased risk of cavitary TB highlighting the role of interleukin-6 in the immunopathology of drug-resistant TB.

Keywords Tuberculosis, HIV, MDR, XDR, Biomarker, Lung cavitation, Inflammation

*Correspondence:
Aida Sivro
sivroaida@gmail.com
[1]Centre for the AIDS Programme of Research in South Africa (CAPRISA), Durban, South Africa
[2]South African Medical Research Council (SAMRC)-CAPRISA-TB-HIV Pathogenesis and Treatment Research Unit, University of KwaZulu-Natal Nelson R Mandela School of Medicine, Durban, South Africa
[3]Department of Medical Microbiology, University of KwaZulu-Natal, Durban, KZN, South Africa
[4]JC Wilt Infectious Disease Research Centre, National Microbiology laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada
[5]Department of Medical Microbiology and Infectious Diseases, University of Manitoba, Winnipeg, MB, Canada

Appendix C: UKZN BREC approval letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

26 May 2022

Miss Slindile Brilliant Lyzeth Ngubane (217016499)
School of Lab Med & Medical Sc
Medical School

Dear Miss Ngubane,

Protocol reference number: **BREC/00004142/2022**
Project title: **Type I, II, and III interferon responses in the female genital tract**
Degree: **MMedSc**

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 26 May 2022. Please ensure that any outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is subject to national and UKZN lockdown regulations, see (http://research.ukzn.ac.za/Libraries/BREC/BREC_Amended_Lockdown_Level_1_Guidelines.sflb.ashx). Based on feedback from some sites, we urge PIs to show sensitivity and exercise appropriate consideration at sites where personnel and service users appear stressed or overloaded.

This approval is valid for one year from 26 May 2022. 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 (2020) (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 noted by a full Committee at its next meeting taking place on 14 June 2022.

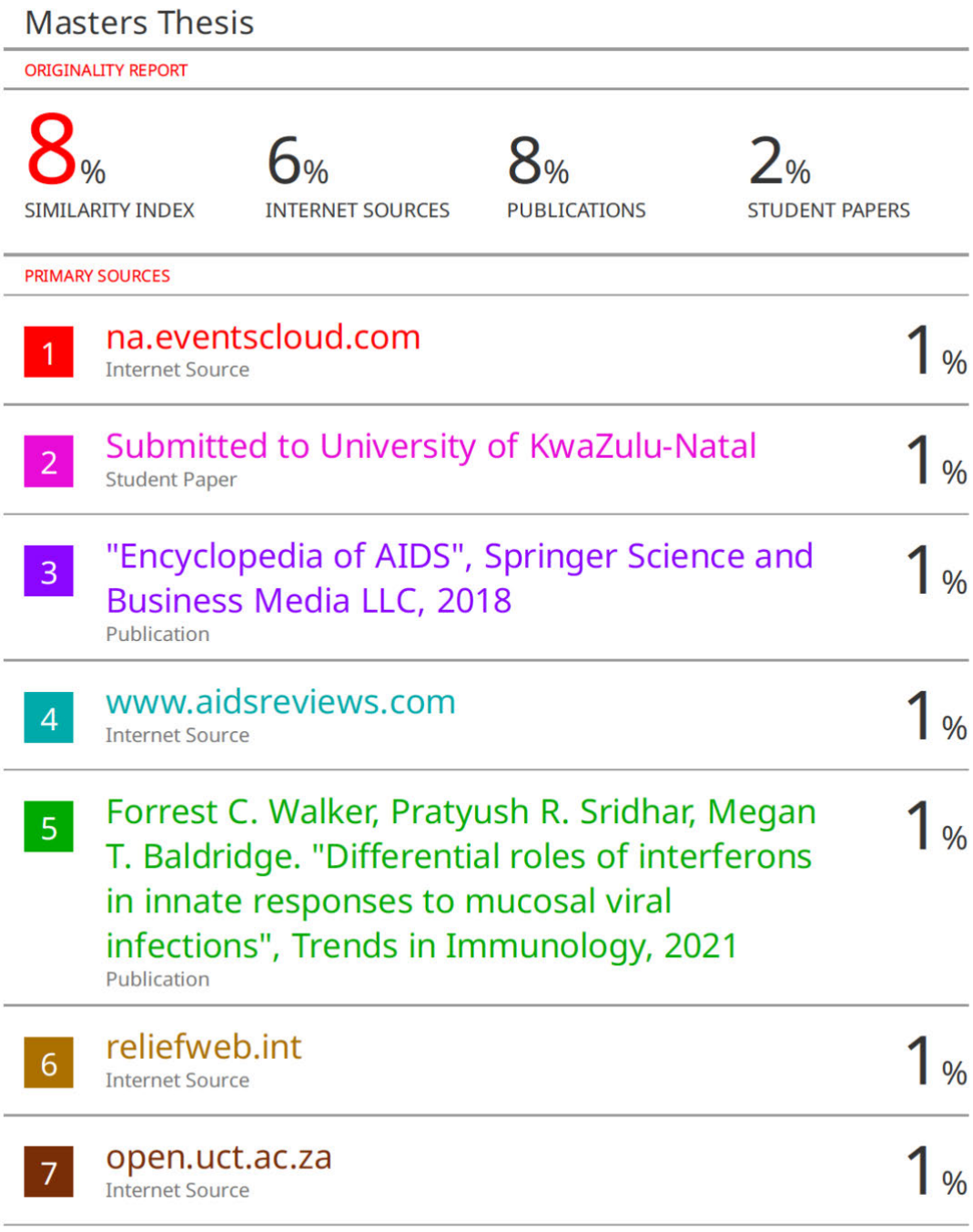
Yours sincerely,

[redacted]

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

Appendix D: Turnitin report



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Appendix E: Community outreach

The CAPRISA research fellows, including myself, visited Sibanesihle High and Ngcedomhlophe High (26 & 27 October 2022) in the Mafakatini area of uMgungundlovu Municipality as well as Reunion Secondary School in Umlazi (15 August 2023). Here, we informed and raised awareness among the learners about HIV, TB, STIs, the risks associated with early sexual debut and teenage pregnancy. Additionally, we answered questions and briefed the learners about our respective projects.

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17 November 2022 | Volume: 10 | Issue: 52

UKZN postgraduate students who provided educational outreach for high school learners.

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Taking Education to Youngsters in Rural Areas

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UKZN postgraduate students from the College of Health Sciences who are also fellows at the Centre for the AIDS Programme of Research in South Africa (CAPRISA) visited Sibanesihle High and Ngcedomhlophe High in the Mafakatini area of the uMgungundlovu Municipality to conduct a community outreach programme.

The students involved were Ms Noluthando Mazibuko-Motau, Ms Nomusa Zondo, Ms Thando Maseko, Ms Senamile Ngema, Mr Lungelo Ntuli, Ms Nonsikelelo Ndlela, Ms Sibongiseni Masondo, Ms Bongeka Mabaso and Ms Silindile Ngubane.

The aim of the outreach project was to inform and raise awareness among learners about the human immunodeficiency virus (HIV), tuberculosis (TB), sexually transmitted infections (STIs), the risks associated with an early sexual debut, and teenage pregnancy.

CAPRISA @CAPRISAOfficial · 23m

@CAPRISAOfficial Patrick Mdletshe head Community programmes & Fellows addressed learners at Reunion Secondary school on #HIVprevention, #endTB and sexual & reproductive health. @NRF_News @HealthZA

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