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The Impact of Semen Exposure on the Immune and Microbial Environments of the Female Genital Tract

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211502126

Submitted in fulfilment of the requirements for the degree of ***Doctor of Philosophy (Medicine)*** in the School of Laboratory Medicine and Medical Science, Department of Medical Microbiology, University of KwaZulu-Natal, Durban, South Africa.

Supervisor: **Dr Lenine Liebenberg**

Co-Supervisor: **Dr Sinaye Ngcapu**

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PREFACE

The experimental work described in this thesis was carried out 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 (UKZN), from January 2016 to December 2019, under the supervision of Dr Lenine JP Liebenberg, PhD, and the co-supervision of Dr Sinaye Ngcapu, PhD.

This study represents the original work conducted by the author and has not otherwise been submitted in any form to any other tertiary institution.

DECLARATION 1 – Author Contributions

I, Janine Jewanraj, declare as follows:

1. That the work described in this thesis has not been submitted to UKZN or any other tertiary institution for the purposes of obtaining an academic qualification, whether by myself or any other party.

2. That my contribution to the project was as follows:

   I participated in the conception and design of the studies. I conducted the laboratory assays to detect the prostate-specific antigen (n=651) in cervicovaginal lavage supernatants using enzyme-linked immunosorbent assays (ELISA). I performed deoxyribonucleic acid (DNA) extractions on cervicovaginal lavage pellet specimens using the QIAGEN DNeasy Extraction Kit and detected Y-chromosome DNA (n=651) in these using real-time polymerase chain reactions. I measured the levels of 48 cytokines (n=650), 9 matrix metalloproteinases (n=144), and 4 tissue inhibitors of metalloproteinases (n=144) in cervicovaginal lavage supernatant specimens by multiplexed ELISA. I analysed and interpreted the data, which a statistician subsequently validated. I wrote the manuscripts, which were reviewed by my supervisor's Dr Liebenberg and Dr Ngcapu, and approved by all co-authors.

Signed: _  __ Date: 1 March 2021

DECLARATION 2 - Plagiarism

I, Janine Jewanraj, declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research.

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

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

4. This thesis does not contain other persons' writing unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
    a. Their words have been re-written, but the general information attributed to them has been referenced.
    b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks and referenced.

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

Signed: [redacted] Date: 4 June 2021

Supervisor's Declaration

I hereby declare that I have read and approved this thesis for submission and believe its contents to be the original work of the candidate, with other work adequately referenced.

Signed: [redacted] Date: 4 June 2021

Signed [redacted] Date: 4 June 2021

DECLARATION 3 – Manuscripts and Publications

This thesis has been submitted to fulfil the requirements for thesis submission by manuscript, which may have at least three papers with the student as the prime author, and at least two of the papers must constitute original research. This thesis includes one published paper and two accepted manuscripts to meet the UKZN PhD requirements for submission by manuscript. The work presented here originated from the CAPRISA 008 tenofovir gel open-label extension trial. The study protocol for the CAPRISA 008 trial can be accessed online at www.caprisa.org/Pages/CAPRISAStudies. Listed below are the manuscripts and publications emanating from this study and the contributions made by all authors to each of the manuscripts.

Manuscript 1:

Jewanraj J, Ngcapu S, and Liebenberg LJP. Semen: A modulator of female genital inflammation and a vector for HIV-1 transmission. *American Journal of Reproductive Immunology; Accepted* (Manuscript ID: AJRI-02-21-043.R1).

Author Contributions:

J.J. conceptualized and wrote the review article. S.N. and L.J.P.L. critically reviewed and revised the manuscript. All authors approved the final submitted manuscript.

Publication 1:

Jewanraj J, Ngcapu S, Osman F, Mtshali A, Singh R, Mansoor LE, Abdool Karim SS, Abdool Karim Q, Passmore JS, and Liebenberg LJP. The impact of semen exposure on the immune and microbial environments of the female genital tract. *Frontiers in Reproductive Health*. 2020;2(8).

Author Contributions:

J.J., S.N., and L.J.P.L. contributed to the conception and design of the study. J.J., L.J.P.L., A.M., and R.S. performed the experiments. J.J., L.J.P.L., and F.O. analysed and interpreted the data. J.J. and L.J.P.L. wrote the manuscript. All authors proofread and approved the final manuscript.

Manuscript 2:

Jewanraj J, Ngcapu S, Osman F, Ramsuran V, Fish M, Mtshali A, Singh R, Mansoor LE, Abdool Karim SS, Abdool Karim Q, Passmore JS, and Liebenberg LJP. Transient association between semen exposure and biomarkers of genital inflammation in South African women at risk of HIV infection. *Journal of the International AIDS Society; Accepted* (Manuscript ID: JIAS-2021-01-0008.R1).

Author Contributions:

J.J., S.N., and L.J.P.L. contributed to the conception and design of the study. J.J., L.J.P.L., A.M., M.F., and R.S. performed the experiments. J.J., L.J.P.L., and F.O. analysed and interpreted the data. J.J. and L.J.P.L. wrote the manuscript. All authors proofread and approved the final manuscript.

Signed: _____ Date: 1 March 2021

CONFERENCE PRESENTATIONS

Oral Presentations

1. **Jewanraj J**, Ngcapu S, Mtshali A, Mansoor LE, Abdool Karim SS, Abdool Karim Q, Passmore JS, and Liebenberg LJP. Impact of semen on the immune and microbial environments of the female genital tract. VIIth Conference of the South African Immunology Society, 17-20 June 2019, Durban, South Africa.
2. **Jewanraj J**, Ngcapu S, Mtshali A, Osman F, Abdool Karim SS, Abdool Karim Q, Passmore JS, and Liebenberg LJP*. Recent semen exposure is associated with elevated genital cytokine concentrations and dysbiosis of the female genital microbiota. Connecting Minds Africa, 25-27 September 2019, Nairobi, Kenya. *Presented by Liebenberg LJP
3. **Jewanraj J**, Ngcapu S, Mtshali A, Osman F, Abdool Karim SS, Abdool Karim Q, Passmore JS, and Liebenberg LJP*. Impact of semen on the immune and microbial environments of the female genital tract. SANTHE Annual Research Consortium, 30 September - 4 October 2019, Nairobi, Kenya. *Presented by Liebenberg LJP

Poster Discussion Presentations

1. **Jewanraj J**, Ngcapu S, Ramsuran V, Fish M, Mtshali A, Mansoor LE, Abdool Karim SS, Abdool Karim Q, Passmore JS, and Liebenberg LJP. High Y-chromosome DNA concentrations are associated with increased cervical cytokine concentrations and activated cervical HIV target cell frequencies. 23rd Virtual International AIDS Conference, 6-10 July 2020.
2. **Jewanraj J**, Ngcapu S, Ramsuran V, Mtshali A, Mansoor LE, Abdool Karim SS, Abdool Karim Q, Passmore JS, and Liebenberg LJP. Y-chromosome DNA concentrations in cervicovaginal fluid are associated with biomarkers of inflammation and increased frequencies of cervical HIV target cells. Immunology 2020, 8-12 May 2020. *Accepted, but the conference was cancelled due to COVID-19.

High Y-chromosomal DNA concentrations are associated with increased cervical cytokine concentrations and activated HIV target cell frequencies

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Introduction

- Semen is the primary vector for HIV transmission to women during condomless sex.
- Semen induces cytokine production and immune cell recruitment at the female genital tract (FGT) to facilitate conception (Sharkey *et al.*, 2012; Robertson,2005).
- Since genital inflammation increases HIV risk in women (Masson *et al.*, 2015; Arnold *et al.*, 2016; Gosmann *et al.*, 2017; Anahtar *et al.*, 2015), semen-induced alterations at the FGT may have implications for HIV risk.
- We investigated the contribution of semen exposure [as measured by Y-chromosome DNA (YcDNA) detection and quantification] on biomarkers of inflammation associated with HIV acquisition.

Methods

- Genital specimens were collected from 152 HIV-negative women over time (n=651 specimens).
- YcDNA was detected using the Human Y-chromosome DNA detection kit and quantified using the Quantifiler Trio DNA quantification kit in cervicovaginal lavage pellet specimens.
- YcDNA concentrations were compared with matched concentrations of 48 cytokines, and epithelial barrier function proteins (MMPs/TIMPs) by multiplexed ELISA, and with frequencies of cervix-derived HIV T cell targets determined by flow cytometry.
- Linear mixed models were used to assess the impact of semen exposure on biomarkers of inflammation over time and were adjusted for other variables related to inflammation or HIV risk (age, STI, Nugent Score, time, randomization arm, and inflammation status).

Higher cervicovaginal YcDNA concentrations were associated with:

Higher concentrations of proinflammatory, chemotactic, growth, and response-related cytokines

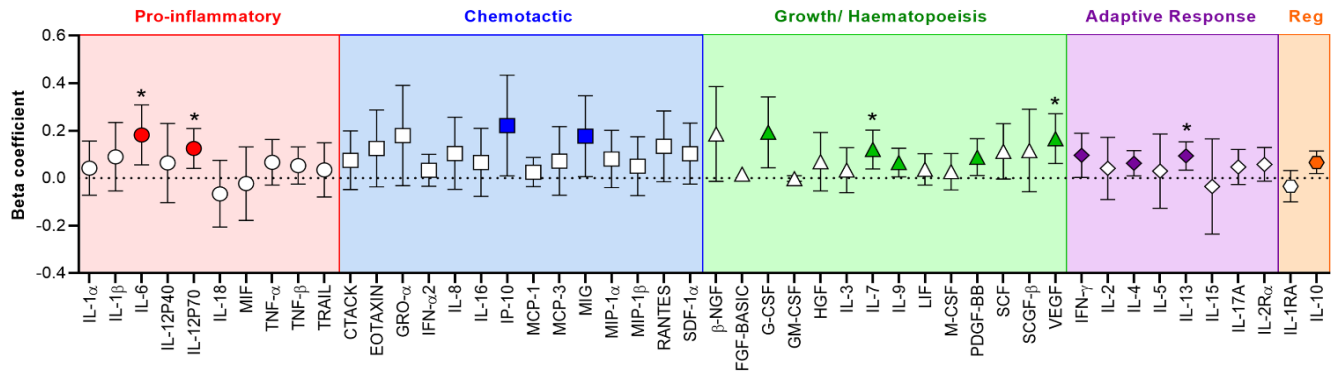

FIGURE 1.

Significant P-values (P<0.05) are indicated by shading and significance after FDR adjustment is indicated by (*). Multivariable linear mixed models adjusted for age, any STI, Nugent Score, randomization arm, and time in study

Higher endocervical CD4 T cell frequencies

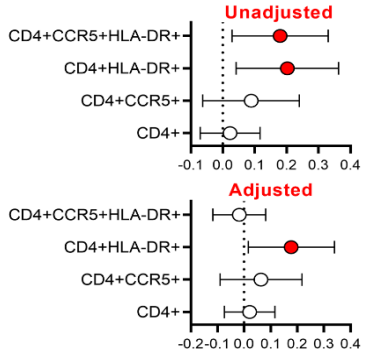

FIGURE 2.

Significant P-values (P<0.05) are indicated by shading. Model 1 adjusted for study arm and time. Model 2 adjusted additionally for age, STIs, Nugent Score, and inflammation.

Higher concentrations of genital tract Matrix Metalloproteinases and their inhibitors

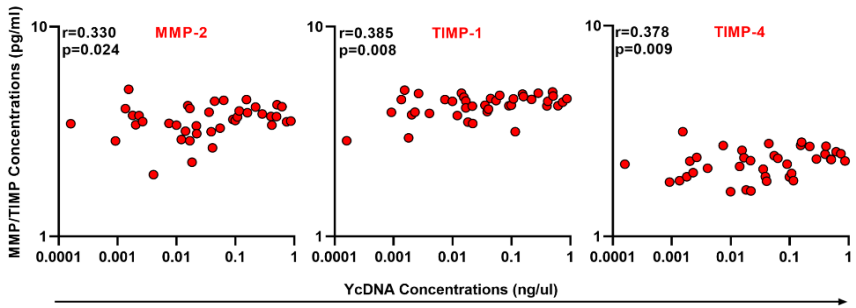

FIGURE 3.

Spearman Rank test P<0.05 was considered significant

Results

- YcDNA detection was associated with marital status (p=0.038), number of pregnancies (p=0.042), frequency of vaginal sex (p=0.008), cohabitation (p=0.027), gonorrhea (p=0.009), and Nugent Score (p=0.006, data not shown).
- A total of 167/218 (77%) genital specimens with detectable YcDNA had a yield sufficient for quantitation.
- In multivariable linear mixed model analyses, higher YcDNA concentrations were associated with elevated concentrations of 13/48 cytokines (**Figure 1**), increased frequencies of activated endocervical CD4 T cells (CD4+HLA-DR+, **Figure 2**); and increased MMP-2, TIMP-1, and TIMP-4 concentrations (**Figure 3**).

Conclusion

- Higher YcDNA concentrations were associated with raised levels of the biomarkers of inflammation assessed: proinflammatory/chemotactic cytokines, MMPs/TIMPs, and activated endocervical CD4 T cell frequencies
- Because genital inflammation increases HIV risk in women, semen-induced alterations at the FGT may have implications for HIV susceptibility in women.

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PRESENTED AT THE 23RD INTERNATIONAL AIDS CONFERENCE (AIDS 2020) | 6-10 JULY 2020

ACKNOWLEDGEMENTS:

ETHICAL APPROVAL

All participants of the CAPRISA 008 trial provided informed consent for the storage of their genital specimens for use in future studies (BFC237/010). This study was approved by the Biomedical Research Ethics Committee at the University of KwaZulu-Natal under the ethics number BE258/19 (**Appendix B**).

DEDICATION

*This thesis is dedicated to my beloved grandmother, the late **Cynthia Govender**. Even though she did not have a formal education, she never stopped sharing her bountiful wisdom, support, and encouragement to study.*

ACKNOWLEDGEMENTS

"*And whatever you do, whether in word or deed, do it all in the name of the Lord Jesus, giving thanks to God the Father through him.*" (***Colossians 3:17****)*

- First and foremost, all glory and honour goes to **God almighty**, who has granted me the knowledge, wisdom, and perseverance to complete this thesis to the best of my ability. These have been both the best and the toughest years of my life, and without his favour and grace, none of this would be possible.

- I wish to express my sincere gratitude to my PhD SUPERvisor **Dr Lenine Liebenberg**. I consider myself extremely fortunate to have had Dr Liebenberg as my mentor and would like to thank her for the constant support, guidance, and encouragement. Completing this thesis would have been impossible without your valuable insights and intellectual contribution. Your enthusiasm and passion for science have inspired me both personally and professionally.

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- To my fiancé, **Nolan**, thank you for your enduring love and believing in me even when I did not believe in myself. I am eternally grateful for your support throughout my academic career.

- A special thanks goes to all **the women that participated in CAPRISA 008 trial** and enabled this research to be conducted.

- Finally, I would like to thank the **DSI-NRF Centre of Excellence** in HIV Prevention for their financial support that enables and encourages young researchers such as myself to pursue a post-graduate degree.

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
A. vaginae	*Atopobium vaginae*
BV	Bacterial vaginosis
BVAB-2	Bacterial vaginosis-associated bacterium 2
β-NGF	Beta nerve growth factor
CAPRISA	Centre for the AIDS Programme of Research in South Africa
CMCs	Cervical mononuclear cells
CVL	Cervicovaginal lavage
CT	*Chlamydia trachomatis*
CI	Confidence interval
CTACK	Cutaneous T cell attracting chemokine
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
Eotaxin	Eosinophil chemotactic protein
PGE	E-series prostaglandins
FDR	False discovery rate
FGT	Female genital tract
FGF-basic	Fibroblast growth factor basic
G. vaginalis	*Gardnerella vaginalis*
GEE	Generalized estimating equation
GI	Genital inflammation
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor

GRO-α	Growth-related oncogene alpha
HGF	Hepatocyte growth factor
HSV	Herpes simplex virus
HIV	Human immunodeficiency virus
IFN	Interferon
IP-10	Interferon gamma-induced protein-10
IL	Interleukin
IL-1RA	Interleukin-1 receptor antagonist
IL-2Rα	Interleukin-2 receptor alpha
IQR	Interquartile range
L. crispatus	*Lactobacillus crispatus*
L. jensenii	*Lactobacillus jensenii*
LIF	Leukaemia inhibitory factor
LRT	Lower reproductive tract
M-CSF	Macrophage colony-stimulating factor
MIP	Macrophage inflammatory protein
MIF	Macrophage migration inhibitory factor
MMPs	Matrix metalloproteinases
Megasphaera 1	*Megasphaera type 1*
MCP	Monocyte chemotactic protein
MIG	Monokine induced by gamma interferon
MG	*Mycoplasma genitalium*
NG	*Neisseria gonorrhoeae*
NF-κB	Nuclear factor kappa B
OR	Odds ratio
PDGF-BB	Platelet-derived growth factor-BB

PCR	Polymerase chain reaction
PrEP	Pre-exposure prophylaxis
P. bivia	*Prevotella bivia*
PTGS2	Prostaglandin-endoperoxidase synthase-2
PSA	Prostate-specific antigen
RT-PCR	real-time PCR
ROC curve	Receiver operating characteristic curve
RANTES	Regulated on activation, normal T cell expressed and secreted
Treg cell	Regulatory T cell
SP	Seminal plasma
STIs	Sexually transmitted infections
SCF	Stem cell factor
SCGF-β	Stem cell growth factor-beta
SDF-1α	Stromal cell-derived factor-1 alpha
Th cells	T helper cells
TSPY	Testis-specific protein Y-encoded
TIMPs	Tissue inhibitors of metalloproteinases
TRAIL	TNF-related apoptosis-inducing ligand
TGF-β	Transforming growth factor-β
TV	*Trichomonas vaginalis*
TNF	Tumour necrosis factor
UKZN	University of KwaZulu-Natal
VEGF	Vascular endothelial growth factor
YcDNA	Y-chromosome DNA

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THESIS CONTEXT

This thesis is comprised of four chapters. **Chapter 1** provides the background and rationale for the studies presented in the subsequent chapters. This chapter includes a comprehensive literature review that discusses the role of semen in modulating female genital inflammation and the related implications for Human Immunodeficiency Virus (HIV) susceptibility in women. The literature review is presented as manuscript number 1 and has been accepted for publication in the American Journal of Reproductive Immunology. This is followed by an overview of the thesis aims and objectives.

Chapter 2 challenged the use of self-reported condom use to gauge vaginal exposure to semen and highlights the need to screen for semen biomarkers in studies of genital mucosal immunity to HIV and other sexually transmitted infections (STIs). Briefly, this study showed that semen exposure measured by Y-chromosome DNA (YcDNA) detection and not with self-reported condom use was related to alterations in the microenvironments of the female genital tract. However, the detection of YcDNA correlated more with barrier-related proteins and bacterial vaginosis-associated bacteria than with cytokines and immune cells commonly linked to increased genital inflammation and HIV acquisition in women. This original research article is publication number 1 and has been published in Frontiers in Reproductive Health: HIV and STIs.

Chapter 3 further describes the impact of more recent semen exposure on biomarkers of female genital inflammation and the persistence of these associations over time. Here, recent semen exposure was detected using a combination of semen biomarkers, including prostate-specific antigen (PSA) detection and the detection and quantification of YcDNA to model the timing of semen exposure at the female genital tract. This study demonstrated that higher YcDNA concentrations and PSA detection, both indicative of recent semen exposure, were associated with a pro-inflammatory response at the female genital mucosa. These semen-associated immune and microbial alterations were shown to wane and equilibrate over time. Although transient, semen-induced inflammation at the female genital tract may significantly impact the risk of HIV infection in high-risk women. This original research article is manuscript number 2 and has been accepted for publication in the Journal of the International AIDS Society.

Chapter 4 summarizes and discusses the major findings of this thesis. It highlights the overall study significance, strengths, and limitations and provides recommendations for future research.

ABSTRACT

Background: Semen is an immunomodulatory fluid that induces mucosal changes at the female genital tract (FGT) for sperm survival and conception. Semen-induced alterations necessary for reproduction may also modulate the inflammatory environment related to HIV risk in women. This thesis investigated the impact of semen exposure on biomarkers of female genital inflammation (GI) and the persistence of these associations over time.

Methods: Stored genital specimens were assessed from HIV-negative women participating in the CAPRISA 008 trial. Cervicovaginal lavage (CVL) samples were screened for Y-chromosome DNA (YcDNA) by real-time PCR as a biomarker of semen exposure within 15 days of genital sampling. Prostate-specific antigen (PSA) detection by ELISA stratified CVLs into semen exposure within 48 hours (PSA+YcDNA+) and between 3-15 days (PSA-YcDNA+). Vaginal cytokine concentrations, matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs) were assessed in CVLs using multiplexed ELISA. Endocervical T-cell frequencies were measured in cytobrushes by flow-cytometry. Vaginal microbes and sexually transmitted infections (STIs) were detected in vulvovaginal swabs by PCR.

Results: Self-reported condom use as a measure of semen exposure was not associated with changes in the FGT microenvironments. Conversely, YcDNA detection predicted significant increases in several cytokines, barrier-related proteins, and *Prevotella bivia* detection (p=0.001). Since YcDNA detection alone was not associated with the immune environment linked to HIV risk, this thesis further investigated the contribution of more recent sex to female GI. PSA detection (semen exposure within 48 hours) was associated with higher YcDNA concentrations (p<0.0001), suggesting a relationship between the timing of semen exposure and vaginal YcDNA concentrations after condomless sex. In support of this, both PSA detection and higher YcDNA concentrations predicted significant increases in several cytokines, barrier-related proteins (MMP-2, TIMP-1, TIMP-4), and higher frequencies of activated CD4+HLA-DR+ T-cells (p=0.032) and CD4+CCR5+HLA-DR+ HIV targets (p=0.046). PSA detection was also associated with increased detection of several bacterial vaginosis (BV)-associated microbes and reduced *Lactobacillus jensenii* detection.

Conclusion: Recent semen exposure contributes to the inflammatory environment associated with HIV risk in women. These studies highlight the need for clinical and immunological studies of STIs and their biomedical interventions to consider semen's contribution to the immune and microbial microenvironments of the FGT.

CHAPTER 1

INTRODUCTION

1.1. Background and Rationale

1.1.1. HIV epidemiology

Although significant strides have been made in treating Human Immunodeficiency Virus (HIV), there is still no cure or effective vaccine against the virus. Annual HIV incidence rates remain unacceptably high, with 1.7 million (1.2 million - 2.2 million) new global HIV infections in 2019 alone (1). Eastern and Southern Africa accounted for approximately 43% (730 000) of all new HIV infections worldwide (1). South Africa, in particular, has the largest HIV seropositive population in the world, with an estimated 7.5 million (6.9 million - 8 million) people living with HIV, approximately 200 000 incident HIV infections, and 72 000 Acquired Immunodeficiency Syndrome (AIDS)-related deaths in 2019 (2). Most infections occur via heterosexual transmission, and women are disproportionately affected by HIV compared to their male counterparts. In high burden areas, young women and girls under 25 years are eight times more likely to be living with HIV than males of the same age (3, 4). Increasing prevention efforts among key target populations, such as young women in sub-Saharan Africa, may substantially reduce HIV transmission rates and help achieve epidemic control.

1.1.2. Factors influencing HIV susceptibility in women

Factors contributing to the higher HIV prevalence observed among women in sub-Saharan Africa include, among others, poverty, gender inequality, gender-based violence, age-disparate relationships with an older male, vaginal douching, STIs, genital inflammation, vaginal microbial diversity, and other biological factors (5-12). Biologically, the surface of the cervicovaginal mucosa is considerably larger than that of the penis and foreskin in men, suggesting greater surface area and increased chances for HIV infection in women (13). Furthermore, since semen and its components are deposited in the vagina during condomless sex and can remain there for up to 15 days, there is an increased potential for exposure to HIV at the FGT (13, 14). Younger women are particularly vulnerable to HIV infection as they are more likely to sustain vaginal microabrasions during condomless sex than older women (15). Additionally, cervical ectopy, the extension of the less resilient single-celled columnar epithelium to the ectocervix, has also been identified as a contributor to HIV susceptibility in younger women (16, 17).

1.1.3. Host defence mechanisms at the female genital tract

Despite the related biological risk factors for HIV infection, the mucosal linings of the FGT also have several defence mechanisms to protect against STIs and other pathogenic microorganisms. During condomless sex, semen is deposited in the lower reproductive tract (LRT), consisting of the vagina and

ectocervix (18-22). The LRT is lined by multiple stratified squamous epithelium layers to provide an impenetrable barrier against invading viruses and bacteria (18-22). Cervicovaginal mucus coats the LRT and acts as a physical barrier to trap pathogens and prevent them from ascending to the upper reproductive tract (23-25). Antimicrobial factors present within the cervicovaginal mucus provide an additional chemical barrier against unwanted pathogens (23-25). The vaginal epithelium is also lined with microbes that contribute to the host defences at the FGT. Commensal bacteria such as *Lactobacillus crispatus* produce lactic acid to acidify the vaginal environment (26-28). These microbes inhibit colonisation by pathogens by lowering the vaginal pH and through competition for nutrients and space (29, 30). Additionally, leukocytes in the female reproductive tract secrete cytokines and recruit other immune cells to the infection site for pathogen clearance (31, 32).

1.1.4. Genital inflammation contributes to HIV susceptibility in women

GI is a natural host immune response involving the upregulation of inflammatory cytokines and immune cell movement to the female genital mucosa for wound healing or protection from infection. However, GI has also been shown to undermine the host defence mechanisms against HIV and increase infection risk in women (10, 33). GI involves the influx of immune cells to the infection site, which may inadvertently increase the availability of HIV susceptible cells at the FGT (34). Studies have defined inflammation by an elevation of pro-inflammatory and chemotactic cervicovaginal cytokines (10, 34, 35). Cervicovaginal cytokines also modulate mucosal barrier function and immune cell recruitment, both of which contribute to the availability of HIV target cells at the FGT (34). Increased microbial diversity and low lactobacillus abundance are also related to increased GI and immune cell infiltration at the female genital mucosa (11, 12). Vaginal microbial diversity increases HIV risk by four-fold in South African women (11). Anahtar *et al*. demonstrated that pathogenic microbes contribute to GI via activation of the nuclear factor kappa B (NF-κB) pathway and lymphocyte recruitment induced by elevated chemokines (12). Specific bacterial taxa (*Prevotella*, *Sneathia*, and other anaerobic bacteria) are also associated with increased mucosal CD4+ T cell numbers in mice (11). GI has additional implications for HIV susceptibility through its association with reduced topical pre-exposure prophylaxis (PrEP) efficacy in women (35). Identifying risky behaviours and factors that influence female GI may help limit their impact on HIV risk. This may also lead to the development of effective biomedical interventions to prevent new HIV infections in women and ultimately curb the HIV pandemic.

1.1.5. Semen exposure may promote female genital inflammation

Semen is primarily studied for its physiological role in reproduction as a delivery system for spermatozoa. It contains several highly immunomodulatory components that interact with the female reproductive tissues to increase the likelihood of pregnancy (36-38). Notably, semen is also the main

vector for HIV transmission to women during coitus (39) and induces alterations in the FGT microenvironments that may influence GI and HIV risk in women. In reproduction, semen contact results in an inflammatory response involving cytokine production [including, among others, interleukin (IL)-1α, IL-6, IL-8, tumour necrosis factor (TNF)-β, monocyte chemoattractant protein (MCP)-1, and granulocyte-macrophage colony-stimulating factor (GM-CSF)] in female tissues (40-44). This upregulation of mucosal cytokines results in leukocyte infiltration for the removal of excess spermatozoa (42). Additionally, bacterial communities present in semen and the foreskin are transferred to the FGT during condomless sex (45-49) and may cause changes in the vaginal microbiome composition. In mammals, rapid neutrophilic leukocytosis occurs in the female reproductive tract to promote microbial homeostasis and clearance of unwanted microorganisms (50, 51). Other semen components may also modulate mucosal barrier integrity and topical PrEP efficacy in women (52-57).

Since excess inflammation may diminish the chances of reproductive success, expansion of the regulatory T cell (Treg) population is induced to facilitate fertilization of the oocyte and embryo implantation (37, 38, 58-60). Semen also contains anti-inflammatory factors, including transforming growth factor (TGF)-β, IL-10, and prostaglandins, which promote a Treg immune response at the FGT for tolerance to the paternal antigens (37, 38, 58-60). These studies demonstrate that semen contains factors that can either promote or inhibit inflammation of the female genital mucosa. Semen appears to induce alterations in the microenvironments of the FGT that are also linked to increased GI and HIV acquisition in women. This implies that although heterosexual HIV transmission rates are relatively low (61-63), semen exposure during condomless sex could contribute to an immune and microbial environment conducive to HIV infection in women. Alternatively, a semen-induced anti-inflammatory response for tolerance to the paternal alloantigen and to facilitate reproduction may also inhibit the clearance of STIs, including HIV at the FGT. These contradictory effects of semen on female genital tissues suggest that more studies are needed to determine semen's impact on the microenvironments of the FGT, particularly in the context of HIV.

1.1.6. Biomarkers of semen exposure within vaginal specimens

Assessments of vaginal exposure to semen rely greatly on self-reported condom use. However, self-reports of condom use and sexual behaviour are fraught with inaccuracies and often lead to the misinterpretation of data (64-66). Routine screening for semen biomarkers in vaginal specimens is necessary for clinical and immunological studies of STIs and their biomedical interventions to assess condom use and semen exposure at the FGT reliably. Biomarkers of semen exposure can be divided into two main categories: biomarkers that detect seminal plasma and those that detect spermatozoa and other cells within semen (67). PSA and YcDNA detection are two of the most well-studied and reliable biomarkers of semen exposure. PSA is a biomarker of exposure to seminal plasma and, therefore, can detect semen exposure from vasectomized men or even those with low sperm counts (68-73). The decay

rate of PSA is relatively quick, and detection in vaginal specimens is usually indicative of semen exposure within 48 hours (68-73). Given its short residence time, PSA detection may be ideal to characterise transient immune alterations at the female genital mucosa. Conversely, YcDNA is a more stable biomarker of semen exposure and can be detected in vaginal specimens up to 15 days after exposure to semen (14, 74, 75). Since YcDNA is detectable in the presence of spermatozoa and for a longer duration, it is useful to determine the likelihood of pregnancy and whether condomless sex occurred within a period longer than two days of sampling, e.g., during clinical trials advising the use of condoms or abstinence (68, 75). Additionally, since YcDNA is detected in the presence of sperm, YcDNA quantities in vaginal specimens could be used as an indicator of male protein concentrations and sperm count and to assess their relative impact on female GI. Furthermore, screening for PSA and YcDNA biomarkers in combination may help describe the persistence of semen-associated alterations at the female genital mucosa.

Here, using biomarkers of semen exposure, this thesis investigated the role of semen in modulating female GI and the related implications for women at high risk of acquiring HIV. These studies contribute to the existing literature on factors that influence GI and HIV risk in women and provide a biological link for the association between condomless sex and increased HIV acquisition. Efforts to develop effective drug-based pre- and post-exposure prophylaxis and an effective HIV vaccine must be accompanied by a better understanding of factors influencing GI. Therefore, the work presented here may facilitate the design of targeted approaches to prevent the further spread of HIV among women.

1.2. Literature review – Manuscript 1

In accordance with the UKZN guidelines, the literature review component of chapter 1 has been drafted for publication to meet the PhD requirements. The manuscript has been accepted for publication in the American Journal of Reproductive Immunology (Manuscript ID: AJRI-02-21-043.R1).

Semen: A modulator of female genital tract inflammation and a vector for HIV-1 transmission

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Abstract

In order to establish productive infection in women, HIV must transverse the vaginal epithelium and gain access to local target cells. Genital inflammation contributes to the availability of HIV susceptible cells at the female genital mucosa and is associated with higher HIV transmission rates in women. Factors that contribute to genital inflammation may subsequently increase the risk of HIV infection in women. Semen is a highly immunomodulatory fluid containing several bioactive molecules with the potential to influence inflammation and immune activation at the female genital tract. In addition to its role as a vector for HIV transmission, semen induces profound mucosal changes to prime the female reproductive tract for conception. Still, most studies of mucosal immunity are conducted in the absence of semen or without considering its immune impact on the female genital tract. This review discusses the various mechanisms by which semen exposure may influence female genital inflammation and highlights the importance of routine screening for semen biomarkers in vaginal specimens to account for its impact on genital inflammation.

Keywords: semen, HIV risk, female genital inflammation, cytokines, vaginal microbiome, immune cells, epithelial barrier integrity

1. Introduction

Despite the advances made in the treatment of Human Immunodeficiency Virus (HIV), the global HIV prevalence remains unacceptably high [1]. The primary determinants of HIV transmission include the accessibility of target cells for infection and viral characteristics such as quantity and fitness. Female genital inflammation contributes to both the availability of HIV target cells and reduced mucosal barrier integrity [2,3]. Genital inflammation, defined by elevated pro-inflammatory and chemotactic cytokines, has also been linked to a three-fold greater risk of acquiring HIV in women [2]. Additionally, microbial dysbiosis contributes to inflammation through increased cytokine production, mucosal barrier disruption, and immune cell recruitment at the female genital tract (FGT) [4-7]. These studies emphasize the role of genital inflammation in HIV acquisition in women and highlight the need to determine factors that contribute to genital inflammation, and then limit their relative impact on HIV risk.

The immune altering capacity of semen is often overlooked in heterosexual HIV transmission and semen is merely considered a vehicle for viral transmission to women during condomless sex [8,9]. Semen induces mucosal changes at the FGT to increase the chances of pregnancy [10-14], and also contains several immunologically active molecules known to both promote and inhibit female genital inflammation [10-13,15-22]. Initially, the presence of semen in the female reproductive tract results in an inflammatory response involving cytokine production and leukocyte recruitment for the removal of excess and abnormal sperm [10,11,20,21]. The alkaline pH of semen and the microbial content of the ejaculate also contribute to alterations in the vaginal microbiome which are known to promote genital inflammation and HIV risk in women [4,5,7,23-28]. A semen-induced pro-inflammatory immune response to prime the female reproductive tract for conception may also promote genital inflammation and HIV acquisition in women [11,20-22].

Conversely, semen also contains factors to help regulate this pro-inflammatory response at the FGT since excessive inflammation may lead to adverse pregnancy outcomes. This results in the induction of a regulatory T cell (Treg) immune response for tolerance to the paternal antigens and to facilitate embryo implantation [16,29-32]. A semen-induced tolerogenic immune response may also inhibit the clearance of HIV and other pathogens at the FGT. Taken together, these studies suggest that semen directly alters the biology of the FGT and may have significant consequences for the risk of HIV infection in women. Here we review the relationship between female genital immunity and male partner semen and its implications for HIV risk in women.

2. Host immune defences to prevent HIV infection at the female genital mucosa

2.1. Innate immune responses at the female genital mucosa

2.1.1. Role of the vaginal epithelium in innate immune defence

During male to female HIV-1 transmission, viral particles present in semen must transverse the vaginal mucus and epithelium to access local cellular targets for infection. However, the FGT has several innate and adaptive immune responses that defend against HIV infection. The innate immune system involves a rapid and non-specific immune response to injury and infection. Tissue-associated phagocytes and intact epithelial barriers are among the primary host defences that serve as physical and chemical barriers against HIV infection [33]. During coitus, semen is deposited in the lower FGT, consisting of the ectocervix and vagina. The lower FGT is lined with several layers of stratified squamous epithelial cells [34,35]. These cells are held together by tight and adherens junctions, which reduce the permeability of the epithelium and prevent viral entry at the lower FGT [35-37]. Furthermore, the lower FGT has superficial layers of vaginal epithelium consisting of cornified epithelial cells that provide an additional layer of protection [38]. The upper FGT includes the fallopian tubes and ovaries, uterus, and the endocervix, each lined with a single layer of columnar epithelial cells held together by tight junctions. Vaginal epithelium thickness is influenced by sex hormone fluctuations during the menstrual cycle phases and with hormonal contraceptive use [39-42]. Increased progesterone has been associated with epithelial thinning at the FGT and a greater risk of HIV infection [41-45]. Tissue-associated phagocytes such as neutrophils engulf and destroy invading pathogens and infected cells through various mechanisms [33,46]. Neutrophils can release their deoxyribonucleic acid (DNA) to form neutrophil extracellular traps that prevent HIV infection through viral inactivation [46]. In addition, epithelial and innate immune cells produce cytokines and induce leukocyte recruitment in response to infection [33,47].

2.1.2. Role of the cervicovaginal mucus in innate immune defence

The cervicovaginal environment is covered in a thick layer of mucus that provides lubrication during coitus, facilitates sperm migration, and acts as a physical and chemical barrier to prevent access to the underlying epithelium [48-52]. Cervicovaginal mucus (CVM) is primarily composed of water and mucin glycoproteins but also contains immunoglobulin (Ig)G, IgA, and several antimicrobial agents which provide additional protection at the female genital mucosa [49,50,53-57]. The lower FGT is populated by commensal microbes that can modify the CVM composition and influence its ability to defend against pathogens. Acidic CVM associated with *Lactobacillus crispatus* dominance and high levels of D-lactic acid can hinder HIV-1 mobility and prevent infection [52,58,59]. Conversely, HIV mobility is significantly increased in CVM derived from women with bacterial vaginosis (BV) [60]. This is likely since *Gardnerella vaginalis*, a common BV-associated microbe secretes sialidase enzymes that degrade the CVM [61]. These findings highlight the complex interplay between the vaginal microbiome and host innate immunity.

2.1.3. Role of the vaginal microbiome in innate immune defence

An optimal vaginal microbiome is dominated by Lactobacilli spp., which exists in a mutualistic relationship with the host and contributes to the immune defences at the FGT [62]. Commensal microorganisms such as *L. crispatus* prevent pathogen colonisation by inhibiting their growth, preventing biofilm formation, lowering the vaginal pH, competing for nutrients and adherence to the epithelium, and by producing antimicrobial agents such as lactic acid, hydrogen peroxide (H_2O_2), and bacteriocin [63-67]. Lactobacilli metabolise glycogen secreted by vaginal epithelial cells to produce L- and D-isomers of lactic acid [67,68]. Physiological concentrations of vaginal lactic acid are sufficient to inactivate BV-associated microbes and other sexually transmitted agents of infection, including HIV [58,59,69-71]. Lactic acid lowers the vaginal pH, enhances the activity of other antimicrobial factors, and upregulates the production of anti-inflammatory cytokines [67,72]. Taken together, these data suggest that a Lactobacillus-dominant vaginal microbiome is highly beneficial and less vulnerable to HIV infection.

2.2. Adaptive immune responses at the female genital mucosa

Adaptive immunity at the FGT involves either cell-mediated or humoral immunity. Cell-mediated immunity involves the removal and destruction of intracellular pathogens and virus-infected cells by T lymphocytes. Antigen-presenting cells process and display antigens to T cells to trigger a pathogen-specific immune response and promote immunological memory. This adaptive immune response is characterised by the involvement of various CD4+ T cells [e.g., T-helper (Th)1, Th2, Treg, T follicular helper (Tfh), and Th17 cells] and CD8+ T cell subsets. Cytotoxic T cells (CD8+) recognise antigens presented on major histocompatibility complex (MHC) class I molecules and directly kill virus-infected cells by inducing apoptosis through perforin and granzymes [73]. Conversely, CD4+ T cells recognise antigens presented on MHC class II molecules and respond by secreting cytokines to activate CD8+ T cells, macrophages, and B cells to destroy infected cells [74,75].

Humoral immunity is mediated by B cells and their secreted antibody products. Antibodies prevent and fight infections by binding to antigens on the pathogen and preventing their entry into host cells, coating the pathogen for phagocytosis, inducing antibody-dependent cell-mediated cytotoxicity, and by activating the complement pathway [76,77]. IgG is the predominant immunoglobulin isotype found in genital secretions of both HIV-infected and uninfected women [78,79]. T cell immunity and the abundance of immunoglobulins at the FGT are highly regulated by sex hormones [73,80].

One to two weeks after infection, effector CD4+, and CD8+ T cells die, leaving behind antigen-specific memory T cells that persist long after infection. Memory T cells mount a rapid immune response upon reinfection with the same pathogen and can be subdivided into central memory cells that circulate between the blood and lymph nodes, and resident and recirculating effector memory cells in non-lymphoid tissue [75,81,82]. Tissue-resident memory T cells (TRMs) reside in mucosal tissues and rapidly respond to local infections by producing cytokines to induce immune cell activation and recruitment at

the FGT [75,83-85]. Although the physiological role of TRMs is to defend against infections, these cells have also been identified as major targets for HIV at the lower FGT [86,87].

3. Genital inflammation increases HIV acquisition risk in women

Although the female genital mucosa has several defences to prevent infection and the probability of heterosexual HIV transmission is relatively low, [9,88] inflammation can increase the risk of HIV acquisition at this site. This is supported by observations of infection by less fit HIV variants in women with genital inflammation than without [89]. Inflammation is the body's natural response to injury or infection and involves the influx of immune cells and their products to the site of infection. However, inflammation also contributes to the availability of HIV susceptible cells at the female genital mucosa. Masson *et al*. demonstrated that genital inflammation, characterised by elevated concentrations in at least 5 of 9 pro-inflammatory cytokines, was associated with a greater risk of HIV infection in South African women [2]. The study also identified specific cytokines [macrophage inflammatory protein (MIP)-1α, MIP-1β, and interferon gamma-induced protein (IP)-10] that were independently associated with HIV seroconversion [2]. The chemokines MIP-1α, MIP-1β, and IP-10 are involved in recruiting HIV target cells to the female genital mucosa [90-93]. Additionally, elevated cervicovaginal cytokines also contribute to HIV risk in women through mucosal barrier disruption [3,94].

A compromised vaginal epithelium facilitates HIV entry and access to local immune cells for infection. Elevated pro-inflammatory cervicovaginal cytokines have been associated with several proteins involved in protease activity, epithelial barrier function, tissue remodelling, and actin cytoskeleton organisation [3]. Arnold *et al*. also demonstrated that increased concentrations of matrix metalloproteinases (MMP)-8 and 9, proteins involved in the remodelling of the extracellular matrix, are associated with raised cytokine biomarkers of inflammation [3]. Elevated levels of MMPs in vaginal fluid from women with BV were also shown to disrupt endocervical epithelial polarization and increase HIV transmigration through the endocervical epithelium [6]. Additionally, a study conducted in mice demonstrated that tissue inflammation induced remodelling of the extracellular matrix and altered CD4+ T cell motility [95]. Tissue remodelling and degradation may result in reduced epithelial barrier integrity thereby facilitating access to HIV target cells at the FGT. Consistent with this, studies have demonstrated an increased risk of HIV infection in women with reduced epithelial barrier function [96-98]. A compromised epithelial barrier may also facilitate microbial translocation [6,94] and vaginal microbial diversity known to increase HIV infection rates in women [4,5,7].

Although a lactobacillus-dominant vaginal microbiome is beneficial to host immunity, South African women tend to have greater microbial diversity [4,5]. Microbial diversity and BV are linked to an increased risk of HIV infection in women [4,5,7] and higher rates of both sexual and vertical HIV transmission [99,100]. Specific BV-associated bacteria (*Prevotella*, *G. vaginalis*, *Sneathia*, *Parvimonas*, and *Gemella*) have

been significantly associated with genital inflammation and an increased risk of HIV acquisition in women [4,5,7,101]. These microbes contribute to inflammation through activation of the nuclear factor kappa B (NF-κB) pathway, increasing genital cytokines, immune cell recruitment, reduced epithelial barrier integrity, and impaired wound healing [4,6,49,102]. These studies highlight the role of genital inflammation in susceptibility to HIV infection in women. A better understanding of factors that modulate genital inflammation is required to prevent HIV transmission in women at high risk of acquiring the virus. Here, considering that HIV is predominantly transmitted to women via heterosexual transmission, we review the potential for semen exposure and condomless sex to foster the genital immune environment linked to HIV risk in women.

4. The structure of the male genital tract and HIV infection

The male genital tract (MGT) is comprised of the penile urethra and the testes (**Figure 1**). In uncircumcised males, the foreskin provides both physical and immunological protection to the glans [103] but is also highly susceptible to HIV infection [104,105]. The outer surface of the foreskin is lined by a double layer of keratinised stratified squamous epithelium that covers the glans/corona and the opening of the penile urethra (meatus) [104,106]. The epithelium of the foreskin is relatively resistant to HIV infection unless microabrasions are induced during condomless sex, which may facilitate access to target cells within the underlying epithelium [104,106,107]. The subpreputial cavity, which is the inside of the foreskin, provides an anoxic and moist microenvironment that harbours a diverse array of anaerobic microbes [27,108-110]. The presence of these anaerobic microbes increases the susceptibility of the neighbouring epithelium and the urethral opening to HIV infection via activation of target cells [108-113]. Additionally, when the penis is erect, the foreskin retracts, exposing the glans and inner foreskin, which are more susceptible to viral infection [114]. The inner foreskin contains HIV target cells that are directly exposed to the vagina during sexual intercourse [105,114-118]. Medical male circumcision involves the surgical removal of the foreskin resulting in a dry keratinised epithelial surface that is more resistant to HIV infection [119-121]. Circumcision also reduces the diversity of the penile microbiota and may decrease HIV acquisition risk in both men and women [108,122-128].

Urine and semen are secreted from the penile urethra, which originates at the bladder and is approximately 20 cm in length and 1-2 cm in diameter [106,117]. In contrast to the foreskin, the urethra is lined with non-keratinised pseudostratified glandular columnar epithelium, which is less resilient to HIV infection [117,129,130]. Given that the epithelium of the penile urethra confers reduced protection against HIV entry and contains a high density of intraepithelial immune cells, this serves as a primary site for infection by sexually transmitted infections (STIs), including HIV [106,107,117,130-133]. The epithelium of the urethra also contains several deep invaginations called the periurethral glands of Littre

[117]. These Littre glands are responsible for pre-ejaculate secretion that neutralises residual urine in the urethral lumen and acts as lubrication during condomless sex [117].

The testes can be divided into two main regions; these are the interstitial spaces between the tubules and the seminiferous tubules [131,134]. The testes are responsible for the production of testosterone [134,135] and spermatogenesis, which occurs in the coiled seminiferous tubules [136-138]. The seminiferous tubules connect to the head of the epididymis and then to the vas deferens via the rete testes [137]. The seminiferous tubules are made up of Sertoli cells that surround the spermatogenic cells and provide essential nutrients to the spermatozoa [134,135]. The peritubular myoid cells are smooth muscle cells that surround the seminiferous tubules of the testis and provide structural integrity to the tubules [137]. Peritubular myoid cells are contractile cells that are involved in the maturation and transport of the spermatozoa into the epididymis [139]. Leydig cells are adjacent to the seminiferous tubules and are the most abundant cells within the interstitial space. These cells are responsible for the production of testosterone and small amounts of oestradiol which facilitate the development of spermatozoa [137].

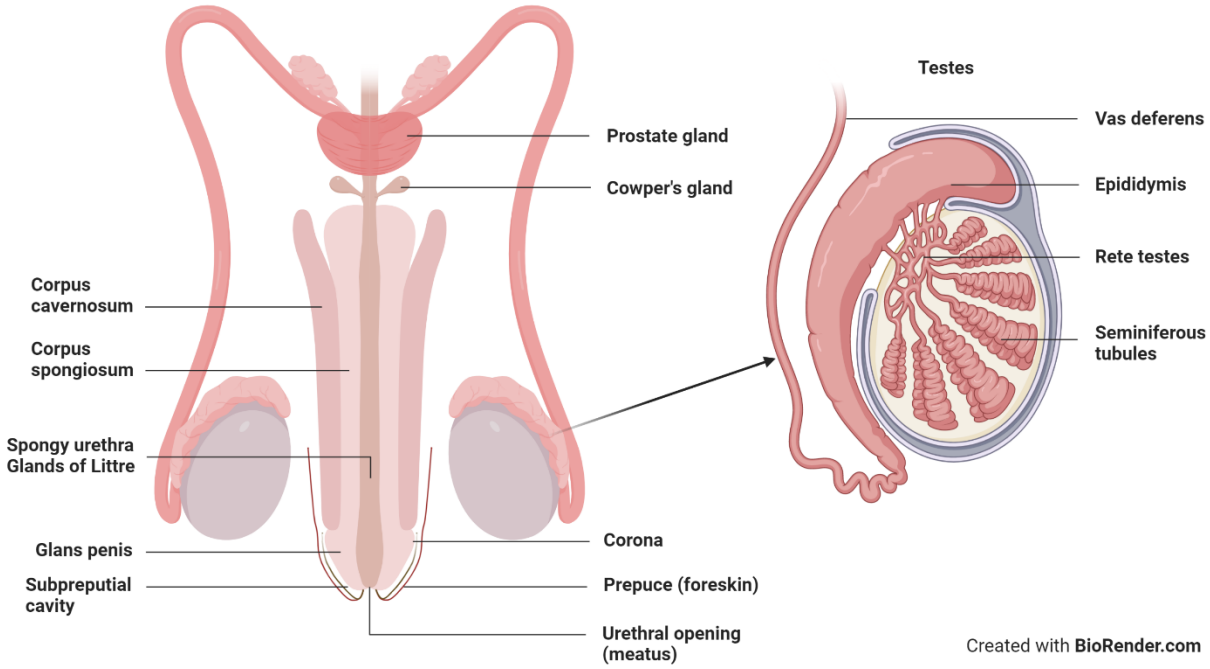

Figure 1. Structure of the male genital tract. The male genital tract is made up of the penile urethra and the testes. The penile urethra is lined with a less resilient non-keratinised pseudostratified glandular columnar epithelium and is a primary site for infection in men. The testes can be divided into two main regions, the seminiferous tubules, and the interstitial spaces between the tubules. The testes are responsible for the production of testosterone and spermatogenesis.

5. Semen composition and implications for HIV infection

Semen contains a mixture of spermatozoa, seminal plasma (SP), microbes, and several bioactive molecules known to both promote and inhibit female genital inflammation. Semen contains secretions from the prostate gland and seminal vesicles [137]. These secretions contain high levels of E-series prostaglandins (PGE) and transforming growth factor (TGF)-β, which are known to have potent immunomodulatory effects [12,16,29-31,140]. TGF-β and PGE2 in semen are commonly associated with anti-inflammatory properties, including suppressing neutrophils, natural killer cells, and dendritic cells (DCs) [29,141,142]. However, in cervical biopsies, PGE2 was shown to stimulate the production of the chemotactic cytokine interleukin (IL)-8 and inhibit the production of the secretory leukocyte peptidase inhibitor, an enzyme with anti-HIV activity [15]. Semen also contains several other cytokines [including IL-1α, IL-1β, IL-2, IL-7, IL-8, IL-10, IL-15, IL-17, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), monocyte chemoattractant protein (MCP)-1, MIP-1α, MIP-1β, regulated on activation, normal T cell expressed and secreted (RANTES), fibroblast growth factor (FGF)-2, growth-related oncogene (GRO)-α, tumour necrosis factor (TNF), vascular endothelial growth factor (VEGF), and fractalkine], hormones, immunoglobulins, and other proteins [10,13,17-20,143,144]. These semen-derived cytokines are involved in immune cell recruitment and the maturation and proliferation of monocytes, T cells, B cells, DCs, and natural killer cells [145-147]. Semen contains high levels of IL-7, which at similar concentrations in cervicovaginal and lymphoid tissues were shown to enhance HIV-1 replication and prevent apoptosis of CD4+ T cells [19,148]. Additionally, semen contains endogenously produced lymphocytes including CD4+ and CD8+ T cells [149]. These semen-derived immune cells are likely transferred to the FGT during coitus and may contribute to the immune alterations observed soon after condomless sex and should be controlled for in studies of genital mucosal immunity to HIV and other STIs. Semen also harbours a diverse array of microbes derived from the penile urethra and upper MGT [24-26]. The most abundant bacterial taxa in semen include among others *Streptococcus, Staphylococcus, Corynebacterium, Lactobacillus, Prevotella, Anaerococcus, Finegoldia,* etc [24-26]. Additionally, protein deposits known as amyloid fibrils have also been identified in semen, their physiological function is to mediate the selection and clearance of damaged sperm [150]. However, these semen-derived amyloid fibrils also greatly enhance HIV infection by facilitating the binding of HIV virions to their cellular targets for infection [151-155]. Importantly, semen composition may be altered in the presence of HIV and other STIs resulting in an increased pro-inflammatory immune response at the FGT, which may further impact HIV susceptibility in women [18,156-161].

6. Contributions of semen to female genital inflammation

6.1. Impact of semen exposure on cytokine biomarkers of FGT inflammation

The immunomodulatory components of semen induce alterations at the FGT to facilitate conception but may also contribute to genital inflammation and HIV risk in women (**Figure 2**) [14,18,19,22,150,153]. Exposure to semen and SP is associated with short-term alterations in several cytokines [including IL-1α, IL-6, IL-8, IL-12p70, TNF-α, TNF-β, IP-10, leukaemia inhibitory factor (LIF), MCP-1, MCP-3, RANTES, GM-CSF, G-CSF, GRO-α, MIP-3α, VEGF, FGF-2, and fractalkine] at the lower and upper FGT [10,11,13,20-22,162-165]. Of particular importance are IL-1α, IL-6, IL-8, TNF-α, MIP-3α, MCP-1, RANTES, and IP-10, which have been used to define female genital inflammation [2,3]. The β-chemokines MIP-1α, MIP-1β, and RANTES are CCR5 ligands that recruit HIV target cells to the FGT but also competitively bind to the CCR5 co-receptor [93]. Vaginal epithelial cells previously exposed to semen had elevated concentrations of MIP-3α (CCL20), a chemokine involved in the recruitment of Langerhans cells to the epithelium [163]. MIP-3α induces chemotaxis of CCR6+ cells, including Th17 cells, the preferential targets for HIV infection [90,166,167], and may therefore increase the availability of HIV susceptible cells at the female genital mucosa. However, in addition to its chemoattractant properties, MIP-3α also exhibits anti-HIV activity through competitive binding to the CCR6 receptor [90,168]. Sharkey *et al*. demonstrated that exposure to semen induced the expression of IL-1β, IL-6, and LIF by endometrial epithelial cells [11]. Expression of these cytokines triggers the recruitment and activation of macrophages, DCs, and neutrophils [11]. Similarly, a study conducted on SP-treated endometrial epithelial cells and stromal fibroblasts demonstrated an upregulation of several cytokines [20]. The presence of semen in the female genital mucosa upregulates the production of pro-inflammatory and chemotactic cytokines [10,11,13,20-22,162-165], with several of these associated with leukocyte recruitment and reduced mucosal barrier integrity [2,3], both significant contributors to the ability of HIV to penetrate and access target cells at the FGT.

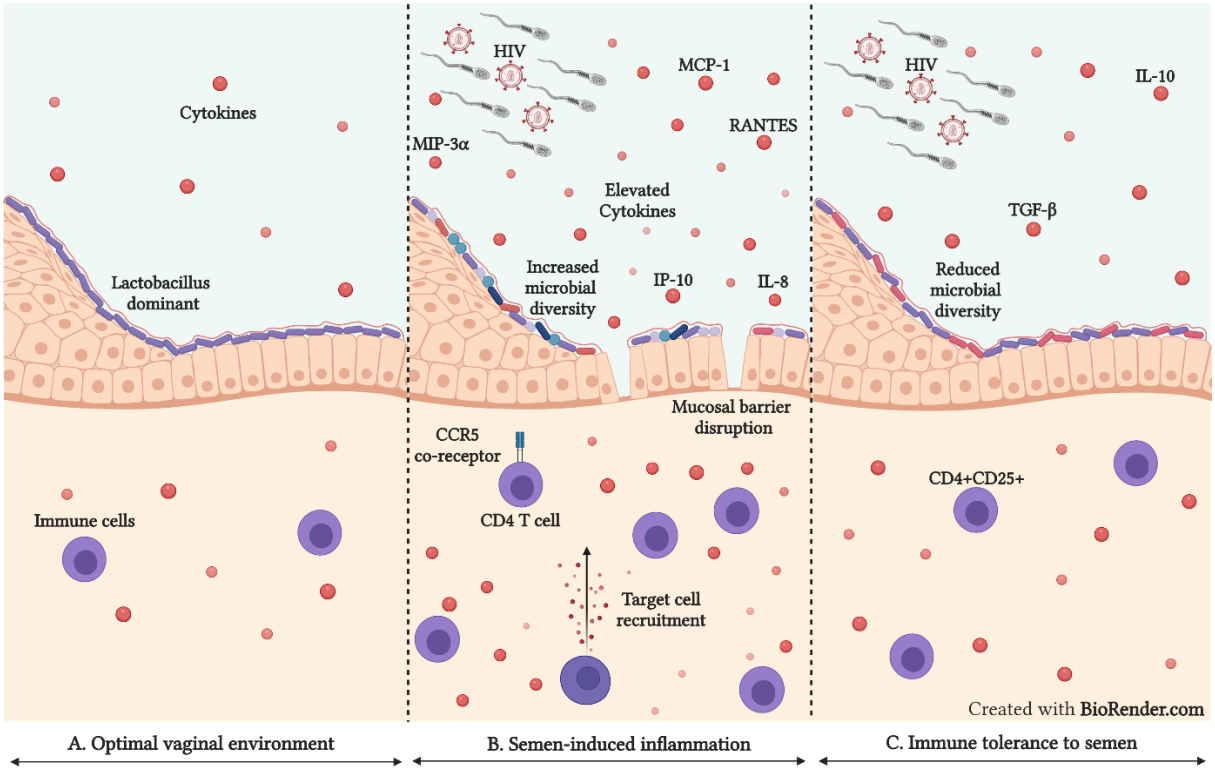

Figure 2. Alterations at the female genital mucosa in response to semen. (A) An optimal vaginal environment contains few cytokines and immune cells. The vaginal microbiome is dominated by Lactobacillus spp. and the mucosal barrier does not contain microabrasions. **(B)** The pro-inflammatory components in semen induce cytokine production and target cell recruitment to the FGT. Semen and condomless sex may induce microabrasions in the epithelial barrier and alterations in the vaginal microbiome. **(C)** The anti-inflammatory components of semen, including TGF-β and IL-10, are associated with fewer cervicovaginal cytokines and expansion of the Treg immune cell (CD4+CD25+) population. Additionally, since homeostasis of the vaginal microbiome is quickly restored after exposure to semen, a tolerogenic immune response to semen may be associated with minor changes in the vaginal microbiome.

6.2. Impact of semen on immune cells at the female genital mucosa

Since semen is initially recognized as foreign in the FGT an immune response is mounted, resulting in cytokine upregulation and the chemotaxis of immune cells. In reproduction, this pro-inflammatory immune response is necessary for the removal of excess and abnormal sperm [29,169]. However, these semen-induced alterations may also increase susceptibility to HIV infection in women. Semen-derived PGE2 has been associated with the recruitment and activation of HIV target cells [162,170]. PGE2 in SP was shown to induce prostaglandin-endoperoxidase synthase-2 (PTGS2) expression in the cervix of women, where it regulates the tolerogenic phenotypes of DCs and macrophages in the postcoital

inflammatory response [11,16]. The expression of PTGS2 in vaginal cells is also related to an increased susceptibility to HIV and other STIs [162]. Recent condomless sex has been associated with an influx of CD14+ macrophages, CD1a+ dendritic cells, and CD8+ T cells to the cervical epithelium and stroma [11]. Additionally, SP treatment significantly induced the chemotaxis of CD14+ monocytes and CD4+ T cells in endometrial epithelial cells and stromal fibroblasts [20]. SP also upregulates the expression of the HIV coreceptor CCR5+ on CD4+ T cells and *in vitro* in HeLa cells [171,172]. Similarly, we have recently demonstrated that higher cervicovaginal Y-chromosome DNA (YcDNA) concentrations and prostate-specific antigen (PSA) detection, both indicative of recent semen exposure, are associated with increased frequencies of activated CD4+HLA-DR+ T cells and CD4+CCR5+HLA-DR+ HIV targets, respectively (Jewanraj *et al*., 2021; accepted).

A Treg immune response is induced soon after semen exposure since prolonged inflammation at the FGT may reduce the odds of fertilization and pregnancy [12,16,30,31,173]. Semen-derived TGF-β and PGE induce a shift from an initial Th1 to a Th2 immune response by promoting Treg cell differentiation and expansion [12,29,30]. The induction of a Treg immune response results in tolerance of the paternal alloantigen at the time of embryo implantation [12,31,32]. Prostaglandins in semen may also upregulate the production of the anti-inflammatory cytokine IL-10 [137]. Consistent with this, we and others have demonstrated elevated cervicovaginal IL-10 concentrations in response to recent semen exposure [13,165]. Additionally, prostaglandins prevent an immune response at the FGT by inhibiting macrophage cytokine production and T cell proliferation [29,30,169,174,175]. Although this induction of immune tolerance may be protective for the paternal alloantigen, this dampened immune response may prevent pathogen clearance at the female genital mucosa.

In addition, studies have demonstrated that prior and prolonged exposure to the same donor's semen improved fertility and reduced preeclampsia rates in women, highlighting the importance of immune tolerance to semen in these contexts [176-180]. Furthermore, a recent study conducted in rhesus macaques demonstrated that repeated vaginal exposure to semen resulted in lower CCR5 expression on CD4+ T cells and reduced infection by Simian Immunodeficiency Virus [181]. These findings suggest that semen exposure to new or multiple concurrent partners may induce a greater and prolonged inflammatory response, which is associated with adverse pregnancy outcomes and possibly an increased risk of HIV transmission [176,177,180-182]. Immune tolerance may be lost on exposure to semen from a new partner, resulting in a more pronounced immune response and suggests a biological link for the relationship between partner concurrency and HIV risk in South African women [183].

6.3. Impact of semen exposure on the vaginal microbiota

BV is a state characterised by a shift in the vaginal microbiome from Lactobacillus dominance to a more diverse spectrum of facultative anaerobes [62,184]. Condomless sex has been associated with BV occurrence [28,185-187] and increases in *Escherichia coli* at the FGT [186,188-190]. Semen contains a diverse array of bacteria that are introduced into the vagina during condomless sex [24-26]. Additionally, the MGT itself (including the penile skin, meatus, glans/corona, and the subpreputial cavity) also contains a diverse array of bacterial taxa that may be transferred to the FGT in the absence of ejaculation and semen exposure [24-28,188,191,192]. A high level of concordance has been observed between the MGT microbiome composition and BV incidence in female partners [27,28,191]. In addition, semen has an alkaline pH range between 7.2-7.8, capable of buffering the acidic pH of vaginal fluid [23,193,194]. This neutralization of the vaginal pH may promote a shift in the vaginal microbiome to a BV-associated state that is conducive to HIV-1 infection [4,5,7,52,69,194,195]. Several factors in semen may also inhibit the activity of extracellular H_2O_2 produced by Lactobacilli species and thus promote the growth of BV-associated microbes [196]. We have demonstrated that recent semen exposure is associated with increased detection of BVAB-2, *Prevotella bivia*, and *G. vaginalis* and with the reduced detection of *Lactobacillus jensenii* in vaginal specimens (Jewanraj *et al*., 2021; accepted) [165]. Increases in other gut-associated microbes have also been observed in the FGT after protected sexual intercourse, suggesting that these alterations in the vaginal microbiota may also be associated with mechanical contamination rather than just semen itself [186,188]. These studies suggest that semen exposure and sexual intercourse may promote a shift in the microbial environments of the FGT that may facilitate HIV infection in women [4,5,7,165].

6.4. Impact of sexual intercourse and semen exposure on the vaginal epithelial barrier

An intact vaginal epithelial barrier is the primary host defence against HIV entry and infection. Reduced epithelial barrier integrity may facilitate HIV access to target cells at the FGT. Colposcopic examination of the vaginal mucosa revealed that friction during consensual sexual intercourse might cause microabrasions in the epithelial barrier [197-199]. Additionally, pro-inflammatory cytokines within semen may also increase the permeability of the vaginal epithelium. Interferon-gamma in semen may increase epithelial permeability by inducing macropinocytosis of tight junction proteins [200]. Semen-derived IL-1β may also increase vaginal epithelium tight junction permeability through the activation of the NF-κβ pathway [201]. Elevated levels of MMPs have also been linked to reduced mucosal barrier integrity, increased cervicovaginal cytokine production, immune cell recruitment at the vaginal mucosa, and increased HIV transmigration [3,6]. We have recently demonstrated that semen exposure is associated with increased concentrations of MMP-2 and their inhibitors in vaginal specimens [165]. An increased HIV incidence has been observed among women with compromised epithelial barrier integrity through the enhanced ability of HIV-1 to penetrate the vaginal epithelium [11,96-98,202].

7. The role of sexual intercourse and semen exposure on topical PrEP efficacy

In addition to its role in female genital inflammation and immune activation, semen exposure and sexual intercourse may also undermine topical pre-exposure prophylaxis (PrEP) efficacy [203-205] and has additional implications for HIV susceptibility in women. The physiological changes that occur during coitus may alter PrEP efficacy by changing the surface area of the vagina and redistributing cervicovaginal fluid and topically applied microbicides [206,207]. In clinical trials, vaginal microbicide gels PRO 2000 and cellulose sulfate failed to confer protection against HIV-1 transmission in women [208,209]. *In vitro* assays demonstrated a significant reduction in the antiviral activity of PRO 2000 gel following sexual intercourse [205]. Tenofovir gel concentrations were also significantly reduced in cervicovaginal lavage and vaginal and cervical tissues after coitus [204]. These findings were likely due to the redistribution of the microbicide gels in the vagina during sexual intercourse.

Semen and SP itself contains several bioactive molecules and may also alter the antiviral activity of microbicides [203,205,210,211]. SP was shown to interfere with the HIV-1 and herpes simplex virus (HSV)-2 inhibitory activity of PRO 2000 and cellulose sulfate microbicides [203,210,211]. Seminal proteins, fibronectin and lactoferrin competitively inhibited the binding of the microbicides to their target on the HSV envelope [211]. The reduced antiviral activity of these microbicides may also be due to electrostatic interactions between cationic SP polyamines and the polyanions of the microbicides [205,210-212]. Zirafi *et al*. demonstrated that seminal amyloids enhance HIV infection and also contribute to the reduced antiviral activity of microbicides [203]. Additionally, we previously demonstrated that recent semen exposure was associated with increased detection of *G. vaginalis* and biomarkers of inflammation in vaginal specimens (Jewanraj *et al*., 2021; accepted), both of which contribute to diminished topical PrEP efficacy in women [213,214]. These studies suggest that sexual intercourse and semen itself may also reduce the efficacy of topical PrEP in women and highlights the need to assess and control for these factors.

8. Biomarkers of semen exposure

Research primarily relies on self-reports of condom use and sexual behaviour, which may lead to inaccurate data interpretation due to reporting bias [215-219]. Although biomarkers of semen exposure were developed for use in forensics, they also have several useful applications in HIV prevention research. Semen biomarkers can be used to control for semen-induced alterations at the FGT, assess condom use in clinical trials, and determine the efficacy of barrier contraceptives and microbicides [165,220-230]. Biomarkers that have been previously used to detect semen in vaginal specimens include PSA, YcDNA, semenogelins, acid phosphatase, and sperm detection by microscopy [165,227-235]. PSA and YcDNA detection are the most well-studied and commonly used biomarkers of semen exposure [236]. PSA is present in high concentrations in semen, and detection in vaginal fluid usually indicates semen exposure

within 48 hours [227,237-240]. We and others have demonstrated that PSA detection in vaginal specimens, a proxy for recent semen exposure, is associated with a pro-inflammatory immune response at the FGT (Jewanraj *et al*., 2021; accepted) [228,230]. Conversely, YcDNA is a more stable biomarker and is detectable in vaginal specimens up to 15 days after coitus [220,232,236,241]. Since YcDNA is detectable in the presence of spermatozoa, it is an ideal measure of the probability of pregnancy [220]. These semen biomarkers may be suitable for different studies depending on the residence time of the biomarker and the study outcome, such as the probability of pregnancy, infection, or genital inflammation. Routine objective screening for semen biomarkers may avoid the discrepancies associated with self-reported data and may lead to more reproducible study outcomes. Additionally, given the immunomodulatory properties of semen, these biomarkers can be used to control for semen's impact on the immune and microbial microenvironments of the FGT.

9. Conclusions

Identifying factors associated with female genital inflammation and limiting their impact on HIV risk is particularly important in high HIV burden areas. Semen is a highly immunomodulatory fluid and is the primary vector for HIV transmission to women during condomless sex. However, most studies of mucosal immunity are conducted in the absence of semen or without consideration of its immune impact on the female genital mucosa. Semen exposure is associated with a short-term inflammatory response at the FGT which is quickly resolved to facilitate immune tolerance to the paternal antigens. Albeit short-lived, a semen-induced pro-inflammatory immune response may promote genital inflammation and HIV risk in women. Additionally, semen and condomless sex may also modulate topical PrEP efficacy and has additional implications for HIV risk in women. Future clinical and immunological studies of HIV and other STIs should consider semen's contribution to the immune and microbial environments of the FGT. We suggest that STI/HIV research may benefit from routine screening for semen biomarkers in vaginal specimens to account for its impact on female genital inflammation.

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Conflict of interests

The authors declare no conflicts of interest.

Author contributions

J.J. conceptualized and wrote the review article. S.N. and L.J.P.L. critically reviewed and revised the manuscript. All authors approved the final submitted manuscript.

Data availability statement

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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1.3. Study Aims

a. To determine the impact of semen exposure on immune and microbial biomarkers of inflammation associated with vaginal HIV transmission in women.
b. To determine the impact of the timing of semen exposure on biomarkers of inflammation linked to vaginal HIV transmission in women and the persistence of these associations over time.

1.4. Objectives

a. To determine semen exposure by detecting Y-chromosome DNA and prostate-specific antigen in cervicovaginal lavage specimens.
b. To assess the impact of semen exposure on cytokine concentrations and immune cell frequencies in the female genital tract.
c. To characterise post-coital alterations in vaginal microbe presence.
d. To investigate the impact of condomless sex on biomarkers of vaginal epithelial barrier integrity.
e. To determine the impact of Y-chromosome DNA concentrations on biomarkers of female genital inflammation.
f. To compare genital immune and microbial profiles between women with evidence of semen exposure within 2 days, semen exposure between 3-15 days, and no semen exposure within the past 15 days of genital sampling.

1.5. Brief overview of the general study design and methodologies

The studies presented in the following chapters included demographic data and genital specimens from sexually active, HIV-negative women between 20-44 years old from the CAPRISA 008 trial. The CAPRISA 008 tenofovir gel open-label extension trial was conducted to investigate the efficacy of integrating tenofovir gel provision into routine family planning services. Women were enrolled in the study between November 2012 and October 2014 from urban and rural CAPRISA clinic sites in KwaZulu-Natal and followed up for an average of 22 months (Mansoor *et al*., 2019).

Potential study participants who met the eligibility criteria were subsequently enrolled in the CAPRISA 008 trial. The inclusion criteria were sexually active women ≥ 18 years, HIV-uninfected, previously took part in the CAPRISA 004 trial, attending family planning services, not pregnant, and willing to use a non-barrier method of contraception. Eligible consenting participants randomly received the 1% tenofovir gel either through family planning clinics (intervention arm) or CAPRISA clinics (control arm). Women attending the family planning clinics had monthly visits for the first three months and then received the gel every 2-3 months at their routine visit, while those attending the CAPRISA clinics received the gel at their monthly follow-up visits. Participants were supplied with single-use, 1% tenofovir gel applicators and advised to apply the first dose of tenofovir gel within 12 hours before coitus and a second dose within 12 hours after coitus, with a maximum number of two gel applications in 24 hours (Mansoor *et al*., 2014, Mansoor *et al*., 2019). HIV and pregnancy tests were conducted at each study visit using the Determine HIV-1/2 (Abbott Laboratories, Lake Bluff, IL, USA) and Uni-Gold Recombigen® (Trinity Biotech, Wicklow, Ireland) HIV rapid tests and the QuickVue One-Step hCG Urine pregnancy test (Quidel Corporation, San Diego, CA, USA). Participants were requested to return all gel applicators, used or not, at each study visit.

Pelvic examinations, blood, and vaginal specimen collections were conducted at the enrollment visit and biannually at months 6, 12, 18, 24, and study exit to conduct safety and gel adherence assessments. Genital specimens were collected for storage to assess safety, risk exposure, gel adherence, activity against other STIs, and resistance. Genital samples, including cervical cytobrushes, CVLs, and vulvovaginal swabs, were collected as outlined in the standard operating procedures and transported to the CAPRISA laboratory for processing and storage (Mansoor *et al*., 2019). All genital specimens were collected under speculum examination. Two cervical cytobrushes were collected as previously reported (Nkwanyana *et al*., 2009). Briefly, the Digene cervical cytobrush was inserted into the endocervical canal, rotated 360º, removed, and stored in a sterile tube containing media supplemented with 10% Fetal Bovine Serum. The cervical cytobrushes were irrigated using a Pasteur pipette and media to collect cervical mononuclear cells. The collection and processing of CVL samples have also been described previously (Bebell *et al*., 2008; McKinnon *et al*., 2018). Briefly, a pipette was used to insert 5 ml sterile saline into the vagina towards the cervical os. The resultant fluid was collected and dispensed into a

sterile container. All CVL samples were transported on ice to the CAPRISA laboratory, where they were subsequently centrifuged, and the resultant cell pellets and supernatants were stored at -80 ºC. Finally, two vaginal swabs were sequentially inserted into the vagina, gently rotated, and placed into sterile tubes containing phosphate-buffered saline.

The study participants provided informed consent for genital specimen storage and possible future research testing (BFC237/010, **Appendix D**). Here, stored cervical cytobrushes were used to assess immune cell frequencies, CVL pellets were used for YcDNA detection and quantification, CVL supernatants were used for PSA detection and to assess soluble protein concentrations, and vaginal swabs were used for STI testing and the detection of BV-associated microbes.

CHAPTER 2

The impact of semen exposure on the immune and microbial environments of the female genital tract

A recent study conducted in CAPRISA 004 trial participants demonstrated that GI is significantly associated with HIV seroconversion in women (Masson *et al*., 2015). Here, GI was characterised by raised levels of pro-inflammatory and chemotactic cytokines in vaginal specimens (Masson *et al*., 2015). Arnold *et al*. further demonstrated that elevated cervicovaginal cytokines are associated with mucosal barrier function and HIV target cell movement to the female genital mucosa. Factors that contribute to GI may also impact HIV susceptibility in women. Semen is the main vector for HIV-1 transmission to women during condomless sex and induces mucosal changes to prime the female reproductive tissues for conception. Semen-induced mucosal alterations during reproduction may also promote GI and HIV acquisition in women. Vaginal exposure to semen is often measured using self-reported condom use. However, since self-reported data is often misreported, the detection of semen biomarkers may be a more reliable and objective way of assessing semen exposure at the FGT.

Chapter 2 investigates the impact of semen exposure, as measured by self-reported condom use and YcDNA detection, on biomarkers of GI associated with HIV risk in women. YcDNA detection in vaginal fluid is a biomarker of semen exposure within the past 15 days (Thurman *et al*., 2016; Zenilman *et al*., 2005; Brotman *et al*., 2010). Here, 31% of participants reporting consistent condom use during the CAPRISA 008 trial had YcDNA evidence of exposure to semen. Furthermore, biomarkers of GI did not differ between women who reported always versus never using a condom during sex. In comparison, YcDNA detection predicted significant increases in cytokine concentrations, biomarkers of epithelial barrier integrity, and the detection of *Prevotella bivia* in vaginal fluid. This study emphasizes the discrepancies associated with self-reported condom use as a measure of semen exposure and highlights the importance of screening for semen biomarkers in clinical and immunological studies of HIV. Here, YcDNA detection was not associated with cytokines or immune cells commonly linked to HIV risk. However, semen-associated alterations observed in mucosal barrier proteins and the vaginal microbiome may still impact HIV susceptibility in women.

Dr Ngcapu, Dr Liebenberg, and I conceptualized and designed the study. I measured the levels of cytokines, barrier-related proteins and detected YcDNA in vaginal specimens as a biological marker of semen exposure. I analysed and interpreted the data, which was subsequently validated by Mrs. Osman, the study statistician. I wrote the manuscript, which was reviewed by my supervisors and approved by all co-authors for publication. The manuscript was published in Frontiers in Reproductive Health: HIV and STIs on 9 November 2020 (**Appendix E**). Aspects of this work have been presented at the VIIth Conference of the South African Immunology Society.

The impact of semen exposure on the immune and microbial environments of the female genital tract

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Abstract

Background: Semen induces an immune response at the female genital tract (FGT) to promote conception. It is also the primary vector for HIV transmission to women during condomless sex. Since genital inflammation and immune activation increase HIV susceptibility in women, semen-induced alterations at the FGT may have implications for HIV risk. Here we investigated the impact of semen exposure, as measured by self-reported condom use and Y-chromosome DNA (YcDNA) detection, on biomarkers of female genital inflammation associated with HIV acquisition.

Methods: Stored genital specimens were collected biannually (mean 5 visits) from 153 HIV-negative women participating in the CAPRISA 008 tenofovir gel open-label extension trial. YcDNA was detected in cervicovaginal lavage (CVL) pellets by RT-PCR and served as a biomarker of semen exposure within 15 days of genital sampling. Protein concentrations were measured in CVL supernatants by multiplexed ELISA, and the frequency of activated CD4+CCR5+ HIV targets was assessed on cytobrush-derived specimens by flow cytometry. Common sexually transmitted infections (STIs) and bacterial vaginosis (BV)-associated bacteria were measured by PCR. Multivariable linear mixed models were used to assess the relationship between YcDNA detection and biomarkers of inflammation over time.

Results: YcDNA was detected at least once in 69% (106/153) of women during the trial (median 2, range 1–5 visits), and was associated with marital status, cohabitation, the frequency of vaginal sex, and Nugent Score. YcDNA detection but not self-reported condom use was associated with elevated concentrations of several cytokines: IL-12p70, IL-10, IFN-γ, IL-13, IP-10, MIG, IL-7, PDGF-BB, SCF, VEGF, β-NGF, and biomarkers of epithelial barrier integrity: MMP-2 and TIMP-4; and with reduced concentrations of IL-18 and MIF. YcDNA detection was not associated with alterations in immune cell frequencies but was related to increased detection of *P. bivia* (OR=1.970; CI 1.309-2.965; P=0.001) at the FGT.

Conclusion: YcDNA detection but not self-reported condom use was associated with alterations in cervicovaginal cytokines, BV-associated bacteria, and matrix metalloproteinases, and may have implications for HIV susceptibility in women. This study highlights the discrepancies related to self-reported condom use and the need for routine screening for biomarkers of semen exposure in studies of mucosal immunity to HIV and other STIs.

Introduction

In sub-Saharan Africa, women account for the majority of Human Immunodeficiency Virus (HIV) infections compared to their male counterparts (1) and remain a key target population for the development of biomedical HIV prevention strategies. The risk of HIV infection in young women is increased in the context of genital inflammation (2, 3), and efforts to better understand the causes of inflammation at the female genital tract (FGT) may inform on the design of targeted approaches to prevent HIV acquisition. HIV requires access to local cellular targets at the FGT to establish productive infection, and cytokine biomarkers of genital inflammation may be linked to HIV risk through their role in cellular recruitment (4). Furthermore, genital cytokine concentrations are also associated with alterations in the integrity of the vaginal epithelium (4), and with the abundance of bacterial vaginosis (BV)-associated microbes at the FGT (5-7), both implicated in susceptibility to HIV infection.

Sex without a condom remains the primary mode of HIV-1 transmission, with semen acting as the major vector for male to female transmission of the virus (8). Semen consists of several pro- and anti-inflammatory factors and functions as a biological modifier at the FGT to facilitate pregnancy and conception (9-11). Semen exposure has been associated with temporary upregulation of cytokines and the recruitment of leukocytes to the cervical epithelium and stroma (9-14). A pro-inflammatory immune response is generally mounted against semen in the FGT, resulting in the removal of excess and abnormal sperm (15, 16). Semen also contains a diverse array of microbial communities and has an alkaline pH, all of which have the potential to alter the vaginal microbiome (17-20). Apart from the immune altering capacity of semen itself, sexual intercourse has been associated with a significant reduction in *Lactobacillus crispatus* (17), increased prevalence of *Gardnerella vaginalis* (21), and may also lead to vaginal epithelial microabrasions (22, 23) that facilitate HIV entry and access to local target cells at the female genital mucosa. These alterations at the FGT may have implications for the risk of HIV acquisition in women.

Semen-associated inflammation may be, however, short-lived, as immune tolerance to paternal alloantigens is induced during reproduction (9, 11, 24, 25). Semen contains anti-inflammatory compounds such as transforming growth factor-β which promotes a shift from a type 1 helper (Th1) to a type 2 helper (Th2) immune response at the FGT, thereby inducing a regulatory T cell (Treg) response (14, 16, 25). Semen also contains high concentrations of prostaglandin E2, which has been shown to inhibit macrophage cytokine production and T cell proliferation (25-27). These anti-inflammatory responses responsible for tolerance to sperm may also inhibit the control of pathogens such as HIV and other sexually transmitted infections (STIs) at the FGT. Taken together, efforts to prevent HIV infection may benefit from a better understanding of the contribution that both pro- and anti-inflammatory properties of semen have on the risk of HIV acquisition in women.

Self-reported condom use is often used as an indication of semen exposure at the FGT. However, this practice is subject to bias, and data are often misreported (28-30). Routine objective screening for the

presence of semen biomarkers as opposed to self-reports of condom use may be useful to reliably assess the frequency of condomless sex e.g., during HIV prevention trials, to assess mucosal immunity to STIs, and to further characterise the impact of semen on the FGT in the context of HIV. Y-chromosome DNA (YcDNA) detection in female genital specimens has previously been used as a reliable biomarker of semen exposure within 15 days of sampling (31-37). Y-chromosome polymerase chain reaction (PCR) is a highly stable, sensitive, and specific method to detect spermatozoa-associated deoxyribonucleic acid (DNA) fragments of the sex-determining region and testis-specific protein Y-encoded (TSPY) genes of the Y-chromosome that are not present on the X-chromosome gene (38-42). Considering the established unreliability of self-reported condom use, we hypothesized that YcDNA detection, but not self-reported condom use will be associated with alterations in biomarkers of inflammation linked to HIV risk in women.

Methodology

Study Design and Population

This longitudinal retrospective study included questionnaire data and stored genital samples from 153 randomly selected HIV-negative women from the CAPRISA 008 trial (43). The CAPRISA 008 trial was an open-label extension trial to assess the effectiveness of delivering tenofovir 1% gel in the context of routine family planning services (43). The women enrolled in this study were aged 20-44 years old, were from urban and rural KwaZulu-Natal, and had previously participated in the parent CAPRISA 004 efficacy trial (44). At the time of initial sampling, all participants had not used 1% tenofovir gel for a minimum of 3 years since exiting the CAPRISA 004 trial and were subsequently provided the tenofovir gel for use throughout the CAPRISA 008 trial, supplied either through CAPRISA clinic sites (control arm) or through family planning services (intervention arm). Genital specimens were collected every 6 months during the two-year trial period (average 5±1 visits). All participants of the CAPRISA 008 trial provided informed consent for the storage of their specimens for use in future studies (BFC237/010). This study was approved by the Biomedical Research Ethics Committee at the University of KwaZulu-Natal under the ethics number BE258/19. YcDNA detection was conducted at the Medical Microbiology Department at the University of KwaZulu-Natal, and all other laboratory assays were conducted at the CAPRISA Mucosal Immunology Laboratory in Durban, South Africa.

Specimen Collection and Processing

Genital specimens including cervical cytobrushes, cervicovaginal lavage (CVL), and vaginal swabs were collected from the participants at each biannual visit. The collection and processing of CVL specimens were previously reported by Bebell *et al* (45). Briefly, a plastic bulb pipette was inserted towards the cervical os through a lubricated speculum. A volume of 5 ml sterile saline was inserted and allowed to bathe the cervix. The resulting fluid accumulated at the posterior fornix and was collected

using the same pipette and dispensed into a sterile conical tube. Thereafter the CVL specimens were transported to the CAPRISA laboratory. At the laboratory, the specimens were centrifuged, and the supernatant was removed and stored in 1 ml aliquots at -80°C.

Cervical cytobrush specimens were collected as previously reported (46). Briefly, a Digene cervical sampler was used to collect cervical mononuclear cells from all participants under speculum examination. The cytobrush was inserted into the endocervical canal and gently rotated 360º to collect cells from the cervical os. The cytobrush specimens were placed into a sterile 15 ml tube (Griener) containing transport medium [Roswell Park Memorial Institute Medium 1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated Fetal Bovine Serum and 5mM glutamine, penicillin, and streptomycin]. Any specimen containing visible blood was discarded.

Vaginal swabs were collected from the posterior fornices and lateral vaginal walls of each participant and tested for the presence of STIs and BV-associated bacteria.

Human Y-Chromosome Detection Assay (PrimerDesign Ltd, UK)

Total DNA was extracted from stored CVL pellet specimens using the MagNAPure LC DNA Isolation Kit I (Roche Applied Science, Indianapolis, IN), according to the manufacturer's instructions. A region of the TSPY1 gene on the Y-chromosome was amplified using the Applied Biosystems® QuantStudio™ 5 RT-PCR System (Thermo Fisher Scientific). YcDNA concentrations were determined from a 1:4 standard curve dilution series. The amplification of the Y-chromosome within 36 cycles was considered a positive result. The negative control (containing no DNA) and an extraction control (PrimerDesign Ltd, UK) were included in each run. Detection of the Y-chromosome and analysis of the results was performed as outlined in the manufacturer's protocol (PrimerDesign Ltd, UK). YcDNA is reported to be stable in the FGT for up to 15 days after sex (31-33) and served as a biomarker of semen exposure in this study.

Quantification of soluble protein biomarkers of inflammation in genital fluid

Concentrations of 48 cytokines, 9 matrix metalloproteinases (MMPs), and 4 tissue inhibitors of metalloproteinases (TIMPs) were measured in undiluted CVL supernatant specimens, according to the manufacturer's instructions. The concentrations of each analyte was measured using the Bio-Plex Pro Human Cytokine, MMP, and TIMP kits and a Bio-Plex Array Reader (Bio-Rad Laboratories) as previously reported (3). The cytokine panel included interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-16, IL-17, IL-18, IL-1 receptor antagonist (IL-1RA), IL-2 receptor α (IL-2Rα), cutaneous T cell attracting chemokine (CTACK), growth related oncogene (GRO)-α, hepatocyte growth factor (HGF), interferon (IFN)-γ, IFN-α2, leukemia inhibitory factor (LIF), monocyte chemotactic protein (MCP)-3, macrophage migration inhibitory factor (MIF), monokine induced by gamma interferon (MIG), β-nerve growth factor (NGF), stem cell factor (SCF), stem cell growth factor (SCGF)-β, stromal cell-derived factor (SDF)-1α, tumor

necrosis factor (TNF)-α, TNF-β, TNF-related apoptosis-inducing ligand (TRAIL), fibroblast growth factor (FGF)-basic, eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage (GM)-CSF, macrophage (M)-CSF, interferon gamma-induced protein (IP)-10, MCP-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor BB (PDGF-BB), regulated on activation, normal T cell expressed and secreted (RANTES) and vascular endothelial growth factor (VEGF). The MMP and TIMP panels included MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12, MMP-13, TIMP-1, TIMP-2, TIMP-3, and TIMP-4. Cytokine data were available for all visits (n=679), while MMP/TIMP data was only generated at baseline (n=145, **Supplementary Figure 1**). The sensitivity of these kits ranged between 0.2 and 45.4 pg/ml for the cytokines and between 1 and 450 pg/ml for each of the MMPs measured in this study. 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.

STI and microbe detection

Vaginal swab specimens were used for STI and microbe detection at the National Health Laboratory Services, Inkosi Albert Luthuli Central Hospital Academic Complex (47). Multiplex PCR amplification was performed on the ABI® 7500 platform from Applied Biosystems (Thermo Fisher Scientific) and using the FTD (Fast-track diagnostics) STD9 kit according to the manufacturer's instructions. The kit contained primers and TaqMan probes that were designed from highly conserved regions of genetic sequences for pathogens associated with STIs, namely *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Gardnerella vaginalis*, *Mycoplasma genitalium*, and Herpes simplex virus (HSV)-1/2. Concentrations of two Lactobacilli strains, *Lactobacillus crispatus* and *Lactobacillus jensenii* (Assay ID Ba04646245_s1, Ba04646258_s1) and BV-associated bacteria i.e., *Gardnerella vaginalis*, *Prevotella bivia*, BVAB2, and *Atopobium vaginae* (Assay ID Ba04646236_s1, Ba04646278_s1, Ba04646229_s1, Pa04646150_s1, respectively) were measured using Applied Biosystems™ TaqMan® assays. All reactions were run on an ABI® 7500 platform from Applied Biosystems (Thermo Fisher Scientific) RT-PCR machine. STI data was available for all visits (n=676), while data on BV-associated bacteria was available for all visits but baseline (n=534, **Supplementary Figure 1**). Gram stain microscopy was used to assess for BV by Nugent Score (48). Women were diagnosed as negative, intermediate, or having BV (Nugent Score 0-3, 4-6, and 7-10, respectively).

Investigation of immune cell frequency

Cervical cytobrush specimens were used to measure the dynamics and frequency of activated (CD38+ or HLA-DR+) or replicating (Ki67+) T cells (CD3+CD4+ or CD3+CD8+) and CD4+CCR5+ targets for HIV replication using multiparametric flow cytometry. Data acquisition was conducted using an LSRII flow cytometer (BD Immunocytometry Systems) and analysed using FlowJo Software version 9.9 (Tree Star, C, US). Gates differentiating negative and positive populations were set by fluorescence minus one staining. Specimens with a cervical CD3+ T cell event count below 100 were excluded from the analysis. The gating strategy is represented in **Supplementary Figure 2**.

Statistical considerations

The Shapiro-Wilk normality test was conducted to determine the distribution of the data. The Mann-Whitney U test was used to compare continuous variables, and the Fisher's exact test was used to compare proportions between the groups at baseline. Questionnaire data were available for 146 participants at baseline, and linear regression models were used to investigate the relationship between self-reported condom use (always vs never) and biomarkers of inflammation [cytokine concentrations (pg/ml), MMP/TIMP concentrations (pg/ml) and immune cell frequencies (%)] at baseline. Soluble protein concentrations were log10-transformed and immune cell frequencies were converted to proportions to ensure normality. Additionally, linear mixed models accounting for repeated measures were used to assess the relationships between YcDNA detection and cytokine concentrations and immune cell frequencies over time. A generalized estimating equation (GEE) model using a logit link and accounting for repeated measures was used to determine the impact of semen exposure on vaginal microbe presence over time. The unadjusted models controlled for study arm, i.e., CAPRISA or family planning services, and time in the study. Multivariable models were adjusted for variables associated with inflammation or HIV risk such as study arm, time in study, Nugent Score, participant age, presence of STIs, the number of vaginal sex acts in the last 30 days, and genital inflammation status. Genital inflammation status was defined by the median cytokine concentration across all visits for each participant in the upper quartile of the distribution of cytokine concentrations (as calculated using the entire dataset) (2). Given that genital inflammation is a linear combination of cytokines, this variable was not controlled for in cytokine analyses. P-values were adjusted for multiple comparisons using the Benjamini-Hochberg method. All tests were conducted at the 5% level of significance. Statistical analyses were performed using GraphPad Prism version 8.3.1 (GraphPad Software, San Diego, CA), STATA version 15.0 (StataCorp., College Station, Texas, USA), and SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

Results

Baseline characteristics of the study population

Demographic data was available for 95% (146/153) of all women at baseline. Overall, the median age of the population was 28 years (interquartile range [IQR] 25-33 years; **Table 1**), with 39% of women having detectable YcDNA in their genital fluid at baseline (57/146 women). More women with detectable YcDNA were married (24.6% vs 10.1%, P=0.038), living with their partner (33.3% vs 16.9%, P=0.027), and reported seeing their partner more often (36.8% vs 21.6%, P=0.017) than those without detectable YcDNA. Additionally, YcDNA detection was associated with a higher median number of lifetime pregnancies [median 2 (IQR 1–3) vs median 1 (IQR 1–2), respectively, P=0.042], and the number of vaginal sex acts in the 30 days prior to sampling [median 5 (IQR 3-10) vs median 4 (IQR 2–6), respectively, P=0.008]. Of the women reporting to have always used a condom during sex, 31% (17/54) had detectable YcDNA in their vaginal specimens, highlighting the discrepancies related to self-reported condom use. Gonorrhoeae detection was significantly associated with YcDNA detection (8.8% vs 0%, respectively, P=0.009). Women with detectable YcDNA also had a higher median Nugent Score [median 3 (IQR 1–7) vs median 1 (IQR 0–3), respectively, P=0.006].

Biomarkers of inflammation were not distinguished by self-reported condom use

Linear regression models were used to investigate the reliability of self-reported condom use as a measure of semen exposure. Biomarkers of female genital inflammation were compared between women self-reporting always (n=54) and never using a condom (n=20) at their baseline visit. Multivariable linear regression models were adjusted for age, any STI, Nugent Score, the number of vaginal sex acts in the past 30 days, randomization arm, and inflammation status. Neither cytokine concentrations, MMP concentrations, nor immune cell frequencies differed between the groups after multivariable adjustments (**Supplementary Tables 1-3**, respectively).

Table 1. Baseline participant characteristics by YcDNA detection in female genital specimens.

Characteristics	Level	Overall (N=146)	YcDNA+ (N=57)	YcDNA- (N=89)	P-Value
Age (years)	Median (IQR)	28 (25 - 33)	29 (25 - 35)	28 (25 - 30)	0.632
Educational level [% (n)]	Primary School	39.0% (57)	43.9% (25)	36.0% (32)	0.060
	HS complete	54.1% (79)	56.1% (32)	52.8% (47)	
	Tertiary complete	4.8% (7)	0	7.9% (7)	
	Less than primary	2.1% (3)	0	3.4% (3)	
Relationship status [% (n)]	Married	15.8% (23)	24.6% (14)	10.1% (9)	**0.038**
	Stable partner	82.9% (121)	73.7% (42)	88.8% (79)	
	Casual Partner	1.4% (2)	1.8% (1)	1.1% (1)	
Study arm [% (n)]	Intervention	47.3% (69)	50.9% (29)	44.9% (40)	0.502
	Control	52.7% (77)	49.1% (28)	55.1% (49)	
Age of regular/stable partner (years)	Median (IQR)	32 (28 - 37)	32 (28 - 38)	32 (29 - 36)	0.633
Number of lifetime pregnancies	Median (IQR)	2 (1 - 2)	2 (1 - 3)	1 (1 - 2)	**0.042**
Number of vaginal sex acts in the last 30 days	Median (IQR)	4 (2 - 8)	5 (3 - 10)	4 (2 - 6)	**0.008**
Partner HIV status [% (n)]	Positive	2.1% (3)	3.5% (2)	1.1% (1)	0.281
	Negative	65.8% (96)	70.2% (40)	62.9% (56)	
	Unknown	32.2% (47)	26.3% (15)	36.0% (32)	
Partner circumcision [% (n/N)]	Yes	32.8% (41/125)	27.5% (14/51)	36.5% (27/74)	0.542
	No	64.8% (81/125)	70.6% (36/51)	60.8% (45/74)	
	Unknown	2.4% (3/125)	2.0% (1/51)	2.7% (2/74)	
Partner living together [% (n)]	Yes	23.3% (34)	33.3% (19)	16.9% (15)	**0.027**
	No	76.7% (112)	66.7% (38)	83.1% (74)	
How often do you see regular partner [% (n/N)]	Daily	27.6% (40/145)	36.8% (21/57)	21.6% (19/88)	**0.017**
	Weekly	42.8% (62/145)	47.4% (27/57)	39.8% (35/88)	
	Monthly	26.9% (39/145)	14.0% (8/57)	35.2% (31/88)	
	< Monthly	2.8% (4/145)	1.8% (1/57)	3.4% (3/88)	
Contraceptive type [% (n)]	Depo-provera	57.5% (84)	63.2% (36)	53.9% (48)	0.105
	Oral contraceptive	21.9% (32)	17.5% (10)	24.7% (22)	
	Nur-isterate	14.4% (21)	8.8% (5)	18.0% (16)	
	Other	6.2% (9)	10.5% (6)	3.4% (3)	
Male condom use [% (n)]	Always	37.0% (54)	29.8% (17)	41.6% (37)	0.178
	Sometimes	49.3% (72)	50.9% (29)	48.3% (43)	
	Never	13.7% (20)	19.3% (11)	10.1% (9)	
HSV-2 antibodies [% (n)]	Positive	88.4% (129)	86.0% (49)	89.9% (80)	0.106
	Negative	9.6% (14)	8.8% (5)	10.1% (9)	
	Equivocal	2.1% (3)	5.3% (3)	0	
Human Papillomavirus [% (n)]	No	48.6% (71)	50.9% (29)	47.2% (42)	0.735
	Yes	51.4% (75)	49.1% (28)	52.8% (47)	
Any STIs [% (n/N)]	No	81.9% (118/144)	78.9% (45)	83.9% (73/87)	0.509
	Yes	18.1% (26/144)	21.1% (12)	16.1% (14/87)	
Neisseria Gonorrhoeae	No	96.5% (139/144)	91.2% (52)	100.0% (87/87)	**0.009**
	Yes	3.5% (5/144)	8.8% (5)	0	
Chlamydia trachomatis	No	93.1% (134/144)	93.0% (53)	93.1% (81/87)	1.000
	Yes	6.9% (10/144)	7.0% (4)	6.9% (6/87)	
Trichomonas vaginalis	No	95.1% (137/144)	96.5% (55)	94.3% (82/87)	0.704
	Yes	4.9% (7/144)	3.5% (2)	5.7% (5/87)	
Mycoplasma genitalium	No	95.8% (138/144)	94.7% (54)	96.6% (84/87)	0.681
	Yes	4.2% (6/144)	5.3% (3)	3.4% (3/87)	
Bacterial vaginosis [% (n/N)]	Median (IQR)	2 (0-4)	3 (1-7)	1 (0-3)	**0.006**
Negative	0-3	74.6% (106/142)	61.4% (35/57)	83.5% (71/85)	**0.001**
Intermediate	4-6	10.6% (15/142)	10.5% (6/57)	10.6% (9/85)	
BV	7-10	14.8% (21/142)	28.1% (16/57)	5.9% (5/85)	

YcDNA detection was associated with alterations in protein biomarkers of inflammation

Considering the potential unreliability in self-report of condom use, given that 31% of women who reported consistent condom use also had YcDNA evidence of recent condomless sex (**Table 1**), we determined whether a biomarker of semen exposure may be a better indicator of immune alterations at the FGT in response to semen. YcDNA detection within female genital specimens was used as a biomarker of semen exposure within 15 days prior to genital sampling (31-33). Linear mixed models were used to compare cytokine concentrations over time and linear regression models were used to compare MMP/TIMP concentrations at baseline between women with detectable YcDNA (semen exposure) and those without (no detectable semen exposure). Women with detectable YcDNA had significantly increased concentrations of IL-12p70 (β=0.202; CI 0.146, 0.258; P<0.001), IP-10 (β=0.230; CI 0.094, 0.366; P=0.001), MIG (β=0.160; CI 0.052, 0.267; P=0.004), β-NGF (β=0.180; CI 0.048, 0.311; P=0.008), IL-7 (β=0.168; CI 0.099, 0.236; P<0.001), PDGF-BB (β=0.062; CI 0.005, 0.120; P=0.035), SCF (β=0.107; CI 0.031, 0.182; P=0.006), VEGF (β=0.252; CI 0.186, 0.318; P<0.001), IFN-γ (β=0.065; CI 0.000, 0.130; P=0.049), IL-13 (β=0.126; CI 0.087, 0.166; P<0.001), IL-10 (β=0.094; CI 0.063, 0.124; P<0.001), and reduced concentrations of IL-18 (β=-0.095; CI -0.184, -0.006; P=0.036) and MIF (β=-0.166; CI -0.259, -0.072; P=0.001; **Figure 1A**) after adjusting for age, any STI, Nugent Score, the number of vaginal sex acts in the past 30 days, time in study, and randomization arm. These associations between YcDNA detection and concentrations of IL-12p70, MIF, IP-10, MIG, β-NGF, IL-7, SCF, VEGF, IL-13, and IL-10 remained significant even after false discovery rate (FDR) adjustments. The concentrations of MMPs and TIMPs were compared among women with detectable YcDNA and those without at baseline. YcDNA detection was associated with elevated concentrations of MMP-2 (β=0.419; CI 0.084, 0.753; P=0.015), and TIMP-4 (β=0.328; CI 0.042, 0.614; P=0.025; **Figure 1B**) after adjusting for age, any STI, Nugent Score, the number of vaginal sex acts in the past 30 days, inflammation status, and randomization arm.

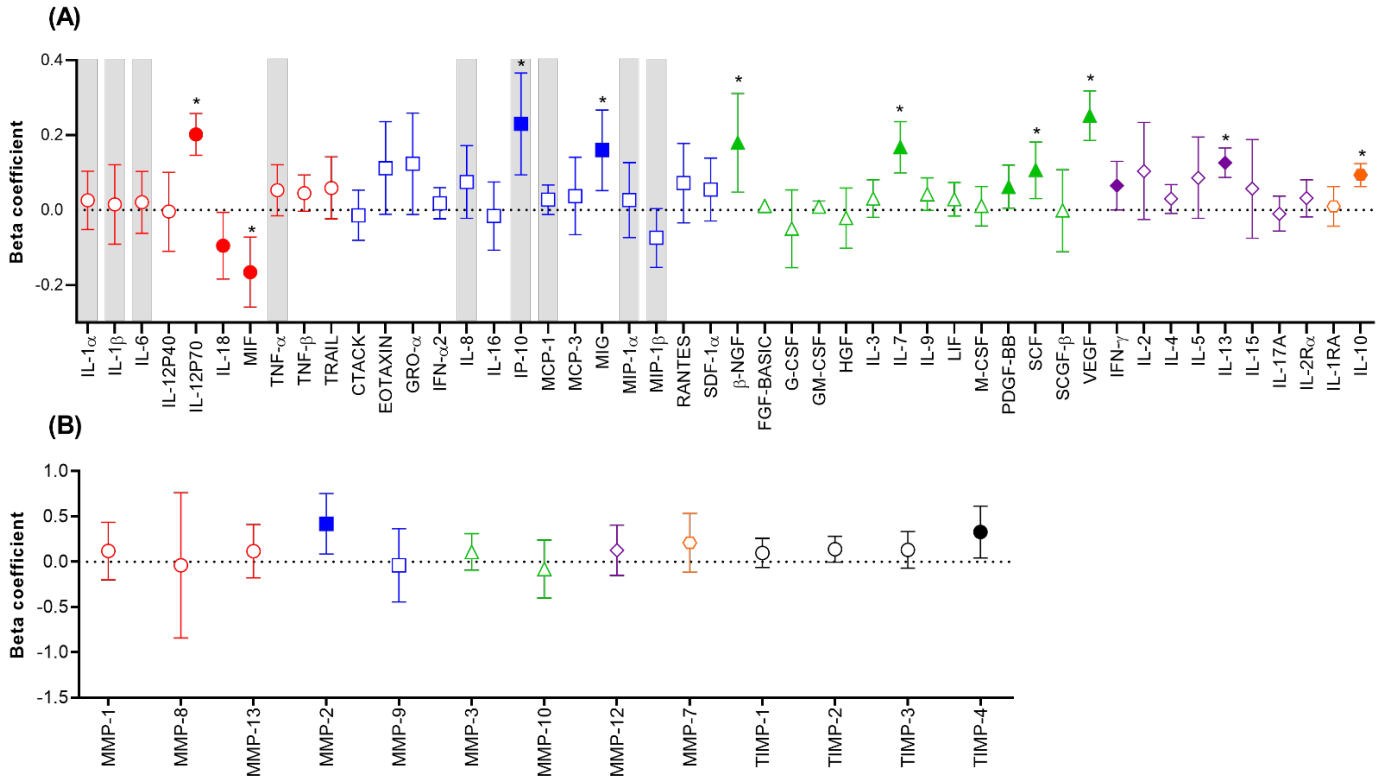

Figure 1. Association between protein biomarkers of inflammation and YcDNA detection in female genital specimens. β-coefficients and corresponding P-values for cytokine associations were determined using multivariable linear mixed models adjusting for age, any STI (*C. trachomatis, N. gonorrhoea, T. vaginalis, M. genitalium*), Nugent Score, number of vaginal sex acts in the past 30 days, randomization arm, and time in study. β-coefficients and corresponding P-values for MMP/TIMP associations were determined using multivariable linear regression models adjusting for age, any STI (*C. trachomatis, N. gonorrhoea, T. vaginalis, M. genitalium*), Nugent Score, number of vaginal sex acts in the past 30 days, randomization arm, and inflammation status. β-coefficients are depicted by shapes and error bars indicate the 95% CI. Significant P-values (P<0.05) are indicated by filled symbols and significance after FDR adjustment is indicated by (*). (**A)** Cytokines are ordered according to functions: pro-inflammatory (red circles), chemotactic (blue squares), growth/haematopoiesis (green triangles), adaptive response (purple diamonds), and regulatory (orange hexagons) cytokines. Gray shadings represent the nine cytokines/chemokines previously associated with the definition of genital inflammation and/or in demonstrating its association with the risk of HIV infection (2, 3). (**B)** MMPs are grouped according to their functions: collagenases (red circles), gelatinases (blue squares), stromelysins (green triangles), macrophage elastase (purple diamond), matrilysin (orange hexagon), and TIMPs are represented by black circles.

Increased detection of BV-associated microbes at the FGT linked to semen exposure

GEE models were used to determine whether semen exposure was linked to an increased presence of BV-associated microbes at the FGT. Women with detectable YcDNA had a significantly increased presence of *P. bivia* (OR=1.970; CI 1.309, 2.965; P=0.001; **Table 2**) compared to those without, after adjusting for age, any STI, the number of vaginal sex acts in the past 30 days, inflammation status, time in study, and randomization arm. This association between YcDNA detection and increased presence of *P. bivia* maintained significance after FDR adjustments (P=0.007).

Table 2. Comparison of vaginal microbes between women with and without detectable YcDNA.

Microbe	OR (95% CI)	P-Value	FDR	OR (95% CI)	Adj P-Value	FDR
L. crispatus	1.083 (0.766 - 1.529)	0.653	0.653	1.082 (0.763 - 1.534)	0.659	0.659
L. jensenii	0.752 (0.514 - 1.099)	0.141	0.237	0.736 (0.506 - 1.070)	0.109	0.189
A. vaginae	0.666 (0.379 - 1.171)	0.158	0.237	0.647 (0.370 - 1.130)	0.126	0.189
BVAB2	1.141 (0.797 - 1.633)	0.472	0.566	1.136 (0.792 - 1.631)	0.489	0.586
G. vaginalis	1.427 (0.990 - 2.058)	0.057	0.171	1.362 (0.942 - 1.968)	0.100	0.189
P. bivia	1.954 (1.312 - 2.911)	**0.001**	**0.006**	1.970 (1.309 - 2.965)	**0.001**	**0.007**

OR and 95% CI were determined using a GEE model with a logit link to account for repeated measures. The unadjusted model controlled for randomization arm and time. The adjusted model additionally controlled for age, the number of vaginal sex acts in the past 30 days, any STI (*C. trachomatis, N. gonorrhoea, T. vaginalis, M. genitalium*), and inflammation status. Significant P-values (P<0.05) are indicated in bold font.

The presence of semen was not associated with immune cell recruitment at the FGT

Since alterations in mucosal cytokines and microbial microenvironments are associated with increased frequency of local HIV-susceptible cells (2, 6), we assessed the impact of semen exposure on the pool of available T cell targets at the FGT. Linear mixed models were used to compare immune cell frequencies between women with detectable YcDNA and those without. Immune cell frequencies were similar between women with detectable YcDNA in their vaginal specimens and those without (**Figure 2**).

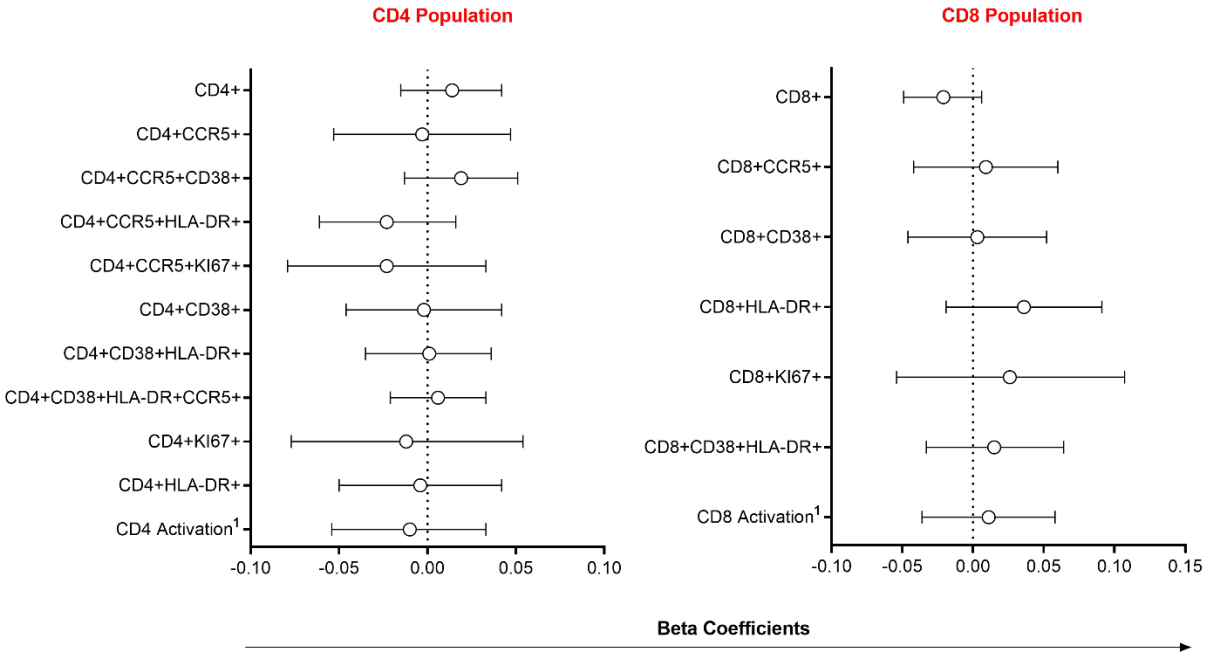

Figure 2. Association between immune cell frequencies and YcDNA detection in female genital specimens. β-coefficients and corresponding P-values were determined using multivariable linear mixed models adjusted for age, any STI (*C. trachomatis, N. gonorrhoea, T. vaginalis, M. genitalium*), Nugent Score, number of vaginal sex acts in the past 30 days, inflammation status, time in study and randomization arm. β-coefficients are depicted by shapes and error bars indicate the 95% CI. [1]Activation refers to cells expressing CCR5, HLA-DR, and/or CD38.

Discussion

Studies have demonstrated that semen contains several bioactive molecules with the ability to alter vaginal flora, induce cytokine production, and immune cell recruitment to the FGT after condomless sex (11-13, 17, 18, 25, 49-53). However, few studies investigated the impact of semen exposure on biomarkers of female genital inflammation in relation to HIV acquisition risk. Genital inflammation in women has been linked to an increased susceptibility to HIV infection (2), if semen exposure alters biomarkers of inflammation, then women may be at greater risk of acquiring the virus. Here we demonstrate that semen exposure as measured by YcDNA detection, but not self-report of condom use, had a greater association with biomarkers of epithelial barrier integrity and modulation of BV-associated bacteria than with the cytokine and immune cell responses related to female genital inflammation and HIV risk.

Traditionally, HIV prevention trials and reproductive health studies rely greatly on self-reported data despite acknowledgement of over-reporting (28-30, 54, 55). This study demonstrated a high level of

discordance between self-reported condom use and the detection of semen biomarkers in vaginal specimens. In this study, almost a third of the women reporting consistent condom use with their partner had detectable YcDNA in their genital specimens. The challenges associated with inaccurate reporting of condom use among women are established and include consistency of condom use, incorrect condom use, condom failure, social desirability bias, and recall bias, to name a few (56-62). However, women without detectable YcDNA may either represent those who did use condoms, those who abstained from sex within 15 days, or those who had condomless sex later than 15 days prior to genital sampling. Condom use was over-reported in this study, highlighting the need for routine objective screening for the presence of semen as a biomarker of condomless sex in future HIV prevention studies.

YcDNA detection was associated with marital status, a higher median number of reported vaginal sex acts in the past 30 days, living with or often seeing a partner, and a higher number of lifetime pregnancies compared to YcDNA negative women. The increased presence of semen markers in CVLs from women in stable relationships may be due to several factors, including reduced HIV/STI risk perception and/or an inability to negotiate condom use (63), and late use or early removal of condoms. Additionally, a greater frequency of coital episodes has been associated with increased odds of condomless sex in women (64). A greater number of coital acts with an infected partner may also increase the potential for exposure to sexually transmitted pathogens. Here, gonorrhoeae was associated with YcDNA detection in women. Gonorrhoeae is sexually transmitted and condomless sexual intercourse with an infected partner is a major risk factor for acquiring the infection (65). However, YcDNA detection was not associated with the other STIs measured, which may be due to a relatively low prevalence of each STI (NG, CT, TV, and MG) in this study. Women with detectable YcDNA in their genital specimens also had a significantly higher median Nugent Score, suggesting that condomless sex is associated with alterations in the vaginal microbiome. These findings are highly consistent with another study reporting that Nugent Scores were significantly associated with the presence of semen in vaginal specimens (66).

Here we investigated the impact of semen exposure on biomarkers of inflammation associated with HIV acquisition in women. YcDNA detection in female genital specimens was used as a biomarker of semen exposure within 15 days of genital sampling (31-33). YcDNA detection at the FGT predicted significantly higher levels of 11/48 cytokines, and reduced concentrations of two, IL-18, and MIF. Increased concentrations of IL-18 and MIF have previously been implicated in male infertility and reduced sperm motility (67, 68). During reproduction, altered immune responses at the FGT may promote reduced concentrations of these cytokines to facilitate conception. The increase in concentrations of several cytokines is consistent with other studies reporting that semen exposure is associated with cytokine upregulation at the FGT (9, 10, 12, 13, 69). Here, semen exposure was associated with both a pro-

inflammatory (IFN-γ, IL-12p70, and IP-10) and anti-inflammatory (IL-10) immune response at the FGT (2, 70, 71). These data support the potential for an initial inflammatory response at the FGT required for embryo implantation and removal of defective sperm, followed by a quick shift to an anti-inflammatory immune response defined by the secretion of IL-10, which may function to promote tolerance to the paternal antigens (14, 15, 25, 72-75). Further, increased concentrations of MIP-1α, MIP-1β, IP-10, and IL-8 have previously been associated with HIV risk in the CAPRISA 004 trial (2). Of these, YcDNA detection was associated only with significant increases in IP-10 in this study, suggesting a limited relationship between semen exposure and those cytokines commonly known to increase the risk of HIV acquisition in women. However, considering that YcDNA is detectable up to 15 days after semen exposure, a biomarker of more recent semen exposure may better characterise the initial pro-inflammatory cytokine response at the FGT which may have implications for HIV risk.

An intact epithelial barrier is a primary host defence against HIV entry and infection. MMPs are proteolytic zinc-dependent enzymes responsible for the degradation and remodelling of the epithelial barrier and have been associated with elevated genital cytokine concentrations (4, 76). YcDNA detection was associated with significant increases in MMP-2 and its regulator TIMP-4. TIMP-4 was likely upregulated at the FGT in response to the high concentrations of MMP-2, since it prevents the activity of MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9 (77, 78). Friction during sexual intercourse has also been associated with microabrasions at the FGT (22, 23). Increased production of MMPs and TIMPs in response to semen exposure and/or friction during condomless sex may compromise the integrity of the female genital epithelial barrier, thereby facilitating HIV entry and access to local target cells. In support of this hypothesis, several studies have demonstrated increased HIV incidence among women with reduced epithelial barrier integrity (79-82). Given that MMPs/TIMPs are only a small subset of proteins that function in maintaining epithelial barrier integrity, further studies are needed using an expanded panel of barrier proteins to reliably assess the impact of condomless sex on the vaginal epithelium.

Recent studies have suggested that vaginal bacteria can also contribute to genital inflammation known to increase HIV risk in women (5, 6). Here, semen exposure was associated with a significantly increased presence of *P. bivia* at the FGT. Semen has an alkaline pH and raises the acidic pH of the vagina to 7.0 or higher after sexual intercourse without a condom, this may favour the growth of BV-associated microbes (20, 83). Additionally, semen also contains a diverse array of microbial communities that have the potential to alter the vaginal microbial composition (17-19). A study conducted in young South African women demonstrated that a diverse vaginal microbiome dominated by anaerobic bacteria was associated with a four-fold greater risk of acquiring HIV (6). Given that YcDNA detection was associated with an increased

presence of *Prevotella*, which has previously been related to HIV risk (6), semen-induced alterations in the vaginal microbiome may have implications for HIV susceptibility in women.

Since HIV requires access to local target cells to establish productive infection, we assessed the impact of YcDNA detection on endocervical T cell frequencies. Here, YcDNA detection was not associated with significant alterations in HIV target cell frequencies at the FGT. This lack of an association between YcDNA detection and endocervical T cell alterations may be due to the longer range of semen detection. Additionally, Th17 cells that are preferential targets for HIV infection (84) and Treg cell populations which may be induced by semen for tolerance to the paternal antigens (16, 85, 86), were not assessed in this study.

The strength of this study lies in the abundance of immunological and microbial data to assess the impact of semen exposure on the FGT in longitudinal analyses. Few studies have investigated the impact of semen exposure at the FGT in the context of HIV. Here, we used a biomarker of semen exposure to reliably assess the impact of condomless sex on multiple biomarkers of inflammation, including those previously associated with HIV risk in women. However, considering potential variations in immune alterations during a period of up to 15 days after semen exposure, comparisons with a biomarker of more recent semen exposure may be required to better assess semen-induced alterations at the FGT. This study was limited by the yield of cervix-derived T cells required to assess both immune activation and regulation, and further investigation is necessary to determine whether YcDNA detection is associated with alterations in endocervical Treg and Th17 cell populations. Here, common BV-associated microbes were assessed using PCR which limits the detection of semen-associated alterations to those specific microbes. The use of 16S rRNA gene sequencing may provide a more comprehensive picture of the impact of semen exposure on the vaginal microbiome. The study was limited in the ability to control for other factors associated with alterations in the immune and microbial environments of the FGT, including the use of vaginal insertive products, menstruation, contraceptive use, etc. Nonetheless, this study demonstrates that semen exposure is associated with immune and microbial changes at the FGT that may have implications for HIV susceptibility in women, and additional studies are required to further characterise these alterations, assess their robustness, and confirm the relative impact on HIV risk.

Here, YcDNA detection, but not self-report of condom use, was associated with shifts in the immune and microbial profiles of the FGT. Although this biomarker of condomless sex <15 days of sampling was not generally associated with the cytokines and immune cells commonly implicated in raised HIV risk, it was, however, associated with biomarkers of epithelial barrier integrity and an increased presence of *P. bivia* which may still have implications for HIV susceptibility in women. This study provides insight into the impact of semen exposure on the FGT and underscores the importance of further studies to better understand the kinetics of these alterations following semen exposure. Taken together, this study emphasises the

reliability of biomarkers of semen exposure over self-report in analyses of female genital immunity and highlights the importance of incorporating biomarkers of semen exposure and controlling for such evidence of condomless sex in future STI/HIV prevention studies. Understanding the specific contribution of semen to a vaginal immune environment conducive to HIV infection may advise the design of targeted biomedical approaches to prevent HIV infection in women.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

J.J., S.N., and L.J.P.L contributed to the conception and design of the study. J.J., L.J.P.L., A.M., and R.S. performed the experiments. J.J., L.J.P.L., and F.O. analysed and interpreted the data. J.J., S.N., L.J.P.L., J.S.P., Q.A.K., L.E.M and S.S.A.K wrote the manuscript

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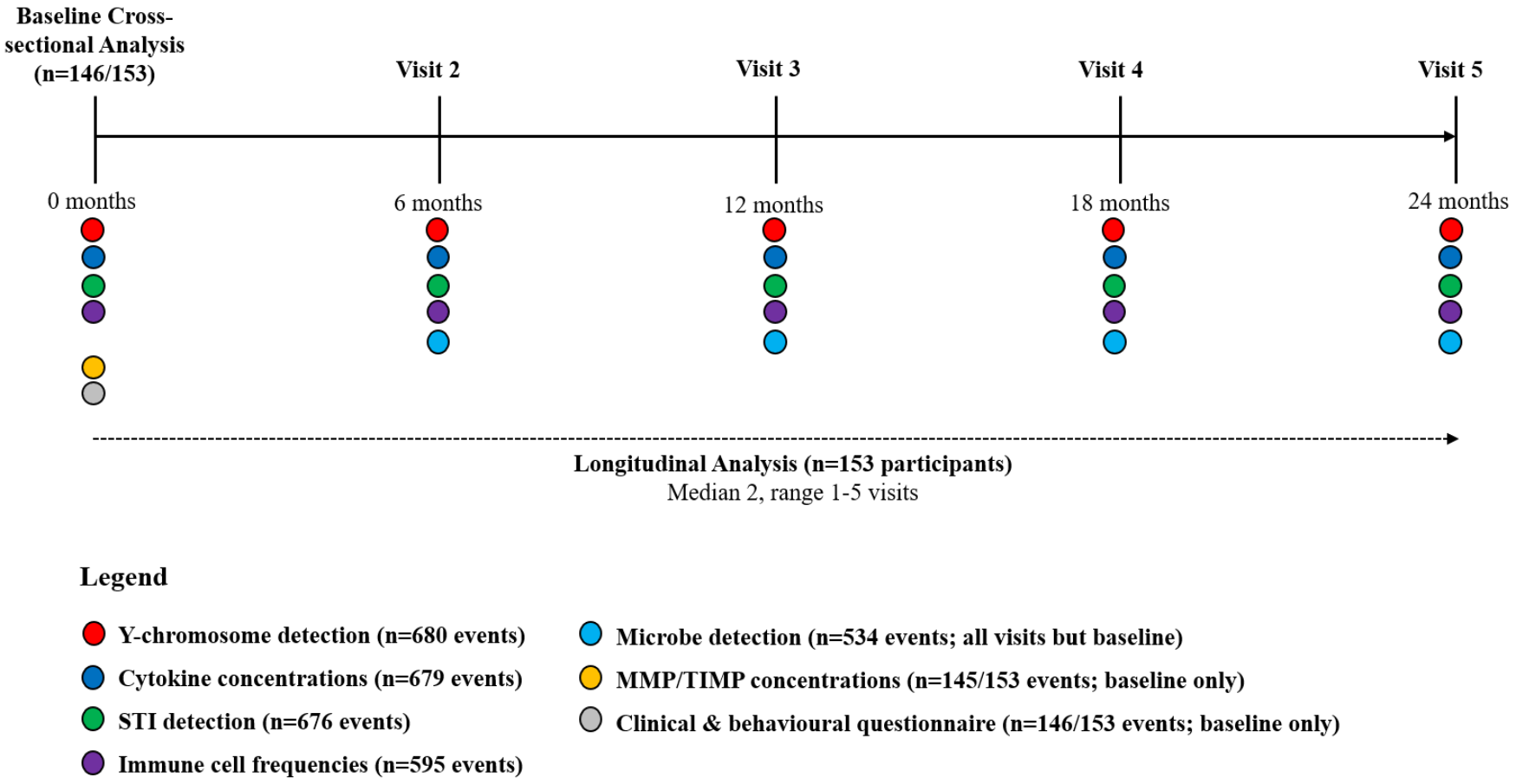
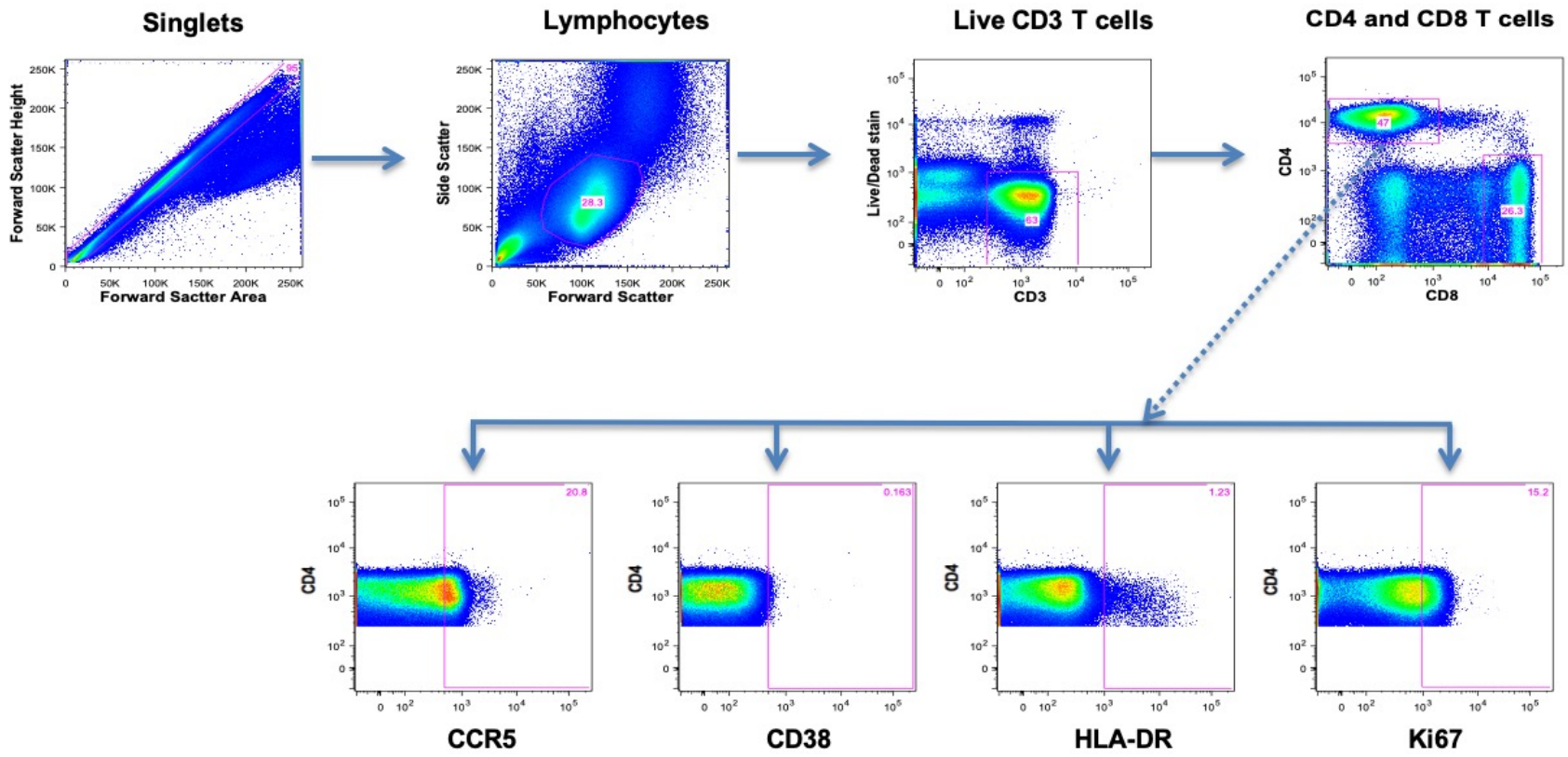


Supplementary Figure 1. Graphical representation of the data available at baseline and longitudinally for CAPRISA 008 trial participants. This study included 153 participants from the CAPRISA 008 trial. Genital specimens were collected from participants biannually during the 2-year trial (median 2, range 1-5 visits). The dataset includes all participants with available YcDNA data. Seven of the 153 participants had YcDNA data for all visits but baseline and were therefore excluded from cross-sectional baseline analysis but were included in the longitudinal analyses. MMP/TIMP and questionnaire data were only available at baseline, and microbe PCR data were available at all visits but baseline.



Supplementary Figure 2. Representative flow cytometry gating plot for the assessment of T cell activation. A singlet gate was used to exclude cell doublets. Live cells were identified, followed by lymphocytes, and the CD3+ T cell population. The CD3+ T cell population was divided into CD4+ and CD8+ T cell subsets. The expression of activation markers (CD38+, HLA-DR+), the marker of proliferation (Ki67+), and the HIV co-receptor for entry (CCR5+) was assessed on CD4+ and CD8+ T cells.

Supplementary Table 1. Baseline associations between cytokine concentrations and women reporting never using a condom during intercourse with their partner.

	BIVARIABLE					MULTIVARIABLE				
		95% CI					95% CI			
Cytokines	**β-coefficient**	**Lower**	**Upper**	**P Value**	**FDR**	**β-coefficient**	**Lower**	**Upper**	**P Value**	**FDR**
IL-1α	0.099	-0.209	0.408	0.524	0.954	0.018	-0.300	0.336	0.912	0.975
IL-1β	0.061	-0.393	0.516	0.788	0.954	-0.060	-0.501	0.380	0.785	0.975
IL-6	0.037	-0.229	0.304	0.781	0.954	-0.031	-0.322	0.260	0.832	0.975
IL-12p40	0.011	-0.348	0.371	0.950	0.954	-0.006	-0.417	0.404	0.975	0.975
IL-12p70	0.054	-0.159	0.267	0.617	0.954	0.027	-0.202	0.256	0.815	0.975
IL-18	0.194	-0.162	0.551	0.280	0.954	0.147	-0.227	0.521	0.436	0.975
MIF	0.241	-0.154	0.635	0.228	0.954	0.155	-0.251	0.561	0.448	0.975
TNF-α	0.028	-0.202	0.258	0.808	0.954	-0.017	-0.258	0.224	0.889	0.975
TNF-β	0.066	-0.119	0.252	0.478	0.954	0.052	-0.158	0.262	0.622	0.975
TRAIL	0.110	-0.216	0.435	0.504	0.954	0.049	-0.282	0.379	0.769	0.975
CTACK	0.080	-0.177	0.337	0.537	0.954	0.022	-0.250	0.295	0.871	0.975
EOTAXIN	0.182	-0.257	0.621	0.411	0.954	0.109	-0.375	0.593	0.654	0.975
GRO-α	0.075	-0.462	0.613	0.781	0.954	0.126	-0.445	0.697	0.661	0.975
IL-8	-0.047	-0.401	0.308	0.793	0.954	-0.070	-0.450	0.310	0.714	0.975
IL-16	0.080	-0.233	0.394	0.612	0.954	0.011	-0.335	0.357	0.950	0.975
IP-10	-0.173	-0.681	0.336	0.500	0.954	-0.119	-0.618	0.379	0.634	0.975
MCP-1	0.046	-0.109	0.202	0.554	0.954	0.073	-0.103	0.248	0.411	0.975
MCP-3	0.112	-0.224	0.447	0.510	0.954	0.108	-0.272	0.489	0.571	0.975
MIG	0.045	-0.430	0.519	0.851	0.954	0.084	-0.410	0.578	0.736	0.975
MIP-1α	0.143	-0.188	0.475	0.391	0.954	0.031	-0.283	0.345	0.844	0.975
MIP-1β	-0.042	-0.360	0.276	0.793	0.954	-0.038	-0.383	0.306	0.825	0.975
RANTES	0.183	-0.186	0.552	0.326	0.954	0.236	-0.157	0.630	0.234	0.975
IFN-α2	0.067	-0.102	0.236	0.429	0.954	0.049	-0.138	0.237	0.600	0.975
SDF-1α	0.145	-0.139	0.428	0.312	0.954	0.108	-0.201	0.418	0.488	0.975
β-NGF	0.057	-0.447	0.561	0.821	0.954	0.080	-0.458	0.618	0.768	0.975
FGF BASIC	-0.003	-0.059	0.054	0.928	0.954	-0.022	-0.078	0.034	0.433	0.975
G-CSF	0.072	-0.326	0.469	0.721	0.954	-0.011	-0.448	0.425	0.960	0.975
GM-CSF	-0.017	-0.059	0.025	0.421	0.954	-0.025	-0.072	0.022	0.298	0.975
HGF	0.027	-0.314	0.367	0.877	0.954	-0.094	-0.439	0.250	0.587	0.975
IL-3	0.063	-0.177	0.303	0.603	0.954	0.068	-0.207	0.343	0.622	0.975
IL-7	0.102	-0.154	0.358	0.431	0.954	-0.008	-0.224	0.208	0.942	0.975
IL-9	0.005	-0.156	0.166	0.954	0.954	-0.038	-0.199	0.123	0.639	0.975
LIF	0.083	-0.112	0.278	0.400	0.954	0.030	-0.166	0.226	0.762	0.975
M-CSF	0.075	-0.138	0.287	0.485	0.954	0.057	-0.171	0.286	0.618	0.975
PDGF-BB	0.112	-0.078	0.302	0.245	0.954	0.072	-0.115	0.258	0.445	0.975
SCF	0.026	-0.344	0.395	0.891	0.954	0.036	-0.350	0.422	0.853	0.975
SCGF-β	0.044	-0.343	0.431	0.822	0.954	-0.049	-0.463	0.365	0.815	0.975
VEGF	0.048	-0.230	0.326	0.730	0.954	0.012	-0.288	0.312	0.936	0.975
IFN-γ	0.036	-0.149	0.221	0.701	0.954	-0.023	-0.210	0.165	0.809	0.975
IL-2	0.128	-0.328	0.585	0.577	0.954	0.095	-0.422	0.611	0.716	0.975
IL-4	0.024	-0.136	0.184	0.763	0.954	-0.040	-0.173	0.093	0.554	0.975
IL-5	0.336	-0.042	0.715	0.081	0.954	0.321	-0.075	0.717	0.110	0.975
IL-13	0.083	-0.088	0.255	0.336	0.954	0.033	-0.137	0.203	0.698	0.975
IL-15	0.219	-0.281	0.720	0.385	0.954	0.323	-0.232	0.877	0.249	0.975
IL-17A	-0.013	-0.164	0.138	0.863	0.954	-0.036	-0.196	0.125	0.657	0.975
IL-2rα	0.005	-0.176	0.186	0.954	0.954	-0.017	-0.210	0.177	0.862	0.975
IL-10	0.024	-0.094	0.143	0.683	0.954	-0.002	-0.123	0.118	0.972	0.975
IL-1RA	0.034	-0.123	0.190	0.668	0.954	0.029	-0.130	0.188	0.715	0.975

β-coefficients and corresponding P-values were determined using linear regression models. Bivariable regression models were adjusted for randomization arm. Multivariable regression models were adjusted for age, any STI (*C. trachomatis. N. gonorrhoea. T. vaginalis. M. genitalium*), Nugent Score, number of vaginal sex acts in the past 30 days, and randomization arm.

Supplementary Table 2. Baseline associations between MMP/TIMP concentrations and women reporting never using a condom during intercourse with their partner.

	BIVARIABLE					MULTIVARIABLE				
		95% CI					95% CI			
MMP/ TIMP	**β-coefficient**	**Lower**	**Upper**	**P Value**	**FDR**	**β-coefficient**	**Lower**	**Upper**	**P Value**	**FDR**
MMP-1	0.148	-0.338	0.634	0.545	0.796	0.067	-0.444	0.578	0.793	0.836
MMP-2	0.275	-0.294	0.844	0.338	0.796	0.239	-0.270	0.749	0.352	0.836
MMP-3	0.103	-0.241	0.447	0.551	0.796	0.129	-0.217	0.475	0.458	0.836
MMP-7	0.040	-0.488	0.568	0.882	0.891	-0.119	-0.699	0.462	0.684	0.836
MMP-8	-0.584	-1.897	0.729	0.378	0.796	-0.791	-2.013	0.432	0.201	0.836
MMP-9	-0.172	-0.859	0.515	0.619	0.796	-0.332	-0.889	0.225	0.238	0.836
MMP-10	0.202	-0.291	0.695	0.416	0.796	0.103	-0.406	0.612	0.687	0.836
MMP-12	-0.175	-0.720	0.371	0.525	0.796	-0.051	-0.536	0.435	0.836	0.836
MMP-13	-0.033	-0.513	0.447	0.891	0.891	-0.121	-0.600	0.358	0.615	0.836
TIMP-1	0.182	-0.086	0.451	0.180	0.360	0.216	-0.058	0.491	0.120	0.240
TIMP-2	-0.058	-0.305	0.190	0.643	0.643	-0.025	-0.293	0.242	0.850	0.850
TIMP-3	0.212	-0.090	0.515	0.166	0.360	0.263	-0.069	0.596	0.118	0.240
TIMP-4	0.189	-0.240	0.618	0.383	0.511	0.206	-0.257	0.668	0.378	0.504

β-coefficients and corresponding P-values were determined using linear regression models. Bivariable regression models were adjusted for randomization arm. Multivariable regression models were adjusted for age, any STI (*C. trachomatis, N. gonorrhoea, T. vaginalis, M. genitalium*), Nugent Score, number of vaginal sex acts in the past 30 days, inflammation status, and randomization arm.

Supplementary Table 3. Baseline associations between immune cell frequencies and women reporting never using a condom during intercourse with their partner.

	BIVARIABLE					MULTIVARIABLE				
		95% CI					95% CI			
Immune cell subsets	**β-coefficient**	**Lower**	**Upper**	**P value**	**FDR**	**β-coefficient**	**Lower**	**Upper**	**P value**	**FDR**
CD4+	-0.051	-0.132	0.029	0.209	0.502	-0.042	-0.136	0.051	0.370	0.757
CD4+CCR5+	0.137	0.001	0.273	0.049	0.444	0.118	-0.029	0.265	0.113	0.639
CD4+CCR5+CD38+	0.070	-0.027	0.168	0.152	0.489	0.072	-0.034	0.179	0.180	0.639
CD4+CCR5+HLA-DR+	0.081	-0.033	0.195	0.163	0.489	0.075	-0.044	0.195	0.213	0.639
CD4+CCR5+KI67+	0.059	-0.102	0.220	0.465	0.620	0.044	-0.116	0.205	0.578	0.757
CD4+CD38+	-0.012	-0.130	0.105	0.834	0.834	-0.008	-0.133	0.117	0.896	0.896
CD4+CD38+HLA-DR+	0.037	-0.039	0.112	0.335	0.620	0.034	-0.051	0.120	0.424	0.757
CD4+CD38+HLA-DR+CCR5+	0.060	-0.006	0.126	0.074	0.444	0.056	-0.019	0.131	0.138	0.639
CD4+KI67+	0.052	-0.120	0.224	0.545	0.654	0.036	-0.149	0.222	0.694	0.757
CD4+HLA-DR+	0.031	-0.088	0.150	0.605	0.660	0.026	-0.100	0.151	0.683	0.757
CD4 Total Activation[1]	0.037	-0.055	0.128	0.424	0.620	0.020	-0.081	0.121	0.694	0.757
CD8+	0.021	-0.059	0.101	0.602	0.923	0.010	-0.081	0.101	0.828	0.963
CD8+CCR5+	0.055	-0.079	0.189	0.415	0.923	0.068	-0.079	0.216	0.359	0.963
CD8+CD38+	0.013	-0.117	0.142	0.846	0.923	0.010	-0.136	0.156	0.891	0.963
CD8+HLA-DR+	-0.001	-0.148	0.145	0.988	0.988	-0.008	-0.170	0.153	0.917	0.963
CD8+CD38+HLA-DR+	0.010	-0.092	0.113	0.840	0.923	0.004	-0.113	0.120	0.948	0.963
CD8+KI67+	0.127	-0.070	0.324	0.200	0.923	0.070	-0.145	0.284	0.515	0.963
CD8 Total Activation[1]	0.011	-0.088	0.110	0.830	0.923	-0.003	-0.114	0.108	0.963	0.963

β-coefficients and corresponding P-values were determined using linear regression models. Bivariable regression models were adjusted for randomization arm. Multivariable regression models were adjusted for age, any STI (*C. trachomatis, N. gonorrhoea, T. vaginalis, M. genitalium*), Nugent Score, number of vaginal sex acts in the past 30 days, inflammation status, and randomization arm. [1]Total activation refers to cells expressing CCR5, HLA-DR, KI67, and/or CD38.

CHAPTER 3

Transient association between semen exposure and biomarkers of genital inflammation in South African women at risk of HIV infection

Exposure to semen from an infected male partner is the primary mode of HIV transmission in women (Royce *et al*., 1997). Semen contains several components that alter the immune responses at the female reproductive tract for conception but may also promote an immune and microbial environment conducive to HIV infection in women. The findings in chapter 2 demonstrated that semen exposure measured by YcDNA was associated more with alterations in mucosal barrier proteins and *P. bivia* detection than with the cytokines or immune cells related to HIV risk in women. Considering the transience of semen-associated immune responses (Sharkey *et al*., 2012; Rametse *et al*., 2018), and that YcDNA can be detected in vaginal fluid for up to 15 days post-exposure (Thurman *et al*., 2016; Zenilman *et al*., 2005; Brotman *et al*., 2010), a biomarker of more recent sex may better characterise a short-lived inflammatory response at the FGT with implications for HIV risk.

Chapter 3 investigates the contribution of more recent semen exposure and YcDNA quantities on the inflammatory environment associated with HIV seroconversion in women. Vaginal exposure to semen was initially determined using YcDNA detection. Since PSA is a biomarker of semen exposure within 48 hours (Thurman *et al*., 2016; Bahamondes *et al*., 2008; Lilja, 1993), the addition of PSA further stratified the participant groups into semen exposure within 48 hours (PSA+YcDNA+), semen exposure within 3-15 days (PSA-YcDNA+), and no semen exposure (PSA-YcDNA-). The levels of YcDNA in vaginal fluid were also assessed to determine the relationship between male protein concentrations and female GI. This study demonstrated that higher YcDNA concentrations were also specifically related to more recent semen exposure (i.e., PSA detection). Both higher YcDNA concentrations and PSA detection correlated with higher levels of several cytokines, barrier-related proteins, and increased HIV target cell frequencies at the FGT. Additionally, PSA detection was also related to BVAB2, *P. bivia*, and *G. vaginalis* detection and reduced presence of *Lactobacillus jesenii*. Here, recent semen exposure was associated with an inflammatory response at the female genital mucosa; however, these responses were reduced by 3-15 days post semen exposure. Albeit short-lived, changes in the vaginal immune and microbial environments induced by recent semen exposure may promote HIV acquisition in high-risk women.

Dr Ngcapu, Dr Liebenberg, and I conceptualized and designed the study. I conducted the laboratory assays to detect cervicovaginal cytokine concentrations, biomarkers of epithelial barrier integrity and to determine semen exposure in vaginal specimens by YcDNA and PSA detection. I analysed and interpreted the data, which was subsequently validated by Mrs. Osman, the study statistician. I wrote the manuscript, and all authors proofread and approved the final version. Aspects of this manuscript have been presented as a poster discussion at the 23rd Virtual International AIDS Conference, 6-10 July

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Transient association between semen exposure and biomarkers of genital inflammation in South African women at risk of HIV infection

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Abstract

Introduction: Semen induces mucosal changes in the female reproductive tract to improve pregnancy outcomes. Since semen-induced alterations are likely short-lived and genital inflammation is linked to HIV acquisition in women, we investigated the contribution of recent semen exposure on biomarkers of genital inflammation in women at high HIV risk and the persistence of these associations.

Methods: We assessed stored genital specimens from 152 HIV-negative KwaZulu-Natal women who participated in the CAPRISA 008 trial between November 2012 and October 2014. During the two-year study period, 651 vaginal specimens were collected biannually (mean five samples per woman). Cervicovaginal lavage (CVL) was screened for prostate-specific antigen (PSA) by ELISA, while Y-chromosome DNA (YcDNA) detection and quantification were conducted by RT-PCR, representing semen exposure within 48 hours (PSA+YcDNA+) and semen exposure within 3-15 days (PSA-YcDNA+). Soluble protein concentrations were measured in CVLs by multiplexed ELISA. T-cell frequencies were assessed in cytobrushes by flow-cytometry, and vulvovaginal swabs were used to detect common vaginal microbes by PCR. Linear mixed models adjusting for factors associated with genital inflammation and HIV risk were used to assess the impact of semen exposure on biomarkers of inflammation over multiple visits.

Results: Here, 19% (125/651) of CVLs were PSA+YcDNA+, 14% (93/651) were PSA-YcDNA+, and 67% (433/651) were PSA-YcDNA-. Semen exposure was associated with how often women saw their partners, the frequency of vaginal sex in the past month, HSV-2 antibody detection, current gonorrhea infection, and Nugent Score. Both PSA detection (PSA+YcDNA+) and higher cervicovaginal YcDNA concentrations predicted increases in several cytokines, barrier-related proteins (MMP-2, TIMP-1, and TIMP-4), and activated CD4+CCR5+HLA-DR+ T-cells (β=0.050; CI 0.001-0.098; p=0.046) and CD4+HLA-DR+ T-cells (β=0.177; CI 0.016-0.339; p=0.032), respectively. PSA detection was specifically associated with raised pro-inflammatory cytokines (including IL-6, TNF-α, IP-10, and RANTES), and with the detection of BVAB2 (OR=1.755; CI 1.116-2.760; p=0.015), *P. bivia* (OR=1.886; CI 1.102-3.228; p=0.021), and *G. vaginalis* (OR=1.815; CI 1.093-3.015; p=0.021).

Conclusions: More recent semen exposure was associated with raised levels of inflammatory biomarkers and the detection of BV-associated microbes, which declined by 3-15 days post-exposure. Although transient, semen-induced alterations may have implications for HIV susceptibility in women.

Introduction

Semen induces mucosal alterations at the female reproductive tract to improve pregnancy outcomes [1-3]. However, the contribution of semen-associated changes to Human Immunodeficiency Virus (HIV) risk in women is unclear. Although an optimal vaginal environment has several defences to prevent infection, genital inflammation limits host defences [4-7] and increases HIV infection risk, even by less fit viruses [4, 8]. Biomarkers of genital inflammation include elevated cervicovaginal cytokines, immune cell recruitment, alterations in barrier-related proteins, and increased microbial diversity [4-7]. Semen contains several bioactive molecules and a diverse array of microbial communities [9-12], which may alter HIV susceptibility in women by promoting genital inflammation. A better understanding of the female immune response during condomless sex and the semen properties that promote genital inflammation may aid in designing effective biomedical HIV prevention strategies in women.

Biomarkers that detect semen within vaginal specimens may be beneficial to characterise the effects of semen exposure on female genital inflammation and HIV risk *ex vivo*. Prostate-specific antigen (PSA) and Y-chromosome deoxyribonucleic acid (YcDNA) detection are well-characterised biomarkers of semen exposure [13-18]. PSA is produced by the prostate gland, and detection in vaginal fluids at concentrations ≥1 ng/ml can be used to assess semen exposure at the female genital tract (FGT) even from vasectomized males [17-19]. In women, PSA is present for a short duration following condomless sex, and detection indicates semen exposure within 48 hours of cervicovaginal sampling [14, 18]. Alternatively, the measurement of YcDNA can be used as a more stable marker of semen exposure [15, 16]. YcDNA detection involves polymerase chain reaction (PCR) amplification of the testis-specific protein Y-encoded (TSPY) gene region and the sex-determining region Y (SRY) gene region in the Y-chromosome [15, 20]. YcDNA can be detected in vaginal fluid up to 15 days after condomless sex [15, 16, 21]. Furthermore, since YcDNA is detectable in the presence of spermatozoa, YcDNA quantities at the FGT may indicate sperm count and seminal protein concentrations.

Considering the transience of semen-associated immune alterations in the FGT [1, 22], a pro-inflammatory immune response to semen exposure may be better characterised using a biomarker of recent condomless sex. Here, we hypothesized that more recent semen exposure and higher cervicovaginal YcDNA concentrations would be associated with the inflammatory environment related to HIV risk in women. To test this hypothesis, we compared immune and microbial markers of genital inflammation among women with evidence of semen exposure within 48 hours (PSA+YcDNA+), 3-15 days (PSA-YcDNA+), and no semen exposure within 15 days prior to genital sampling (PSA-YcDNA-). Additionally, we investigated the association between markers of genital inflammation and cervicovaginal concentrations of YcDNA, which may also reflect more recent sex and male protein concentrations at the FGT.

Methods

Study population and design

We enrolled 152 HIV-negative women between 20-44 years of age from KwaZulu-Natal who participated in the CAPRISA 008 study [23-25]. Women participated in the CAPRISA 008 study over two years, between November 2012 and October 2014, and were followed up for an average of 22 months [23, 25]. Demographic data were assessed at baseline, and vaginal specimens [including cervicovaginal lavage (CVL), cytobrushes, and vulvovaginal swabs] were collected at enrollment and biannually at months 6, 12, 18, 24, and study exit (average 5±1 visit; 651 genital specimens) as previously reported [24-27]. Semen biomarkers were measured in vaginal specimens collected at each of the multiple study visits per participant. The detection of semen biomarkers indicated whether semen exposure occurred between 0-2 or 3-15 days before specimen collections at the respective visits. Participants provided informed consent for the specimen storage and use in future studies (BFC237/010). This study protocol was approved by the University of KwaZulu-Natal Biomedical Research Ethics Committee (BE258/19).

Screening for semen in vaginal fluids

PSA ELISA

The Human PSA-total ELISA Kit (SIGMA-ALDRICH™) was used to detect PSA in CVL supernatants according to the manufacturer's protocol. Briefly, 100 µl of samples, standards, and negative controls were examined in duplicate. The CVL specimens were considered PSA positive if detectable concentrations were above the lowest standard's average concentration. Absorbance was measured at 450 nm with a VersaMax™ ELISA Microplate Reader. Detection of PSA within vaginal specimens indicated semen exposure within 48 hours before genital sampling.

YcDNA detection and quantification

DNA extraction was conducted on CVL pellets using the MagNAPure LC DNA Isolation Kit I (Roche Applied Science, Indianapolis, IN), as instructed by the manufacturer. The Human Y-chromosome Quantification Kit (PrimerDesign Ltd, UK) was used to detect a TSPY1 gene region present on the Y-chromosome within the extracted DNA. The Y-chromosome primer/probe mix and the *PrecisionFAST*™ Mastermix were used according to the manufacturer's instructions (PrimerDesign Ltd, UK). Amplification was conducted on the Applied Biosystems® QuantStudio™ 5 real-time (RT)-PCR System (Thermo Fisher Scientific). Detection of YcDNA within vaginal specimens indicated semen exposure within 15 days before genital sampling [15, 16]. The Quantifiler™ Trio DNA Quantification Kit from Applied Biosystems™ (Thermo Fisher Scientific) was used to quantify YcDNA concentrations in YcDNA+ CVL specimens as outlined in the manufacturer's protocol. The

assay simultaneously quantified the total amount of amplifiable human DNA and human male DNA in 10 µl of the sample.

Investigation of soluble biomarkers of genital inflammation

The levels of genital cytokines and barrier-related proteins were assessed in CVL supernatants using multiplexed ELISA assays. Cytokine concentrations were measured using the Bio-Plex Pro™ Human Cytokine 21-Plex and 27-Plex kits (Bio-Rad Laboratories) as previously described (**Table S1**) [28]. Concentrations of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) were quantified using the MMP 9-Plex, and TIMP 4-Plex kits (Bio-Rad Laboratories), respectively, as instructed by the manufacturer. MMP/TIMP measurements were performed on baseline samples (n=136; **Figure S1**). All analyte concentrations were measured using the Bio-Plex® 200 system (Bio-Rad Laboratories, Inc., USA).

Investigation of immune cell frequencies

Multiparametric flow cytometry was used to assess the expression of activation markers (CD38+ or HLA-DR+), the maker of proliferation (Ki67+), and the HIV co-receptor (CCR5+) on CD4+ T cells in cervical cytobrush-derived specimens. The viability of cervical mononuclear cells (CMCs) was determined using the LIVE/DEAD™ Fixable Dead Cell Staining Kit (Invitrogen) as outlined in the manufacturer's protocol. CMCs were treated with antibody-conjugated fluorophores, washed, and fixed (**Table S2**). Data were acquired on an LSRII flow cytometer (BD Immunocytometry Systems) and analysed with FlowJo™ Software version 9.9 (Tree Star, C, US). The gating strategy was previously published [24].

Detection of sexually transmitted infections (STIs) and vaginal microbes

Genital swabs were used to detect common STI pathogens and vaginal microbes, as previously described (**Table S3**) [29]. Multiplex PCR amplification was conducted to detect STI pathogens using the Fast-track Diagnostics STD9 detection kit, as outlined in the manufacturer's protocol. Common vaginal microbes were detected using Applied Biosystems™ TaqMan® assays [29]. All reactions were conducted using the Applied Biosystems 7500 RT-PCR machine (Thermo Fisher Scientific). Data on STI detection in vaginal specimens were available for all visits (n=648), while data on bacterial vaginosis (BV)-associated bacteria were generated for all visits except baseline (n=515; **Figure S1**). BV was diagnosed using Nugent scoring by Gram stain microscopy [30].

Statistical considerations

Statistical analyses were performed using GraphPad Prism version 8.4.3 (GraphPad Software, San Diego, CA), STATA version 15.0 (StataCorp., College Station, Texas, USA), and SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Continuous variables and proportions were compared at baseline using the Kruskal-Wallis test and the Chi-square or Fisher's exact test, respectively. Longitudinal analyses included one model with YcDNA concentrations as the main exposure variable and a complementary model with PSA/YcDNA categories as the main exposure variable. CVL specimens were classified in terms of PSA and YcDNA detection as PSA-YcDNA-, PSA+YcDNA+, and PSA-YcDNA+. The Mann-Whitney test was used to determine whether YcDNA concentrations differed significantly between PSA+YcDNA+ and PSA-YcDNA+ specimens at baseline. Receiver operating characteristic (ROC) curve analysis was used to plot the lowest cut-off YcDNA concentration that predicted PSA positivity. Linear regression models were used to determine the impact of PSA+YcDNA+ and PSA-YcDNA+ events on MMP/TIMP concentrations compared to PSA-YcDNA- events at baseline. Spearman's Rank correlation was used to determine the relationship between concentrations of YcDNA and MMPs/TIMPs at baseline. Linear mixed models (LMMs) adjusting for repeated measures were used to assess the impact of PSA+YcDNA+ and PSA-YcDNA+ events on cytokine concentrations and immune cell frequencies compared to PSA-YcDNA- events over multiple visits. Generalized estimating equation (GEE) models using a logit link were used to compare microbe presence between PSA+YcDNA+, PSA-YcDNA+, and PSA-YcDNA- groups over multiple visits. Multivariable LMMs adjusted for the contribution of study arm (CAPRISA or family planning clinics), time in the study, Nugent Score, participant age, STIs, the frequency of vaginal sex in the past month, and genital inflammation status [4, 28]. The Benjamini-Hochberg method was used to calculate the false discovery rate (FDR).

Results

Study population

Baseline clinical and demographic data are reported for 137/152 women with both PSA and YcDNA data available (**Table 1**). The study participants' overall median age was 28 years [interquartile range (IQR) 25-33 years]. Semen exposure was determined by YcDNA detection (YcDNA+), with PSA detection further stratifying participant groups into semen exposure within 48 hours of sampling (PSA+YcDNA+) and between 3-15 days of sampling (PSA-YcDNA+). At baseline, 28% (38/137) of women were PSA+YcDNA+, 14% (19/137) were PSA-YcDNA+, and 58% (80/137) were PSA-YcDNA-, suggesting no semen exposure within 15 days of sampling. Semen exposure was associated with how often women saw their partners (p=0.038), the frequency of vaginal sex in the past month (p=0.006), Herpes simplex virus (HSV)-2 detection (p=0.047), current gonorrhea infection (p=0.018),

and Nugent Score (p=0.003). Higher median Nugent Scores were specifically driven by semen exposure within 48 hours compared to no semen exposure [median 3 (IQR 1-7) vs. median 1 (IQR 0-3), respectively, p=0.003]. Further, women with evidence of semen exposure within 3-15 days reported more frequent acts of vaginal sex in the last month than women with no evidence of semen exposure [median 8 (IQR 4-10) vs. median 4 (IQR 2-6), respectively, p=0.017].

Table 1. Baseline characteristics of the study participants grouped by the timing of semen exposure.

		No Semen Exposure	**Semen Exposure**		
Characteristics	Level	PSA-YcDNA- (n=80)	Within 48 hours PSA+YcDNA+ (n=38)	Within 3-15 days PSA-YcDNA+ (n=19)	p-value
Median Age (IQR)		28 (25 - 30)	29 (25 - 35)	29 (24 - 33)	0.543
Study arm [% (n)]	Intervention	43.8 (35)	55.3 (21)	42.1 (8)	0.458
	Control	56.3 (45)	44.7 (17)	57.9 (11)	
Age of sexual debut (year)	Median (IQR)	18 (17 - 19)	18 (16 - 20)	17 (16 -18)	0.120
Age at menarche	Median (IQR)	14 (13 - 16)	15 (13 - 16)	14 (13 - 16)	0.917
Number of lifetime partners	Median (IQR)	3 (2 - 4)	2 (1 - 3)	2 (1 - 4)	0.184
Relationship status [% (n)]	Married	10.0 (8)	28.9 (11)	15.8 (3)	0.103
	Stable partner	88.8 (71)	68.4 (26)	84.2 (16)	
	Casual partner	1.3 (1)	2.6 (1)	0	
How often you see your partner [% (n)]	Daily	19.0 (15/79)	34.2 (13)	42.1 (8)	**0.038***
	Weekly	40.5 (32/79)	47.4 (18)	47.4 (9)	
	Monthly	36.7 (29/79)	18.4 (7)	5.3 (1)	
	< Monthly	3.8 (3/79)	0	5.3 (1)	
Male condom use [% (n)]	Always	42.5 (34)	26.3 (10)	36.8 (7)	0.345
	Sometimes	47.5 (38)	55.3 (21)	42.1 (8)	
	Never	10.0 (8)	18.4 (7)	21.1 (4)	
Vaginal sex acts in the last 30 days	Median (IQR)	4 (2 - 6)	4 (3 - 11)	8 (4 - 10)‡	**0.006***
Partner circumcision [% (n)]	Yes	36.8 (25/68)	24.2 (8/33)	33.3 (6/18)	0.709
	No	60.3 (41/68)	72.7 (24/33)	66.7 (12/18)	
Partner HIV status [% (n)]	Positive	1.3 (1)	2.6 (1)	5.3 (1)	0.565
	Negative	62.5 (50)	73.7 (28)	63.2 (12)	
	Unknown	36.3 (29)	23.7 (9)	31.6 (6)	
Herpes simplex virus 2 [% (n)]		89.6 (69/77)	92.1 (35)	73.7 (14)	**0.047***
Human papillomavirus [% (n)]		50.0 (40)	50.0 (19)	47.4 (9)	0.978
***Neisseria gonorrhoeae* [% (n)]**		0	10.5 (4)	5.3 (1)	**0.018***
***Chlamydia trachomatis* [% (n)]**		7.7 (6/78)	10.5 (4)	0	0.356
***Trichomonas vaginalis* [% (n)]**		5.1 (4/78)	2.6 (1)	5.3 (1)	0.815
***Mycoplasma genitalium* [% (n)]**		3.8 (3/78)	5.3 (2)	5.3 (1)	0.925
Bacterial vaginosis (Nugent Score)	Median (IQR)	1 (0 - 3)	3 (1 - 7)†	1 (0 - 6)	**0.003***
Negative [% (n)]	0 - 3	84.2 (64/76)	57.9 (22)	68.4 (13)	**0.004***
Intermediate [% (n)]	4 - 6	9.2 (7/76)	7.9 (3)	15.8 (3)	
BV [% (n)]	7 - 10	6.6 (5/76)	34.2 (13)	15.8 (3)	

The Chi-square and Fisher's exact tests were used to compare proportions between the groups as deemed appropriate. Continuous data were assessed by Kruskal-Wallis tests to compare differences between no semen exposure (PSA-YcDNA-), semen exposure within 48 hours (PSA+YcDNA+), and semen exposure within 3-15 days (PSA-YcDNA+), Dunn's post-testing was applied to adjust for multiple comparisons. Significant differences between no semen exposure and semen exposure within 48 hours are indicated by (†), while differences between no semen exposure and exposure within 3-15 days are indicated by (‡). Significant p-values ($p<0.05$) are indicated by (*). PSA: Prostate-specific antigen; YcDNA: Y-chromosome DNA; IQR: interquartile range; HIV: Human Immunodeficiency Virus; BV: Bacterial vaginosis

Higher cervicovaginal YcDNA concentrations reflect more recent semen exposure

In women with evidence of condomless sex, we assessed the relationship between YcDNA concentrations and the timing of semen exposure. At baseline, only 70% (40/57) of YcDNA+ specimens (i.e., PSA+YcDNA+ and PSA-YcDNA+ specimens) had a yield sufficient for quantitation. Women with detectable genital PSA (PSA+YcDNA+; n=30) had significantly higher YcDNA concentrations than women without [PSA-YcDNA+; n=10; median 0.077 ng/µl (IQR 0.020-0.314) vs. 0.002 ng/µl (0.002-0.017), respectively; p<0.0001; **Figure 1**].

Since higher YcDNA concentrations were observed soonest after semen exposure, it suggests a potential for a threshold YcDNA concentration to serve as a proxy for the timing of semen exposure before genital sampling. ROC curve analysis suggests a YcDNA concentration cutoff of 0.005 ng/µl predicts PSA positivity with the greatest sensitivity [87.4%, confidence interval (CI) 81.2, 93.6] and with a specificity of 62.3% (CI 50.9, 73.8; **Table S4**; **Figure S2**).

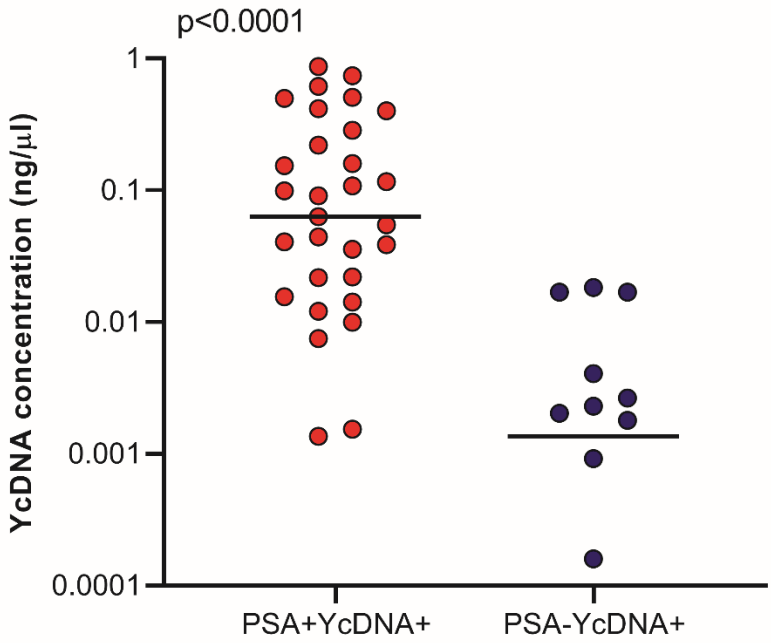

Figure 1. Relationship between YcDNA concentrations and timing of semen exposure prior to cervicovaginal sampling. The Mann-Whitney U test was used to compare YcDNA concentrations between women with evidence of semen exposure within 48 hours (PSA+YcDNA+; n=30) and 3-15 days (PSA-YcDNA+; n=10) at baseline. PSA: Prostate-specific antigen; YcDNA: Y-chromosome DNA

Semen-associated alterations to cervicovaginal cytokines

Since semen's impact on the FGT is likely short-lived [1, 22], we hypothesized that higher YcDNA concentrations and semen exposure within 48 hours would be associated with greater alterations in cervicovaginal cytokines (**Table S1**). Multivariable LMMs were used to compare YcDNA concentrations and cytokine levels over multiple visits (n=167 genital specimens). Higher YcDNA concentrations were associated with elevated concentrations of 12/48 cytokines (**Figure 2a**). The strongest associations were observed between higher YcDNA concentrations and IL-13 (β=0.093; CI 0.034, 0.152; p=0.002) and VEGF (β=0.165; CI 0.060, 0.269; p=0.002), both maintaining statistical significance after FDR adjustment.

Cytokine concentrations were also compared between PSA+YcDNA+ specimens (n=124) and PSA-YcDNA+ specimens (n=93) relative to PSA-YcDNA- specimens (n=433) over multiple visits. PSA+YcDNA+ events were significantly associated with increases in 18/48 cytokines and with reductions in two cytokines relative to PSA-YcDNA- events (**Figure 2b**). The strongest associations were observed with IL-12p70 (β=0.321; CI 0.252, 0.390), IL-13 (β=0.211; CI 0.162, 0.261), IL-7 (β=0.254; CI 0.167, 0.341), VEGF (β=0.404; CI 0.323, 0.486), MIF (β=-0.244; CI -0.364, -0.125), and IL-10 concentrations (β=0.144; CI 0.106, 0.182; all $p<0.001$). In comparison, PSA-YcDNA+ specimens only had increased concentrations of IP-10 (β=0.212; CI 0.030, 0.395; p=0.023) and IL-3 (β=0.074; CI 0.006, 0.141; p=0.034).

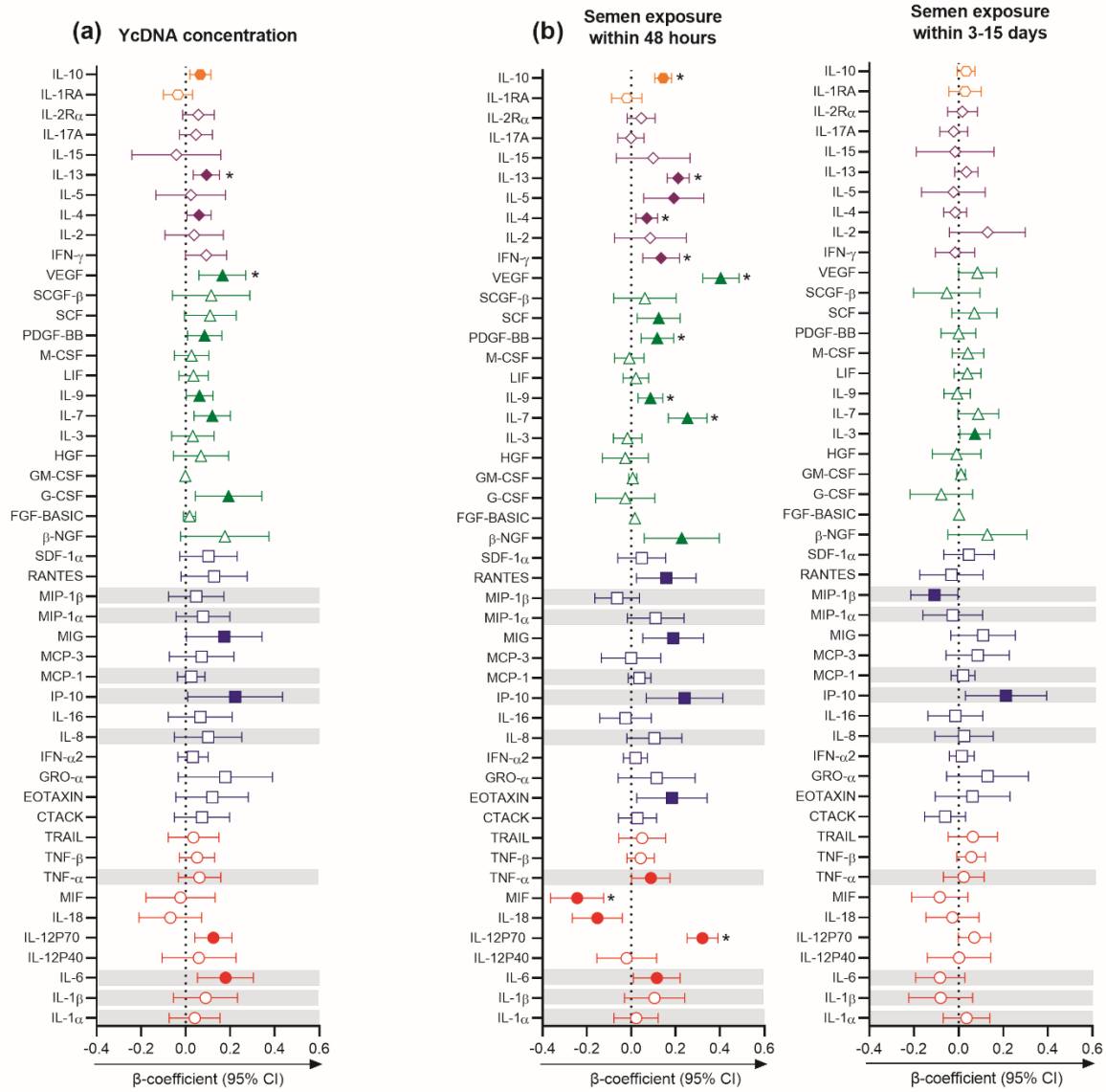

Figure 2. Associations between semen exposure and cervicovaginal cytokine concentrations. Multivariable linear mixed models controlling for study arm, time in study, participant age, the frequency of vaginal sex in the past month, sexually transmitted infections (*Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis, and Mycoplasma genitalium*), and Nugent Score were used to determine the association between semen exposure and vaginal cytokine concentrations over multiple visits. **(a)** Associations between YcDNA concentrations and cervicovaginal cytokine concentrations over multiple visits (n=167 genital specimens). **(b)** Longitudinal comparison of cytokine concentrations between semen exposure within 48 hours (PSA+YcDNA+ specimens; n=124) and 3-15 days (PSA-YcDNA+ specimens; n=93) relative to no semen exposure (PSA-YcDNA- specimens; n=433). Cytokines are ordered according to their functions: pro-inflammatory (red circles), chemotactic (blue squares), growth/haematopoiesis (green triangles), adaptive response (purple diamonds), and regulatory (orange hexagons) cytokines. Grey shadings represent the cytokines previously associated with genital inflammation and in demonstrating its association with HIV risk in this cohort [4, 28]. β-coefficients are depicted by shapes and error bars indicate the 95% CI. Filled shapes indicate significant p-values ($p<0.05$), and significance after FDR adjustment is indicated by (*). Table S1 contains a list of abbreviations for the 48 cytokines measured in this study. YcDNA: Y-chromosome DNA; CI: Confidence interval

Semen-associated alterations to barrier-related proteins

MMPs and their regulators, TIMPs are involved in the remodelling of the extracellular matrix. Increased expression of MMPs is associated with elevated genital cytokines, epithelial barrier disruption, and HIV transmigration [5, 31]. Therefore, we compared cervicovaginal YcDNA concentrations with MMP/TIMP concentrations at baseline (n=40). Moderate positive correlations were observed between YcDNA concentrations and the levels of MMP-2 (r=0.330; CI 0.039, 0.570; p=0.024), TIMP-1 (r=0.385; CI 0.102, 0.611; p=0.008), and TIMP-4 (r=0.378; CI 0.093, 0.605; p=0.009; **Figure 3**).

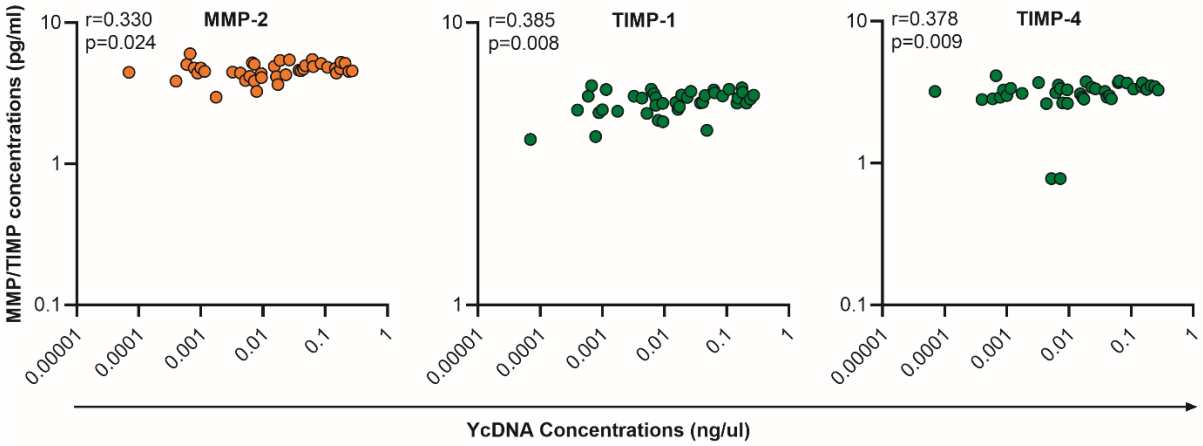

Figure 3. Correlations between YcDNA concentrations and MMP/TIMP concentrations at baseline. Spearman's Rank correlations were used to determine the relationship between YcDNA concentrations and MMP/TIMP concentrations at baseline (n=40). Orange circles represent MMPs, and green circles represent their inhibitors (TIMPs). MMP: Matrix metalloproteinase; TIMP: Tissue-inhibitors of metalloproteinases; YcDNA: Y-chromosome DNA

Similarly, PSA+YcDNA+ CVLs (n=37) had higher concentrations of MMP-2 (β=0.506; CI 0.119, 0.892; p=0.011), TIMP-1 (β=0.230; CI 0.043, 0.417; p=0.016), and TIMP-4 (β=0.466; CI 0.125, 0.808; p=0.008; **Figure 4**). No significant associations were observed with semen exposure within 3-15 days (PSA-YcDNA+ CVLs; n=19).

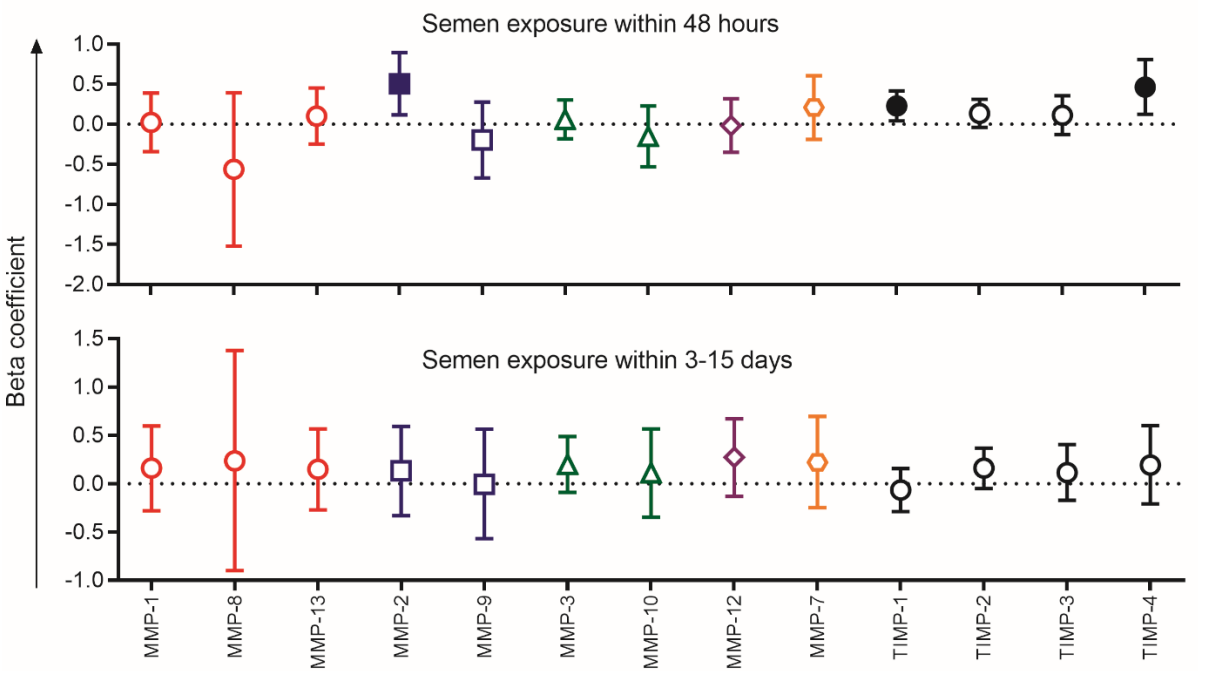

Figure 4. Associations between MMP/TIMP concentrations and timing of semen exposure at baseline. Multivariable linear regression models were used to compare MMP/TIMP concentrations at baseline between women with evidence of semen exposure within 48 hours (n=37) and 3-15 days (n=19) relative to those with no semen exposure within the 15 days (n=80) before genital sampling. Models were adjusted for age, sexually transmitted infections (*Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis, and Mycoplasma genitalium*), Nugent Score, the frequency of vaginal sex in the past month, study arm, and inflammation status. β-coefficients are depicted by shapes and error bars indicate the 95% confidence intervals. Filled shapes indicate significant p-values ($p<0.05$). MMPs are grouped according to their function: collagenases (red circles), gelatinases (blue squares), stromelysins (green triangles), macrophage elastase (purple diamond), matrilysin (orange hexagon), and TIMPs are represented by black circles. MMP: Matrix metalloproteinase; TIMP: Tissue-inhibitors of metalloproteinases

Semen-associated alterations to endocervical immune cell frequencies

Since HIV must gain access to local target cells for infection, we investigated whether YcDNA concentrations and the timing of semen exposure were associated with alterations in endocervical immune cell frequencies. In multivariable LMMs, higher YcDNA concentrations were associated with increased frequencies of the activated CD4+HLA-DR+ T cell populations (n=145 genital specimens; β=0.177; CI 0.016, 0.339; p=0.032; **Figure 5a**).

Additionally, semen exposure within 48 hours (n=108 genital specimens) was associated with higher frequencies of activated HIV target cells (CD4+CCR5+HLA-DR+; β=0.050; CI 0.001, 0.098; p=0.046; **Figure 5b**). No significant associations were observed with semen exposure within 3-15 days (n=85 genital specimens).

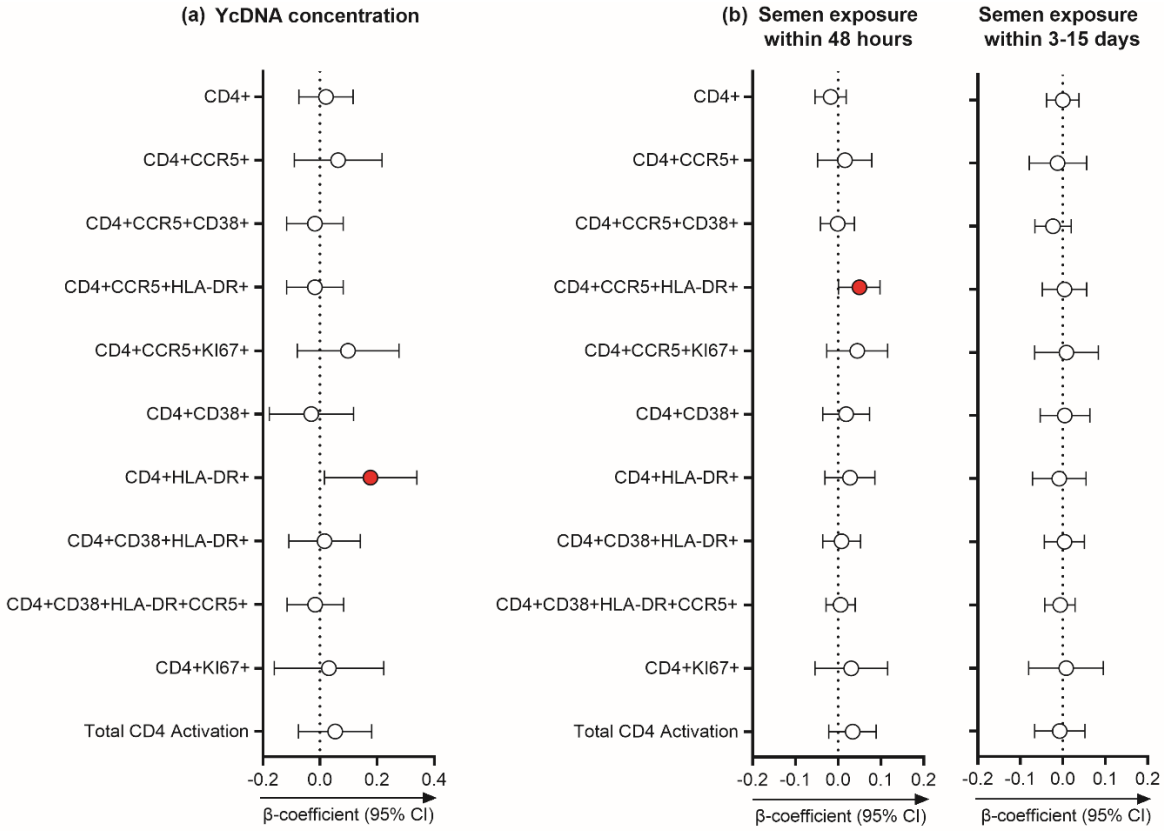

Figure 5. Associations between semen exposure and endocervical immune cell frequencies. Linear mixed models adjusting for the time in study, study arm, participant age, the frequency of vaginal sex in the past month, sexually transmitted infections (*Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis, and Mycoplasma genitalium*), inflammation status, and Nugent Score were used to determine the relationship between semen exposure and endocervical T cell frequencies over multiple visits. **(a)** Associations between YcDNA concentrations and immune cell frequencies over multiple visits (n=145 genital specimens). **(b)** Longitudinal comparison of immune cell frequencies between semen exposure within 48 hours (PSA+YcDNA+ specimens; n=108) and 3-15 days (PSA-

YcDNA+ specimens; n=85) relative to no semen exposure (PSA-YcDNA- specimens; n=375). β-coefficients are depicted by circles and error bars indicate the 95% CI. Red filled circles indicate significant p-values ($p<0.05$). Total CD4 activation refers to cells expressing CCR5, HLA-DR, and/or CD38. YcDNA: Y-chromosome DNA; CI: Confidence interval

Semen-associated alterations to vaginal microbes

Since vaginal microbial diversity is associated with HIV infection in women [6, 7], we examined the contribution of YcDNA concentrations and the timing of semen exposure on vaginal microbe detection over multiple visits [6, 7]. In adjusted GEE models, higher YcDNA concentrations were associated with reduced detection of *G. vaginalis* in vaginal specimens [n=133; odds ratio (OR)=0.269; CI 0.095, 0.764; p=0.014; **Figure 6a**].

Semen exposure within 48 hours (n=87 genital specimens) was associated with more frequent detection of BVAB2 (OR=1.755; CI 1.116, 2.760; p=0.015), *P. bivia* (OR=1.886; CI 1.102, 3.228; p=0.021), *G. vaginalis* (OR=1.815; CI 1.093, 3.015; p=0.021), and reduced detection of *L. jensenii* (OR=0.515; CI 0.319, 0.831; p=0.007; **Figure 6b**) over multiple visits. Semen exposure within 3-15 days was only associated with increased detection of *P. bivia* (n=74 genital specimens; OR=2.142; CI 1.248, 3.676; p=0.006).

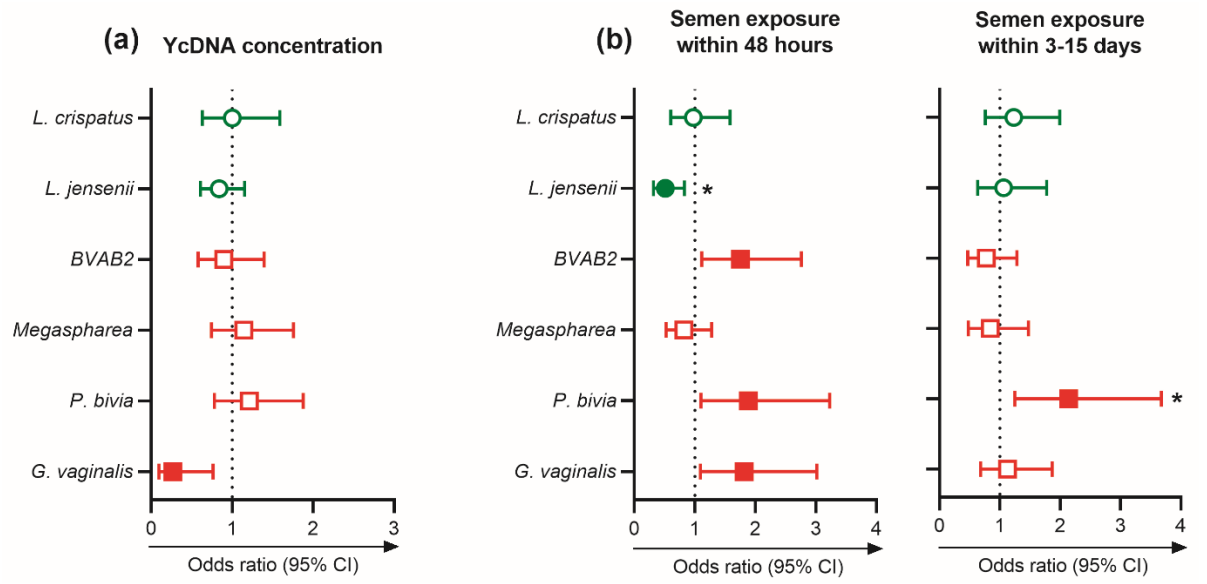

Figure 6. Semen-associated alterations to post-coital vaginal microbes. GEE models were used to determine the association between semen exposure and microbe presence over multiple visits. **(a)** Comparisons between YcDNA concentrations and vaginal microbe presence (n=133 genital specimens). **(b)** Microbe presence was compared between semen exposure within 48 hours (PSA+YcDNA+ specimens; n=87) and 3-15 days (PSA-YcDNA+ specimens; n=74) relative to no semen exposure within 15 days (PSA-YcDNA- specimens; n=354) before genital sampling. Models

adjusted for participant age, the frequency of vaginal sex in the past month, sexually transmitted infections (*Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis, and Mycoplasma genitalium*), inflammation status, time in the study, and study arm. Odds ratios are depicted by shapes and error bars indicate the 95% CI. Filled shapes indicate significant p-values ($p<0.05$), and (*) indicates significance after FDR adjustment. YcDNA: Y-chromosome DNA; CI: Confidence interval; *L. crispatus*: *Lactobacillus crispatus*; *L. jensenii*: *Lactobacillus jensenii*; BVAB2: Bacterial vaginosis-associated bacterium 2; *P. bivia*: *Prevotella bivia*; *G. vaginalis*: *Gardnerella vaginalis*

Discussion

We have previously demonstrated that semen exposure within the previous 15 days, measured by YcDNA detection alone, was associated more with alterations in barrier-related proteins and *P. bivia* detection than with the immune environment commonly linked to HIV risk in women [24]. Considering the transience of semen-associated immune responses [1, 22], we further investigated the contribution of more recent semen exposure to genital inflammation in women at high HIV risk. Here, PSA detection and higher YcDNA concentrations, both suggesting more recent semen exposure, were associated with elevated cervicovaginal cytokines, barrier-related proteins, increased detection of BV-associated microbes, and higher HIV target cell frequencies in vaginal specimens (**Figure 7**). These findings suggest that semen-associated immune responses at the FGT are transient and highlights the importance of considering the timing of genital sampling after condomless sex.

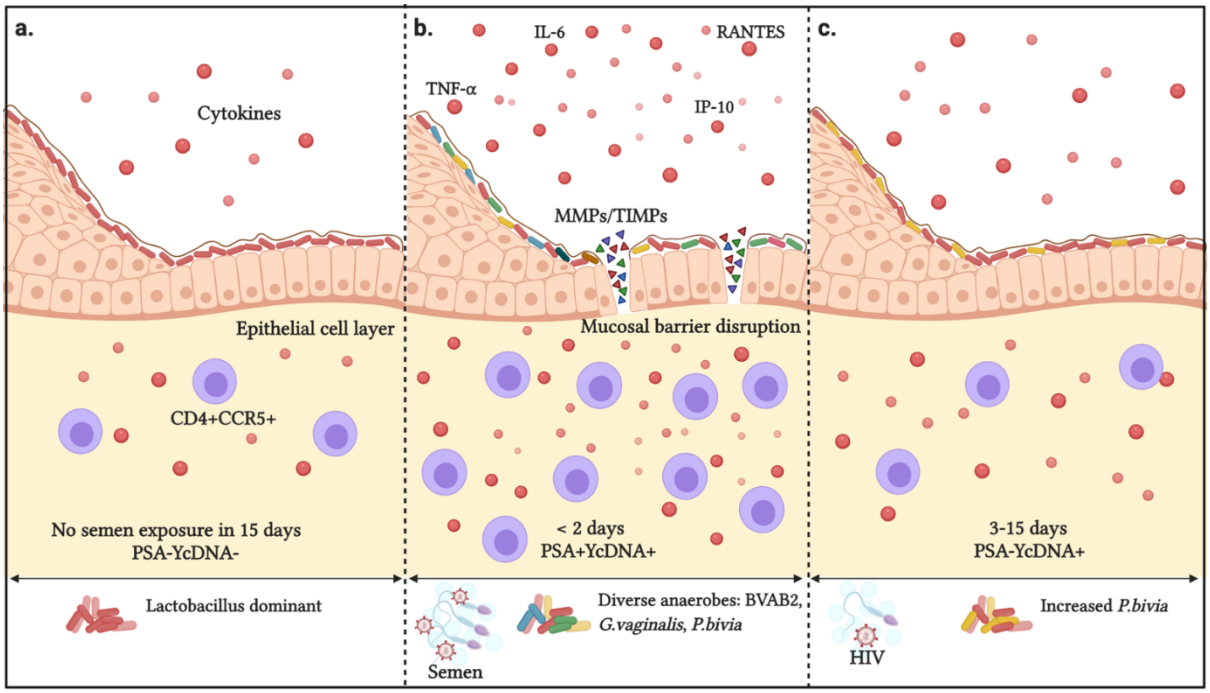

Figure 7. Graphical representation of semen-associated alterations at the female genital mucosa. **(a)** An optimal vaginal environment in the absence of semen exposure. Here, the vaginal microbiome is dominated by Lactobacillus, few cytokines, and immune cells are present at the FGT, and the vaginal

epithelial barrier is intact. **(b)** In this study, recent semen exposure < 2 days before genital sampling and higher YcDNA concentrations were associated with elevated concentrations of several cytokines, MMPs/TIMPs, BV-associated microbes, and HIV target cell recruitment at the female genital mucosa compared to no semen exposure within 15 days. **(c)** In comparison, semen exposure within 3-15 days was only associated with moderate alterations in cervicovaginal cytokine concentrations and increased detection of *P. bivia* compared to no semen exposure within 15 days. PSA: Prostate-specific antigen; YcDNA: Y-chromosome DNA; BVAB2: Bacterial vaginosis-associated bacterium 2; *P. bivia*: *Prevotella bivia*; *G. vaginalis: Gardnerella vaginalis*; HIV: Human Immunodeficiency Virus; IL-6: Interleukin-6; TNF-α: Tumour necrosis factor-alpha; RANTES: regulated on activation, normal T cell expressed and secreted; IP-10: Interferon gamma-induced protein-10; MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinases

At baseline, more than a third of the women had detectable YcDNA, indicating semen exposure within a range of 15 days before genital sampling. Similar to our previous report [24], YcDNA detection was associated with the frequency of physical interaction with the partner, the frequency of vaginal sex in the past month, current gonorrhoeae infection, and higher BV Nugent Score. The timing of semen exposure was specifically related to the number of vaginal sex acts reported and the BV Nugent Score. Semen exposure within 3-15 days allowed for a longer range of semen detection and was therefore associated with a greater number of coital episodes in the last month. Semen exposure within 48 hours was associated with higher Nugent Scores, suggesting that condomless sex is linked to a short-term increased presence of BV-associated microbes at the FGT.

We assessed the relationship between YcDNA quantities and the timing of semen exposure. Concentrations of YcDNA at the FGT may indicate the amount of seminal proteins and sperm count in the ejaculate of the male partner during condomless sex. These data demonstrated that YcDNA concentrations are also related to the timing of semen exposure since PSA detection was associated with higher YcDNA concentrations. The Quantifiler™ Trio DNA Quantification Kit used in this study correctly predicted PSA positivity in vaginal specimens with even minimal levels of YcDNA. YcDNA concentrations could be useful in studies requiring the use of a single semen biomarker, which also indicates the timing of semen exposure. Further studies are needed to confirm the feasibility of using YcDNA concentrations to determine more recent semen exposure (i.e., PSA detection) before genital sampling.

Semen exposure within 48 hours and higher YcDNA concentrations were associated with raised levels of several cytokines, while semen exposure within 3-15 days was associated with increased concentrations of only two cytokines (IL-3 and IP-10). Although transient, elevated concentrations of IL-6, TNF-α, IP-10, RANTES, and IL-10 associated with more recent semen exposure have also been related to increased HIV risk in women [4, 26, 32]. Of particular importance are the chemokines, with

raised IP-10 independently associated with HIV seroconversion and T cell recruitment [4, 33], and RANTES, a CCR5 ligand, involved in both the blocking of HIV binding to CCR5 on target cells and the recruitment of these target cells to the FGT [34].

Similar to our previous findings' semen-exposure was associated with increased concentrations of MMPs and their inhibitors [24]. Here, we further demonstrated that PSA detection, and higher YcDNA concentrations, in particular, were associated with elevated concentrations of MMP-2, TIMP-1, and TIMP-4. Importantly, there was only a moderate positive correlation between YcDNA concentrations and MMP/TIMP biomarkers of inflammation. However, these same MMPs/TIMPs were also significantly associated with PSA detection in multivariable linear regression models. Increased MMPs/TIMPs may signify wound healing since microabrasions are introduced at the FGT during coitus [35] and may also impact HIV risk in women through reduced mucosal barrier integrity and/or target cell recruitment [5].

Our findings confirmed that more recent semen exposure was also associated with increased frequencies of activated endocervical CD4 T cells (CD4+HLA-DR+ and CD4+CCR5+HLA-DR). Studies suggest that semen exposure at the FGT is associated with an initial pro-inflammatory response resulting in leukocyte recruitment to remove excess and abnormal sperm [1, 36]. This may explain the increase in CD4+HLA-DR+ T cell frequencies in response to higher YcDNA concentrations observed in this study, which may also indicate higher sperm counts. Others have reported that this inflammatory response dissipates within 48 to 72 hours, and a regulatory T cell immune response is mounted to facilitate conception [2, 3, 37]. This may also explain why semen exposure within 3-15 days was not related to immune cell alterations in this study. Activated endocervical CD4 T cells are putative HIV target cells, and increased frequencies of these cells may promote heterosexual transmission of HIV from an infected male partner.

Pathogens, STIs, and commensal microbes present in semen and at the male genital tract [11, 12, 38, 39] are transferred to the FGT during condomless sex and could alter the vaginal microbial composition [11, 40]. Surprisingly, higher concentrations of cervicovaginal YcDNA were associated with reduced detection of *G. vaginalis.* Further studies are required to determine reasons for this association e.g., the contribution of semen-derived antimicrobial activities, the impact of semen on *G. vaginalis* growth dynamics, etc. Conversely, semen exposure within 48 hours was associated with increased detection of several BV-associated microbes (BVAB2, *P. bivia*, and *G. vaginalis*) and reduced detection of *L. jensenii*, while women exposed to semen within 3-15 days only had an increased detection of *P. bivia* in vaginal specimens. Studies have demonstrated that a diverse vaginal microbiome with increased Prevotella and reduced *L. crispatus* is associated with a higher risk of HIV acquisition among women [6, 7]. This is due to the ability of BV-associated microbes to induce cytokine production and the recruitment of activated CD4+ T cells, both of which were observed in this study [6, 7].

A strength of this study was the use of recent semen biomarkers and the wealth of immune and microbial data to reliably assess the effect of semen exposure on the female genital mucosa over multiple visits. To our knowledge, this is the first study to assess the impact of YcDNA concentrations and the timing of semen exposure on markers of genital inflammation related to HIV risk in women. Given that semen itself contains several endogenously produced cytokines and CD4+ T cells from the male partner [10, 22, 41, 42], a limitation of this study was the inability to determine whether the elevated immune responses detected were from semen itself or an immune response elicited at the FGT. However, Chen *et al*., demonstrated that endometrial epithelial cells and stromal fibroblasts treated with seminal plasma had raised levels of several cytokines after adjusting for endogenous seminal plasma cytokines [43]. Similar to our findings, seminal plasma exposure was associated with elevated G-CSF, IL-6, TNF-α, and VEGF among others [43]. This study distinguished cytokines produced by the FGT from endogenous seminal plasma cytokines and suggests that the immune markers detected here were mounted by the FGT and not due to residual seminal components.

Conclusions

This longitudinal study demonstrates that semen exposure induces a transient pro-inflammatory immune response at the FGT which may significantly impact HIV risk in women. Here, PSA detection and higher concentrations of YcDNA, both indicative of recent semen exposure, were associated with increases in cervicovaginal cytokines and barrier-related proteins, recruitment, and activation of endocervical CD4 T cells, and alterations in vaginal microbes. These findings emphasize the need for studies of genital mucosal immunity to STIs such as HIV to consider the contribution of semen exposure to the FGT immune and microbial environments, preferably using a biomarker of recent semen exposure. This study also highlights the importance of consistent condom use, particularly in settings where women are at increased risk of acquiring HIV.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

J.J., S.N., and L.J.P.L. contributed to the conception and design of the study. J.J., L.J.P.L., A.M., M.F., and R.S. performed the experiments. J.J., L.J.P.L., and F.O. analyzed and interpreted the data. J.J. and L.J.P.L. wrote the manuscript. All authors have read and approved the final manuscript.

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Supplementary data

Table S1. List of cytokines measured in cervicovaginal lavage supernatant specimens.

Abbreviation	Cytokine Name
IL-1α	interleukin-1 alpha
IL-1β	interleukin-1 beta
IL-2	interleukin-2
IL-3	interleukin-3
IL-4	interleukin-4
IL-5	interleukin-5
IL-6	interleukin-6
IL-7	interleukin-7
IL-8	interleukin-8
IL-9	interleukin-9
IL-10	interleukin-10
IL-12p40	interleukin-12 p40
IL-12p70	interleukin-12 p70
IL-13	interleukin-13
IL-15	interleukin-15
IL-16	interleukin-16
IL-17	interleukin-17
IL-18	interleukin-18
IL-1RA	interleukin-1 receptor antagonist
IL-2Rα	interleukin-2 receptor alpha
CTACK	cutaneous T cell attracting chemokine
GRO-α	growth-related oncogene alpha
HGF	hepatocyte growth factor
IFN-γ	Interferon-gamma
IFN-α2	interferon alpha-2
LIF	leukaemia inhibitory factor
MCP-3	monocyte chemotactic protein-3
MIF	macrophage migration inhibitory factor
MIG	monokine induced by gamma interferon
β-NGF	beta nerve growth factor
SCF	stem cell factor
SCGF-β	stem cell growth factor-beta
SDF-1α	stromal cell-derived factor-1alpha
TNF-α	tumour necrosis factor-alpha
TNF-β	tumour necrosis factor-beta
TRAIL	TNF-related apoptosis-inducing ligand
FGF-basic	basic fibroblast growth factor
eotaxin	eosinophil chemotactic protein
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
M-CSF	macrophage colony-stimulating factor
IP-10	interferon gamma-induced protein-10
MCP-1	monocyte chemotactic protein-1
MIP-1α	macrophage inflammatory protein-1 alpha
MIP-1β	macrophage inflammatory protein-1 beta
PDGF-BB	platelet-derived growth factor-BB
RANTES	regulated on activation, normal T cell expressed and secreted
VEGF	vascular endothelial growth factor

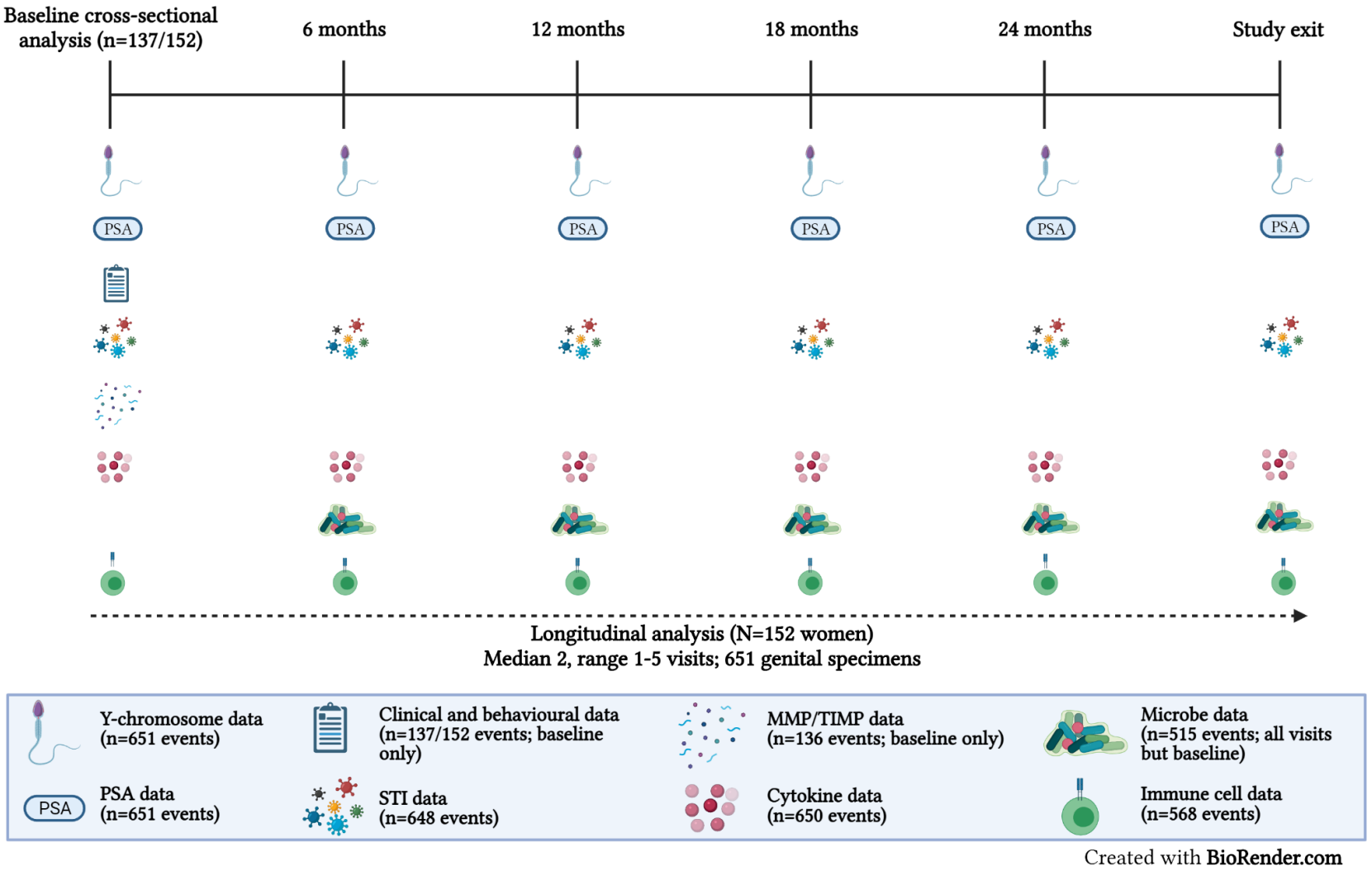

Figure S1. Graphical representation of the data available at baseline and longitudinally for CAPRISA 008 study participants. This study included 152 female participants from the CAPRISA 008 trial. Genital specimens were collected at baseline and longitudinally at months 6, 12, 18, 24, and study exit during the two-year trial (median 2, range 1-5 visits). The dataset included all baseline and longitudinal genital specimens with both YcDNA and PSA data available (n=651 genital specimens). Clinical and behavioural data are reported for the 137/152 women with both PSA and YcDNA data available at baseline. MMP/TIMP data were only available at baseline visits (n=136), and microbe data were available at all visits but baseline (n=515). PSA: Prostate-specific antigen; STI: sexually transmitted infection; MMP: matrix metalloproteinase; TIMP: tissue inhibitor of metalloproteinases

Table S2. Flow cytometry information on the antibody clones, fluorophores, and suppliers.

Antibody	**Source**	**Identifier**
Mouse anti-human CD14, Pacific Blue conjugated, clone M5E2	BD	BD Biosciences Cat# 558121, RRID:AB_397041
Mouse anti-human CD19, Pacific Blue conjugated, clone HIB19	BioLegend	BioLegend Cat# 302232, RRID:AB_2073118
Mouse anti-human CD8, Fluorescein isothiocyanate (FITC) conjugated, clone SK1	BioLegend	BioLegend Cat# 344704, RRID:AB_1877178
Mouse anti-human CD3, Allophycocyanin tandem dye (APC-H7) conjugated, clone SK7	BD	BD Biosciences Cat# 560275, RRID:AB_1645476
Mouse anti-human CD4, Peridinin-chlorophyll protein-cyanine 5.5 (PerCP-Cy5.5) conjugated, clone RPA-T4	BD	BD Biosciences Cat# 560650, RRID:AB_1727476
Mouse anti-human CD38, eFluor655 conjugated, clone HB7	eBioscience (Thermofisher)	Thermo Fisher Scientific Cat# 95-0388-42, RRID:AB_1724053
Mouse anti-human HLA-DR, Phycoerythrin (PE) conjugated, clone L243	BD	BD Biosciences Cat# 347401, RRID:AB_2629277
Mouse anti-human KI-67, Brilliant violet 700 (BV700) conjugated, clone Ki-67	BioLegend	BioLegend Cat# 350515, RRID:AB_11218996
Mouse anti-human CCR5, Allophycocyanin (APC) conjugated, clone 2D7/CCR5	BD	BD Biosciences Cat# 556903, RRID:AB_398619
LIVE/DEAD Fixable Violet Dead Cell Stain (Intracellular amines)	Invitrogen	Cat#34955

Table S3. List of common STI pathogens and other vaginal microbes measured in vulvovaginal swabs.

Abbreviation	**STI pathogen/ vaginal microbe**
CT	*Chlamydia trachomatis*
TV	*Trichomonas vaginalis*
NG	*Neisseria gonorrhoeae*
MG	*Mycoplasma genitalium*
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
L. crispatus	*Lactobacillus crispatus*
L. jensenii	*Lactobacillus jensenii*
G. vaginalis	*Gardnerella vaginalis*
P. bivia	*Prevotella bivia*
BVAB2	Bacterial Vaginosis-Associated Bacterium 2
Megasphaera 1	*Megasphaera type 1*
A. vaginae	*Atopobium vaginae*

Table S4. Sensitivity and specificity for the YcDNA concentration cutoff value of 0.005 ng/µl.

YcDNA concentration cutoff value		**PSA**		**Accuracy (95% CI)**
		Positive	Negative	
0.005 ng/µl	Positive	97	26	Sensitivity = 87.4% (81.2 – 93.6) Specificity = 62.3% (50.9 – 73.8) PPV = 78.9% (71.7 – 86.1) NPV = 75.4% (64.3- 86.6)
	Negative	14	43	

PPV: predictive value; NPV: negative predictive value; PSA: prostate-specific antigen; YcDNA: Y-chromosome DNA; CI: confidence interval

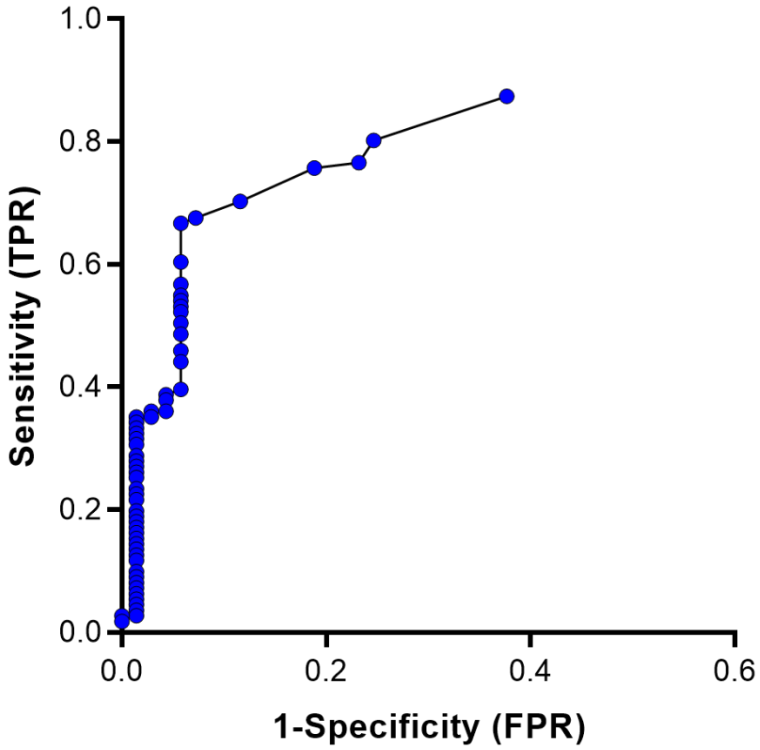

Figure S2: ROC curve for all YcDNA concentration cutoff values. Cutoff values ranged between 0.005 ng/µl and 1.700 ng/µl. TPR: true positive rate (sensitivity); FPR: false positive rate (1-specificity).

CHAPTER 4

4.1. Summary and discussion of the main findings

The ability of semen to induce mucosal changes in female tissues has been extensively studied in reproduction (37, 38, 41, 42). Less studied, however, is the contribution of semen-induced alterations to the GI profile known to modulate HIV risk in women (10, 34). Raised levels of inflammatory cytokines, compromised epithelial barrier integrity, immune cell recruitment and activation, and increased microbial diversity have all been linked to increased GI and HIV acquisition in women (10-12, 34). Semen-induced alterations to these biomarkers of inflammation may very well promote a genital immune milieu conducive to HIV infection in women. Vaginal exposure to semen is often assessed by self-reported condom use; however, considering the established unreliability of self-reported data (64-66), a biological marker of semen exposure may better characterise semen-associated alterations at the FGT. Therefore, we compared the impact of semen exposure as measured by a semen biomarker and self-reported condom use on inflammatory markers linked to HIV risk in women. We then further investigated the contribution of more recent sex on female GI and the persistence of these associations over time.

Chapter 1 detailed the background and rationale for these studies. This chapter included a literature review highlighting the various mechanisms by which exposure to semen may alter biomarkers of female GI and impact HIV risk. These included semen-associated alterations in cervicovaginal cytokines, FGT mucosal barrier integrity, immune cell recruitment, and the vaginal microbiome composition. Additionally, the role of semen in modulating the activity of topical PrEP at the female genital mucosa was discussed. This manuscript emphasized the need for clinical and immunological studies of STIs and their biomedical interventions to consider the immunomodulatory contributions of semen to the FGT microenvironments. The manuscript has been accepted for publication at the American Journal of Reproductive Immunology.

The remainder of the thesis presented the laboratory research conducted to address the study's aims to define and determine the persistence of the impact that semen exposure has on the FGT immune and microbial environments linked to HIV risk. First, the data described in **Chapter 2** challenged the utility of self-reported condom use over semen biomarkers in studies of genital mucosal immunity. The detection of YcDNA in vaginal fluid was used to indicate semen exposure within the 15 days before genital sampling. Here, a third of the women reporting always using a condom during intercourse had YcDNA present in their genital specimens, highlighting the inconsistencies associated with self-reported data. These discrepancies observed with self-reported condom use may be attributed to multiple factors, including recall bias, reduced STI risk perception among women in stable relationships, the inability to safely negotiate condom use, and incorrect or inconsistent condom use (76). Furthermore, YcDNA detection but not self-reported condom use predicted significant alterations

in the microenvironments of the FGT, i.e., higher levels of several soluble proteins (MMPs and cytokines) and more frequent detection of *P. bivia*.

MMPs are protein biomarkers of epithelial barrier remodelling and repair associated with increased HIV risk in women (34). Alterations in these barrier-related proteins may signal compromised epithelial barrier integrity caused by semen exposure and/or condomless sex, which may facilitate HIV entry at the female genital mucosa. Additionally, semen exposure within 15 days was related to *P. bivia* detection, which has previously been associated with increased GI and HIV acquisition in women (11). Prevotella was shown to increase activated genital CD4+ T cell numbers in a mouse model (11). YcDNA detection also correlated with increased levels of several growth factors [nerve growth factor (NGF)-β, IL-7, platelet-derived growth factor (PDGF)-BB, stem cell factor (SCF), and vascular endothelial growth factor (VEGF)], chemokines [monokine induced by gamma interferon (MIG) and interferon-gamma induced protein (IP)-10], pro-inflammatory (IL-12p70), anti-inflammatory (IL-10), and adaptive response cytokines [interferon (IFN)-γ and IL-13]. Of the cytokines previously related to GI (10, 34), only IP-10 was elevated upon exposure to semen. Also, neither self-reported condom use nor YcDNA detection was associated with changes in endocervical HIV T cell frequencies. Taken together, these data demonstrated the inadequacies associated with self-reports of condom use versus the use of semen biomarkers in studies of genital mucosal immunity. However, the limited overlap in biomarkers associated with YcDNA detection and with HIV risk in CAPRISA study participants suggest either minimal association between semen exposure and the biomarkers linked to HIV risk in women or perhaps transience in the associations that could not be described due to the long 15-day range for semen detection with YcDNA (14, 74, 75).

Because studies have demonstrated that semen exposure is related to short-term alterations at the female genital mucosa (42, 43), a biomarker of more recent condomless sex may better characterise semen-associated alterations at the FGT. **Chapter 3** examined the contribution of more recent semen exposure on biomarkers of female GI and assessed the persistence of these associations over time. Here, using the combination of PSA and YcDNA detection as measures of semen exposure, cervicovaginal lavages were classified as semen exposure with 48 hours (PSA+YcDNA+), semen exposure within 3-15 days (PSA-YcDNA+), and no semen exposure (PSA-YcDNA-) within the 15 days before genital sampling. Additionally, this chapter assessed YcDNA quantities as an indicator of male protein concentrations at the FGT and investigated its relative impact on biomarkers of inflammation. PSA detection was significantly associated with higher YcDNA concentrations at the FGT. These data suggest a link between higher YcDNA quantities and more recent sex and the potential for higher YcDNA concentrations to serve as an alternative measurement of recent semen exposure before genital sampling. Assessing YcDNA concentrations may be useful in studies requiring the use of a single semen biomarker that indicates male protein concentrations and sperm counts in vaginal specimens after condomless sex and additionally predicts the timing of semen exposure. However, multiple factors may

influence the amount of detectable YcDNA in the FGT following condomless sex, including variations in partner sperm count, genital hygiene practices, natural drainage of semen after sex, menstruation, etc. (74). Therefore, further studies with a larger sample size and various YcDNA quantification kits are needed to confirm the feasibility of using YcDNA concentrations to determine the timing of vaginal exposure to semen.

PSA detection and higher YcDNA concentrations, both indicative of recent semen exposure, were related to higher levels of vaginal cytokines, barrier-related proteins, and increased HIV target cells at the female genital mucosa. Importantly, several growth factors and adaptive cytokines, not previously associated with genital inflammation and HIV risk in this cohort of women (10), were associated with recent semen exposure. These growth-related and adaptive cytokines are likely upregulated to prime the female reproductive tract for conception and pregnancy, as reviewed by Dimitriadis *et al*. (77). Recent semen exposure also predicted significant increases in several cytokines associated with increased HIV risk in women, including IL-6, TNF-α, IP-10, regulated on activation, normal T cell expressed and secreted (RANTES), and IL-10 (10, 78, 79). Elevated pro-inflammatory cytokines modulate HIV risk in women through immune cell recruitment and reduced barrier integrity at the female genital mucosa (10, 34). In support of this, higher YcDNA concentrations and semen exposure within 48 hours were also associated with elevated concentrations of barrier-related proteins (MMP-2, TIMP-1, and TIMP-4) and higher levels of activated endocervical HIV target cells (CD4+HLA-DR+ and CD4+CCR5+HLA-DR). In order to establish productive infection in women, HIV requires access to local target cells for infection. These data suggest that recent semen exposure contributes to a genital immune milieu conducive to HIV infection in women. PSA detection also predicted significantly higher Nugent scores, the detection of BV-associated microbes (BVAB2, *P. bivia*, and *G. vaginalis*), and reduced detection of *L. jensenii*. The presence of these BV-associated bacteria at the FGT has previously been related to increased HIV risk in South African women (11, 12, 80). Importantly, all participants of the CAPRISA 008 trial received 1% tenofovir gel to assess the efficacy of integrating tenofovir gel provision into routine family planning services (81). *G. vaginalis* which was associated with recent semen exposure, was previously shown to reduce microbicide efficacy through tenofovir gel metabolism (80). These findings suggest that microbial diversity induced by condomless sex and/or recent semen exposure has additional implications for HIV risk in women through reduced topical PrEP efficacy.

In comparison, semen exposure within 3-15 days (PSA-YcDNA+) was associated with raised levels of only two cytokines (IL-3 and IP-10), only one of which was previously associated with HIV risk in this cohort (10). Additionally, no significant associations were observed between semen exposure within 3-15 days and immune cell frequencies and barrier-related proteins, while only minimal alterations in the vaginal microbiome (i.e., presence of *P. bivia*) were detected.

These findings suggest that the semen-induced alterations observed with YcDNA detection alone in chapter 2 were specifically driven by recent semen exposure within 48 hours. In further support of this, PSA detection, i.e., semen exposure within 48 hours, was associated with elevated concentrations of cytokines additional to that observed by YcDNA detection alone. Similarly, PSA detection in chapter 3 was associated with the detection of additional BV-associated microbes and increases in HIV target cells, not observed in chapter 2. Semen exposure within 48 hours was associated with alterations in the immune and microbial markers of inflammation linked to HIV risk in women. These associations were transient and declined by 3-15 days post semen exposure. These data highlight the importance of considering the timing of genital sampling after condomless sex and controlling for semen's impact on mucosal immunity, preferably using a biomarker of recent semen exposure.

4.2. Significance

These studies contribute to the existing literature on the biological factors that influence GI and HIV risk in women. This is particularly important since identifying and controlling for the factors that contribute to GI could likely limit their impact on HIV acquisition in women. Here, recent semen exposure was shown to contribute to the inflammatory milieu conducive to HIV infection in women. These studies highlight the need for clinical and immunological studies of STIs to control for semen's contribution to the immune and microbial environments of the FGT. Findings from these studies may also facilitate the design of biomedical interventions that consider semen's immune impact on the FGT and prevents HIV transmission among women in high burden areas. For example, the use of films or lubricants that contain anti-inflammatory compounds may help circumvent the initial semen-induced inflammatory response at the FGT. However, additional studies are required to identify specific semen-derived factors that modulate this inflammatory response at the FGT in order to develop more targeted therapies. Given that recent condomless sex/semen exposure is associated with increased detection of BV-associated microbes, regular use of probiotics may help to maintain vaginal health and restore microbial homeostasis after condomless sex. Additionally, since most semen microbes are likely derived from the male genital tract (47, 82), medical male circumcision may also help reduce the diversity of the penile microbiota and lower rates of HIV transmission in both men and women (83, 84). These findings also support consistent condom use and emphasize the need for structural interventions to encourage the use of condoms, particularly in settings where women are at increased risk of acquiring HIV.

4.3. Strengths

A strength of these studies lies in their longitudinal design and the abundance of immunological and microbial data. Cervicovaginal cytokines, barrier-related proteins, common vaginal microbes, immune cell frequencies, and semen biomarkers were measured in vaginal specimens collected biannually during the CAPRISA 008 two-year study period (mean five samples per woman). The wealth of data facilitated the reliable and comprehensive assessment of semen's impact on the immune and microbial environments of the female genital mucosa over time. The use of semen biomarkers also permitted more accurate assessments of semen's impact on the FGT while avoiding the discrepancies associated with self-reported data. Additionally, the use of two different semen biomarkers in combination helped to characterise the persistence of semen-associated alterations at the female genital mucosa. These studies not only confirmed semen's immune altering capacity at the FGT but also demonstrated that these alterations are short-lived and highlight the importance of considering the timing of genital sampling after condomless sex. Concentrations of YcDNA in vaginal specimens were also identified as a potential semen biomarker that may indicate not only male protein concentrations and possibly sperm count but also the timing of semen exposure and its relative impact on the female genital mucosa.

4.4. Limitations

Semen contains several microbes derived from the penile urethra and foreskin as well as endogenously produced cytokines and immune cells (43, 47, 85-87). A limitation of these studies was the inability to determine the proportion of the biomarkers detected here that may have been sourced from the male partner. However, since the genital fluid collected was from female participants, the detection of male-derived immune and microbial factors would likely be minimal and presumably, as these data suggest, even lower with longer durations between genital sampling and semen exposure. These studies were limited by the amount of cervix-derived T cells required to assess other important HIV target cell populations such as Th17 cells, Th22 cells, tissue-resident memory T cells, antigen-presenting cells (including dendritic cells and macrophages), and neutrophils (88, 89). However, since activated CD4+CCR5+ T cells are putative HIV target cells, the inclusion of CCR5 and activation markers in the panel may still adequately describe a relationship between semen exposure and cellular biomarkers of HIV risk. Common vaginal microbes were detected using PCR, limiting the investigation of semen-associated alterations in the vaginal microbiome to those specific microorganisms. The use of 16S rRNA gene sequencing may provide a deeper understanding of post-coital alterations to the vaginal microbiome. Additionally, MMPs are only a subset of proteins that maintain the integrity of the epithelial barrier. An expanded panel of barrier-related proteins may provide a better understanding of the impact that semen exposure and friction during sex have on the vaginal epithelium. However, despite these limitations, these studies nonetheless provide a comprehensive understanding of the

impact of semen exposure on the immune and microbial environments of the FGT linked to HIV risk and the persistence of these associations over time.

4.5. Future directions and recommendations

Future studies of semen's impact on the female genital mucosa should incorporate additional assessments of the Th17 cell population, the primary targets for HIV infection, additional barrier-related proteins (e.g., tight junction proteins), and 16S rRNA gene sequencing to assess semen-induced alterations in the vaginal microbiome comprehensively. Studies of immune tolerance to semen (90-92) imply that exposure to semen from new or multiple concurrent partners may be associated with increased GI and HIV acquisition in women. *In vitro* models can be used to assess immune tolerance to semen by culturing cytobrush-derived cervical mononuclear cells from female participants and exposing these cells to partner semen and/or semen from an unrelated male. Additionally, *ex vivo* assessments of immune tolerance to semen may involve Y-chromosome sequencing to distinguish semen from different males in vaginal fluid from women with multiple concurrent partners. Complementary assessments of immune and microbial biomarkers of inflammation in both these *in vitro* and *ex vivo* models may provide a biological link between partner concurrency and increased HIV risk in women and may highlight the importance of monogamy in high HIV burden areas (93). Additionally, in these *ex vivo* studies, we were unable to determine the contribution of endogenous semen-derived factors to the immune and microbial markers detected. *In vitro* models could be used to assess and adjust for semen-derived proteins (i.e., cytokines and MMPs/TIMPs) and immune cells in future studies. *In vitro* models could also assist in identifying the specific seminal components that modulate these inflammatory responses to limit its impact on HIV acquisition risk in women. Here, YcDNA concentrations were also identified as a potential biomarker to assess the male protein concentrations, sperm counts, and timing of semen exposure in vaginal specimens after condomless sex. Further studies involving semen analysis are warranted to confirm the reliability of using YcDNA concentrations to predict male protein concentrations and sperm count at the FGT. Additionally, more studies must be conducted in a larger cohort and with various YcDNA quantification kits to assess the feasibility of using YcDNA concentrations to predict the timing of semen exposure before genital sampling.

4.6. Conclusions

These studies confirmed that vaginal exposure to semen is associated with elevated biomarkers of inflammation linked to HIV risk in women. Self-reported condom use was an unreliable measure of semen exposure and may lead to inaccurate data interpretation. These findings underscore the need to screen for semen biomarkers to confirm condom use and semen exposure in HIV prevention trials.

Here, semen exposure within 48 hours was associated with raised levels of several cytokines, barrier-related proteins, increases in activated HIV target cell frequencies, and the detection of BV-associated bacteria in vaginal fluid. However, these associations were short-lived and dissipated by 3-15 days after exposure to semen. Nonetheless, these findings suggest that semen induces alterations at the FGT that may increase HIV infection risk in women. This thesis highlights the importance of consistent condom use, particularly in settings where women are at increased risk of acquiring HIV. This thesis also highlights the importance of considering the timing of genital sampling after condomless sex and controlling for semen by using a biomarker of recent semen exposure. Furthermore, these findings emphasize the need for clinical and immunological studies of STIs and their biomedical interventions to consider the contribution of semen to the immune and microbial environments of the FGT.

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APPENDIX

Appendix A: Co-authored manuscript not included in the thesis

Liebenberg LJP, Passmore JS, Osman F, **Jewanraj J**, Mtshali A, Gerardo Garcia-Lerma J, Heneine W, Holder A, Archary D, Ngcapu S, Sivro A, Mansoor LE, Abdool Karim Q, Abdool Karim SS, and McKinnon LR. Genital immune cell activation, and tenofovir gel efficacy in women at risk of HIV infection. *Cell Reports Medicine; Submitted.*

Contributions:

I conducted laboratory assays to assess the levels of cytokines and MMPs in CVL supernatant specimens. I reviewed and approved the final version of the manuscript.

Appendix B: Other poster presentations

1. **Jewanraj J**, Rositch A, Mhlungu S, Mtshali A, Osman F, Abdool Karim SS, Abdool Karim Q, Passmore JS, and Liebenberg LJP. Immune mechanisms for the relationship between high-risk HPV infection and the risk of HIV infection. 32nd International Papillomavirus Conference, 2-6 October 2018, Sydney, Australia.

CAPRISA

Immune Mechanisms for the Relationship Between High Risk HPV Infection and the Risk of HIV Infection

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

Janine Jewanraj [1,6], Anne Rositch [3], Sanele Mhlungu [1], Andile Mtshali [1], Farzana Osman [1], Salim Abdool Karim [1,2], Quarraisha Abdool Karim [1,2], Jo-Ann Passmore [1,4,5], and Lenine Liebenberg [1,6]

[1] Centre for the AIDS Programme of Research in South Africa (CAPRISA), Durban, South Africa. [2] Department of Epidemiology, Columbia University, New York City, NY, USA. [3] Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA. [4] Institute of Infectious Disease and Molecular Medicine (IDM), University of Cape Town, Cape Town, South Africa. [5] National Health Laboratory Service, South Africa. [6] University of KwaZulu-Natal, Department of Medical Microbiology, Durban, South Africa.

Introduction

In Sub-Saharan Africa women are disproportionately affected by both Human Papillomavirus (HPV) and Human Immunodeficiency Virus (HIV). Several studies have shown the association between high risk HPV (HR-HPV) and an increased risk of HIV acquisition. However, few have investigated whether pro-inflammatory immune responses associated with HR-HPV infection, contribute to a genital immune environment known to increase the risk of HIV infection.

Methods

The Roche Linear Array was used to detect the presence of 37 HPV genotypes in CVL pellets of 167 HIV-uninfected women enrolled in the CAPRISA 008 trial. Cytokine and Matrix Metalloproteinase concentrations were measured in matching CVL supernatants using multiplex ELISA technology. The frequencies of activated ($CD38^+$ and/or $HLA\text{-}DR^+$) or proliferating ($Ki67^+$) T cells ($CD3^+$), NK cells ($CD56^+CD16^+$), and HIV target cells ($CCR5^+CD4^+$) were assessed on complementary cytobrush-derived specimens by flow cytometry.

Results

Table 1. Participant demographics according to HPV status (N=167)

Variable		Overall (N=167) % (n)	HPV+ (N=85) % (n)	HPV- (N=82) % (n)	P-value
Age mean (SD)		29.6 (5.5)	28.4 (4.9)	30.9 (5.9)	0.004*
Age (years)					
	(20-25)	18.6% (31)	20.0% (17)	17.1% (14)	0.032
	(25-29)	41.3% (69)	49.4% (42)	32.9% (27)	
	(≥ 30)	40.1% (67)	30.6% (26)	50.0% (41)	
Sexual Behaviour median (IQR)					
Age of Sexual Debut		18 (16 - 19)	18 (16 - 19)	18 (16 - 20)	0.323
Age of regular/stable partner		32 (28 - 37)	34 (30 - 37)	32 (28 - 36)	0.005*
Age of first partner		21 (19 - 24)	20 (19 - 24)	21 (19 - 24)	0.426
Number of live births		1 (1 - 2)	1 (1 - 2)	2 (1 - 3)	0.015*
Age at first pregnancy		19 (17 - 22)	19 (17 - 21)	19.5 (18 - 23)	0.191
Age when started menstruating		14 (13 - 16)	14 (13 - 15)	14 (13 - 16)	0.701
Number of lifetime partners		2 (2 - 4)	3 (2 - 4)	2 (1 - 4)	0.264
Number of vaginal sex acts in the last 30 days		4 (2 – 8)	4 (3 – 8)	4 (2 – 6)	0.110
Living with regular partner					
	(Yes)	23.4% (39)	16.5% (14)	30.5% (25)	0.026*
	(No)	76.6% (128)	83.5% (71)	69.5% (57)	
Use of Male Condoms					
	(Always)	37.7% (63)	34.1% (29)	41.5% (34)	0.638
	(Sometimes)	48.5% (81)	45.1% (37)	45.1% (37)	
	(Never)	13.8% (23)	13.4% (11)	13.4% (11)	

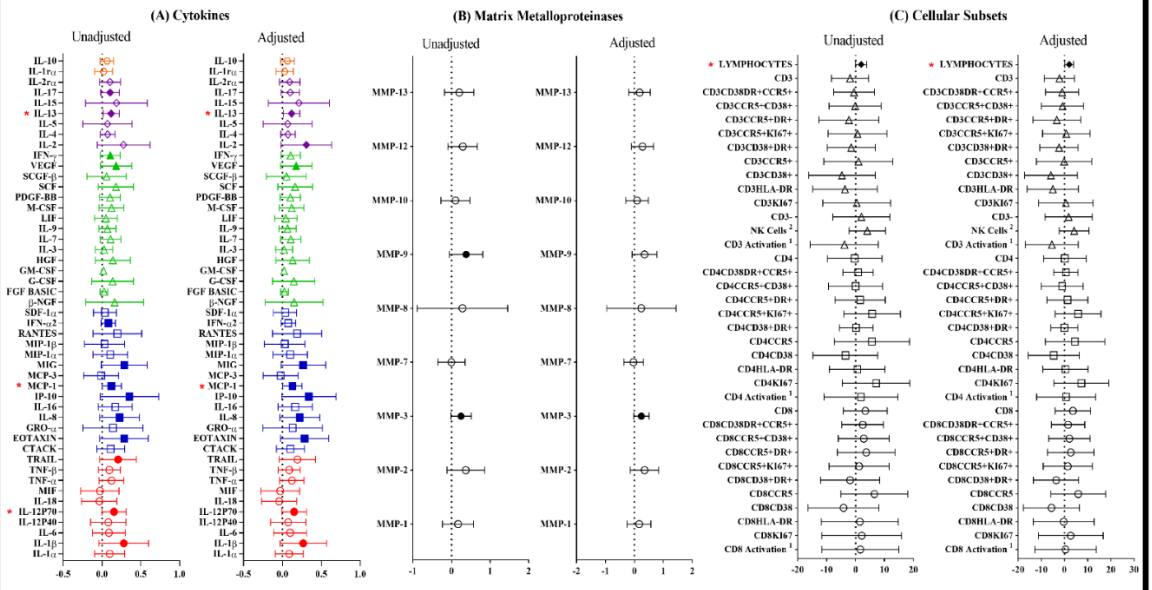


Figure 1. Association between HR-HPV and protein (A&B) and cellular (C) biomarkers of genital inflammation. β-coefficients and corresponding P-values were determined using linear regression models. B-coefficients are indicated by shapes and error bars indicate 95% confidence intervals. Significant P-values (P<0.05) are indicated by (*) and shading, whilst trends (P<0.1) are indicated by shading only. [1]Activation refers to cells expressing CCR5, HLA-DR and/or CD38. [2]NK cells refers to those expressing $CD56^+CD16^+$ markers. Cytokines are ordered according to functionality: pro-inflammatory, chemokines, growth factors, adaptive response and anti-inflammatory cytokines.

Conclusion

This study demonstrated a significant association between HR-HPV and cellular and cytokine biomarkers of inflammation. Genital inflammation and increased mucosal immune cells have been associated with increased risk of HIV acquisition. Longitudinal investigations are warranted to confirm the biological mechanisms by which persistent HR-HPV infection increase HIV acquisition in women.

Acknowledgements

I would like to thank the following for funding my research and conference attendance:

1. CAPRISA-DST-NRF Centre of Excellence in HIV Prevention
2. African Health Research Institute for awarding me the Connect Africa Travel Scholarship.
3. International Papillomavirus Society for flights and conference registration.

2. Masondo S, **Jewanraj J**, Ngcapu S, Osman F, Mtshali A, Mansoor LE, Abdool Karim SS, Abdool Karim Q, Passmore JS, Liebenberg LJP. The impact of TGF-β on the genital immune environment associated with HIV risk in young women. Virtual HIV Research for Prevention Conference, 27-28 January and 3-4 February 2021. *Presented by S Masondo

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*]

[1] Centre for the AIDS Programme of Research in South Africa (CAPRISA), Durban, South Africa. [2] University of KwaZulu-Natal, Department of Medical Microbiology, Durban, South Africa. [3] Department of Epidemiology, Columbia University, New York City, NY, USA. [4] Institute of Infectious Disease and Molecular Medicine (IDM), University of Cape Town, Cape Town, South Africa. [5] National Health Laboratory Service, South Africa.

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.

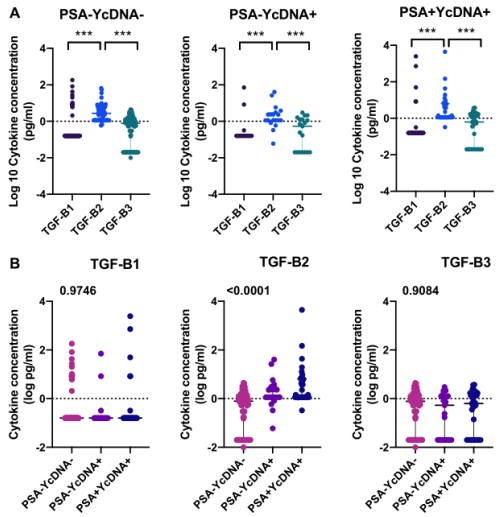
Conclusion

- 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.

HIV VIRTUAL
HIV RESEARCH FOR PREVENTION
27 & 28 JAN | 3 & 4 FEB 2021

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.

Appendix C: Ethics approval letters

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

05 June 2019

Ms J Jewanraj (211502126)
School of Laboratory Medicine and Medical Sciences
College of Health Sciences
JANINE.JEWANRAJ@GMAIL.COM

Dear MS Jewanraj

Protocol: Impact of semen on the immune and microbial environments of the female genital tract
Degree: PhD
BREC Ref No: BE258/19

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received 20 March 2019.

The study was provisionally approved pending appropriate responses to queries raised. Your response received on 30 April 2019 to BREC letter dated 26 April 2019 has been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have been met and the study is given **full ethics approval** and may begin as from 05 June 2019. Please ensure that site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is valid for one year from **05 June 2019.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

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

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

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

The sub-committee's decision will be **noted** by a full Committee at its next meeting taking place on **11 June 2019.**

Yours sincerely

[redacted]

Prof D Wassenaar
Acting Chair: Biomedical Research Ethics Committee

cc: Postgrad administrator: dudhrajhp@ukzn.ac.za SINAYE.NGCAPU@UKZN.AC.ZA Supervisor: LENINE.LIEBENBRG@CAPRISA.ORG

Biomedical Research Ethics Committee
Professor V Rambiritch (Chair)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 2486 **Facsimile:** +27 (0) 31 260 4609 **Email:** brec@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

06 May 2020

Ms J Jewanraj (211502126)
School of Laboratory Medicine and Medical Sciences
College of Health Sciences
JANINE.JEWANRAJ@GMAIL.COM

Dear MS Jewanraj

Protocol: Impact of semen on the immune and microbial environments of the female genital tract
Degree: PhD
BREC Ref No: BE258/19

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	**05 June 2020**
Expiration of Ethical Approval:	**04 June 2021**

I wish to advise you that your application for Recertification received on 05 May 2020 for the above protocol has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

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

The committee will be notified of the above approval at its next meeting to be held on 09 June 2020.

Yours sincerely

[redacted]

……..
Ms A Marimuthu
(for) Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

cc: postgrad administrator: dudhrajhp@ukzn.ac.za
Supervisor: lenine.liebenbrg@caprisa.org
sinaye.ngcapu@ukzn.ac.za

Appendix D: Informed consent form for specimen storage and possible future research testing

CAPRISA 008
Open-Label Randomized Controlled Trial to Assess the Implementation Effectiveness and Safety of 1% Tenofovir Gel Provision through Family Planning Services in KwaZulu-Natal, South Africa

Version 2.0
23 November 2011

INFORMED CONSENT FOR SPECIMEN STORAGE FOR POSSIBLE FUTURE RESEARCH

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

PRINCIPAL INVESTIGATORS:
Professor Quarraisha Abdool Karim, Professor Salim S Abdool Karim, Dr Leila E Mansoor
2nd Floor Doris Duke Medical Research Institute
Nelson R Mandela School of Medicine
Private Bag 7, Congella 4013, Durban, South Africa
PHONE: 031-260 4550

Introduction
You have decided to take part in CAPRISA 008. During this study blood, urine and genital specimens will be collected from you for the purposes of this study. Specimens that are left over following completion of all testing required for CAPRISA 008 may be useful for future research. You are being asked to consent to the storage of your blood and genital specimens for possible future research that may or may not be related to this study.

This consent form gives you information about the collection, storage, and use of your blood and genital specimens for possible future research. Feel free to talk to the study staff about any questions you may have. If you agree to the storage of your blood and genital specimens for possible future research, you will be asked to sign this consent form. We will give you a copy of this form to keep.

You can still take part in CAPRISA 008 even if you decide not to sign this form. If you decide not to sign this form, the specimens described below will be collected from you and after all the study related testing has been completed all your remaining specimens will be destroyed.

HOW WILL YOU GET THE BLOOD AND VAGINAL SPECIMENS FROM ME?
The study staff will take blood from you when you come for the following study visits: at enrolment, 6 month, 12 month, 18 month, 24 month and your last study visit. At each of these visits we will take about 30 ml or about 6 teaspoons of blood with a needle from your arm for the study. This blood is needed to carry out the regular tests for the CAPRISA 008 research study. If you agree to have your specimens stored for possible future research, we will store the remainder of this blood after the tests for this study have been completed for research that is not related to this study.

In addition, as part of this study, you will have a pelvic examination when you come for the following study visits: at enrolment, 6 month, 12 month, 18 month, 24 month and your last study visit and we will collect genital specimens. These specimens will be stored and may be used during, or at the end of the study, to recheck or conduct additional tests to help us understand how the gel works better. If you have not agreed to your specimens being stored for future testing not related to this study, these specimens will be destroyed after all the study related tests have been completed. If you agree to have your specimens stored for possible future research, these genital specimens will be kept and used for possible future research that is not related to this study.

HOW WILL YOU USE MY STORED BLOOD AND VAGINAL SPECIMENS?
The stored samples may be used for future research, to confirm test results, or to make sure that the samples tested have all come from the same person. If new methods of testing become available, the samples may be used to see if these new tests give the same results. We may test your cells, proteins, other chemicals in your body and your genes (DNA). Some of the samples will also be tested to see how your nutritional status may be interacting with HIV infection. Your samples may be analyzed in laboratories outside of South Africa. Your blood or genital specimens will not be sold or used in products that make money for the researchers or anyone else. Virus obtained from your sample may be used in vaccine research and development. Any studies that use your specimens in future research will be

reviewed by the Biomedical Research Ethics Committee of the Nelson R. Mandela School of Medicine, University of KwaZulu-Natal.

We do not plan to contact you or your regular doctor with any results from tests done on your stored blood or vaginal specimens. This is because research tests are often experimental and we don't think the results will be useful for making decisions about your health. Additionally these tests will be done in a way that will make it extremely difficult to link the test results to you. You will not be compensated for use of your stored specimens.

All future research studies using your specimens will be reviewed first by the CAPRISA Scientific Review Committee and a special committee at the University of KwaZulu-Natal Biomedical Research Ethics Committee.

HOW LONG WILL YOU KEEP MY BLOOD AND GENITAL SPECIMENS?
There is no time limit on how long your blood and vaginal specimens will be stored.

HOW WILL MY BLOOD AND GENITAL SPECIMENS BE STORED?
Your blood and genital specimens will be stored at special facilities that are designed to store blood and genital specimens safely and securely. The storage facilities are based at the CAPRISA Research Laboratory, Doris Duke Medical Research Institute, Nelson R Mandela School of Medicine, University of KwaZulu-Natal. The storage facilities are designed so that only authorized researchers can have access to the blood and genital specimens. Some CAPRISA staff responsible for the storage facilities will need to have access to your blood specimens in order to store them and to keep track of where they are, but these staff will not have any information that can identify you.

DOES STORAGE OF MY BLOOD AND VAGINAL SPECIMENS BENEFIT ME?
It is unlikely that there will be any direct benefits to you from tests done on your stored specimens. There may be benefits to society of doing research on your stored blood and/or genital specimens. These benefits may include learning more about HIV infection or other diseases.

WHAT ARE THE RISKS?
There are few risks related to storing your blood and genital specimens. When future tests are done on the stored blood and genital specimens, there is a small but possible risk to your privacy.

WHAT ABOUT CONFIDENTIALITY?
In order to keep your information private, your blood and genital specimens will be labelled with a code. Your personal information, such as name, address, and phone number, will not be placed on the specimens. Only the research clinic where you come for study visits will be able to link the storage code with your personal information.

In the future, when researchers are given your stored specimens to study, they will be given only the code; they will not be given your personal information.

The results of tests done on your stored specimens will not be included in your health records. Every effort will be made to keep your personal information confidential, but we cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law.

WHAT ARE MY RIGHTS?
Allowing your blood and genital specimens to be stored for future research is completely voluntary. You may decide not to have any blood or genital specimens stored for future research. You can still be in this research study or any future study if you decide not to allow your specimens to be stored for future research. However, specimens will still be collected from you and stored in order to complete what is needed for this study.

Even if you decide now that your blood and genital specimens can be stored for possible future research, you may change your mind at any time. If you change your mind, you must contact your study doctor or nurse and let them know that you do not want your blood and genital specimens used for future research. The reverse may also be true, that is, you may not want to agree to have your blood and genital specimens stored now but change your mind at a later date. Should you reconsider agreeing to your specimens being stored you should feel free to discuss with the study staff at any time during the study including at the end of the study. Your blood and genital specimens in storage will be destroyed after all study related testing has been completed, included quality assurance testing should you not agree to storage of your blood and/or genital specimens for future research.

WHAT DO I DO IF I HAVE QUESTIONS?
If you ever have any questions about the storage of your blood or genital specimens you should contact Professor Quarraisha Abdool Karim or Professor Salim S Abdool Karim at 031-260 4550, CAPRISA, Second Floor Doris Duke Medical Research Institute, Durban or Dr Janet Frohlich at 033-260 6851, CAPRISA Vulindlela Clinic, Mafakathini or Dr Leila Mansoor at 031- 260 4641 at the CAPRISA eThekwini Clinic.

If you have questions about your rights as a research participant, you should contact the Biomedical Research Ethics Administration, University of KwaZulu-Natal, Research Office, Westville Campus, Govan Mbeki Building, Private Bag X 54001, Durban, 4000, KwaZulu-Natal, SOUTH AFRICA. Tel: 27 31 2604769 - Fax: 27 31 2604609, Email: BREC@ukzn.ac.za.

Or you can write to the South African Medicines Control Council (MCC): The Registrar: SA Medicines Control Council, Department of Health, Private Bag X828, PRETORIA, 0001 Fax: (012) 323-4474, e-mail: labusa@health.gov.za

SIGNATURES
Please carefully read the statements below and think about your choice. No matter what you decide it will not affect your care or your participation in CAPRISA 008.

I agree to have blood and genital specimens taken for the purpose of storage and testing for future research related to HIV and other infections.

_____ Yes

_____ No

_____	_____	_____
Participant Name (Print)	Participant Signature	Date
_____	_____	_____
Study Staff Conducting Consent Discussion (print)	Staff Signature	Date
_____	_____	_____
Witness Name (print) (If participant is unable to provide a signature)	Witness Signature	Date

The section below is to be completed by the person who administered the informed consent

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

Appendix E: Publication 1: The impact of semen exposure on the immune and microbial environments of the female genital tract. *Frontiers in Reproductive Health*. 2020;2(8).

frontiers in Reproductive Health

ORIGINAL RESEARCH
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The Impact of Semen Exposure on the Immune and Microbial Environments of the Female Genital Tract

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Background: Semen induces an immune response at the female genital tract (FGT) to promote conception. It is also the primary vector for HIV transmission to women during condomless sex. Since genital inflammation and immune activation increase HIV susceptibility in women, semen-induced alterations at the FGT may have implications for HIV risk. Here we investigated the impact of semen exposure, as measured by self-reported condom use and Y-chromosome DNA (YcDNA) detection, on biomarkers of female genital inflammation associated with HIV acquisition.

Methods: Stored genital specimens were collected biannually (mean 5 visits) from 153 HIV-negative women participating in the CAPRISA 008 tenofovir gel open-label extension trial. YcDNA was detected in cervicovaginal lavage (CVL) pellets by RT-PCR and served as a biomarker of semen exposure within 15 days of genital sampling. Protein concentrations were measured in CVL supernatants by multiplexed ELISA, and the frequency of activated CD4+CCR5+ HIV targets was assessed on cytobrush-derived specimens by flow cytometry. Common sexually transmitted infections (STIs) and bacterial vaginosis (BV)-associated bacteria were measured by PCR. Multivariable linear mixed models were used to assess the relationship between YcDNA detection and biomarkers of inflammation over time.

Results: YcDNA was detected at least once in 69% (106/153) of women during the trial (median 2, range 1–5 visits), and was associated with marital status, cohabitation, the frequency of vaginal sex, and Nugent Score. YcDNA detection but not self-reported condom use was associated with elevated concentrations of several cytokines: IL-12p70, IL-10, IFN-γ, IL-13, IP-10, MIG, IL-7, PDGF-BB, SCF, VEGF, β-NGF, and biomarkers of epithelial barrier integrity: MMP-2 and TIMP-4; and with reduced concentrations of IL-18 and MIF. YcDNA detection was not associated with alterations in

immune cell frequencies but was related to increased detection of *P. bivia* (OR = 1.970; CI 1.309–2.965; $P = 0.001$) at the FGT.

Conclusion: YcDNA detection but not self-reported condom use was associated with alterations in cervicovaginal cytokines, BV-associated bacteria, and matrix metalloproteinases, and may have implications for HIV susceptibility in women. This study highlights the discrepancies related to self-reported condom use and the need for routine screening for biomarkers of semen exposure in studies of mucosal immunity to HIV and other STIs.

Keywords: Y-chromosome DNA, semen, genital inflammation, HIV, cytokines, microbes, matrix metalloproteinases, immune cells

INTRODUCTION

In sub-Saharan Africa, women account for the majority of Human Immunodeficiency Virus (HIV) infections compared to their male counterparts (1) and remain a key target population for the development of biomedical HIV prevention strategies. The risk of HIV infection in young women is increased in the context of genital inflammation (2, 3), and efforts to better understand the causes of inflammation at the female genital tract (FGT) may inform on the design of targeted approaches to prevent HIV acquisition. HIV requires access to local cellular targets at the FGT to establish productive infection, and cytokine biomarkers of genital inflammation may be linked to HIV risk through their role in cellular recruitment (4). Furthermore, genital cytokine concentrations are also associated with alterations in the integrity of the vaginal epithelium (4), and with the abundance of bacterial vaginosis (BV)-associated microbes at the FGT (5–7), both implicated in susceptibility to HIV infection.

Sex without a condom remains the primary mode of HIV-1 transmission, with semen acting as the major vector for male to female transmission of the virus (8). Semen consists of several pro- and anti-inflammatory factors and functions as a biological modifier at the FGT to facilitate pregnancy and conception (9–11). Semen exposure has been associated with temporary upregulation of cytokines and the recruitment of leukocytes to the cervical epithelium and stroma (9–14). A pro-inflammatory immune response is generally mounted against semen in the FGT, resulting in the removal of excess and abnormal sperm (15, 16). Semen also contains a diverse array of microbial communities and has an alkaline pH, all of which have the potential to alter the vaginal microbiome (17–20). Apart from the immune altering capacity of semen itself, sexual intercourse has been associated with a significant reduction in *Lactobacillus crispatus* (17), increased prevalence of *Gardnerella vaginalis* (21), and may also lead to vaginal epithelial microabrasions (22, 23) that facilitate HIV entry and access to local target cells at the female genital mucosa. These alterations at the FGT may have implications for the risk of HIV acquisition in women.

Semen-associated inflammation may be, however, short-lived, as immune tolerance to paternal alloantigens is induced during reproduction (9, 11, 24, 25). Semen contains anti-inflammatory compounds such as transforming growth factor-β which promotes a shift from a type 1 helper (Th1) to a type 2 helper (Th2) immune response at the FGT, thereby inducing a regulatory T cell (Treg) response (14, 16, 25). Semen also contains high concentrations of prostaglandin E2, which has been shown to inhibit macrophage cytokine production and T cell proliferation (25–27). These anti-inflammatory responses responsible for tolerance to sperm may also inhibit the control of pathogens such as HIV and other sexually transmitted infections (STIs) at the FGT. Taken together, efforts to prevent HIV infection may benefit from a better understanding of the contribution that both pro- and anti-inflammatory properties of semen have on the risk of HIV acquisition in women.

Self-reported condom use is often used as an indication of semen exposure at the FGT. However, this practice is subject to bias, and data are often misreported (28–30). Routine objective screening for the presence of semen biomarkers as opposed to self-reports of condom use may be useful to reliably assess the frequency of condomless sex e.g., during HIV prevention trials, to assess mucosal immunity to STIs, and to further characterize the impact of semen on the FGT in the context of HIV. Y-chromosome DNA (YcDNA) detection in female genital specimens has previously been used as a reliable biomarker of semen exposure within 15 days of sampling (31–37). Y-chromosome polymerase chain reaction (PCR) is a highly stable, sensitive, and specific method to detect spermatozoa-associated deoxyribonucleic acid (DNA) fragments of the sex-determining region and testis-specific protein Y-encoded (TSPY) genes of the Y-chromosome that are not present on the X-chromosome gene (36, 38–41). Considering the established unreliability of self-reported condom use, we hypothesized that YcDNA detection, but not self-reported condom use will be associated with alterations in biomarkers of inflammation linked to HIV risk in women.

METHODOLOGY

Study Design and Population

This longitudinal retrospective study included questionnaire data and stored genital samples from 153 randomly-selected HIV negative women from the CAPRISA 008 trial (42). The CAPRISA 008 trial was an open-label extension trial to assess

the effectiveness of delivering tenofovir 1% gel in the context of routine family planning services (42). The women enrolled in this study were aged 20–44 years old, were from urban and rural KwaZulu-Natal, and had previously participated in the parent CAPRISA 004 efficacy trial (43). At the time of initial sampling, all participants had not used 1% tenofovir gel for a minimum of 3 years since exiting the CAPRISA 004 trial and were subsequently provided the tenofovir gel for use throughout the CAPRISA 008 trial, supplied either through CAPRISA clinic sites (control arm) or through family planning services (intervention arm). Genital specimens were collected every 6 months during the 2-year trial period (average 5 ± 1 visits). All participants of the CAPRISA 008 trial provided informed consent for the storage of their specimens for use in future studies (BFC237/010). This study was approved by the Biomedical Research Ethics Committee at the University of KwaZulu-Natal under the ethics number BE258/19. YcDNA detection was conducted at the Medical Microbiology Department at the University of KwaZulu-Natal, and all other laboratory assays were conducted at the CAPRISA Mucosal Immunology Laboratory in Durban, South Africa.

Specimen Collection and Processing

Genital specimens including cervical cytobrushes, cervicovaginal lavage (CVL), and vaginal swabs were collected from the participants at each biannual visit. The collection and processing of CVL specimens was previously reported by Bebell et al. (44). Briefly, a plastic bulb pipette was inserted toward the cervical os through a lubricated speculum. A volume of 5 ml sterile saline was inserted and allowed to bathe the cervix. The resulting fluid accumulated at the posterior fornix and was collected using the same pipette and dispensed into a sterile conical tube. Thereafter the CVL specimens were transported to the CAPRISA laboratory. At the laboratory the specimens were centrifuged, and the supernatant was removed and stored in 1 ml aliquots at −80°C.

Cervical cytobrush specimens were collected as previously reported (45). Briefly, a Digene cervical sampler was used to collect cervical mononuclear cells from all participants under speculum examination. The cytobrush was inserted into the endocervical canal and gently rotated 360° to collect cells from the cervical os. The cytobrush specimens were placed into a sterile 15 ml tube (Griener) containing transport medium [Roswell Park Memorial Institute Medium 1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated Fetal Bovine Serum and 5 mM glutamine, penicillin, and streptomycin]. Any specimen containing visible blood was discarded.

Vaginal swabs were collected from the posterior fornices and lateral vaginal walls of each participant and tested for the presence STIs and BV-associated bacteria.

Human Y-Chromosome Detection Assay (PrimerDesign Ltd, UK)

Total DNA was extracted from stored CVL pellet specimens using the MagNAPure LC DNA Isolation Kit I (Roche Applied Science, Indianapolis, IN), according to the manufacturer's instructions. A region of the TSPY1 gene on the Y-chromosome was amplified using the Applied Biosystems® QuantStudio™ 5 RT-PCR System (Thermo Fisher Scientific). YcDNA concentrations were determined from a 1:4 standard curve dilution series. The amplification of the Y-chromosome within 36 cycles was considered a positive result. The negative control (containing no DNA) and an extraction control (PrimerDesign Ltd, UK) were included in each run. Detection of the Y-chromosome and analysis of the results was performed as outlined in the manufacturer's protocol (PrimerDesign Ltd, UK). YcDNA is reported to be stable in the FGT for up to 15 days after sex (31–33) and served as a biomarker of semen exposure in this study.

Quantification of Soluble Protein Biomarkers of Inflammation in Genital Fluid

Concentrations of 48 cytokines, 9 matrix metalloproteinases (MMPs), and 4 tissue inhibitors of metalloproteinases (TIMPs) were measured in undiluted CVL supernatant specimens, according to the manufacturer's instructions. The concentrations of each analyte was measured using the Bio-Plex Pro Human Cytokine, MMP, and TIMP kits and a Bio-Plex Array Reader (Bio-Rad Laboratories) as previously reported (3). The cytokine panel included interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-16, IL-17, IL-18, IL-1 receptor antagonist (IL-1RA), IL-2 receptor α (IL-2Rα), cutaneous T cell attracting chemokine (CTACK), growth related oncogene (GRO)-α, hepatocyte growth factor (HGF), interferon (IFN)-γ, IFN-α2, leukemia inhibitory factor (LIF), monocyte chemotactic protein (MCP)-3, macrophage migration inhibitory factor (MIF), monokine induced by gamma interferon (MIG), β-nerve growth factor (NGF), stem cell factor (SCF), stem cell growth factor (SCGF)-β, stromal cell-derived factor (SDF)-1α, tumor necrosis factor (TNF)-α, TNF-β, TNF-related apoptosis-inducing ligand (TRAIL), fibroblast growth factor (FGF)-basic, eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage (GM)-CSF, macrophage (M)-CSF, interferon gamma-induced protein (IP)-10, MCP-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor BB (PDGF-BB), regulated on activation, normal T cell expressed and secreted (RANTES) and vascular endothelial growth factor (VEGF). The MMP and TIMP panels included MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12, MMP-13, TIMP-1, TIMP-2, TIMP-3, and TIMP-4. Cytokine data were available for all visits ($n = 679$), while MMP/TIMP data was only generated at baseline ($n = 145$, **Supplementary Figure 1**). The sensitivity of these kits ranged between 0.2 and 45.4 pg/ml for the cytokines and between 1 and 450 pg/ml for each of the MMPs measured in this study. 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.

STI and Microbe Detection

Vaginal swab specimens were used for STI and microbe detection at the National Health Laboratory Services, Inkosi Albert Luthuli Central Hospital Academic Complex (46). Multiplex PCR amplification was performed on the ABI® 7500 platform from Applied Biosystems (Thermo Fisher Scientific) and using the FTD (Fast-track diagnostics) STD9 kit according to the manufacturer's instructions. The kit contained primers and TaqMan probes that were designed from highly conserved regions of genetic sequences for pathogens associated with STIs, namely *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Gardnerella vaginalis*, *Mycoplasma genitalium*, and Herpes simplex virus (HSV)-1/2. Concentrations of two Lactobacilli strains, *Lactobacillus crispatus* and *Lactobacillus jensenii* (Assay ID Ba04646245_s1, Ba04646258_s1) and BV-associated bacteria i.e., *Gardnerella vaginalis*, *Prevotella bivia*, BVAB2, and *Atopobium vaginae* (Assay ID Ba04646236_s1, Ba04646278_s1, Ba04646229_s1, Pa04646150_s1, respectively) were measured using Applied Biosystems™ TaqMan® assays. All reactions were run on an ABI® 7500 platform from Applied Biosystems (Thermo Fisher Scientific) RT-PCR machine. STI data was available for all visits ($n = 676$), while data on BV-associated bacteria was available for all visits but baseline ($n = 534$, **Supplementary Figure 1**). Gram stain microscopy was used to assess for BV by Nugent Score (47). Women were diagnosed as negative, intermediate, or having BV (Nugent Score 0–3, 4–6, and 7–10, respectively).

Investigation of Immune Cell Frequency

Cervical cytobrush specimens were used to measure the dynamics and frequency of activated (CD38+ or HLA-DR+) or replicating (Ki67+) T cells (CD3+CD4+ or CD3+CD8+) and CD4+CCR5+ targets for HIV replication using multiparametric flow cytometry. Data acquisition was conducted using a LSRII flow cytometer (BD Immunocytometry Systems) and analyzed using FlowJo Software version 9.9 (Tree Star, C, US). Gates differentiating negative and positive populations were set by fluorescence minus one staining. Specimens with a cervical CD3+ T cell event count below 100 were excluded from the analysis. The gating strategy is represented in **Supplementary Figure 2**.

Statistical Considerations

The Shapiro-Wilk normality test was conducted to determine the distribution of the data. The Mann-Whitney *U*-test was used to compare continuous variables, and the Fisher's exact test was used to compare proportions between the groups at baseline. Questionnaire data were available for 146 participants at baseline, and linear regression models were used to investigate the relationship between self-reported condom use (always vs. never) and biomarkers of inflammation [cytokine concentrations (pg/ml), MMP/TIMP concentrations (pg/ml) and immune cell frequencies (%)] at baseline. Soluble protein concentrations were log10-transformed and immune cell frequencies were converted to proportions to ensure normality. Additionally, linear mixed models accounting for repeated measures were used to assess the relationships between YcDNA detection and cytokine concentrations and immune cell frequencies over time. A generalized estimating equation (GEE) model using a logit link and accounting for repeated measures was used to determine the impact of semen exposure on vaginal microbe presence over time. The unadjusted models controlled for study arm, i.e., CAPRISA or family planning services, and time in the study. Multivariable models were adjusted for variables associated with inflammation or HIV risk such as study arm, time in study, Nugent Score, participant age, presence of STIs, the number of vaginal sex acts in the last 30 days, and genital inflammation status. Genital inflammation status was defined by the median cytokine concentration across all visits for each participant in the upper quartile of the distribution of cytokine concentrations (as calculated using the entire dataset) (2). Given that genital inflammation is a linear combination of cytokines, this variable was not controlled for in cytokine analyses. *P*-values were adjusted for multiple comparisons using the Benjamini-Hochberg method. All tests were conducted at the 5% level of significance. Statistical analyses were performed using GraphPad Prism version 8.3.1 (GraphPad Software, San Diego, CA), STATA version 15.0 (StataCorp., College Station, Texas, USA), and SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Baseline Characteristics of the Study Population

Demographic data was available for 95% (146/153) of all women at baseline. Overall, the median age of the population was 28 years [interquartile range [IQR] 25–33 years; **Table 1**], with 39% of women having detectable YcDNA in their genital fluid at baseline (57/146 women). More women with detectable YcDNA were married (24.6 vs. 10.1%, $P = 0.038$), living with their partner (33.3 vs. 16.9%, $P = 0.027$), and reported seeing their partner more often (36.8 vs. 21.6%, $P = 0.017$) than those without detectable YcDNA. Additionally, YcDNA detection was associated with a higher median number of lifetime pregnancies [median 2 (IQR 1–3) vs. median 1 (IQR 1–2), respectively, $P = 0.042$], and the number of vaginal sex acts in the 30 days prior to sampling [median 5 (IQR 3–10) vs. median 4 (IQR 2–6), respectively, $P = 0.008$]. Of the women reporting to have always used a condom during sex, 31% (17/54) had detectable YcDNA in their vaginal specimens, highlighting the discrepancies related to self-reported condom use. Gonorrhoeae detection was significantly associated with YcDNA detection (8.8 vs. 0%, respectively, $P = 0.009$). Women with detectable YcDNA also had a higher median Nugent Score [median 3 (IQR 1–7) vs. median 1 (IQR 0–3), respectively, $P = 0.006$].

TABLE 1 | Baseline participant characteristics by YcDNA detection in female genital specimens.

Characteristics	Level	Overall (*N* = 146)	YcDNA+ (*N* = 57)	YcDNA– (*N* = 89)	P-Value
Age (years)	Median (IQR)	28 (25–33)	29 (25–35)	28 (25–30)	0.632
Educational level [% (n)]	Primary School	39.0% (57)	43.9% (25)	36.0% (32)	0.060
	HS complete	54.1% (79)	56.1% (32)	52.8% (47)	
	Tertiary complete	4.8% (7)	0	7.9% (7)	
	Less than primary	2.1% (3)	0	3.4% (3)	
Relationship status [% (n)]	Married	15.8% (23)	24.6% (14)	10.1% (9)	**0.038**
	Stable partner	82.9% (121)	73.7% (42)	88.8% (79)	
	Casual Partner	1.4% (2)	1.8% (1)	1.1% (1)	
Study arm [% (n)]	Intervention	47.3% (69)	50.9% (29)	44.9% (40)	0.502
	Control	52.7% (77)	49.1% (28)	55.1% (49)	
Age of regular/stable partner (years)	Median (IQR)	32 (28–37)	32 (28–38)	32 (29–36)	0.633
Number of lifetime pregnancies	Median (IQR)	2 (1–2)	2 (1–3)	1 (1–2)	**0.042**
Number of vaginal sex acts in the last 30 days	Median (IQR)	4 (2–8)	5 (3–10)	4 (2–6)	**0.008**
Partner HIV status [% (n)]	Positive	2.1% (3)	3.5% (2)	1.1% (1)	0.281
	Negative	65.8% (96)	70.2% (40)	62.9% (56)	
	Unknown	32.2% (47)	26.3% (15)	36.0% (32)	
Partner circumcision [% (n/N)]	Yes	32.8% (41/125)	27.5% (14/51)	36.5% (27/74)	0.542
	No	64.8% (81/125)	70.6% (36/51)	60.8% (45/74)	
	Unknown	2.4% (3/125)	2.0% (1/51)	2.7% (2/74)	
Partner living together [% (n)]	Yes	23.3% (34)	33.3% (19)	16.9% (15)	**0.027**
	No	76.7% (112)	66.7% (38)	83.1% (74)	
How often do you see regular partner [% (n/N)]	Daily	27.6% (40/145)	36.8% (21/57)	21.6% (19/88)	**0.017**
	Weekly	42.8% (62/145)	47.4% (27/57)	39.8% (35/88)	
	Monthly	26.9% (39/145)	14.0% (8/57)	35.2% (31/88)	
	< Monthly	2.8% (4/145)	1.8% (1/57)	3.4% (3/88)	
Contraceptive type [% (n)]	Depo-provera	57.5% (84)	63.2% (36)	53.9% (48)	0.105
	Oral contraceptive	21.9% (32)	17.5% (10)	24.7% (22)	
	Nur-isterate	14.4% (21)	8.8% (5)	18.0% (16)	
	Other	6.2% (9)	10.5% (6)	3.4% (3)	
Male condom use [% (n)]	Always	37.0% (54)	29.8% (17)	41.6% (37)	0.178
	Sometimes	49.3% (72)	50.9% (29)	48.3% (43)	
	Never	13.7% (20)	19.3% (11)	10.1% (9)	
HSV-2 antibodies [% (n)]	Positive	88.4% (129)	86.0% (49)	89.9% (80)	0.106
	Negative	9.6% (14)	8.8% (5)	10.1% (9)	
	Equivocal	2.1% (3)	5.3% (3)	0	
Human Papillomavirus [% (n)]	No	48.6% (71)	50.9% (29)	47.2% (42)	0.735
	Yes	51.4% (75)	49.1% (28)	52.8% (47)	
Any STIs [% (n/N)]	No	81.9% (118/144)	78.9% (45)	83.9% (73/87)	0.509
	Yes	18.1% (26/144)	21.1% (12)	16.1% (14/87)	
Neisseria Gonorrhoeae	No	96.5% (139/144)	91.2% (52)	100.0% (87/87)	**0.009**
	Yes	3.5% (5/144)	8.8% (5)	0	
Chlamydia trachomatis	No	93.1% (134/144)	93.0% (53)	93.1% (81/87)	1.000
	Yes	6.9% (10/144)	7.0% (4)	6.9% (6/87)	
Trichomonas vaginalis	No	95.1% (137/144)	96.5% (55)	94.3% (82/87)	0.704
	Yes	4.9% (7/144)	3.5% (2)	5.7% (5/87)	
Mycoplasma genitalium	No	95.8% (138/144)	94.7% (54)	96.6% (84/87)	0.681
	Yes	4.2% (6/144)	5.3% (3)	3.4% (3/87)	
Bacterial vaginosis [% (n/N)]	Median (IQR)	2 (0–4)	3 (1–7)	1 (0–3)	**0.006**
Negative	0–3	74.6% (106/142)	61.4% (35/57)	83.5% (71/85)	**0.001**
Intermediate	4–6	10.6% (15/142)	10.5% (6/57)	10.6% (9/85)	
BV	7–10	14.8% (21/142)	28.1% (16/57)	5.9% (5/85)	

Significant P-values (P < 0.05) are indicated by bold font.

Biomarkers of Inflammation Were Not Distinguished by Self-Reported Condom Use

Linear regression models were used to investigate the reliability of self-reported condom use as a measure of semen exposure. Biomarkers of female genital inflammation were compared between women self-reporting always ($n = 54$) and never using a condom ($n = 20$) at their baseline visit. Multivariable linear regression models were adjusted for age, any STI, Nugent Score, the number of vaginal sex acts in the past 30 days, randomization arm, and inflammation status. Neither cytokine concentrations, MMP concentrations, nor immune cell frequencies differed between the groups after multivariable adjustments (**Supplementary Tables 1–3**, respectively).

YcDNA Detection Was Associated With Alterations in Protein Biomarkers of Inflammation

Considering the potential unreliability in self-report of condom use, given that 31% of women who reported consistent condom use also had YcDNA evidence of recent condomless sex (**Table 1**), we determined whether a biomarker of semen exposure may be a better indicator of immune alterations at the FGT in response to semen. YcDNA detection within female genital specimens was used as a biomarker of semen exposure within 15 days prior to genital sampling (31–33). Linear mixed models were used to compare cytokine concentrations over time and linear regression models were used to compare MMP/TIMP concentrations at baseline between women with detectable YcDNA (semen exposure) and those without (no detectable semen exposure). Women with detectable YcDNA had significantly increased concentrations of IL-12p70 ($\beta = 0.202$; CI 0.146, 0.258; $P < 0.001$), IP-10 ($\beta = 0.230$; CI 0.094, 0.366; $P = 0.001$), MIG ($\beta = 0.160$; CI 0.052, 0.267; $P = 0.004$), β-NGF ($\beta = 0.180$; CI 0.048, 0.311; $P = 0.008$), IL-7 ($\beta = 0.168$; CI 0.099, 0.236; $P < 0.001$), PDGF-BB ($\beta = 0.062$; CI 0.005, 0.120; $P = 0.035$), SCF ($\beta = 0.107$; CI 0.031, 0.182; $P = 0.006$), VEGF ($\beta = 0.252$; CI 0.186, 0.318; $P < 0.001$), IFN-γ ($\beta = 0.065$; CI 0.000, 0.130; $P = 0.049$), IL-13 ($\beta = 0.126$; CI 0.087, 0.166; $P < 0.001$), IL-10 ($\beta = 0.094$; CI 0.063, 0.124; $P < 0.001$), and reduced concentrations of IL-18 ($\beta = -0.095$; CI −0.184, −0.006; $P = 0.036$) and MIF ($\beta = -0.166$; CI −0.259, −0.072; $P = 0.001$; **Figure 1A**) after adjusting for age, any STI, Nugent Score, the number of vaginal sex acts in the past 30 days, time in study, and randomization arm. These associations between YcDNA detection and concentrations of IL-12p70, MIF, IP-10, MIG, β-NGF, IL-7, SCF, VEGF, IL-13, and IL-10 remained significant even after false discovery rate (FDR) adjustments. The concentrations of MMPs and TIMPs were compared among women with detectable YcDNA and those without at baseline. YcDNA detection was associated with elevated concentrations of MMP-2 ($\beta = 0.419$; CI 0.084, 0.753; $P = 0.015$), and TIMP-4 ($\beta = 0.328$; CI 0.042, 0.614; $P = 0.025$; **Figure 1B**) after adjusting for age, any STI, Nugent Score, the number of vaginal sex acts in the past 30 days, inflammation status, and randomization arm.

Increased Detection of BV-Associated Microbes at the FGT Linked to Semen Exposure

GEE models were used to determine whether semen exposure was linked to an increased presence of BV-associated microbes at the FGT. Women with detectable YcDNA had a significantly increased presence of *P. bivia* (OR=1.970; CI 1.309, 2.965; $P = 0.001$; **Table 2**) compared to those without, after adjusting for age, any STI, the number of vaginal sex acts in the past 30 days, inflammation status, time in study, and randomization arm. This association between YcDNA detection and increased presence of *P. bivia* maintained significance after FDR adjustments ($P = 0.007$).

The Presence of Semen Was Not Associated With Immune Cell Recruitment at the FGT

Since alterations in mucosal cytokines and microbial microenvironments are associated with increased frequency of local HIV-susceptible cells (2, 6), we assessed the impact of semen exposure on the pool of available T cell targets at the FGT. Linear mixed models were used to compare immune cell frequencies between women with detectable YcDNA and those without. Immune cell frequencies were similar between women with detectable YcDNA in their vaginal specimens and those without (**Figure 2**).

DISCUSSION

Studies have demonstrated that semen contains several bioactive molecules with the ability to alter vaginal flora, induce cytokine production, and immune cell recruitment to the FGT after condomless sex (10–13, 17, 18, 25, 48–51). However, few studies investigated the impact of semen exposure on biomarkers of female genital inflammation in relation to HIV acquisition risk. Genital inflammation in women has been linked to an increased susceptibility to HIV infection (2), if semen exposure alters biomarkers of inflammation, then women may be at greater risk of acquiring the virus. Here we demonstrate that semen exposure as measured by YcDNA detection, but not self-report of condom use, had a greater association with biomarkers of epithelial barrier integrity and modulation of BV-associated bacteria than with the cytokine and immune cell responses related to female genital inflammation and HIV risk.

Traditionally, HIV prevention trials and reproductive health studies rely greatly on self-reported data despite acknowledgment of over-reporting (28–30, 52, 53). This study demonstrated a high level of discordance between self-reported condom use and the detection of semen biomarkers in vaginal specimens. In this study, almost a third of the women reporting consistent condom use with their partner had detectable YcDNA in their genital specimens. The challenges associated with inaccurate reporting of condom use among women are established and include: consistency of condom use, incorrect condom use, condom failure, social desirability bias, and recall bias, to name a few (54–60). However, women without detectable YcDNA may either

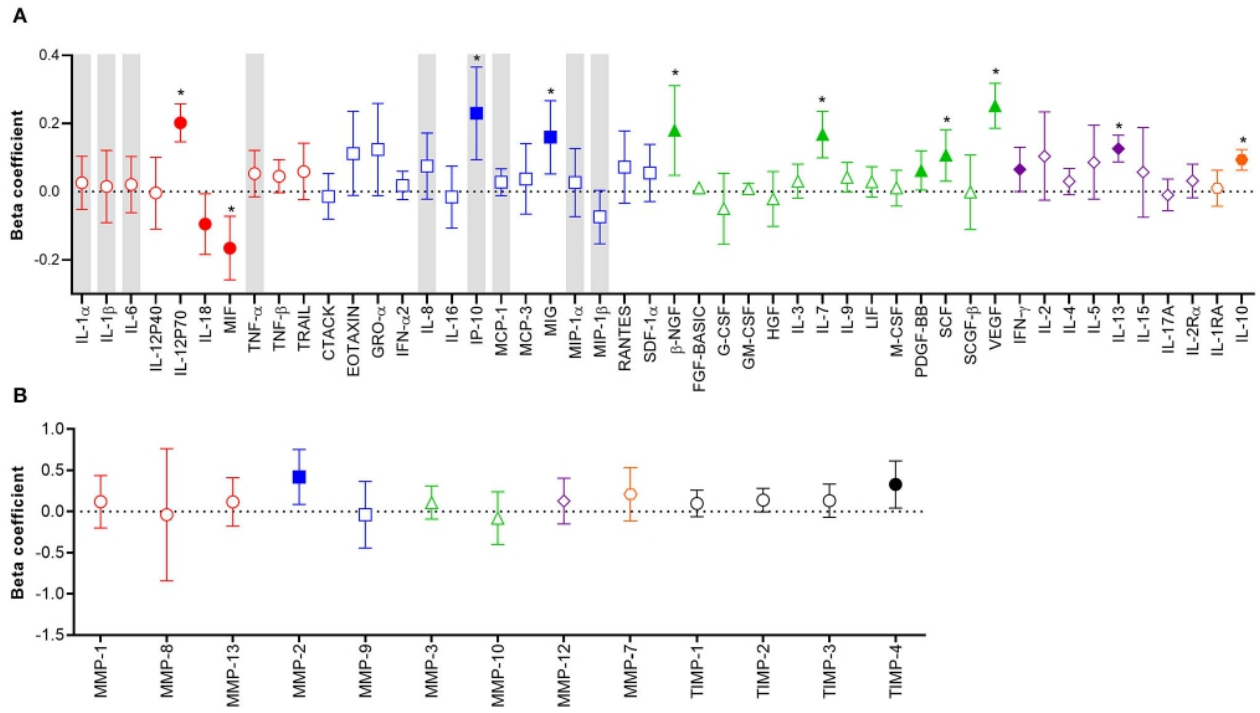

FIGURE 1 | Association between protein biomarkers of inflammation and YcDNA detection in female genital specimens. β-coefficients and corresponding *P*-values for cytokine associations were determined using multivariable linear mixed models adjusting for age, any STI (*C. trachomatis, N. gonorrhoeae, T. vaginalis, and M. genitalium*), Nugent Score, number of vaginal sex acts in the past 30 days, randomization arm, and time in study. β-coefficients and corresponding *P*-values for MMP/TIMP associations were determined using multivariable linear regression models adjusting for age, any STI (*C. trachomatis, N. gonorrhoeae, T. vaginalis, and M. genitalium*), Nugent Score, number of vaginal sex acts in the past 30 days, randomization arm, and inflammation status. β-coefficients are depicted by shapes and error bars indicate the 95% CI. Significant *P*-values ($P < 0.05$) are indicated by filled symbols and significance after FDR adjustment is indicated by (*). **(A)** Cytokines are ordered according to functions: pro-inflammatory (red circles), chemotactic (blue squares), growth/haematopoiesis (green triangles), adaptive response (purple diamonds), and regulatory (orange hexagons) cytokines. Gray shadings represent the nine cytokines/chemokines previously associated with the definition of genital inflammation and/or in demonstrating its association with the risk of HIV infection (2, 3). **(B)** MMPs are grouped according to their functions: collagenases (red circles), gelatinases (blue squares), stromelysins (green triangles), macrophage elastase (purple diamond), matrilysin (orange hexagon), and TIMPs are represented by black circles.

TABLE 2 | Comparison of vaginal microbes between women with and without detectable YcDNA.

Microbe	OR (95% CI)	*P*-Value	FDR	OR (95% CI)	Adj *P*-Value	FDR
L. crispatus	1.083 (0.766–1.529)	0.653	0.653	1.082 (0.763–1.534)	0.659	0.659
L. jensenii	0.752 (0.514–1.099)	0.141	0.237	0.736 (0.506–1.070)	0.109	0.189
A. vaginae	0.666 (0.379–1.171)	0.158	0.237	0.647 (0.370–1.130)	0.126	0.189
BVAB2	1.141 (0.797–1.633)	0.472	0.566	1.136 (0.792–1.631)	0.489	0.586
G. vaginalis	1.427 (0.990–2.058)	0.057	0.171	1.362 (0.942–1.968)	0.100	0.189
P. bivia	1.954 (1.312–2.911)	**0.001**	**0.006**	1.970 (1.309–2.965)	**0.001**	**0.007**

OR and 95% CI were determined using a GEE model with a logit link to account for repeated measures. The unadjusted model controlled for randomization arm and time. The adjusted model additionally controlled for age, the number of vaginal sex acts in the past 30 days, any STI (C. trachomatis, N. gonorrhoeae, T. vaginalis, M. genitalium), and inflammation status. Significant P-values (P < 0.05) are indicated by bold font.

represent those who did use condoms, those who abstained from sex within 15 days, or those who had condomless sex later than 15 days prior to genital sampling. Condom use was over-reported in this study, highlighting the need for routine objective screening for the presence of semen as a biomarker of condomless sex in future HIV prevention studies.

YcDNA detection was associated with marital status, a higher median number of reported vaginal sex acts in the past 30 days, living with or often seeing a partner, and a higher number of lifetime pregnancies compared to YcDNA negative women. The increased presence of semen markers in CVLs from women in stable relationships may be due to several factors, including

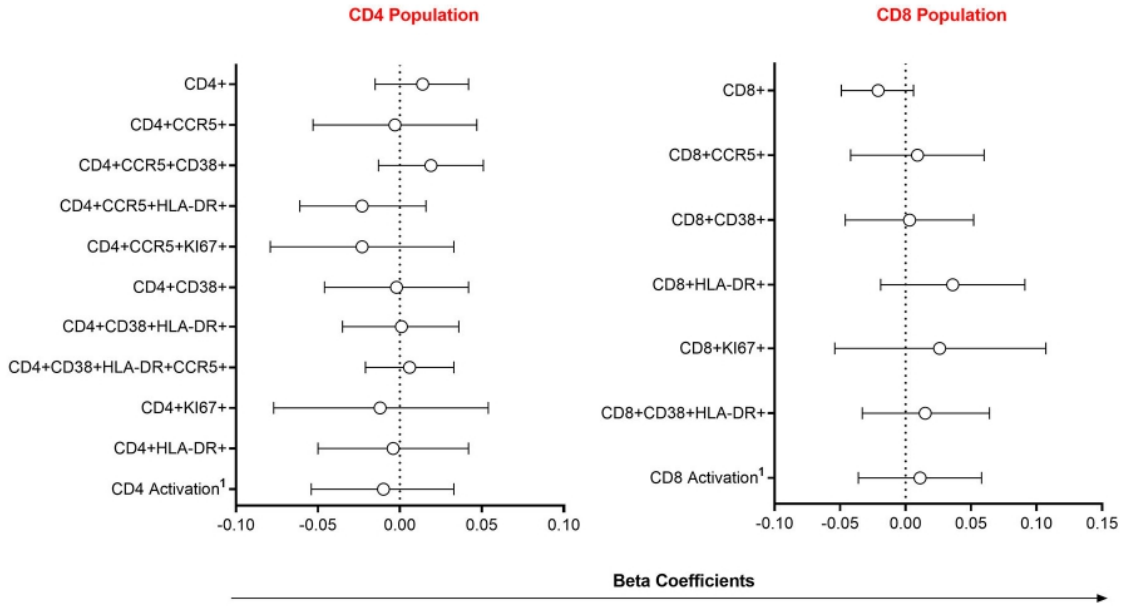

FIGURE 2 | Association between immune cell frequencies and YcDNA detection in female genital specimens. β-coefficients and corresponding *P*-values were determined using multivariable linear mixed models adjusted for age, any STI (*C. trachomatis, N. gonorrhoeae, T. vaginalis, and M. genitalium*), Nugent Score, number of vaginal sex acts in the past 30 days, inflammation status, time in study, and randomization arm. β-coefficients are depicted by shapes and error bars indicate the 95% CI. [1]Activation refers to cells expressing CCR5, HLA-DR and/or CD38.

reduced HIV/STI risk perception and/or an inability to negotiate condom use (61), and late use or early removal of condoms. Additionally, a greater frequency of coital episodes has been associated with increased odds of condomless sex in women (62). A greater number of coital acts with an infected partner may also increase the potential for exposure to sexually transmitted pathogens. Here, gonorrhoeae was associated with YcDNA detection in women. Gonorrhoeae is sexually-transmitted and condomless sexual intercourse with an infected partner is a major risk factor for acquiring the infection (63). However, YcDNA detection was not associated with the other STIs measured, which may be due to a relatively low prevalence of each STI (NG, CT, TV, and MG) in this study. Women with detectable YcDNA in their genital specimens also had a significantly higher median Nugent Score, suggesting that condomless sex is associated with alterations in the vaginal microbiome. These findings are highly consistent with another study reporting that Nugent Scores were significantly associated with the presence of semen in vaginal specimens (64).

Here we investigated the impact of semen exposure on biomarkers of inflammation associated with HIV acquisition in women. YcDNA detection in female genital specimens was used as a biomarker of semen exposure within 15 days of genital sampling (31–33). YcDNA detection at the FGT predicted significantly higher levels of 11/48 cytokines, and with reduced concentrations of two, IL-18, and MIF. Increased concentrations of IL-18 and MIF have previously been implicated in male infertility and reduced sperm motility (65, 66). During reproduction, altered immune responses at the FGT may promote reduced concentrations of these cytokines to facilitate conception. The increase in concentrations of several cytokines is consistent with other studies reporting that semen exposure is associated with cytokine upregulation at the FGT (9, 10, 12, 13, 67). Here, semen exposure was associated with both a pro-inflammatory (IFN-γ, IL-12p70, and IP-10) and anti-inflammatory (IL-10) immune response at the FGT (2, 68, 69). These data support the potential for an initial inflammatory response at the FGT required for embryo implantation and removal of defective sperm, followed by a quick shift to an anti-inflammatory immune response defined by the secretion of IL-10, which may function to promote tolerance to the paternal antigens (14, 15, 25, 70–73). Further, increased concentrations of MIP-1α, MIP-1β, IP-10, and IL-8 have previously been associated with HIV risk in the CAPRISA 004 trial (2). Of these, YcDNA detection was associated only with significant increases in IP-10 in this study, suggesting a limited relationship between semen exposure and those cytokines commonly known to increase the risk of HIV acquisition in women. However, considering that YcDNA is detectable up to 15 days after semen exposure, a biomarker of more recent semen exposure may better characterize the initial pro-inflammatory cytokine response at the FGT which may have implications for HIV risk.

An intact epithelial barrier is a primary host defense against HIV entry and infection. MMPs are proteolytic zinc-dependent enzymes responsible for the degradation and remodeling of the epithelial barrier and have been associated with elevated genital cytokine concentrations (4, 74). YcDNA detection was associated with significant increases in MMP-2 and its regulator TIMP-4.

TIMP-4 was likely upregulated at the FGT in response to the high concentrations of MMP-2, since it prevents the activity of MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9 (75, 76). Friction during sexual intercourse has also been associated with microabrasions at the FGT (22, 23). Increased production of MMPs and TIMPs in response to semen exposure and/or friction during condomless sex may compromise the integrity of the female genital epithelial barrier, thereby facilitating HIV entry and access to local target cells. In support of this hypothesis, several studies have demonstrated increased HIV incidence among women with reduced epithelial barrier integrity (77–80). Given that MMPs/TIMPs are only a small subset of proteins that function in maintaining epithelial barrier integrity, further studies are needed using an expanded panel of barrier proteins to reliably assess the impact of condomless sex on the vaginal epithelium.

Recent studies have suggested that vaginal bacteria can also contribute to genital inflammation known to increase HIV risk in women (5, 6). Here, semen exposure was associated with a significantly increased presence of *P. bivia* at the FGT. Semen has an alkaline pH and raises the acidic pH of the vagina to 7.0 or higher after sexual intercourse without a condom, this may favor the growth of BV-associated microbes (20, 81). Additionally, semen also contains a diverse array of microbial communities that have the potential to alter the vaginal microbial composition (17–19). A study conducted in young South African women demonstrated that a diverse vaginal microbiome dominated by anaerobic bacteria was associated with a 4-fold greater risk of acquiring HIV (6). Given that YcDNA detection was associated with an increased presence of *Prevotella*, which has previously been related to HIV risk (6), semen-induced alterations in the vaginal microbiome may have implications for HIV susceptibility in women.

Since HIV requires access to local target cells to establish productive infection, we assessed the impact of YcDNA detection on endocervical T cell frequencies. Here, YcDNA detection was not associated with significant alterations in HIV target cell frequencies at the FGT. This lack of an association between YcDNA detection and endocervical T cell alterations may be due to the longer range of semen detection. Additionally, Th17 cells that are preferential targets for HIV infection (82) and Treg cell populations which may be induced by semen for tolerance to the paternal antigens (16, 83, 84), were not assessed in this study.

The strength of this study lies in the abundance of immunological and microbial data to assess the impact of semen exposure on the FGT in longitudinal analyses. Few studies have investigated the impact of semen exposure at the FGT in the context of HIV. Here, we used a biomarker of semen exposure to reliably assess the impact of condomless sex on multiple biomarkers of inflammation, including those previously associated with HIV risk in women. However, considering potential variations in immune alterations during a period of up to 15 days after semen exposure, comparisons with a biomarker of more recent semen exposure may be required to better assess semen-induced alterations at the FGT. This study was limited by the yield of cervix-derived T cells required to assess both immune activation and regulation, and further investigation is necessary to determine whether YcDNA detection is associated with alterations in endocervical Treg and Th17 cell populations. Here, common BV-associated microbes were assessed using PCR which limits the detection of semen-associated alterations to those specific microbes. The use of 16S rRNA gene sequencing may provide a more comprehensive picture of the impact of semen exposure on the vaginal microbiome. The study was limited in the ability to control for other factors associated with alterations in the immune and microbial environments of the FGT, including the use of vaginal insertive products, menstruation, contraceptive use, etc. Nonetheless, this study demonstrates that semen exposure is associated with immune and microbial changes at the FGT that may have implications for HIV susceptibility in women, and additional studies are required to further characterize these alterations, assess their robustness, and confirm the relative impact on HIV risk.

Here, YcDNA detection, but not self-report of condom use, was associated with shifts in the immune and microbial profiles of the FGT. Although this biomarker of condomless sex <15 days of sampling was not generally associated with the cytokines and immune cells commonly implicated in raised HIV risk, it was, however, associated with biomarkers of epithelial barrier integrity and an increased presence of *P.bivia* which may still have implications for HIV susceptibility in women. This study provides insight into the impact of semen exposure on the FGT and underscores the importance of further studies to better understand the kinetics of these alterations following semen exposure. Taken together, this study emphasizes the reliability of biomarkers of semen exposure over self-report in analyses of female genital immunity and highlights the importance of incorporating biomarkers of semen exposure and controlling for such evidence of condomless sex in future STI/HIV prevention studies. Understanding the specific contribution of semen to a vaginal immune environment conducive to HIV infection may advise the design of targeted biomedical approaches to prevent HIV infection in women.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Biomedical Research Ethics Committee at the University of KwaZulu-Natal. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JJ, SN, and LL contributed to the conception and design of the study. JJ, LL, AM, and RS performed the experiments. JJ, LL, and FO analyzed and interpreted the data. JJ, SN, LL, J-AP, QA, LM, and SA wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frph.2020.566559/full#supplementary-material

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