The impact of TGF-β on the genital immune environment associated with HIV risk in young women

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PREFACE

The experimental work described in this thesis was conducted at the Centre for the AIDS Programme of Research in South Africa (CAPRISA), Doris Duke Medical Research Institute, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa, under the supervision of Prof. Lenine Liebenberg.

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

[redacted]

Sibongiseni Masondo Date: 9 January 2025

[redacted]

Prof. Lenine Liebenberg Date: 9 January 2025

PLAGRIASIM DECLARATION

I, Sibongiseni Masondo, declare as follows:

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

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

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

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

   a. Their words have been rewritten, but the general information attributed to them has been referenced.

   b. Their exact words were used, and their writing was placed inside quotation marks and referenced.

Signed [redacted]

DEDICATION

To God Almighty.

"' For I know the plans I have you,' declares the Lord, 'plans to prosper you and not to harm you, plans to give you a hope and a future.'"

Jeremiah 29:11

ACKNOWLEDGEMENTS

I would like to express my appreciation to:

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- The CAPRISA Mucosal Laboratory staff and a special thanks to the CAPRISA fellows for their valuable contribution, support and encouragement.

- The participants of the CAPRISA 008 trial who enabled this project to be possible.

- The DSI-NRF Centre of Excellence in HIV Prevention for the financial support.

- My family and friends, thank you for the love, prayers, and support. Thank you for always believing in and encouraging me, especially during difficult times.

ETHICS DECLARATION

The Biomedical Research Ethics Committee approved this study of the University of KwaZulu-Natal (BREC 00001814/2020).

Table of Contents

LIST OF ACRONYMS

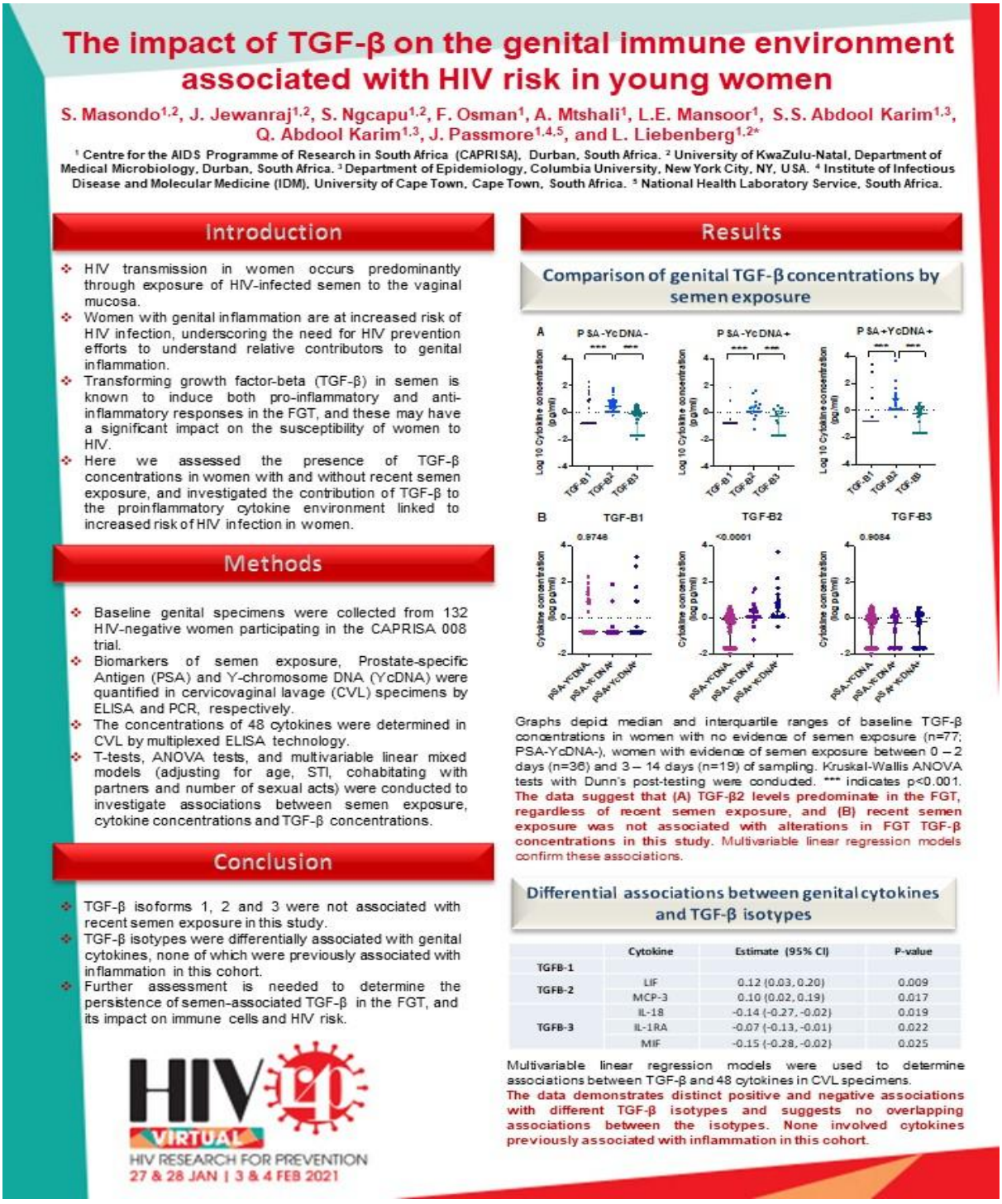
AIDS	Acquired Immunodeficiency Syndromes
APC	Allophycocyanin Tandem Dye
BASIC-FGF	Basic Fibroblast Growth Factor
BV	Bacterial Vaginosis
CAPRISA	Centre for the AIDS Programme of Research in South Africa
CCR5	C-C Chemokine Receptor Type 5
CD	Cluster of Differentiation
CTACK	Cutaneous T Cell- Attracting Chemokine
CVL	Cervicovaginal Lavage
CXCR3	C-X-C Motif Chemokine Receptor 3
ELISA	Enzyme-Linked Immunosorbent Factor
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FGT	Female Genital Tract
FITC	Fluorescein Isothiocyanate
FOXP3	Forkhead Box P3
FRT	Female Reproductive Tract
G-CSF	Granulocyte Colony-Stimulating Factor
GEC	Genital Epithelial Cells
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GRO-α	Growth-Regulated Oncogene-Alpha
HB-EGF	Heparin-Binding EGF-Like Growth Factor
HGF	Hepatocyte Growth Factor
HLA	Human Leucocyte Antigen
HRP	Horse-Radish Peroxidase
IFN-α	Interferon Alpha
IFN-γ	Interferon Gamma
IgG/A/M	Immunoglobulins G/A/M
IL	Interleukin
IL-1Ra	Interleukin-1 Receptor Antagonist
IP-10	Interferon Gamma-Induced Protein 10
IQR	Interquartile Range
LIF	Leukaemia Inhibitory Factor
LIF	Leukaemia Inhibitory Factor
LTBP	Latent TGF Binding Proteins
LTR	Long Terminal Repeats
MCP-1	Monocyte Chemoattractant Protein 1
MCP-3	Monocyte Chemotactic Protein 3
M-CSF	Macrophage Colony-Stimulating Factor
MIF	Macrophage Migration Inhibitory Factor
MIG	Monokine Induced Gamma Interferon
MIP-1α	Macrophage Inflammatory Protein-1 Alpha
MMP	Matrix Metalloproteinase
MRT	Male Reproductive tract
NF-kB	Nuclear Factor-kappa B
NOD	Nucleotide-binding oligomerization domain

NK	Natural Killer
PAMP	Pathogen-Associated Molecular Patterns
PBS	Phosphate-Buffered Saline
PDGF	Platelet-Derived Growth Factor
PGE	Prostaglandin E
PRR	Pattern Recognition Receptors
PSA	Prostate-Specific Antigen
RANTES	Regulated Upon Activation, Normal T Cell Expressed and Secreted
RPMI	Roswell Park Memorial Institute Medium
SA-PE	Streptavidin Phycoerythrin
SCF	Stem Cell Factor
SCGF-β	Stem Cell Growth Factor Beta
SDF-1α	Stromal Cell-Derived Factor-1 Alpha
SLPI	Secretory Leucocyte Protease Inhibitors
SMAD	Suppressor of Mothers Against Decapentaplegic
STAT	Signal Transducer and Activator of Transcription
STI	Sexually Transmitted Infection
TCR	T Cell Receptor
TGF-β	Transforming growth factor-beta
TIMP	Tissue Inhibitors of Matrix Metalloproteinases
TNF-α	Tumour necrosis factor-alpha
TNF-β	Tumour necrosis factor-beta
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
Treg	Regulatory T Cells
TβR	TGF-β Receptor
UNAIDS	Joint United Nations Programme on HIV/AIDS
VEGF	Vascular endothelial growth factor
α	Alpha
β	Beta
β-NGF	Nerve Growth Factor Beta
γ	Gamma

LIST OF TABLES

LIST OF FIGURES

Sibongiseni Masondo, Janine Jewanraj, Sinaye Ngcapu, Farzana Osman, Andile Mtshali, Leila Mansoor, Salim Abdool Karim, Quarraisha Abdool Karim, Jo-Ann Passmore, Lenine Liebenberg. The impact of TGF-β on the genital immune environment associated with HIV risk in young women. Journal of the International AIDS Society (4th HIV Research for Prevention conference (HIVR4P // Virtual)), 27 & 28 January | 3 & 4 February 2021. (Poster presentation).



The impact of TGF-β on the genital immune environment associated with HIV risk in young women

S. Masondo[1,2], J. Jewanraj[1,2], S. Ngcapu[1,2], F. Osman[1], A. Mtshali[1], L.E. Mansoor[1], S.S. Abdool Karim[1,3], Q. Abdool Karim[1,3], J. Passmore[1,4,5], and L. Liebenberg[1,2*]

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Introduction

- HIV transmission in women occurs predominantly through exposure of HIV-infected semen to the vaginal mucosa.
- Women with genital inflammation are at increased risk of HIV infection, underscoring the need for HIV prevention efforts to understand relative contributors to genital inflammation.
- Transforming growth factor-beta (TGF-β) in semen is known to induce both pro-inflammatory and anti-inflammatory responses in the FGT, and these may have a significant impact on the susceptibility of women to HIV.
- Here we assessed the presence of TGF-β concentrations in women with and without recent semen exposure, and investigated the contribution of TGF-β to the proinflammatory cytokine environment linked to increased risk of HIV infection in women.

Methods

- Baseline genital specimens were collected from 132 HIV-negative women participating in the CAPRISA 008 trial.
- Biomarkers of semen exposure, Prostate-specific Antigen (PSA) and Y-chromosome DNA (YcDNA) were quantified in cervicovaginal lavage (CVL) specimens by ELISA and PCR, respectively.
- The concentrations of 48 cytokines were determined in CVL by multiplexed ELISA technology.
- T-tests, ANOVA tests, and multivariable linear mixed models (adjusting for age, STI, cohabitating with partners and number of sexual acts) were conducted to investigate associations between semen exposure, cytokine concentrations and TGF-β concentrations.

Conclusion

- TGF-β isoforms 1, 2 and 3 were not associated with recent semen exposure in this study.
- TGF-β isotypes were differentially associated with genital cytokines, none of which were previously associated with inflammation in this cohort.
- Further assessment is needed to determine the persistence of semen-associated TGF-β in the FGT, and its impact on immune cells and HIV risk.

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Results

Comparison of genital TGF-β concentrations by semen exposure

Graphs depict median and interquartile ranges of baseline TGF-β concentrations in women with no evidence of semen exposure (n=77; PSA-YcDNA-), women with evidence of semen exposure between 0 – 2 days (n=36) and 3 – 14 days (n=19) of sampling. Kruskal-Wallis ANOVA tests with Dunn's post-testing were conducted. *** indicates p<0.001. **The data suggest that (A) TGF-β2 levels predominate in the FGT, regardless of recent semen exposure, and (B) recent semen exposure was not associated with alterations in FGT TGF-β concentrations in this study.** Multivariable linear regression models confirm these associations.

Differential associations between genital cytokines and TGF-β isotypes

	Cytokine	Estimate (95% CI)	P-value
TGFB-1			
TGFB-2	LIF	0.12 (0.03, 0.20)	0.009
	MCP-3	0.10 (0.02, 0.19)	0.017
TGFB-3	IL-18	-0.14 (-0.27, -0.02)	0.019
	IL-1RA	-0.07 (-0.13, -0.01)	0.022
	MIF	-0.15 (-0.28, -0.02)	0.025

Multivariable linear regression models were used to determine associations between TGF-β and 48 cytokines in CVL specimens. **The data demonstrates distinct positive and negative associations with different TGF-β isotypes and suggests no overlapping associations between the isotypes. None involved cytokines previously associated with inflammation in this cohort.**

Sibongiseni Masondo, Janine Jewanraj, Sinaye Ngcapu, Farzana Osman, Andile Mtshali, Leila Mansoor, Salim Abdool Karim, Quarraisha Abdool Karim, Jo-Ann Passmore, Lenine Liebenberg. The impact of TGF-β on immune responses at the female reproductive tract. International Union of Immunological Societies (18th International Congress of Immunology, Cape Town), 27 November – 2 December 2023. (Poster Presentation).

The impact of TGF-β on immune responses at the female reproductive tract

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Introduction

- HIV transmission occurs predominantly through exposure of infected semen to the vaginal mucosa.
- Women with genital inflammation are at increased risk of HIV infection, underscoring the need for HIV prevention efforts to understand relative contributors to genital inflammation.
- Transforming growth factor-beta (TGF-β) in semen is known to induce both pro-inflammatory and anti-inflammatory responses in the FGT, and these may have a significant impact on the susceptibility of women to HIV infection.
- Here we investigated the relationship between TGF-β and cytokine and cellular biomarkers of inflammation in the female reproductive tract linked to increased risk of HIV infection in women.

Methods

- The study included a subset of 132 CAPRISA 008 trial participants with biannual sampling of genital specimens (N=641 visits).
- The presence of prostate-specific antigen (PSA) in cervicovaginal lavage (CVL) was determined by ELISA and indicated semen exposure within 48 hours of genital sampling.
- Multiplex ELISA assays were used to determine the concentrations of TGF-β isoforms and 48 other cytokines in CVL.
- Flow cytometry was conducted to identify activated CD4+ T cell populations among cervical mononuclear cells collected from cytobrushes.
- Multivariable linear mixed models assessed associations between TGF-β concentrations and semen exposure and other biomarkers of inflammation.

Results

TGF-β1 & 2 are associated with reduced CD4 T cell activation

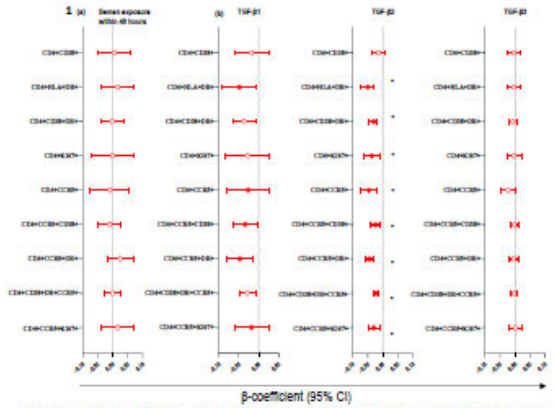


Figure 1: Association between immune cell frequencies and TGF-β levels. (a) Association between semen exposure within 48 hours (PSA) and immune cell frequencies at the FRT. (b) Associations between TGF-β isoforms and FRT immune cell frequencies were investigated in cervicovaginal lavage specimens (n=641) by multivariable linear mixed models controlling for participant age, any STI, BV Nugent Score, study arm and time in study.

TGF-β1 & 3 are associated with a decrease in FRT cytokines

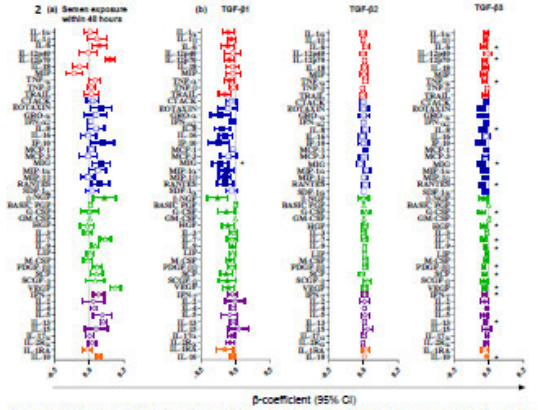


Figure 2: Associations between TGF-β and other cytokine concentrations in cervicovaginal fluid. (a) Association between semen exposure within 48 hours (PSA) and genital cytokines at the FRT. (b) Associations between TGF-β and other cytokines at the FRT were investigated in cervicovaginal lavage specimens (n=641) by multivariable linear mixed models controlling for participant age, the number of vaginal sex acts in the past 30 days, any STI, BV Nugent Score, study arm and time in study.

PSA is not associated with TGF-β

- PSA detection was associated with neither TGF-β1, TGF-β2 nor TGF-β3 concentrations at the FRT.
- Among the 132 participants, irrespective of PSA status, at baseline, TGF-β2 concentrations were significantly higher than TGF-β3 concentrations.

Conclusion

- TGF-β concentrations were not associated with PSA detection in this study, which may suggest a normalization of TGF-β levels in the genital fluid within 48 hours after intromission.
- Further interrogation is required to determine the dynamics of intromitted or endogenous TGF-β and inflammatory biomarkers, the persistence of their immune impact, and the relation to HIV risk.

ABSTRACT

Background: The HIV pandemic has disproportionally affected young women living in sub-Saharan Africa, with most new infections transmitted via condomless sex. Semen exposure is shown to increase several cytokines, cellular and barrier-related biomarkers of inflammation associated with HIV acquisition in women. The predominance of the anti-inflammatory transforming growth factor-beta (TGF-β) cytokine is well established in semen, and regulation of the cervical immune response is meant to facilitate conception. In this study, we investigated the contribution of TGF-β to the genital inflammatory profile linked to HIV risk in women.

Methods: This study included a subset of 132 CAPRISA 008 trial participants with a biannual sampling of genital specimens (N=641 visits). The presence of prostate-specific antigen (PSA) in cervicovaginal lavage (CVL) was determined by ELISA and indicated the likelihood of condomless sex and semen exposure within 48 hours of genital sampling. Multiplex ELISA assays were used to determine the concentrations of TGF-β isoforms 1, 2, 3 and 48 other cytokines in CVL specimens. Flow cytometry was conducted to identify activated (CD38+, HLA-DR+, CCR5+ and/or Ki67+) CD4+ T cell populations among cervical mononuclear cells collected from cytobrushes. Multivariable linear mixed models assessed associations between TGF-β concentrations and semen exposure and with cellular and cytokine biomarkers of inflammation.

Results: TGF-β isoform concentrations were similar in CVL specimens with and without evidence of recent semen exposure. Further, independent of semen exposure, TGF-β1 detection and TGF-β3 concentrations were associated with significant decreases in multiple FRT cytokine concentrations. TGF-β1 detection and TGF-β2 concentrations significantly reduced multiple populations of activated $CD4^+$ T cells at the FRT.

Conclusion: Although TGF-β isoforms were differentially expressed in the FRT and differed in the nature of their individual associations with local cytokine concentrations and cellular frequencies, their general relationship with reduced levels of genital cytokines and immune cells attests to their documented immunomodulatory effects. TGF-β concentrations were not associated with PSA detection, which likely indicates a normalisation of TGF-β levels in genital fluid within 48 hours after intromission. Although TGF-β concentrations were independently associated with dampening local cellular and cytokine levels, the previously observed relationship between semen exposure and increased levels of inflammatory biomarkers was maintained in the cohort. Further interrogation is required to determine the dynamics of intromitted or endogenous TGF-β and inflammatory biomarkers, the persistence of their immune impact, and the relation to HIV risk.

CHAPTER 1

Introduction

INTRODUCTION

1.1 The global HIV burden

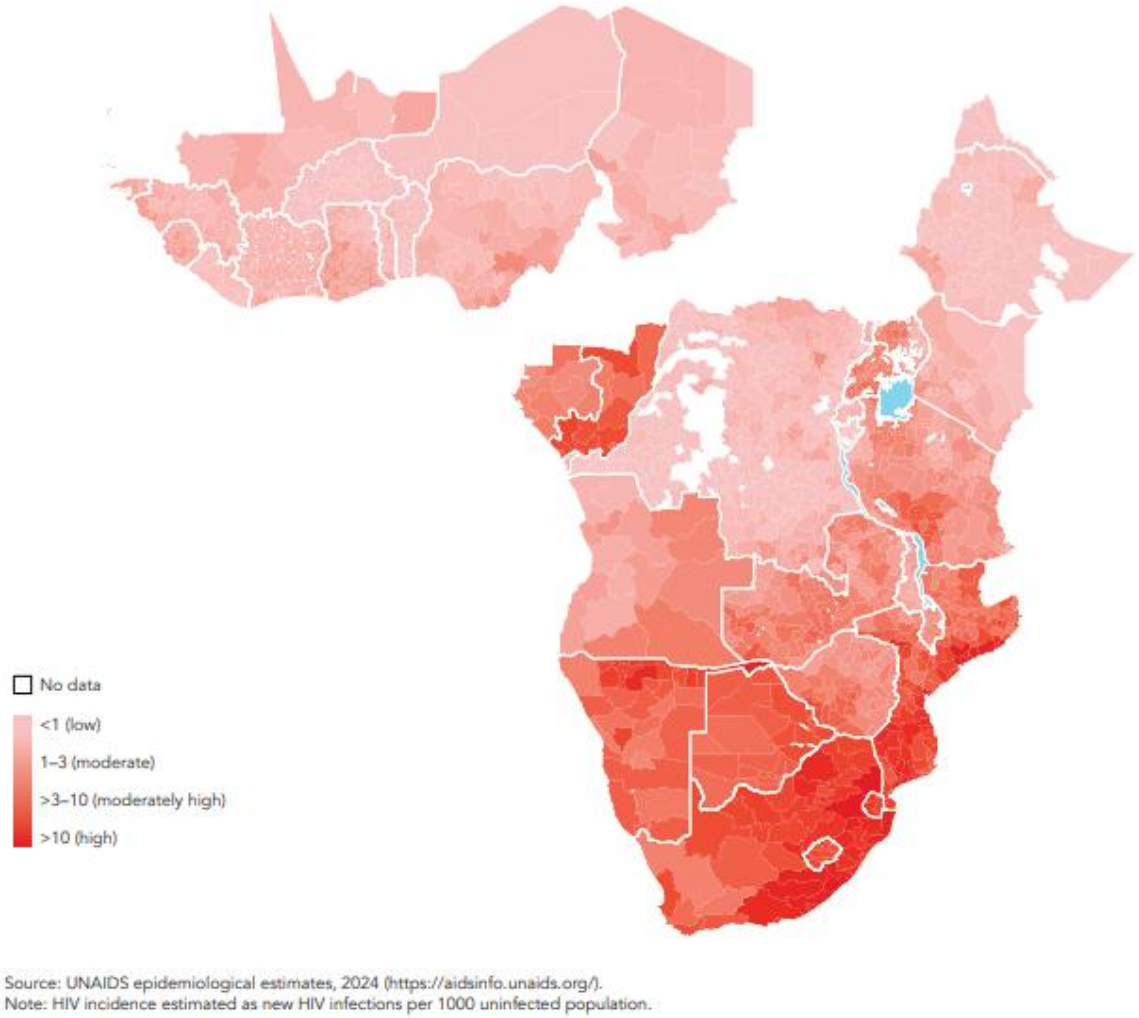
Human immunodeficiency virus (HIV) infection, if not controlled, can result in a severe immunocompromised condition known as AIDS (Acquired Immune Deficiency Syndrome) (Stevenson, 2003, Maina *et al*., 2016). Today, HIV infection represents one of the leading public health pandemics, with 84.2 million people infected globally and an estimated 39.9 million people worldwide living with HIV in 2023 (UNAIDS, 2024). Even though the rates of newly-infected people have recently decreased, over 1.3 million people still have new infections, and 630 000 AIDS-related deaths were reported worldwide last year (UNAIDS, 2024). Among the 39.9 million people living with HIV, 86% reported knowing their HIV status, and 5.6 million people were not aware of their HIV status (UNAIDS, 2024). To combat and end the HIV pandemic by 2030, UNAIDS has set a guideline for 2025 that calls for 95-95-95 targets for HIV testing, HIV treatment and viral suppression (Frescura *et al*., 2022). This indicates that 95% of people living with HIV know their HIV status, 95% of those people are receiving anti-retroviral therapy, and 95% of those people receiving treatment are virally suppressed (Frescura *et al*., 2022).

1.2 Sub-Saharan HIV epidemic

Globally, Eastern and Southern Africa accounts for half of the population living with HIV (UNAIDS, 2022) and has 140 000 new infections in 2023 (UNAIDS, 2024). In this region, the prevalence is disproportionally high in young women (Karim and Baxter, 2019), with young women aged 15-19 years accounting for six in seven new HIV infections in 2021 (UNAIDS, 2022). Young women aged 15-24 years are twice as likely to be living with HIV than young men of the same age category (UNAIDS, 2022). In sub-Saharan Africa, the virus is transmitted predominantly through heterosexual sex (Hunter, 1993), and inflammation of the female reproductive tract (FRT) has been identified as a biological factor increasing the risk of HIV acquisition (Masson et al., 2015). Though the incidence rate of HIV is on the decrease, in sub-Saharan Africa, it is exceptionally high amongst adolescent girls and young women (UNAIDS, 2024). With AIDS being the leading cause of death in sub-Saharan Africa**,** combating the risk of HIV acquisition will go a long way to reducing the death toll of people living with HIV in sub-Saharan Africa.

1.3 South African HIV epidemic

South Africa is one of the sub-Saharan countries with one of the highest HIV prevalence, with a rate of 17.1% (UNAIDS, 2024). Women aged 15 to 49 have almost double the HIV prevalence (22.6%) compared to their male counterparts with 11.5% (UNAIDS, 2024). With high prevalence and incidence rates in South Africa (Figure 1.1), the death toll is also on the increase. A survey conducted among South Africans indicated that the use of condoms has decreased in 2022 compared to the previous years (UNAIDS, 2024), suggesting the need for more effective HIV prevention strategies among young people, particularly young women.



Source: UNAIDS epidemiological estimates, 2024 (https://aidsinfo.unaids.org/).
Note: HIV incidence estimated as new HIV infections per 1000 uninfected population.

Figure 1.1. The HIV incidence rate in sub-Saharan Africa among young women and adolescent girls (15-24 years) in 2021. Figure from UNAIDS epidemiological estimates, 2024 (UNAIDS epidemiological estimates, 2024 (https://aidsinfo.unaids.org/).

1.4 Biological factors affecting HIV

The relationship between genital inflammation and HIV risk is likely facilitated by cytokine-mediated recruitment of HIV target cells to the mucosa of the vagina and damage to the integrity of the epithelial barrier facilitating HIV entry (Shukair *et al*., 2013, Passmore *et al*., 2016). Male-to-female HIV transmission involves a multiplex of inhibitory and facilitatory molecules that regulate infection risk during intercourse (Hladik and Doncel, 2010), and semen-associated alterations in the FRT may promote sexual transmission of HIV (Doncel *et al*., 2014). With genital inflammation associated with an increased risk of HIV acquisition, this review will focus specifically on the components of semen and particularly the role of TGF-β in modulating the FRT immune response.

The risk of HIV infection differs within a population. It depends on several biological, clinical, structural, and behavioural factors, e.g., multiple sexual partners, sexual violence, inconsistent condom use, and other sexually transmitted infections (Belle and Gamedze, 2019). Recently, genital inflammation has been identified as a biological factor that significantly increases the risk of HIV infection in young women (Masson *et al*., 2015). Cytokine biomarkers of genital inflammation have since been associated with the recruitment of HIV-susceptible target cells, a compromised mucosal barrier, and a dysbiotic genital microbiota (Anahtar *et al*., 2015, Gosmann *et al*., 2017, Arnold *et al*., 2016), suggesting multiple potential mechanisms for the relationship between genital inflammation and HIV risk. With the HIV epidemic evolving, identifying the causes of genital inflammation and understanding the manner of its impact on HIV risk is of utmost importance to the design of targeted approaches to reducing the risk of sexual transmission of HIV.

During condomless sex, HIV-1 is transmitted to women either cell-free or cell-associated via semen (Doncel *et al*., 2014). Semen is primarily responsible for transporting spermatozoa to the female reproductive tract (FRT) (Doncel *et al*., 2014) and promoting conception through changes in the FRT mediated through its bioactive molecule content (Sharkey *et al*., 2012b). Both pro- and anti-inflammatory responses are elicited by semen in the FRT (Sharkey *et al*., 2007, Sharkey *et al*., 2012b), and both have the potential to impact the susceptibility of women to HIV significantly. Signalling molecules found in seminal plasma interact with FRT epithelial cells and can induce pro-inflammatory gene expression, leukocyte recruitment, and activation of adaptive and innate immune responses (Robertson, 2005, Robertson *et al*., 2009b, Sharkey *et al*., 2012b). Semen exposure results in the chemotaxis of leukocytes such as macrophages, dendritic cells and T lymphocytes, including $CD4^+$ T cells targets for HIV infection (Sharkey *et al*., 2012b) that assist in endometrial receptivity for embryo implantation (Schjenken and Robertson, 2020). It also elicits a robust anti-inflammatory cytokine response to support the immune tolerance required to facilitate embryo implantation (Robertson, 2005). Semen facilitates this by promoting the development of regulatory T cells (Treg), which are essential for immune tolerance during implantation and development of the placenta (Robertson *et al*., 2018).

This dampening of FRT immune responses may also contribute to an immune environment with sub-optimal immunity to HIV and other STI.

Transforming growth factor-beta (TGF-β) is a common bio-active molecule in semen, responsible primarily for promoting immune tolerance in the FRT through its involvement with Treg and activation and inhibition of natural killer cells (Robertson *et al*., 2002, Lisco *et al*., 2012, Sharkey *et al*., 2012a, Doncel *et al*., 2014). TGF-β consists of three isoforms of similar structures (Pickup *et al*., 2013) but with varying impacts on immune cells (Sharkey *et al*., 2012a). Although their signals are transduced using the same signalling pathways (Cheifetz *et al*., 1987, Hachim *et al*., 2018), a different gene encodes each isoform (Hachim *et al*., 2018). The TGF-β found in semen is synthesised in the male seminal vesicle and is only activated in the FRT after ejaculation (Tremellen *et al*., 1998, Sharkey *et al*., 2012a). TGF-β and prostaglandins are linked to the differentiation of naïve CD4+ CD25+ cells to Treg cells expressing the transcription factor FOXP3+ (Robertson *et al*., 2013). Regulatory T cells suppress the inflammatory cell-mediated immune response (Rudensky, 2011, Robertson et al., 2013, Theron et al., 2017b). They are central to regulating immune tolerance (Baratelli *et al*., 2005, Rudensky, 2011, Robertson *et al*., 2013). By indirectly regulating T and NK cell responses, TGF-β-mediated immune tolerance to seminal fluid could increase HIV risk, presumably through limited immunity to sexually transmitted infections at the FRT.

TGF-β has documented pro-inflammatory properties that may also impact HIV-1 susceptibility. TGF-β in semen promotes the differentiation of CD4+ Th17 HIV target cells and the subsequent induction of inflammatory cytokines (Luo *et al*., 2017). In immortalised ectocervical cells, TGF-β treatment elicits the pro-inflammatory cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL) -6, and IL-8 (Sharkey *et al*., 2012a). Elevation of these cytokines in women is associated with genital inflammation, and increased concentrations of IL-8 have been associated with HIV risk (Masson *et al*., 2015). TGF-β3 is responsible for upregulating an array of chemokine and inflammatory cytokine genes that are also upregulated by seminal plasma in ectocervical cells *in vivo* and *in vitro* (Sharkey *et al*., 2012a, Doncel *et al*., 2014).

Considering the predominance of TGF-β in semen, the potential for its pro-inflammatory and anti-inflammatory properties to impact HIV risk, and evidence for semen exposure to induce inflammation in the FRT, this study tests the hypothesis that TGF-β concentrations will be higher in cervicovaginal secretions of women with evidence of recent semen exposure and assesses the relationship between TGF-β and cytokine, cellular and barrier biomarkers of the inflammation associated with HIV risk in women. Further, this study aims to understand the persistence of semen-associated TGF-β and that of related immunological responses in the FGT to inform on the development of strategies to curb the genital inflammation associated with HIV risk.

CHAPTER 2

Literature Review

2.1 The immune responses at the female reproductive tract

The female reproductive tract is organised into two parts, the upper and the lower part. The vaginal canal and the ectocervix make up the lower part, and the upper part is composed of the endocervix and the uterus. A mucus barrier lines the endocervix. The barrier of the FRT is the first line of defence against pathogens entering through the lower FRT. The endocervical epithelium makes up the mucosal barrier. The mucus secreted by the epithelium is vital for maintaining the vaginal mucosal barrier (Lacroix *et al*., 2020). The cervicovaginal mucus is an integral part of the defence against pathogens as it houses endometrial fluid with cells and molecules of different kinds, which contribute to immune defence, cells like neutrophils that excrete nucleic acid, exfoliated epithelial cells, mucin-associated fatty acids and other proteins **(**Lacroix *et al*., 2020). The genital epithelial cells (GEC) located in the FRT express pattern recognition receptors (PRR), which include nucleotide-binding oligomerization domain (NOD)-like receptors and toll-like receptors (TLR) that can sense foreign pathogens and rapidly alert both the adaptive and innate immune cells of an invading pathogen. (Nguyen *et al*., 2014). When alerted of the incoming pathogens, the adaptive and innate systems respond via inflammation, phagocytosis, cell recruitment, and other means (Hickey et al., 2011).

The innate system optimises the use of soluble factors secreted by the epithelial cells to curb the outgrowth of microbes (Wira and Fahey, 2004). Other factors secreted by epithelial cells with bactericidal effects include secretory leucocyte protease inhibitors (SLPI), defensins, lactoferrin, enzyme lysozyme, and antimicrobial peptides (Wira and Fahey, 2004). The innate system is also comprised of innate cells such as dendritic cells (Clark et al., 2000), macrophages (Hirayama *et al*., 2017), natural killer cells (Vivier *et al*., 2008), and monocytes, among other cells (Vijay, 2018). Toll-like receptors (TLR), which are part of the PRR family, play a crucial role in innate immunity as they recognise pathogen-associated molecular patterns (PAMPs) and can induce an inflammatory response (Takeuchi and Akira, 2010, Vijay, 2018). TLRs are expressed by various immune cells and are responsible for recognising pathogens introduced to the host. These TLRs are essential for an effective innate response and subsequent adaptive responses.

The adaptive immune system works through the presentation of antigen-specific T-cells or B-cell secretion (Marshall *et al*., 2018). In the FRT, adaptive immunity involves pathogen-specific responses driven by antigen presentation by dendritic cells, macrophages, epithelial cells and Langerhans cells of the endometrium and the cervix (Reis Machado *et al*., 2014) to T cells and B cells. The adaptive system also contributes to the defence against pathogens by releasing secretory immunoglobulins such as IgG, IgM and IgA that play an essential role in strengthening the mucosal barrier from infection (Fahrbach

et al., 2013, Wang *et al*., 2014, Lacroix *et al*., 2020). With genital inflammation being associated with HIV acquisition risk, understanding the contributors to genital inflammation and their relative impact on HIV risk is of utmost importance

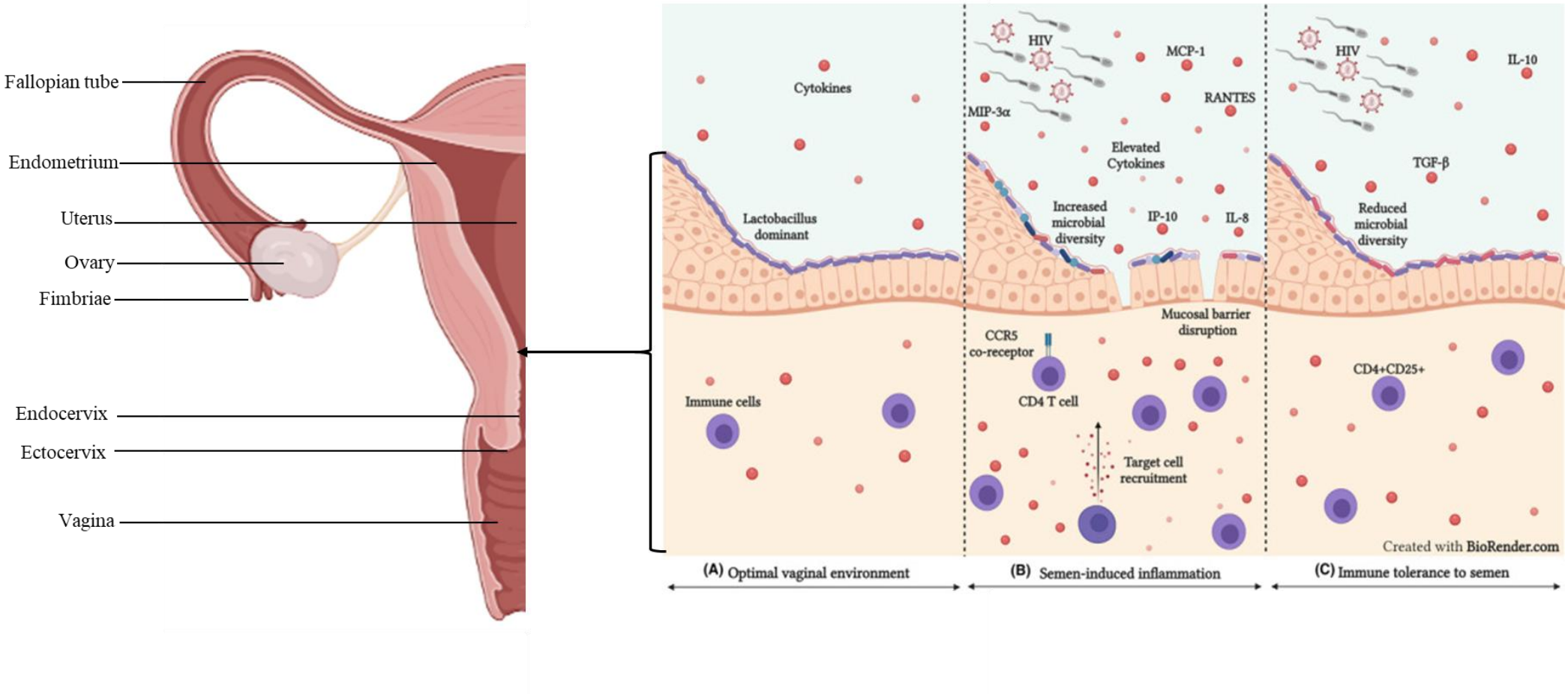


Figure 2.1. The female reproductive tract structure. The FRT contains the uterus, endometrium, endocervix, ectocervix and the vagina. (A) The optimal vaginal environment for the vagina with a few cytokines and a balanced lactobacillus dominant species. (B) Semen is introduced into the FRT, and the environment changes with a disrupted mucosal barrier. (C) The FRT has reached immune tolerance against semen, and the barrier is intact.

2.2 Genital inflammation and HIV acquisition risk

The risk of HIV acquisition is not only attributed to behavioural factors such as multiple sexual partners, sexual frequencies, and the use of condoms, but also biological factors such as genital inflammation, sexually transmitted infections (STIs), and the vaginal microbiome (Anahtar *et al*., 2015, Freeman *et al*., 2006, McKinnon *et al*., 2018). Inflammation is established as an immune response to tissue injury and stimuli such as infectious agents (Aggarwal *et al*., 2006). It may be acute and therapeutic and thus beneficial to the host, or it may emerge as a chronic condition and harmful to the host (Aggarwal *et al*., 2006, Ahmed *et al*., 2017). Genital inflammation, defined by an elevation in five of nine pro-inflammatory cytokines and chemokines above the 75th percentile, has been associated with an increased risk of HIV acquisition (Masson *et al*., 2015). Pro-inflammatory cytokines upregulate HIV infection by promoting the recruitment of target cells, such as endocervical T cells (Arnold *et al*., 2016). These cytokines also disrupt the tight junctions in the epithelial junctions in the epithelial cells and reduce the barrier integrity (Nazli *et al*., 2010, Passmore *et al*., 2016). Some pro-inflammatory cytokines, e.g. tumour necrosis factor-alpha (TNF-α), are also responsible for inducing the expression factor nuclear factor (NK)-KB, which binds to the HIV-long terminal repeat (LTR) and induces viral production (Duh *et al*., 1989, Herbein *et al*., 2010). Furthermore, HIV acquisition risk has been associated with concentrations of the individual cytokines IL-8, induced protein (IP)-10, interferon-gamma (IFN-γ), and macrophage inflammatory protein (MIP)-1α and -1β (Masson *et al*., 2015). MIP-1α and MIP-1β are natural ligands of the HIV coreceptor CCR5, The chemokines recruit CCR5-expressing CD4^{+} T cells and enhance the potential for HIV infection (Walker *et al*., 2015). Inflammatory cytokines have been associated with mucosal barrier proteins (Arnold *et al*., 2016) and promote local recruitment of activated CD4^{+} T cells (Rodriguez-Garcia *et al*., 2014), thereby fostering an environment conducive to establishing a productive HIV infection (Liebenberg *et al*., 2017, Passmore *et al*., 2016). Understanding the many factors involved in genital inflammation may help inform biomedical interventions needed to curb the risk of acquiring HIV in young women.

There are many contributors to genital inflammation, e.g. microbiota composition, sexually transmitted infections, contraception, vaginal insertive practices, and others (Jewanraj *et al*., 2021a, Robertson and Sharkey, 2016). The microbiota found in the FRT is a significant determinant of FRT health. Although unique in every woman, a low-diversity microbial community dominated by Lactobacillus species is typically associated with a "healthy" vagina. In contrast, dysbiosis (an imbalance in the microbial community) is associated with elevated concentrations of the pro-inflammatory cytokines and cervical HIV target cells that contribute to increased susceptibility to HIV infection (Torcia, 2019, Mtshali *et al*., 2021). Sexually transmitted infections (STIs) are another major contributor to genital inflammation. Even though genital inflammation is vital for the clearance of infection, it may disrupt or further the infected epithelial layer, allowing the sexually transmitted pathogens to infect other areas. When STI

infection is not cleared, the infection may lead to susceptibility to HIV (Passmore *et al*., 2016). Contraceptives have been associated with HIV risk (Heffron *et al*., 2012). Vaginal insertive practises are employed for various reasons, and multiple studies have demonstrated adverse effects of these on genital health, e.g. inflammation, micro-abrasions, and lesions have been associated with the vaginal practice. (Jewanraj *et al*., 2021a). These vaginal insertive practices are mainly used for or during intercourse facilitating a breeding ground for genital inflammation. Semen has been associated with genital inflammation (Jewanraj *et al*., 2021b), and this association has implications for HIV risk.

2.3 Semen and the FRT immune response

The primary biological functions of semen include delivering the spermatozoa to the FRT for reproduction (Robertson, 2005) and promoting both the integrity and survival of the sperm to transport it from the male reproductive tract (MRT) to the FRT (Suarez and Pacey, 2006). Semen contains several molecules to support these functions, such as prostaglandins, cytokines, enzymes, glycans, sex steroid hormones, and defensins (Aumüller and Riva, 1992, Schjenken and Robertson, 2020). In the FRT, these molecules can initiate modifications in cellular gene expression, resulting in changes to cell composition, function, and the structure of localised tissues and tissues surrounding the tract, such as the ovaries, peripheral lymphoid organs, and spleen (Robertson, 2005). Changes to the FRT due to seminal plasma infiltration may result in modifications which last longer than the initial oocyte fertilisation. Understanding the ways in which semen exposure affects the FRT immune and microbial environment and the subsequent impact on HIV risk is of utmost importance.

Seminal plasma originates from the epididymis, rete testis and the male accessory glands that contain seminal vesicles, bulbourethral and prostate glands (Figure 1.2) (McGraw *et al*., 2015). Various cytokines and growth factors secreted from these tissues and transferred during ejaculation have been identified as playing a crucial role in embryo development. These cytokines found in semen which include interleukin (IL)- 1α, IL-1β, IL-2, IL-4, IL-6, IL-8, leukaemia inhibitory factor (LIF), macrophage colony-stimulating factor (M-CSF), macrophage inflammatory protein (MIP)- 1α, MIP-1β, interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), and granulocyte colony-stimulating factor (G-CSF), transforming growth factor alpha (TGF-α), TGF-β, granulocyte macrophage-colony stimulating factor (GM-CSF), insulin, insulin-like growth factor (IGF)-I and II, heparin-binding EGF-like growth factor (HB-EGF), and epidermal growth factor (EGF), and are partially regulated in the oviduct and uterine epithelium by semen exposure (Maegawa *et al*., 2002, Politch *et al*., 2007, Bromfield *et al*., 2014).

Semen may also be involved in the permeability of the epithelial barrier in the FRT since it contains large quantities of IL-1β, a cytokine affecting epithelial barrier permeability through nuclear transcription factor (NF-κB) activation (Al-Sadi and Ma, 2007). This, in turn, may lead to HIV penetrating the barrier and accessing local immune cell targets (Sharkey *et al*., 2012b).

The mechanisms through which the suppression of immune responses in the FRT are mediated by semen are not clearly established but appear to be related to the high concentration of prostaglandin (PG) and TGF-β in the semen. These modulators are considered the primary constituents responsible for inducing an inflammatory response in the FRT (Robertson *et al*., 2009b). Prostaglandins can induce both anti- and pro-inflammatory cytokines production, such as interleukin-8 (IL-8) and interleukin-10 (Denison *et al*., 1999). Prostaglandins, mainly prostaglandin E (PGE), act as a tolerance-inducing agent in the FRT by protecting the male gametes from inflammatory damage (Robertson and Sharkey, 2016). PGE2 also plays an integral part in mediating immune function. In conjunction with TGF-β and IL-15, PGE2 can induce tolerance of the paternal and fetal antigens, leading to Treg cell differentiation and expansion in the FRT (Robertson *et al*., 2009a). The role of TGF-β in the FRT needs to be studied to identify its function in immune induction and suppression of immune responses.

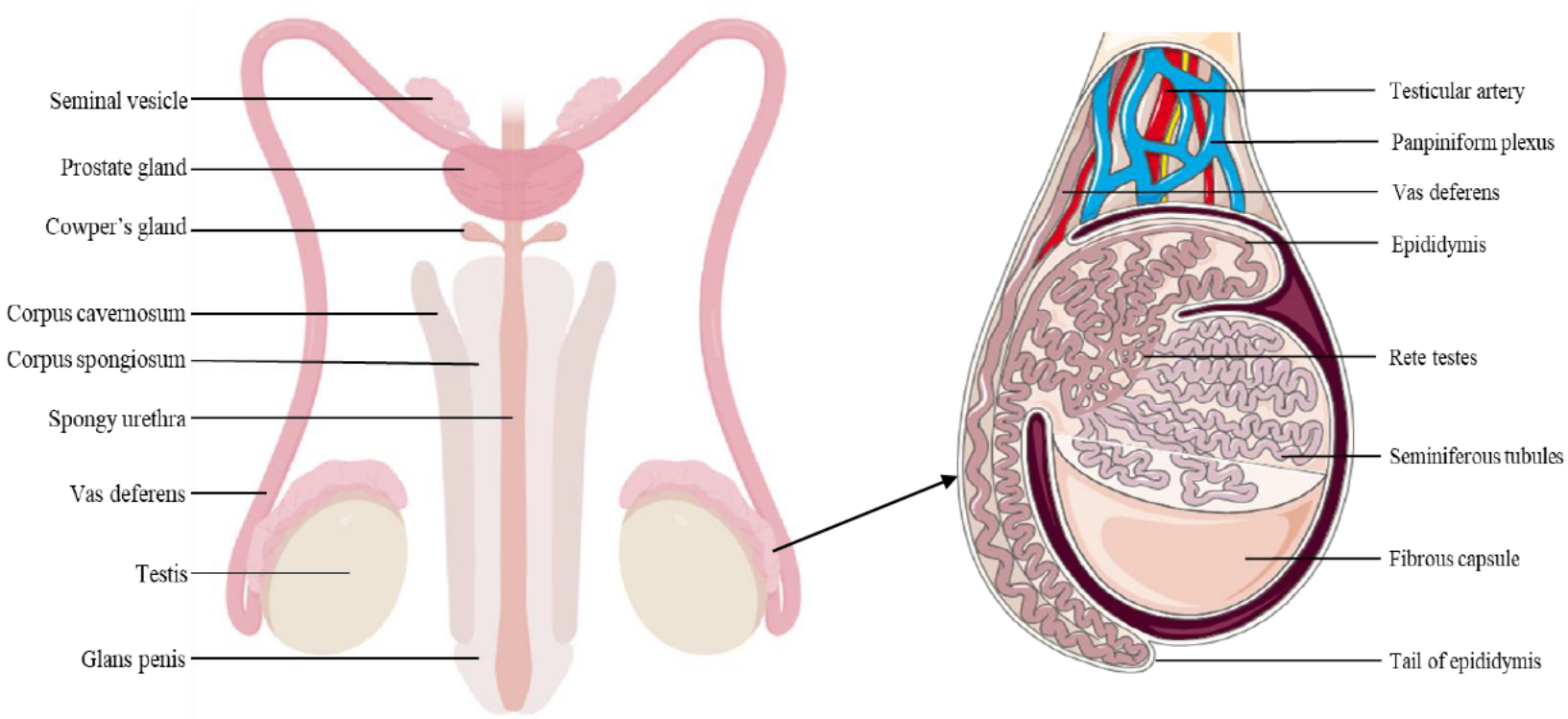


Figure 2.2. The male reproductive tract structure. The MRT consists of the testes, prostate gland, and the penis. The testes are made up of the epididymis and the seminiferous tubules. The MRT is vital for reproduction as it transfers sperm-containing semen from the penis to the FRT during coitus. Structures were created using software from Biorender.com and Servier.com, respectively.

2.4 Transforming growth factor-beta and its properties

TGF-β is a signalling agent in semen and is involved in the genital cytokine inflammatory response in the cervix (Sharkey *et al*., 2012a). This pleiotropic cytokine has multiple inflammatory and regulatory activities (Sanjabi *et al*., 2009). It is produced and expressed by cells with varying impacts on the immune system (Worthington *et al*., 2012). TGF-β controls several cell functions, such as proliferation, embryonic development, differentiation, wound healing, and angiogenesis (Deng *et al*., 2024). This cytokine consists of three homologous isoforms (TGF-β1, TGF-β2 and TGF-β3), all of which belong to the TGF-β superfamily with numerous members including inhibins, activins, bone morphogenetic proteins, and growth differentiation factors (Chang *et al*., 2002). Although the same signalling pathways transduce their signals (Cheifetz *et al*., 1987, Hachim *et al*., 2018), each isoform is encoded by a different gene (Hachim *et al*., 2018), and they are responsible for various functions when expressed.

To achieve its biological activity, TGF-β binds to the target cells through the type II receptor, activating the type I receptors (Theron et al., 2017a). This, in turn, phosphorylates and activates the SMAD transcription factors (Smad 2 and Smad 3) (Theron et al., 2017a). After the Smad 2 and -3 are phosphorylated, they bind to co-Smad 4, which is translocated to the nucleus where gene expression is modulated (Theron et al., 2017a). TGF-β isoforms are synthesised in the latent form and are activated before binding to the type II receptors. Before binding to receptors, TGF-β bound to latency-associated protein (LAP) and latent TGF binding proteins (LTBP) and require activation to cleave the LAP and LTBP (Shi *et al.*, 2011). This protein must be dissociated from TGF-β to convert to its active form, bind to its receptors, and perform its functions (Shi *et al*., 2011). Proteolytic processes are required to activate TGF-β, such as enzymatic digestion or extreme pH (Nocera and Chu, 1995). Enzymes such as tissue- and urokinase-type plasminogen activators, subtilisin-like endoproteases, and plasmin activate TGF-β in the male reproductive tract (Chu and Kawinski, 1998). Activation of semen-derived TGF-β following sexual intercourse is facilitated by exposure to the acidic pH and proteolytic enzymes in the FRT environment (Tremellen *et al*., 1998, Sharkey *et al*., 2012a). After activation, high-affinity binding occurs between TGF-β receptor (TβR) II and the TGF-βs, and TβRI is phosphorylated (Omwandho *et al*., 2010). It is well established that TGF-β2 binds weakly to TβRII and requires an accessory receptor to first bind with it to achieve the same level of high-affinity binding as TGF-β1 and TGF-β3 (Villarreal *et al*., 2016).

TGF-β isoforms are synthesised by different cells in the male reproductive tract. The Leydig and Sertoli cells are responsible for TGF-β1, TGF-β2 and TGF-β3 expression (Wang *et al*., 2021). The Sertoli cells are somatic cells located in the tubules of the testis. They provide nutritional support for

spermatogenesis by taking up nutrients from the bloodstream for male germ cell development (Silva *et al*., 2022). Spermatogenesis is the sperm being produced from the primordial germ cells (Gilbert and Barresi, 2000). The Leydig cells are the predominant extra-tubular somatic cells found in the interstitial tissue of the testes (Silva *et al*., 2022). These cells produce androgens that initiate, maintain, and regulate spermatogenesis (Smith and Walker, 2014). TGF-β isoforms also facilitate steroidogenesis (Lui *et al*., 2003), the multiphase process synthesising steroid hormones from cholesterol (Miller and Bose, 2011). TGF-β1 also regulates the production of immature cells testicular Sertoli cells and may be linked to spermatogenic epithelial development (Wang *et al*., 2021). Of the three isoforms, TGF-β1 is predominately expressed by immune cells; however, all the isoforms appear to have interchangeable roles *in vitro* (Mukumbang, 2017).

2.4.1 Anti-inflammatory effects of TGF-β and implications for HIV risk in women

TGF-β regulates several cell types through multiple mechanisms. These include, for example, TGF-β-mediated suppression of effector T helper (Th) cell differentiation, differentiation of regulatory T cells from naïve T cells, inhibition of B and T cell proliferation, suppression of innate cell function such as natural killer cells, macrophages and dendritic cells, and inhibiting the production of effector cytokines interferon-gamma (IFN-γ), interleukin 2 and 4 (IL-2 and IL-4), (Cottrez and Groux, 2001, Laouar *et al*., 2005, Werner *et al*., 2000, Strobl and Knapp, 1999, Ruegemer *et al*., 1990).

The ability of TGF-β to inhibit T-cell activation occurs through the interference of TCR signalling (Chen *et al*., 2003a). TGF-β inhibits Th1 and Th2 subset differentiation by obstructing the expression of transcription factors GATA-3 and T-bet (Gorelik *et al*., 2000; Gorelik *et al*., 2002). TGF-β inhibits Th1 by blocking another transcription factor, Stat4, activated by the IL-2 signalling response. TGF-β also stimulates the conversion of naive CD4+ T cells to Foxp3+ regulatory T cells that inhibit activation and proliferation of effector T cells (Chen *et al*., 2003b). Low levels of TGF-β promote the expression of FoxP3, which determines the anti-inflammatory response of Tregs, and suppress the production of the IL-23 receptor, which is essential for the generation of Th17 cells (Zhou et al., 2008, Schon and Weiskirchen, 2014). High levels of TGF-β1 can also be expressed by Th17 cells, and this cytokine can sustain Th17 cells *in vivo* through an autocrine mechanism (Gutcher *et al*., 2011). Additionally, IL-1β and TNF-α are found to promote the Th17 cell development mediated by TGF-β and IL-6 (Veldhoen *et al*., 2006). TGF-β1 exhibits its anti-inflammatory properties by inhibiting macrophage activation via the Smad3 pathways and inhibiting expression of the activation markers of macrophages (Werner *et al*., 2000). TGF-β2 inhibits the transcriptional regulators in the context of reduced IFN-γ production (Batlle and Massagué, 2019). Lastly, TGF-β3's capacity to inhibit the differentiation of CD4+ T cells that express FoxP3 shows its anti-inflammatory role (Shah and Qiao, 2008) and ability to inhibit the

production of antibodies and B cell proliferation (Tsuchida et al., 2017). Sex hormones modulate TGF-β production and ensure various aspects of immunosuppression at certain stages in the menstrual cycle (Takahashi *et al*., 1994; Wira and Rossoll, 2003). The expression of TGF-β during the luteal phase is increased alongside upregulation of specific proteases (Kane *et al*., 2008).

Studies have shown that Treg expansion in early pregnancy has been associated with seminal plasma exposure (Robertson, 2005, Robertson *et al*., 2002). For embryo implantation and placental development to occur, a change in the immune environment must take place to accommodate the antigens of the conceptus (Robertson *et al*., 2002). It might be argued that exposure to semen in early pregnancy could increase the capacity of the FRT to accommodate the semi-allograft of the foetus if the tolerance to mucosal immunity is induced against the antigens deposited alongside seminal TGF-β (Robertson *et al*., 2002). This promotes tolerance to the embryo and increases receptivity to the embryo implantation (Robertson *et al*., 2013). The mechanisms of this suppression by semen are still unknown; however, they may be linked, in part, to high TGF-β concentrations and prostaglandin (Robertson *et al*., 2002; Robertson *et al*., 2009a). Treg contribute to immune deficiency or a lack of immune activation.

2.4.2 Pro-inflammatory effects of TGF-β and implications for HIV risk in women

Although TGF-β is well-known for its immunoregulatory functions, its pro-inflammatory role is less explored (Marek *et al*., 2002). In interleukin 6 (IL-6) presence, TGF-β directs Th 17 cell differentiation and promotes continuous inflammatory autoimmune conditions (Korn *et al*., 2009, Sanjabi *et al*., 2009). High levels of TGF-β1 can also be expressed by Th17 cells, and this cytokine can sustain Th17 cells *in vivo* through an autocrine mechanism (Gutcher *et al.*, 2011). Additionally, IL-1β and TNF-α are found to promote the Th17 cell development mediated by TGF-β and IL-6 (Veldhoen *et al.*, 2006). TGF-β3, which is generated by Th17 cells in development, depends on IL-23, which, when combined with IL-6, causes Th17 cells to become extremely pathogenic. (Lee *et al.*, 2012). TGF-β stimulates the release of inflammatory cytokines, which include IL-6 and TNF-α (Marek *et al.*, 2002, Sharkey *et al.*, 2012a). TGF-β induced GM-CSF and IL-6 in ectocervical epithelial cells, demonstrating TGF-β's ability to signal pro-inflammatory cytokine synthesis (Sharkey *et al*., 2012a).

TGF-β in semen may also impact HIV-1 in women through its association with increased inflammatory responses at the FRT (Doncel *et al*., 2014). TGF-β in semen could propagate HIV-1 by suppressing the innate immune activity of natural killer cells (Jiang *et al*., 2018), promoting the differentiation of CD4+ Th17 cells and the induction of inflammatory cytokines (Luo *et al*., 2017). A study observed that TGF-

β upregulates CCR5 and CXCR3 during HIV-1 infection, potentially increasing activated memory CD4 (Yim *et al*., 2023).

Semen has been shown to facilitate the profile of genital inflammation and HIV risk (Jewanraj *et al*., 2021b). Considering the documented abundance of TGF-β in semen and its ability to both inhibit and elicit an inflammatory response in the female reproductive tract (FRT), further studies to understand the mechanisms involved and the consequences of inflammation and HIV risk may contribute to the design of targeted approaches to reduce HIV risk in women. Further, TGF-β isoforms and their independent functions in the FRT need better understanding to inform on targeted strategies to mitigate genital inflammation.

Hypothesis:

Semen exposure increases TGF-β levels in the female reproductive tract, with waning levels over time. TGF-β concentrations in the FRT will correspond with an altered cytokine profile and cellular biomarkers of inflammation, with implications for HIV risk.

Aim:

To assess the effects of TGF-β on the genital inflammation associated with HIV risk in women.

Objectives:

- To assess the persistence of TGF-β concentrations in the female reproductive tract after semen exposure and
- To determine the relationship between TGF-β and cytokine and cellular biomarkers of inflammation and regulation in the female reproductive tract.

CHAPTER 3

Methodology

METHODOLOGY

3.1 Study design

The study was conducted in a convenience sample of 132 women participating in the CAPRISA 008 randomised controlled trial (Mansoor *et al.*, 2019), an open-label extension trial integrating tenofovir-containing oral pre-exposure prophylaxis (PrEP) into routine family planning services as an HIV prevention tool. The CAPRISA 008 trial was conducted at the CAPRISA eThekwini and Vulindlela family clinics in KwaZulu-Natal (South Africa) and followed HIV-negative women for over two years, between November 2012 and October 2014. The participants were previously enrolled in the CAPRISA 004 1% tenofovir gel trial (Abdool Karim *et al.*, 2010). They were provided with tenofovir gel to assess adherence and HIV incidence rates within the context of family planning and CAPRISA clinics. Every six months, pelvic exams were conducted, and genital specimens (cervicovaginal lavage, cervicovaginal swabs, and cervical cytobrushes) were collected under speculum examination. Laboratory assays were performed at the CAPRISA Mucosal Immunology laboratory in Durban, South Africa, and included matching longitudinal measurements of TGF-β and other cytokines, cellular activation, and the semen biomarkers PSA (prostate-specific antigen) and YcDNA (Y-chromosome DNA) in genital specimens. The Biomedical Research Ethics Committee from the University of Kwa-Zulu Natal approved this study (BREC/00001814/2020), and participants provided informed consent for the future use of their specimens for research purposes. Stored genital specimens collected biannually (mean 5 visits) from 132 HIV-negative women participating in the CAPRISA 008 tenofovir gel open-label extension trial were included in this study.

3.2 Cervicovaginal collection and processing

Cervicovaginal lavages (CVL), cervical cytobrushes, and vaginal swab specimens were collected under speculum examination at enrolment and biannual visits as previously described (Bebell *et al.*, 2008, Liebenberg *et al*., 2011, Jewanraj *et al*., 2021b). A 5 ml sterile phosphate-buffered saline (PBS) volume was inserted through the speculum. The cervix was bathed with sterile PBS and then aspirated. The aspirated cervicovaginal lavage fluid was transferred on ice to the CAPRISA laboratory for processing by centrifugation. CVL specimens were stored at -20°C until required to quantify soluble proteins. Cervical mononuclear cells were collected by inserting Digene cervical samplers into the endocervix and rotated once 360°. The cytobrush was immediately placed into a 3ml antibiotic (Penicillin and Streptomycin)-containing RPMI and transferred on ice to the CAPRISA laboratory for processing (Jewanraj *et al*., 2021b). A vulvovaginal swab was collected from the participant's lateral vaginal walls and posterior fornices. Undiluted specimens were transported to the CAPRISA laboratory and were

stored at -20°C until required to test for the presence of bacterial vaginosis, BV-related bacteria, and sexually transmitted infections (STIs).

3.3 PSA measurements

Prostate-specific antigen (PSA) is a widely used and reliable biomarker for semen exposure. The presence of this biomarker was assessed in the CVL supernatants. According to the manufacturer's instructions, the Sigma-Aldrich Human PSA-total ELISA (Enzyme-linked immunosorbent assay) kit was used to measure PSA concentration in 642 CVL supernatants. The tests were conducted in duplicate with a detection limit of 0.94 ng/ml and a 60 ng/ml upper limit detection (Jewanraj *et al.*, 2021). Figure 3.1 is a summarised version of the PSA ELISA protocol.

3.3.1 Sample preparation

Sample diluent Buffer B was diluted using the 5-fold dilution with deionised water prior to starting the experiment. Samples were diluted 2-fold using Sample Diluent Buffer B.

3.3.2 Standard preparation

The standards were prepared by adding 400 µl of diluent B into item C to prepare a 50 ng/ml solution. The powder was dissolved and gently mixed. To prepare a 2500 pg/ml standard solution, a PSA-total standard (50 µl) was taken from item C and added to 1X Assay Diluent B. From the 1X Assay Diluent B, a volume of 300 µl was added to tubes used for the serial dilution (7 tubes). In the first serial dilution tube, 50 µl of the standard solution was added. The first tube was vortexed, and 200 µl was added to the second tube. This process was repeated until the seventh tube was complete, and each tube was thoroughly mixed before the transfer to the next tube. The 1X Assay Diluent B was used as a blank (zero standard).

3.3.3 Biotinylated Detection Antibody Preparation

The Detection antibody vial was briefly vortexed prior to use. A volume of 100 µl was added to a 1X Diluent Buffer B to prepare the detection antibody concentrate. The mixture was gently mixed by pipetting up and down quickly. The detection antibody was diluted by adding 15 800 µl of Buffer B to make 16 ml of detection antibody.

3.3.4 HRP-Streptavidin Concentrate Preparation

The HRP-Streptavidin concentrate vial was briefly spun before use. A volume of 19 960 µl was added to 40 µl of HRP-Streptavidin concentrate to make up 20 ml of HRP-Streptavidin solution. This solution was mixed well.

3.3.5 Running the assay

All the samples and reagents were brought to room temperature. In a 96-well plate, a 100 µl volume of each sample and each standard were added into specific wells. The wells were covered and incubated at room temperature for 2.5 hours whilst shaken gently.

After incubation, the solution was discarded, and the plate was washed four times with 1x Wash Solution at the wash station. The wash buffer was removed and decanted from the plate. A volume of 100 µl of Biotinylated Detection Antibody (already prepared) was added to each well. The plate was incubated for 1 hour and shaken gently.

After incubation, the solution was discarded, and the wash step was repeated. A 100 µl of HRP-Streptavidin was added to each well plate. The plate was incubated for 45 minutes while shaken gently. The solution was discarded, and the wash step was repeated. A 100 µl of ELISA Colorimetric TMB Reagent volume was added to each well. The plate was incubated for 30 minutes, and the plate was shaken gently. Stop solution (50 µl) was added to each well. Data was immediately acquired at 450 nm on a VersaMax™ ELISA Microplate Reader.

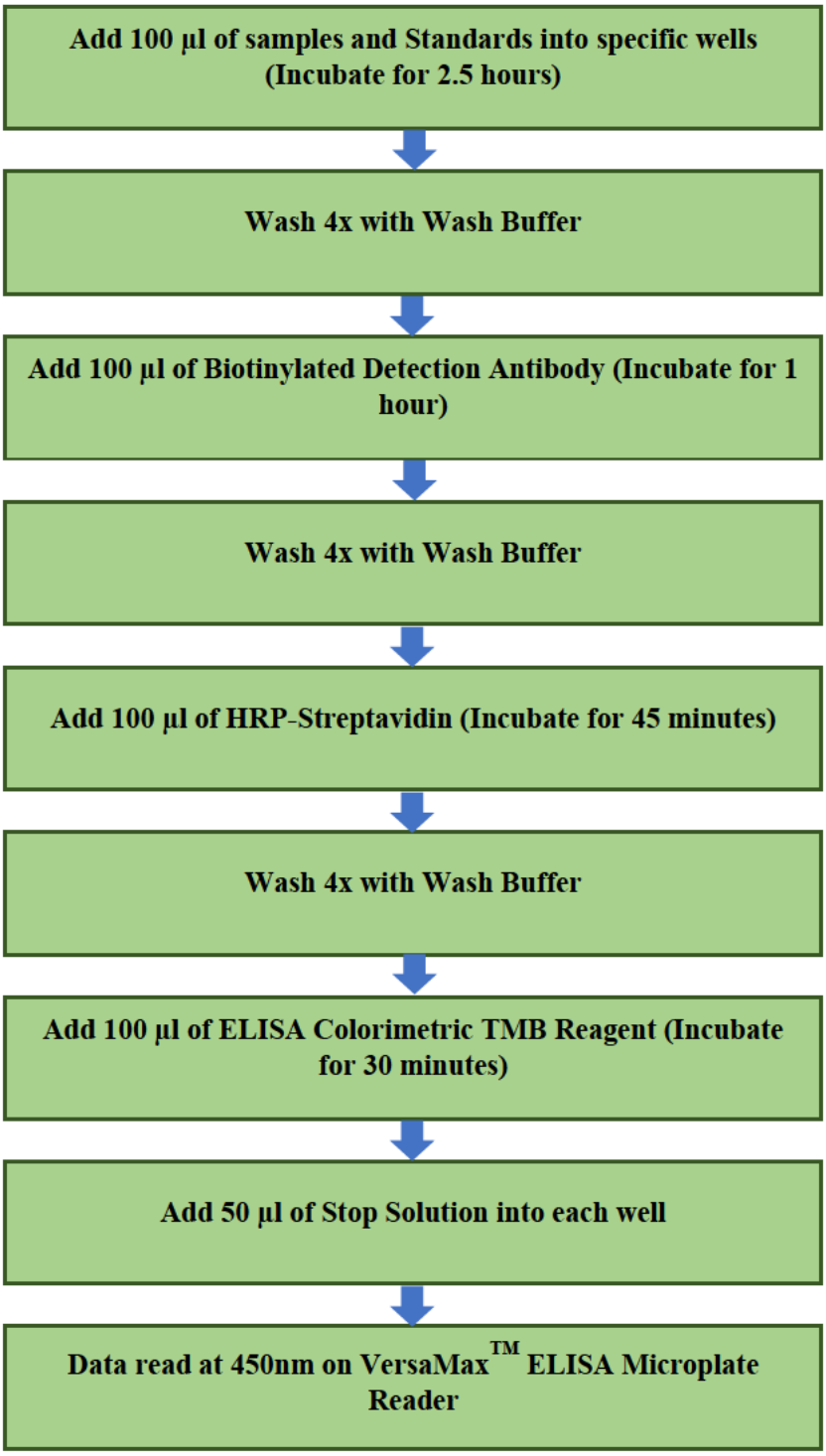


Figure 3.1. Prostate-Specific Antigen ELISA summarised protocol.

3.4 Quantification of cytokines and matrix metalloproteinases

Cervicovaginal soluble proteins were quantified for all visits, as previously described by Jewanraj *et al*. (2021b). Briefly, multiplex Enzyme-Linked Immunosorbent Assay (ELISA) assays were conducted to quantify 51 cytokines (BASIC-FGF, CTACK, EOTAXIN, G-CSF, GM-CSF, GRO-α, HGF, IFN-α, IFN-G, IP-10, IL-1α, IL-1β, IL-1Ra, IL-2Rα, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL12 (p70), IL-13, IL-15, IL-16, IL-17, IL-18, LIF, M-CSF, MIP-1α MIF, MCP-1, MCP-3, MIG, β-NGF, PDGF, RANTES, SCF, SCGFβ, SDF-1α, TNF-β, TRAIL, TNF-α, VEGF, and TGF-β1, TGF-β2 and TGF-β3) in CVL supernatants using the Bio-Plex Pro TGF-β Panel, Group II 21-Plex Panel and Bio-Plex Pro Human Cytokine Group I 27-Plex Panels according to manufacturer instructions (Bio-Rad Laboratories, Inc; Hercules, Ca, USA). Additionally, multiplex ELISA assays were used to determine the concentrations of matrix metalloproteinase (MMP) (MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12, and MMP-13) biomarkers of wound healing and epithelial barrier integrity using the MMP 9-Plex kits according to manufacturer instructions (Bio-Rad Laboratories, Inc; Hercules, Ca, USA).

3.5 Detection of TGF-β in CVL supernatant specimens

The Bio-Plex Pro TGF-β Assay (Bio Rad Laboratories, Inc; Hercules, Ca, USA) was used to measure TGF-β concentrations in 642 CVL supernatants following the manufacturer's protocol detailed below. Data was collected using a Bio-Plex 200 machine and analysed using Bio-Plex Manager V6 Software (Bio Rad Laboratories, Inc; Hercules, Ca, USA).

3.5.1 *Preparation of samples*

All samples were kept on ice until ready to be used. The CVL was diluted with the lavage buffer in which it was stored. Dilutions were prepared in a 2 ml microcentrifuge tube.

3.5.2 *Reconstitution of single vials of standards*

The vial containing the lyophilised standard was reconstituted by tapping the vial gently and adding 500 µl of sample diluent to the vial. The vial was gently vortexed for 5 seconds and incubated for 30 minutes on ice.

3.5.3 *Preparation for the standard dilution series*

Nine polypropylene tubes (1.5 ml) were labelled blank and S1-8. The specific volumes of standard diluent were added to each tube. The reconstituted standards were gently vortexed before removing any volumes. In the S1 tube, 128 µl was added to the 72 µl of standard diluent. The tubes were vortexed for 5 seconds, and then 50 µl was transferred from the tube labelled S1 to the tube labelled S2 and vortexed afterwards. Series dilution was continued until the S8 tube, as shown in **Figure 3.2**.

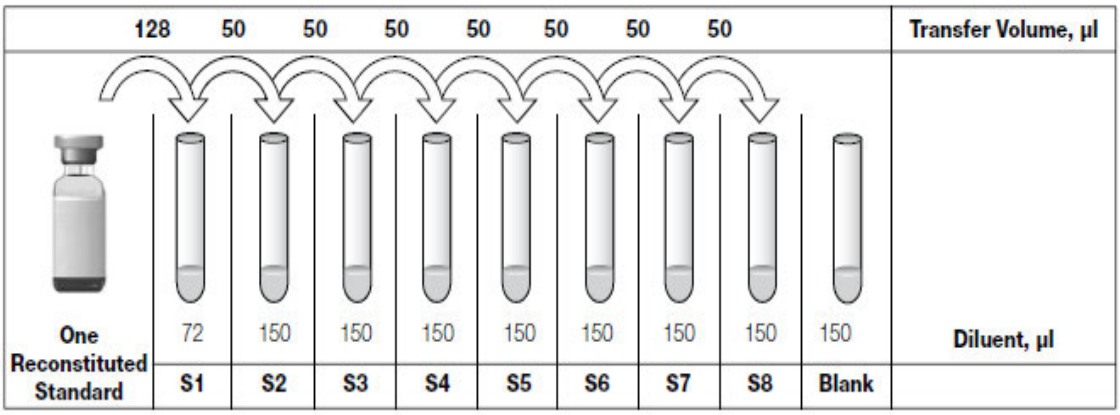


Figure 3.2. Image representing the serial dilutions for TGF-β standard.

3.5.4 *Preparation of the coupled beads*

The coupled beads were diluted to 1x concentration from 20x stock. The tubes were kept on ice. Assay buffer (5 472 µl) was added into a polypropylene tube (15 ml), and for 30 seconds, the stock coupled beads were vortexed at medium speed. The coupled beads were diluted to 1x by pipetting 288 µl to the tube (15 ml). Each of the wells in the assay plate had a total volume of assay buffer of 50 µl.

3.5.5 *Preparation of the detection antibody*

The detection antibody was prepared 10 min prior to use. The stock detection antibodies were vortexed at medium speed for 20 seconds; then, a 30-second spin was done to obtain the volume in its entirety at the bottom of the tube. The detection antibodies were diluted (1x) by adding 150 µl into a 2850 µl Detection Antibody Diluent volume in a 5 ml polypropylene tube. The tube was vortexed before use and covered with aluminium foil.

3.5.6 *Preparation of Streptavidin-PE (SA-PE)*

The SA-PE was prepared ten minutes before use. In a 15 ml tube, 60 µl of SA-PE (100X) was added to 5490 µl Assay buffer to produce a 1x solution in a 5 ml polypropylene tube. The tube was vortexed and covered with aluminium foil before use.

3.5.7 *Running the assay*

All assay components were kept at room temperature before use. Pipettes were calibrated, and new tips were used for every volume transfer. All the incubation steps required for the assay were conducted at 850 ± 50 rpm on a shaker and were protected from the light. A Bio-Plex Pro II wash station was used for every wash option (**Table 3.1**).

Table 3.1. Summary of the wash station protocol

Assay Step	**Bio-Plex Wash station Magnetic Program**
Add coupled beads to plate	Magnetic Wash x2
Sample incubation	Magnetic Wash x3
Detection antibody incubation	Magnetic Wash x3
Streptavidin-phycoerythrin incubation	Magnetic Wash x3

A volume of 50 µl coupled beads was transferred into each assay plate well. The plate was washed twice with 100 µl Bio-Plex wash buffer (**Table 3.1**). Thereafter, 50 µl of the sample, standards, controls, and blanks were transferred to their respective locations in the assay. The plate was then covered with sealing tape and aluminium foil and was kept at room temperature for 2 hours at 850 ± 50 rpm.

After incubation, the wells were washed thrice with 100 µl of wash buffer (100 µl) (**Table 3.1**). A volume of 25 µl detection antibodies was added to each well using a reagent reservoir and multichannel pipet. A new sheet of sealing tape was used to cover the plate, and aluminium foil was used to protect it from the light. The plate was kept at room temperature for 1 hour at 850 ± 50 rpm.

After the incubation with detection antibodies, the plate was washed thrice using 100 µl wash buffer per well (**Table 3.1**). The diluted SA-PE (1x) was added at 50 µl per well using a multichannel pipette and a reagent reservoir. New sealing tape and foil were used to cover the plate and were kept at room temperature for 30 minutes at 850 ± rpm.

A 100 µl volume of wash buffer per well was used to wash the plate (**Table 3.1**) before adding 125 µl Assay Buffer was required for data acquisition. The plate was placed on a shaker at room temperature for 30 seconds at 850 ± 50 rpm before data acquisition on a BioPlex200 Array Reader (BioRad, Hercules, California, USA).

Data collection was conducted using the Bio-Plex Manager software version 6. Sample protein concentrations were calculated from standard curves using a five-parameter logistic regression formula. Cytokine and MMP concentrations below the lower limit of detection were reported as half of the minimum concentration measured for each analyte. Likewise, concentrations above the detectable limit were recorded as double the maximum concentration measured for each analyte. To reduce the impact of inter-plate variability, all CVL specimens collected from each participant over time were run on the same assay plate. Intra-plate and inter-plate variability were assessed to detect significant differences between duplicate or inter-plate wells, respectively, and Spearman rho ≥ 0.8 and non-significant p-values were considered acceptable.

3.6 Immune cell quantification

Multiparametric flow cytometry on cervical cytobrush specimens was conducted to assess the frequency of activated (HLA-DR+ or CD38+) or replicating (Ki67+) CD4+CCR5+ HIV-susceptible T cell targets. The gating strategy used was previously described by Jewanraj *et al.* (2020).

3.6.1 *Immune marker surface staining*

When the processing was complete, cells isolated from 100 µl were inserted in a 96-well plate for staining. The samples were then bathed with Phosphate Buffered Saline and placed in the wells. At 2100 rpm (833 xg), the cells were centrifuged at 4°C. The supernatants were removed, and the pellets were resuspended in 200 µl PBS, then centrifuged at 4°C at 2100 rpm (833 xg) for 3 minutes. Viable cells were identified using the live/dead stain Fixable Violet Cell Stain kit (VIVID, Molecular probes, Life Technologies, USA). A 1:40 dilution using dH_2O was done to prepare the Vivid dye, and it was further diluted using PBS. This solution was then added to each well that had the cell pellets. The assay plate was incubated for 20 minutes in the darkness at 4°C. The pellets were resuspended in 200 µl Wash Buffer (1% FBS in PBS) in each well. At 4°C, the plate was centrifuged at 2100 rpm for 3 minutes in Eppendorf 5810 Centrifuge (Eppendorf AG, Hamburg, Germany) and the supernatant was then discarded. The extracellular part of the mononuclear cells were stained with anti-conjugated fluorochromes: Allophycocyanin Tandem Dye (APC-Cy7)-labelled anti-CD19 (BD Bioscience), Fluorescein Isothiocyanate (FITC)-labelled anti-CD8 (BioLegend), Peridinin-Chlorophyll Protein-Cyanine 5.5 (PerCP-Cy5.5)-labelled anti-CD4(BD Bioscience), Pacific Blue labelled-anti-CD19 (BD Bioscience), eF605-labelled anti-CD16 (BD Bioscience), and Phycoerythrin Cyanin 7 labelled anti-CD56 (BD Bioscience). The antibodies were titred and inserted into each well (2 µl). Anti-CD4 was

the exception; only 1 µl was inserted into each well. The plate was incubated for 20 minutes at 4°C in the darkness.

3.6.2 *Immune marker intracellular staining*

After incubation, the cells were resuspended gently in 200 µl Wash Buffer (1% FBS in PBS) and centrifuged at 2100 rpm (833 xg) for 3 minutes at 4°C. The supernatant was discarded, the pellets were treated with Cytofix/Cytoperm (100 µl), and incubation was conducted for 20 minutes at 4°C in the dark. Perm Wash Buffer (BD Biosciences) (150 µl) was inserted into the plate wells. The plate was centrifuged for 3 minutes at 2100 rpm at 4°C. The supernatant was discarded, and Allophycocyanin-labelled anti-CCR5 (10 µl) was added with 2 µl of these antibodies: Phycoerythrin (PE)-labelled anti-HLA-DR (Bioscience), Brilliant Violet 700 (BV700)-labelled anti-76 (BioLegend). The plate was incubated at 4°C for 20 minutes in the dark. Following incubation, Wash Buffer (200 µl) was inserted into each plate well and centrifuged at 4°C for 20 minutes at 2100 rpm (833 xg). The supernatant was discarded, and the BD Cell Fix (100 µl) was inserted to resuspend the pellet. The cells were moved to a fluorescence-activated cell sorting (FACS) tube. The wells of the plate were washed with BD Cell Fix (50 µl) and moved to FACS tubes.

3.6.3 *Compensation bead preparation*

The compensation beads were prepared as follows: the beads were vortexed at high speed, and one drop of each positive and negative beads was inserted into each FACS tube. Antibodies (2 µl) were added to each FACS tube.

3.6.4 *Data acquisition*

The LSRII flow cytometer (BD Immunocytometry Systems) was used to collect data, and FlowJo Software version 9.9 (Tree Star, C, US) was used for data analysis.

3.7 Statistical considerations

CVL specimens were classified according to PSA detection, with "PSA+" indicating exposure to semen within 0-48 hours of sampling and "PSA-" indicating no exposure to semen within 0-48 hours of sampling. Soluble protein concentrations were log-transformed. Mann-Whitney tests were used to compare median TGF-β concentrations in baseline PSA+ and PSA- specimens and between continuous TGF-β subunits at baseline. Fisher's exact tests were used to compare proportions at baseline. Linear regression models assessed the relationship between baseline TGF-β concentrations, controlling for participant age, any STI, BV Nugent Score, time in study, and study arm. Linear regression models were used to investigate the relationship between TGF-β concentrations and inflammation biomarkers,

including cytokine concentrations (log_{10} pg/ml), MMP concentrations (log_{10} pg/ml), and immune cell frequencies at baseline. Linear mixed models were used to compare the relationship between TGF-β concentrations, biomarkers of semen exposure, and cytokine concentrations in women with evidence of recent sex over time. Linear mixed models adjusted for the age of the participants, STI presence, number of vaginal sex acts in the past month, study arm, and Nugent score. Adjustment for multiple comparisons was performed using a step-down approach (Columb and Sagadai, 2006). Statistical analyses were performed using GraphPad Prism Version 8.43.3 (GraphPad Software, San Diego, CA) and STATA version 15.0 (StataCorp., College Station, TX, USA). P-values less than 0.05 were considered significant.

CHAPTER 4

Results

RESULTS

4.1 Characteristics of the study population

The study included a convenience sample of 132 young, HIV-negative women participating in the randomised, controlled, open-label extension CAPRISA 008 trial (Mansoor *et al.*, 2019). The median age of the study population was 28 years [interquartile range (IQR) 25-33 years] and included a similar representation of women in the trial's intervention arm (n=70; TFV gel provided by family planning clinics) and control arm (n=62; TFV gel provided by CAPRISA clinics; Table 1). Recent condomless sex was determined by PSA detection in CVL specimens, indicative of either semen exposure (PSA+) or no semen exposure (PSA-) within 0-2 days prior to genital sampling. At baseline, PSA was detected in 27% (36/132) of the study participants, and detection was significantly associated with relationship status (p=0.0112). Furthermore, PSA was detected in 16.6% (8/48) of women who reported consistent condom use.

Table 4.1. Demographics of study participants according to PSA status.

Demographics	**Level**	**Overall (N=132)**	**PSA+ (N=36)**	**PSA- (N=96)**	**P-value**
Age (years)	Median (IQR)	28 (25-33)	29 (25-35)	28 (25-33)	0.3113
Study arm [%(n)]	Intervention	53.0% (70)	41.7% (15)	57.3% (55)	0.1212
	Control	47.0% (62)	58.3% (21)	42.7% (41)	
Relationship status [%(n)]	Married	16.7% (22)	30.6% (11)	11.5% (11)	**0.0112**
	Stable partner	82.6% (109)	69.4% (25)	87.5% (84)	
	Casual partner	0.8% (1)	0	1.0% (1)	
Age of regular/stable partner	Median (IQR)	32 (28-37)	32 (28-41)	32 (28-37)	0.4375
Number of vaginal sex acts in the last 30 days	Median (IQR)	4 (2-8)	4 (2-8)	4 (3-10)	0.1262
Partner HIV status [%(n)]	Positive	2.3% (3)	2.8% (1)	2.1% (2)	0.2180
	Negative	66.7% (88)	75.0% (27)	63.5% (61)	
	Unknown	31.1% (41)	22.2% (8)	34.4% (33)	
Regular partner living together [%(n)]	Yes	25.0% (33)	36.1% (13)	20.8% (20)	0.1126
	No	75.0% (99)	63.9% (23)	79.2% (76)	
Use of male condom [%(n)]	Always	36.4% (48)	22.2% (8)	41.7% (40)	0.0881
	Sometimes	49.2% (65)	58.3% (21)	45.8% (44)	
	Never	14.4% (19)	19.4% (7)	12.5% (12)	
Any discharge-associated STI [%(n)]	Yes	19.2% (25)	25.0% (27)	17.0% (16)	0.3255
	No	80.8% (105)	75.0% (9)	83.0% (78)	
Inflammation status [%(n)]	Yes	31.8% (42)	36.1% (13)	30.2% (29)	0.5346
	No	68.2% (90)	63.9% (23)	69.8% (67)	

PSA, prostate-specific antigen; IQR, interquartile range; STI, sexually transmitted infection: Chlamydia, Gonorrhoea, Trichomonas, Neisseria gonorrhoeae and/or Mycoplasma genitalium; inflammation status defined as per McKinnon et al., (2018); p-values <0.05 were considered statistically significant.

4.2 TGF-β2 predominates at the FRT

First, baseline detection of the three TGF-β isoforms was compared in cervicovaginal fluid of the 132 participants. TGF-β1 was observed above the limit of detection in 46/132 participants (35%), less frequently than both TGF-β2 (95/132; 72%) and TGF-β3 (95/132; 72%). Considering the predominance of specimens with TGF-β1 concentrations below the detectable limit in this study (35%; **Figure 4.1**), TGF-β1 detection was assessed as a binary variable in subsequent statistical analyses and thus excluded from comparisons of continuous variables, e.g. the magnitude of cytokine responses (**Figure 4**).

Median baseline concentrations of TGF-β2 and TGF-β3 were observed at 0.57 Log_{10} pg/ml (IQR -1.52 to 0.79 Log_{10} pg/ml) and -0.08 Log_{10} pg/ml (IQR -2.3 to 0.19 Log_{10} pg/ml), respectively (**Figure 4.1A**). TGF-β2 concentrations were significantly higher than TGF-β3 (p<0.0001), and this pattern was mirrored in baseline PSA+ (n=36; **Figure 4.1B**) and PSA- (n=96; **Figure 4.1C**) specimens.

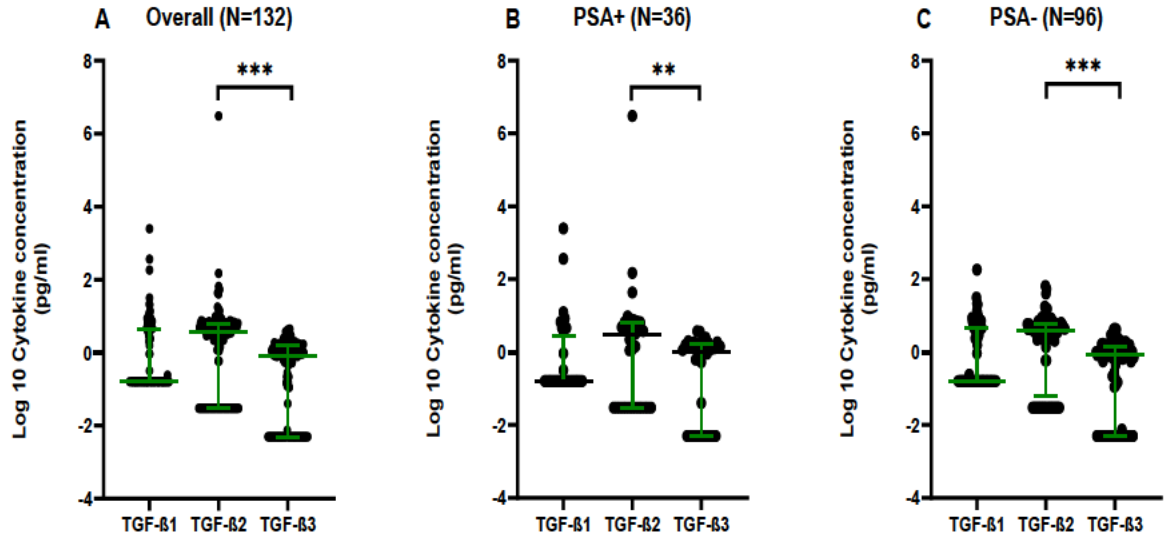


Figure 4.1. TGF-β in cervicovaginal lavage specimens of women with and without recent semen exposure. Dot plots depict baseline median and interquartile ranges of TGF-β concentrations in all participants (**A**) and in women with (**B**; n=36; PSA+) and without evidence of semen exposure within 0 – 2 days of sampling (**C**; n=96; PSA-). Concentrations of TGF-β1 are included for visualisation purposes, with only the concentrations of the continuous variables (TGF-β2 and TGF-β3) compared using non-parametric Mann-Whitney tests. P-values <0.05, <0.01 or <0.001 are indicated by asterisks (*), (**), or (***), respectively.

4.3 Associations between FRT TGF-β isoform concentrations and PSA detection

The baseline levels of individual TGF-β isoforms were then compared in women with and without PSA (**Figure 4.2**). No significant difference was observed in TGF-β1 detection between PSA+ (11/36 specimens, 30.6%) and PSA- specimens (35/96 specimens, 36.5%) at baseline (p=0.682; **Figure 4.2A**). Additionally, no significant differences in median TGF-β2 concentrations were observed between specimens with (median 0.485 Log_{10} pg/ml; IQR -1.523, 0.818 Log_{10} pg/ml) and without detectable PSA (median 0.595 Log_{10} pg/ml; IQR -1.199, 0.784 Log_{10} pg/ml; p=0.6222; **Figure 4.2B**); nor in median TGF-β3 concentrations observed between PSA+ and PSA- specimens (median -0.009 Log_{10} pg/ml, IQR -2.301, 0.232 Log_{10} pg/ml and median -0.081 Log_{10} pg/ml, IQR -2.301, 0.156 Log_{10} pg/ml, respectively; p=0.436; **Figure 4.2C**). Multivariable models confirmed that PSA detection was associated with neither TGF-β1, TGF-β2 nor TGF-β3 at the FRT during the 2-year trial, controlling for study arm and time in study (simple model), or, additionally for participant age, any current STI, and BV Nugent Score; N=641 specimens; **Table 4.2**).

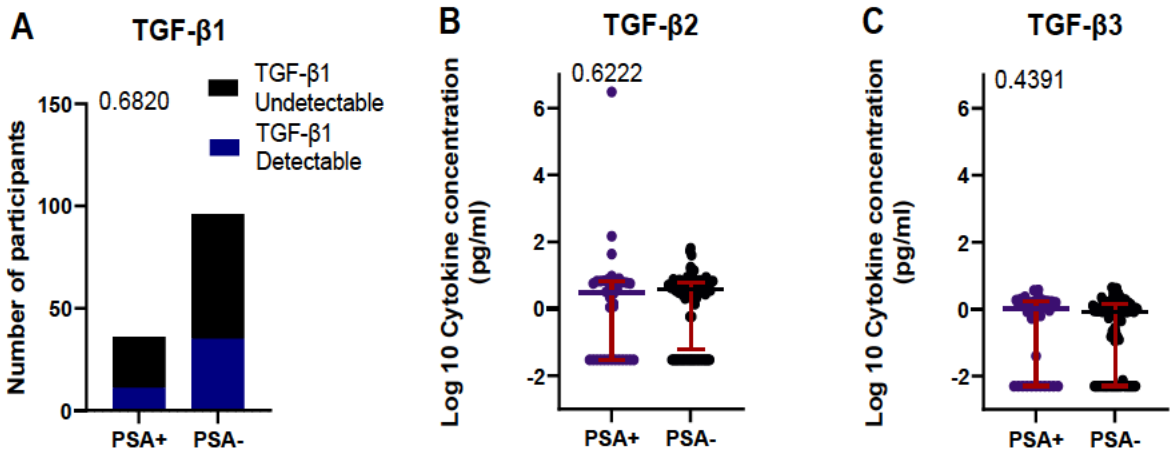


Figure 4.2. Relationship between TGF-β isoforms and PSA detection in female genital specimens. **(A)** The contingency graph depicts detectable and undetectable levels of TGF-β1. Dot plots depict median and interquartile ranges of TGF-β2 **(B)** and TGF-β3 concentrations **(C)** in women with detectable (PSA+; n=36) and undetectable PSA (PSA-; n=96) in cervicovaginal lavage specimens. Non-parametric Mann-Whitney tests compared continuous variables; categorical data were compared using Fisher's exact test. P-values <0.05 were considered significant.

Table 4.2. Association between TGF-β isoform levels and PSA detection during CAPRISA 008 trial (N=132 women, 641 tests).

	Simple Model[1]		Expanded Model[2]	
Continuous Variables	**β-coefficient (95% CI)**	**P-value**	**β-coefficient (95% CI)**	**P-value**
TGF-β2	-0.010 (-0.180 – 0.161)	0.910	0.014 (-0.161 - 0.190)	0.874
TGF-β3	-0.075 (-0.257 – 0.1075)	0.421	-0.042 (-0.232 - 0.148)	0.664
Binary Variables	**Odds Ratio (95% CI)**	**P-value**	**Odds Ratio**	**P-value**
TGF-β1	1.14 (0.560 – 2.326)	0.715	1.198 (-0.534 – 0.930)	0.596

Continuous and Binary variables were modelled in linear mixed models and random effects models, respectively. [1]Simple and [2]Expanded models adjusted for, respectively, CAPRISA 008 study arm time in study, age, STIs, BV Nugent score, CAPRISA 008 study arm, and time in study.

4.4 Associations between TGF-β and soluble biomarkers of genital inflammation and epithelial integrity

The associations between TGF-β and cervicovaginal cytokines during the study were investigated by linear mixed models adjusting for PSA detection, participant age, Nugent Score, STI presence, time in the study and study arm (**Figure 4.3**). Independently of PSA detection, TGF-β1 detection and TGF-β3 concentrations were associated with decreases in the concentrations of multiple FRT cytokines (**Figure 4.3**). The association between TGF-β1 detection and reduced MIG concentrations (β = -0.2264; CI -0.347, -0.106; P-value = 0.000) maintained statistical significance after adjustment for multiple comparisons, as did associations between TGF-β3 concentrations and that of IL-6, IL-12p70, TNF-a, IL-8, MIG, RANTES, G-CSF, HGF, IL-7, IL-9, PDGF-ββ, SCF, VEGF, IFN-γ, IL-13 and IL-10 (**Figure 4.3**). Additionally, the odds of detecting TGF-β1 were higher in women without genital inflammation as defined by (Masson *et al*., 2015) (OR 0.243; CI 0.101, 0.588; P-value = 0.002), and concentrations of TGF-β3 were also significantly higher in women without genital inflammation (β = -0.197, CI -0.386, -0.008; P-value = 0.041). In contrast, TGF-β2 concentrations were associated with increased MCP-1 concentrations (β = 0.027; CI 0.005, 0.048; P-value = 0.015), but only before adjusting for multiple comparisons, and TGF-β2 concentrations were not associated with genital inflammation status in this study (β = -0.026; CI -0.2012, 0.151; P-value = 0.773).

Investigations of TGF-β isoforms and matrix metalloproteinase biomarkers of genital epithelial barrier integrity identified a relationship between TGF-β3 concentration and reduced concentrations of MMP-

2 (β = -0.171; CI -0.333 - 0.009; P-value = 0.038); but the statistical association did not withstand adjustment for multiple comparisons (**Table 4.3**).

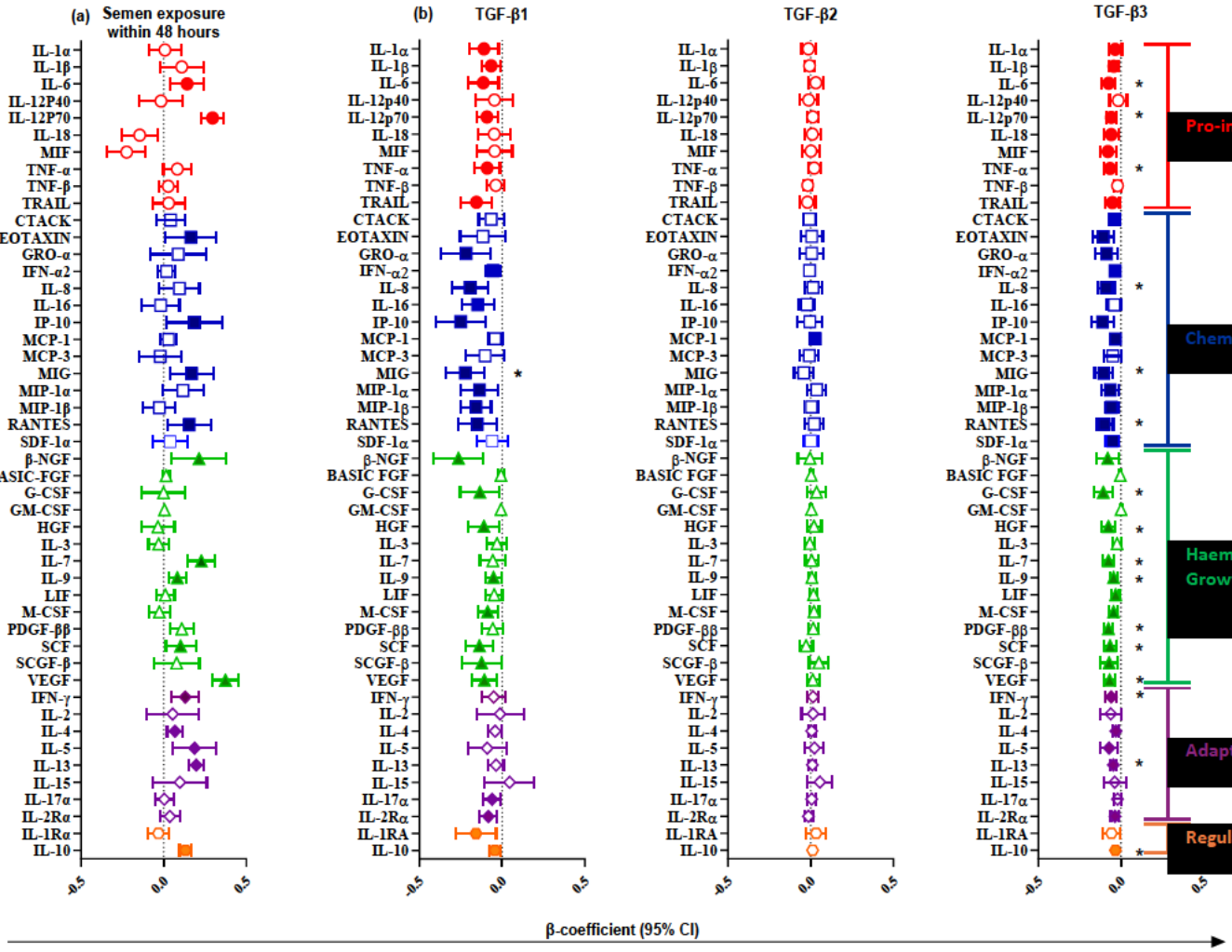


Figure 4.3. Associations between TGF-β and other cytokine concentrations in cervicovaginal fluid. (a) Association between semen exposure within 48 hours (PSA) and genital cytokines at the FRT. **(b)** Associations between TGF-β and other cytokines at the FRT were investigated in cervicovaginal lavage specimens (n=641) by multivariable linear mixed models controlling for participant age, BV Nugent Score, any STI, the number of vaginal sex acts in the past 30 days, study arm and time in study. Shapes and error bars depict β-coefficients and 95% confidence intervals. Shaded shapes depict P-values <0.05. Associations maintaining statistical significance after adjustment for multiple comparisons are indicated by black asterisks. Cytokine functional groupings: pro-inflammatory (red circles), chemotactic (blue squares), haematopoiesis/growth (green triangles), adaptive response (purple diamonds), and regulatory (orange hexagons) cytokines.

Table 4.3. Association between TGF-β isoform and matrix metalloproteinase concentrations during the CAPRISA 008 trial.

TGF-β	Variables	β-coefficient[1]	95% Confidence interval	P-value
			Lower - Upper	
TGF-β1	MMP-1	-0.095	-0.418 - 0.229	0.567
	MMP-2	-0.163	-0.547 - 0.222	0.407
	MMP-3	-0.110	-0.335 - 0.115	0.338
	MMP-7	-0.294	-0.640 - 0.051	0.095
	MMP-8	-0.435	-1.312 - 0.442	0.331
	MMP-9	-0.378	-0.789 - 0.032	0.071
	MMP-10	-0.013	-0.356 - 0.331	0.943
	MMP-12	-0.223	-0.516 - 0.070	0.135
	MMP-13	-0.236	-0.563 - 0.090	0.156
TGF-β2	MMP-1	0.014	-0.116 - 0.144	0.834
	MMP-2	0.029	-0.125 - 0.183	0.715
	MMP-3	0.006	-0.085 - 0.097	0.902
	MMP-7	0.019	-0.121 - 0.159	0.788
	MMP-8	0.054	-0.302 - 0.411	0.765
	MMP-9	-0.014	-0.203 - 0.174	0.880
	MMP-10	0.034	-0.104 - 0.172	0.628
	MMP-12	-0.027	-0.147 - 0.094	0.666
	MMP-13	-0.001	-0.133 - 0.132	0.993
TGF-β3	MMP-1	0.007	-0.132 - 0.147	0.918
	MMP-2	-0.171	-0.333 - 0.009	**0.038**
	MMP-3	0.032	-0.067 - 0.131	0.523
	MMP-7	-0.125	-0.273 - 0.022	0.096
	MMP-8	-0.172	-0.555 - 0.211	0.380
	MMP-9	-0.041	-0.246 - 0.163	0.693
	MMP-10	-0.093	-0.240 - 0.054	0.214
	MMP-12	-0.059	-0.190 - 0.072	0.379
	MMP-13	-0.108	-0.248 - 0.032	0.132

[1]Adjusting for participant age, PSA result, STIs, BV Nugent score, CAPRISA 008 study arm, time in study.

4.5 FRT TGF-β concentrations associated with reduced immune cell frequencies

The relationships between immune cell frequencies and TGF-β levels during the trial were assessed using multivariable linear mixed models controlling for age, PSA detection, any STI, Nugent Score, study arm and time in the study **(Figure 4.4b)**. TGF-β1 detection and TGF-β2 concentrations were associated with reduced numbers of several activated CD4+ T cell populations at the FRT. The significance in the concentration of TGF-β1 was not maintained. The TGF-β1 detection was associated with reduced numbers of proliferating cells, HIV target makers and T cell activation cells.

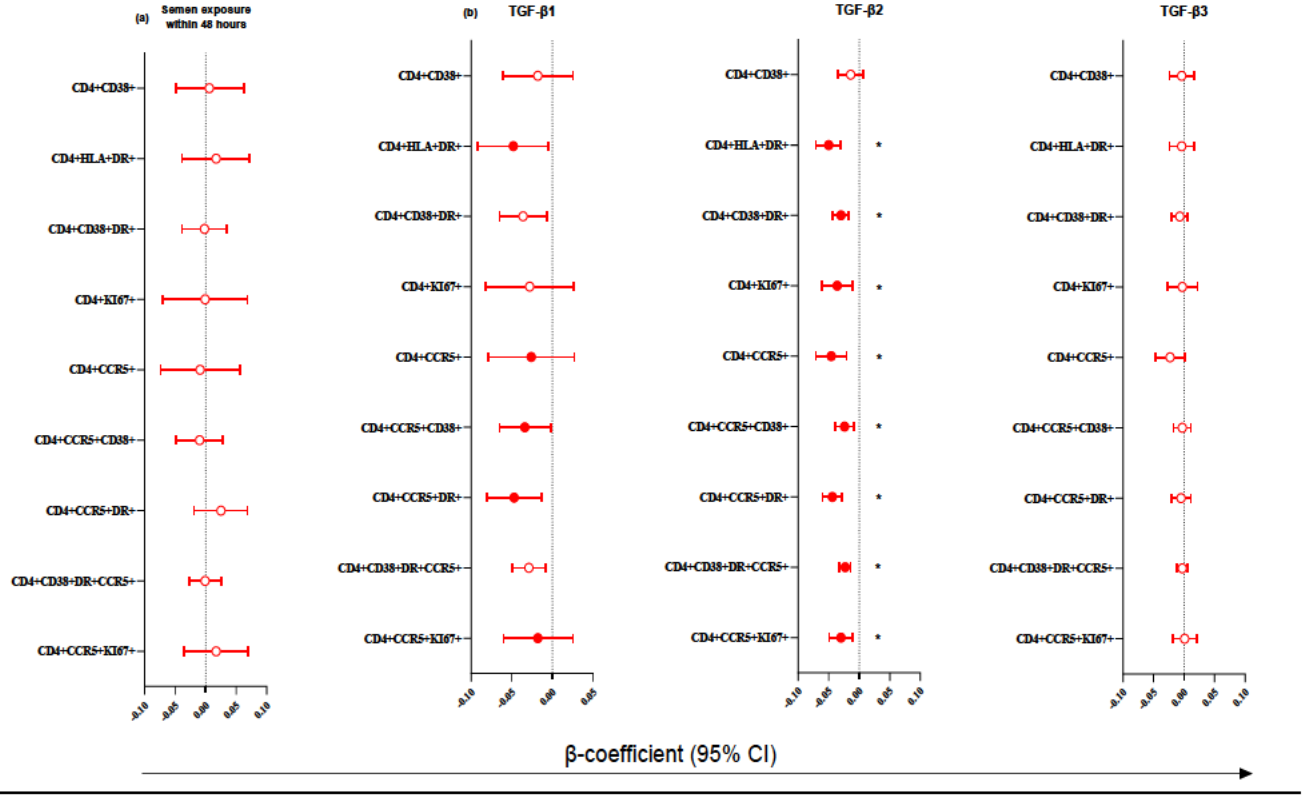


Figure 4.4. Association between immune cell frequencies and TGF-β levels.
(a) Association between semen exposure within 48 hours (PSA) and immune cell frequencies at the FRT. **(b)** Associations between TGF-β isoforms and FRT immune cell frequencies were investigated in cervicovaginal lavage specimens (n=641) by multivariable linear mixed models controlling for participant age, any STI, BV Nugent Score, study arm and time in study. Shapes and error bars depict β-coefficients and confidence intervals. Shaded shapes depict P-values <0.05, and associations maintaining statistical significance after adjustment for multiple comparisons are indicated by black asterisks. The CD4 T cell receptor markers go as follows: immune activation markers (CD38+ or HLA-DR+), cell proliferation marker (Ki67+), and HIV co-receptor (CCR5+).

CHAPTER 5

Discussion

DISCUSSION

In addition to its function of transporting spermatozoa to the FRT, semen contains biological modulators that influence changes in the FRT to facilitate conception (Robertson, 2005). While TGF-β in the FRT is reported to be associated with both pro-inflammatory and immunomodulatory functions, in this study, observations of immunomodulatory functions predominated, with TGF-β isoforms demonstrating differential but all negative associations with several genital cytokines, cellular frequencies, and biomarkers of epithelial barrier integrity. Interestingly, even though it is well established that TGF-β concentrations are high in semen, semen exposure as measured by PSA detection was not associated with the detection or magnitude of TGF-β isoforms, and associations between TGF-β and reduced levels of pro-inflammatory biomarkers at the FRT were observed independently of semen exposure.

In light of the observed relationship between TGF-β and declining pro-inflammatory cytokine concentrations, previous data from this population of women demonstrating an association between semen exposure and significant increases in pro-inflammatory, chemotactic, and growth-related cytokines, $CD4^+$ T cell frequencies, and biomarkers of epithelial compromise (Jewanraj *et al*., 2021b) seem counterintuitive and warrant further investigation. One reason for this apparent contradiction could be that intromitted TGF-β may be short-lived at the cervix (<48 hours), and the related anti-inflammatory effects are insufficient to limit the inflammatory profile associated with HIV risk in this population. Alternatively, this inflammatory profile may reflect the sustained recruitment and support of modulatory Tregs initiated by TGF-β intromission or endogenous production on sex and semen exposure. However, both TGF-β decay and Treg frequencies were not measured in this study and require future investigation to understand better the relationship between sex and semen exposure and the genital immune environment.

TGF-β is a pleiotropic cytokine with a wide variety of functions depending on the isoform, with isoforms differentially involved in wound healing, immune cell function and cell growth differentiation and growth (Huang *et al.*, 2014). TGF-β1 is an important immunoregulatory cytokine responsible for the proliferation and inhibition of T regulatory cells, regulating and maintaining immune tolerance (Yang *et al.*, 2021, Yoshimura *et al.*, 2010). TGF-β2 is involved in the growth and division of cells and is associated with tumour suppression (Lindsay *et al.*, 2012). TGF-β3 is the least documented isoform of the three in human studies, with limited reports describing production in B cells, $CD4^+$, $CD8^+$ and γδ T cells (Okamura *et al.*, 2015) and linking it to the pathogenesis of autoimmune diseases (Okamura *et al.*, 2015). In our study of TGF-β in genital fluid, TGF-β1 was predominantly undetectable, and TGF-

β2 concentrations were higher than TGF-β3 levels. TGF-β is not only expressed in the MRT but is actively expressed in the FRT and modulated by the menstrual cycle in the endometrial and stromal cells. TGF-β, when abnormally elevated in the endometrium, may lead to infertility (Tang *et al*., 2005). Several studies report normal concentrations of TGF-β1 and TGF-β2 at different menstrual cycles in CVLs and serum, presumably related to wound healing and inflammation (Gargiulo et al. (2004) (Jones et al., 2006). (Jones et al., 2006) demonstrated that TGF-β1 and TGF-β2 are upregulated during wound repair, and TGF-β3 is upregulated afterwards. The concentrations of TGF-β1 and TGF-β2 found in the CVL in our study would not have been affected by the different stages of the menstrual cycle since participants were using hormonal contraceptives.

Previous work from our group documented that recent semen exposure was associated with elevated levels of biomarkers of inflammation linked to HIV risk (Jewanraj *et al*., 2021b). Of course, the contribution of intromitted cytokines to this profile was unclear, and further study is needed to identify partner factors that may contribute to this. Despite the established predominance of TGF-β in semen, our work demonstrated that the isoforms are not elevated in women with evidence of semen exposure within two days of sampling. A study by Sharkey *et al.* (2016) observed TGF-β1 as the most prevalent isoform in the seminal plasma, TGF-β3 followed by half the amount of TGF-β1, and the TGF-β2 represented the lowest isoform concentration in seminal plasma (Sharkey *et al.*, 2016). This is in contrast to our observations of the detectability and magnitude of TGF-β concentrations at the FGT, where TGF-β1 was predominantly undetectable in CVL, and TGF-β2 concentrations were higher than TGF-β3 levels, irrespective of PSA status.

The strong negative associations between TGF-β levels and inflammatory biomarkers in this study, irrespective of PSA detection, contradicts reports from *in vitro* studies that show an increase in pro-inflammatory cytokine production from endocervical cells on exposure to TGF-β isoforms (Tremellen *et al*., 1998, Sharkey *et al*., 2012a). Sharkey *et al.* (2012) reported that GM-CSF and IL-6 were induced in the FRT in the presence of all TGF-β isoforms (Sharkey *et al*., 2012a). TGF-β is well known for its immunosuppressive role, and the induction of T regulatory cells plays an important role (Wang *et al.*, 2023). These Tregs suppress various physiological and pathological immune responses; part of this is the suppression of inflammatory cytokines (Sakaguchi *et a*l., 2009). The decrease in immune response may result from immune tolerance in the FRT since semen, particularly TGF-β and prostaglandin, have been associated with inducing immune tolerance. Unfortunately, Treg frequencies or other biomarkers of immune tolerance were not assessed in this study, and further research is required to support this theory.

It is also possible that, *in vivo*, TGF-β decays soon after its introduction to the FRT, very early within the two-day band in which PSA is detectable. *In vitro* studies have demonstrated an elevation of genital cytokines after 8 hours and a decline after 24 hours (Rametse *et al*., 2018, Sharkey *et al*., 2012a), and another showed an increase in TGF-β1 after seminal exposure to the FRT after 2-6 hours and 10–14-hour intervals (Nakra *et al.*, 2016). This may align with the requirement of transient acid exposure for activation (Nocera and Chu, 1995), while the FRT is generally maintained at a low pH. The half-life of activated TGF-β was 100 minutes in an animal study (rats). However, latent TGF-β had a greater half-life, indicating how quickly activated TGF-β is cleared (Wakefield *et al*., 1990). In human studies, TGF-β is shown to generate a short half-life of 2-3 minutes (Gallo-Oller *et al*., 2020), which speaks to how quickly TGF-β is cleared.

The strong negative associations between TGF-β levels and inflammatory biomarkers in this study, irrespective of PSA detection, attest to the immunomodulatory function of TGF-β in the FRT (Agarwal *et al.*, 1994, Worthington *et al.*, 2012, Yoshimura *et al.*, 2010). TGF-β1 and TGF-β3 were associated with a reduction in multiple FRT cytokines. TGF-β2 was only associated with a reduction in MCP-1, a chemotactic cytokine responsible for the regulation of infiltration and migration of macrophages/monocytes, natural killer cells and memory T lymphocytes (Yoshimura *et al.*, 1989, Deshmane *et al.*, 2009). A reason for the differential relationships between TGF-β isoforms and other cytokine concentrations could be related to receptor-binding differences between the TGF-β isoforms. TGF-β1 and TGF-β3 bind to the same TGF-β receptor (TβR) II that is involved in phosphorylating TβRI, but TGF-β2 binds weakly to TβRII. TGF-β2 binds to betaglycan (TGF-β co-receptor), which assists the isoform in binding to TβRII at a higher affinity (Villarreal *et al.*, 2016). Cells that fail to express betaglycan lack the robust response they offer for TGF-β1 and TGF-β3 (Villarreal *et al*., 2016).

A few limitations are noted in this study. Firstly, there was no way to differentiate between endogenous TGF-β in the FRT or intromitted TGF-β from the male reproductive tract (MRT). Further studies are required to fully comprehend the contribution of semen-associated TGF-β to the immune profile of the FRT. Secondly, given that the level of TGF-β in semen varies depending on several circumstances, including concurrent HIV/STI, the matching seminal sample from the partners of the recruited study participants could have provided additional insight. Thirdly, the lack of association between seminal biomarkers (PSA) and TGF-β levels in women suggests that the 48hr range of PSA detection was inadequate for the study's purposes, and further studies are required to effectively time and track the activation and waning of TGF-β activity in the FRT. Fourthly, the CVL's dilution factor was unknown and could not be adjusted for. Further, the lack of Treg profiling in the FRT limited the examination of TGF-β-activated Tregs and immune tolerance. Taken together, the study describes the differential genital expression of TGF-β isoforms in young South African women, confirms their relation to local cytokine concentrations and cellular frequencies, and details further avenues of research required to

better define the contribution of sex, semen exposure and other partner factors the immune environment associated with HIV risk in young women in South Africa.

CHAPTER 6

Conclusion

CONCLUSION

In conclusion, although TGF-β isoforms were differentially expressed in the FRT and differed in their individual associations with local cytokine concentrations and cellular frequencies, their general relationship with reduced levels of genital cytokines and immune cells attests to their documented immunomodulatory effects. TGF-β concentrations were not associated with PSA detection, which likely indicates a normalisation of TGF-β levels in genital fluid within 48 hours after intromission. Although TGF-β concentrations were independently associated with dampening local cellular and cytokine levels, the previously observed relationship between semen exposure and increased levels of inflammatory biomarkers was maintained in the cohort. Further interrogation is required to determine the dynamics of intromitted or endogenous TGF-β and inflammatory biomarkers, the persistence of their immune impact, and the relation to HIV risk.

CHAPTER 7

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