Characterization of Female Genital Tract Immunological Markers and Molecular Vaginal Microbiota: Relevance for risk of HIV Transmission and HIV Prevention

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Identificatie van vaginale immunologische merkers en moleculaire microbiota: potentieel belang voor HIV transmissie en HIV preventie

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Front cover: Turkana girl from Loyiangalani, Turkana County - Kenya

Back cover: Women of Olloilalei, Kajiado County - Kenya

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This thesis is dedicated to my brother Harry Ebale, you are dearly missed.

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I am sure you have read one iteration or another of this quote “no one can whistle a symphony. It takes a whole orchestra to play it” Halford E. Luccock (I have no idea who this person is but academic integrity requires us to reference, sindio?). No matter how many times you hear it, it never loses its truth, there is very little that we accomplish on our own. This PhD is no different. There are many, many people to thank. I also know for sure that I will forget some of them (*and remember just after I submit it for print*). If your name is not here and you have supported me in one way or another, charge it to my head and not my heart. Insert your name here. Thank you very much _____. This #PhDjourney has been a really long one and these are the people I would like to thank for walking with me. The members of this orchestra.

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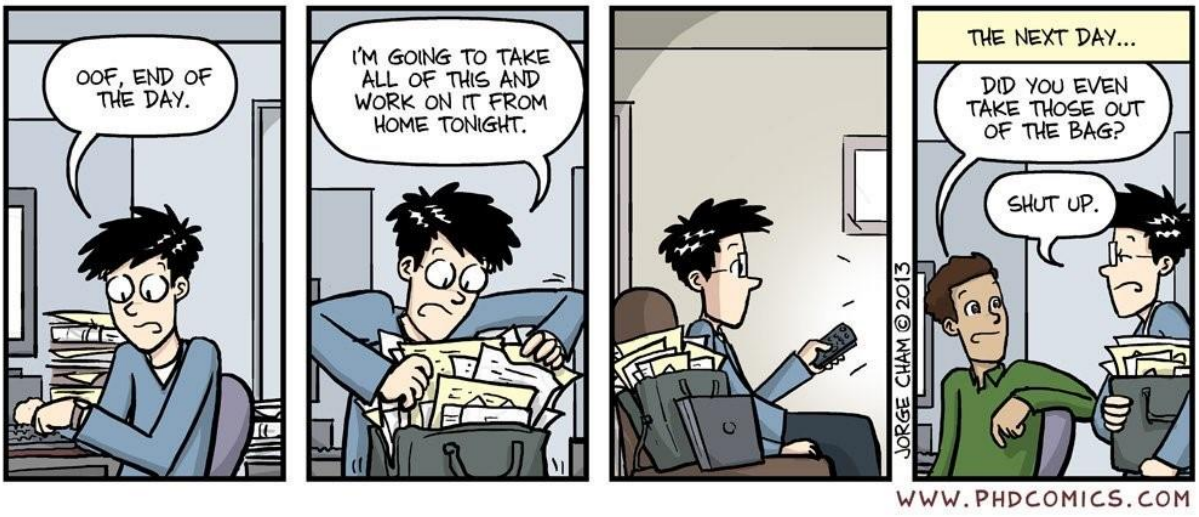

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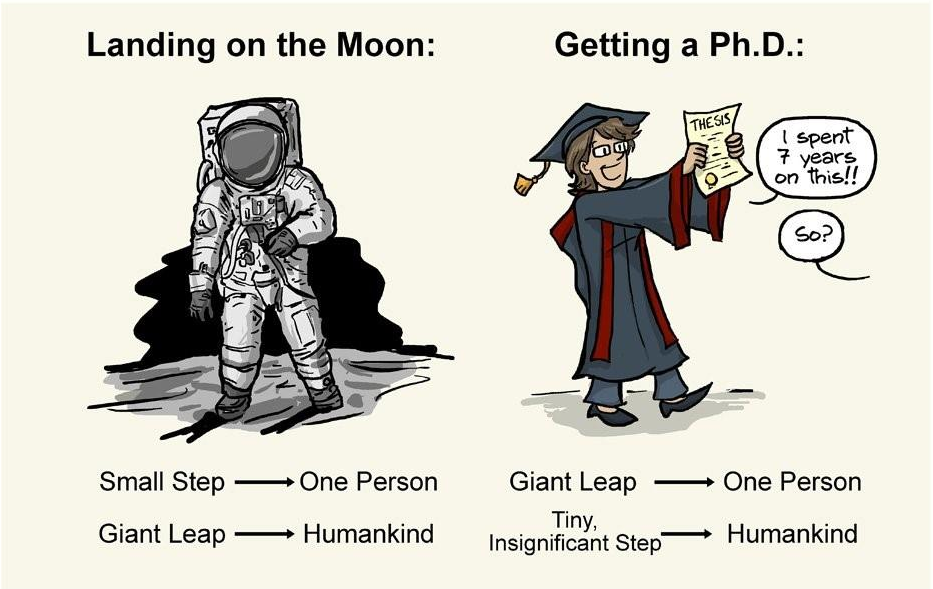

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List of Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
APCs	Antigen-Presenting Cells
ARVs	Antiretrovirals
AV	Aerobic Vaginitis
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
BV	Bacterial Vaginosis
CAPRISA	Centre for AIDS Programme of Research in South Africa
cART	Combination Antiretroviral Therapy
CCR5	Chemokine Receptor Type 5
CD	Cluster of Differentiation
CDC	Centers for Disease Control
CRF	Circulating Recombinant Forms
CS	Cellulose Sulphate
CT	*Chlamydia trachomatis*
CTL	Cytotoxic T-Cell
CV	Coefficient of Variation
CVL	Cervicovaginal Lavage
CXCR4	Chemokine Receptor Type 4
DCs	Dendritic Cells
DIV	Desquamative Inflammatory Vaginitis
DMPA	Depot Medroxyprogesterone Acetate
DNA	Deoxyribonucleic acid
ECS	Endocervical Secretions
EDCTP	European and Developing Countries Clinical Trials Partnership
ELISA	Enzyme-linked Immunosorbent Assay
FCS	Foetal Calf Serum
FDA	Food and Drug Administration
FGT	Female Genital Tract
FITC	Fluorescein Isothiocyanate
FRT	Female Reproductive Tract
FSH	Follicle-Stimulating Hormone
GALT	Gut-Associated Lymphoid Tissue
G-CSF	Granulocyte Colony-stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony-stimulating Factor
H_2O_2	Hydrogen Peroxide
HBD2	Human Beta Defensin 2

hCG	Human Chorionic Gonadotropin
HEC	Hydroxyethylcellulose
HGF	Hepatocyte Growth Factor
HIV	Human Immunodeficiency Virus
HLA-DR	Human Leukocyte Antigen – antigen D Related
HPV	Human Papilloma Virus
HSV-2	Herpes Simplex Virus 2
HTLV	Human T-cell Leukemia Virus
ICC	Intra-class Correlation Coefficient
ICRHK	International Centre of Reproductive Health Kenya
IFN	Interferons
IL	Interleukin
IL-1RA	Interleukin-1 Receptor Antagonist
IN	Integrase
IP-10	IFN-γ –induced protein
ISGs	Interferon-Stimulated Genes
ITM	Institute of Tropical Medicine
LAV	Lymphadenopathy-associated Virus
LH	Luteinizing Hormone
LLD	Low Limit of Detection
LLOQ	Lower Limit of Quantitation
LTR	Long Terminal Repeat
MA	Matrix Protein
MCP-1	Monocyte Chemoattractive Protein-1
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
MIP-1β	Macrophage Inflammatory Protein 1 Beta
MPO	Myeloperoxidase
MSD	Meso Scale Discovery
MSM	Men Who Have Sex with Men
N9	Nonoxynol-9
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NG	*Neisseria gonorrhoeae*
NK	Natural Killer
NNRTIs	Non-nucleoside Reverse Transcriptase Inhibitors
NOD	Nucleotide-binding Oligomerization Domain
NRTIs	Nucleoside/nucleotide Reverse Transcriptase Inhibitors
OPV	Oral Polio Vaccine

PAMPS	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PC	Principal Component Analysis
PCR	Polymerase Chain Reaction
PMTCT	Prevention of Mother-To-Child Transmission
PR	Protease
PrEP	Pre-Exposure Prophylaxis
PRRs	Pathogen Recognition Receptors
PSA	Prostate-Specific Antigen
QC	Quality Control
RANTES	Regulated upon Activation, Normal T cell Expressed and Secreted
RLRs	RIG-1-like receptors
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
RU	Rinda Ubuzima
SIV	Simian Immunodeficiency Virus
SLPI	Secretory Leukocyte Protease-Inhibitor
SoPs	Standard Operational Procedures
SSA	Sub-Saharan Africa
STD	Sexually Transmitted Diseases
STI	Sexually Transmitted Infection
TasP	Treatment as Prevention
TCID	Tissue Culture Infective Dose
TER	Trans-Epithelial Resistance
TLRs	Toll-like Receptors
TNF	Tumor Necrosis Factor
TV	Trichomonas Vaginalis
ULD	Upper Limit of Detection
ULOQ	Upper Limit of Quantitation
UNAIDS	The Joint United Nations Programme on HIV/AIDS
UTI	Urinary Tract Infection
VMB	Vaginal Microbiota
VMMC	Voluntary Medical Male Circumcision
VOICE	Vaginal and Oral Interventions to Control the Epidemic
WHO	World Health Organization
Wits RHI	Wits Reproductive Health and HIV Institute
WST-1	Water-soluble tetrazolium-1

1 Rationale, Objectives and Outline

1.1 Rationale

Worldwide, women are disproportionately affected by the HIV pandemic. In Sub-Saharan Africa, this lopsided effect of the HIV pandemic is even greater with 60% of those living with HIV being women (1). The explanation for this could be biological, with a greater mucosal surface area exposed to the virus in women compared to men during penetrative vaginal sexual intercourse, higher average viral load in men compared to women (2), lack of circumcision of their male sexual partners (3), female genital tract inflammation due to other sexually transmitted infections (4, 5) and association with developmental factors such as cervical ectopy in young women (6). Sociocultural reasons are also responsible as in the case where the most effective HIV prevention method if properly used, the condom, is largely male initiated; economic disadvantage among women that leads to unsafe sexual behaviour (7, 8); the risk of or actual exposure to gender based violence that disempowers women and reduces their ability to negotiate for safe sex (9-11); high school drop-out rates (12, 13) and high risk sexual relationships between young women and much older men (14). Women, therefore, are left vulnerable to HIV infection even within the context of stable relationships where there is less incentive to use protection during sexual intercourse yet concurrent relationships continue to exist (15, 16).

The female genital tract (FGT) can be divided into the upper FGT (endocervix, uterus, fallopian tubes) and the lower FGT (ectocervix and vagina). The lower FGT is composed of a multi-layered squamous epithelium covered by a protective mucous layer that serves to trap and get rid of pathogens from the vaginal tract. The upper FGT is covered by a single layer columnar epithelium. A small region, the transformation zone, exists where the ecto- and endo-cervix meet. The “normal”, healthy lower FGT is colonized by Lactobacilli species (specifically *L. crispatus*, *L. iners, L. vaginalis, L. jensenii* and *L. gasseri*) that are thought to help in producing bactericidal activity, keeping the pH low and producing lactic acid (17), hence providing protection from pathogenic organisms (18). Bacterial vaginosis (BV) is a condition in which this natural vaginal microbiota (VMB) environment is upset and the vagina is characterized by an overgrowth of diverse (facultative) anaerobic bacteria. BV is nowadays described as a polymicrobial dysbiosis with high presence of species from the following taxa: *G. vaginalis, Atopobium vaginae*, *Prevotella* spp., *Eggerthella* spp., *Mobiluncus* spp., *Lachnospiraceae* spp., and several more [2]. Though not a pathogenic condition on its own, BV has been associated with an increased risk of pre-term birth and miscarriage (19), pelvic inflammatory disease (20) and increased acquisition *and* transmission of sexually transmitted infections (STIs) including HIV (21, 22).

Mucosal immune responses in the healthy FGT are under hormonal influence making them relevant to reproductive and physiological functions that include fertilization, foetus implantation, pregnancy and child birth. This implies that variations in cellular and soluble immune mediators can be expected in the course of the menstrual cycle and other reproductive phases that are characterized by changing levels of reproductive hormones. This

includes reduction of specific immune responses to allow for accommodation of foreign spermatozoa and the implantation of a semi-allogeneic foetus. At the same time, both the innate and adaptive arms of the mucosal immune system function to protect against viral and bacterial infections within the context of "normal" or healthy vaginal microbiota. This interplay between reproductive and immune functions means that characterization of female genital tract immunity must always be contextualized.

There has been paucity of data regarding vaginal microbiota and immunological data especially for African women who, as previously mentioned bear a disproportionate burden of HIV infection. The studies discussed in this thesis were carried out as a preamble to and later as part of the multi-country Vaginal Biomarkers Study that sought to bridge that data gap and generate new knowledge necessary for understanding FGT immunology and for the development of interventions to improve women's health in Africa and beyond. When clinical trials with first and second generation vaginal microbicides for the prevention of HIV transmission essentially failed, methodologies for assessing the safety of candidate microbicides had to be reviewed. These early microbicide candidates, HIV membrane-destroying surfactants (N9 and SAVVY) and HIV cell binding-blocking polyanions (CS, PRO2000 and Carageenan), had proven anti-HIV activity *in vitro* with reasonable *in vitro* safety profiles, except nonoxylnol-9 that had already shown signs of vaginal toxicity in previous studies (23-25). It was therefore surprising that these compounds not only failed to demonstrate efficacy against HIV transmission *in vivo* but one (N9) actually potentially increased the risk of infection after frequent use in placebo-controlled trials (26, 27). In 2014, the US FDA released non-binding guidance https://www.fda.gov/downloads/drugs/guidances/ucm328842.pdf for the development of "intravaginal drug products that reduce the risk of HIV acquisition" (i.e. vaginal microbicides). The guidance applied to microbicides that could be formulated as gels, creams, tablets, films, drug-impregnated sponges and drug-impregnated vaginal rings. In addition to other guidance, the FDA stated that "sponsors should assess a candidate vaginal microbicide for the potential to cause cervicovaginal inflammation or epithelial breakdown." By their recommendation, N9 should actually be used in these assessments as a positive control due to its known toxicity. The FDA also explicitly recommends for testing for "static and cidal activity of candidate microbicides on normal resident microflora such as *Lactobacilli*". Our studies contribute to the body of knowledge relevant for these safety aspects of candidate microbicides by describing "baseline" female genital tract cellular and soluble immune markers of inflammation in several distinct populations (e.g. healthy non-pregnant women, adolescent girls and women who engaging in vaginal practices among others) but also the vaginal microbiota in sexually active Caucasian and African women.

1.2 Thesis Objectives

- To characterize vaginal bacterial species and concentrations of soluble vaginal immune mediators over time in our European and African study populations
- To describe host correlates of vaginal microbiota and immune mediators
- To describe the implications of host correlates of vaginal bacteria and immune mediators on HIV prevention interventions for women

1.2 Thesis Outline

Chapter 1 This chapter provides context and the rationale for the work done as part of this PhD and outlines out three objectives.

Chapter 2 Here, I give a general introduction on HIV/AIDS looking at the epidemiology of the epidemic, describing the virus and finally an overview of biomedical (a mixture of clinical and medical) HIV prevention strategies such as treatment, vaccines, voluntary medical male circumcision, pre-exposure prophylaxis and microbicides. In this, and the next chapter, we focus on evidence that was available during our work and new data is dicussed in the general discussion section. There are, however, occasional references to new data for the purposes of proper contextualization.

Chapter 3 The thesis then narrows down to heterosexual HIV transmission, focusing on the female genital tract. I describe the menstrual cycle; innate and adaptive immunity in the female genital tract and the vaginal microbiota. With this background covered, HIV transmission at the genital mucosa is discussed.

Chapter 4 This chapter describes the first of our experimental work where we looked at soluble and cellular biomarkers of inflammation in the lower female genital tract of Caucasian women from Antwerp, Belgium.

Chapter 5 Following our success with the work with Antwerp women, we went ahead to do a cross-sectional analysis of immunological markers and vaginal microbiota in 430 women from Kenya, Rwanda and South Africa. The results are described in this chapter.

Chapter 6 Two cohorts (one without bacterial vaginosis and the other with incident bacterial vaginosis) that were a subset of the 430 women from Sub-Saharan Africa provided longitudinal data on both genital tract immunology and vaginal microbiome description.

Chapter 7 I finally have a discussion on how everything fits together and give perspectives on how we can move from the specifics of the relevance of our findings for HIV prevention microbicide development to general HIV prevention but also make a case for a coordinated and wholesome approach as opposed to uni-focussed approaches.

2 HIV/AIDS: An Introduction

2.1 Epidemiology

In the 30 months preceding July 1981, there were increasing reports of *Pneumocystis* jiroveci pneumonia (previously referred to as "Pneumocystis carinii") and a rare cancer, Kaposi's Sarcoma, among previously healthy homosexual men in New York and California (28). Although clinicians could not explain sudden influx of these diseases and other serious infections in these groups of patients, it soon became evident that they had impaired cellular immunity. The Centers for Disease Control (CDC) then came up with the term Acquired Immune Deficiency Syndrome (AIDS) to describe this condition previously referred to as 'Kaposi's sarcoma and opportunistic infections in previously healthy persons' (29). At the same time, considerable effort was dedicated by different research groups to the search for the etiologic agent responsible for this condition. In 1983, Françoise Barre-Sinoussi then working in Prof. Luc Montagnier's laboratory at the Institut Pasteur in Paris reported that they had isolated a T-lymphotrophic retrovirus from a patient with lymphadenopathy and at risk for AIDS (30). They named the virus lymphadenopathy-associated virus (LAV) and postulated it to be the causative agent of AIDS. Dr. Robert Gallo who had previously isolated human T-cell leukemia viruses types 1 and 2 (HTLV-I and HTLV-II) was also involved in the search for the cause of AIDS and in 1984, his group reported the isolation of a new retrovirus from patients with AIDS which they named HTLV-III (31, 32). The term AIDS-related groups of viruses was also used on retroviruses isolated from AIDS patients (33, 34). Later on, LAV, HTLV-III and the AIDS-related group of viruses were all suspected (and this was later confirmed) to be the same virus and were renamed Human Immunodeficiency Virus (HIV) in 1986 (35). From 1985 on, only two years after HIV was defined as the causative agent for AIDS, commercial enzyme-linked immunosorbent assay (ELISA) kits were available for testing blood-donor samples for the HIV-antibody (36-38). These kits were useful in ensuring non-contaminated blood transfusion but would also be useful later on in sentinel sites for monitoring incident HIV cases and HIV prevalence worldwide.

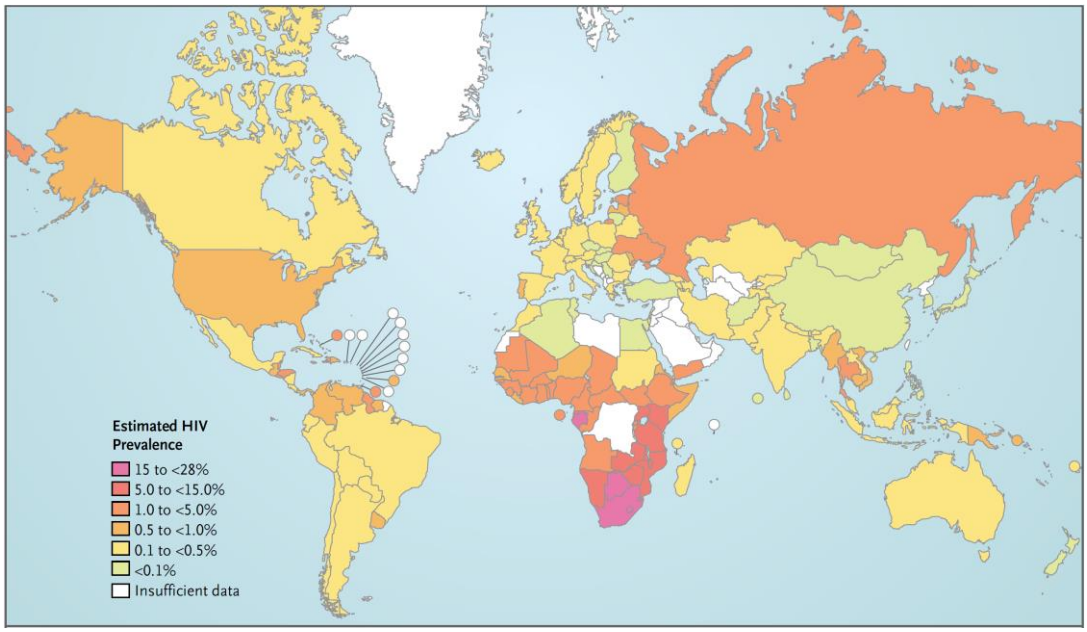


Figure 1. World map of prevalence of HIV infection as at 2012 (adapted from (39)). Sub-Saharan Africa has the highest burden of disease with Southern Africa mostly affected in terms of disease burden and HIV prevalence.

Over 30 years since the initial reports that led to the discovery of the virus, HIV/AIDS has become a major global epidemic. The World Health Organization (WHO) reported that by the year 2014, a total of 71 million people worldwide had been infected by HIV and 34 million of them had died. There are regional disparities in the global HIV burden (Figure 1). In 2016, approximately 70% of the 36.7 million people living with HIV worldwide were in Sub-Saharan Africa (40). A global, multi-sectoral response to the AIDS epidemic has, however, resulted in significant reductions in AIDS-related deaths globally and a slight reduction in the number of new HIV infections (40). The 2017 **UN**AIDS Data report attributes the decline in AIDS-related mortality to the scale up of anti-retroviral therapy with 60% of people living with HIV in Eastern and Southern Africa on treatment in 2016 (40). This is still some way off from reaching the targeted 90% coverage and HIV/AIDS remains a gendered epidemic with more women than men living with HIV, especially in Sub-Saharan Africa (Figure 2).

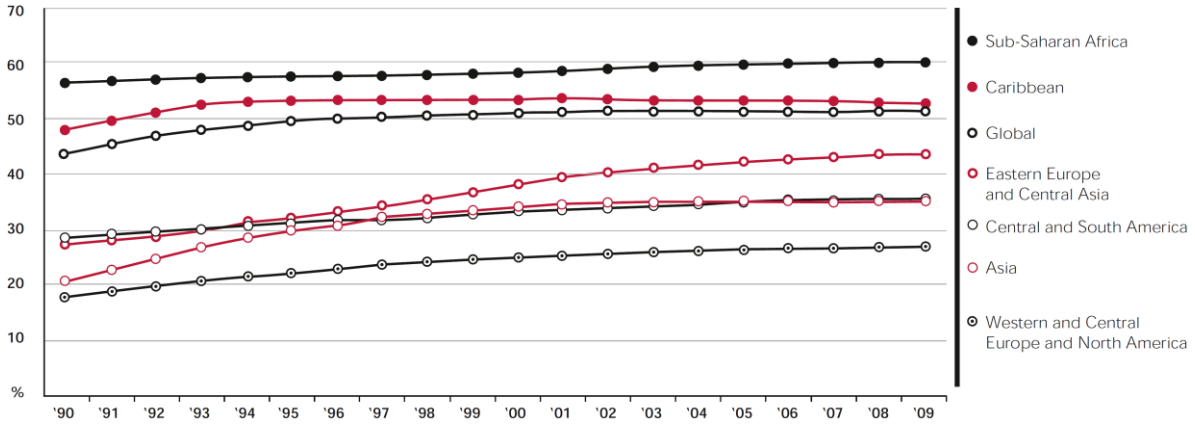


Figure 2 Proportion of people 15 years and older living with HIV who are women, 1990 – 2009 (1)

2.2 Origin and Classification

The origin of HIV has long been a subject of intense debate. However, recent advances in science have allowed analyses including non-invasive testing of wild ape as well as human samples that led to agreement on the origin of HIV. Phylogenetic analyses of virus sequences show that both HIV species (HIV-1 and HIV-2) came about as a result of cross-species transfer of the exogenous lentivirus, Simian Immunodeficiency Virus (SIV), from non-human primates to humans in Africa (41). Claims that transmission from chimpanzees to humans occurred in the Democratic Republic of Congo when chimpanzee tissue was used to prepare the oral polio vaccine (OPV) were disproved when remnant OPV samples tested negative for both SIV and chimpanzee DNA (42). It turns out that OPV was prepared using macaque tissue, a primate whose SIV is not closely related to HIV (phylogenetically) in the way that chimpanzee SIV is. Based on what is known about lentivirus transmission, but with no direct evidence, it is most likely that initial human infection occurred due to mucosal exposure to non-human primate blood and/or fluids, such as would happen during the hunting and handling of these animals in Central Africa. These analyses have also shown that following zoonosis, HIV transmission between humans probably went on for up to seven decades before being picked up as an epidemic in the early 80s. Independent cross-species transmission events led to the establishment of four distinct groups of HIV-1.

Group M is believed to have first infected humans in the early 1900s (43, 44) and to date remains the pandemic form of HIV. Epidemiological evidence points to the capital of the Democratic Republic of Congo (Kinshasa) as the epicentre of the epidemic. This evidence includes the discovery of early strains of the virus (44, 45) and identification of different subtypes of group M viruses (A, B, C, D, F, G, H, J, K) in tissue samples from people who inhabited the large, fast-growing city as early as 1959 (44). From here, these viruses spread to different parts of the world and in some cases of superinfection of individuals with viruses of different subtypes, new circulating recombinant forms (CRFs) were generated (Figure 3). These CRFs incorporate parts of different HIV subtype viruses in their genomes and currently number 88 (https://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html). Work has been done that makes it possible to trace the evolution and distribution of these virus strains across the world. CRF01, for example, probably originated from Central Africa but spread through Thailand to become the predominant virus in the HIV epidemic in Southeast Asia (46). Subtype B also started off in Africa, spread into Haiti and then the United States of America and the rest of the western world where it is the predominant type (47). In Southern Africa where HIV prevalence is the highest, subtype C is highly predominant accounting for over 90% of HIV cases in urban areas (48-51) and is mostly transmitted heterosexually. Clade C viruses also dominate East Africa, especially Ethiopia, Djibouti and Tanzania, together with clade A viruses, mostly in Kenya (52).

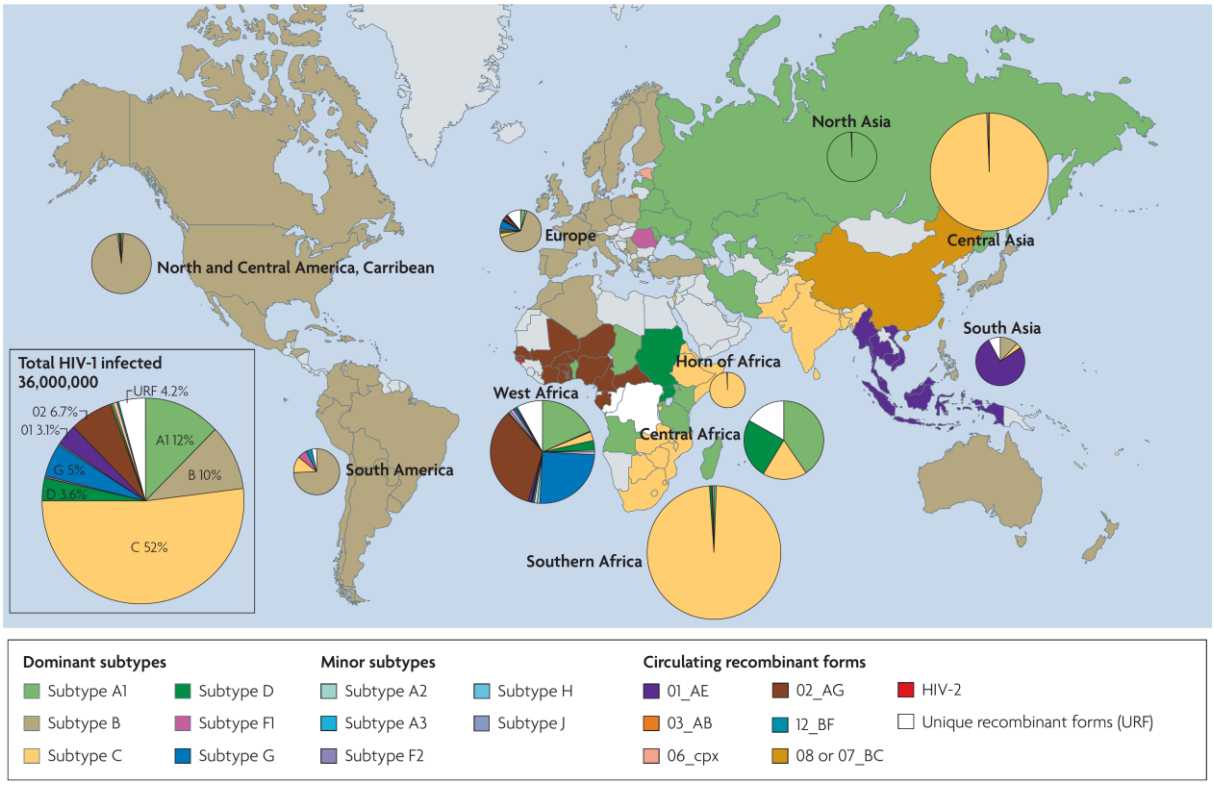


Figure 3. Distribution of HIV subtypes around the world - 2007 (adapted from (48)) The frequency of each HIV-1 subtype and recombinant form was estimated in each country based on published findings. The countries are colour-coded based on the dominant HIV-1 group main (M) subtype. The countries coloured grey have a low level of HIV-1 prevalence or were not represented in the scientific literature related to HIV-1 subtype prevalence. The pie charts depict the proportion of each subtype or recombinant form in each geographical region. The size of the pies is proportional to the number of HIV-1 infected individuals in that particular region.

Group N was first identified in 1998 and has since been restricted to a few infections in Cameroon (53, 54). Both group M and N viruses were derived from SIVcpz*Ptt* strains which infect chimpanzees from Southern Cameroon (55, 56). **Group O** was discovered in 1990 and is also restricted to a few countries in West Africa (57-60). There is no conclusive data as to whether these group O viruses are of chimpanzee or gorilla origin but they are more widely spread compared to group N and P. Even more scarce is the **group P** virus only identified in two individuals of Cameroonian origin since 2009 (61, 62). It is believed that this virus was transferred to humans through a gorilla infected with SIVgor that possibly came from an infected chimpanzee.

HIV-2, on the other hand, has its origins from the sooty mangabey (SIVsmm) in West Africa where infections have largely been documented (63-65). As with HIV-1, independent events of cross-species transmission are believed to have led to generation of eight different groups

of the virus (A, B, C, D, E, F, G & H). Figure 4 illustrates known species of SIV and points at which cross species transfer occurred leading to HIV-1 and HIV-2 infections in humans as discussed. This thesis focuses on HIV-1 which causes over 98% of HIV infections worldwide.

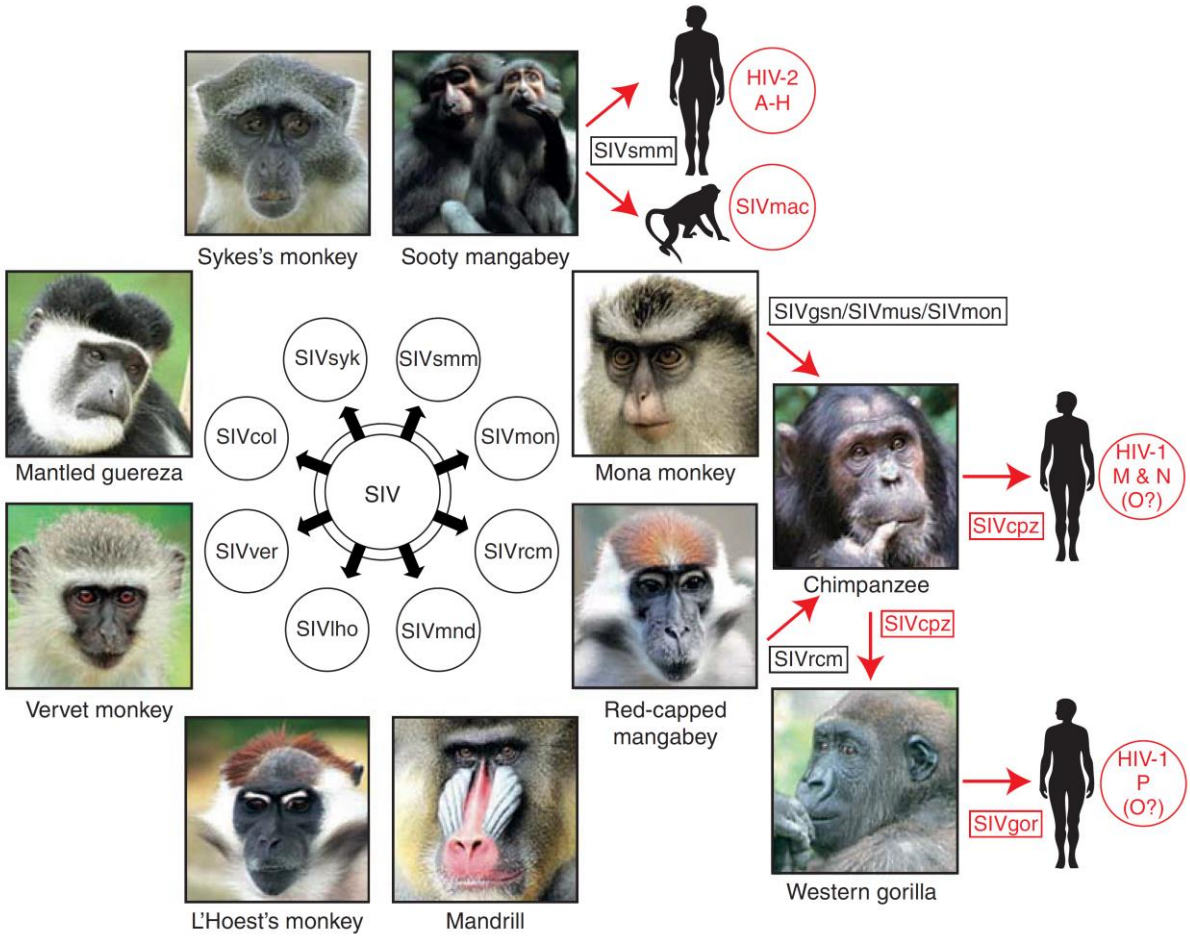


Figure 4. Origins of human immunodeficiency viruses. Old World monkeys are naturally infected with more than 40 different lentiviruses, termed simian immunodeficiency viruses (SIVs) with a suffix to denote their primate species of origin (SIVsmm from sooty mangabeys; SIVmon – mona monkey; SIVrcm – red-capped mangabey; SIVmnd – the mandrill; SIVho – L'Hoest's monkey; SIVver – the vervet monkey; SIVcol – the mantled guereza; SIVsyk – Sykes's monkey; SIVmac – macaques; SIVcpz – chimpanzee; SIVgor – gorilla; SIVgsn – spot nosed monkey and SIVmus – mustached monkey). Several of these SIVs have crossed the species barrier to great apes and humans, generating new pathogens. Known examples of cross-species transmissions, as well as the resulting viruses, are highlighted in red (adapted from (66))

2.3 HIV Structure and Genome

HIV is a retrovirus. These are RNA viruses that replicate through a DNA intermediate utilizing the reverse transcriptase enzyme that transcribes the RNA genome into double-stranded DNA. This DNA is integrated into the genome of the infected cell allowing for replication. Both HIV-1 and HIV-2 are in the sub-family of retroviruses called lentiviruses (or "slow" viruses). These are named so because of their long incubation periods. Unlike endogenous retroviruses that

are transmitted vertically from one generation to another, HIV is an exogenous retrovirus with horizontal transmission from an infected individual to a susceptible host (67-69).

2.3.1 HIV Morphology

At the core of the HIV virion is the p24 capsid that envelopes the two copies of the RNA genome; key virus proteins such as reverse transcriptase (RT), protease (PR) and integrase (IN) and; accessory proteins such as Vif, Vpr and Vpu (Figure 5). This capsid is surrounded by the p17 matrix protein that is anchored onto the virus lipoprotein membrane that forms its envelope. This membrane is formed during the process of viral budding from its host cell and contains host cell proteins that are important in subsequent adherence to other cells. It is also made up of the viral surface glycoprotein gp120 and the transmembrane protein gp 41. The mature virion measures about 120 nm in diameter.

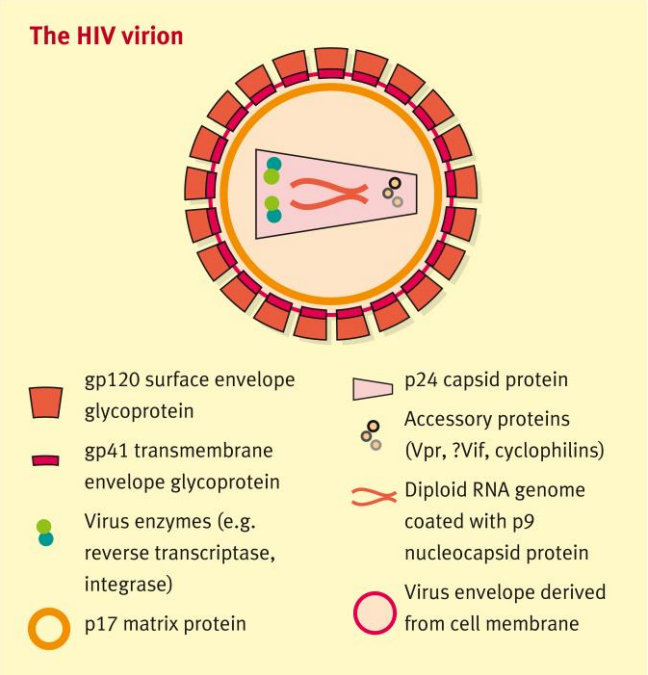


Figure 5. Structure of the HIV-1 virion (adapted from (67)). The virus particle has an envelope containing the surface glycoprotein gp120 and the transmembrane protein gp41. Beneath the p17 matrix protein is the p24 capsid protein that contains the viral genetic material and other essential (RT, IN, PR) and accessory (Vif, Vpr, Vpu) proteins.

2.3.2 Genetic structure

The HIV genome is about 9.8kb in size and, like other retroviruses, has three main structural genes: *gag* (group specific antigen), *env* (envelope) and *pol* (polymerase). The 5' and 3' ends of the genome are made up of long terminal repeats (LTR) that do not code for any viral proteins. Six other genes (*vif*, *vpu*, *vpr*, *tat*, *rev* and *nef*) code for regulatory and accessory proteins necessary for viral replication and infection. The functionality of these genes is as illustrated in figure 6 and described below.

The ***gag*** gene codes for a polyprotein (Pr55) that is then cleaved by the viral protease after the virus buds out of the host cell into several different proteins. These proteins are the p17 matrix protein (MA) that lines the inner part of the virus envelop acting as an anchor for the gp41 transmembrane protein; the p24 capsid that encloses the virion core; the p9 nucleocapsid that coats the viral RNA genome and the small p6 protein with functionality in viral budding *vpr* incorporation. The ***env*** gene also codes for a precursor glycoprotein (gp160) that gets split, this time by cellular proteases, into the highly variable gp120 glycoprotein that lines the outer surface of the virus particle and gp41 that is a transmembrane protein. The ***pol*** gene gets transcribed as a result of a frame-shifting in the *gag* gene readthrough and codes for the enzymes utilized by the virus – reverse transcriptase, protease and integrase.

The accessory protein ***vif*** (viral infectivity factor) plays a central role in ensuring replication of HIV in the host cells. Target cells contain an endogenous inhibitory factor known as APOBEC3G. This factor functions by deaminating cytosine to uracil in viral mRNA leading to protein degradation and non-formation of proviral DNA. *Vif* binds to and degrades APOBEC3G allowing for generation of viable viral genetic material. ***Vpr*** (viral protein R) has several functionalities including the promotion of cell cycle arrest, transportation of the pre-integration viral complex into the nucleus, stimulation of HIV LTR and influencing the expression of NK cells receptors on infected cells. ***Vpu*** functions to counteract cellular proteins such as tetherin that would otherwise bind new viruses and inhibit them from being released from their host cells.

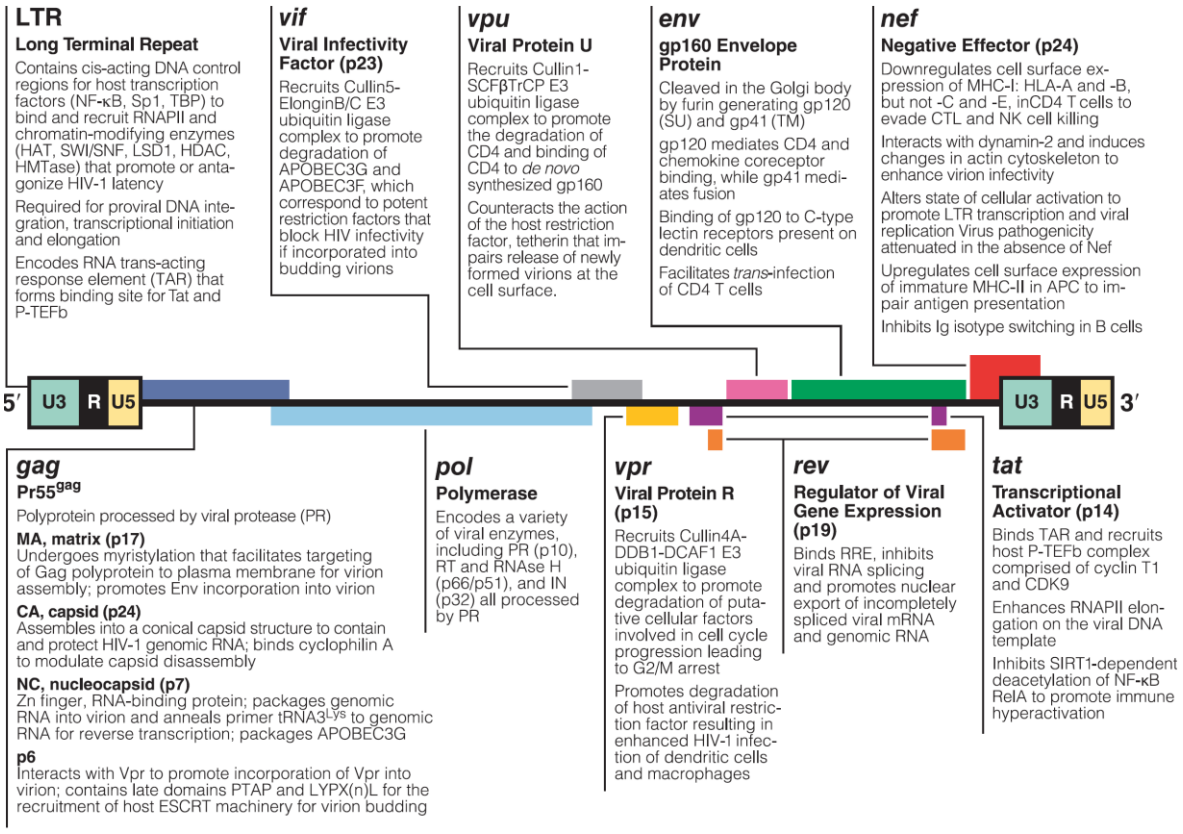


Figure 6. HIV-1 genome and gene functionalities (adapted from (70))

The transactivator of transcription (***Tat***) regulatory protein binds to nascent viral mRNA at the TAR region of the 5' LTR end and greatly stimulates viral transcription. The regulator of viral protein expression (***Rev***) functions as a nucleus export factor that controls the switch between early regulatory proteins expression, encoded by multiple spliced (ms) mRNA to late-stage structural protein expression. This is done by binding to a specific RNA structure in the Env, the Rev-Responsive Element (RRE) thus facilitating the export of unspliced and single-spliced RNA species out of the cellular nucleus and subsequent translation into structural proteins. Lastly, by attracting lymphocytes to infected macrophages, the Negative regulatory factor (***Nef***) increases the number of host cells that get infected. Nef also interferes with cell-cell interaction in immune responses by downregulating the expression of several cellular factors such as CD4, IL-2 receptors and MHC class I proteins on infected cells (67).

2.4 HIV Replication Cycle

Replication of HIV occurs in its target cells which are cells that express CD4 on their surface. The different steps of its lifecycle are illustrated in figure 7 below.

Step 1 Fusion Binding of HIV to its host cell is initiated when the viral glycoprotein gp120 comes into proximity with and is attached to another glycoprotein CD4 (cluster of differentiation 4) receptor that is found on the surface of immune cells. To complete fusion and initiate viral entry into the host cell, another co-receptor is required. HIV utilizes two principal co-receptors for this process; the CCR5 chemokine receptor, highly expressed on monocytes/macrophages (and to a lesser extent on CD4 T cells) and the CXCR4 chemokine receptor primarily found on CD4 T lymphocytes (and much less on macrophages). Viruses that utilize the CCR5 co-receptor are the predominant type in the HIV pandemic and are referred to as R5 or macrophage-tropic viruses. X4 viruses are the ones that utilize the CXCR4 co-receptor with a preference for T lymphocytic cell lines (which, unlike primary T cells, express only CXCR4 and no CCR5). After establishing an infection, viruses are able to switch their co-receptor usage from CCR5 to CXCR4 for their propagation in the host in advanced clinical stages. There are other chemokine co-receptors that have been postulated but these two are the major ones utilized by HIV.

Step 2 Entry Binding of the HIV glycoproteins to the CD4 receptor and corresponding co-receptor induces a conformational change in the transmembrane protein that exposes a hydrophobic region in its structure. gp41 is then able to insert its N-terminal into the host cell membrane completing the fusion process and allowing for the release of the viral capsid into the host cell cytoplasm.

Step 3 Reverse transcription Once inside the cytoplasm, the viral capsid dissociates releasing its contents. The reverse transcriptase (RT) enzyme that is within the capsid then initiates the process of converting the single-stranded viral genomic RNA material into double-stranded DNA. This is an interesting process that utilizes the host tRNA Lys3 as a primer that binds to the viral RNA primer binding site and initiates DNA synthesis (71). The RT enzyme has both polymerase activity that synthesizes DNA and RNase H activity that degrades the viral genomic

RNA in the RNA-DNA duplex. Research has shown that both actions necessarily have to be carried out by the same RT enzyme but that in a virion, there could be multiple copies of RT some of which only have polymerase and others RNase activity (72, 73). These work together to generate dsDNA in the host cell cytoplasm. The presence of multiple "template" RNA and "template switching" is an important characteristics that forms the basis of recombination, which is a crucial factor in rapid viral evolution and adaptation to immune and drug pressure.

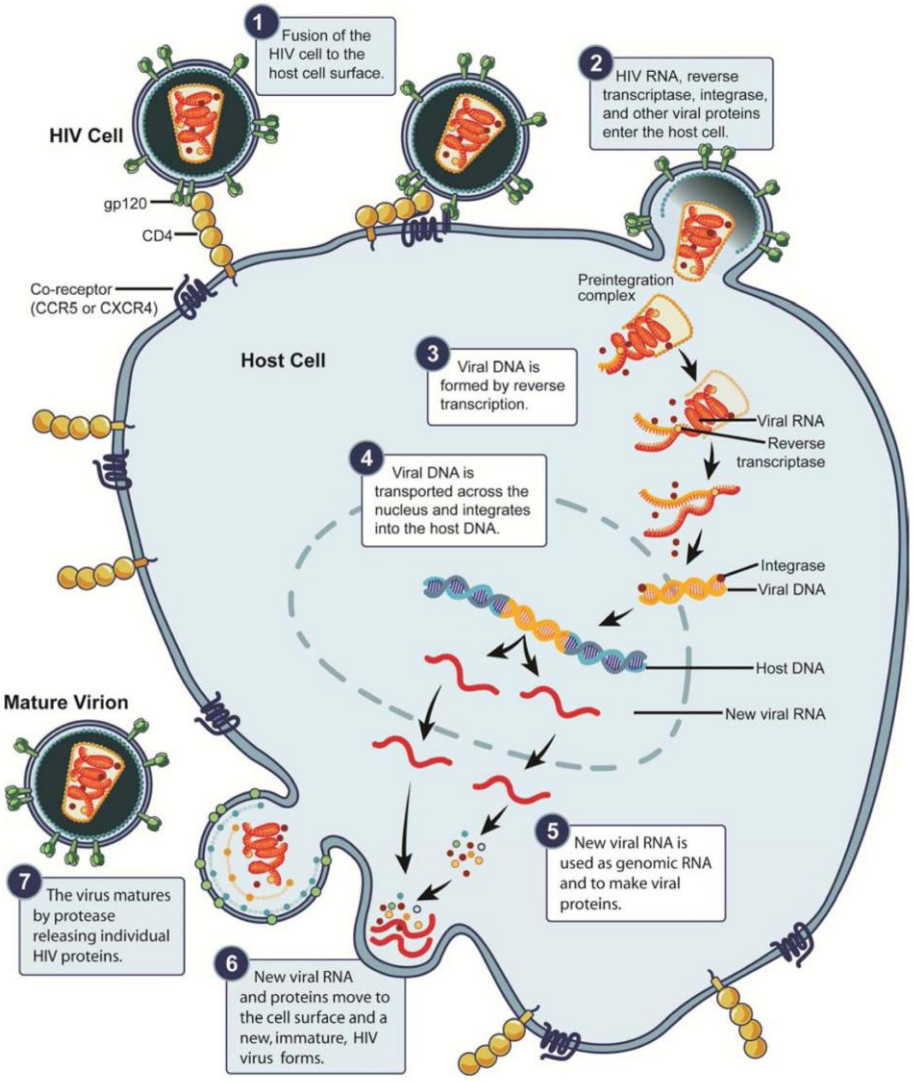


Figure 7. HIV replicative cycle (adapted from (74)). **Cell Entry** –The first step of cell entry is the attachment of the HIV envelope glycoprotein gp120 onto human chemokine receptors (CCR5 or CXCR4) on the CD4 cell surface. After the initial attachment, the next step requires fusion of the viral and cell membranes, allowing the viral proteins to enter into the cytoplasm. **Reverse Transcription** – After cell entry as HIV is a retrovirus, the virus's RNA template transcribes into a double-stranded viral DNA in the presence of the enzyme reverse transcriptase. **Integration –** The viral double-stranded DNA produced after reverse transcription is then transported into the cellular nucleus. In the presence of the integrase enzyme, a multi-step process allows the integration of viral DNA into host genome, and ultimately formation of proviruses. **Formation of Infectious Virons by HIV Proteases –** After successful integration of viral DNA into the host genome and formation of proviral proteins, the next step of the

HIV-1 life cycle is the cleavage of these polyproteins and formation of infectious virions. The viral enzyme protease is the key element for this process.

Step 4 Nuclear transfer and integration into host genome Newly synthesized double stranded viral DNA is closely associated with both viral and host proteins in a high molecular weight pre-integration complex (PIC). This complex crosses over from the host cell cytoplasm into the nucleus in an active process through nuclear pore complexes that involves existing nuclear import pathways (75-77). The viral proteins Vpr (78-80), integrase (81-84) and nuclear transport signals on the p17 matrix protein (85) are actively involved in this nuclear transfer process. It is because of its ability to cross nuclear membranes that HIV can replicate in metabolically active non-dividing cells such as macrophages and dendritic cells while still actively replicating in dividing cells. Once the pre-integration complex is in the nucleus, the viral integrase and cellular enzymes catalyse the process of removing nucleotides from the 3' end of the viral DNA, attacking of phosphodiester bonds in the target host DNA and subsequent joining of the staggered 3' end of the viral DNA to the, now exposed, 5' end of the target host DNA completing viral integration into the host chromosome. Several experiments in different cell types have shown that integration occurs most favourably in actively transcribing regions of the host chromosomes (86-90). This allows for effective transcription of viral gene before the infected cell gets destroyed either by the host immune system or by the inherent toxicity caused by the infection.

Step 5 Viral genome expression In an active infected host cell, cellular RNA polymerase II treats the integrated viral genome as it would any of its native genes and generates viral mRNA transcripts. Early on in the transcription process, fully spliced viral RNA is transported to the cytoplasm using endogenous cellular pathways and translated there to produce the viral regulatory proteins Tat and Rev. Nef is also an early, and functional product of viral genome expression. After translation, Tat and Rev return into the nucleus. Tat then functions by binding to the Tat-responsive region (TAR) on the viral mRNA LTR region and greatly enhances production of early viral proteins by cellular mechanisms. Rev binds to the Rev-responsive element (RRE) (91, 92) and is responsible for the transport of full length unspliced or incompletely spliced viral mRNA (that would have otherwise been restricted or degraded in the nucleus) to the cytoplasm for translation. The action of Rev allows for a switch from the early phase to late-phase viral gene expression and the late gene expression products include the structural proteins Env, Gag and Pol but also the accessory proteins Vif, Vpr and Vpu.

Step 6 Virion formation and budding The rough endoplasmic reticulum (RER) is the site of translation of both Env and Vpu. Gag proteins, on the other hand, are translated on free ribosomes in the cytoplasm otherwise called cytosolic polysomes. These viral structural, enzymatic and accessory proteins are trafficked to the cellular plasma membrane where they assemble in a process that is believed to be regulated by Gag (93-97). This assembly process also involves the formation of the capsid structure that includes two genomic RNA molecules. As the assembled virus buds from the host cell, it incorporates the viral Env glycoproteins gp120 and gp41 in parts of the cell membrane with glycoproteins derived from the host cell

but also the cellular protein cyclophilin that is believed to protect the virus from inhibition by Trim 5α after it infects another cell. Host-derived molecules such as MHC I and II, ICAM 1 and HLA DR are also incorporated into the virion by unknown mechanisms. It is possible that this occurs as a consequence of the virus preferentially budding from the "lipid raft" structures that are enriched for these molecules, because the rafts are involved in immunological (and viral) synapse formation?

Step 7 Maturation and spread The virion that buds from the infected host cell is immature and non-infective. In this virion, the Gag molecules are packed in a radial arrangement and this is reorganized upon maturation. Maturation occurs when the viral protease cleaves the Gag and GagPol polyproteins into fully processed MA, CA, NC, p6, PR, RT and IN proteins (98, 99). The viral capsid core that is formed following maturation then contains all the essential proteins and genetic material necessary for the next round of infection once the infectious particle comes into contact with an uninfected target cell.

The mechanism summarized above describes the cell-free spread of HIV where new mature virus particles diffuse through physiological fluids before infecting new cells. The observation of preferred virion budding at sites of cell-to-cell contact (100) confirmed the suspicion of a different modality of HIV cell-to-cell spread that may or may not be more efficient than cell-free spread, especially in the context of immune (101-104) or drug pressure. Different studies then showed the presence of close contact between infected and uninfected cells (CD4+ T-cells, macrophages and dendritic cells) and the formation of what was referred to as the *virological synapse* (VS) that allowed for cell-to-cell transfer of HIV (105-108). In certain instances, one infected cell can have multiple contact points with uninfected cells referred to as *polysynapses*. It's important to note that the immune cell interactions that HIV-1 takes advantage of are part of normal immune surveillance where DCs or T-cells scan the body for foreign antigens and are activated to mount an immune response to the same. Some viral glycoproteins such as Env can also induce cell-to-cell interaction by its interaction with the CD4 T-cell receptor in an uninfected cell (109). Proposed mechanisms of viral transfer through these T-cell synapses include i, budding and fusion with closely opposed membrane ii, movement of viral material along nanotubes and fusion iii, budding, surfing along filopodia then fusion iv, cell-cell fusion to give syncytia and v, budding and endocytosis (110).

2.5 HIV Pathogenesis

For individuals not on effective treatment, the life course of the virus and the accompanying immune responses and disease in the host follow a general pattern as outlined in figure 8 below and explained below.

2.5.1 Acute phase

HIV infection starts locally with activated and resting CD4+ T cells that are close to the site of exposure being targeted. Usually, a single founder virion will infect a single CD4+ T cell and

there will be gradual increase in viral diversity in response to immune pressure in different body compartments. This is followed by local expansion of the virus populations, movement to the nearest lymphoid organs before one ends up with a systemic infection. Movement from a local to systemic infection likely happens in one of four ways i) simple diffusion of cell-free virus from areas of high concentrations to neighbouring tissues and/or organs with fewer viruses ii) virus transfer to lymphoid organs by dendritic cells iii) viral migration by infected CD4+ T cells iv) targeted transfer to the gut-associated lymphoid tissue (GALT) through the transmitted Env protein that mimics the α4β7 integrin (111). Regardless of relocation mechanism, early infection results in massive systemic loss of CD4+ T cells especially in the GALT. CD4+ T cell depletion has also been correlated with increased genital IL-1β, IL-6 and IL-8 (112).

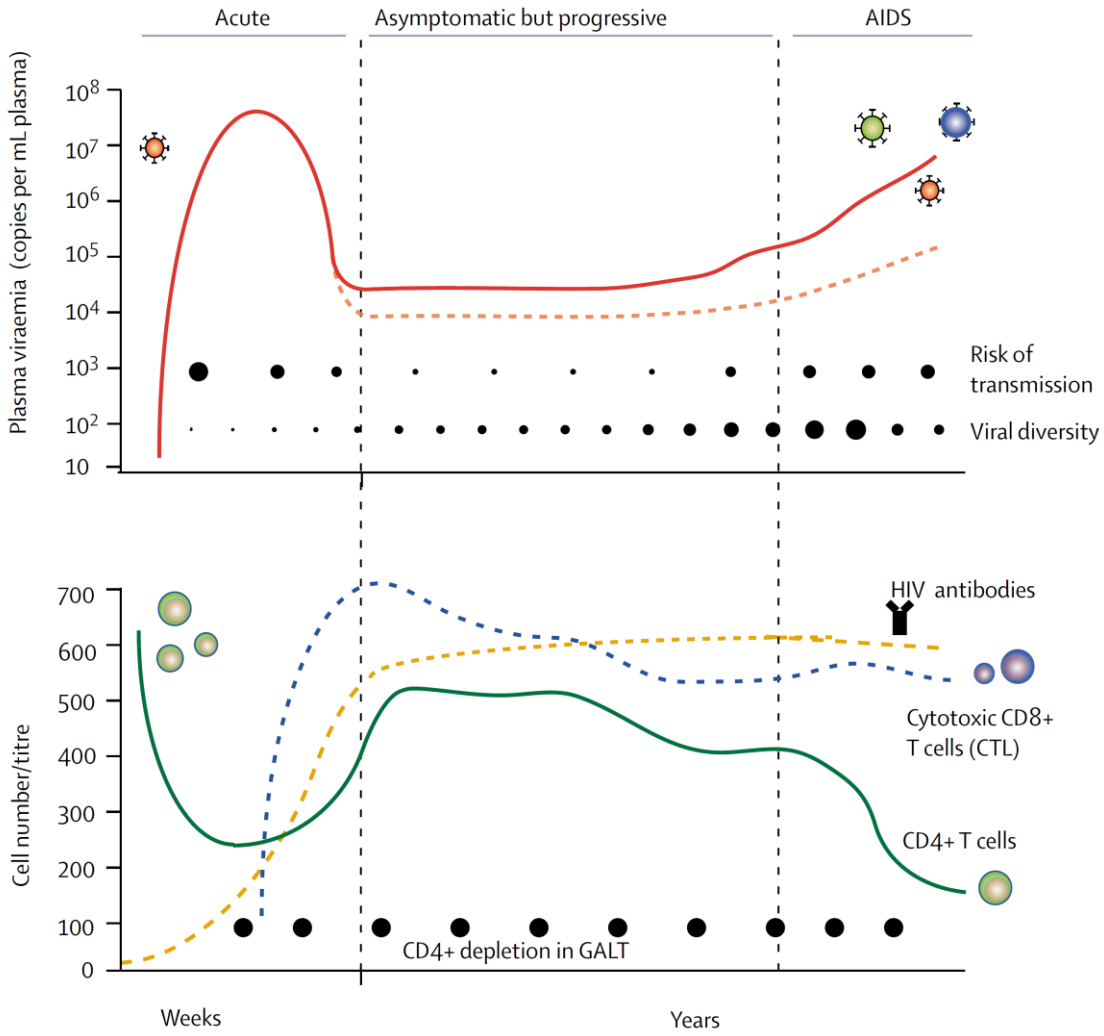


Figure 8. Stages of HIV-1 infection (adapted from (113)). Plasma viraemia (red lines, top panel). Full vs. dotted red lines in the top panel show how viral set points can differ between two individuals. HIV-1 specific antibodies (orange line, bottom panel). Cytotoxic CD8+ T lymphocytes (blue line, bottom panel). CD4+ T cells (green line, bottom panel). Viral diversity increases throughout diseases (closed circles, top panel).

In the first four or so weeks following infection, there is rampant viral replication with viral loads reaching upwards of 10^7 copies per millilitre of plasma. In many, but not all, infected individuals, there are observations of flu-like symptoms - fever, skin rash, fatigue, night sweats, joint aches, sore throat, diarrhoea and enlarged lymph nodes during this phase. The risk of HIV transmission is extremely high at this point because of the high viral load and inadequate immune response. Many people will not even know that they have been infected at this point. When viremia peaks, an adaptive immune response is observed; both anti-HIV antibodies and CD8+ T cells responses to peptides expressed on the surfaces of HIV-1 infected cells. Because of this immune response but also the exhaustion of activated CD4+ T cells, there is a drop in viremia, followed by partial recovery of CD4+ T cells. With this drop of viremia, there is a drop of risk of HIV transmission.

2.5.2 Asymptomatic, but progressive, phase

What follows then are years of a largely asymptomatic infection phase. During this period, immune pressure keeps viral replication at a steady phase, preventing viral flare up as was seen during the acute phase. But that is not to say that things are quiet as there is continual infection and death of CD4+ T cells. The other characteristic of this chronic phase of HIV infection is systemic immune activation that is thought to be initiated during the acute phase of infection (as GALT CD4+ T cells are rapidly depleted) due to translocation of microbial products across the damaged intestinal mucosa (114, 115).

2.5.3 Symptomatic, Acquired Immune Deficiency Syndrome (AIDS) phase

Ongoing CD4+ T cell depletion does get to a tipping point (estimated at approximately 200 cells per µL) where the host immune system gets overwhelmed. At this stage, the patient is said to have an acquired immune deficiency syndrome (AIDS), which is different from mere HIV infection. This takes an average of about 10 years for most individuals but there are a subset of individuals (about 5%), referred to as 'rapid progressors' who get here within 2 to 5 years. On the other extreme, about 5-10% of HIV infected individuals will remain asymptomatic for over 10 years without treatment. These ones are referred to as long-term non-progressors. Once this tipping point is reached, there is a rapid rise in viremia due to loss of control by the immune system, the individual becomes susceptible to multiple opportunistic infections and if not treated, the patient eventually dies.

2.6 HIV/AIDS Clinical Management: Testing and Treatment

From 1985, only two years after HIV was defined as the causative agent for AIDS, commercial enzyme-linked immunosorbent assay (ELISA) kits were available for testing blood-donor samples for the HIV-antibody (36-38). These kits were useful in ensuring non-contaminated blood transfusion but would also be useful later on in sentinel sites used to monitor incident HIV cases and HIV prevalence worldwide.

The multifaceted assault on HIV has made significant gains over the last three decades mainly on the treatment aspect that has seen the development of antiretrovirals that arrest virus replication essentially making HIV infection a chronic disease. This is however not sufficient as toxic side effects of long term drug use exist and patients are not permanently cured. In the early days, ARV therapy was recommended when the patient's CD4 cell count fell below 200 cells per millilitre. Subsequent studies (116-118) that showed the benefits of early treatment i.e. lower chances of opportunistic infections, mortality and onward HIV transmission, as well as much lower toxicity and pill burden of more recent drug combinations, resulted in the current test-and-treat strategy where it is recommended for a HIV-positive individual to start on antiretroviral treatment as soon as they are diagnosed. There are four main classes of antiretroviral drugs that function to inhibit HIV-1 at different points in its life cycle as indicated in figure 9 below.

2.6.1 Entry or fusion inhibitors

As previously discussed, fusion and then entry are the first steps of the HIV replication cycle and are dependent on the viral gp41 protein and host CCR5 co-receptor among others. These two are the targets of the first class of ARVs that we describe. Maraviroc is a CCR5 co-receptor antagonist and enfuvirtide (T 20) binds to the two heptad repeat sequences (HR1 and HR2) of the gp41 protein preventing the conformational change that allows viral entry into the host cell.

2.6.2 Reverse transcriptase inhibitors (RTIs)

This class contains the largest number of drugs and has two sub-classes that target different sites of the same viral enzyme – nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs). When nucleoside analogues of the DNA building blocks (thymidine, guanosine, cytidine and adenosine) are incorporated into the process of viral DNA synthesis by the reverse transcriptase enzyme, the process is halted because they lack the 3'-hydroxil group and viral replication cannot continue (i.e. "chain terminating" action of NRTI). Further, nucleotide (monophosphorylated nucleoside) analogues were developed with better efficiency compared to the nucleosides that required activation by triple phosphorylation. This first group are the NRTIs and include the following US Food and Drug Administration (FDA)-approved ARVs lamivudine, zidovudine, emtricitabine, abacavir, tenofovir disoproxyl fumarate, enteric coated didanosine and stavudine. The second group of NNRTIs function differently in that they bind to an allosteric hydrophobic pocket close to the catalytic site of the RTI causing it to undergo a conformational change that affects it's binding to its template blocking subsequent activity and HIV replication. ARVs in this group are rilpivirine, etravirine, delavirdine, efavirenz and nevirapine.

2.6.3 Integrase inhibitors

This class of drugs consist of compounds that inhibit the integration of viral DNA strands into the host cell DNA within the nucleus. ARVs in this class are few - raltegravir, dolutegravir and elvitegravir.

2.6.4 Protease inhibitors (PIs)

The tail end of the HIV life cycle utilizes the protease enzyme for viral assembly and maturation. These are the steps targeted by the PIs – amprenavir, tipranavir, indinavir, saquinavir, lopinavir, ritonavir, fosamprenavir calcium, darunavir, atazanavir sulphate and nelfinavir mesylate.

HIV-1 has the ability to develop resistance against monotherapy with some of these classes of drugs and as a result, these drugs are usually administered in combination to increase their potency but also reduce drug toxicity of some classes. There are many more drugs under development and these will hopefully result in drugs with less toxicity that are tolerable for HIV-infected individuals.

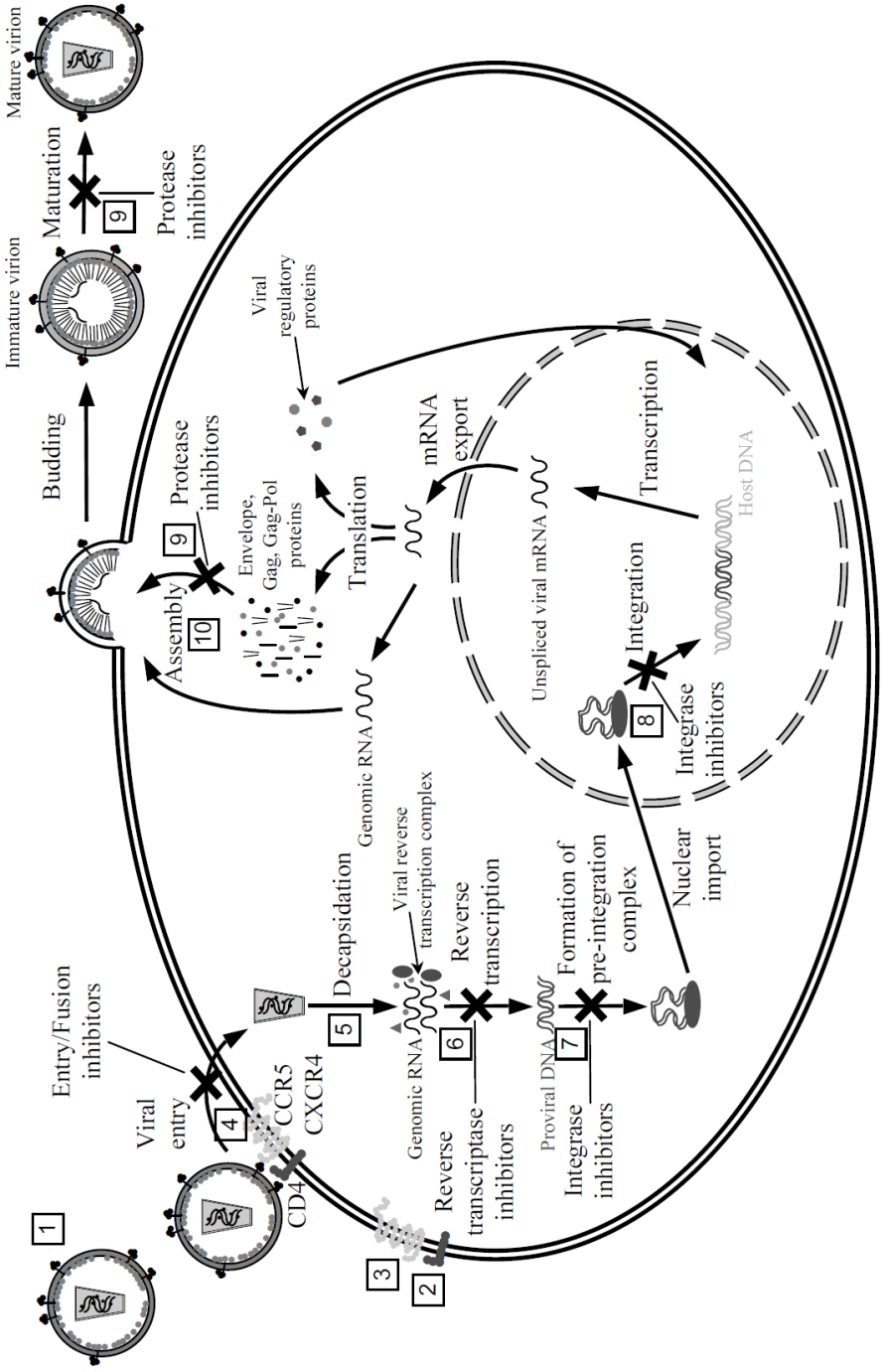


Figure 9. Stages in the HIV life cycle targeted by antiretrovirals (adapted from (119)). The first group of drugs (entry inhibitors) stop the virus from entering the host cell; the second block the process of reverse transcription of genomic RNA to proviral DNA; integrase inhibitors function by targeting the process of integration of viral DNA with host cell DNA within the nucleaus; finally, protease inhibitors target the last step of the HIV life cycle and inhibit viral assembly and maturation. Administering these drugs in combination reduces the chances of the virus escaping drug pressure by developing resistance against a single class of compounds.

2.7 Biomedical HIV Prevention Strategies

2.7.1 Treatment as prevention (TasP)

Three seminal studies have informed this strategy; HPTN 052, the PARTNER study and the Opposites Attract study (116, 117, 120). Together, these studies looked at virtually thousands of sex acts without condoms or oral pre-exposure prophylaxis (PrEP) among gay and heterosexual discordant couples and found zero cases of HIV transmission when the HIV positive partner in the relationship was on ARV treatment and was virally suppressed i.e. viral load below 200 copies per millilitre of blood. These findings support the TasP strategy and led to the Undetectable = Untransmittable (U=U) campaign https://www.preventionaccess.org/ . The thrust of this message should be to put HIV positive individuals on treatment as soon as possible, support them to access the services they need and to remain adherent to treatment. For couples, it is important to note that it takes up to 6 months for someone on treatment to be virally supressed and hence to reduce the risk of HIV transmission. Therefore, other HIV prevention measures (such as PrEP) should be utilized in that season of high risk. Obviously, HIV acquisition can also occur when the HIV positive partner is not adherent to his/her treatment or the HIV negative partner has multiple partners outside of the relationship with the virally supressed partner.

This concept is not exactly new though and it is the same logic that has been applied in prevention of mother-to-child transmission of HIV (PMTCT) averting over 1.5 million new infections among exposed infants since the mid-nineties. Vertical HIV transmission can occur during pregnancy, in child birth and during breastfeeding. In effective PMTCT programmes, HIV positive mothers are put on ARVs during these periods of risk and this strategy reduces the risk of HIV transmission to their infants from 15-45% to below 5%.

2.7.2 Voluntary medical male circumcision

Evidence from randomized control studies in South Africa (3), Uganda (121) and Kenya (122) showed that medical circumcision reduced the risk of female-to-male sexual HIV transmission by approximately 60%. This is most likely due to the fact that the inner surface of the penile foreskin is unkeratinized and rich in HIV target cells but also because of reduction in HIV infection co-factors such as STIs (123, 124). Based on this, voluntary medical male circumcision (VMMC) was recommended by WHO and UNAIDS as an *additional* strategy for HIV prevention especially in high HIV burden settings with low circumcision rates. Care should be taken to prevent risk compensation e.g. reduction in condom use by sexually active males as a result of belief in the partial protection that is afforded by VMMC as this would reverse its potential gains.

2.7.3 Vaccines

On the vaccine front, the Thai trial provided a long overdue proof of concept on the viability of a prophylactic HIV/AIDS vaccine. This trial utilized a canarypox virus vector to deliver env,

gag and protease subunits of the HIV-1 clade B virus and achieved 31% protection – modest, but the best results achieved so far (125). No phase III trial has been completed since the Thai trial. There is ongoing work that is building on the results of this clinical trial but also other strategies such as application of broadly neutralizing antibodies against multiple strains of HIV. Pipeline efforts are summarized in figure 10 below but the reality is that it's likely going to take a while before we have a viable HIV vaccine.

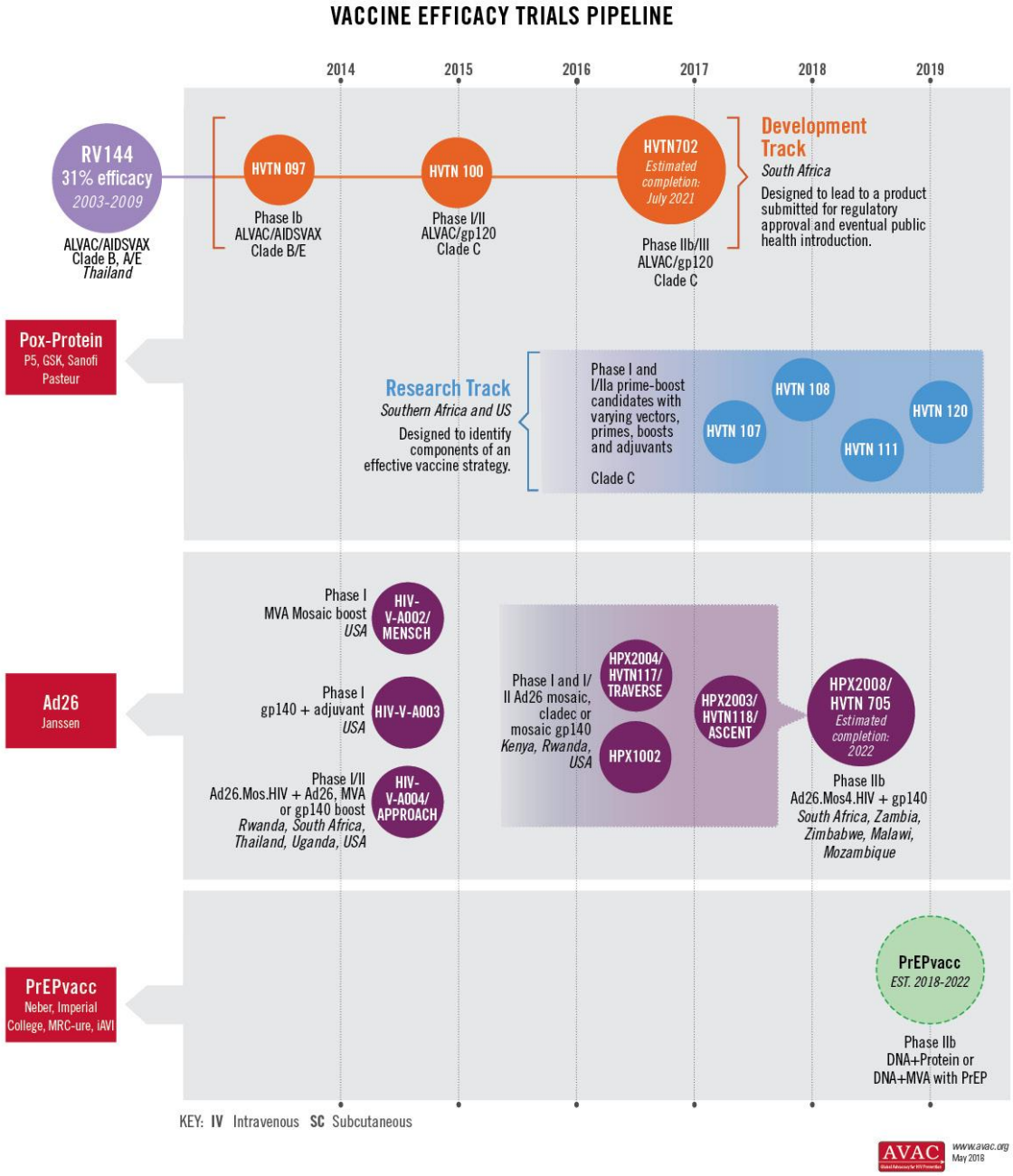


Figure 10. Vaccine efficacy trials pipeline. *AVAC 2018*. This figure shows the RV 144 Thai trial that had 31% efficacy, the other trials that are building up on that results but also other studies evaluating strategies such as broadly neutralizing antibodies that would work against multiple viral strains.

2.7.4 Oral pre-exposure prophylaxis (PrEP)

Oral HIV pre-exposure prophylaxis (PrEP) is the 'new kid on the block'. This involves HIV negative individuals taking anti-HIV drugs to keep themselves from being infected by the virus. Recent studies have shown oral PrEP to be effective in preventing HIV transmission when taken by men who have sex with men (126-128) and heterosexual discordant couples (129) during their seasons of risk. Following this evidence, the US FDA approved Truvada (tenofovir disoproxyl fumarate + emtricitabine) for HIV prevention among adults at high risk in the year 2012. As at April 2018, there were 2 global guidelines on PrEP, 28 national guidelines, 40 regulatory approvals and over 240,000 PrEP users globally. Clearly, PrEP is picking up as a HIV prevention strategy but there are real implementation challenges that will need to be overcome if this strategy is to make an impact on the HIV epidemic. One of the biggest challenges with PrEP is adherence. As indicated in figure 11 below that includes results from both oral and topical PrEP, those who did not take PrEP in these trials were not protected. Unfortunately, many of these were women, and young women at that. Lessons learnt from early PrEP implementation programmes such as these ones https://www.prepwatch.org/providing-oral-prep/ from a demonstration project in resource-limited settings will be useful for future success. Clearing houses such as PrEP watch are a best practice to be emulated in terms of consolidating evidence and resources to support global coordination of HIV prevention.

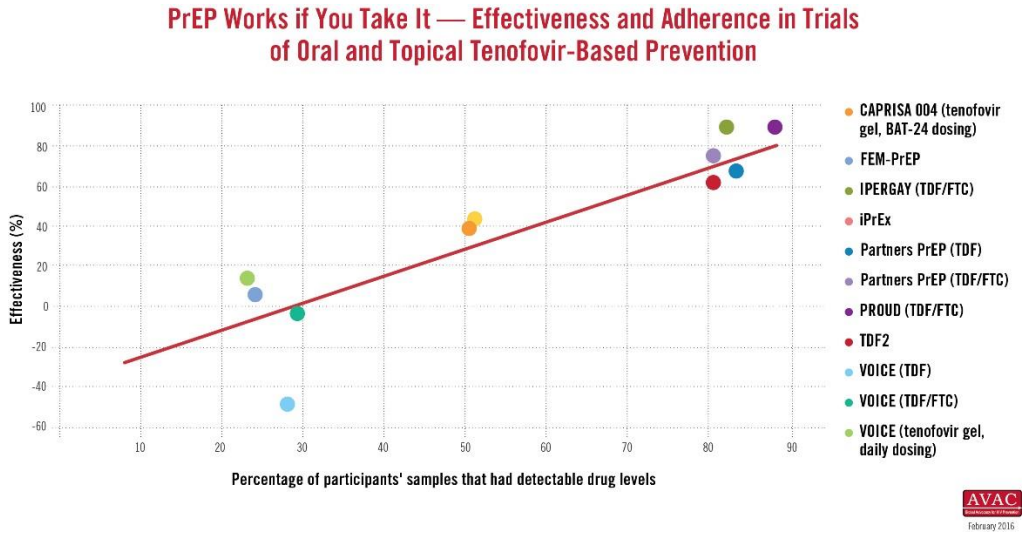


Figure 11. Adherence to PrEP and effectiveness. *AVAC 2016.* Studies such as the PROUD and IPERGAY studies demonstrated very high effectiveness of daily oral and on-demand PrEP in HIV prevention among men who have sex with men because they took the drug as prescribed. Effectiveness was however, very low among girls and young women in the VOICE and FEM-PrEP studies mainly due to struggles with adherence for different reasons.

2.7.5 Topical microbicides

The development of effective topical anti-HIV microbicides is an important prevention strategy that augments existing preventive measures and one that would be particularly

beneficial for women. Microbicides are formulated as gels, creams, films, sponges, suppositories, implants, physical barriers or vaginal rings. This variety of formulations allow end users to select what works best for them and their sexual partners. Importantly, they provide a female-controlled option for HIV prevention that is much needed given the gender disparity with HIV infection and the inability of women, in many circumstance, to negotiate for condom (or any other protective mechanism) use. They can be applied either in the vaginal cavity or rectally before and/or after sexual intercourse and provide protection in case of exposure to HIV (figure 12 below). First generation microbicides (surfactants) however failed to provide protection during clinical trials and one, N-9, even resulted in increased risk of HIV acquisition by the users (27). This was partially attributed to the fact that these compounds resulted in increased mucosal inflammation that results in recruitment of HIV-target cells. Surfactants function by disrupting the cell membrane of pathogens such as HIV and were expected to function in the same way at the vaginal mucosal surface. Product development then moved to second generation compounds such as polyanions, acidifiers and monoclonal antibodies. Examples of these were cellulose sulphate and Curraguard®. These ones were supposed to block the HIV attachment to the host cells but they failed as well and did not demonstrate significant protection in clinical trials. Current candidate topical microbicides depend on antiretrovirals to exert their protection against HIV infection. Proof of principle with this class of compounds was given in the CAPRISA 004 clinical trial where 1% tenofovir gel used before and after sex reduced the risk of HIV acquisition by 39% and HSV-2 acquisition by 51% (130). Protection was even higher (54%) among women with better adherence to the gel. Subsequent trials, VOICE and FACTS 001 (131, 132) however, failed to confirm these positive results – mainly due to challenges with adherence. The latest success with vaginal microbicides came from the monthly dapivirine ring that showed modest protection (31% and 27% reduction in HIV-1 incidence respectively) in two phase III clinical trials – The Ring Study (133) and ASPIRE (134). Interim results from the open-label studies (DREAM and HOPE) that followed these trials showed even higher adherence by women using the ring and better protection (54% for both). The dapivirine ring is currently undergoing review for regulatory approval by the European Medical Agency and other global and national agencies. A summary of microbicide compounds, their safety results and current status is given in table 1.

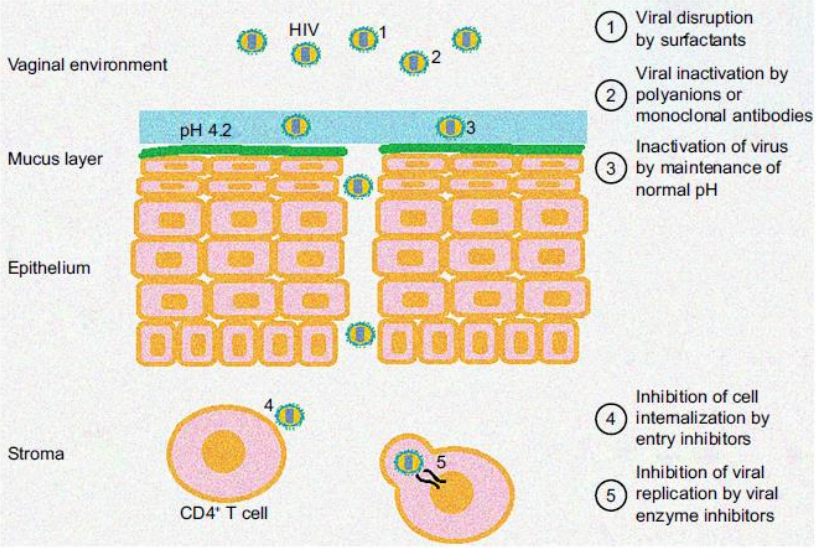


Figure 12. Action sites of different microbicides (adapted from (135))

Table 1 Vaginal formulations for the prevention of sexual transmission of HIV. Adapted (*and modified*) from (135)

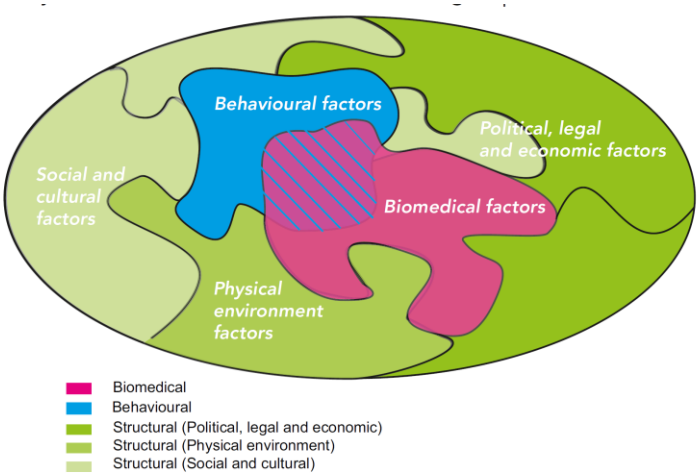
Mechanism of action	Microbicide	Pharmaceutical form	Animal tests	Clinical trials	Current status
Surfactants	Nonoxynol-9	Gel Film	-	Not safe Not effective	Rejected
	Savvy gel®	Gel	-	Safe Not effective	Rejected
Acidifier	Buffer Gel®	Gel	-	Safe Not effective	Rejected
Polyanions	Carraguard®	Gel	-	Safe Not effective	Its use as a carrier is being assessed
	PRO 2000®		Safe Effective	Safe Not effective	Rejected
	VivaGel®		Safe Effective	Not safe	Rejected
gp 120-neutralizing monoclonal antibody	Vitamin B_{12}	Gel	Safe Dose-dependent effectiveness	-	Large amounts are required and it is very expensive to produce
Innate molecule - Blocks HIV-mediated envelope fusion	Cyanovirin-N	Gel	Safe Effective	-	Candidate for clinical trials It has been expressed and purified from transgenic plants
		Probiotics (genetically modified *Lactobacillus jensenii* strain 1153 - 1666 expressing Cyanovirin-N)	Safe and positive for the vaginal environment Effective	-	In clinical trials
Entry inhibitors	Maraviroc	Gel (hydroxyethyl cellulose)	Safe Effective	-	Its period of effectiveness must be increased
		Gel (silicone)	Higher and sustained concentrations	-	Candidate for clinical trials
		Intravaginal ring	Safe		Controlled release over 28 days

Table 1 *(Continued)*

Mechanism of action	Microbicide	Pharmaceutical form	Animal tests	Clinical trials	Current status
Viral enzyme inhibitors	Tenofovir / tenofovir disoproxil fumarate	Gel	Safe Effective	Safe Effective	The first microbicide that demonstrated efficacy in women
		Intravaginal ring	Safe Effective	Safe	In clinical trials It provides lasting protection in animals
		Nanoparticles (into a film)	Safe	-	Controlled release over 24 hours Further evaluation is needed
	MIV-I50	Gel	Effective	Safe	In clinical trials
		Intravaginal ring	Effective	-	Candidate for clinical trials
	Dapivirine	Gel	Safe Effective	Safe	In clinical trials
		Intravaginal ring	Safe Effective	Safe Effective	Controlled release over 28 days Has demonstrated efficacy in women
		Film	Safe Effective	Safe	In clinical trials

All these HIV prevention strategies discussed above should not be administered in isolation but rather in the context of *combination* HIV prevention. This means that target populations should have access to HIV testing services; should be counselled on correct and consistent use of condoms; should be screened and treated for sexually transmitted infections that increase the risk of HIV infection and; be advised on safer sex practices such as the reduction of multiple sexual partners. Other aspects of combination prevention are illustrated in this 2010 UNAIDS paper(136) (figure 13).

The concept of combination is behind ongoing research work to develop co-formulated multipurpose products that would lower the risk of acquisition of HIV and other STIs but also function as contraceptives. For all these strategies, it is useful to understand different factors that would modulate their efficacy. This PhD project contributes to that body of knowledge.



Biomedical intervention strategies to reduce exposure, transmission and/or infection

Male and female condom provision
Drug treatment including opioid substitution therapy, needle and syringe provision
Male circumcision
Biomedical prophylaxis – ARVs in PMTCT services, post exposure prophylaxis, etc.
Appropriate and accessible STI services, ART for prevention
Blood safety, standard precautions in health care setting
etc.

Behavioural intervention strategies to promote individual risk reduction

HIV testing and risk reduction counseling
Behaviour change communication to promote partner reduction, condom use, uptake of HIV testing and counseling, etc.
HIV education

Interpersonal communication, including peer education and persuasion
Social marketing of prevention commodities
Cash incentives for individual risk avoidance
etc.

Social and cultural intervention strategies

Community dialog and mobilization, to demand services; for AIDS competence, etc.
Stigma reduction programmes
Advocacy and coalition building for social justice

Media and interpersonnal communication to clarify values, change harmful social norms
Education curriculum reform, expansion and quality control
Support youth leadership
etc.

Political, legal and economic strategies

Human rights programming
Prevention diplomacy with leaders at all levels
Community Microfinance/microcredit
Training/advocacy with police, judges, etc.
Policies re. access to condoms (schools, prisons etc.)

Review and revise workplace policies
Stakeholder analysis & alliance building
Strategic advocacy for legal reform
Regulation/deregulation, taxes
etc.

Intervention strategies addressing physical environment:

Housing policy and standards
Enhance farming, other modes of subsistence, for food securit
Infracstructure development – transportation, communications, etc.

Figure 13 Combination intervention strategies to prevent HIV infection. UNAIDS 2010. Because of the complex, multifactorial nature of the HIV epidemic, effective HIV prevention and an arrest of the epidemic will require a response that goes beyond the biomedical strategies that reduce exposure and transmission to include behavioural and structural interventions that promote risk reduction and provide a condusive environment with access to treatment and prevention services.

3 Heterosexual HIV Transmission

Heterosexual HIV Transmission

Sub-Saharan Africa bears the biggest burden of HIV infections (40) and transmission is predominantly through heterosexual intercourse. The focus of our project is male-to-female and not female-to-male HIV transmission, and even more specifically factors that increase vulnerability to HIV infection through the female genital tract. In understanding and dealing with some factors (such as bacterial vaginosis) that increase female susceptibility to infection, female-to-male transmission of HIV will also be reduced (137). This does not preclude the fact that women also engage in receptive anal sex (138). Although reporting of anal sexual intercourse is variable in Sub-Saharan African women (138, 139), it should also be considered in HIV prevention programs for the simple reason that the rectal mucosa is rich in HIV target cells (140, 141) and receptive anal sex has a higher HIV transmission risk compared to both insertive anal sexual intercourse (142) and vaginal sexual intercourse (143, 144). It has also been shown that co-colonization of both the rectum and vagina by protective lactobacilli is associated with a lower risk of dysbiosis in the vagina as opposed to when only either the vagina or rectum is colonized by healthy bacteria (145).

3.1 The Female Genital Tract: The First Frontier

The mucosal surface of the female genital tract is the first point of contact between the infective cell-associated or cell-free virus present in seminal fluid during heterosexual vaginal intercourse. It would benefit our discussion then to understand the structure and normal physiological functions of the FGT before delving into how these affect HIV acquisition (and onward transmission) in women.

FGT Anatomy

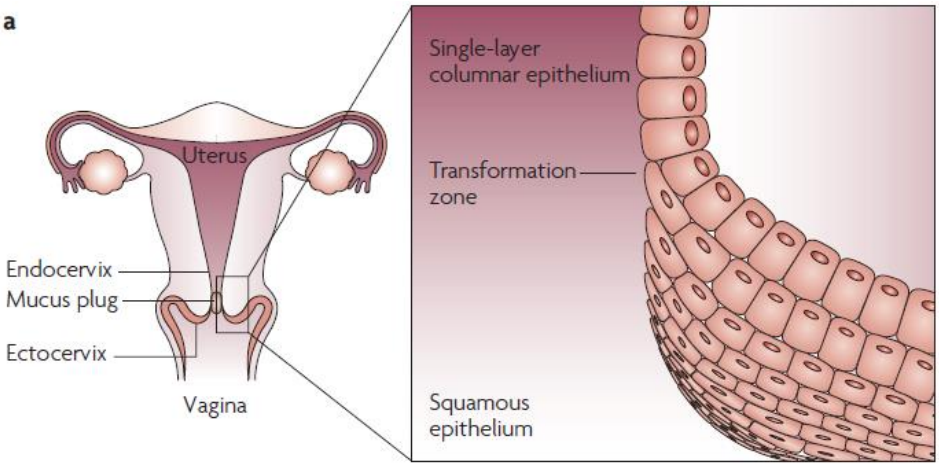


Figure 13 Female genital tract anatomy (adapted from (146)). In women, viral invasion occurs mostly through the non-keratinized squamous epithelium of the vagina and ectocervix, as well as through the single-layer columnar epithelium of the endocervix. The endocervical canal is filled with mucus, providing a barrier against the ascent of pathogens. However, ovulation is accompanied by hydration and alkalinization of the mucus plug, possibly decreasing its barrier function. Infection in women can

also ensue when HIV-1 invades the single layer columnar epithelium of the rectum following receptive anal intercourse.

The female genital tract technically runs from the vaginal opening all the way to the fallopian tubes that extend bi-directionally from the uterine cavity (figure 13). The FGT can be further divided into two parts: the fallopian tubes, uterus and endocervical canal that constitute the *upper FGT* and the ectocervix and vagina that are described as part of the *lower FGT*. The upper FGT is lined by a single layer of tall, columnar mucin-secreting epithelial cells with tight junctions between the cells. These tight junctions are lacking at the points at which the cells join the basal membrane figure 14 (147). As part of normal growth and development, hormonal changes result in enlargement of muscles that underlie the endocervical canal. When this happens, the endocervical mucosa at the lower end of the canal is pushed outwards exposing the columnar epithelium to the vaginal vault. This condition is called *cervical ectopy*.

The lower FGT lining is composed of a multi-layered squamous non-keratinized epithelium covered by a protective mucous layer that serves to trap and get rid of pathogens from the vaginal tract. This squamous epithelium has three regions; a) basal cells b) parabasal and intermediate cells and c) apical superficial cells that are mature. Tight junctions are characteristic of the two lower regions but lack in the apical epithelial cells that line the lower FGT figure 14 (147). The cells in the three regions have differential responses when stimulated by hormones present in the FGT at different developmental points (discussed later on). Structural support for the FGT mucosa is provided by stromal fibroblasts that are underneath the epithelium that lines the FGT.

The region where the endo- and ectocervix meet is referred to as the transformation zone and here, there is a change from the single layer to the multi-layered epithelium. The opening of the endocervical canal into the vagina is referred to as the cervical os and it separates the vaginal vault from the uterine cavity. The cervical os is normally blocked by a highly viscous mucous plug. The cells of the FGT secrete mucous that coats the FGT lining forming a physical as well as a biochemical barrier from pathogenic invasion. Contents of the cervical mucous include mucin glycoproteins, antibodies and other antibacterial proteins and peptides.

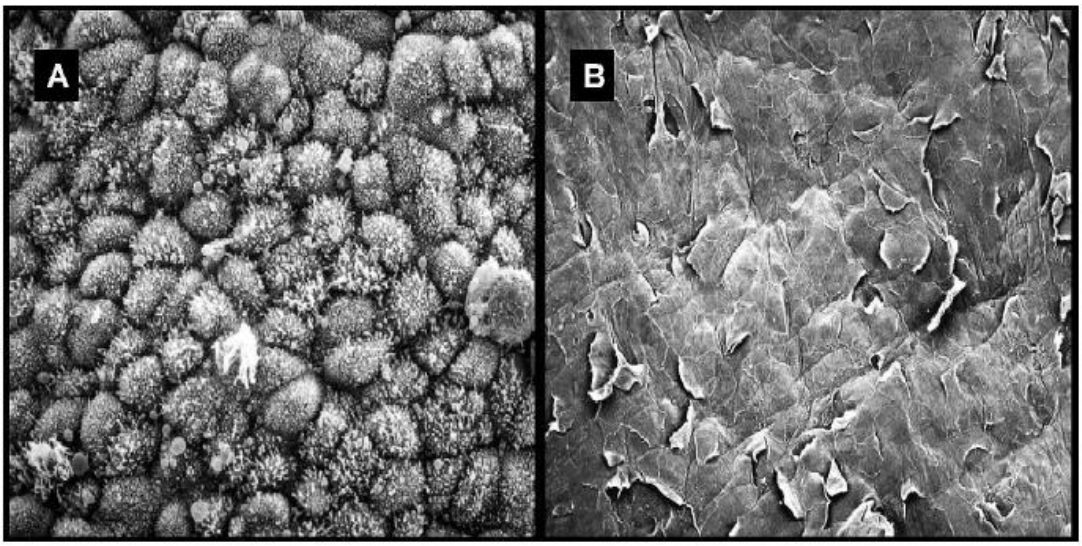


Figure 14 Endocervical and Ectocervical/Vaginal mucosal lining (adapted from (147)). Human endocervical and ectocervical luminal surfaces viewed by scanning-electron microscopy. A) The endocervical luminal surface showed an intact, cohesive epithelial cell layer. B) In contrast, the ectocervical luminal surface was covered with flattened and loosely attached epithelial cells. Original magnification 32000 (A) and 3200 (B).

3.1.1 Menstrual cycle

The menstrual cycle is part of the normal reproductive occurrences in females that is timed from the first day of menstrual bleeding to when menses begins in the cycle that follows. On average, the menstrual cycle lasts 28 days although it could be longer or shorter in different women. This cycle can be divided into two phases 1) the follicular or *proliferative* phase whose length varies from anywhere between 10 to 16 days. This lasts from the first day of menses until ovulation. 2) The luteal or *secretory* phase is predictably 14 days in most women from just after ovulation to the end of the cycle.

This rhythmic and cyclic process that ends with either pregnancy or the degeneration and removal of the uterus lining is under hormonal control. Briefly, the hypothalamous region of the brain releases the gonadotropin-releasing hormone that in turn stimulates the pituitary gland to release both the follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH then induce production of estradiol (the predominant form of Oestrogen) and progesterone from the ovaries (figure 15). These hormones then are of interest in the study of reproductive and other functions of the female genital tract from menarche all the way to menopause.

During the follicular phase of the menstrual cycle, hormonal changes (specifically, high oestrogen) result in recruitment and maturation of follicles from the ovary until one dominant follicle releases the mature egg at ovulation that roughly occurs mid-cycle. The granular cells that are not released with the egg are luteinized and combine with other cells and the stroma to form the corpus luteum. It is in this phase that the highest peak of estradiol is observed

(figure 15), followed by a sharp drop before ovulation with the second peak occurring in the luteal phase. Progesterone is produced by the corpeus luteum in the luteal phase and it prepares the lining of the uterus for reception and development of the fertilized ovum. At this time, the endometrium becomes thicker and more enriched with blood vessels. Both estradiol and progesterone levels taper off at the end of the cycle ahead of menses as the corpus luteum that primarily produces them in this phase degenerates. Menses occurs when there is no fertilization of the ovum with the hormonal changes (drop in levels of both estradiol and progesterone) resulting in contraction of the smooth uterine muscles and shedding off of the uterine lining. Menstrual fluid contains endometrial tissue, red blood cells, enzymes and other FGT exudates.

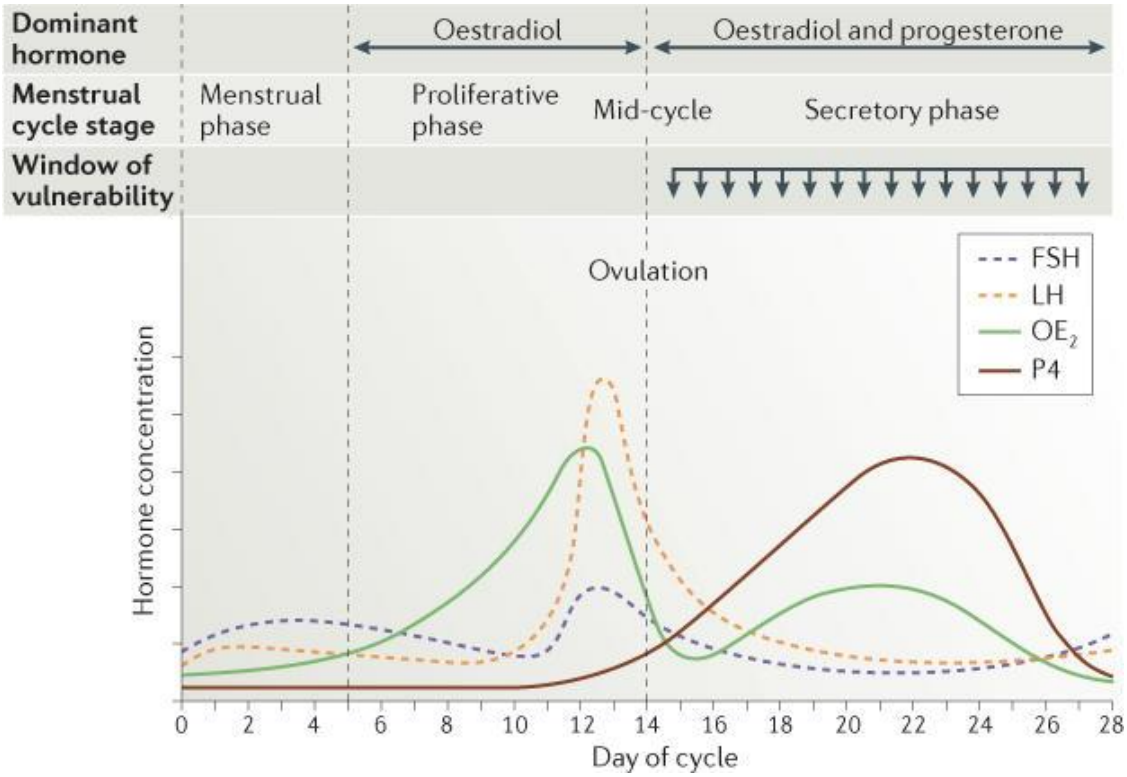


Figure 15. The menstrual cycle (adapted from (148)). The 28-day menstrual (ovarian) cycle is divided into four stages — menstrual phase, proliferative phase, mid-cycle (ovulation) and secretory phase — that are characterized by cyclic changes in hormone levels. Day 0 is defined by the onset of menstrual bleeding, which lasts for 3–5 days in most women. Menses is followed by the proliferative phase, during which the endometrial lining is reconstituted. Follicle-stimulating hormone (FSH) produced by the anterior pituitary gland induces oestradiol (OE2) production by the ovary. OE2 levels increase during the proliferative phase and peak before mid-cycle (ovulation), followed by a rapid drop in concentration. Rising OE2 levels stimulate luteinizing hormone (LH) production by the anterior pituitary, the levels of which surge in the late-proliferative phase within 24–36 hours of the OE2 peak, leading to ovulation and increasing progesterone (P4) synthesis. At the same time, FSH levels increase by a smaller amount. Both LH and FSH levels rapidly drop in the early secretory phase. After ovulation, the concentrations of P4, and to a lesser extent OE2, which are both produced by the corpus luteum in response to LH, steadily increase before peaking at mid-secretory phase. Both FSH and LH levels remain low throughout the secretory phase. In the absence of fertilization, OE2 and P4 levels drop,

which leads to endometrial shedding and the onset of menses. Immune changes in the FRT that occur as a result of cyclic changes in hormone levels create an optimal environment for successful fertilization and implantation during the secretory phase. This environment of regulated immune responses creates a 'window of vulnerability' during this phase, with permissive conditions for the entry and survival of pathogens.

3.1.2 Protective immunity in the female genital tract

The female genital tract is bi-functional: 1) It plays a reproductive function as the site where male spermatozoa are deposited resulting in eventual fertilization of the mature female oocyte. 2) The female genital tract is also immunologically active and the upper FGT is not sterile as previously thought (149-157). This local immune system is necessary to protect the body against invading pathogens that may be sexually or otherwise transmitted. Both the humoral and cell-mediated arms of the immune system are functional in the female genital tract and we discuss both here. What is unique is that mucosal immunity must be effected in such a way that it protects the body from infection but at the same time remains tolerant of allogeneic cells from both the male sexual partner and the ensuing fœtus as well as commensal bacteria that colonize the vaginal vault. This delicate dance that is the regulation of mucosal immunity in the female genital tract is directed by the sex hormones estradiol and progesterone. In the sections that follow, we will discuss different aspects of both innate and adaptive immunity and how these are influenced by reproductive hormones in the context of possible HIV infection.

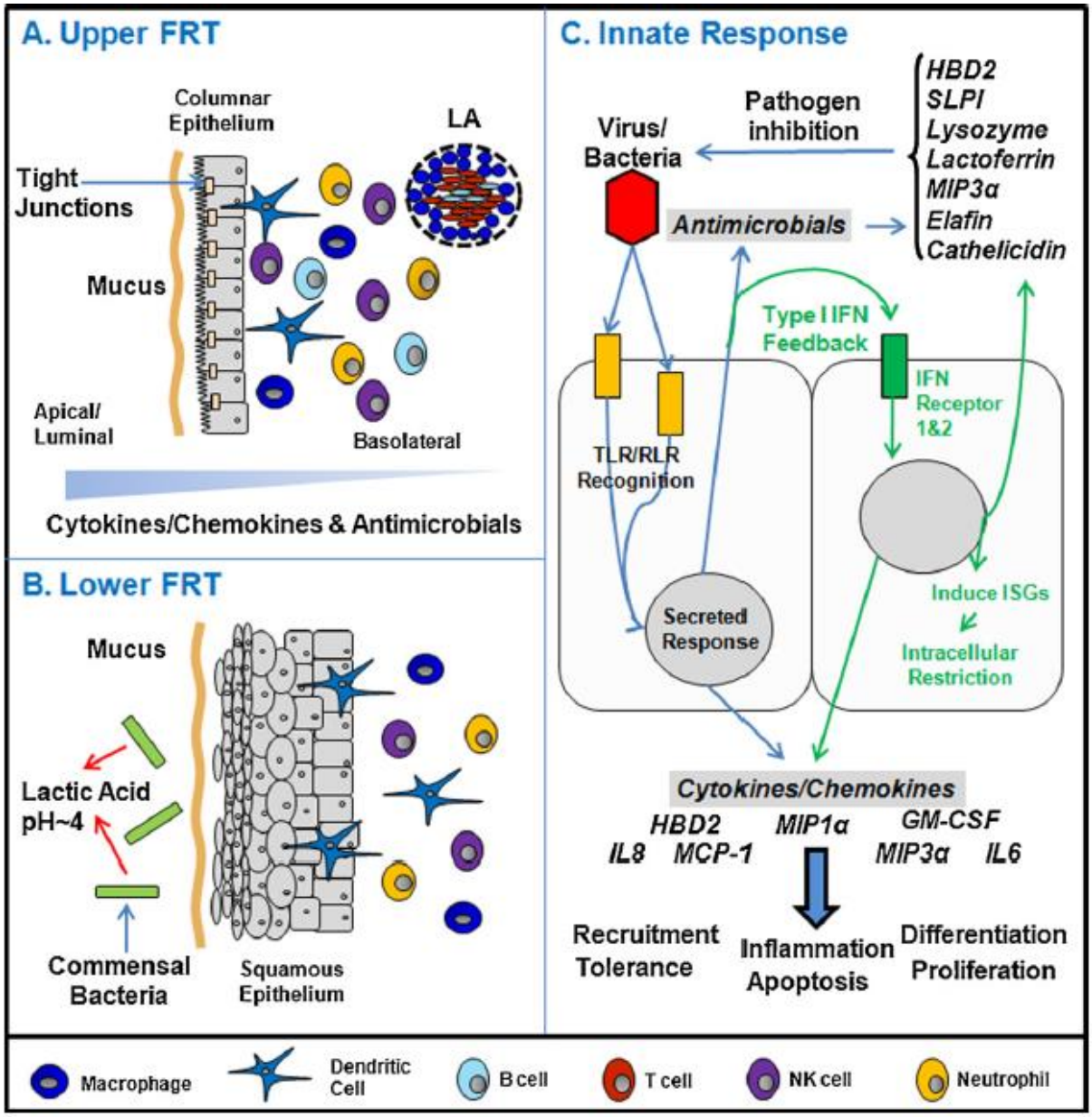


Figure 16. Innate and adaptive immunity at the FGT (adapted from (158)). Schematic of the major components of the mucosal innate immune system in the human female reproductive tract (female reproductive tract). Panel A: the upper female reproductive tract, consisting of the Fallopian tubes, uterine endometrium and endocervix is lined by a single layer of columnar epithelial cells linked by tight junctions. Overlying the cells is a protective mucus layer (Section 2). Secretion is generally preferential towards the apical/luminal compartment with a gradient across the epithelial layer from lumen to tissue. Underlying the epithelial cells are innate and adaptive immune cells (Section 3). Also shown are lymphoid aggregates (LA) that are unique to the uterus. Panel B: the lower female reproductive tract, consisting of the ectocervix and vagina is covered by a layer of stratified squamous epithelial cells (Sections 2 and 3). Similar to the upper female reproductive tract, epithelial cells are protected by a mucus layer. The lower female reproductive tract has a resident commensal bacterial population that produces lactic acid thus lowering vaginal pH (Section 2). Below the epithelial layer are innate and adaptive immune cells. Panel C: the innate immune response of upper female reproductive tract epithelial cells to an invading pathogen. Epithelial cells express a panel of Toll-like receptors (TLRs)

and RIG-like receptors (RLRs) that allow them to recognize and respond to bacteria or viruses. The Type I interferon (IFN) response (green arrows, middle section of panel C) is a potent defense system in female reproductive tract cells (Sections 4 and 5). Additionally, in response to pathogens and sex hormones, antimicrobials and cytokines/chemokines are secreted to confer broad spectrum protection.

Innate immunity in the female genital tract

Physical and biochemical barriers

The innate immune response at the FGT mucosa offers the first level of protection to the body from invasive pathogens. It is constitutively active and includes mucosal barriers and other protective processes that do not necessarily lead to memory of the pathogen for future response. The first and basic level of protection in the FGT is the *epithelial barrier*. As previously discussed, the upper FGT is lined with a single layer of columnar epithelial cells that are held together by tight and adherens junctions that make it impenetrable to certain pathogens though it remains vulnerable to others such as HIV which can productively infect intraepithelial target myeloid and T cells (159). A multi-layered squamous epithelium covers the lower FGT making its barrier function more robust than the upper FGT. Even though the apical cells in the lower FGT don't, cells in the basal and parabasal layers are held together by tight junctions. The fact that the upper layer of the epithelial cells in the lower FGT are continuously shed may reduce the chances of establishment of infection by some pathogens. A second level of physical and biochemical barrier function is the *mucous* that lines the mucosal surface of the FGT. Mucins present in genital mucous can trap pathogens making it a physical barrier to infection in the genital tract (160, 161). HIV motility (through diffusion) is, for example, over 1000 times slower in genital mucous from healthy women as compared to its potential motility in water (162). Mucous in the lower FGT has also been shown to work with immunoglobulins to increase barrier function and provide further protection from viral infection at the mucosa (163, 164). It also contains secreted antimicrobial proteins, such as secretory leukocyte peptidase inhibitor (SLPI), lactoferrin, lysozyme, defensins and high-mobility group nucleosomal-binding domain 2 (HMG N2) (165, 166). Finally, commensal bacteria in the lower FGT break down glucose to produce lactic acid that keeps *vaginal pH low* and unsuitable for pathogen survival. Some of these bacteria also produce hydrogen peroxide (H_2O_2) that is antimicrobial in nature especially for gram-negative bacterial pathogens (167).

Hormonal effects on physical and biochemical barriers

During the proliferative phase of the menstrual cycle, increasing levels of estradiol are associated with a gradual thickening of the uterine epithelium that then reduces late in the secretory phase. On the other hand, *in vitro* treatment of cultured primary uterine cells and differentiated cell lines (ECC1) with estradiol and progesterone demonstrated that estradiol, but not progesterone, reduced transepithelial resistance (TER) which is a measure of the integrity of the monolayer formed by these cells due to the presence of tight junctions (168). A similar observation was made when vaginal-ectocervical cells from pre- and post-

menopausal women were treated with estradiol (169, 170). Estradiol has been shown to affect the expression of both occludin and claudin proteins (171, 172) and this possibly causes a relaxation of the tight junctions in the epithelial barrier *in vivo*. It is possible that pathogens (including HIV) could also take advantage of these changes for easier penetration of the FGT mucosa to establish infections.

Expression of mucin genes that produce the mucin glycoproteins that are a major component of the FGT mucous also varies in the course of the menstrual cycle affecting mucous properties (160, 161). During the proliferative phase and ovulation, the mucous is thin and watery with low viscosity allowing for sperm movement into the upper FGT. In the secretory phase, this mucous becomes thick and viscous and prevents movement from the lower into the upper FGT (173). Changes in the vaginal microbiome that occur in the course of the menstrual cycle are discussed further on.

Pattern recognition receptors (PRRs)

Epithelial and immune cells in the female genital tract express both surface and cytosolic pattern recognition receptors that recognize and bind to pathogen-associated molecular patterns (PAMPS) activating innate immune responses. These PAMPS are ligands that are repeated in many bacteria, viruses and fungi and can be specifically recognized by pattern recognition receptors that function alone or others that form dimers to increase specificity and variety (174-176). Different studies have shown epithelial cells from the female genital tract express different pattern recognition receptors such as Toll-like receptors (TLRs) 1-9 (149, 177, 178), RIG-1-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). TLR7 and RIG-1 recognize HIV (179, 180). Variability in PRR expression has also been established with bacterial receptors TLR2, TLR4, NOD1 and NOD2 finding higher expression in the upper FGT compared to the lower FGT (177, 181, 182). Wira and colleagues postulated that this may imply damped responses to bacterial pathogens in the lower FGT that is inhabited by commensal bacteria but a higher level of immune alertness to bacterial infection in the upper FGT (148). Sensitivity to viral invasion, on the other hand, is even in both the upper and lower FGT as suggested by a more constant expression of viral-sensitive TLRs TLR3,TLR7, TLR8 and TLR9 (183).

Hormonal effects on pattern recognition receptors expression and function

The expression of TLR2, TLR6, TLR9 and TLR10 was found to be lower in human uterine tissue during the proliferative compared to the secretory phase (184-187). *In vitro*, estradiol was shown to decrease the expression of TLR2 and TLR6 by vaginal epithelial cell lines (VK2 cells HPV-16 E6/E7 transformed) as well as *TLR4* mRNA expression by fibroblasts from the human uterus (188). These fibroblasts demonstrated a higher level of expression of TLR4 when exposed to progesterone that is present in vivo during the secretory phase of the menstrual cycle (185). Further, the expression of pro-inflammatory cytokine interleukin (IL)-6 and chemokines IL-8 and migration inhibitory factor (MIF) by uterine epithelial cells upon stimulation by TLR agonists had been shown to be inhibited by estradiol (189). Estradiol also

stops the expression of tumor necrosis factor (TNF), human beta-defensin 2 (HBD2), IL-8 and nuclear factor NF-κB that are normally stimulated by IL-1β (190). Estradiol can exert its effect on the NF-κB pathway directly by preventing its transport from the cellular cytoplasm to the nucleus or by stopping the degradation of NF-κB inhibitors (191, 192). Indirectly, it can function by inducing SLPI that then inhibits NF-κB (193). The overall picture that then emerges is one whereby estradiol dampens inflammation in the female genital tract during the secretory phase of the menstrual cycle.

Cellular effectors of innate immunity

At the cellular level, the innate immunity is also active in the female genital tract. Leukocytes form 6-20% of the total number of cells in the tissues of the female genital tract (194). Most of these leukocytes are in the upper FGT and even there, the numbers are more in women who are pre-menopausal compared to post-menopausal women. Innate leukocytes include macrophages, dendritic cells, natural killer (NK) cells and neutrophils. *Macrophages* are present in all tissues of the FGT in small amounts (194) but they are largely found in the endometrium and connective tissue (153). *Dendritic cells (DCs)* function in the same way as the macrophages meaning that they utilize the process of phagocytosis to ingest pathogens and then destroy them by acidic digestion. Interstitial DCs are found among the stromal cells that underlie the single epithelial layer of the upper FGT as well as Langerhans cells in between the multiple cells of the squamous epithelium in the lower FGT (195). *NK cells* are the majority (up to 70%) of leukocytes in the upper FGT where they function as pro-inflammatory agents by producing chemokines and cytokines that activate macrophages (196). The NK cells in the FGT are different from the ones found in blood, as they express CD9, CD69 and CD94 markers suggesting that they may be functionally different from blood-based NK cells. Actually, these markers are also differentially expressed on NK cells throughout the FGT as well (196). Lastly, we have *neutrophils* that are found in both the upper and lower FGT with a higher concentration in the former (153). These neutrophils express Toll-like receptors 1-9 and serve their protective function by phagocytosis; releasing antimicrobial proteins such as Trappin-2/Elafin, α-defensins, phospholipases and cytokines and by producing oxidative compounds (197).

Hormonal effects on cellular effectors of innate immunity

The numbers of macrophages and NK cells in the endometrium are regulated by both estradiol and progesterone (196, 198). These two hormones do not seem to have an effect on the numbers of macrophages and NK cells in the lower FGT (153, 196). The number of neutrophils peak during menses when they serve both an immune function – cytotoxic activity and pro-inflammatory cytokines production and reproductive function – supporting the degeneration of endometrial tissue by releasing elastase that activates extracellular matrix metalloproteinases.

Soluble (secreted) innate immunity

Cytokines are defined as low molecular weight polypeptides (~5-20 kDa) with a typically short half-life (199) that are produced by a variety of cells and function to mediate intercellular communication. Chemokines are a specific class of cytokines that function by attracting leukocytes to the site of inflammation or production. Because of this, chemokines are known as chemo-attractants. Upon secretion, cytokines bind to receptors on target cell surfaces triggering a signaling cascade that results in a plethora of possible effects such as cell differentiation, cell maturation, cellular apoptosis, immune cell recruitment, inflammation, multiplication of surface receptors for other molecules, increased release of the same cytokine and release of other cytokines and/or antimicrobial proteins among other effects. Cytokines are potent in the sense that they are able to carry out their biological functions at extremely low concentrations. Importantly, different types of cytokines may serve the same function and this is known as redundancy. A single cytokine may have the ability to exert its effect (to varying degrees) on many different types of cells (pleitropy) just as a single cytokine can be produced by many different types of cells. It is also possible that one cell type could express different types of receptors for the same cytokine. When cytokines act on cells within the vicinity of their points of release, they are classified as *paracrine*. When their action is on the cells that release them, they are classified as *autocrine*. Other cytokines exert their function in regions of the body far away from where they are released and these are classified as *endocrine*. Majority of cytokines are either paracrine or autocrine.

Antimicrobial peptides are another group of secreted modulators of innate immunity which are peptides made up of less than 100 amino acids with activity against pathogens under physiological conditions. Secretion of these peptides by epithelial and immune cells occurs both constitutively and upon stimulation by pathogens via pathogen recognition receptors. They are normally broad-spectrum in their microbicidal function with demonstrated activity against viruses, bacteria and fungi (200, 201). Antimicrobial peptides can tackle infections directly or indirectly by either killing or preventing the growth of microorganisms invading the host (202). Additionally, these peptides also serve as chemo-attractants whose secretion results in an influx of both innate and adaptive immune cells to the female genital tract.

Immunity in the FGT is also effected by the secretion of cytokines, chemokines and antimicrobial peptides that collectively function to activate both the innate and adaptive immune systems in order to provide protection from infection (153) or to directly kill the invading pathogen (152) In the FGT, all these polypeptides are produced by epithelial cells, T-cells and macrophages either constitutively or in response to stimulation of different arms of the immune system. Research has shown that cytokine secretion in the uterus by epithelial cells is mostly done towards the apical mucosal surface resulting in many of the effects we have previously described including differentiation, maturation and attraction of immune cells into the uterine lumen (150). Epithelial cells in the FGT have been shown to produce various cytokines including granulocyte-macrophage colony-stimulating factor (GM-CSF), colony-

stimulating factor-1 (CSF-1), tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, IL-8, leukemia inhibitory factor (LIF) and transforming growth factor beta (TGF-β) (203-205). *In vitro* studies in which uterine epithelial cell lines ECC1 were stimulated with TLR2, TLR4 and TLR9 agonists demonstrated expression of IL-6, IL-8 and monocyte chemoattractive protein-1 (MCP-1) (178). Exposure of primary uterine epithelial cells to the synthetic double-stranded ribonucleic acid (dsRNA) poly (I:C) that binds to TLR3 (206) caused these cells to express pro-inflammatory cytokines TNF-α, IL-6, GM-CSF and G-CSF, but also induced the expression of the chemokines IL-8, MCP-1 and MIP-1β (149). Chemokines such as IL-8 and RANTES (regulated upon activation, normal T cell expressed and secreted) are secreted by epithelial cells and they function by attracting immune cells into the female genital tract (149, 150, 207, 208). This chemokine gradient that is caused by apical secretion of IL-8 serves a protective role in the FGT as it results in neutrophil migration across the epithelium as well as higher levels of α-defensins (209). MIP3α, for example, is secreted by uterine epithelial cells and it attracts B cells, memory T cells and immature bone marrow-derived dendritic cells (210, 211). On the other hand, secretion of these soluble immune mediators could also occur into the sub-epithelial compartment where they would then influence the development and function of the immune cells that reside there (212, 213).

Following infection by either bacteria or viruses Type 1 interferons (IFNs) are rapidly induced (149, 214) and this results in the paracrine or autocrine up-regulation of interferon-stimulated genes (ISGs). Because almost all cells express IFNs and their receptors as well as the large number of ISGs, down-stream innate immunity effects of IFN induction are numerous and widespread (214). In the FGT, it is still unknown which IFN subtype (IFNα, IFNβ, IFNκ, IFNω, IFNε) is predominantly secreted in which compartment and by which cells. Trifonova and colleagues have however shown that vaginal epithelial cell lines but not primary human vaginal cells have the ability to secrete IFN (215). Patel and colleagues also demonstrated IFNβ secretion by primary human uterine epithelial cells as well as the uterine epithelial cell line ECC-1 in response to TL3 agonist poly (I:C) (216)

Antimicrobial peptides and cytokines/chemokines secreted by uterine cells include Trappin-2/Elafin, SLPI, human α-defensin-5 (HBD5), β-defensins 1-4, MIP-1α, MIP-1β, RANTES, stromal cell-derived factor-1α (SDF-1α)and MIP-3α (149, 189, 217-224). *In vitro*, uterine epithelial cell lines ECC1 have been shown to express human β-defensins HBD1 and HBD2, SLPI, lysozyme, MIP-3α, Trappin-2/Elafin, cathelicidin, IFN-β and other IFN-β-stimulated genes whose products have direct virucidal effects (149, 153, 225). Pivarcsi and colleagues demonstrated the *in vitro* secretion of the antimicrobial peptide hBD2 by the vaginal epithelial cell lines PK E6/E7 (226). hBD2 forms pores in the membranes of pathogenic bacteria resulting in their death (202) but it also has chemotactic properties. MIP-1α, MIP-1β, RANTES and SDF-1 can inhibit the binding of HIV-1 to both of its co-receptors (CCR5 and CXCR4) on host cells (227). SLPI is one of the peptides with broad-spectrum effects with demonstrated activity against bacteria (*Staphylococcus aureus*), fungi (*Candida albicans*) and RNA virus (HIV-1) (228-230). As reviewed in 2013 by Aboud *et al* (231), there are antiproteases expressed in the FGT epithelial

layer that are associated with reduced susceptibility to HIV infection by women who remain seronegative despite long-term, high-risk exposure to HIV such as is common in sex workers with multiple clients who may not be using condoms consistently. Focusing on serpins and cystatins, Aboud and colleagues demonstrate that these antiproteases have the ability to interfere with HIV transmission and the establishment of a productive infection if transmission succeeds. Through different mechanisms, the actions of these antiproteases result in attenuated inflammation, reduced immune activation with subsequent interference with migration of HIV target cells to the FGT mucosa. These antiproteases can also function by directly interfereing with the ability of HIV to replicate or bind to the mebrane of host target cells. It is not far-fetched then to attribute observed *in vitro* HIV-1 and HSV-2 inhibitory activity of cervico-vaginal lavage (CVL) fluids to these proteins especially because there has been correlation of inhibition to concentration of some of these antimicrobial peptides (232, 233). What is interesting though is that even though secretions from the upper FGT directly inhibit pathogens of the FGT such as *N. gonorrhoeae* and the others mentioned above, they do not have the same effect on *Lactobacillus crispatus* (234) which is a commensal bacterium that predominantly colonizes the lower FGT in healthy women. The exact mechanism of this differential effect in the FGT is yet to be elucidated and other studies have shown that several *Lactobacillus* strains can actually induce secretion of β-defensin 2 by epithelial cells from the intestines (235).

Hormonal effects on soluble innate immunity

Different studies have shown contrasting results about levels of cytokines (IL-6), chemokines (IL-8) and antimicrobial peptides (SLPI, HBD2, HNP1-3 and lactoferrin) in the lower FGT during the proliferative, ovulatory or secretory phases of the menstrual cycle depending on the method used for sample collection (cervical vaginal lavage (CVL), Dacron swabs or tampons) (217, 236-238) (Schumacher 1973). Notably, the antimicrobial peptides (SLPI, HBD2, HNP1-3 and lactoferrin) are suppressed by estradiol mid-cycle and remain so over a period of 7-10 days (236). This is thought to provide a conducive reproductive environment preventing excessive immune reaction to spermatozoa and the foetus in the event of successful fertilization. The downside to this period of immune suppression is that it makes the woman vulnerable to sexually transmitted infections as discussed by Wira and Fahey (239). Reproductive hormones could directly influence the secretion of these cytokines or exert their effect indirectly through underlying stromal cells in the FGT. Some studies have demonstrated production of hepatocyte growth factor (HGF) by uterine stromal cells under the influence of estradiol. HGF then regulates the secretion of TNFα and MIP3α by uterine epithelial cells (240-242). GM-CSF, TGF-β and TNF-α are among the secreted factors whose release is under the direct influence of sex hormones (203, 243-245). IFNβ upregulation of ISGs in human uterine epithelial cells has been shown to be unaffected by estradiol *in vitro* (216). Data from a different group indicates that expression of local mediators of inflammation, IL-8 and MCP-1 by human endometrial cells is suppressed by the reproductive hormone progesterone (246).

Ovine progesterone has been shown to induce ISGs expression in the uterus both apically and in the sub-epithelial compartment (247) but it also dampens the expression of IFNα in plasmacytoid DCs (248) making the overall effect in the FGT unclear. Although data is conflicting on the levels of different soluble markers of innate immunity in the course of the menstrual cycle, what is not in dispute is the fact that these markers fluctuate over time and that these changes are influenced by reproductive hormones.

Adaptive immunity in the female genital tract

As opposed to how innate immunity functions, adaptive immunity is pathogen-specific and after initial exposure, the immune system develops memory that ensures a more enhanced immune reaction during subsequent exposures to the same pathogen. Intra- or extracellular pathogens are processed and presented on the surface of antigen-presenting cells (APCs) to lymphocytes. This triggers an immune reaction that aims to get rid of the pathogen or stop its establishment in the host. APCs in the FGT include macrophages, dendritic cells, Langerhans cells as well as epithelial cells. FGT effectors of adaptive immunity are either T-lymphocytes (specifically CD4+ and CD8+ T cells) or immunoglobulins that are synthesized and secreted by B cells.

T lymphocytes form the majority of leukocytes in the female genital tract accounting for 30-60% of leukocytes in all FGT tissues (194). When activated, cytotoxic CD8+ T cells recognize *intracellular* pathogens through peptides expressed on MHC I molecules on the surface of these infected cells. These immune cells (CD8+ T cells) then release granzymes and perforins that destroy infected cells by cytolysis or by inducing apoptosis (249). CD4+ T cells are activated by antigens from *extracellular* pathogens presented (by APCs) on MHC II molecules and differentiate into one of specific sub-types: Th1 – responsible for cell-mediated adaptive immunity, Th2 – humoral adaptive immune response, Treg – regulatory T cells, Th17, Th9 or follicular helper T cells (250). Their differentiation and action is dependent on and mediated by the cytokine milieu in their immediate vicinity. CD4+ T cells then exert their protective immune function by further cytokine production with resulting cytotoxicity and antibody production by relevant cells. CD4+ T cells also release IFN-γ that activates CD8+ T cells to destroy cells infected by viruses such as HIV (251).

In the absence of inflammation, the transformation zone and the area surrounding it is mostly made up of CD4+ and CD8+ T lymphocytes as well as macrophages. CD8+ T cells are more predominant in the stroma or in between the epithelial cells in this region of the upper FGT compared to CD4+ T cells (194, 252). A while ago, Yeaman and colleagues (253) described the presence of unique lymphoid aggregates (LA) in the stratum basalis of the uterine endothelium. These aggregates are made up of a core of B cells surrounded by T cells (mostly CD8+CD4-) with an outer halo of macrophages/monocytes. In women without infections these aggregates are unique to the endometrium and are not found in the endocervix or lower FGT. Wira and colleagues postulate that these clusters may be a way of preserving the T cell repertoire and memory cells during menstruation (148). CD4+ and CD8+ T cells numbers in the

lower FGT are more or less equal (254). The lower FGT mucosa also has CD1a+ cells (LCs) as well as DCs (255). In the lower FGT, clusters of immune cells (memory CD4+ or CD8+ T cells, B cells, DCs and macrophages) have been shown to form after herpes simplex virus 2 (HSV2) infection but it's yet to be established whether or not these clusters are under hormonal influence (256).

B lymphocytes are present in all FGT tissues but in low amounts (194). Mature B cells (plasma cells) that produce both IgG and IgA are found in higher frequencies in the cervix compared to the vagina. In the female genital tract, IgG levels have been found to be higher than IgA levels (257, 258). The polymeric form of IgA (pIgA) found in the FGT is transported to the FGT lumen in secreted form (S-IgA) by the polymeric immunoglobulin receptor (pIgR) whose expression on epithelial cells is upregulated by cytokines produced by activated T-cells (257). The origin of IgG found in the FGT is from both local B cells as well as from circulation. Because it is found in its monomeric form, it's unlikely that IgG is transported via pIgR. When the antibodies bind to the target antigens the antigens are eliminated through the complement system or antibody-dependent cell-mediated cytotoxicity.

Hormonal regulation of adaptive immunity

As with some aspects of innate immunity, cellular adaptive immunity is somewhat downregulated in the FGT during the phase of the menstrual cycle when ovulation and foetus implantation occur and this too makes the woman more susceptible to HIV infection (259-261). The presence and localization of these cells in the lower FGT is not affected by the menstrual cycle as they are in the upper FGT (262). This also applies to cytotoxic T-cell (CTL) activity in the FGT that has been demonstrated throughout the menstrual cycle, i.e. CD8+ CTL activity, but not number of CD8+ T cells, is reduced in the upper FGT after secretory phase of menstrual cycle (261, 262), while CTL activity remains the same in lower FGT regardless of phase in menstrual cycle (263). Lymphoid aggregates in the upper FGT are under the influence of sex hormones as they were smaller (300-400 cells) during the proliferative phase, growing in size to approximately 3000 to 4000 cells during the secretory phase of the menstrual cycle when CTL activity is suppressed.

The secretion of humoral components of the immune system into the FGT varies by site (fallopian tubes, uterus, cervix and vagina) in the course of the menstrual cycle even when these sites are exposed to the same hormones produced at specific points in the menstrual cycle (264-266). Notably, IgG, IgA and lactoferrin levels are at their lowest levels in mucous in the lower FGT midway through the menstrual cycle (during ovulation) with higher levels at the beginning and end of the cycle (267-271). IgA and IgG suppression during ovulation is most likely so that the humoral immune response to the allogeneic sperms is muted. Oral contraceptives also repress the levels of these proteins (271). Sex hormones also influence the expression of pIgR by epithelial cells in the FGT and by doing so they modulate the levels of immunoglobulins in FGT secretions (272). Specifically, pIgR production in the uterine

epithelium is highest in the secretory phase of the menstrual cycle but if very minimal in the vaginal epithelium (265, 273).

3.2 Vaginal microbiota

The final aspect of the female genital tract that is relevant to HIV (and other STIs) transmission that we shall consider is the *vaginal microbiota*. The study of vaginal microbiota is at once an old and nascent field. It is old in the sense that knowledge of the microorganisms that colonize the vaginal mucosa is over a century old. It can also be considered new because the emergence of new, specifically molecular, techniques to further characterize these organisms continues to reveal new and exciting knowledge especially in the context of their relevance for health outcomes – specifically sexual and reproductive health. By definition, the microbiota on the vaginal mucosal surface include bacteria, fungi, unicellular protozoa and viruses. These microorganisms could be symbiotic – that live in complete harmony with their host and are mutually beneficial; commensals – that benefit from the host; pathobionts – which are symbionts that under certain conditions can cause disease or; parasitic – that are considered harmful to the host because of their ability to cause disease. Additionally, these microorganisms are found, to varying degrees, in both the upper and lower female genital tract. The focus of our work, however, will be on the bacteria that are found on the vaginal mucosal surface in health and in disease conditions.

3.2.1 The healthy vaginal microbiota

From as early as 1894 when the German gynecologist Albert Döderlein used microscopy to describe the microbiota in vaginal secretions, lactobacilli have been shown to be the dominant bacterial species in the healthy human vagina from menarche to menopause, a state known as eubiosis. A recent comparative study (274) reports that the *Lactobacillus* species typically comprise over 70% of the local resident bacteria in the human vagina while for other non-human mammals, this figure stands at less than 1%. Different hypotheses have been advanced to explain this unique aspect of the, now known, protective and dominant nature of vaginal lactobacilli but none of them is conclusive yet. These hypothesis include the 'disease-risk hypotheses' where a relatively high risk of STIs and obstetric complications by the human female exerts a selective pressure that resulted in the high lactobacilli presence (275); a 'reproductive phase' hypothesis that attributes high lactobacilli colonization to a continuous 28-day reproductive cycle that sees a *Lactobacillus* peak when estrogen levels are high - a feature that is missing in many non-human mammals with short breeding seasons; the 'common function hypothesis' that attributes protection to other bacteria and mechanisms other than lactobacilli; the diet-related hypothesis that postulates that high starch levels in human diet results in increased glycogen in the vagina with consequent proliferation of vaginal lactobacilli (274) and; a recent hypothesis by Mario Vaneechoutte that links a possible semi-aquatic phase in human development to lactobacilli dominance that would have been necessary as it results in low vaginal pH offering protection from infection in an environment with salty water (276).

Up until the year 2002 when Burton and Reid (277) published their work that included molecular techniques in the analysis of bacterial flora in menopausal women, identification of vaginal microbiota was done using gram stains, clue cells and culture-based methods. Vaginal bacterial isolates would be grown on (selective or non-selective) media and then phenotypically identified. Non-selective media such as MacConkey agar, mannitol salt agar and tryptic soy base with 5% sheep blood agar allows for the identification of a broad range of both aerobic and anaerobic bacteria as these media support the growth of many microorganisms. If one wanted to culture a specific species of bacteria and not another, they would then use selective media that supports the growth of the bacteria of interest while inhibiting other bacteria from growing. Lactobacilli for example are selectively cultured on Rogosa and Sharpe media which, interestingly, doesn't support the growth of *Lactobacillus iners*. This meant that *L. iners* which grows better on human bilayer Tween (HBT) agar (the same media used to isolate *Gardnerella vaginalis*) was underappreciated for a long time. Since 2002, molecular techniques such as sequencing, polymerase chain reaction (PCR), DNA fingerprinting and DNA hybridization allowed for a more detailed and unbiased characterization of vaginal microbiota, confirming what had been discovered before but also allowing for identification of other, hitherto unidentified, bacterial species in the human vagina under different conditions. What has remained is the general agreement on the dominance of lactobacilli in health. The most common lactobacilli species found in the vagina are *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus iners*, *Lactobacillus jensenii* and *Lactobacillus vaginalis* (278-283). It remains unknown why other Lactobacillus species such as *L. acidophilus* and *L. helveticus* are not part of this dominant group yet they have access to the female genital tract. It's important to note here that there are other studies in different locations that point to possible significant roles played by other species such as *L. plantarum* and *L. fermentum* in Bulgarian women (284), *L. reuteri*, *L. fermentum* and *L. salivaris* in Indian women (285) as well as *L. pentosus*, *L. fermentum*, *Enterococcus faecalis* and *Weisella (Lactobacillus) viridescens* in a study in South Africa (286). Additionally, there is sufficient evidence that other non-*Lactobacillus* species bacteria, some of which are largely associated with dysbiosis in the female genital tract, are also present in the vaginal mucosa of 'healthy' or asymptomatic women but in low quantities. These include *Gardnerella vaginalis*, *Atopobium vaginae* and *Prevotella bivia* among others (287).

Lactobacilli are Gram-positive, facultative anaerobes that dominate the microbiota on different human body sites. Vaginal lactobacilli are special in that it seems that they have evolved and adapted over time to the special niche that is the vaginal vault. A comparative functional genomic analysis by Mendes-Soares (288) demonstrated that vaginal lactobacilli have relatively smaller genomes with lower GC content compared to other non-vaginal lactobacilli. These lactobacilli also over- or under-expressed certain proteins; evidence of their adaptation. As an example, vaginal lactobacilli have been shown to have more stress proteins, mannose/glycogen utilization pathways, sortase-dependent proteins, toxin-antitoxin systems and transcription factors compared to lactobacilli in the gastrointestinal tract. Additionally, this study observed an overrepresentation of genes involved in intracellular trafficking,

secretion and vesicular transport; translation, ribosomal structure and biogenesis; carbohydrate transport and metabolism; defence mechanisms; cell motility; energy production and conversion among others. Gene clusters involved in secondary metabolites biosynthesis, transport and catabolism; coenzyme transport and metabolism; nucleotide transport and metabolism were underrepresented. Uniquely, *L. iners* has several genes that haven't yet been described in the other dominant vaginal lactobacilli that may explain its different behavior starting from the growth requirements discussed above to its presence in conditions of dysbiosis that we discuss further on. These genes include an iron-sulfur cluster, alkaline shock proteins, various heat-shock proteins, cold-shock proteins, universal stress proteins and σ-factors (289). Some studies have been done on the mechanisms by which lactobacilli remain dominant and provide benefits to the human host and these are discussed below and summarized in figure 17.

Glycogen utilization and lactic acid production

Infants born by vaginal delivery acquire their microbiota from contact with the mother's vaginal and fecal microbes (290). For those born through cesarean section, acquisition of initial microbiota is from the mother's skin, caregivers and the environment. For a few months after birth and then again from puberty to menopause, there is a clear correlation between vaginal glycogen content and lactobacilli presence (17). Both of these lack (or are reduced) just before puberty and after the onset of menopause. A study by Mirmonsef et al (291) demonstrated correlation between high vaginal glycogen content, high lactobacilli (*L. crispatus* and *L. jensenii* but not *L. iners*) and low pH (4.4). pH was higher (5.8) in vaginal samples with a lower glycogen content . Investigation into the role of reproductive hormones (estrogen and progesterone) in synthesis and accumulation of glycogen in the female genital tract has yielded mixed results. Glycogen content has been shown to be up to tenfold higher during the early secretory compared to the proliferative phase of the menstrual cycle under the synergistic effect of both estrogen and progesterone (292). Other studies, however, have not found a correlation between glycogen levels and sexual reproductive hormones in the FGT (293, 294). In terms of protection, the reduction of glycogen to maltose and glucose results in the production of lactic acid that greatly lowers vaginal pH (to between 4 and 4.5) creating an environment that is not conducive for the survival of many pathogens. Lactic acid has antimicrobial activity against a wide range of vaginal pathogens including Chlamydia trachomatis (295), uropathogenic Escherichia coli (296), Neisseria gonorrhoeae (297), herpes simplex virus type 2 (HSV-2) (298) and HIV-1 (299). Specifically, lactic acid has been shown to enhance the ability of mucus at the cervicovaginal surface to slow down and trap HIV-1 virions (162).

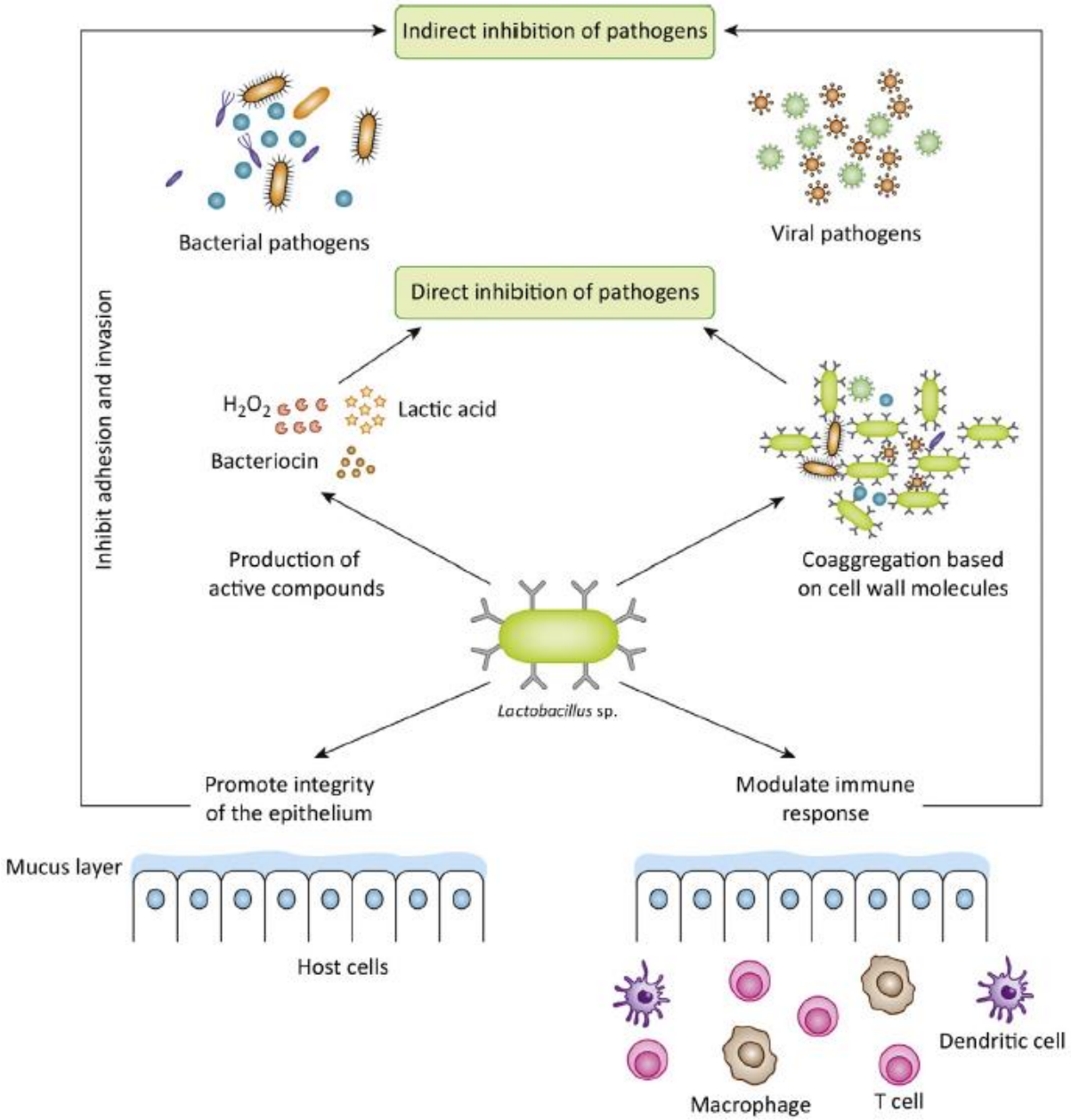


Figure 17 Mode of action of Lactobacillus species. Adapted from (300) Lactobacilli are able to inhibit pathogens in a direct manner by co-aggregation or by producing active components such as bacteriocins, lactic acid and hydrogen peroxide (H_2O_2)*. In addition, they promote the integrity of the epithelium by stimulating mucus secretion and modulating the immune response, which are indirect ways of inhibiting viral and bacterial pathogens. *H_2O_2 production as a defence mechanism *in vivo* is a subject of recent debate and is further discussed below.

The subject that remains contentious is whether lactobacilli have the capacity to directly metabolize glycogen to glucose or whether they rely on an initial degradation that is done by host enzymes such α-amylase to simpler forms that can then be utilized by lactobacilli. Different studies have provided evidence *in vitro* (301-306) as well as genomic (307-309) evidence for and against the ability of different lactobacilli species to degrade glycogen. The recent excellent review on the vaginal microbial community by Vaneechoutte (276) cites an

old review (reference number 80 in (276)) that provides somewhat convincing evidence that lactobacilli degrade glycogen. In newborn girls, vaginal pH abruptly drops from around 5.7 at birth to 4-4.5 within four days of birth immediately the vagina was colonized by lactobacilli species (then referred to as 'Döderlein's bacilli'). What is not clear about the other line of argument that supports prior degradation by human enzymes is why the resulting sugars (maltose, trimaltose or tetramaltose) would be used selectively by vaginal lactobacilli and not by the other bacteria present in the vaginal vault. More work needs to be done to clarify this.

Hydrogen peroxide (H_2O_2) production

Peroxidases (such as myeloperoxidase – MPO, from neutrophils or monocytes) in combination with H_2O_2 are able to oxidise halides (such as chloride) into an oxidant that can potently attack oxidisable sites in susceptible microorganisms – especially those that produce little or no H_2O_2-scavenging enzymes such as catalase. There is experimental as well as epidemiological evidence regarding the microbicidal role that H_2O_2-producing lactobacilli play against vaginal pathogens (310, 311). For example, *in vitro* inhibition of *Gardnerella vaginalis* by lactobacilli was demonstrated at an optimum pH of 5.0 to 6.0 and toxicity attributed to H_2O_2 since i, MPO and chloride addition to the co-culture restored toxicity when lactobacilli concentration had been reduced to a non-toxic level ii, toxicity was not observed when the researchers used non H_2O_2-producing lactobacilli and iii, catalase inhibited observed toxicity (312). Eschenbach and colleagues found H_2O_2-producing facultative *Lactobacillus* species in majority (96%) of healthy women but only in 6% of women with bacterial vaginosis (BV) (313). In the same study, anaerobic lactobacilli that do not produce H_2O_2 were found on a larger (36%) proportion of women with BV compared to normal women (4%).

Other groups have, however, questioned the direct role of H_2O_2 as a protective agent in the human vagina; and that with reasonable cause for doubt. The main thrust of their argument is that there isn't sufficient concentrations of H_2O_2 under normal physiological conditions in the vagina for it to function as a bactericidal agent. The vagina is an anaerobic environment thought to have insufficient levels of oxygen necessary for production of H_2O_2. Under anaerobic conditions, lactobacilli will preferentially produce lactate as a fermentation product as opposed to H_2O_2 that is produced when lactobacilli are grown in aerobic conditions. Different studies growing lactobacilli in aerated conditions report H_2O_2 levels between 0.05mM to 3.29mM (111, 106, 112). But physiological concentrations of H_2O_2 are below 100 µM, concentrations that have been shown to lack the capacity to inhibit several BV-associated bacteria *in vitro* (109). Only when H_2O_2 concentrations were increased to 100 mM (50-fold higher than what lactobacilli are able to produce and 5000 times higher than the estimated concentration of H_2O_2 *in vivo*) was inactivation observed but even then, it was more potent for lactobacilli compared to BV-associated bacteria.

The question that then remains is, is H_2O_2 one of the protective mechanisms of lactobacilli or not? The answer may lie somewhere in between a yes and a no. *In vitro* experiments using both metronidazole-resistant and susceptible *Gardnerella vaginalis* demonstrated that its

inhibition by *Lactobacillus* species was greater when co-cultures were done under static, anaerobic conditions (similar to *in vivo* vaginal conditions) as opposed to agitated, aerobic conditions (314). At low pH (±4), lactic acid accounted for majority (60-95%) of the inhibitory activity compared to H_2O_2 (0-30%). This study suggests that it is possible that H_2O_2 plays a protective role under physiologic conditions but that its role may be somewhat inferior to the role played by lactic acid in protection. The individual activity of these compounds was confirmed in a different study (315). Interestingly, this last study also demonstrated synergy between lactic acid and H_2O_2 with enhanced killing activity by H_2O_2 in the presence of lactic acid. Further studies will be needed to understand the role of H_2O_2 with clarification as to whether it plays a functional protective role *in vivo* or it is merely a marker of the presence of bacteria that are known to be beneficial to women's health.

Adhesion to host cells

Another protective mechanism thought to be utilized by lactobacilli is their loose adherence to the vaginal epithelium. Different studies have shown the ability of vaginal Lactobacillus species to stop mucosal adhesion by pathogens through competition or by displacement. This has been demonstrated for *Staphylococcus aureus*, Group B streptococci (316), *Pseudomonas aeruginosa, Klebsiella pneumonia* and *E. coli* (317), *G. vaginalis* and *P. bivia* (318), Trichomonas vaginalis (319) as well as HSV-2 (316, 320). Recent genomic studies on *Lactobacillus* species have described putative adhesin genes that may potentially explain this protective mechanism of lactobacilli (321, 322).

Coaggregation with pathogens

Beyond adherence to the vaginal epithelium, there is evidence of protection by lactobacilli through coaggregation to pathogens. Younes and collegues demonstrated strong adhesion between vaginal lactobacilli strains and three pathogenic *Staphylococcus aureus* strains (323). This bonding occurred rapidly and was actually stronger between the probiotics and pathogenic strains than it was between staphylococcal bacteria in the experiment. A different group demonstrated the ability of human vaginal lactobacilli to coaggregate *Streptococcus agalactiae* and *Staphylococcus aureus in vitro* in a way that could be protective against urogenital tract infections (324).

Bacteriocin production

Bacteriocins are proteinaceous substances secreted by bacteria with activity against other microorganisms. It is a widely held notion that bacteriocins are generally effective against microorganisms closely related to the bacteria but it has recently been shown that some such as L23, fermenticin HV6b and lactocillin among others are effective against Gram-positive and Gram-negative bacteria residing in the female genital tract and other microorganisms such as *Candida* that are unrelated to them including viruses (325-329). Stoyancheva and colleagues specifically demonstrated production of active bacteriocins by *Lactobacillus* species isolated

from human vaginal samples with the ability to inhibit the development of pathogenic microorganisms (329). Bacteriocins generally exert their function at two levels i, at cell envelope level where they could, for example, inhibit peptidoglycan synthesis or act directly by forming pores on the cell membranes compromising integrity, causing leakage and resulting in eventual cell death. ii, at the intracellular level whether they affect gene expression and protein production (reviewed in (325)). The field would benefit from further characterization of these bacteriocins in the female genital tract.

Immune modulation as a protective mechanism?

There is not too much data on the immunomodulatory role of vaginal lactobacilli. *In vitro* data point more towards the fact that lactobacilli do not stimulate inflammatory immune reaction from vaginal epithelial cells and may in fact result in reduction of cytokine secretion after TLR stimulation of these cells (330). Another *in vitro* study showed that probiotic lactobacilli, *L. rhamnosus* GR-1 and *L. reuteri* RC-14, suppress the inflammatory response of vaginal epithelial cell lines (VK2/E6E7) to *Candida albicans* by putatively inhibiting NF-κB signalling (188). *L. iners* is an outlier (again!) in this aspect as it has been shown to up-regulate PRR signalling pathways in human primary vaginal cells (V9) and increase TNF expression at RNA, but not protein, level (331).

3.2.2 Vaginal dysbiosis

When the normal conditions I have described are disrupted, there is an imbalance in the vaginal bacteria microbiota, resulting in an overgrowth of pathogenic bacteria, a condition generally described as *dysbiosis*. Two relatively unknown or uncommon dysbiotic states marked by a dominance of the enteric facultative anaerobes *Escherichia coli*, *Staphylococcus aureus* and group B Streptococcus (*S. agalactiae*) are aerobic vaginitis (AV) and desquamative inflammatory vaginitis (DIV). As recently reviewed by Donders *et al* (332), AV is an inflammatory condition with yellow to green thick vaginal discharge with a rotten smell and some women with AV experience painful sexual intercourse. Under the microscope, vaginal smears from women with AV have evidence of leucocytes and immature epithelial cells. AV prevalence is low (between 7 and 12%) but it has been associated with other STIs such as HIV, HPV and *Chlamydia trachomatis* infection as well as other sequelae such as pre-term birth. DIV is a chronic condition with a purulent discharge, vestibule-vaginal irritation and as with AV, associated with painful coitus (333). When vaginal secretions of women with DIV are observed microscopically, one will notice immature epithelial cells as well as an increase in inflammatory cells. Opinions on the clinical relevance of these two conditions as separate and important syndromes are divided with the balance tilting away from their independent recognition. The third dysbiosis state most widely prevalent and recognized is bacterial vaginosis (BV) and this is also the focus of my study and discussion in this thesis.

Bacterial vaginosis (BV)

The history of bacterial vaginosis goes back to over 50 years ago when, after the Second World War, there was a massive increase in what was then known as "non-specific vaginitis". In 1955, Gardner and Dukes (334) presented results from their study where the infection of 92% of those diagnosed with bacterial vaginitis was attributed to *Haemophilus vaginalis*. *Haemophilus vaginalis* was later renamed *Gardnerella vaginalis* after Gardner. Later, reference to this syndrome changed from 'vaginitis' to 'vaginosis' indicating the lack of inflammation in BV that is characteristic of vaginitis (335). A further reference to this condition as bacterial vaginosis was indicative of the fact that it wasn't caused by fungi or other parasites and it is a term that is still widely used despite numerous suggestions to change the designation (336).

Definition

Based on years of culture-dependent and microscopic work, and more recently on microbial characterization by molecular techniques, BV is generally defined as a clinical condition characterized by a reduction in vaginal lactobacilli and an overgrowth of (facultative) anaerobic bacteria. Cultured-based studies described the replacement of the dominant lactobacilli described above in healthy women with a mixture of diverse anaerobes dominated by the Gram-variable rods of *Gardnerella vaginalis* (334, 337) but including Gram-negative anaerobic rods *Prevotella* spp (338), Gram-positive anaerobic cocci such as *Peptostretococcus* spp and curved Gram-positive anaerobic rods *Mobiluncus* spp (339). Molecular techniques have led to an expansion of the bacteria associated with BV to include other (new) fastidious and non-cultivable bacteria such as BV-associated bacteria (BVAB) 1, 2 and 3 that belong to the *Lachnospiraceae* family; *Eggerthella* spp, *Atopobium vaginae* and *Lactobacillus iners*. Identification of these and the other abundant taxa in vaginal samples by molecular methods was recently reviewed by van de Wijgert *et al* (340). Interestingly, recent molecular studies actually show that even the well-known *Gardnerella vaginalis* can be further defined as a taxon that is made up of at least four different species (341-343). PCR-based studies have also shown that *Lactobacillus* spps. are 50- to 100-fold lower in BV samples compared to samples from healthy women while BV-associated species such as *G. vaginalis* and *A. vaginae* increase by approximately 350- to 500-fold (344). It remains to be clarified whether the overall vaginal bacterial load increases with BV compared to a healthy vaginal environment.

BV diagnosis

There are three broad ways of diagnosing BV; i, Amsel criteria, ii, Nugent scoring and iii, using molecular methods. The Amsel criteria (345) are the most widely used for clinical diagnosis of BV. These criteria require that at least three of the following four conditions are fulfilled for one to be classified as being BV-positive 1. thin, homogenous vaginal discharge 2. A fishy volatile amine odor when potassium hydroxide is added to vaginal secretions; 3. vaginal pH>4.5; and 4. The presence of squamous epithelial cells covered with bacteria (clue cells) on

wet mount microscopy. We note that not all BV cases are characterized by these markers proposed by Amsel. The second method of BV diagnosis, Nugent scoring, is semi-quantitative and relies on Gram staining of vaginal smears followed by microscopy and subsequent scoring (346). Gram-positive rods are scored 0 if missing and 4 when their quantity is high. Gram-negative coccobacilli forms are scored 0 if missing and 4 when their quantity is high. Finally, curved Gram-negative rods are scored 0 if missing and 2 if in high quantity. These scores are then added up and a score of 0-3 is termed as a normal (or healthy) microbiome, 4-6 is for intermediate microbiome and 7-10 is positive for BV. Though it does require some level of skill, this method is more objective than the first and is mostly used in research settings. For over a decade now, molecular methods have been used to characterize the vaginal microbiome especially in research settings. As previously discussed, there are a variety of molecular methods used to characterize the vaginal microbiome but sequencing is the most commonly used method followed by PCR then DNA fingerprinting techniques, phylogenetic DNA microarrays and hybridization to oligonucleotide probes coupled to beads (340). The 16S-rDNA is the most widely used target gene for the reason that it has a number of highly conserved regions (allowing for amplification of multiple species from complex vaginal samples) and eight variable regions (allowing for species differentiation in the context of complexity). Quantitative PCR, 16S-rDNA gene sequencing, clone library analysis, DNA hybridization and microarray techniques have been used for BV diagnosis targeting specific species such as *G. vaginalis*, *A. vaginae*, *Mycoplasma hominis* and *Lactobacillus* spps. (347-352). In one of those studies (348), qPCR for *A. vaginae* and *G. vaginalis* provided the highest predictive value for BV as diagnosed by Nugent scoring (95% sensitivity and 99% specificity). Indeed, one of the conclusions in the review by van de Wijgert *et al* was that molecular methods by and large correlate with the Nugent score but less so with Amsel criteria.

Epidemiology and risk factors

Description of the global epidemiology of BV by different groups is influenced by the diagnostic method used. The 1993 review by Phillip Mead that looked at prevalence rates based on clinical criteria reported BV prevalence ranging from 4% to 61% (353). Women included in their analyses consisted of gynecologic patients, college students, adolescents in correctional facilities, women visiting STD clinics and obstetric patients all based in the USA. Mead estimated asymptomatic BV cases to be anywhere between 30 to 75% of all cases. A later review by Kenyon *et al* (354) looked at global data and summarized prevalence of BV as classified by the more reproducible Nugent scoring system. In summary, BV prevalence was reported to be highest in certain parts of Sub-Saharan Africa (e.g. 68.3% in Mozambique) and lowest in Europe (e.g. 5.9% in Ireland) and Asia. As a caveat, they also reported sub-regions within those wider geographical definitions that went against the grain i.e. low prevalence in Sub-Saharan countries such as Burkina Faso (6.4%) and high prevalence in Tibet (51.6%). Secondly, there was considerable variations in BV prevalence between ethnic groups in many of those regions. Specifically, Black and Hispanic women were reported to have the highest prevalence of BV irrespective of their location. van de Wijgert *et al* in their review of studies

that looked at studies that characterized the vaginal microbiota using molecular methods; Black African and African-American women as well as U.S. Hispanic women were more likely to have bacterial diversity (characteristic of BV) and less likely to have lactobacilli (except *L. iners*) (340). A very recent analysis, however, gives reason for caution in interpreting results of microbiota assessments. Using Nugent scoring, Beamer *et al* reported a higher (36%) prevalence of BV in black compared to white women (8%). However, when they excluded women with asymptomatic BV and other STIs, there were no racial differences in microbial density and frequencies as assessed by both culture-based and molecular methods. They go ahead to conclude that it is possible then that some of the differences seen in microbiota between white and black women could be due to the fact that some microbiota are associated with asymptomatic BV or other STIs that are more prevalent in back as compared to white women.

Debate on the etiology of BV is still ongoing. Because it is polybacterial in nature, there has been some difficulty in identifying a single causal agent. The review by van de Wijgert *et al* identified 25 taxa that were present in at least 50% of the studies (340). Risk factors that have been associated with BV include ethnicity and geographical location as discussed above, smoking, douching, intrauterine device use, menses, number and frequency of sexual contacts, non-circumcision of male sexual partner, low vitamin D levels, poverty, chronic stress and even genetic factors. It is important to add here that many of these risk factors are not causal in and of themselves and they could simply be indicators of other underlying causal or explanatory factors. As discussed in the review on global epidemiology by Kenyon and colleagues (354), a number of these factors do not universally explain BV acquisition. Male circumcision, for example, may explain low BV prevalence in West Africa but not in Asia where circumcision rates are low. Being African has been associated with high BV rates in the US but low prevalence in some studies carried out in Africa calls for caution in classifying African race as a risk factor.

BV treatment and sequelae

Treatment of BV is generally done with broad spectrum antibiotics – metronidazole and clindamycin – that are administered orally or topically (intravaginal) over 5 to 7 days with up to 90% cure rates (355, 356). Other studies have demonstrated efficacy co-treatment of BV-positive women with oral clindamycin and vaginal metronidazole and probiotics (357, 358). Overall, side effects are fewer when topical treatment is used. The biggest issue with BV treatment is recurrence. Between 50-70% of women experience BV recurrence within 3-6 months and up to 80% of them suffer from recurrence in the long-term (356, 359). The reason for this is not absolutely clear. It may be that BV-associated bacteria relapse because they grow in a biofilm that protects them from drug and immune action; that these bacteria are reintroduced to the vaginal vault by the women's sexual partners or from other reservoirs such as the rectum in the same woman; that lactobacilli species do not become established after pathogenic bacteria are cleared by treatment – making the case for co-treatment with

probiotics; that the risk factors that resulted in BV in the first place such as vaginal practices or smoking are still present; or even that viruses that target lactobacilli (bacteriophages) are activated or transmitted to the erstwhile treated woman. Work is ongoing to develop effective BV treatment with little or no relapse.

In the meantime, it is of great public health concern that we understand BV well in order to prevent it or effectively treat it. This is because BV – even with intermediate microbiota – has been associated with increased risk of acquisition and transmission of STIs including HIV, pre-term birth, pelvic inflammatory disease and maternal and neonatal infections (360). These and other sequelae are further discussed in general discussion section the context of the results of our experimental work.

3.3 HIV transmission at the FGT mucosa

At a population level, HIV infection following unprotected heterosexual intercourse is estimated to occur in between 3 to 50 out of 10,000 sexual acts (361). This puts the probability of infection at about 0.1 to 1% in monogamous heterosexual couples (362) and is a testament to the relative efficiency with which HIV infection at the mucosal surface is inhibited under normal conditions. But infection does occur and the target cells are those that express the main receptor, CD4, and either the CCR5 or CXCR4 co-receptors. Basal and parabasal epithelial cells of the ectocervix express both CCR5 and CD4 (363) Leukocytes on the lower FGT also express CCR5 and CD4 (363). Expression of these chemokine receptors makes cells in both the upper and lower FGT susceptible to infection by HIV. CCXR4, CCR5 and CD4 receptor expression in epithelial cells in the uterus varies with menstrual cycle (364). All are upregulated after fertilization (365).

3.3.1 Mechanisms of infection across the cervicovaginal mucosal barrier

Experimental evidence using SIV in macaques shows that cervicovaginal epithelial barrier penetration after exposure happens quite rapidly – within 30 minutes to 1 hour (366). Several mechanisms have been proposed to explain how HIV crosses the epithelial barrier in the female genital tract (367) i) cell-associated or cell-free viruses could go through breaches in the genital mucosa such as the microabrasions that result from sexual intercourse. This way, these viruses have direct access to target cells residing beneath the mucosal surface such as leukocytes, stromal T cells, dendritic cells and macrophages ii) cell-free virus could also be taken in through transcytosis across polarized epithelial cells, from apical to basal cells where they are released to infect target leukocytes. Maybe because of the lack of receptor and co-receptor expression, surface epithelial cells do not get infected by HIV. If HIV enters into these cells, it is degraded before it is passed on to the next cell layer. The virus could however reach the lower layers of the epithelial barrier via the small gaps that exist between squamous epithelial cells iii) virions could also be taken from the luminal surface into the sub-epithelial surface by dendritic cells. Langerhans cells can internalize HIV into their cytoplasmic organelles and by doing so, exit the basal end with intact, infectious viruses.

3.4 Relevance of mucosal immunity and vaginal microbiota to HIV transmission

Regardless of the mechanism(s) leading to infection, there is a rapid expansion of infection from the small founder population proximal to the site of exposure to HIV resulting in systemic infection before the adaptive arm of the immune system can response to contain infection. Initial observations in macaques demonstrated that this rapid expansion of viral infection is fueled by a pro-inflammatory innate immune reaction (368). Chemoattractant chemokines such as MIP-3α produced my plasmacytoid dendritic cells in the cervicovaginal mucosa attracts CD4+ T cells that are infected and further disseminate the viruses. Treatment of the macaques with glycerol monolaurate that inhibits production of MIP-3α and other pro-inflammatory cytokines, protected these macaques from infection by both HIV and SHIV despite repeated intravaginal exposure to the virus. More recently, Deruaz and colleagues (369) used humanized mice to demonstrate that HIV could be restricted to the female genital tract if they blocked the ability of leukocytes to respond to chemoattractants. Proteases at the FGT mucosa such as myeloblastin that function to enhance the production of chemoattractants (IL-8 and MCP-1) by endothelial cells have been shown to be downregulated in HIV-esposed seronegative sex workers from Abidjan (370). The balance of such proteases and their regulatory inhibitors and their importance in immunity at the genital mucosa is discussed in a little more detail in the general discussion section of this thesis. These studies agree with observations in the CAPRISA 004 clinical trial where women with higher baseline (pre-product use) genital inflammation had higher incidences on HIV infection compared to women who lacked inflammation (371). Among young South African women, it was observed that those with vaginal dysbiosis characterized by increased diversity in microbial species and reduced levels of *Lactobacillus* species were four times more likely to acquire HIV compared to those with *Lactobacillus crispatus* dominance in their vaginal microbiome (372). All these studies show the connection between mucosal immune, the vaginal microbiota and HIV transmission. Candidate microbicide compounds that cause inflammation, even sub-clinically so, can increase the risk of infection. Understanding the relationship between vaginal microbiota and genital tract immunity is also important for the same reason. If certain bacterial species are protective and others not, then candidate microbicide products should not disrupt the vaginal microbiome in way that leans towards promotion of inflammation. Additionally, as discussed before, there are numerous endogenous and exogenous variables that shift the immune balance in the genital tract. These need to be understood so as to understand periods during which women are most at risk of HIV infection but also to know when to attribute inflammation (and by extension increased HIV risk) to candidate microbicide compounds or these other factors.

4 Searching for lower female genital tract soluble and cellular biomarkers: defining levels and predictors in a cohort of healthy Caucasian women

Title: Searching for lower female genital tract soluble and cellular biomarkers: defining levels and predictors in a cohort of healthy Caucasian women

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4.1 Abstract

Background:

High concentrations of pro-inflammatory cytokines have been previously observed in the genital fluids of women enrolled in microbicide trials and may explain observed increased HIV transmission in some of these trials. Although the longitudinal nature of these studies allows within-subject comparisons of post-product levels to baseline levels, the fact that the physiologic variations of these cytokines and other markers of immune activation are not fully defined in different populations, makes it difficult to assess changes that can be directly attributed to microbicide use as opposed to other biological and behavioural factors.

Methods:

Cervicovaginal lavage samples were collected from 30 healthy Caucasian and assayed for concentrations of ten cytokines/chemokines - inflammatory cytokines Interleukin-1α (IL-1α), IL-1β, IL-6 and IL-12(p70); anti-inflammatory cytokine IL-1 receptor antagonist (IL-1RA); CC chemokine macrophage inflammatory protein 1 beta (MIP-1β); CXC chemokines IFN-γ – induced protein (IP-10) and IL-8; growth factors granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF); total protein content and; two antimicrobial proteins - elafin and β-defensin using a multiplex immunoassay and ELISA. Cellular markers were characterized by flow cytometry on mononuclear cells collected from the endocervix using flocked swabs. The following antibody-combination was used for direct staining: CD14 APC (1.98µg/ml)/ Viaprobe (PerCP) / HLA-DR FITC (0.99µg /ml)/ / CD3 PE (0.48 µg/ml). Bacterial quantification for total *Lactobacillus* species, *L. crispatus, L. iners, L. jensenii*, *L. gasseri, G. vaginalis,* and *A. vaginae* was performed using quantitative PCR.

Results:

Ectopy, menstrual cycle phase, prostate-specific antigen and presence of leucocytes in endocervical cells' supernatant were associated with the concentrations of cyto-/chemokines in cervicovaginal secretions. Approximately 3% of endocervical cells collected were monocytes of which a median of 52% (SD = 17) expressed both CD4 and CCR5 markers. Approximately 1% of the total cells were T-cells with a median of 61% (SD = 10) CD4 and CCR5 expression. Around 5% of the monocytes and 16% of the T-cells expressed the immune activation marker HLA-DR. Higher percentages of T-cells were associated with greater quantities of IL-1RA, GM-CSF and elafin. No significant associations were observed between any of the bacterial species over more than one soluble marker. Women with *Lactobacillus crispatus* and *Lactobacillus jensenii* present showed a negative association with cellular inflammatory markers.

Conclusion:

We demonstrate the presence of selected soluble and cellular immune activation markers and identify their predictors in the female genital tract of healthy women. Future clinical trials

should consider ectopy, sexual activity, menstrual cycle phase and presence of bacterial species as possible confounders when evaluating the possible inflammatory effects of microbicide compounds.

4.2 Introduction

In the HIV prevention campaign, safe and effective anti-HIV microbicides would offer a discrete protection option when applied vaginally by women before and/or after sexual intercourse but also rectally by men who have sex with men (MSM). Though modest, recent success with the CAPRISA 004 tenofovir gel trial that conferred 39% protection against HIV infection in sexually active women in South Africa (130) provided proof of concept and a much needed impetus in the field of anti-HIV microbicide development although a subsequent study in a different population (VOICE) failed to show effectiveness of the vaginal tenofovir gel (373). Oral antiretrovirals (ARVs) have also been shown to be effective pre-exposure chemoprophylactic agents in MSM (374) as well as in heterosexual discordant couples (375). Previously, clinical trials with first generation vaginal microbicide compounds including surfactants and entry/fusion inhibitors either showed no effectiveness or resulted in an increased risk of HIV infection in the subjects who used them although they had proven antiviral activity *in vitro* (26, 376, 377). It was postulated that the increased infection risk could have been partially due to microbicide-induced mucosal inflammation that results in attraction and activation of CD4+ immune cells, which are prime targets for HIV infection. Excessive inflammation and compound toxicity could also compromise the cervical and vaginal epithelial integrity exposing sub-epithelial target cells like dendritic cells (DC's), CD4+ T-lymphocytes and macrophages to HIV (378, 379). Either way, a better understanding of the mucosal immunity and its modulating factors in different human populations is essential for designing better anti-HIV microbicides and for characterizing drug effects on HIV transmission.

Efforts are therefore underway to define soluble and cellular biomarkers that could be used in microbicide trials to assess sub-clinical mucosal inflammation and hence increase product safety (380-382). Quantification of soluble biomarkers including pro- and anti-inflammatory cytokines found in female genital tract (FGT) secretions has been done in cervicovaginal lavage (CVL) samples or endocervical secretions (ECS) samples collected using different types of swabs or sponges. Considerable data has been generated on cytokine concentrations in the FGT secretions of HIV-positive (112, 383), high risk HIV-negative (383), low risk HIV-negative (383), microbicide trial participants (384) and even healthy women (382). However, methodological variations in sample collection, processing and assay still present a challenge for comparison of data between different studies (385), a challenge also encountered in cytokine measurement in serum and plasma samples (386, 387). Although attempts have been made to standardize cytokine measurement in the vaginal and blood serum context (388) and baseline confidence intervals have been published for some of them in CVLs (388), normative values for these cytokines and other markers of immune activation have not been defined in all populations making it hard to assess cytokine variations that can be attributed to microbicide use as opposed to age, hormonal changes during the menstrual cycle, vaginal tract infection and exposure to semen among other factors.

Definition of cellular markers of immune activation in the FGT of healthy women is also of paramount importance not only because some of these cells are targets for initial HIV infection, but in addition, their recruitment to mucosal surfaces propagates local HIV replication and subsequent systemic dissemination due to normal immune trafficking mechanisms (389, 390). The healthy female genital tract is also home to certain bacterial species that create an acidic environment hostile to pathogens. Imbalance in the vaginal microbiome can result in bacterial vaginosis (BV) that has been associated with greater susceptibility to HIV infection (391). To our knowledge, there is no study to date that correlates the above mentioned soluble markers of immune activation with cellular immune activation markers, the vaginal microbiome and clinical data all of which are part of the complex FGT environment targeted in microbicide trials. Generation of data on the ranges of cytokines/chemokines, local cell populations and the factors that affect their expression in healthy women is needed for assessing the safety of future microbicide candidates.

In this study, we report concentrations of 12 selected cytokines, chemokines and growth factors as well as β-defensin and the anti-protease elafin in CVLs from healthy women representative of a typical early phase I trial population and establish clinical factors associated with their immunoassay detection. These soluble markers were selected studies based on an initial run with 27 analytes with results from two different populations in the USA and Rwanda (392). We also characterize by flow cytometry the proportion of T-cells and monocytes, as well as their expression of the activation marker HLA-DR and the HIV co-receptor CCR5 in the endocervical canal of these women. The association between the vaginal microbiome composition with these soluble and cellular factors is also examined.

4.3 Subjects and methods

4.3.1 Ethical statement

IRB approval was obtained from the Institute of Tropical Medicine and from the Ethics Committee at the University Hospital of Antwerp. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. All study participants gave their written informed consent.

4.3.2 Study subjects

Thirty women aged between 19 and 38 years were recruited at the Institute of Tropical Medicine (ITM) in Antwerp, Belgium using a previously described recruitment strategy for a classical healthy population for a phase I microbicide trial (393). Briefly, we developed and circulated recruitment flyers among the mostly Belgian students in the Master in Tropical Medicine course for doctors and nurses. Study participants were also recruited from personnel at the ITM not involved in the study. Some of the study participants had participated in Phase I studies at the ITM and had agreed to be wait-listed for future studies. These women were not pregnant, did not use any hormonal contraception for the duration of the study, they did not have vaginal infections at screening and had a regular menstrual cycle. Sexual activity was allowed and condoms were provided. Women were screened and then scheduled for five follow-up visits on days 7 (+/- 2 in the follicular phase) and 21 (+/- 2 in the luteal phase) of the three subsequent menstrual cycles. At each visit, a written questionnaire was completed by the women about their sexual activity over the three days preceding the day of sampling.

4.3.3 Sample collection

A clinician collected three high vaginal specimens at each visit, using flocked synthetic swabs (COPAN Innovation, Italy). Two swab specimens were used for quantitative PCR for vaginal bacterial species testing (394) and the third for prostate-specific antigen (PSA) testing. The swabs were stored at 2-8°C until transport to the laboratory, where they were stored dry at -20°C until testing.

For cervicovaginal lavage samples, 10 ml normal saline at room temperature was flushed using a sterile pipette over the cervix and the lateral vaginal walls. This fluid was aspirated from the posterior fornix using the same pipette and collected in a 15 ml falcon tube that was then put in a cool box with ice (2-8°C). To collect endocervical cells, a flocked swab was inserted into the endocervical canal and gently turned over 360°. The swab was then removed and placed in a falcon tube with 10 ml phosphate buffered saline (PBS) with 1% foetal calf serum (FCS), L-glutamine (200mM) and penicillin/streptomycin (10.000U/ml). This procedure was repeated with a second flocked swab that was placed in the same tube as the first one. The samples were stored in a cool box together with the CVL samples and immediately transported to the laboratory for processing.

4.3.4 Sample processing

Sample processing was started within one hour of sample collection for CVL and within 30 minutes for endocervical cell samples. CVL samples were centrifuged at 1000 x g for 10 minutes at 4°C to get rid of debris and the supernatant (~9ml) was aliquoted into five fractions of approximately 1.8ml each and stored at -80°C. Processing of the endocervical swab for cellular markers involved addition of 50 µl of 1M DL-Dithiothreitol (Sigma-Aldrich, Belgium) to the 10 ml medium and incubation for 15 minutes at 37°C to dissolve the mucus. The tube was then gently vortexed to release the cells from the swab tips, after which the swab tips were removed and cells centrifuged at 1000 x g for 10 minutes at 4°C. After leucocyte and haemoglobin testing using dipsticks, the supernatant was discarded and the cell pellet resuspended in 1 ml cell culture medium. Twenty five microliters was then removed for cell counting before analysis was done by flow cytometry.

4.3.5 Clinical and laboratory diagnostic tests

Estimates of the pH of vaginal secretions were determined by the study doctor using colour-fixed indicator sticks pH-Fix 3.6 – 6.1 (Macherey-Nagel GmbH & Co KG, Duren, Germany). These pH strips are colour-coded with graduations at pH points 3.6, 4.1, 4.4, 4.7, 5.0, 5.3, 5.6 and 6.1 and sample pH values are determined by comparing the test fields to the colour block (accuracy ±0.1 pH). Vaginal smears were examined using the Nugent scoring system in which a Nugent score of 7-10 is consistent with BV, 4-6 is an intermediate score and 0-3 reflects a normal vaginal microbiome (346). Participants were also tested for the following sexually transmitted infections (STIs): Trichomoniasis and Candidiasis using wet mount analysis; *Chlamydia trachomatis* - CT and *Neisseria gonorrhoeae* – NG using commercial nucleic acid amplification assays (BD Probetec SDA assay from Becton Dickinson and positive results confirmed with Amplicor CT/NG Roche).

PSA testing was performed on vaginal secretions using the Seratec® PSA semiquant assay (Seratec Diagnostica, Göttingen, Germany). A volume of 500 µl of PSA buffer was added to the thawed swab and was shaken for 2 hours. After centrifugation of 300 µl for 1 min at 13000 x g, 200 µl of supernatant was used for testing according to the manufacturer's instruction.

The presence of leucocytes (sensitivity, 20 – 25 cells/µl as trace) and haemoglobin (sensitivity, 10 red blood cells/µl) in the supernatant of endocervical cells samples was tested using the 5+NL Servotest® test strips (Servoprax® GmbH, Wesel, Germany).

4.3.6 Cytokine and chemokine measurement

Concentrations of the inflammatory cytokines Interleukin-1α (IL-1α), IL-1β, IL-6 and IL-12(p70); anti-inflammatory cytokine IL-1 receptor antagonist (IL-1RA); CC chemokine macrophage inflammatory protein 1 beta (MIP-1β); CXC chemokines IFN-γ –induced protein (IP-10) and IL-8; growth factors granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) in CVL samples were analysed using the Bio-

Plex™ human cytokine assay kit (Bio-Rad Laboratories NV-SA, Nazareth, Belgium) according to the manufacturer's instructions. Briefly, the lyophilized standard was reconstituted for 30 minutes on ice with 500 µl PBS containing 0.5% bovine serum albumin (BSA) and then serially diluted (1 in 4). Magnetic beads coupled with unique capture antibodies were then prepared in assay buffer and kept on ice. The assay plate was pre-wetted with 100 µl assay buffer and drained using vacuum filtration. The coupled beads were vortexed for 30 seconds and 50 µl added to each well in the assay plate. After two plate washes with 100 µl wash buffer, 50 µl of standards, samples and controls (all containing 0.5 % BSA) were added to each well in the assay plate. The plate was then sealed, covered with aluminium foil and incubated on a shaker (500 rpm) at room temperature for 30 minutes. After this period, the plate was washed thrice with 100 µl wash buffer using vacuum filtration and 25 µl of biotinylated detection antibodies added to each well. The plate was sealed, covered with aluminium foil again and incubated under the same conditions as before for 30 minutes. After three washes, 50 µl of streptavidin-PE was added to the wells and incubated on a shaker (500 rpm), at room temperature for 10 minutes. After incubation, the plate was washed 3 times as before, 125 µl assay buffer added to each well, covered and shaken for 4 minutes at 500 rpm. Fluorescence data was collected using the Bio-Plex™ array reader and the Bio-Plex™ Manager 5.0 software used to calculate cytokine concentrations using a weighted five-parameter logistic curve-fitting method on the four-fold dilution series of the standard provided with the kit.

Elafin and β-defensin were measured by ELISA kits from R&D Systems (Minneapolis, MN) and Phoenix Pharmaceuticals (Burlingame, CA), respectively, following manufacturers' instructions. Optical densities were read at 450 nm with a second reference filter of 570 nm using a Victor2 multilabel reader and WorkOut Software (PerkinElmer, Waltham, MA). For the elafin assay, all CVLs were tested at a 250-fold dilution in duplicates (samples were pre-diluted in PBS with 1% BSA and then diluted 5-fold directly on the plate using the manufacturer's supplied reagent diluent). For the β-defensin assay, all CVLs were tested at a 100-fold dilution. The samples were pre-diluted 25-fold in 1% BSA/PBS and then diluted 4-fold on the ELISA plate using the manufacturer-supplied assay diluent. Samples with values below or above the assay detection ranges were repeatedly tested at lower or higher dilutions to obtain accurate protein measurements. A quality control (QC) sample was prepared by pooling CVL samples and running an aliquot of the QC pool on each elafin and β-defensin plate to assess inter-assay reproducibility. The inter-batch CV% of the QC values was 9% for the elafin ELISA kits and 18% for the β-defensin ELISA kits. The intra-assay CV% (mean+/-SD) assessed for duplicate samples measurements was 6.8 +/- 5% for the elafin and 6.6 +/- 7.8% for the β-defensin assay. Elafin and β-defensin concentrations were normalized to total protein determined by a BCA assay (Thermo Scientific, Rockford, IL) using the Victor 2 counter. For total protein, all samples were tested in duplicates at a 5-fold dilution in PBS and retested either undiluted or 5-fold diluted if values were below or above detection range, respectively. The total protein intra-assay CV% was 0-10% (mean +/-SD=2.4+/- 2%).

4.3.7 Flow cytometry

Endocervical cells were transferred into FACS tubes at 2 x 10^5 cells per tube and spun at 590 x g for 5 minutes before the supernatant was removed. Direct staining was done by incubating the cells with labelled antibodies for 20 to 30 minutes at 4°C. All antibodies were from BD-biosciences, unless otherwise stated. The following antibody-combination was used: CD14 APC (1.98µg/ml)/ Viaprobe (PerCP) / HLA-DR FITC (0.99µg /ml)/ / CD3 PE (0.48 µg/ml). After washing in PBS, the cells were fixed with 1% paraformaldehyde solution.

When the number of cells was sufficient, the same samples were also used for indirect staining. We aimed for 2*10^5 cells per staining. This was determined using tripan blue staining of 25µl of sample followed by microscopy counting. If the total amount of PBMC-like cells (not epithelial cells) allowed 2*10^5 for each staining, then all panels were used. However, if cell counts were really low (e.g. in case of bad sample quality), then the primary stains were prioritized. Cells were first incubated with primary antibody (anti-CCR5 [20µg/ml]; Biolegend) for 30 minutes on ice before being washed in PBS and incubated for 20 minutes with secondary antibody (biotin labelled goat anti-mouse [20µg/ml]). After another washing step, streptavidin-PE [20µg/ml] was added. The samples were further washed and mixed with diluted mouse serum. Finally, cells were directly labelled with antibodies before FACS analysis using a BD FACScalibur instrument. The following antibody-combinations were used: CD3 APC (1.98µg/ml)/ Viaprobe (PerCP)/ CD4 FITC (0.12 µg/ml) / CCR5 PE and CD14 APC(1.98µg/ml)/ Viaprobe (PerCP)/ CD4 FITC (0.12 µg/ml) / CCR5 PE. Isotype controls were used to set gates. For analysis, the cells were gated on the population that was CD3+ or CD14+. Analysis was done using the FlowJo software (version 8.8.4 TreeStar, Inc., Ashland, OR, USA).

4.3.8 Bacterial species quantification

Quantitative PCR for total *Lactobacillus* species, *L. crispatus, L. iners, L. jensenii*, *L. gasseri, G. vaginalis,* and *A. vaginae* were performed as described in Jespers *et al* (394). Briefly, the primers were synthesized by Eurogentec, Seraing, Belgium. The 25 µl PCR mixture contained QuantiTect SYBR Green PCR (Qiagen, Venlo, the Netherlands) with the exception of the PCR mixture for *L. vaginalis* which contained Thermo Scientific Absolute SYBR Green Mix (ABgene, Epsom, UK), 5 µl DNA extract, primers, and Milli-Q water. The amplification reactions were performed using the Corbett Life Science Rotor-Gene™ 6000 (Qiagen, Venlo, the Netherlands). For each of the organisms standard curves were constructed. A total of 6 standards were prepared by a tenfold dilution and within a range of 10^2 copies/5 µl to 10^7 copies/5 µl. The quantitative result obtained with the qPCR was expressed in number of copies/5 µl and was back calculated taking into account the total specimen elute volume, the volume extracted, the DNA extract volume obtained, and volume of DNA amplified.

4.3.9 Data analysis

The lower and upper limits of quantitation for each soluble marker were defined as the lowest and highest concentration of their standards within acceptable recovery ranges (70-130%).

CVL samples with soluble marker concentrations below the lower detection limit in the Bio-Plex™ assay were assigned concentrations midway between the lower limit of quantitation (LLOQ) and zero. Those above the upper detection limit were assigned values twice the upper limit of quantitation (ULOQ). In the analyses for associations with different factors that could influence the expression of these soluble markers in the FGT, $\log_{10}$ transformed values were used. To characterize the variation of the soluble markers over time between and within women, we calculated the intra-class correlation coefficient (ICC) for the analytes using the random effects model with the equation ICC = $sigma_B^2/(sigma_B^2+sigma_W^2)$ where $sigma_B^2$ is the variance between women and $sigma_W^2$ is the within woman variance in log-transformed soluble marker concentrations. A high ICC in this context means that there is relatively more inter-woman soluble marker concentration variation than intra-woman variation i.e. the analytes are more constant for each woman compared to the total variation. ICC values were not calculated for IL-12 and GM-CSF because of the high percentage of samples that were below the detection limit.

We modelled analyte concentrations based on presence of ectopy, recent sexual activity as determined by PSA detection, menstrual cycle phase, haemoglobin presence, leucocyte presence as well as the presence of specific bacteria in the vaginal cavity and percentages of cervical cellular markers. All analyses were carried out using mixed effect linear (IL-1α, IL-1β, IL-6, IL-1RA, MIP-1β, IP-10, IL-8, G-CSF, elafin and β-defensin) or logistic regression models (for IL-12(p70) and GM-CSF) with random effects for women and fixed effects for assay plate to correct for inter-assay variability. Logistic regression models were used for IL-12(p70) and GM-CSF because 47% and 67% of the samples respectively were below the detection limit precluding means-based analyses. Each predictor was assessed for association with soluble marker concentration or presence and all significant variables were then included in a multiple predictor model. The model was simplified using stepwise exclusion until only the significant predictors remained in the model. All analyses were carried out using STATA software (version 11 College Station, Texas, USA) and graphs plotted using GraphPad Prism software (version 5.02, GraphPad Prism Software, San Diego, California, USA).

4.4 Results

4.4.1 Cohort demographics

Cohort characteristics are presented in Table 1. Four of the women (13%) had a sexual preference for the same gender and all of those self-reported to be sexually active during the study. Of the remaining 26 women with a male sexual partner preference, 69% reported sexual activity during the study. Ectopy, classified as small, moderate or large by the study doctor following a standardized protocol (395), was present in 20 out of the 30 women enrolled in the study. Seven of the 20 cases were classified as small (Table 1). After screening, 84 (60%) of the total visits were during the follicular phase (day 7) of the menstrual cycle and 57 (40%) during the luteal phase (day 21).

Table 1. Demographic, clinical exam and behavioural data of study population (N = 30)

Demographic, clinical exam and behavioural data	
	N (%)
Race	
Caucasian	30 (100)
Sexually active during study	
Yes	22 (73)
No	8 (27)
Contraception	
None	12 (40)
Intrauterine cupper device	1 (3)
Condoms	17 (57)
Cervical ectopy	
Absent	10 (33)
Small	7 (23)
Moderate	12 (40)
Large	1 (4)
Partner preference	
Male	26 (87)
Female	4 (13)
Age average (range)	27 (19 – 38)

4.4.2 Clinical and laboratory diagnostic tests

Twelve samples (8.5%) were found to be PSA positive (Table 2), an indication of recent sexual activity. All but one of the women with positive PSA tests self-reported sexual activity during the study period. Leucocytes were detected in the supernatant of 52% and haemoglobin in 42% of the endocervical cells samples taken (Table 2). All women enrolled in the study were asymptomatic for vaginal infections with negative lab results (Trichomoniasis, Candidiasis, CT and NG) during screening and all but one of them did not have BV during the follow-up visits. Intermediate Nugent scores (4 and 6) were registered at single time points (visit 4) for two different participants (participant 33 and participant 21, respectively). Even though a Nugent score of 6 is, by definition, intermediate, participant 21 was classified as having BV based on her clinical presentation and the raised pH of 6.1.

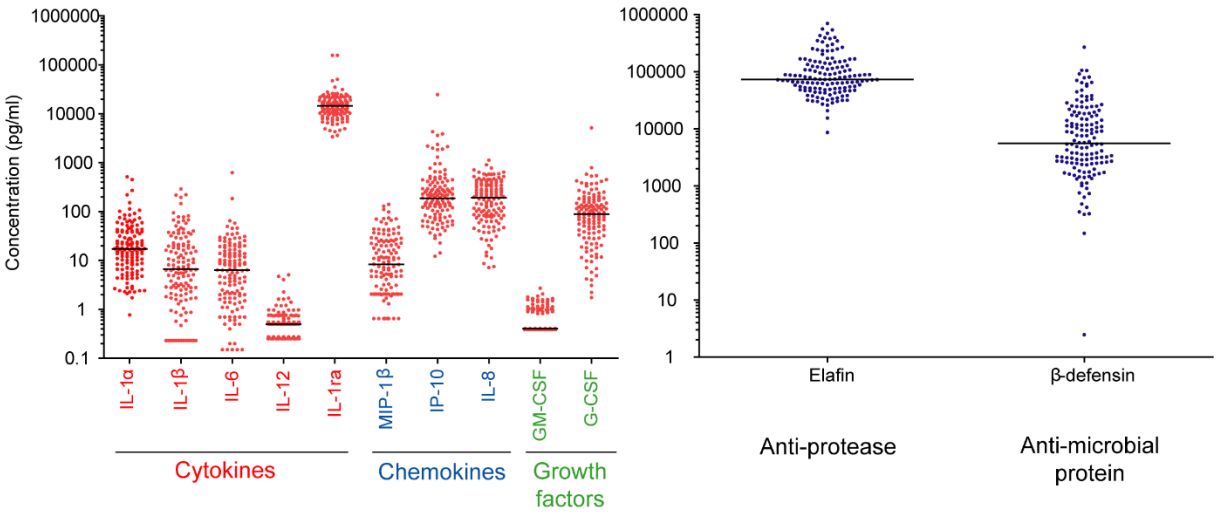
Table 2. Clinical laboratory data from samples of study population (N = 141)

Clinical lab data			No. of samples (%)
Vaginal secretions	**pH**		
		3.6	98 (69.5)
		4.1	42 (29.8)
		6.1	1 (0.7)
	Nugent score		
		0	133 (94.3)
		1	5 (3.6)
		2	0 (0.0)
		3	1 (0.7)
		4	1 (0.7)
		5	0 (0.0)
		6	1 (0.7)
		> 6	0 (0.0)
	PSA positive		
		Yes	12 (8.5)
		No	129 (91.5)
Cells supernatant	**Leucocyte presence** [b]		
		Yes	70 (52)
		No	65 (48)
	Haemoglobin presence [b]		
		Yes	57 (42)
		No	78 (58)

[b] 6 missing values
PSA: prostate-specific antigen

4.4.3 Distribution of soluble markers concentrations in CVL

As can be seen in figure 1, the most readily measurable cytokines, chemokines and growth factors (Figure 1A) in CVL showed a median concentration in the pg/ml protein range while the anti-inflammatory IL1-RA (Figure 1A), the anti-protease elafin as well as β-defensin (Figure 1B) were in the ng/ml range. All analytes evaluated were detected in the majority of the CVL samples, except for IL-12(p70) and GM-CSF that were below the LLOQ in at least 40% of the samples.



Kyongo et al._Figure 1

Figure 1. Distribution of soluble marker concentrations in lavage samples

Cytokines, chemokines and growth factors were measured in CVL samples using the Bio-Plex™ assay while Elafin and β-defensin were quantified using ELISA. Each data point represents a single sample and the line through data points represents the median concentration.

Details of the mean, median, range and SD as well as percentages detected for each soluble marker in the women in our cohort are given in table 3.

Table 3. Soluble markers concentrations in CVL samples of study population

Soluble marker	Percentage detected	CVL concentration (pg/ml)
Pro-inflammatory cytokines		
IL-1α	99	17.51 (0.77 – 513.04) [a] 35.73 (67.27) [b]
IL-1β	90	6.73 (0.23 – 289.98) 23.05 (44.19)
IL-6	96	6.70 (0.15 – 624.10) 17.08 (57.33)
IL-12(p70)	53	0.50 (0.25 – 5.10) 0.61 (0.71)
Anti-inflammatory cytokines		
IL-1RA	99	14,424 (3,372 – 156,688) 16,119 (14,554)
CC chemokines		
MIP-1β	76	8.40 (0.65 – 140.90) 18.65 (25.48)
CXC chemokines		
IP-10	100	185.55 (12.23 – 24,659) 592.47 (2,247)
IL-8	100	203.80 (7.20 – 1,128) 247.12 (204.90)
Growth factors		
GM-CSF	33	0.405 (0.39 – 2.71) 0.68 (0.45)
G-CSF	100	93.36 (1.75 – 5,138) 166.74 (460.74)
Antimicrobial proteins		
Elafin	100	73,465 (8,591 – 704,092) 117,706 (116,832)
β-defensin	100	5,553 (2.45 – 269,839) 16,216 (29,961)
Total protein [c]	100	0.1724 (0.012 – 2.084) 0.246 (0.2312)

[a] Median (minimum - maximum)
[b] Mean (SD)
[c] mg/ml

4.4.4 Longitudinal variation of soluble markers concentrations in CVL

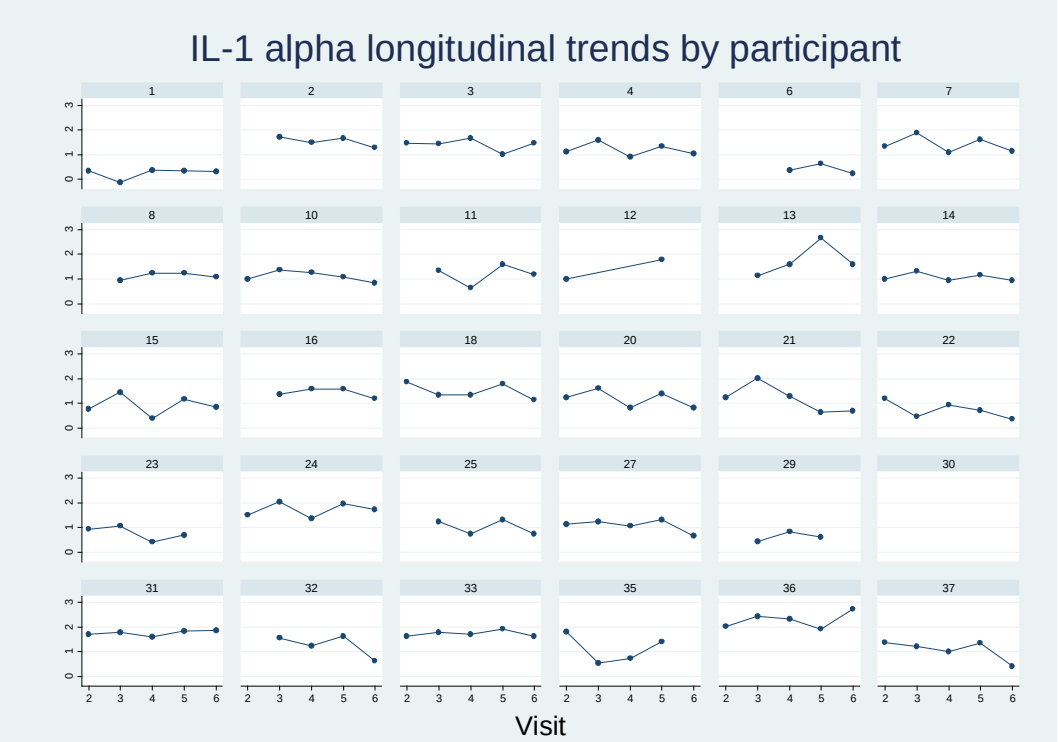
Inter- and intra-woman variation in soluble marker concentration differed from one analyte to another. For most of the soluble markers the inter-woman variation was higher than the variation between repeated samplings at different time points for the same woman (ICC>0.50). As seen in table 4, the ICC values were highest with IL-8, elafin and β-defensin meaning that for these three analytes, their concentrations were relatively more constant

over time in each woman and the inter-woman variation was higher. G-CSF had the lowest ICC value (0.41) indicating a relatively large variation between repeated sampling for the same woman compared to the variation in the analyte concentrations between women. Longitudinal trends for each analyte and each woman are shown in Supporting Figure S1.

Table 4. Intra-class correlation coefficients (ICC) for soluble markers concentrations in CVL

Soluble marker	ICC
Il-1α	0.60
IL-1β	0.59
IL-6	0.51
IL-12	ND [a]
IL-1ra	0.50
MIP-1β	0.61
IP-10	0.53
IL-8	0.70
GM-CSF	ND [a]
G-CSF	0.41
Elafin	0.72
β-defensin	0.63

[a] Not done due to the high percentage of samples in which the cytokine concentrations were below the detection limits of our assay.


IL-1 alpha longitudinal trends by participant
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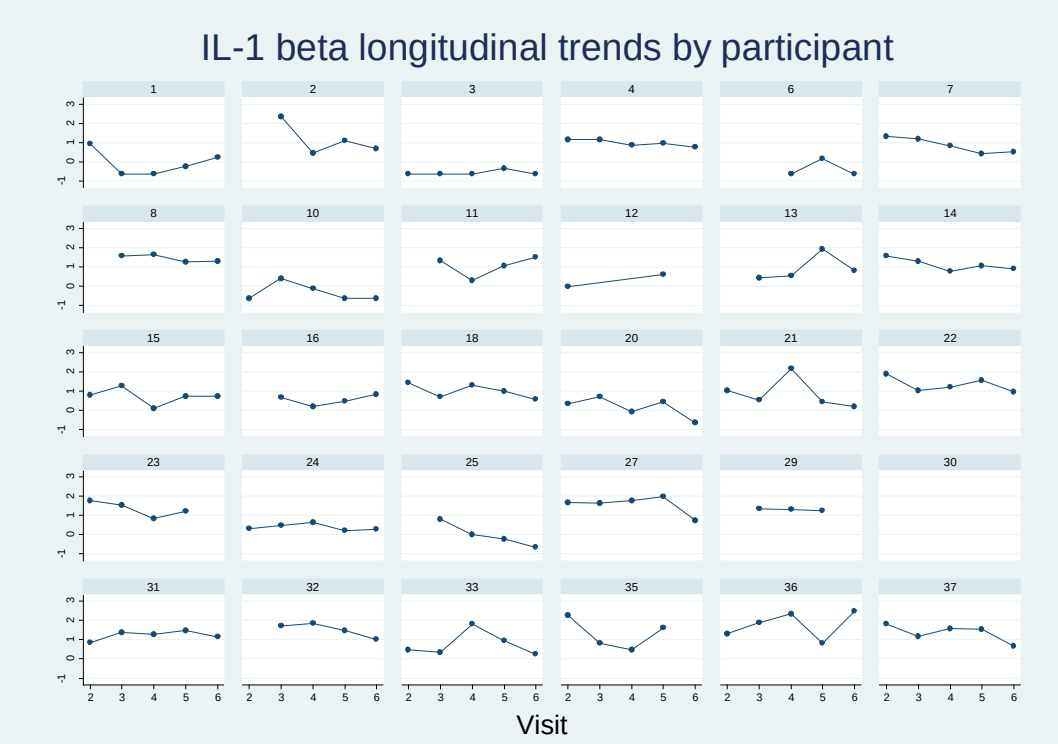
IL-1 beta longitudinal trends by participant
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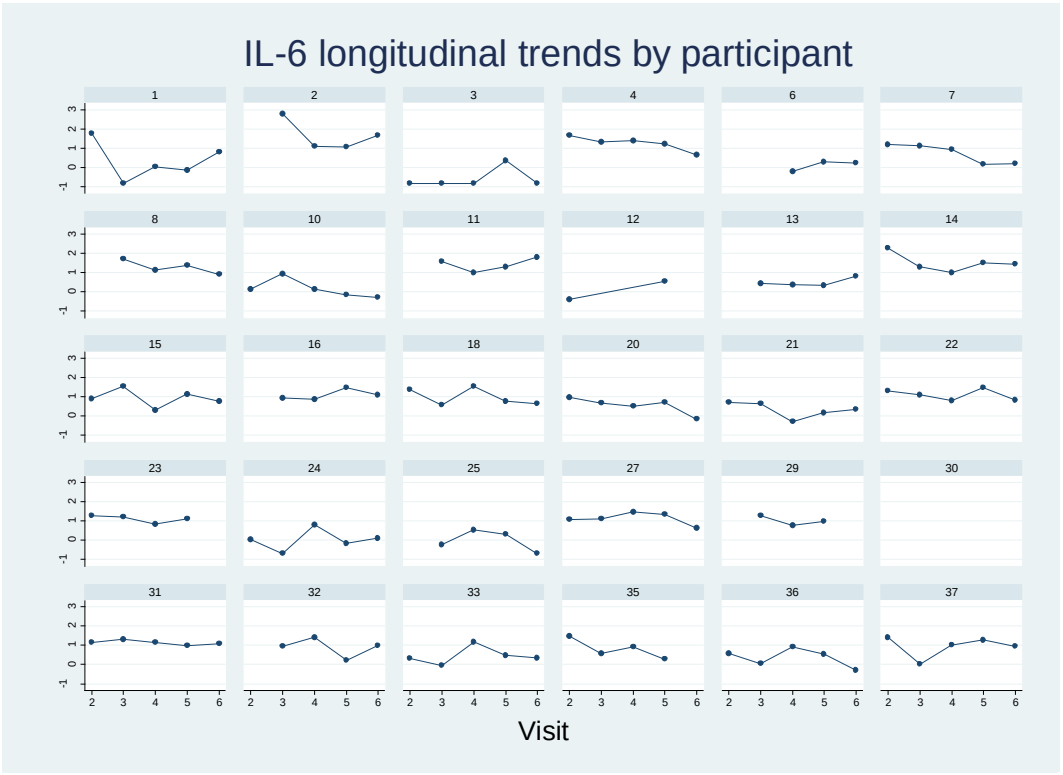
IL-6 longitudinal trends by participant
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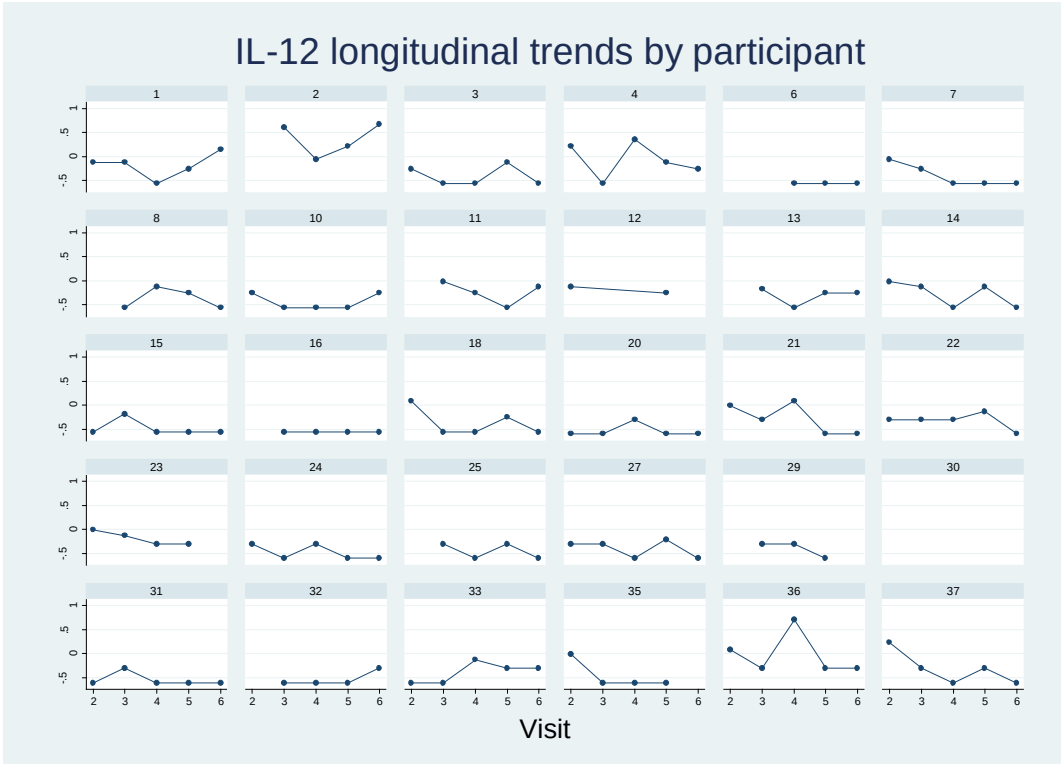
IL-12 longitudinal trends by participant
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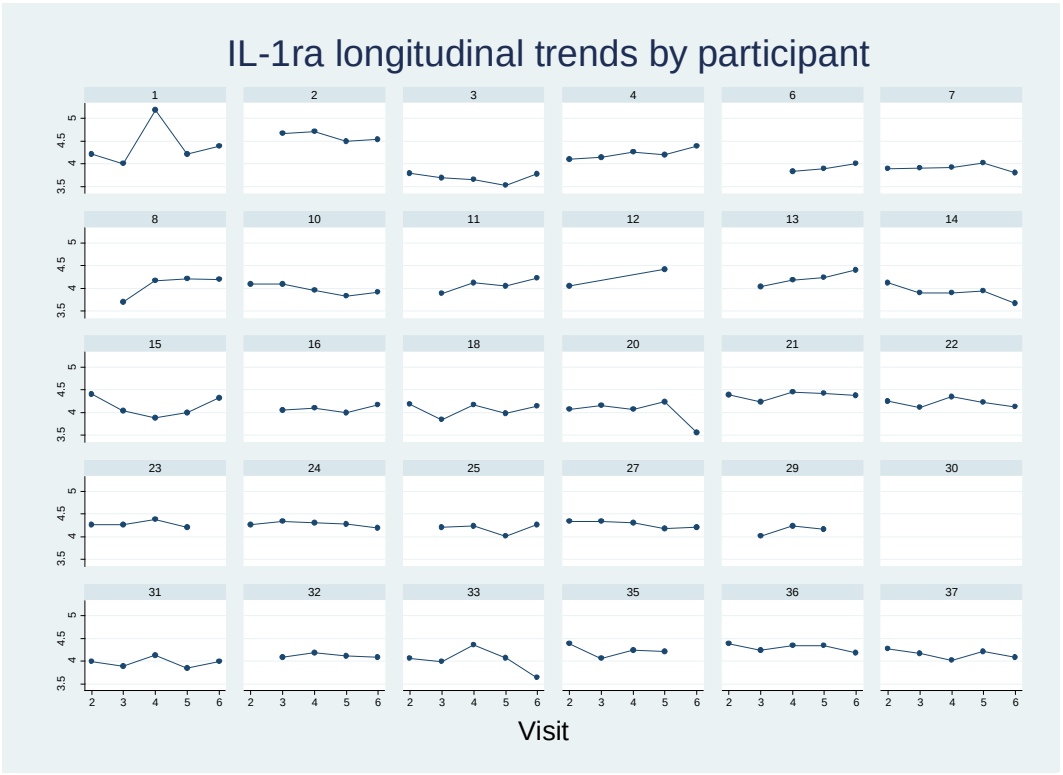
IL-1ra longitudinal trends by participant
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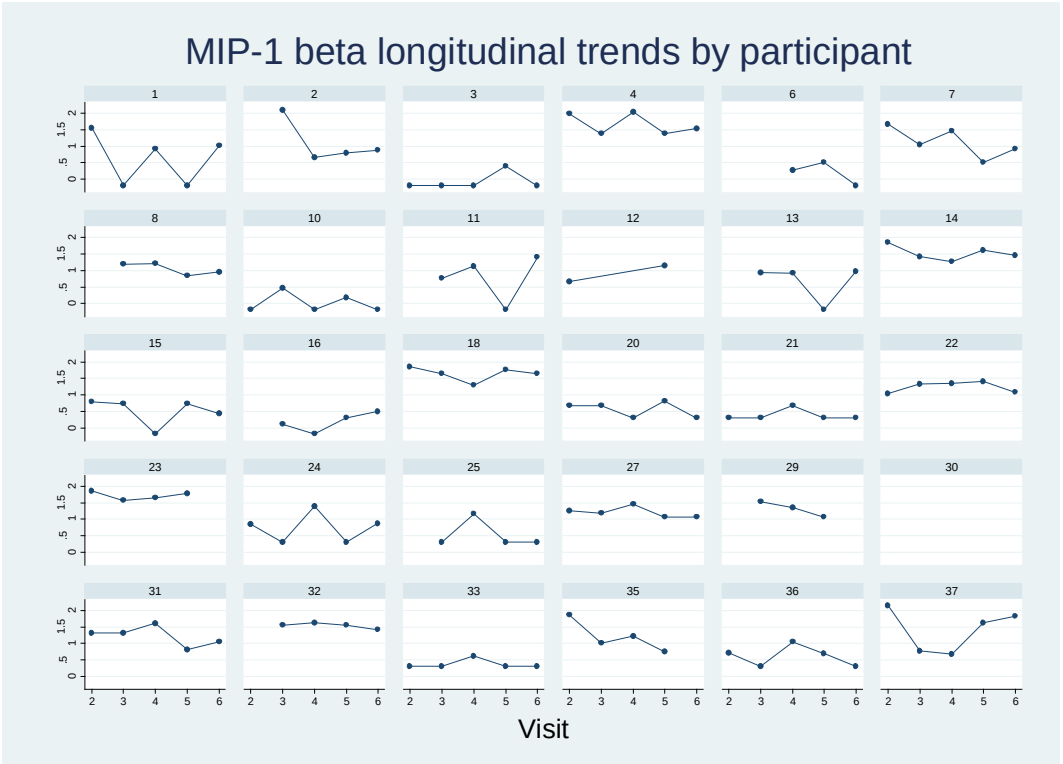
MIP-1 beta longitudinal trends by participant
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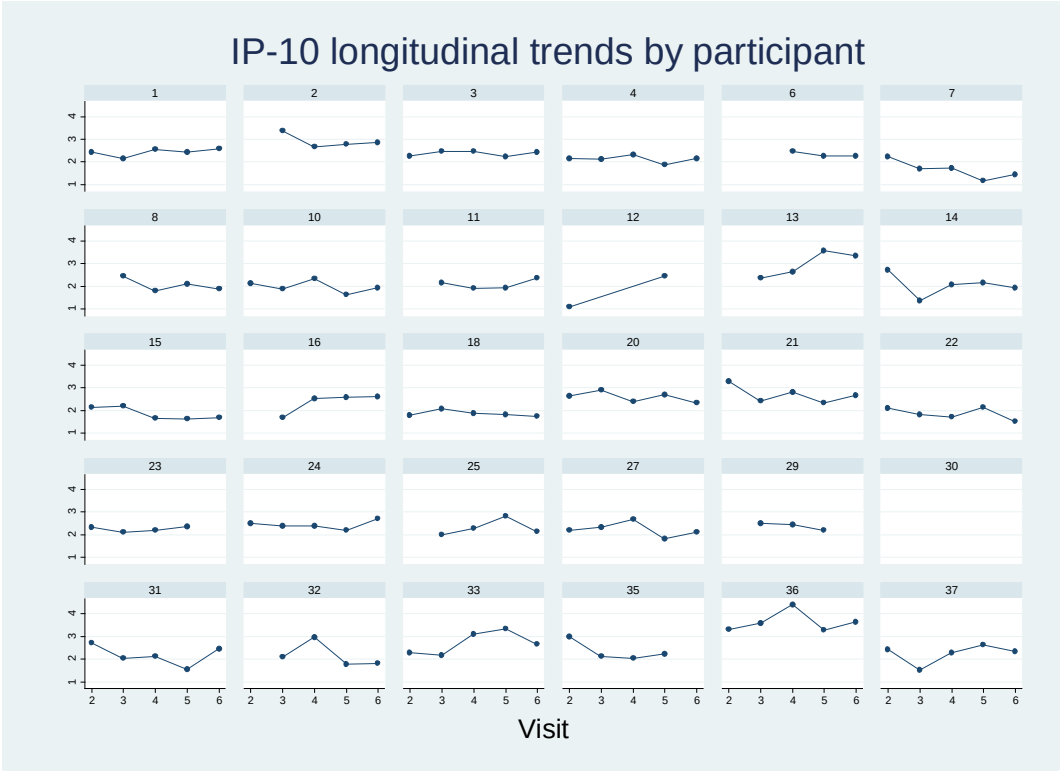
IP-10 longitudinal trends by participant
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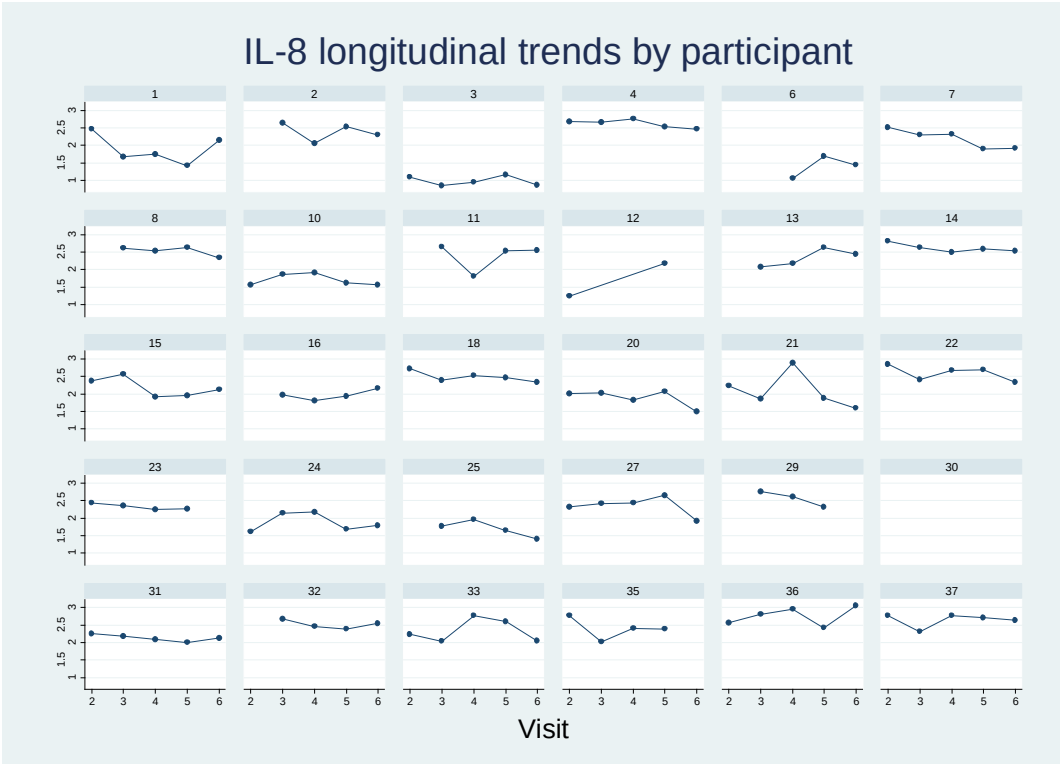
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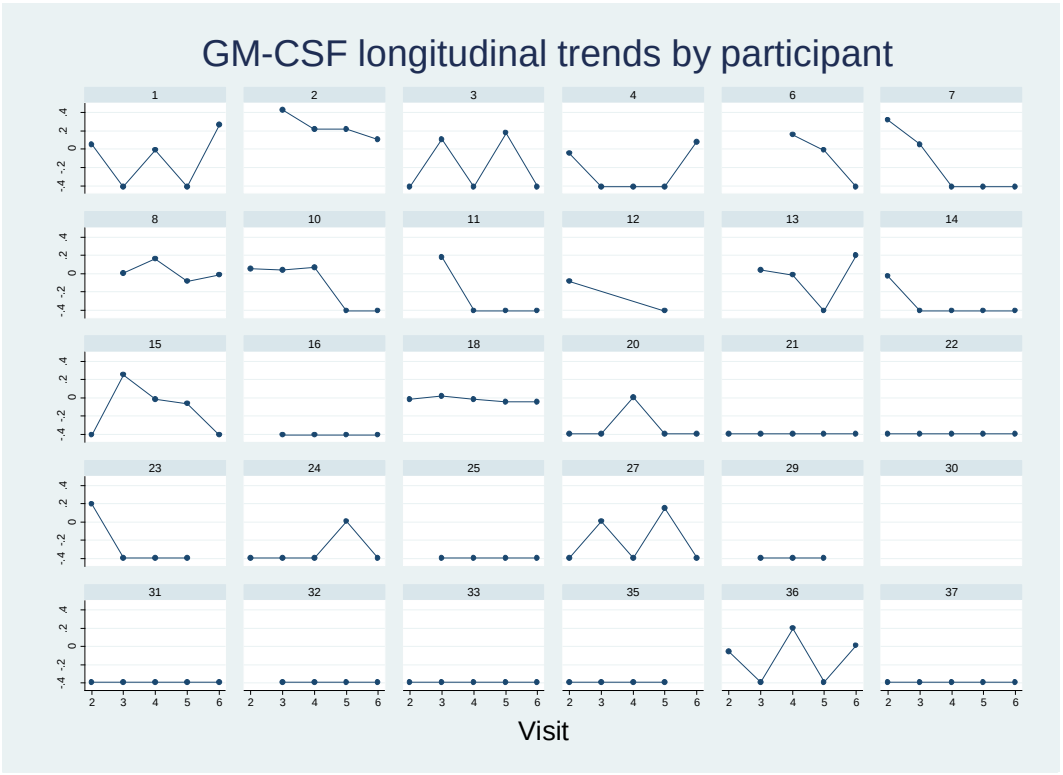
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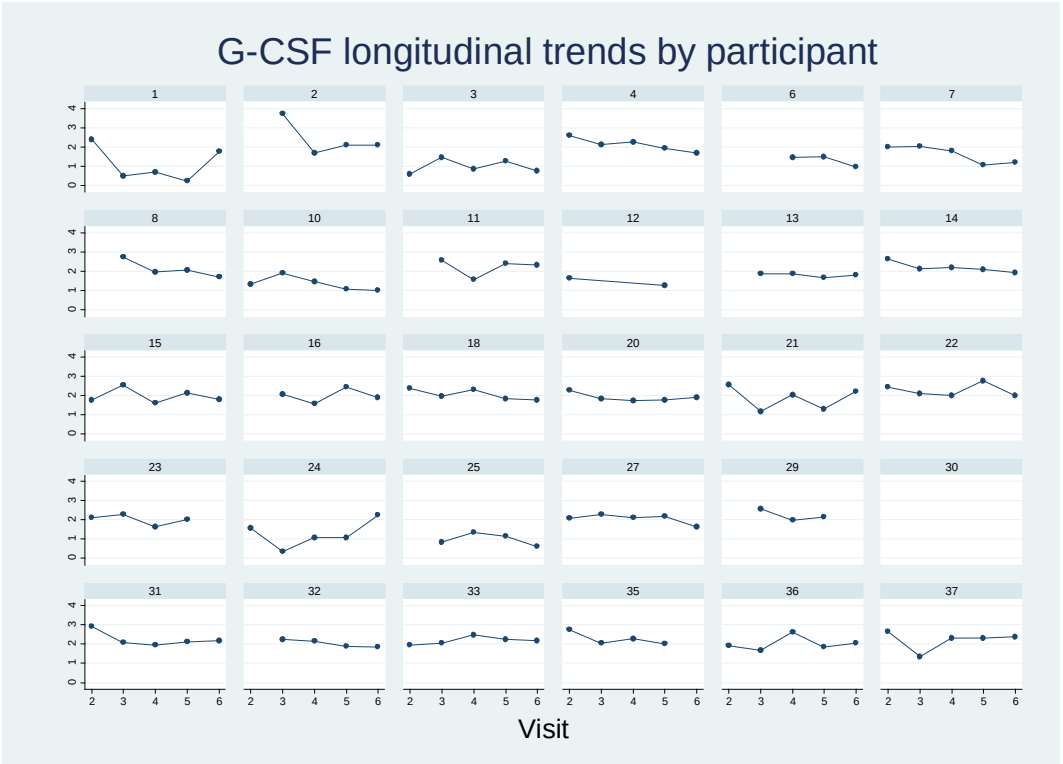
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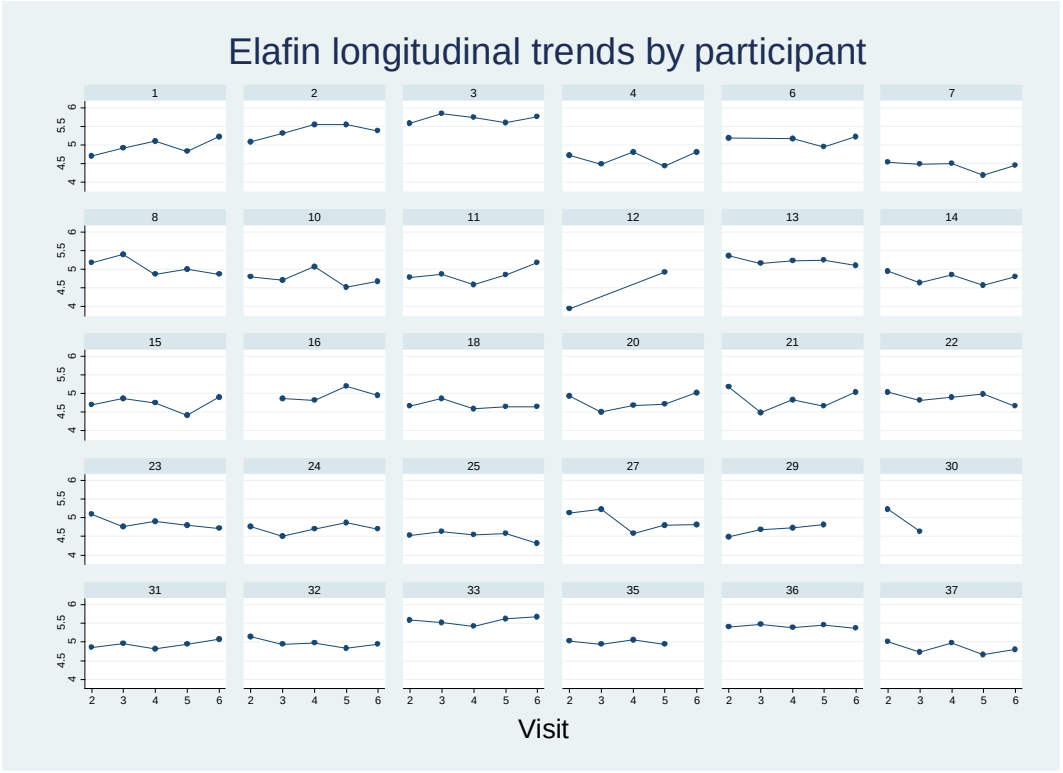
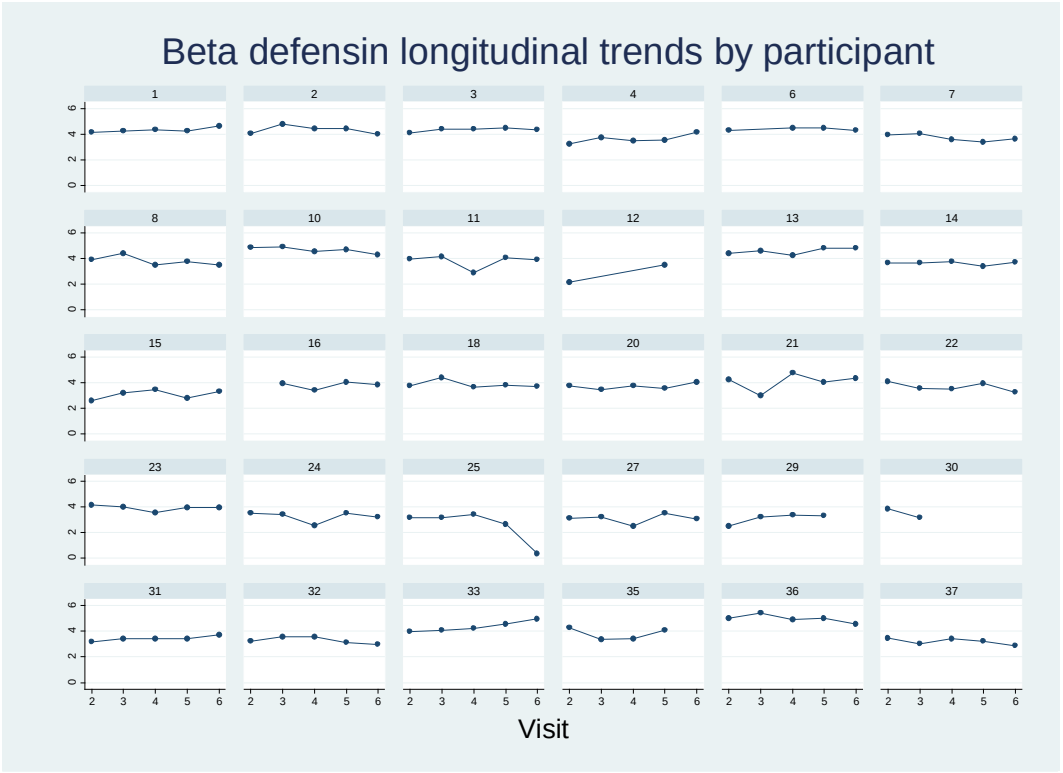


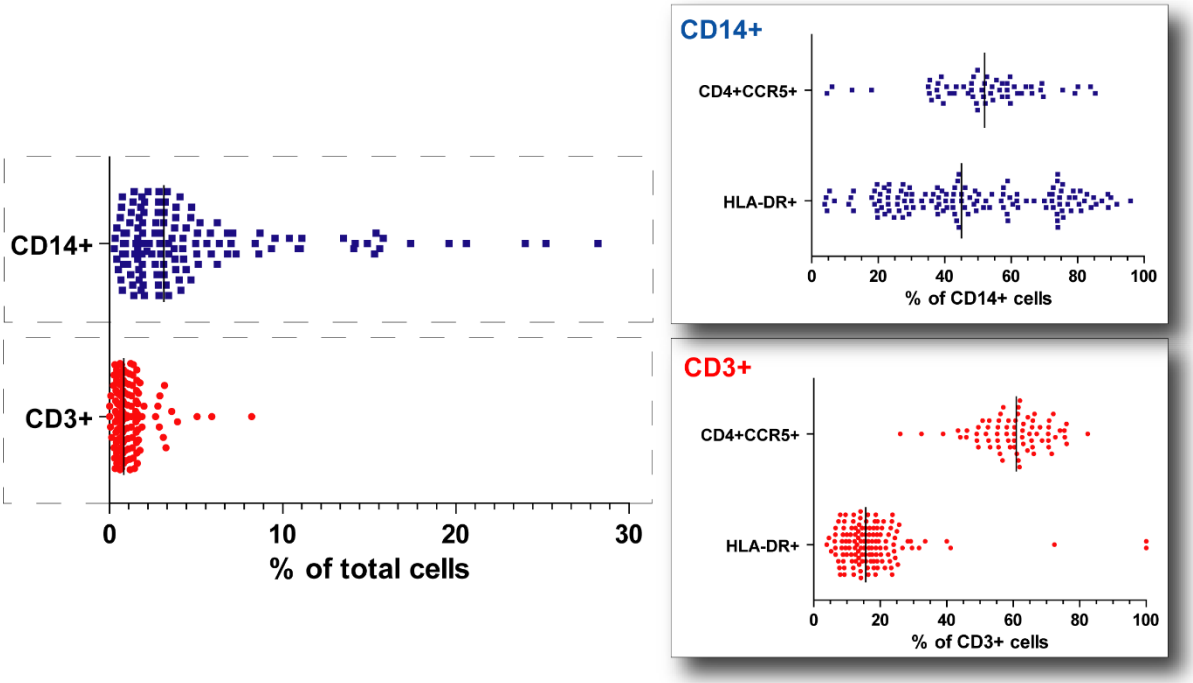


Supporting Figure S1. Longitudinal trends of soluble markers concentrations in CVL by participant

Longitudinal trends for each of the analytes (IL-1α, IL-1β, IL-6, IL-12, IL-1RA, MIP-1β, IP-10, IL-8, GM-CSF, G-CSF, elafin and β-defensin) for each participant are shown. Each point on the X-axis is the visit number by participant and the Y-axis is the concentration of the soluble marker in CVL [log(pg/ml)].

4.4.5 Characterization of immune cellular markers in the endocervical canal

On average, 3.1% of endocervical cells collected using swabs from the study population were monocytes of which 52% expressed both CD4 and CCR5 receptors (Figure 2). Of the total cells, a median of 0.8% were T-cells with 60.9% combined CD4 and CCR5 expression. Even though the women in our study were asymptomatic for FGT infections, a significant median proportion (45.7%) of the monocytes and 15.7% of the T-cells collected from their FGT expressed the immune activation marker HLA-DR.



Kyongo et al._Figure 2

Figure 2. Characterization of endocervical leucocyte markers on cells sampled using flocked swabs

First, viable cells were selected by gating for Viaprobe-negative cell populations on a Viaprobe/SSC plot. Next, viable T cells and monocytes were respectively gated on a CD3-SSC and CD14-SSC plot, to select CD3+ and CD14+ cells. Finally, the percentages of HLA-DR+ or CD4+/CCR5+ cells were gated on a HLA-DR histogram or CD4/CCR5 plot. Three point one percent of endocervical cells collected using swabs were monocytes. Fifty two percent of these cells expressed both the CD4 and CCR5 receptors. Of the total cells, 0.8% were T-cells with 60.9% combined CD4 and CCR5 expression. Even though the women in our study were asymptomatic, a significant proportion (45.7%) of the monocytes and 15.7% of the T-cells collected from their FGT expressed the immune activation marker HLA-DR. All percentages refer to median levels in the study population.

4.4.6 Associations with concentrations of soluble markers in CVL

Clinical associations

Cervical ectopy was strongly and consistently associated with higher levels of the pro-inflammatory IL-1β, IL-8, IL-6, MIP-1β and G-CSF soluble markers in CVL (Table 5).

The levels of IL-1α and β-defensin were elevated in the CVL of women at day 21 of their menstrual cycle compared to day 7. In contrast, day 21 samples had lower IL-1RA and MIP-1β concentrations compared to day 7 samples. Lower levels of IP-10 were also observed in day 21 samples with statistical significance only in the univariate regression model.

In CVL samples of women with detectable white blood cells, higher levels of IL-1α, IL-1β and G-CSF and lower levels of IP-10 were quantified.

The presence of PSA in vaginal secretions and the presence of haemoglobin in endocervical cells' supernatant showed single, independent associations with higher IP-10 and IL-1RA concentrations in CVL, respectively.

Vaginal microbiome associations

No significant associations were observed between any of the species over more than one soluble marker (Table 5). Single associations were observed between the presence of *Lactobacillus iners*, a non-H_2O_2 producer and IL-8. GM-CSF appeared to be higher in samples from women with *Lactobacillus gasseri.* Women with *Lactobacillus crispatus* and *Lactobacillus jensenii* present showed a negative association with cellular inflammatory markers (Table 6) but no association with soluble inflammatory markers.

Endocervical cellular associations

In the samples with higher percentages of CD3 positive T-cells, concentrations of IL-1RA, GM-CSF and elafin were also higher. Higher percentages of CD3+CD4+CCR5+ cells were weakly associated with lower levels of elafin.

4.4.7 Associations with expression of cellular markers in the endocervix

The presence of ectopy was associated with a higher percentage of total monocytes and CD3+CD4+CCR5+ cells but a lower percentage of activated monocytes (Table 6). As with soluble markers, fluctuations with the menstrual cycle was observed with the cellular markers. Specifically, total monocytes, CD3+HLA-DR+ and CD3+CD4+CCR5+ cells were higher on day 21 compared to day 7 of the menstrual cycle. The presence of haemoglobin in the cells' supernatant was associated with a higher percentage of activated monocytes.

The impact of bacterial species on the cellular markers in the endocervical canal in our cohort was only seen with the presence of both *L. crispatus* and *L. jensenii* that was associated with reduced total T-cells, CD3+HLA-DR+ and CD3+CD4+CCR5+ cells in our cohort.

Table 5. Association coefficients between soluble markers and their clinical, vaginal microbiome and endocervical cellular predictors

	IL-1α	IL-1β	IL-6	IL-12[a]	IL-1RA	MIP-1β	IP-10	IL-8	GM-CSF[a]	G-CSF	IL-1RA:IL-1(α+β)	Elafin	β-defensin
Cervical ectopy		0.75*	0.89*			0.56*		0.48*		0.67*			
PSA presence			0.36$				0.30*						
Menstrual cycle phase[b]	0.23*				-0.08*	-0.14*	-0.13$				-0.24*		0.12*
Haemoglobin					0.07*								
Leucocytes	0.24*	0.40*			-0.13$		-0.25*			0.31*	-0.36*		
***L. crispatus* & *L. jensenii* presence**													
***L. iners* presence**		0.52$						0.25*					
***L. gasseri* presence**									3.36*				
***G. vaginalis* & *A. vaginae* presence**			-0.46$										
% T-cells					0.05*				1.94*			0.04*	
% Monocytes													
CD3+HLADR+													
CD14+HLADR+													
CD3+CD4+CCR5+										0.01$		-0.01*	
CD14+CD4+CCR5+													

[a] Associations between IL-12(p70), GM-CSF and their predictors were modelled using logistic regression and their association in the table described using odds ratios
[b] Luteal (day 21) vs. follicular (day 7) phase

Data on soluble marker concentrations was log-transformed before analysis. *Association coefficients representing associations that remained statistically significant in multivariate models. $ Coefficients representing univariate associations.

PSA: prostate-specific antigen

Table 6. Association coefficients between endocervical cellular markers and their clinical and vaginal microbiome predictors

	% T-cells	% Monocytes	CD3+HLADR+	CD14+HLADR+	CD3+CD4+CCR5+
Cervical ectopy		2.67*		-16.81*	8.49*
PSA presence					
Menstrual cycle phase [a]		1.95*	5.27*	-7.97[$]	5.02*
Haemoglobin				9.38*	
Leucocytes					7.5[$]
***L. crispatus* & *L. jensenii* presence**	-0.45*		-6.94*		-6.11*
***L. iners* presence**					
***L. gasseri* presence**					
***G. vaginalis* & *A. vaginae* presence**					

[a] Luteal (day 21) vs. follicular (day 7) phase

Data on soluble marker concentrations was log-transformed before analysis. No associations were found with CD14+CD4+CCR5+ cells. *Association coefficients representing associations that remained statistically significant in multivariate models. [$] Coefficients representing univariate associations.

PSA: prostate-specific antigen

4.4.8 Participants with intermediate Nugent scores

The two women with intermediate Nugent scores on their fourth visits to the clinic (participants 21 and 33) had unique expression profiles of specific soluble markers and bacterial species colonization. Specifically, IL-1β, IL-8 , IL-12(p70) and MIP-1β expression in these women peaked at visit 4. Visit 4 was also the time point at which the highest quantity of *A. vaginae* and *L. iners* but not *G. vaginalis* were detected in the vaginal swab samples of participant 21 (Figure 3). The bacterial species profile of participant 33 was slightly different with *G. vaginalis* and *L. iners* but not *A. vaginae* peaking at visit four. Interestingly, IL-6 expression dipped in participant 21 but peaked in participant 33 at that time point.

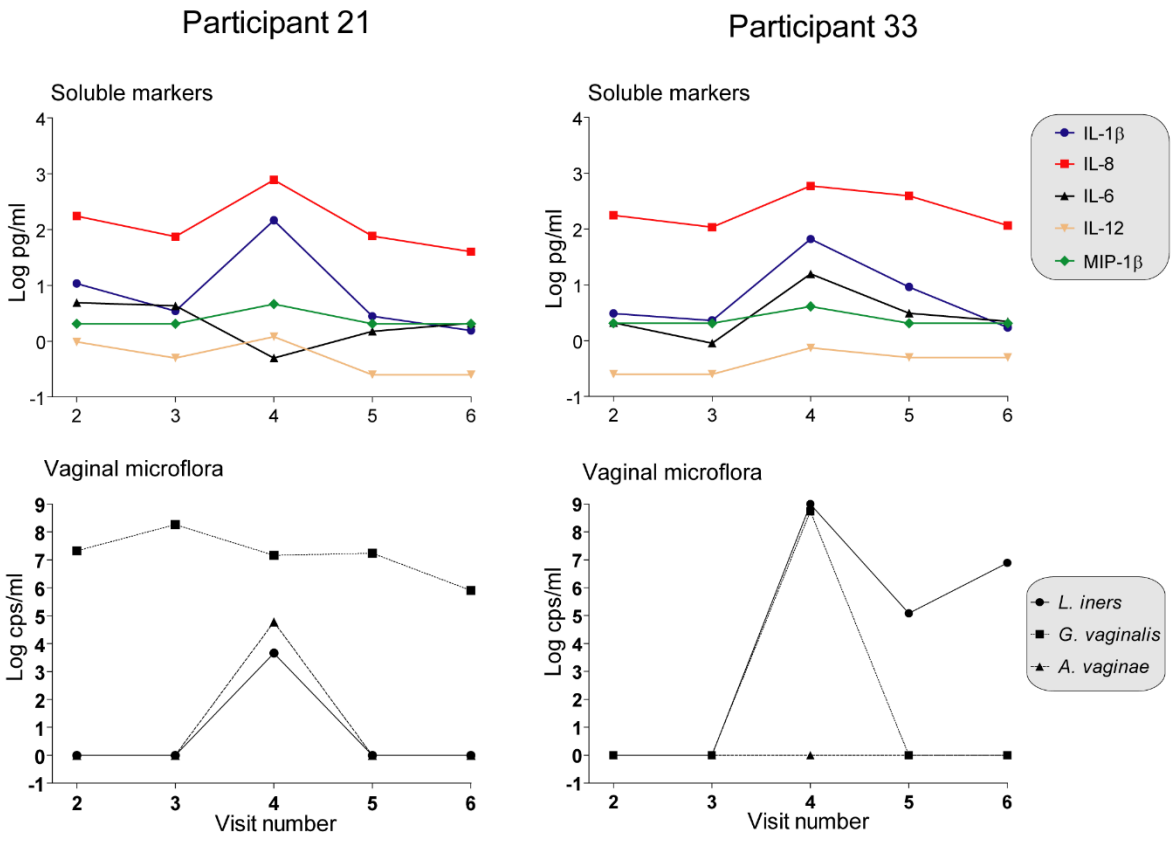


Fig 3 - Kyongo et al.

Figure 3. Case profiles of longitudinal trends of selected bacterial species counts and soluble biomarkers for women with intermediate Nugent scores on visit 4

Two women with intermediate Nugent scores on their fourth visits to the clinic (participants 21 and 33) had unique expression profiles of specific soluble markers and bacterial species colonization. Specifically, IL-1β, IL-8 and modestly IL-12(p70) and MIP-1β expression in these women peaked at visit 4. Interestingly, visit 4 was also the time point at which *A. vaginae* and *L. iners* peaked for participant 21 and *G. vaginalis* and *L. iners* peaked for participant 33. Of note, IL-6 expression dipped in participant 21 but peaked in participant 33 at visit four.

4.5 Discussion

Characterization of soluble and cellular biomarkers and the factors that influence their secretion in the FGT is undoubtedly important for the field of anti-HIV microbicide development. Our study describes normative expression levels of a selected panel of soluble biomarkers in the lower FGT of a typical healthy Caucasian population for an early phase I microbicide trial. These biomarkers were selected based on comparison of an initial Bio-Plex™ run with 27 analytes with results from two different populations in Rwanda and the United States of America as described elsewhere (392). In addition we characterized cervical T-cells and monocytes, which are markers of cellular immunity, sensitive to inflammation and potentially susceptible to HIV. Finally, we investigated whether characteristics such as ectopy, menstrual cycle phase, bacterial species and the presence of PSA, leucocytes and haemoglobin in endocervical secretions were associated with levels of soluble and cellular biomarkers in the female genital mucosa.

Not all selected soluble biomarkers were detected in all samples. This observation is in agreement with other studies where, for example, GM-CSF and IL-12(p70) were also below the LLOQ for the majority of samples regardless of sampling site (endocervix or vagina) or specimen collection method (396). Separately, in the cervical secretion samples of female adolescents in the US collected by Weck-cel® sponges, 39% of samples analysed for IL-12(p70) were below the detection limit (397). In a different study by Lieberman *et al* (382), however, IL-12(p70) was readily detectable in all endocervical secretion samples of healthy, non-pregnant women. These discrepant results may be reflective of methodological differences between the studies and highlight the need of developing standardised methods that should be used for analyte detection if results are to be favourably compared between studies for eventual selection of biomarkers to be used in clinical trials.

Cervical ectopy is a condition in which a proportion of the ectocervix is lined by columnar epithelium instead of the multi-layered squamous epithelium usually found in the mature ectocervix. A thinner mucosal barrier would imply greater vulnerability to physical trauma during coitus leading to inflammation and also exposure of sub-mucosal HIV target cells. It is not surprising therefore that previous studies have found ectopy to be a probable risk factor for HIV infection (6, 398, 399). The strong association of cervical ectopy with the mainly pro-inflammatory soluble markers in our study is consistent with a recent study that found higher levels of pro-inflammatory cytokines/chemokines in the CVL of healthy young women with predominantly columnar and metaplastic ectocervical epithelium compared to predominant squamous epithelium (400) including IL-1β, IL-6 and IL-8 as observed in our study. Previously, *in vitro* experiments with non-stimulated immortalized cell lines of endocervical origin showed higher expression of IL-6, IL-8 and M-CSF compared with cell lines of ectocervical origin under the same conditions (401) corroborating observations seen in women with ectopy.

Based on a general consistency between anamnestic data and testing for PSA, this marker was found to be a reliable measure of recent sexual activity in our study population. In our cohort,

57% of the women reported condom use. However, the single incidence where the FGT secretions tested positive for PSA even though the subject did not report sexual activity indicates the importance of verification of self-reported data by laboratory methods especially in the context of microbicide trials. Previous reports of ectopic prostatic tissue in the upper and lower FGT (402-404) that could explain exceptional detection of PSA in semen-free vaginal samples (405) call for caution in interpretation of results. That said, intercourse is known to disrupt the vaginal ecosystem as constituents of seminal plasma optimize conditions to promote conception. For example, vaginal pH is increased after coitus due to the higher pH of semen, with semen also promoting the influx of leucocytes and Langerhans cells into the FGT (406, 407). Changes in vaginal bacterial species can also be expected as during coitus, bacteria colonising the perineum could be transferred into the vagina. The observation in our study of increased concentrations of IP-10 (and univariately IL-6) in the presence of PSA are in agreement with a recent study that reported increased leukocyte recruitment and pro-inflammatory cytokine mRNA expression in ectocervical tissue exposed to seminal fluid during coitus (408). This pro-inflammatory effect was independent of the physical effects of coitus as it was not seen in controls who used condoms. *In vitro*, seminal plasma induced IL-6 production in ectocervical cells but not vaginal or endocervical cells (406). These changes, coupled with the physical effects of coitus, can lead to greater susceptibility to infection upon exposure to HIV and should also be taken into account in clinical trials that also assess the effects of microbicide compounds on the FGT.

Our study also recorded variations in concentrations of specific soluble and cellular immune modulators with the menstrual cycle. These variations are not surprising given the hormonal changes occurring in the female genital tract during the course of the menstrual cycle. Wira and Fahey even suggested a window of viral infectivity on days 14-23 of the menstrual cycle during which FGT immunity is suppressed by sex hormones (239). In the CVL of healthy pre-menopausal women, Al-Harthi and colleagues (409) found five-fold higher expression of IL-6 and IL-1β in the follicular compared to the luteal phase of the menstrual cycle. In contrast, the same group found equal expression of the same cytokines in the CVL of HIV-seropositive pre-menopausal women with significant elevation only seen during menses (410). Our study group did not show differences in the expression of these two cytokines between the follicular and luteal phases. While HIV infection could explain differences observed in their studies and ours, a significant difference is that our study controlled for ectopy which was associated with both IL-1β and IL-6 expression and could have confounded their analyses. Fleming *et al* (411) also demonstrated maximal β-defensin-1 mRNA expression in the endometrium during the mid-secretory phase and maximal β-defensin-2 mRNA expression during menstruation. Another study showed positive correlation of CVL levels of M-CSF with serum levels of E2 and the E2/P ratio (412) and increased levels of IL-1β and TGFβ2 (412). Estradiol, a hormone which is increased together with progesterone in the luteal phase, has previously been shown to increase mRNA expression of human β-defensin-2 by uterine epithelial cells while at the same time inhibiting the expression of the pro-inflammatory TNF-α, IL-6 and IL-8 *in vitro* (189). This probable two-sided effect of the sex hormone is also seen in our study where IP-10 and MIP-

1β were decreased on day 21 compared to day 7 of the menstrual cycle. It remains unknown, however, what role progesterone played in these fluctuations and future *in vitro* studies would benefit from assessing the combined effects of these sex hormones to gain a better understanding of the *in vivo* situation. Of note, we demonstrated increased expression of IL-1α and β-defensin and decreased expression of IL-1RA in the luteal compared to the follicular phase of the menstrual cycle, highlighting the need to take into account the menstrual cycle time point during microbicide trials. Differential counts of *L. crispatus* (0.22 log higher) and *L. iners* (0.83 log lower) on day 21 compared to day 7 of the menstrual cycle were also observed in women in our study population in whom *L. crispatus*, *L. iners*, *L. jensenii* and *L. gasseri* were present in at least 4 of 5 study visits as described elsewhere by Jespers *et al* (394).

Detection of haemoglobin in cervicovaginal secretions is not unusual even when sampling is done outside menses. Haemoglobin was detected in 64% of ECS by Lieberman *et al* (382) although there was no significant association with any of the analytes tested in their study. An earlier study documented significantly higher concentrations of IL-10 and IL-12(p70) in blood-contaminated ECS samples compared to non-contaminated samples (397). Haemoglobin presence in cells supernatant was positively but weakly associated with IL-1RA and activated monocytes in our population. These results need to be interpreted with caution as it is possible that soluble and cellular markers of systemic origin leak into the FGT due to trauma from sampling and are not reflective of a local immune response at the female genital mucosa. Leucocyte presence that is a normal marker of inflammation was unsurprisingly positively associated with pro-inflammatory analytes IL-1α, IL-1β and G-CSF in CVL and negatively with IL-1RA. A recent study also found a strong correlation between the neutrophil marker myeloperoxidase (MPO) and G-CSF that supports neutrophil function (413).

Bacterial vaginosis, a condition in which the vagina is colonized by anaerobic bacteria instead of the protective lactobacilli species, has been associated with increased susceptibility to STIs and HIV infection (167, 414, 415). In our study population, the presence of both *G. vaginalis* and *A. vaginae* was inversely correlated with IL-6. This partial dampening of the immune response could possibly explain the presence of these BV related organisms in healthy asymptomatic women. The two participants (21 and 33) with intermediate Nugent scores had distinct vaginal microbiome and soluble marker expression profiles. Participant 21 complained of a vaginal itch, had a white watery discharge on examination and a vaginal pH of 6.1 during her fourth visit to the clinic. She was treated with 150mg Diflucan for clinical candidiasis and improved on the next day. These clinical symptoms combined with a Nugent score of six led to her classification as having BV. The Nugent scores during her other visits were all zero. Interestingly, a longitudinal assessment of the CVL concentrations of the soluble immune modulators in participants 21 and 33 show similar trends (figure 3); IL-1β, IL-8 and modestly IL-12(p70) and MIP-1β all peak during visit 4 when these participants had intermediate Nugent scores. These observations are partly in agreement with a different study where both IL-1β and IL-1RA were found to be significantly higher in women with intermediate Nugent scores compared to women with normal Nugent scores (388). Cauci *et al* (416) showed 13 fold vaginal

IL-1β in women with BV in association with anti-*Gardnerella vaginalis* hemolysin (Gvh) IgA response. They concluded that the induction of the pro-inflammatory cytokine IL-1β might be a necessary event to elicit an innate immune response to control anaerobic genital tract infections and that high levels of vaginal IL-1β were associated with mounting of an antigen-specific mucosal immune response in women with bacterial vaginosis (417). A recent *in vitro* model of vaginal bacterial colonization showed that in contrast to *L. crispatus*, BV-associated *P. bivia* and especially *A. vaginae* induce increased production of pro-inflammatory chemokines (e.g. IL-8) (418). Importantly, the peaks in pro-inflammatory cytokines observed in these two participants in our study coincide with peaks of both *A. vaginae* and *L. iners* for participant 21 and *G. vaginalis* and *L. iners* for participant 33 *(*figure 3). In participant 21, IL-6 concentrations dipped during visit 4 when both *A. vaginae* and *G. vaginalis* were present corroborating the inverse association seen before. *G. vaginalis* was constantly present in this participant and she only developed clinical symptoms when *A. vaginae* was also present. In contrast, in participant 33 IL-6 peaked during this visit but there was also no peak of *A. vaginae*, suggesting that *A. vaginae* and *G. vaginalis* work in concert to dampen IL-6 expression and cause BV.

The presence of both *L. crispatus* and *L. jensenii* was not related to any of the cytokines or chemokines and had an inverse association with the total percentage of T-cells, CD3+HLA-DR+ and CD3+CD4+CCR5+ cells. This is in agreement with the knowledge that the *Lactobacillus* species are the major constituents of the healthy vaginal microbiota in women and that they have consistently been associated with absence of vaginal symptoms, reduced risk of STIs and a healthy pregnancy outcome (419, 420). In our healthy women the presence of *L. iners,* a non-H_2O_2 producing bacterium, was associated with increased IL-1β and IL-8. *L. iners* is present in high numbers in women with and without BV as demonstrated by Jespers *et al* (394). In contrast to studies carried out in North America and Europe where *L. crispatus* dominates, *L. iners* has been shown to be the predominant species in a study in Nigeria (421). Unpublished data from Tanzania also shows predominance in *L. iners* (422) in spite of a normal flora as defined by Nugent. Assuming that *L. crispatus* is the key flora of a healthy vagina, the lack of it may be related to the high BV population prevalence in Tanzania. In addition, Srinivasan and colleagues showed that concentrations of *L. iners* increase after antibiotic treatment for BV, suggesting that it fills in for bacteria successfully eradicated by treatment (423) and that women who have been previously treated for BV, may be at higher risk for recurrence. Associations between the different types of vaginal flora in this study population are described elsewhere by Jespers *et al* (394).

A potential weakness of the study is that ectopy was determined by observation and not by photography and computer-assisted measurement. The associations with ectopy however remained strongly significant in the multivariate analyses ruling out the probability that they were chance associations. Additionally, scoring of ectopy for all participants was done by a single physician using a documented manual, thereby excluding variation due to subjective interpretation/classification. Finally, statistical significance was retained in the associations

even when only the moderate and large ectopies were considered as present and the small ones classified as absent (data not shown). Given the relatively large number of possible predictors and outcome measures, only associations which were observed across several soluble markers in CVL are expected to be reproducible. Single associations could be due to chance and should be confirmed in further studies.

Soluble marker concentrations described in this study can act as reference values for further studies in women with similar or different profiles. Comparison of concentrations across studies will however only be feasible once sample collection, processing and measurement methods are standardized. Most studies use traditional ELISAs or multiplex immunoassays for soluble markers detection. The main advantage of using multiplex assays for detection of soluble biomarkers is that very small volumes of samples are needed to detect multiple analytes and this is especially beneficial in the case of ECS where eluted sample volumes are limited. Establishing the normative ranges of soluble and cellular biomarkers in healthy populations allows for the selection of specific ones with distinct expression profiles under conditions of immune activation and whose variations can be universally attributed to specific predictors and hence can be reliably used as indicators of product safety within the setting of microbicides clinical trials. In future, clinical trials should consider ectopy, sexual activity as determined by PSA presence, menstrual cycle phase and presence of bacterial species in the FGT as possible confounders when evaluating the possible inflammatory effects of microbicide compounds in the FGT.

5 A cross-sectional analysis of selected genital tract immunological markers and molecular vaginal microbiota in Sub-Saharan African women with relevance to HIV risk and prevention.

Title: A cross-sectional analysis of selected genital tract immunological markers and molecular vaginal microbiota in Sub-Saharan African women with relevance to HIV risk and prevention.

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Keywords: Vaginal inflammation, Immune activation, Female genital tract, HIV, Bacterial vaginosis, *Lactobacillus*, Microbiota, *G. vaginalis*, Reproductive health, Prevention

Key Points

We identify IP-10 suppression as a potential mechanism of immune evasion by bacterial vaginosis (BV)-associated bacteria and describe the non-inflammatory nature of *L. crispatus* and *L. vaginalis.* Future prevention interventions targeting these factors could influence HIV susceptibility in African women.

5.1 Abstract

Background

Data on vaginal immune mediators, microbial composition and their modulating factors in Sub-Saharan African women are limited.

Methods

Cervicovaginal lavage (CVL) samples from 430 sexually active women from Kenya, South Africa and Rwanda were analyzed for twelve soluble immune mediators. Ten bacterial species were quantified in vaginal swab samples. Bacterial vaginosis (BV) was defined by Nugent scoring. We compared the anti-HIV activity of CVL samples from BV-positive to those from BV-negative women.

Results

Pregnant women, adolescents, women engaging in traditional vaginal practices and HIV-positive women differed in soluble markers compared to reference groups of HIV-negative women. Cervical mucus, cervical ectopy, abnormal discharge and having multiple sex partners were each associated with an increase in inflammation mediators. Interleukin (IL)-1α, IL-1β, IL-6, IL-12 and IL-8 were elevated and the IL-1RA/(IL-1(α+β) ratio decreased in women with BV. Interferon gamma-induced protein (IP)-10 was decreased in BV-positive compared to BV-negative women. *Lactobacillus crispatus* and *Lactobacillus vaginalis* were associated with decreased pro-inflammatory cytokines and each BV-associated species with increased pro-inflammatory cytokines. The *in vitro* anti-HIV activity of CVLs from BV-positive women was stronger than that of BV-negative women.

Conclusion

We found significant associations of factors that can influence HIV susceptibility with the levels of soluble immune mediators in the vaginal environment of healthy, sexually active women. These factors should be considered when establishing normative levels or pathogenic cut-offs of biomarkers of inflammation and associated risks in African women.

5.2 Introduction

The majority of HIV transmission in Sub-Saharan Africa (SSA) is through heterosexual contact and young women have very high HIV incidence rates (424). Immune activation in the female genital tract (FGT) is associated with the secretion of pro-inflammatory cytokines and chemokines by mucosal epithelial cells. Concomitant attraction of cells expressing the HIV receptor and co-receptors to the FGT mucosa enhances susceptibility to infection (376). Indeed, increased levels of soluble markers of inflammation were observed in the FGT of South African women who acquired HIV in the CAPRISA 004 vaginal microbicide trial (425).

There is a paucity of data on the clinical and epidemiological factors associated with immunological markers and consequently risk of HIV acquisition in various groups of women from SSA. Hormonal variation during the menstrual cycle is accompanied by a transient immune suppression that is necessary to ensure successful fertilization and embryo implantation in the uterus (239). Hormonal differences may therefore result in different mucosal immunological profiles in pregnant women and adolescent girls as compared to non-pregnant adult women. Differential exposure to mucosal infections (426) and other behavioural factors that alter HIV acquisition risk such as traditional vaginal practices and having multiple sexual partners might also have an impact on mucosal immunology in the FGT. SSA has the highest prevalence of bacterial vaginosis (BV) (354) which has been associated with greater susceptibility to HIV infection (427) and increased female-to-male HIV-1 transmission (137). All these factors work in concert and are best studied together for a holistic view of mucosal immunology in the FGT.

We set out to characterize twelve soluble immune markers in the FGT of 430 women from three SSA countries differentially affected by the HIV pandemic. We then explored epidemiological, physiological, behavioural and clinical factors potentially associated with these immune markers. This included correlations with quantitative PCR (qPCR) data of vaginal microbiota. Finally, we evaluated the *in vitro* anti-HIV activity of cervicovaginal lavage (CVL) samples from BV-positive women compared to BV-negative women.

5.3 Methods

5.3.1 Study participants

HIV-negative pregnant women, adolescents, women engaging in traditional vaginal practices, sex workers and a group of HIV-positive women on combination antiretroviral therapy (cART) were compared to a reference group of adult HIV-negative women at low-risk for HIV acquisition. The present paper focuses on the data collected at the enrolment visit, i.e. baseline data of the study participants.

A total of 430 sexually active women were enrolled in 2010-2011 and followed up for eight months at the International Centre of Reproductive Health Kenya (ICRHK), Mombasa, Kenya; the Wits Reproductive Health and HIV Institute (Wits RHI), Johannesburg, South Africa and Rinda Ubuzima (RU), Kigali, Rwanda. The composition of the cohort was 400 HIV-negative women and 30 non-pregnant HIV-positive women (22-35 years), the latter recruited from public HIV treatment clinics in Kigali, Rwanda. The HIV-negative women included the following subgroups: 219 adult women (18-35 years) who were at a low risk for HIV acquisition, did not engage in traditional vaginal practices and were not pregnant, recruited in Kenya and South Africa henceforth referred to as *the reference group*; 60 pregnant women (18-40 years), recruited from local family planning clinics (Mombasa and Johannesburg); 60 non-pregnant adolescents (16-17 years), recruited from youth-friendly family planning services (Mombasa and Johannesburg); 31 non-pregnant women (19-33 years) engaging in traditional vaginal practices i.e. they used substances (cloth/lemon juice/detergents) other than water and/or fingers to clean, dry or tighten the vagina on a regular basis, recruited from health centres in inner-city Johannesburg and the surrounding communities; and 30 non-pregnant women (22-33 years) at high risk for HIV acquisition, recruited from the sex worker community in Kigali, Rwanda, using community mobilizers.

Written informed consent was sought at screening, eligibility assessed and women were tested for HIV infection according to national guidelines, reproductive tract infections (RTIs) (*Chlamydia trachomatis* (CT) by PCR, *Neisseria gonorrhoea* (NG) by PCR - BD Probetec SDA assay from Becton Dickinson and positive results confirmed with Amplicor CT/NG Roche, *Trichomonas vaginalis* (TV) by the InPouch TV culture system, candida by wet mount, HSV-2 and syphilis both in serum), urinary tract infection (UTI) and cervical dysplasia by Pap smear. Women who were eligible and gave consent were enrolled (visit 1) soon after the last day of their menstrual period (a maximum of two months after the screening visit). At this visit, women were interviewed about their sexual activity, vaginal hygiene practices, condom use and sexual partners. A physical and vaginal speculum examination was carried out. After this first visit, women were scheduled to visit the clinic on day 23 (visits 2 and 4) and on day 9 (visits 3, 5, 6 and 7) of their menstrual cycle.

Participants were eligible for inclusion in the study if they were: in good physical and mental health; able and willing to participate in the study as required by the protocol; able and willing

to give informed consent (and assent for minors) according to national guidelines, including parental consent for adolescents; HIV-negative at screening as confirmed by rapid HIV testing unless confirmed HIV-positive for inclusion in the 'HIV-positive women' group. Pregnant women were only included in the study in the 'pregnant women' group if they were ≤14 weeks pregnant as determined by abdominal ultrasound. HIV-positive women were included if they had been on cART for at least 6 months, were currently asymptomatic and had a CD4 count of more than 350 cells/µl.

The exclusion criteria for participation in the study included meeting one or more of the following criteria: history of hysterectomy and other genital tract surgery in the three months prior to the screening visit; never had penetrative vaginal intercourse; enrolled in HIV prevention trials involving investigational products; confirmed internal and/or external genital warts at screening and/or enrolment; currently breastfeeding and less than 6 months post-partum at the time of enrolment and pregnant (unless for inclusion in the 'pregnant women' group). Women with STIs were not excluded from the study.

5.3.2 Ethical approval

All women provided their written informed consent or assent for minors and consent of legal representatives. The study protocol was approved by the Ethical Review Committee, Kenyatta National Hospital, Kenya; the Human Research Ethics Committee (Medical), University of the Witwatersrand, South Africa; the Rwanda National Ethics Committee, Rwanda; the Institutional Review Board of the Institute of Tropical Medicine (ITM), Belgium; and the ethics committees of the Ghent University Hospital in Ghent and the Antwerp University Hospital in Antwerp, Belgium. In addition the study was approved by the National Council of Science and Technology, Kenya; and the National AIDS Control Commission, Rwanda.

5.3.3 Clinic visits and procedures

The rapid HIV antibody tests, wet mount microscopy for candidiasis, BV by Amsel criteria, a urine dipstick test for UTI and a urine hCG test for pregnancy were performed on site following local or national guidelines and their results made available immediately. A physical and pelvic exam was also carried out. HIV and RTI risk reduction counselling and testing were performed according to local standard operational procedures (SOPs) based on national guidelines and women were encouraged to bring their partner for couple counselling. Women newly diagnosed with HIV or found to be pregnant were referred for appropriate care in public clinics. Condoms were provided free-of-charge at this and all subsequent visits.

The results of the remaining diagnostic tests (CT, NG, TV, syphilis and HSV-2) were reviewed two weeks later (results visit) and the Pap smear results as soon as they were available. Women received treatment for RTIs/UTIs as needed, according to national guidelines. A re-assessment of eligibility was also performed.

A vaginal smear sample for BV-testing was also collected. The vaginal pH was determined by the study physician using colour-fixed indicator strips pH-Fix 3.6-6.1 (Macherey-Nagel GmbH & Co KG, Duren, Germany). Two high vaginal swab specimens were collected using flocked synthetic swabs (COPAN Innovation, Italy). The swab specimens were used for quantification of vaginal bacterial species by quantitative PCR (qPCR) and for prostate-specific antigen (PSA) testing. The swabs were stored in a cool box with ice at 2-8°C before transport to the laboratory where they were stored dry at -80°C until testing. For cervicovaginal lavage (CVL) samples, 10 ml normal saline at room temperature was flushed over the cervix and the lateral vaginal walls. This fluid was aspirated from the posterior fornix using the same pipette and collected in a 15 ml falcon tube that was then put in a cool box with ice (2-8°C) and immediately transported to the laboratory for processing. A mid-stream urine sample was collected for testing for UTIs and pregnancy as in the screening visit.

5.3.4 Laboratory procedures

5.3.4.1 Sample processing

CVL sample processing was started within a maximum of one hour after sample collection. CVLs were centrifuged at 1,000 x g for 10 minutes at 4°C and the supernatant (~9ml) was aliquoted into three fractions; two of approximately 4ml each and one of 1ml. The aliquots were stored at -80°C at each of the study sites. CVL samples and vaginal swabs were shipped in batches using a temperature-monitored dry shipper to the central laboratory at the ITM in Antwerp, Belgium where they were stored at -80°C before analysis for soluble markers of inflammation or use in the CVL antiviral assay.

DNA extraction from two vaginal swab specimens (per woman) was performed by thawing the swabs at room temperature for 30 minutes. After thawing, 1,200 µl of diluted phosphate buffered saline (PBS) (1 part PBS and 9 parts saline) at pH 7.4 was added to each swab and gently vortexed for 15 seconds. One milliliter of each swab suspension was pooled into a final volume of 2 ml. An aliquot of 250 µl was used for DNA extraction using the Abbott *m*24*sp* automated extraction platform (Abbott, Maidenhead, United Kingdom) according to the manufacturer's instructions. 200 µl of eluted DNA was stored at -80 °C until use in the qPCR assays.

5.3.4.2 Quantification of soluble immune mediators in CVLs

Concentrations of the cytokines Interleukin (IL)-1α, IL-1β, IL-6 and IL-12(p70); CC chemokine MIP-1β; CXC chemokines interferon (IFN)-γ-induced protein (IP-10) and IL-8; growth factors granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) in CVL samples were analysed at the Institute of Tropical Medicine (ITM) in Antwerp, Belgium using the Bio-Plex™ human cytokine assay kit (Bio-Rad Laboratories NV-SA, Nazareth, Belgium) as previously described (428). Elafin, SLPI, IL-1RA and total protein concentrations in CVL samples were measured at the Laboratory of Genital Tract Biology,

Brigham and Women's Hospital, Boston, MA, USA. Elafin and SLPI were quantified using ELISA kits from R&D Systems (Minneapolis, MN) following manufacturers' instructions.

Fluorescence data was collected using the Bio-Plex™ array reader and the Bio-Plex™ Manager 5.0 software used to calculate cytokine concentrations using a weighted five-parameter logistic curve-fitting method on the four-fold dilution series of the standard provided with the kit. The assay linearity ranges, low limit of detection (LLD) and inter-assay coefficient of variation (CV% = 100*SD/mean) based on a quality control sample assessment on each assay plate are provided in supplementary table 4.

Optical densities were read at 450 nm with a second reference filter of 570 nm using a Victor2 multi-label reader and WorkOut Software (PerkinElmer, Waltham, MA). Anti-inflammatory cytokine IL-1RA was measured using the Meso Scale Discovery (MSD) multiplex platform and Sector Imager 2400 (MSD, Gaithersburg, MD). The MSD Discovery Workbench Software was used to convert relative luminescent units into protein concentrations (pg/ml) using interpolation from several log calibrator curves. Total protein measurement in CVL samples was done by a bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL) using the Victor 2 counter. Samples were first screened at one dilution (1:2,500 for elafin, 1:500 for SLPI, and 1:50 for IL-1RA) followed by a repeated measurement with dilution adjusted to fit the assay linearity. Samples were tested undiluted for the total protein BCA assay. Samples with initial values below or above the assay detection ranges for all these analytes were repeatedly tested at lower or higher dilutions to obtain accurate protein measurements. The assay linearity ranges, LLD and inter-assay CV based on a quality control CVL pool assessment on each plate are provided in supplementary table 4. The CV% (mean ±SD) assessed for duplicate measurements of all study samples was 18.2 ± 10.8% for elafin, 17.6 ± 11.6% for SLPI, and 8.4 ± 5.0% for IL-1RA.

5.3.4.3 Characterization of vaginal microbiota

Total *Lactobacillus* species, *L. crispatus, L. iners, L. jensenii*, *L. gasseri* and *L. vaginalis;* the BV related species *A. vaginae, G. vaginalis* and *P. bivia;* and *Escherichia coli* were measured and expressed as bacterial counts/ml using qPCR. Vaginal Gram-stained smears were scored at the ITM using the Nugent method (Nugent score 7-10: positive for BV; 4-6: intermediate; 0-3: normal microbiota) (346).

For total *Lactobacillus* species, *L. crispatus, L. iners, L. jensenii*, *L. gasseri,* and *L. vaginalis*, the 25 µl PCR mixture contained 12.5 µl Rotor-Gene SYBR Green RT-PCR Master mix (Rotor-Gene SYBR Green PCR Kit, Qiagen, Venlo, the Netherlands), 5 µl DNA extract, 0.5-1.0 µM of their respective primers (Integrated DNA Technologies, Leuven, Belgium), and RNase-Free Water provided with the Rotor-Gene SYBR Green PCR kit. The amplification reactions were performed using the Rotor Gene Q MDx 5 plex (Qiagen, Venlo, the Netherlands). The qPCR reactions for *A. vaginae, G. vaginalis, P. bivia* and *Escherichia coli* were performed in a final volume of 10 µl, containing 5 µl of LightCycler 480® SYBR Green I Master (Roche Applied

Science, Basel, Switzerland), 0.2-1.25 µM of their respective primers (Eurogentec, Liège, Belgium) and 2 µl of DNA extract. Amplification was carried out using the LightCycler480® platform and the LightCycler® 480 Software Version 1.5 (Roche, Basel, Switzerland).

For each of the organisms, standard curves were constructed. A total of 6 standards were prepared by a tenfold dilution of the DNA stock in HPLC grade water. The DNA of the lactobacilli were extracted from cultures of *L. crispatus* LMG 9479^T , *L. gasseri* LMG 9203^T, *L. iners* LMG 18914^T, *L. jensenii* LMG 6414^T and *L. vaginalis* LMG 12891^T grown at 35°C ±2°C on Columbia agar base (BBL, BBL, Becton Dickinson, Erembodegem, Belgium) + 5% horse blood under anaerobic conditions (Anaerocult A, Merck, VWR International, Leuven). The DNA was extracted from cultures of *A. vaginae* CCUG 38953^T, *G. vaginalis* ATCC14018^T, *E. coli* ACM1803^T grown on TSA + 5% sheep blood (Becton Dickinson, Erembodegem, Belgium) and *P. bivia* ATCC29303^T grown on Columbia agar (Becton Dickinson, Erembodegem, Belgium) at 37°C ±2°C under anaerobic conditions (BugBox, LedTechno, Heusden-Zolder, Belgium). After extraction, the DNA concentrations were determined using NanoDrop (Thermo Fisher scientific, Erembodegem, Belgium). The genomic concentrations were calculated using the described genomic sizes of the type strains. Both the standard curve and samples were run in duplicate. The number of bacteria was expressed as bacteria/ml.

5.3.4.4 CVL antiviral assay

A TZM-bl indicator cell assay was used to determine the intrinsic antiviral activity of CVL. The optimal, non-toxic CVL dilution utilized in this experiment was determined by the WST-1 proliferation assay described in sub-section 5.3.4.5.

To determine the antiviral activity of cervicovaginal fluid, 100 µl of CVL (at a 1:4 dilution) was pre-incubated for one hour (37°C/7% CO_2) with 10^4 TZM-bl cells in 50 µl medium supplemented with 30 µg/ml Diethylaminoethyl (DEAE)-dextran per well in a 96-well flat-bottomed tissue culture plate. TZM-bl cells (also called JC53BL-13) are a HeLa cell line that were genetically manipulated to express CD4 and CCR5 and contain integrated reporter genes for firefly luciferase and *E. coli* β-galactosidase under control of an HIV-1 LTR. Once TZM-bl cells are infected, viral Tat induces luciferase (Luc) reporter gene expression that can be quantified by luminescence. Subsequently, 50 µl virus (subtype B HIV-1_{BaL} or subtype C HIV-1_{VI829}) at 200 tissue culture infective dose $(TCID)_{50}$ was added to each well for a final CVL dilution of 1:8 (a dilution previously determined to be non-toxic to TZM-bl cells). After 48 hours (37°C/7% CO_2), 125 µl of the culture supernatant was removed, 75 µl of Steadylite HTS (Perkin-Elmer, Life Sciences, Zaventem, Belgium) added and luciferase activity measured using a TriStar LB941 luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Antiviral activity was expressed as a percentage of growth in the positive control wells containing cells, virus and control buffer (normal saline with 200 µg/ml bovine serum albumin). All conditions were tested in triplicate. We also compared the concentrations of the soluble immune markers in the CVLs of these BV-negative versus BV-positive women.

5.3.4.5 Water-soluble tetrazolium-1 (WST-1) proliferation assay

The potential toxicity of CVL samples and the control buffer was tested using the WST-1 proliferation assay (Roche Diagnostics GmbH, Germany) as previously described (429). TZM-bl cells (10^4 cells/well) were plated in a 96-well flat-bottomed plate and CVL or control buffer at the same dilution as in the CVL antiviral assay was added. Cell proliferation reagent was added after 48 hours and cell viability measured compared with untreated control cultures.

5.3.5 Data analysis

Data analysis was performed using SAS 9.4 and R 3.0.1 according to a data analysis plan which was prepared prior to data analysis.

To ensure comparability across plates, we assigned to values below (above) the quantification limit, half (twice) of the lowest (highest) average accepted value of the standard series across plates. Women with missing data on Nugent scores and/or immune mediators were excluded from relevant analyses. All soluble marker data were log-transformed before analysis. In addition to the individual soluble marker concentrations, we analysed the IL-1RA/IL-1(α+β) ratio. Distributions of soluble immune markers were examined overall and by group.

Comparisons among different study groups with respect to mean levels of soluble markers were performed using t-tests. In this analysis, pregnant, adolescent and women engaging in vaginal practices were each compared to the reference groups from their respective countries. Women in the sex workers and HIV-positive groups from Rwanda were compared to the combined reference groups from both Kenya and South Africa because we did not have a reference group recruited in Rwanda.

We performed a principal component (PC) analysis (430) to describe the associations among the soluble markers. SLPI was excluded from the PC analysis because it was only measured in a sub-section of the women for budgetary reasons. Three main PCs each accounted for at least 10% of the variability and were therefore further analyzed to identify important associations with candidate determinants to be selected for further regression analysis.

Bivariate associations between the *a priori* selected candidate determinants and the three main PCs as well as each of the proteins and the IL-1RA/IL-1(α+β) ratio were determined using simple linear regression analysis (supplementary table 1). All determinants that were significantly ($p \leq 0.05$) associated with the three main PCs were then assessed in a multiple linear regression model. The model was simplified using stepwise deletion to determine the main factors associated with relevant soluble markers in the FGT. The final multiple regression model (table 2) retained all independent, influential variables associated with any of the main principal components and was subsequently applied to each of the soluble markers, the PC scores and the IL-1RA/IL-1(α+β) ratio. If two determinants were strongly correlated (e.g. PSA and recent vaginal sex), the most influential variable was retained in the model. This final model was used to describe the effects of each of the determinants, while correcting for

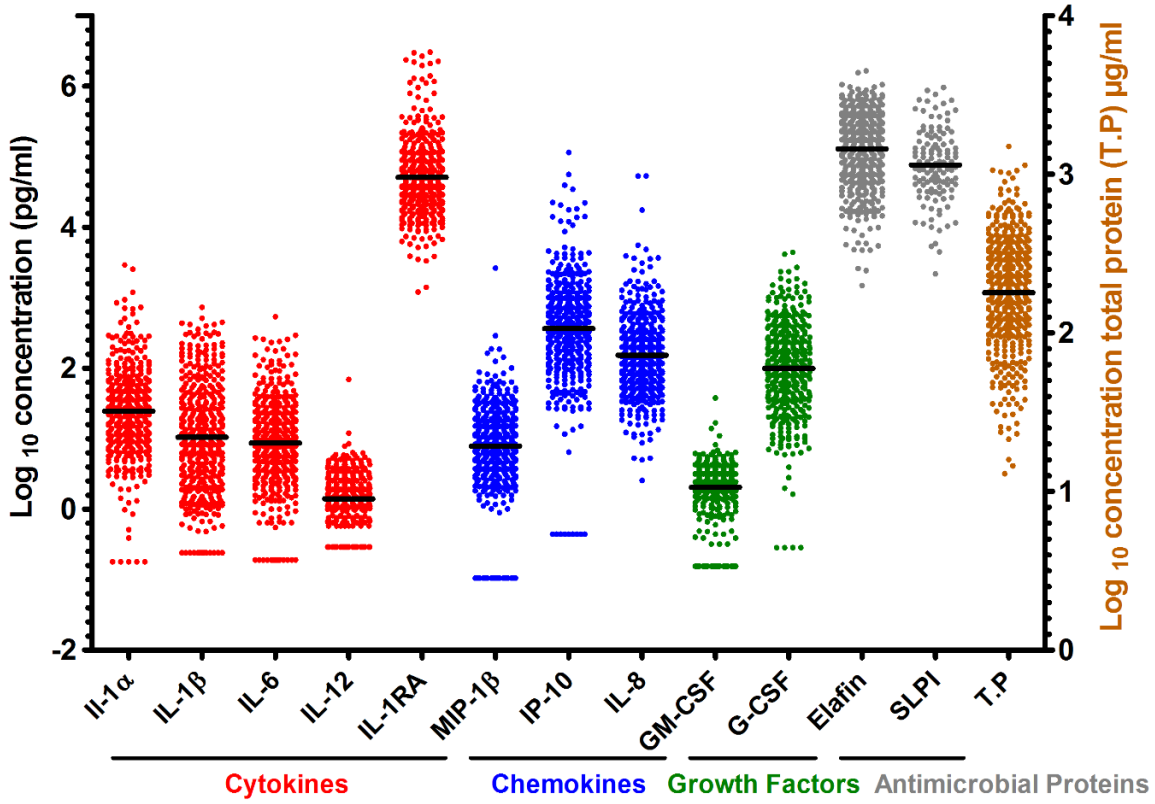
possible confounders. RTIs were not included in the final analysis mainly because they were assessed (and treated) at a different time point (screening visit) while the soluble markers were assessed at baseline (enrolment visit). Associations between the presence/absence of vaginal microbiota species and mean soluble marker concentrations were assessed using t-tests. Concentrations of soluble markers of inflammation in samples used for the CVL antiviral activity tests were analysed using the Wilcoxon rank-sum test.

5.4 Results

The median age of the study groups ranged from 25-26 years except for the adolescents who had a median age of 16 years (by design) and the HIV-positive women who had a median age of 30 years. In all groups, except the sex workers, the majority (>80%) of women reported having a single sex partner in the three months preceding the first study visit. The average baseline prevalence of BV by Nugent score was 33% in the reference groups and it was similar in pregnant women and adolescents (both 30% on average), 37% in women engaging in vaginal practices, 68% in sex workers and 48% in HIV-positive women. The complete sociodemographic, behavioural and clinical characteristics of the study population by group are published elsewhere (431).

5.4.1 Differences of immune mediators between the study groups

The distribution of the all cytokines, chemokines, growth factors and antimicrobial proteins varied widely among women (supplementary figure 1). Some significant differences between the groups were noted when analysis was restricted to women without BV (highlighted in bold in table 1). IL-1α for pregnant women in South Africa and IL-6 and MIP-1β for the vaginal practices group were elevated compared to their respective reference groups. Pregnant women from Kenya had significantly lower levels of elafin compared to their reference group. Adolescents from South Africa had significantly lower levels of IL-12 and MIP-1β. HIV-positive women had a distinct immunological profile with half of the immune mediators measured (IL-1β, IL-6, IL-12, IL-1RA, MIP-1β and IL-8) significantly higher than those in the reference group.



Supplementary figure 1. Distribution of the concentrations of soluble immunological markers in cervicovaginal lavage samples collected on visit 1 from women enrolled in the EDCTP Vaginal Biomarkers Study. Quantification of the soluble markers was done using the Bio-Plex™ multiplex assay except for IL-1RA, elafin and SLPI that were quantified using single ELISAs and total protein using the BCA assay. Each data point represents an individual sample from a single woman with the median concentration represented by the black line across the data points. IL: interleukin, IL-1RA: IL-1 receptor antagonist, MIP-1β: macrophage inflammatory protein 1β, IP-10: interferon gamma-induced protein 10, GM-CSF: granulocyte-macrophage colony-stimulating factor, G-CSF: granulocyte colony-stimulating factor, SLPI: secretory leukocyte protease-inhibitor, T.P: total protein

Table 1. Log $_{10}$ concentrations and analysis of expression of soluble immunological markers between study groups and sites

	Reference groups		**Pregnant women**		**Adolescents**		**Vaginal practicesπ**	**Sex workers**	**HIV-positive women**
	Kenya N=110 mean (SD)	South Africa N=109 mean (SD)	Kenya N=30 mean (SD)	South Africa N=30 mean (SD)	Kenya N=30 mean (SD)	South Africa N=30 mean (SD)	South Africa N=31 mean (SD)	Rwanda N=30 mean (SD)	Rwanda N=30 mean (SD)
Cytokines (pg/ml)									
IL-1α	1.21 (0.60)	1.43 (0.54)	1.48 (0.81)*	***1.76 (0.69) *****	1.22 (0.58)	1.35 (0.55)	1.44 (0.60)	1.48 (0.62)	1.46 (0.63)
IL-1β	0.94 (0.82)	1.00 (0.71)	0.92 (0.70)	1.09 (0.65)	0.83 (0.65)	0.87 (0.75)	1.32 (0.79)*	1.16 (0.81)	***1.31 (0.73)****
IL-6	0.83 (0.72)	0.93 (0.57)	0.86 (0.54)	0.88 (0.49)	0.90 (0.52)	0.78 (0.64)	***1.24 (0.57)****	1.09 (0.67)	***1.18 (0.52)****
IL-12 (p70)	0.06 (0.40)	0.22 (0.30)	0.04 (0.36)	0.09 (0.36)	-0.04 (0.38)	***-0.01 (0.36)****¥**	0.33 (0.33)	0.26 (0.34)	***0.38 (0.25) *****
IL-1RA	4.59 (0.62)	4.74 (0.43)	4.70 (0.68)	4.94 (0.50)*	4.48 (0.50)	4.58 (0.41)	4.79 (0.51)	4.83 (0.39)	***4.90 (0.49)****
Chemokines (pg/ml)									
MIP-1β	0.88 (0.75)	0.91 (0.45)	0.69 (0.68)	0.78 (0.51)	0.65 (0.63)	***0.63 (0.45)****¥**	***1.18 (0.42)****	1.18 (0.53)*	***1.16 (0.42) ****
IP-10	2.38 (0.71)	2.69 (0.64)	2.333 (0.69)	2.86 (0.52)	2.47 (0.70)	2.37 (0.70)*¥	2.85 (0.62)	2.65 (0.72)	2.81 (0.97)
IL-8	2.15 (0.67)	2.19 (0.46)	2.10 (0.58)	2.21 (0.45)	2.01 (0.48)	1.93 (0.54) **¥	2.34 (0.58)	2.40 (0.73)*	***2.42 (0.42) ****
Growth factors (pg/ml)									
GM-CSF	0.29 (0.46)	0.31 (0.46)	0.29 (0.37)	0.44 (0.22)	0.20 (0.43)	0.32 (0.22)	0.45 (0.30)	0.32 (0.23)	0.15 (0.48)
G-CSF	1.94 (0.70)	2.01 (0.62)	1.91 (0.59)	1.93 (0.61)	1.94 (0.52)	1.90 (0.55)	2.29 (0.54)*	1.91 (0.77)	2.21 (0.59)
Antimicrobial proteins (pg/ml)									
Elafin	5.06 (0.48)	5.19 (0.54)	***4.79 (0.59)****¥**	5.21 (0.49)	5.08 (0.41)	5.03 (0.66)	5.31 (0.37)	5.12 (0.48)	5.13 (0.63)
SLPI†	4.79 (0.61)	4.92 (0.44)	4.83 (0.65)	5.13 (0.64)	4.89 (0.49)	5.10 (0.24)	4.85 (0.45)	4.86 (0.26)	ND
Total protein (µg/ml)									
	8.09 (0.40)	8.38 (0.27)	8.12 (0.28)	8.44 (0.29)	8.09 (0.33)	8.28 (0.31)	8.29 (0.33)	8.31 (0.31)	***8.41 (0.35)****

The numbers within the tables are log $_{10}$ values of concentrations of soluble immunological markers measured in pg/ml or µg/ml as detailed in the first column.
Pregnant women, adolescents and women engaging in vaginal practices (other than just vaginal washing) are compared to the reference groups in their respective countries. Sex workers and HIV-positive women from Rwanda are compared to the combined reference groups from Kenya and South Africa.
*: p≤0.050, **: p≤0.010, ***: p≤0.001. Associations in bold remain positive when analysed in BV-negative women (Nugent score 0-3) (see supplementary table 3).
π Women who used substances (cloth/lemon juice/detergents) other than water and/or fingers to clean, dry or tighten the vagina on a regular basis
¥ Negative association. All other associations are positive
† Only measured on a subset of 119 women (38, 35, 6, 9, 12, 3, 11, 5, 0 in each group respectively).
‡ IL-1RA, elafin, and total protein not measured for one woman
ND Not determined

5.4.2 BV, ectopy, cervical mucus, vaginal discharge and vaginal washing are associated with pro-inflammatory immune mediators

Women with BV were shown to have higher levels of the pro-inflammatory cytokines IL-1α, IL-1β, IL-6, IL-12 and IL-8 compared to BV-negative women (table 2). Conversely IP-10, IL-1RA/IL-1(α+β) ratio and total protein were lower in women with BV. A lower IL-1RA/IL-1(α+β) ratio is reflective of reduced attenuation of the inflammatory response caused by IL-1α and IL-1β. The presence of cervical mucus on speculum exam was associated with an increase in pro-inflammatory IL-6, MIP-1β and G-CSF but a decrease in anti-inflammatory IL-1RA. The presence of cervical ectopy was strongly associated with increased IL-1β, IL-6, IL-12, MIP-1β, IL-8, G-CSF and also total protein levels. Abnormal vaginal discharge on speculum exam was associated with increased IL-1β, IP-10, IL-8, G-CSF and total protein. Even though detection of PSA was associated with an increase in IL-1α and IL-12 in the bivariate analysis (supplementary table 1), these associations were not significant in the final regression model. An increase in the number of sexual partners was associated with increased IL-1α and IL-6. There was a trend towards an increase in IL-1α, IL-1β and total protein in women with a history of vaginal washing during bathing but the levels of these markers were decreased when the washing was reported to be recent. Reproductive tract infections (at screening) were positively associated with IL-1RA and total protein (supplementary table 1a) but these associations were not significant in the multiple regression analyses.

Table 2. Multiple regression analysis of determinants of soluble immunological markers

	Cervical mucus	**Cervical ectopy**	**Cervical epithelial abnormalities**	**Abnormal vaginal discharge**	**PSA**	**No. of sex partners**	**Vaginal washing**	**Bacterial vaginosis**
IL-1α				x (+)		* (+)	* (a)	*** (+)
IL-1β		*** (+)		** (+)			** (a)	*** (+)
IL-6	* (+)	*** (+)		x (+)		**(+)		** (+)
IL-12		*** (+)	x (+)					*** (+)
IL-1RA	* (-)	x (+)					x (-)	
MIP-1β	* (+)	*** (+)						
IP-10				*** (+)		x (+)		*** (-)
IL-8		*** (+)		** (+)		x (+)		** (+)
GM-CSF								
G-CSF	* (+)	*** (+)	x (+)	** (+)				x (+)
Elafin		x (-)						
SLPI								x (-)
Total protein		* (+)		* (+)			* (a)	** (-)
IL1RA/IL1(α+β)								*** (-)

x: p≤0.100, *: p≤0.050, **: p≤0.010, ***: p≤0.001.
(-): negative association, (+): positive association, (a): mixed (negative & positive) associations
PSA: prostrate-specific antigen
Final determinants selected based on multiple regression analysis on the principal components
Determinant categories: Cervical mucus on speculum examination (Absent/Present), Ectopy (No/yes), Cervical epithelial abnormalities (No/Yes), Abnormal vaginal discharge on speculum examination (No/Yes), PSA (No/Yes), Number of sex partners last 3 months (0/1/>1), Vaginal washing when bathing (No/Yes, but not recent/Yes, recent), BV by Nugent score (BV-negative, Intermediate, BV-positive)
This final multiple regression model also corrects for the study group because of the differences in soluble marker concentrations observed between the groups.

Supplementary table 1a. Statistical significance of association between soluble immune mediator concentrations over all possible determinants.

	IL-1α	IL-1β	IL-6	IL-12	IL-1RA	MIP-1β	IP-10	IL-8	GM-CSF	G-CSF	Elafin	SLPI	Total Protein
Site (Kenya, South Africa, Rwanda)	**	*	**	***	***	***	***	**			**		***
Group (6 groups)	*	*	**	***	**	***		***		*			
Group & site (9 study groups)	**	*	**	***	***	***	***	**			**		***
Age				*** (+)		*** (+)		** (+)					
Lifetime no. of sexual partners	** (+)	* (+)	* (+)	** (+)		** (+)	** (+)	** (+)		** (+)	*** (+)	* (+)	* (+)
No. of sexual partners within 3 months	** (+)				** (+)	* (+)		** (+)					
Parity		*** (+)	*** (+)	*** (+)		*** (+)		*** (+)		** (+)			
Contraception						*				***			
Vaginal discharge		*** (+)	* (+)				* (+)	*** (+)		*** (+)	* (-)		
Vaginal epithelial abnormalities													
Cervical epithelial abnormalities		** (+)	** (+)	*** (+)	* (+)	*** (+)		** (+)		*** (+)			** (+)
Colposcopic findings				** (+)		* (+)		* (+)					
Ectopy		*** (+)	*** (+)	*** (+)	**(+)	*** (+)	** (+)	*** (+)		*** (+)			*** (+)
Reproductive tract infections					* (+)								* (+)
HSV-2								* (+)			* (+)		
Recent vaginal sex											* (-)		* (-)
Prostate-specific antigen	* (+)			* (+)									
Vaginal washing	*	*		*	*	*	**				**		***
Product used to wash/clean/dry/tighten vagina	*										**		
BV by Nugent score	*** (+)	*** (+)	* (+)	*** (+)			*** (-)	** (+)					** (-)
Vaginal pH		*** (+)		*** (+)			*** (-)	* (+)	** (-)		*** (-)		*** (-)
Cervical mucus on speculum examination		* (+)	** (+)		* (-)	*** (+)		* (+)		*** (+)			
Cervical mucus colour	* (-)	* (+)	*** (+)		** (-)	*** (+)		* (+)		** (+)	* (-)		* (-)

Note: *: p≤0.050, **: p≤0.010, ***: p≤0.001. Association indicated when clear: (-): negative association, (+): positive association.
Group (6 groups): Reference groups, pregnant women, adolescents, vaginal practices, sex workers and HIV-positive women
Group & site (9 study groups): Reference groups (Kenya & South Africa), pregnant women (Kenya & South Africa), adolescents (Kenya & South Africa), vaginal practices, sex workers and HIV-positive women
Reproductive tract infections: *Chlamydia trachomatis* (CT), *Neisseria gonorrhoea* (NG) *Trichomonas vaginalis* (TV), HSV-2 and syphilis
HSV-2: Herpes simplex virus 2
BV categories: BV-negative (Nugent score 0-3), Intermediate (Nugent score 4-6) and BV-positive (Nugent score 7-10)
Vaginal pH: 3.6, 4.1, 4.4, 4.7, 5.0, 5.3, 5.6 and 6.1
Cervical mucus colour: none (no mucus mucus visible at os), clear, other colour

Supplementary table 1b. Statistical significance of association between soluble immune mediator concentrations over all possible determinants.

	IL-1RA / IL1(α+β)	PCS 1	PCS 2	PCS 3
Site (Kenya, South Africa, Rwanda)		***	**	
Group (6 groups)		***		
Group & Site (9 study groups)		***	**	
Age		** (+)		
Lifetime no. of sexual partners		*** (+)		
No. of sexual partners within 3 months		* (+)	* (+)	
Parity		*** (+)	*** (+)	
Contraception				
Vaginal discharge	* (-)	** (+)		
Vaginal epithelial abnormalities				
Cervical epithelial abnormalities		*** (+)		
Colposcopic findings		* (+)		
Ectopy		*** (+)	*** (+)	
Reproductive tract infections			***	
HSV-2				
Recent vaginal sex			* (+)	
Prostate-specific antigen				*** (+)
Vagina washing		*		*** (-)
Product used to wash/clean/dry/tighten vagina				***
BV by Nugent score	*** (-)	*** (+)		*** (+)
Vaginal pH	* (-)		*** (+)	*** (+)
Cervical mucus on speculum examination		* (+)	*** (+)	
Cervical mucus colour		* (+)	*** (+)	

Note: *: p≤0.050, **: p≤0.010, ***: p≤0.001. Association indicated when clear: (-): negative association, (+): positive association.

Group (6 groups): Reference groups, pregnant women, adolescents, vaginal practices, sex workers and HIV-positive women

Group & site (9 study groups): Reference groups (Kenya & South Africa), pregnant women (Kenya & South Africa), adolescents (Kenya & South Africa), vaginal practices, sex workers and HIV-positive women

PCS: principal component score

PC 1 had positive loadings for all soluble markers. This component can therefore be considered as equivalent to the total proportion of soluble markers in the sample and it explained 42% of the data variability. PC 2 contrasted the amounts of IL-6 and MIP-1β (positive loadings) with those of IL-1α, IL-1RA, IP-10 and elafin (negative loadings). This component explained 14% of the data variability. PC 3 contrasted the amounts of IL-1α, IL-1β, and IL-1RA (positive loadings) with those of IP-10, GM-CSF, and elafin (negative loadings). This component explained 10% of the variability in the data.

Reproductive tract infections: *Chlamydia trachomatis* (CT), *Neisseria gonorrhoea* (NG) *Trichomonas vaginalis* (TV), HSV-2 and syphilis

HSV-2: Herpes simplex virus 2

BV categories: BV-negative (Nugent score 0-3), Intermediate (Nugent score 4-6) and BV-positive (Nugent score 7-10)

Vaginal pH: 3.6, 4.1, 4.4, 4.7, 5.0, 5.3, 5.6 and 6.1.

Cervical mucus colour: none (no mucus mucus visible at os), clear, other colour

5.4.3 Unique associations of individual bacterial species with soluble immune mediators.

The presence of *L. crispatus* and *L. vaginalis* but not the other lactobacilli species was associated with lower levels of pro-inflammatory cytokines while the BV-associated bacteria *A. vaginae*, *G. vaginalis* and *P. bivia,* and *E. coli*, clearly skewed the pro-inflammatory balance upwards (Table 3). Of note, the IFN-γ-induced chemokine IP-10 was significantly increased in the presence of all the *Lactobacillus* species except *L. gasseri*, but was significantly lower in the CVL of women who had *A. vaginae* and *G. vaginalis* present in their vaginal swab samples. The presence of *L. gasseri* was also associated with increased IL-1RA, IL-12 and GM-CSF, and the presence of *L. jensenii* associated with increased GM-CSF concentrations. The concentrations of the protective antimicrobial proteins SLPI and elafin were elevated in women with specific *Lactobacillus* species; *L. vaginalis* and *L. jensenii* for SLPI and *L. iners* for elafin.

Table 3. Analysis of associations between soluble immunological markers in cervicovaginal lavage and the presence of vaginal bacterial species in vaginal swab samples

Bacterial species	Lower levels	Higher levels
Lactobacilli		
Lactobacillus crispatus	IL-8* IL-1α** IL-1β** GM-CSF* IL-12***	IP-10*
Lactobacillus vaginalis	IL-1α* MIP-1β* IL-12** IL-1β***	SLPI*IP-10***
Lactobacillus iners		IP-10* Elafin*
Lactobacillus jensenii		GM-CSF* SLPI** IP-10***
Lactobacillus gasseri		IL-1RA* IL-12*GM-CSF**
BV-associated bacteria		
Gardnerella vaginalis	IP-10***	IL-8* IL-12** IL-1α*** IL-1β***
Atopobium vaginae	IP-10***	IL-8* IL-12** IL-1α*** IL-1β***
Prevotella bivia		IL-1β** IL-8**
Other		
Escherichia coli		IL-1RA* GM-CSF* IL-12** IL-12*** IL-6*** IL-8*** G-CSF*** IP-10*** MIP-1β***

*: p≤0.050, **: p≤0.010, ***: p≤0.001.
BV: bacterial vaginosis

5.4.4 Anti-HIV activity of CVL *in vitro* and associations with presence or absence of BV

BV has previously been associated with an increased risk of HIV acquisition. We hypothesized that CVLs from women with BV would have decreased anti-HIV activity compared with CVLs from women without BV. To test this hypothesis, we selected 15 women with a Nugent score of 0 (BV-negative) and 14 women with a Nugent score of 7-10 (BV-positive) and compared the CVL antiviral activity of these two groups. Contrary to our expectations, the CVL-antiviral activity against HIV_{BaL} was slightly but significantly greater in the BV-positive women compared to the BV-negative women and the same trend was observed with HIV_{VI829} but the difference was not statistically significant (figure 1A). CVLs from BV-positive women had significantly higher levels of IL-1α, IL-1β, IL-6 and IL-8 but lower levels of IP-10 and SLPI (figure 1B)

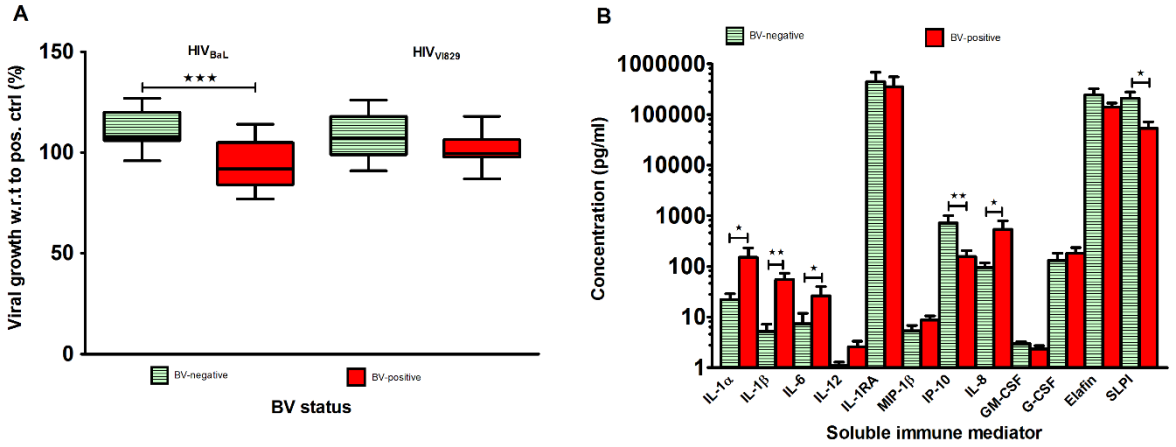


Figure 1 Cervicovaginal lavage (CVL) antiviral activity and corresponding soluble marker concentrations of selected CVL samples from women with or without bacterial vaginosis (BV). **A.** Percentage infection of TZM-bl cells by HIV_{BaL} (subtype B) and HIV_{VI829} (subtype C) in the presence of CVL at a 1:8 final dilution. Indicated viral growth concentration is compared to control wells with 1:8 final dilution of the same control buffer (normal saline) used for CVL collection. The box plots depict the median (bold line), 25th and 75th percentiles (box) and range (whiskers). All CVL samples were assayed in triplicate. ***: p≤0.001 **B.** Concentrations of soluble immune mediators in corresponding aliquots of CVLs used in CVL antiviral assay. *: p≤0.050, **: p≤0.010

For this experiement, we purposively selected BV-negative women with high lactobacilli counts and BV-positive women with low lactobacilli counts. The BV-negative women had high counts of *L. crispatus* and *L. vaginalis*, as well as lower levels of other lactobacilli, but no or low counts of *G. vaginalis*, *A. vaginae*, and *Prevotella* spp. The BV-positive women had high counts of *G. vaginalis*, *A. vaginae*, and *Prevotella* spp., high counts of *L. iners* (higher than the BV-negative women), but no or low counts of all other lactobacilli. *E. coli* counts were similar in both groups (supplementary table 2).

Supplementary table 2. pH, Nugent scores and bacterial counts at visit 1 for women used in CVL antiviral activity assay

Participant	pH	Nugent Score	Total lactobacilli	*L. crispatus*	*L. iners*	*L. jensenii*	*L. gasseri*	*L. vaginalis*	*P. bivia*	*G. vaginalis*	*A. vaginae*	*E. coli*
BV-Negative												
1006-R	4.1	0	16383840	22575520	355200	174560	0	10	49	0	0	0
1053-R	4.4	0	18779360	22568320	0	10	0	0	10	0	0	0
1050-R	4.1	0	308705280	343613600	0	3390240	841120	64960	227	25640	0	20480
1064-R	3.6	0	2702560	3606240	0	0	0	0	0	0	0	0
1071-R	3.6	0	197018400	269372640	447840	0	0	153600	315	0	0	66320
2038-R	5	0	1864051840	1173857920	0	0	0	1833440	656	0	0	32000
2117-R	4.7	0	209579520	239378560	193600	1738080	0	243520	343	0	0	0
2186-A	4.4	0	1542049600	1776754720	0	0	0	10	370	0	0	81320
2131-A	3.6	0	800430240	665880000	767680	1036000	10	265600	1612	0	0	186400
2174-R	3.6	0	2644447200	3538097280	152572480	90839040	0	2853600	2460	0	0	573200
2181-R	4.4	0	596383520	707406560	0	0	10	516640	0	0	0	87200
2187-R	4.7	0	111943520	18580480	217630560	66206720	0	1252320	655	0	0	82080
2093-R	4.7	0	577958720	656584480	120637920	0	0	0	10	0	0	0
2174-R	3.6	0	523155680	587784160	10	4876640	0	10	0	0	0	10
2156-R	4.7	0	226582080	163470400	686699040	0	10	450880	314	0	0	48040
BV-positive												
1049-R	5.3	7	39149120	0	163921920	0	0	0	182	9220000	162400000	53120
1167-R	5.3	8	1351360	0	5878880	0	0	0	93	868000	116800000	80960
1226-A	4.7	8	1502880	0	4887520	0	0	0	0	3784000	80800000	83080
1262-P	4.1	8	945280	0	2531520	0	0	0	41640	184800	22240000	0
1241-R	5	9	46783680	0	210989120	0	0	0	586	1968000	53400000	49600
3005-N	5	8	12671680	0	47630080	0	0	0	35840000	59000000	24080000000	0
2050-R	5	8	15869920	0	30863840	0	0	0	180400	8140000	916000000	0
2096-R	5	8	10	0	10	0	0	0	473600	1880000	440400000	95600
2062-A	5	10	7381120	0	32663200	0	0	0	413200	27400000	1312000000	112000
2062-A	5	10	791360	0	2280160	0	0	0	221200	2468000	240400000	44480

2116-R	6	8	3871200	0	15234720	0	0	0	561	23320000	1264000000	104000
2123-R	4,7	8	24632000	0	144631360	0	0	0	1184000	18360000	467600000	71520
2116-R	4,7	8	2440320	0	32929920	0	0	0	2268	23480000	1880000000	291600
2232-A	4,4	7	235892320	0	1576829280	0	0	10020640	7284	35840000	36560000	972000

5.5 Discussion

We extensively characterized the ranges of vaginal soluble immune mediators and their physiological and behavioural determinants in various groups of women from three SSA countries. We also described the associations between vaginal microbiota and soluble markers of inflammation in the vaginal tract with notable lower levels of IP-10 in genital fluids in the context of BV and lower levels of pro-inflammatory markers with specific *Lactobacillus* species. Lastly, our study demonstrated how the *in vitro* anti-HIV activity of cervicovaginal fluid varies depending on the BV-status and the bacterial load of specific species of these women.

Pregnant women (up to 14 weeks gestational age) in Kenya were found to have increased levels of the pro-inflammatory cytokine IL-1α and for the South African women, the anti-inflammatory protein IL-1RA, when compared to the reference group of low-risk HIV negative women. A recent study of Ugandan and Zimbabwean women found higher cervical levels of IL-1β, IL-6, IL-8, VEGF, SLPI and IL-1RA and lower IL-1RA:IL-1β in pregnant versus non-pregnant HIV negative women (432). Kenyan pregnant women in our study showed lower levels of elafin. Elafin has been shown to be expressed in the genital tract of pregnant women (433) and its anti-HIV activity was previously described (223, 434) making it a probable correlate of immunity against HIV infection. In another relatively small study of predominantly White US women (435), IL-1α and IL-1RA were relatively higher in the CVLs of pregnant women with a mean gestational age of 23.5 weeks. Elafin levels were also lower in the CVLs of pregnant women in this study but only when controlled for total protein. A different study (436) among predominantly White US women near-term (35-37 weeks gestational age) also observed higher levels of IL-1α, IL-1β, IL-8 and IL-1RA but lower levels of human beta defensin 2 in pregnant versus non-pregnant women. These studies, performed in geographically spread populations, seem to point towards a state of elevated inflammatory cytokines and decreased antimicrobial/anti-inflammatory protein levels in the FGT in pregnancy, which may partially explain the increased risk of HIV acquisition during pregnancy (437). Clearly then, pregnant women differ from their non-pregnant peers with regard to cervicovaginal immune mediators, but these differences may vary by country or cohort, limiting their extrapolation.

Young women in South Africa have been shown to have a high HIV incidence (424); they may be highly susceptible to HIV infection from both a biological and a behavioural perspective. The observation of lower levels of IL-12 and MIP-1β in adolescents compared to non-pregnant adults in South Africa may therefore seem counter-intuitive. However, it may indicate an immature state of the immune barrier. The lack of higher levels of IL-8 and IL-1β, which are considered the hallmark of vaginal inflammation (384), in these adolescents suggests that the increased risk of HIV infection in South African adolescents might mostly be due to behavioural factors as described elsewhere (438, 439)

Martin-Hilber and colleagues (440) describe vaginal practices as tools used by girls and women to navigate their reproductive life and marital roles. Some of these practices may impact

directly on HIV susceptibility (441). Certain over-the-counter feminine hygiene products also negatively affect vaginal microbiota resulting in increased IL-8 production (442). In our study, women in South Africa who reported using substances other than water to clean, dry or tighten their vagina were found to have higher levels of IL-6 and MIP-1β in the sub-analyses in BV-negative women. Our results on the effect of washing the vagina during bathing suggest that, in the long term, it may result in an inflamed FGT. Vaginal washing has previously been associated with a lower likelihood of *Lactobacillus* isolation from a cohort of HIV-1 seronegative female sex workers from Kenya (443). Our study therefore supports the hypothesis that specific vaginal practices can be potential risk factors for HIV acquisition (444) probably as a result of mucosal inflammation. Of importance is the fact that when women reported having recently washed their vagina, there was a corresponding decrease in the amounts of cytokines. Vaginal washing before sample collection may therefore interfere with the measurement of immunological markers.

HIV-positive women were found to have a generally more inflamed vaginal profile compared to HIV-negative women even after controlling for BV (supplementary table 3). These data agree with previous studies, showing increased soluble pro-inflammatory markers in the genital tracts of HIV-infected individuals (112, 445). It is important to note that the women in our study were on a successful cART regime which means that treatment suppressed viral load but did not fully suppress inflammation in the FGT.

Increased cervical mucus and abnormal vaginal discharge on speculum examination could both be clinical signs of infection in the FGT. As study participants were not re-tested during visit 1, we cannot definitively attribute these signs to infection. Their association with pro-inflammatory cytokines however means that they may increase susceptibility to HIV. Cervical ectopy is characterized by an extension of the columnar epithelium typically found in the endocervix to cover a proportion of the ectocervix that is normally lined by multi-layered squamous epithelial cells (446). The strong association between the presence of cervical ectopy and increased CVL concentrations of IL-1β, IL-6, MIP-1β, IL-8, IL-12 (p-70) and G-CSF in this African population agrees perfectly with our previous observations in a cohort of healthy Caucasian women (428) and that of others (400). It may be explained by previous *in vitro* observations that columnar epithelial cells of endocervical origin produce higher levels of pro-inflammatory mediators than isogenic stratified squamous epithelial cells of ectocervical or vaginal origin (401, 447, 448). An enhanced pro-inflammatory environment in the lower FGT could potentially increase susceptibility to HIV acquisition and may account for reported associations (399, 449) of cervical ectopy with HIV infection.

BV by Nugent score in our study was associated with a significant increase in a number of pro-inflammatory cytokines/chemokines (IL-1α, IL-1β, IL-6, IL-12 and IL-8) extending the findings of other studies (417, 447, 450). Further, in a study on HIV-1 infected female sex workers in Kenya, treatment and elimination of BV by Nugent score was associated with reduced genital tract levels of IL-1β, IL-8 and RANTES (451). The association of a lower IL-1RA/IL-1(α+β) ratio

with BV is indicative of excessive production of IL-1α and IL-1β in the vaginal mucosa (452). Clearly, epithelial and immune cells of the FGT mount an innate immune response after stimulation with bacterial products from the diverse bacteria (reviewed in (453)) that are characteristic of BV. This sub-clinical FGT inflammation may partially explain the increased risk of HIV acquisition associated with BV.

To our knowledge, only one previous Swedish study correlated immunological markers with vaginal microbiota characterized to the individual species level using molecular methods (454) but this has not been investigated in women from SSA. The presence of lactobacilli (specifically *L. crispatus* and *L. vaginalis*) was associated with lower levels of pro-inflammatory cytokines in our study. This is consistent with previous observations about the non-inflammatory nature of specific *Lactobacillus* species (418, 455). These species were also associated with higher levels of the protective antimicrobial proteins SLPI and elafin in our study. SLPI is constitutively expressed in the FGT but it is also induced by NF-kβ activation and reduced in association with BV signs in adolescent African American women (456).

BV is not associated with overt clinical inflammatory symptoms. Our analysis of associations between vaginal microbiota and soluble markers of immunity revealed an interesting result that we think may hint at one of the possible explanations for this dampened inflammation; the presence of BV-associated *G. vaginalis* and *A. vaginae* were both strongly associated with lower levels of IP-10. This chemokine (also known as CXCL10) is one of the ligands for the CXCR3 receptor (457), it is induced by Type II IFN-γ, Type I IFNs-α/β but also TNF-α (458, 459) and functions as a chemoattractant for different immune cells to the site of infection or inflammation. Suppression of IP-10 production or function by pathogenic bacteria has been described before (460-462) though, to our knowledge, not in clinical studies in the context of BV. Fichorova and colleagues recently reported that although IP-10 expression was upregulated by FGT pathogens *P. bivia* and *T. vaginalis* individually, their combination completely suppressed IP-10 expression in culture (463). Even though the presence of *G. vaginalis* and *A. vaginae* were individually associated with lower levels of IP-10 in our study, taken alone these two pathogens did not suppress IP-10 *in vitro* (463). *In vivo* IP-10 suppression may therefore be due to an association of these bacteria with one or a combination of many other bacteria in the FGT of women with BV. It is interesting though that previous reports exist on sialidase and prolidase activity in the genital fluids of some women with BV, which is correlated with degradation of anti-vaginolysin immunoglobulin A (IgA) and low IL-8 immune response (417, 464). The degradation of anti-vaginolysin IgA is achieved by removal of sialic acids from the secretory IgA molecules that makes them more sensitive to proteolytic cleavage (465). Taken together, these results hint at the existence of processes through which BV-associated pathogens influence certain innate and adaptive immune responses. Further research is needed to explore interactions between microbial virulence factors in BV and host immune mediators, and their implications on BV pathogenicity.

CVL anti-HIV activity has been previously reported in both HIV+ and HIV- women and this activity attributed mainly to the cationic fraction of CVL including correlation with antimicrobial peptides (232, 466). Though surprising to us, the observation of modest but significantly higher anti-HIV_{BaL} activity in CVLs from BV-positive women is similar to previous results in slightly different contexts (467, 468). In our study, increased anti-HIV activity in CVLs from BV-positive women cannot be attributed to the two peptides with known anti-HIV properties (elafin and SLPI) as the concentrations of these were lower than in the CVLs from healthy women. It is possible that other antimicrobial proteins that we did not quantify in our CVL samples were responsible for the observed anti-HIV_{BaL} activity especially those, like HBD2, reported to be increased in genital fluids in the context of BV (331, 469). HBD2 correlated positively with *in vitro* HIV inhibition in the study by Keller *et al* but also in a different study with CVL from HIV positive women where MIP-3α was also implicated in HIV-inhibition (232, 470). Caution should always be applied in the interpretation of these results as most vaginal lavage samples constitute a significant dilution of the proteins found *in vivo* and physiological concentrations needed for activity *in vivo* might not be reached *in vitro*. What is clear from our study is that there might be an unidentified substance(s) in BV-positive CVL that influences its ability to inhibit HIV-infection of TZM-bl cells *in vitro*. Differences seen with what was expected in the context of natural BV-HIV dynamics also highlight the limitation of assays using cell lines as opposed to *in vivo* whole-organ responses with possibilities of susceptible cell recruitment and infection. These experiments with BV-positive CVL should be repeated with a larger sample size than what we used in our study so as to confirm their validity and possible implications.

In this study, a high variation in concentrations of the different soluble markers in CVL samples was observed. While multiplex technologies are useful where there is a limitation in sample volumes, natural variation in soluble marker concentrations in FGT samples limit the combination of markers that can be assayed in a single multiplex assay as was observed with IL-1RA in our assays. This limitation can be overcome by grouping high or low-concentration markers together in pre-mixed multiplex panels, by optimizing the multiplex immune assays to fit broad linearity ranges of multiple markers or by using single-target immunoassays where multiple aliquots of individual samples can be diluted appropriately for accurate quantification of high-concentration proteins.

In conclusion, we identified IP-10 suppression as a potential mechanism of immune evasion by BV-associated bacteria and described the non-inflammatory nature of *L. crispatus* and *L. vaginalis.* Future prevention interventions targeting these factors could influence HIV susceptibility in African women.

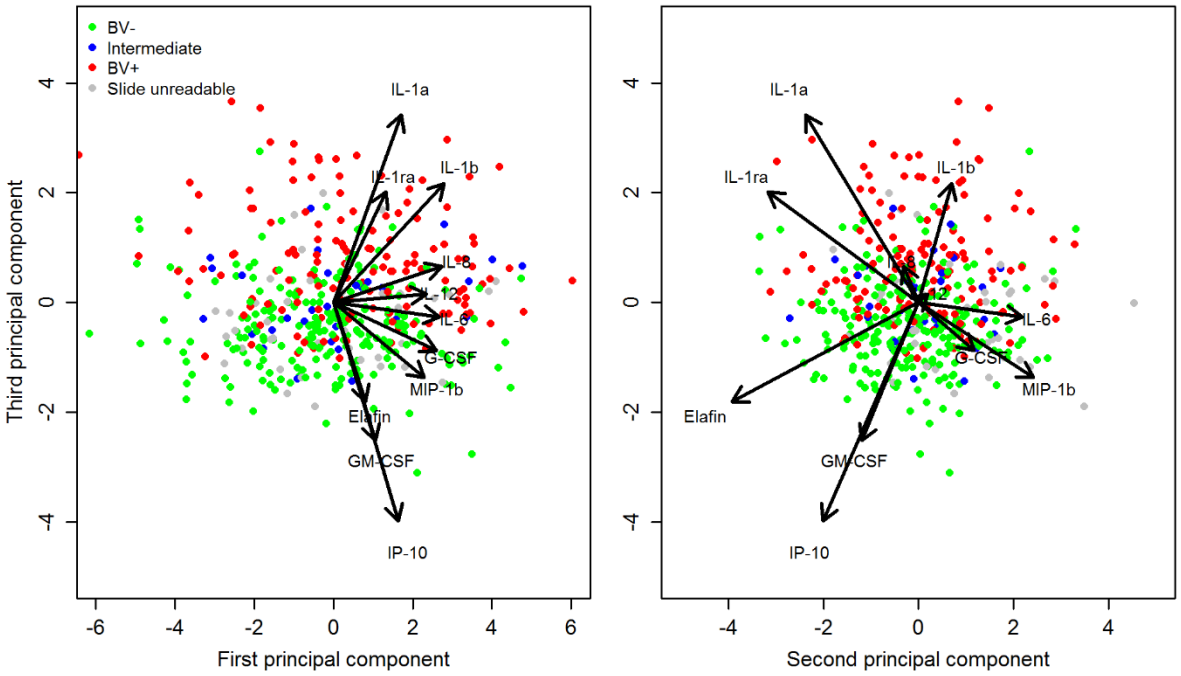
5.9 Supplementary Results

Distribution of soluble markers of inflammation

Nine proteins were quantified using the Bio-Plex™ multiplex assay and for these, 1% (IL-1α), 3% (IL-1β), 3% (IL-6), 14% (IL-12), 4% (MIP-1β), 2% (IP-10), 7% (GM-CSF) and 1% (G-CSF) of the samples assayed were below the lower limit of detection (LLD) for undiluted CVL. For IL-8, 0.5% of the samples were above the upper limit of detection (ULD) of the multiplex immunoassay. IL-1RA was originally included in the Bio-Plex multiplex panel but 70% of the undiluted CVL samples had concentrations above the ULD and the samples were re-analyzed using a single spot Meso Scale Discovery electrochemiluminescence assay with appropriate sample dilutions. The same sample dilution approach was used for the quantification of the antimicrobial proteins elafin and SLPI and total protein in the CVL samples. The range in concentrations of all proteins measured varied widely between women (supplementary figure 1).

Principal component analysis

The results of the principal component analysis on the log10-transformed soluble markers data are summarized in supplementary figure 2. The first three principal components each described at least 10% of the variability of the data and together accounted for 66% of the data variability (supplementary table 5). These three components were selected for further analysis. Scatter plots of the relationships of the z-scores (mean 0, SD 1) of these principal components are shown in supplementary figure 2. The first principal component had positive loadings for all soluble markers. This component can therefore be considered as equivalent to the total proportion of soluble markers in the sample and it explained 42% of the data variability. The second principal component contrasted the amounts of IL-6 and MIP-1β (positive loadings) with those of IL-1α, IL-1RA, IP-10 and elafin (negative loadings). This component explained 14% of the data variability. The third principal component contrasted the amounts of IL-1α, IL-1β, and IL-1RA (positive loadings) with those of IP-10, GM-CSF, and elafin (negative loadings). This component explained 10% of the variability in the data.



Supplementary figure 2. Scatter plot of the first 3 principal components from the principal component analysis of the soluble immunological markers quantified in cervicovaginal lavage samples collected during visit 1 from all women enrolled in the EDCTP Vaginal Biomarkers Study. The colors of the individual dots show the bacterial vaginosis (BV) status based on the Nugent score for each corresponding vaginal swab sample. The arrows show the factor loadings of the soluble markers on the three principal components that cumulatively explain 66% of the variability of the data.

Supplementary table 3. Log $_{10}$ concentrations and analysis of expression of soluble immunological markers between study groups and sites in BV-negative women (Nugent score 0-3)

	Reference groups		**Pregnant women**		**Adolescents**		**Vaginal Practicesπ**	**Sex workers**	**HIV-positive women**
	Kenya N=55 mean (SD)	South Africa N=63 mean (SD)	Kenya N=19 mean (SD)	South Africa N=18 mean (SD)	Kenya N=18‡ mean (SD)	South Africa N=18 mean (SD)	South Africa N=16 mean (SD)	Rwanda N=6 mean (SD)	Rwanda N=13 mean (SD)
Cytokines (pg/ml)									
IL-1α	1.02 (0.54)	1.31 (0.49)	1.23 (0.66)	1.59 (0.45)*	1.21 (0.63)	1.21 (0.38)	1.32 (0.47)	1.33 (0.57)	1.43 (0.46)
IL-1β	0.60 (0.66)	0.79 (0.58)	0.67 (0.62)	0.96 (0.62)	0.80 (0.74)	0.54 (0.72)	1.08 (0.70)	0.64 (0.52)	1.30 (0.77)**
IL-6	0.73 (0.72)	0.84 (0.54)	0.86 (0.55)	0.91 (0.52)	0.91 (0.56)	0.53 (0.69)	1.18 (0.62) *	0.64 (0.46)	1.26 (0.51)*
IL-12 (p70)	-0.01 (0.37)	0.18 (0.33)	-0.05 (0.40)	0.02 (0.33)	-0.10 (0.43)	-0.15 (0.33)**¥	0.20 (0.35)	-0.09 (0.42)	0.35 (0.27)*
IL-1RA	4.58 (0.70)	4.72 (0.42)	4.54 (0.73)	4.86 (0.49)	4.58 (0.60)	4.58 (0.37)	4.74 (0.42)	4.92 (0.36)	4.98 (0.51)*
Chemokines (pg/ml)									
MIP-1β	0.79 (0.68)	0.90 (0.39)	0.57 (0.77)	0.84 (0.39)	0.75 (0.51)	0.55 (0.51)**¥	1.22 (0.46)**	0.86 (0.32)	1.32 (0.45)**
IP-10	2.46 (0.56)	2.86 (0.49)	2.56 (0.70)	2.98 (0.53)	2.54 (0.78)	2.80 (0.37)	3.10 (0.58)	2.90 (0.66)	3.43 (0.85)***
IL-8	2.03 (0.51)	2.14 (0.43)	1.95 (0.54)	2.17 (0.40)	2.07 (0.50)	1.94 (0.56)	2.20 (0.48)	2.04 (0.33)	2.47 (0.41)**
Growth factors (pg/ml)									
GM-CSF	0.31 (0.45)	0.40 (0.39)	0.25 (0.43)	0.45 (0.22)	0.23 (0.35)	0.35 (0.25)	0.35 (0.27)	0.36 (0.21)	0.14 (0.55)
G-CSF	1.84 (0.78)	1.99 (0.64)	1.92 (0.63)	1.91 (0.65)	1.94 (0.58)	1.83 (0.45)	2.30 (0.55)	1.49 (1.11)	2.52 (0.47)**
Antimicrobial proteins (pg/ml)									
Elafin	5.10 (0.46)	5.23 (0.54)	4.82 (0.60)*¥	5.15 (0.45)	5.13 (0.43)	5.46 (0.40)	5.33 (0.39)	5.12 (0.44)	5.47 (0.33)*
SLPI	4.80 (0.58)	4.95 (0.45)	4.99 (0.58)	5.25 (0.59)	4.89 (0.49)	5.10 (0.24)	4.88 (0.50)	4.91 (0.28)	ND
Total protein (µg/ml)									
	8.11 (0.36)	8.40 (0.23)	8.12 (0.27)	8.47 (0.25)	8.20 (0.33)	8.42 (0.21)	8.37 (0.30)	8.31 (0.29)	8.62 (0.20)***

Pregnant women, adolescents and women engaging in vaginal practices (other than just vaginal washing) are compared to the reference groups in their respective countries. Sex workers and HIV-positive women from Rwanda are compared to the combined reference groups from Kenya and South Africa.

Pregnant women, adolescents and women engaging in vaginal practices are compared to non-pregnant women in their respective countries. Sex workers and HIV-positive women from Rwanda are compared to the combined reference groups from Kenya and South Africa.

π Women who used substances (cloth/lemon juice/detergents) other than water and/or fingers to clean, dry or tighten the vagina on a regular basis

*: p≤0.050, **: p≤0.010, ***: p≤0.001.

¥ Negative association. All other associations are positive

† Only measured on a subset of 103 women (34, 31, 5, 7, 12, 3, 7, 4, 0 respectively).

‡ IL-1RA, Elafin, and total protein not measured for one woman

Supplementary table 4. Soluble immune mediators assay linearity ranges, low limit of detection and inter-assay coefficient of variation based on a quality control sample assessment

Analyte	Assay Platform	Manufacturer	Assay Calibrator Range	Low Limit of Detection (LLD): formula	LLD: average ± SD	Inter-assay Coefficient of Variation %*
IL-1α	BioPlex	BioRad Labs NV-SA, Belgium	23,534-0.36 pg/ml	¥	1.0 ± 0.6 (pg/ml)	8.2
IL-1β	BioPlex	BioRad Labs NV-SA, Belgium	31,130-0.48 pg/ml	¥	0.5 ± 0.0 (pg/ml)	7.4
IL-6	BioPlex	BioRad Labs NV-SA, Belgium	25,160-0.38 pg/ml	¥	0.9 ± 0.6 (pg/ml)	8.6
IL-8	BioPlex	BioRad Labs NV-SA, Belgium	26,657-0.41 pg/ml	¥	1.6 ± 1.7 (pg/ml)	15.4
IL-12 (p70)	BioPlex	BioRad Labs NV-SA, Belgium	37,884-0.58 pg/ml	¥	0.6 ± 0.1 (pg/ml)	9.6
GM-CSF	BioPlex	BioRad Labs NV-SA, Belgium	20,314-0.31 pg/ml	¥	1.2 ± 1.1 (pg/ml)	7.1
G-CSF	BioPlex	BioRad Labs NV-SA, Belgium	37,569-0.57 pg/ml	¥	4.4 ± 3.3 (pg/ml)	13.6
MIP-1β	BioPlex	BioRad Labs NV-SA, Belgium	13,960-0.21 pg/ml	¥	1.8 ± 1.5 (pg/ml)	8.9
IP-10	BioPlex	BioRad Labs NV-SA, Belgium	57,400-0.88 pg/ml	¥	17.0 ± 15.3 (pg/ml)	19.6
Elafin	Duoset ELISA	R&D Systems, USA	4,000-15.6 pg/ml	(blank mean + 2 SD)*lowest sample dilution	5.3 ± 7.2 (pg/ml)	34.0
SLPI	Quantikine ELISA	R&D Systems, USA	4,000-15.6 pg/ml	(blank mean + 2.5 SD)*lowest sample dilution	6.1 ± 14.8 (pg/ml)	24.8
IL-1RA	Meso Scale Small Spot	Meso Scale Discovery, USA	200 -0.003 ng/ml	(blank mean + 2.5 SD)*lowest sample dilution	0.1 ± 0.3 (ng/ml)	19.9
Total Protein	BCA assay	Thermo Scientific, USA	2,000-31.25 µg/ml	(blank mean + 3 SD)*lowest sample dilution	66 ± 25 (pg/ml)	6.3

¥ : lowest calibrator concentration on each plate with acceptable recovery percentage range (70-130%)
*(CV% = 100*SD/mean) Based on duplicate measurements of a CVL quality control pool diluted to fit approximately the middle of the assay range for each analyte. We generally considered inter-assay CV < 30% acceptable.

Supplementary table 5. Summary of principal component analysis on the log-transformed soluble markers data; loadings

	Comp. 1	Comp. 2	Comp. 3	Comp. 4	Comp. 5	Comp. 6	Comp. 7	Comp. 8	Comp. 9	Comp. 10	Comp. 11
IL-1α	0.244	-0.336	0.485	0.146	0.407	0.220	-0.113	0.281	0.483		-0.174
IL-1β	0.399	0.102	0.310		0.166			-0.133	-0.238	0.192	0.770
IL-6	0.381	0.313			0.104	-0.102	0.326			0.687	-0.388
IL-12	0.338				-0.488	-0.241	-0.415	0.627	-0.141		
IL-1RA	0.191	-0.453	0.283		-0.602		0.485	-0.255			
MIP-1β	0.326	0.344	-0.196		-0.187	-0.102	-0.265	-0.389	0.661	-0.164	
IP-10	0.233	-0.285	-0.568	-0.175		0.643		0.170		0.126	0.190
IL-8	0.389					0.300	-0.327	-0.425	-0.477	-0.218	-0.429
GM-CSF	0.152	-0.174	-0.377	0.855	0.153	-0.197					
G-CSF	0.370	0.168	-0.121	-0.196	0.244	-0.171	0.493	0.240		-0.620	
Elafin	0.115	-0.560	-0.242	-0.410	0.264	-0.544	-0.208	-0.154		0.122	
P.o.V	0.42	0.14	0.10	0.08	0.07	0.05	0.05	0.04	0.03	0.02	0.01
C.P.o.V	0.42	0.56	0.66	0.74	0.81	0.86	0.90	0.94	0.97	0.99	1.00

Comp.: Component
P.o.V: Proportion of variance
C.P.o.V: Cumulative proportion of variance
The cut-off for significance of the loadings was arbitrarily set at ±0.2

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Conflict of interest

The authors declare no conflict of interest

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6 A longitudinal analysis of the vaginal microbiota and vaginal immune mediators in women from sub-Saharan Africa.

Title: A longitudinal analysis of the vaginal microbiota and vaginal immune mediators in women from sub-Saharan Africa

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6.1 Abstract

In cross-sectional studies increased vaginal bacterial diversity has been associated with vaginal inflammation which can be detrimental for health. We describe longitudinal changes at 5 visits over 8 weeks in vaginal microbiota and immune mediators in African women. Women (N=40) with a normal Nugent score at all visits had a stable lactobacilli dominated microbiota with prevailing *Lactobacillus iners*. Presence of prostate-specific antigen (proxy for recent sex) and being amenorrhoeic (due to progestin-injectable use), but not recent vaginal cleansing, were significantly associated with microbiota diversity and inflammation (controlled for menstrual cycle and other confounders). Women (N=40) with incident bacterial vaginosis (Nugent 7-10) had significantly lower concentrations of lactobacilli and higher concentrations of *Gardnerella vaginalis*, *Atopobium vaginae*, and *Prevotella bivia*, at the incident visit and when concentrations of proinflammatory cytokines (IL-1β, IL-12p70) were increased and IP-10 and elafin were decreased. A higher 'composite-qPCR vaginal-health-score' was directly associated with decreased concentrations of proinflammatory cytokines (IL-1α, IL-8, IL-12(p70)) and increased IP-10. This longitudinal study confirms the inflammatory nature of vaginal dysbiosis and its association with recent vaginal sex and progestin-injectable use. A potential role for proinflammatory mediators and IP-10 in combination with the vaginal-health-score as predictive biomarkers for vaginal dysbiosis merits further investigation.

6.2 Introduction

The vaginal mucosal surface is colonised by a variety of bacterial species and the composition, which has implications for reproductive health, is influenced by both endogenous and exogenous factors (reviewed in (340)). Using culture-dependent and molecular amplification techniques (such as quantitative polymerase chain reaction (qPCR) and next generation sequencing), a 'normal' vaginal microbiota (VMB) has been defined as one dominated by lactic acid-producing *Lactobacillus* species. The clinical condition bacterial vaginosis (BV) is associated with increased diversity and quantity of bacteria and a concomitant decrease in lactobacilli (471). Molecular studies have shown some lactobacilli (notably *L. crispatus*) are more associated with health than others (*L. iners*) because they are associated with a lower risk of developing vaginal dysbiosis (472) or acquiring sexually transmitted infections (STIs) (21).

BV is the most common vaginal dysbiosis (340) and has been associated with adverse clinical outcomes including pre-term birth and miscarriage (19), pelvic inflammatory disease (20), and the acquisition and transmission of STIs including HIV (137, 427, 473, 474). It has been suggested that such adverse outcomes are directly associated with inflammatory or immune activation cascades triggered by vaginal dysbiosis (360). For example, inflammation triggered by vaginal dysbiosis probably attracts CD4 + cells to the cervicovaginal mucosa, thereby increasing the availability of target cells for HIV at the site of viral entry into the body (475).

Sub-Saharan Africa has the highest prevalence of BV (22) but diagnosis is often missed because symptoms are frequently absent or nonspecific, and the microscopic methods necessary for diagnosis by Amsel criteria or Nugent (346) scoring are typically not available. Cross-sectional studies using molecular techniques have confirmed the high prevalence of vaginal dysbiosis in sub-Saharan Africa and have also shown that *L. iners* dominated VMB are more frequent than those dominated by *L. crispatus* (18, 476). Sub-Saharan African women may therefore be less protected from vaginal dysbiosis, even when they have a lactobacilli-dominated VMB.

Information on fluctuations in the VMB over time is limited, but studies (394, 477) have shown the variation within an individual over time is more pronounced than the variation between individuals (478), and that such changes are influenced by the menstrual cycle and can occur rapidly (477, 479).

We conducted a longitudinal cohort study (the Vaginal Biomarkers Study) in 430 women at three sites in Kenya, Rwanda and South Africa (431). The study visits were tightly scheduled to control for the menstrual cycle. We selected 40 women with consistently normal VMB (defined as a Nugent score of 0-3) at five study visits over eight weeks and 40 women who developed BV (Nugent score of 7-10) during the same eight-week period. The primary objective of this sub-study was to describe the vaginal bacterial species and concentrations of vaginal immune mediators in these cohorts over time. Secondary objectives included the determination of host correlates of vaginal bacteria and immune mediators, any associations

between them and those which occurred around the time of incident BV diagnosis. This is the first longitudinal study to describe the composition of the VMB and vaginal immune mediators in quantitative terms over time as well as any associations between them whilst controlling for menstrual cycle and other factors known to be associated with changes in the vaginal micro-environment.

6.3 Results

The cross-sectional characteristics of all 430 women enrolled in the Vaginal Biomarkers Study, including the composition of the VMB, have been previously described (18, 431, 476, 480). In this sub-study, the median age of the 40 women with a consistently normal VMB (reference group) and the 40 women who developed incident BV (incident BV group) was 23 and 24 years respectively (ranges 16-34 years and 16-33 years). The median age at first vaginal intercourse was 17 years for both groups. Half of the women with a consistently normal VMB (53%) and 43% of the women who developed BV had had two or three lifetime sex partners with most (78% and 88%, respectively) having had one sex partner in the last three months. Over fifty percent of the women in both groups had delivered a child at least once. Most women in both groups (80%) currently used contraception: 36% used progestin injections, 13% used combined hormonal pills, 5% were sterilised, and 26% used condoms only. All women in the reference group tested negative for pregnancy, HIV, syphilis, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and *Trichomonas vaginalis*, by design. The baseline herpes simplex virus type 2 (HSV-2) prevalence was 30% in the women with a consistently normal VMB and 33% in the women who developed BV.

Table 1 describes participant characteristics by group over the five study visits for those parameters that were subsequently included in mixed effects regression models as potential confounders of the main associations of interest between VMB bacteria and vaginal immune mediators (see methods). The reference group included HIV-negative adult women (N=16 Kenya, N=16 South Africa), adolescents (N=6 Kenya), and HIV-negative sex workers (N=2 Rwanda). The incident BV group included HIV-negative adult women (N=16 Kenya, N=11 South Africa), adolescents (N=5 Kenya, N=2 South Africa), pregnant women (N=1 Kenya, N=3 South Africa); and HIV-negative sex workers (N=2 Rwanda). The detection of prostate-specific antigen (PSA) in the vagina as a marker of vaginal sex in the last 24-48 hours (481, 482) and self-reported vaginal cleansing in the evening or morning just prior to the study visit were both common (25-57% and 28-53% at different visits, respectively). Clinician-observed abnormal vaginal discharge and cervical mucus were also common, but cervical epithelial findings visible by the naked eye (abrasion, laceration, ecchymosis, petechiae, erythema, or ulcer) were uncommon (occurring in 1-8 women at each visit), throughout the study (Table 1). At all visits, a substantial proportion of women (up to 33% of the women with a consistently normal VMB and up to 68% of the women who developed BV) had a vaginal pH above 4.5, which is considered outside the normal range and is one of the Amsel criteria for the diagnosis of BV (345).

Table 1. The prevalence of participant characteristics at each study visit

Characteristics of women with a normal VMB throughout	**Visit 1 (n=40)**	**Visit 2 (n=40)**	**Visit 3 (n=40)**	**Visit 4 (n=40)**	**Visit 5 (n=40)**
Vaginal PSA present	11 (27.5)	11 (27.5)	10* (25.0)	11 (27.5)	16 (40.0)
Vaginal cleansing during bathing this morning or last night	16 (40.0)	12 (30.0)	11 (27.5)	11 (27.5)	11 (27.5)
Clinician-observed abnormal vaginal discharge	7 (17.5)	10 (25.0)	10 (25.0)	10 (25.0)	16 (40.0)
Clinician-observed cervical mucus present	13 (32.5)	11* (28.2)	15 (37.5)	17 (42.5)	16 (40)
Clinician-observed cervical epithelial abnormalities present†	6 (15.0)	4 (10.0)	4 (10.0)	4 (10.0)	8 (20.0)
Petechiae	5 (12.5)	3 (7.5)	2 (5.0)	3 (7.5)	3 (7.5)
Erythema	1 (2.5)	0	1 (2.5)	1 (2.5)	4 (10.0)
Ecchymosis	0	1 (2.5)	1 (2.5)	0	0
Ulcer	0	0	0	0	1 (2.5)
Vaginal pH <4	8 (20.0)	9 (22.5)	13 (33.3)	11 (27.5)	9 (22.5)
4-4.5	19 (47.5)	20 (50.0)	17 (43.6)	16 (40.0)	22 (55.0)
4.6-5	10 (25.0)	9 (22.5)	6 (15.4)	11 (27.5)	7 (17.5)
>5	3 (7.5)	2 (5.0)	3 (7.5)	2 (5.0)	2 (5.0)
Characteristics of women with incident BV	**Visit 1 (n=40)**	**Visit 2 (n=40)**	**Visit 3 (n=40)**	**Visit 4 (n=40)**	**Visit 5 (n=40)**
Vaginal PSA present	12 (30.8)	18 (48.7)	21 (56.8)	15 (40.5)	14 (43.8)
Vaginal cleansing during bathing this morning or last night	21 (52.5)	15 (38.5)	14 (35.0)	16 (41.0)	13 (33.3)
Clinician-observed abnormal vaginal discharge	5 (20.5)	6 (15.4)*	13 (32.5)	11 (28.2)*	11 (29.0)**
Clinician-observed cervical mucus present	14 (35.0)	10 (25.6)*	10 (25.0)	12 (30.8)*	14 (36.8)**
Clinician-observed cervical epithelial abnormalities present‖	5 (12.5)	3 (7.7)*	2 (5.0)	3 (7.7)*	3 (7.7)*
Petechiae	3 (7.5)	0	1 (2.5)	0	1 (2.6)
Ecchymosis	1 (2.5)	1 (2.6)	0	0	0
Erythema	0	1 (2.6)	0	1 (2.6)	2 (5.1)
Laceration	1 (2.5)	1 (2.6)	0	1 (2.6)	0
Ulcer	0	0	1 (2.5)	0	0
Abrasion	0	0	0	1 (2.6)	0
Vaginal pH <4	4 (10.0)	4 (10.5)	3 (7.5)	1 (1.6)	3 (7.9)
4-4.5	22 (55.0)	16 (42.1)	10 (25.0)	16 (41.0)	10 (26.3)
4.6-5	12 (30.0)	8 (21.1)	13 (32.5)	16 (41.0)	16 (42.1)
>5	2 (5.0)	10 (26.3)	14 (35.0)	6 (15.4)	9 (23.7)

Abbreviations: PSA=prostate specific antigen; VMB=vaginal microbiota. Data are number of women with the characteristic (% of total number of women). Cervical mucus presence includes mild, moderate or abundant mucus. * One missing value. ** Two missing values. † 26 events in 13 participants. ‖ 16 events in ten participants.

6.3.1 VMB bacteria and Candida over time (reference group)

The presence of individual VMB bacteria and *Candida albicans* was determined by qPCR and expressed as $\log_{10}$ genome equivalents (geq) per millilitre (ml). Over the five visits, presence was classified as: never present (0% of visits); sporadically present (1-25% of visits); regularly present (26-74% of visits) and consistently present (75%-100% of visits). The presence of individual *Lactobacillus* species was relatively stable over the five visits in the reference group; i.e. either consistently or never present (Figures 1 and 2). This was particularly true of *L. crispatus*, which was consistently present in 47% of women or never present in 53% of women (Figure 2). In 79% of the women with consistent *L. crispatus*, this was accompanied by a consistent or regular presence of *L. vaginalis* (Figure 1). *L. iners* was consistently present in 75% of women and regularly present in another 10% of women. *L. iners* and *L. crispatus* did occur together at least twice in 35% of women, but women with high concentrations of *L. crispatus* had lower concentrations of *L. iners* and vice versa (Figure 1). *C. albicans*, *L. jensenii* and *L. gasseri* were never present in 60%, 63% and 75% of the women, respectively. *Escherichia coli* was present (but always in a lower concentration than the lactobacilli) at least once in 90% of women, *Prevotella bivia* in 91% of women, *Gardnerella vaginalis* in 58% of women, and *Atopobium vaginae* in only 17% of women.

Figure 1: Presence/absence and concentration of vaginal microbiota bacteria over the eight week study period in women with a Nugent score of 0-3 throughout. Each box depicts one visit for a particular woman. The shading of the box indicates the concentration (in $\log_{10}$ geq/ml) of each taxon with darker colours depicting a higher concentration. If the taxon was absent, the box is white.

Figure 2: Frequency of vaginal microbiota presence over the eight week study period in women with a Nugent score of 0-3 throughout. Data in Y-axis are % of women. Sporadically present: present at 25% or fewer visits; regularly present: present at 26 - 74% of visits; consistently present: present at 75% or more visits.

Figure 1 Presence/absence and concentration of vaginal microbiota bacteria over the eight week study period in women with a Nugent score of 0-3 throughout

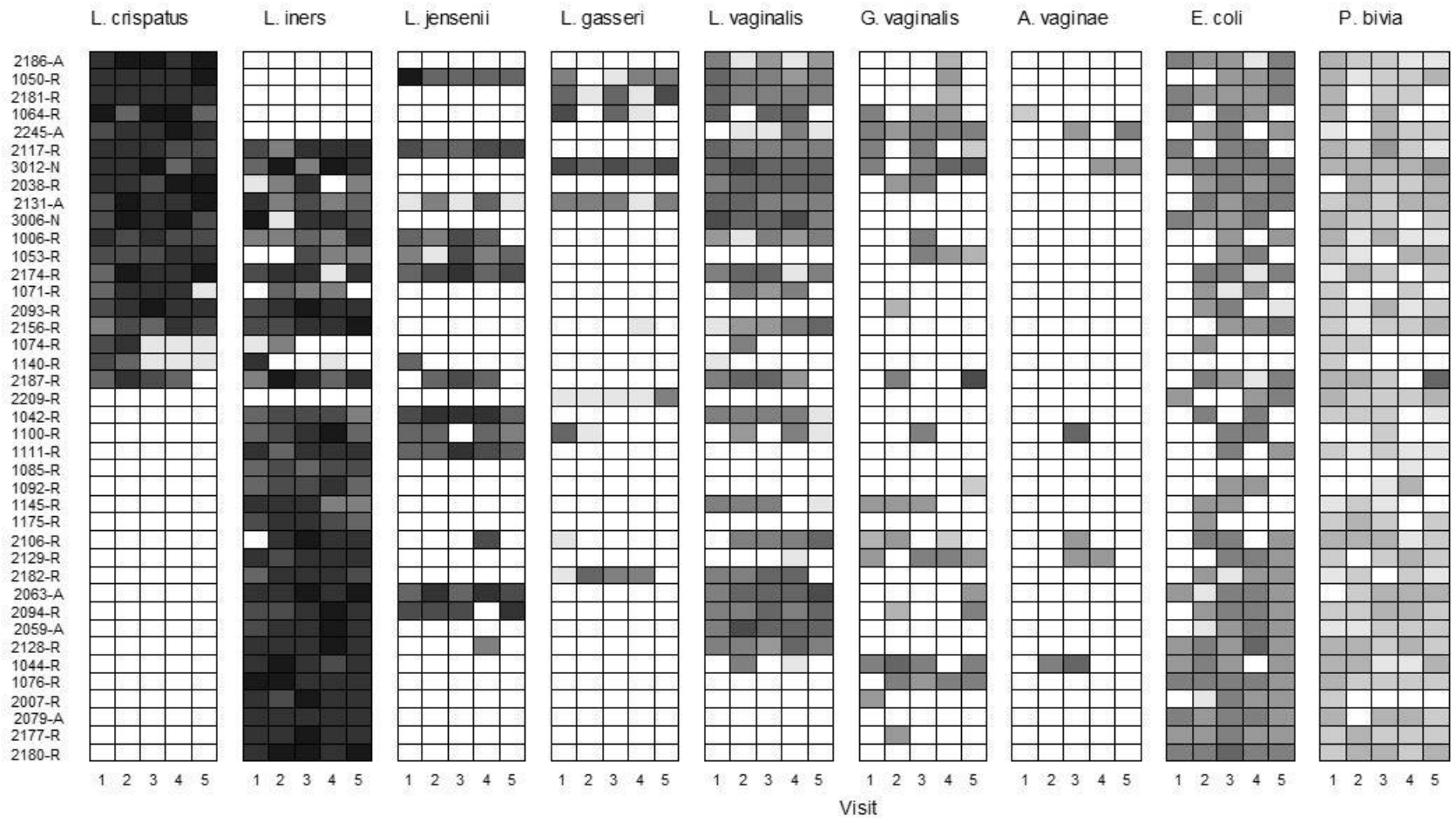
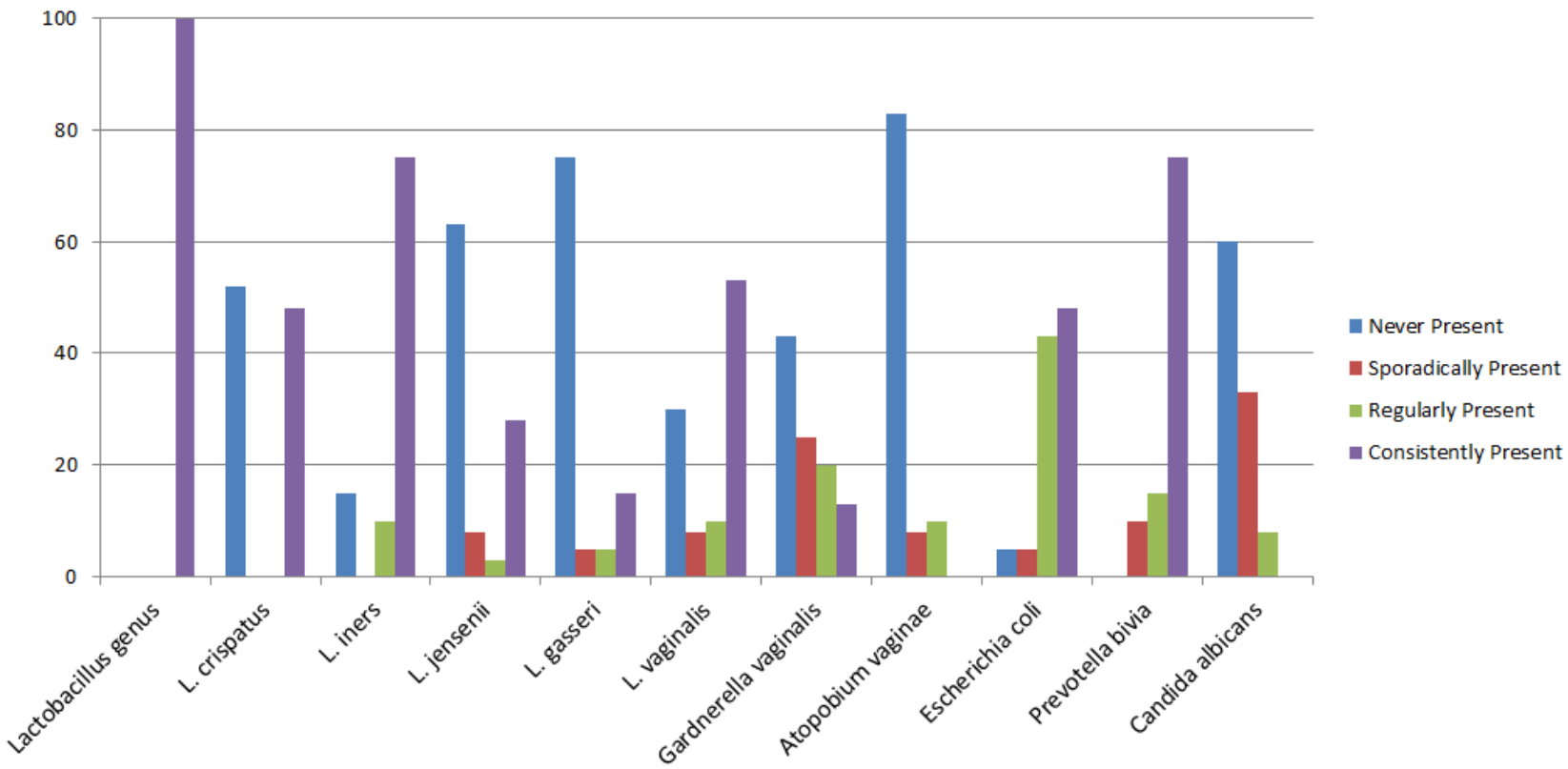


Figure 2 Frequency of vaginal microbiota bacteria presence over the eight week study period in women with a Nugent score of 0-3 throughout



Correlates of longitudinal variations in the concentrations of VMB bacteria were assessed in mixed effects linear regression models for those VMB bacteria that were consistently present in at least 25% of the women in the reference group. Each model had one such VMB bacteria concentration as the outcome, individual women as random effects, and presence or absence of a menstrual cycle, menstrual cycle phase (follicular or luteal phase; see methods), presence of vaginal PSA, and recent vaginal cleansing as fixed effects. It is important to note that all amenorrhoeic women in this sub-study were progestin injection users. The models showed that changes in the concentrations of VMB bacteria over time were larger within women than they were between women, with the exception of *L. jensenii* (Table 2). The mean *Lactobacillus* genus concentration in amenorrhoeic women was lower (-0.55 $\log_{10}$ geq/ml; p=0.023) than the mean concentration in women with a menstrual cycle (Table 2), with *L. crispatus* accounting for the greatest difference (Table 2). The mean *Lactobacillus* genus (-0.39 $\log_{10}$ geq/ml; p=0.010), *L. iners* (-0.75 $\log_{10}$ geq/ml; p= 0.008) and *P. bivia* (-0.38 $\log_{10}$ geq/ml; p=0.045) concentrations were significantly lower at visits with vaginal PSA detected (Table 2). The mean *E. coli* concentration was significantly lower at luteal phase visits compared to follicular phase visits in women with a menstrual cycle (-0.75 $\log_{10}$ geq/ml; p=0.020). There were no significant associations between recent vaginal cleansing and concentration of any VMB bacteria (Table 2).

Table 2. Mean differences in VMB bacteria concentrations in women with a Nugent score of 0-3 during five visits over eight weeks by presence and phase of the menstrual cycle, presence of PSA and recent vaginal cleansing

	Women with cycle, follicular phase visits			Women with cycle, luteal phase visits vs follicular phase visits		Amenorrhoea (all visits) vs women with cycle (all visits)		Visits with PSA present vs not, among follicular phase visits with no recent vaginal cleansing reported		Visits with recent vaginal cleansing reported vs not, among follicular phase visits with PSA absent	
VMB bacteria	**Mean conc[1]**	**SD between[2]**	**SD within[2]**	**Mean diff[3]**	**p[4]**	**Mean diff[5]**	**p[4]**	**Mean diff[6]**	**p[4]**	**Mean diff[7]**	**p[4]**
Lactobacillus genus	7.62	0.60	0.73	0.20	0.124	-0.55	**0.023**	-0.39	**0.010**	-0.27	0.146
L. crispatus	6.76	1.34	1.56	0.49	0.245	-1.33	0.091	-0.30	0.529	-0.05	0.939
L. iners	8.36	0.62	1.29	-0.25	0.356	0.08	0.811	-0.75	**0.008**	-0.32	0.272
L. jensenii	5.79	1.41	1.32	0.85	0.066	-0.30	0.785	0.36	0.476	0.12	0.842
L. vaginalis	5.84	0.92	1.31	-0.19	0.545	0.75	0.192	0.01	0.980	0.50	0.239
E. coli	5.26	<0.001	1.18	-0.75	**0.020**	0.14	0.637	0.10	0.757	-0.26	0.373
P. bivia	3.00	0.31	0.81	-0.16	0.346	0.27	0.199	-0.38	**0.045**	0.03	0.880

Abbreviations: conc=concentration; diff=difference; PSA=prostate-specific antigen; SD=standard deviation; VMB=vaginal microbiota; vs=versus.

1. Expressed in $\log_{10}$ genome equivalents per mL (geq/ml). The expected value for women with a menstrual cycle in the follicular phase of the cycle.
2. The between-women and within-women standard deviations.
3. The mean difference in concentration ($\log_{10}$ geq/ml) between the luteal and follicular phases of the cycle for women with a menstrual cycle.
4. From the mixed effects linear regression models with each item in the first column as the outcome, individual women as random effects, and fixed effects as described in the first row of the table. For women with the bacteria present during at least 75% of visits and excluding the visits during which the bacteria was absent. We only included VMB bacteria that were consistently present (in at least 75% of the visits) in at least 25% of women.
5. The mean difference in concentration ($\log_{10}$ geq/ml) between women with amenorrhoea (all visits) and women with a cycle (all visits).
6. The mean difference in concentration ($\log_{10}$ geq/ml) between visits with PSA present versus not present for visits with the same presence and phase of menstrual cycle and the same vaginal cleansing status.
7. The mean difference in concentration (log10 geq/ml) between visits at which reporting recent vaginal cleansing was reported versus not reported among visits with the same presence and phase of menstrual cycle and the same PSA status.

6.3.2 Vaginal immune mediators over time (reference group)

Concentrations of various cytokines, chemokines, and growth factors were measured in cervicovaginal lavages (CVLs) and expressed in $\log_{10}$ pg/ml (see methods). Mixed effects linear regression models with each immune mediator concentration as the outcome, individual women as random effects, and menstrual cycle presence and phase as fixed effects showed that changes in concentrations of immune mediators over time were larger within women than they were between women, with the exception of interleukin (IL)-1α (Table 3). The mean IL-1α concentration was significantly higher in luteal phase relative to follicular phase visits (0.16 $\log_{10}$ pg/ml; p=0.004) but mean IL-6 (-0.26 $\log_{10}$ pg/ml; p<0.001), CC chemokine macrophage inflammatory protein (MIP)-1β (-0.26 $\log_{10}$ pg/ml; p<0.001) and granulocyte colony-stimulating factor (G-CSF) concentrations (-0.26 $\log_{10}$ pg/ml; p=0.007) were significantly lower. Mean concentrations of IL-8 (0.28 $\log_{10}$ pg/ml; p=0.016), IL-12(p70) (0.15 $\log_{10}$ pg/ml; p=0.038) and MIP-1β (0.34 $\log_{10}$ pg/ml; p=0.013) were higher in amenorrhoeic women compared to women with a menstrual cycle. Further mixed effects linear regression models with each immune mediator as the outcome, individual women as random effects, and presence and phase of the menstrual cycle as fixed effects, were fitted with the following additional fixed effects added (in separate models): vaginal pH category (<4.0, 4.0-4.5, >4.5); presence of abnormal vaginal discharge, cervical mucus, a cervical epithelial finding, or vaginal PSA; and recent vaginal cleansing. Visits with PSA detected had significantly higher mean concentrations of IL-6, IL-12(p70), and CXC chemokines interferon (IFN)-γ-inducible protein (IP-10); visits with a higher vaginal pH had a higher mean concentration of IL-1RA and a lower mean concentration of secretory leucocyte peptidase inhibitor (SLPI); visits with abnormal vaginal discharge had lower mean concentrations of IL-1α, IL-1RA, GM-CSF and elafin; visits with cervical mucus had a lower mean concentration of elafin; and visits with cervical epithelial findings had a lower mean concentration of granulocyte macrophage colony stimulating factor (GM-CSF) (Table 3). Recent vaginal cleansing was not significantly associated with concentrations of any of the immune mediators (data not shown).

Table 3: Mean differences in immune mediator concentrations in women with a Nugent score of 0-3 during five visits over eight weeks by presence and phase of the menstrual cycle (A), clinical characteristics and presence of PSA (B)

A.	**Women with cycle, follicular phase visits**			**Women with cycle, luteal phase vs follicular phase visits**		**Amenorrhoea (all visits) vs women with cycle (all visits)**	
Immune mediators	Mean conc[1]	SD between[2]	SD within[2]	Mean diff[5]	p[3]	Mean diff[6]	p[3]
Total protein	8.17	0.21	0.24	0.03	0.447	0.15	0.071
IL-1α	1.10	0.36	0.30	0.16	**0.004**	0.15	0.253
IL-1β	0.66	0.39	0.51	-0.06	0.521	0.27	0.081
IL-6	0.78	0.42	0.50	-0.32	**<0.001**	0.31	0.055
IL-8	2.01	0.30	0.34	-0.06	0.334	0.28	**0.016**
IL-12(p70)	-0.05	0.16	0.30	-0.00	0.949	0.15	**0.038**
IL-1RA	4.73	0.48	0.57	0.12	0.257	0.07	0.708
IP-10	2.52	0.29	0.42	-0.09	0.258	0.17	0.155
MIP-1β	0.81	0.36	0.37	-0.26	**<0.001**	0.34	**0.013**
GM-CSF	0.32	0.12	0.35	0.02	0.741	0.06	0.340
G-CSF	1.81	0.48	0.53	-0.26	**0.007**	0.33	0.071
Elafin	5.07	0.34	0.37	0.01	0.824	-0.12	0.333
SLPI	4.75	0.33	0.41	0.04	0.611	0.05	0.666

B.	**Visits with vaginal pH 4.0-4.5 or >4.5 vs <4**			**Visits with vaginal discharge vs not**		**Visits with cervical mucus vs not**		**Visits with cervical epith findings vs not**		**Visits with PSA present vs not**	
Immune mediators	4.0-4.5 Mean diff[8]	>4.5 Mean diff[8]	p[4]	Mean diff[9]	P[4]	Mean diff[10]	p[4]	Mean diff[11]	p[4]	Mean diff[7]	p[4]
Total protein	-0.00	-0.16	**0.014**	-0.07	0.150	-0.04	0.386	0.08	0.235	0.02	0.650
IL-1α	0.09	0.01	0.187	-0.13	**0.024**	-0.09	0.080	-0.09	0.266	0.10	0.095
IL-1β	0.14	0.07	0.366	0.07	0.464	-0.04	0.601	-0.07	0.638	0.09	0.390
IL-6	0.08	0.22	0.170	0.16	0.098	0.04	0.671	0.00	0.988	0.21	**0.032**
IL-8	0.09	0.06	0.409	0.06	0.355	-0.02	0.684	-0.08	0.395	0.09	0.188
IL-12(p70)	0.11	0.14	0.097	0.06	0.280	0.01	0.876	0.11	0.154	0.19	**0.001**
IL-1RA	0.05	0.35	**0.015**	-0.25	**0.025**	-0.14	0.132	0.05	0.734	-0.02	0.840
IP-10	0.06	-0.13	0.064	-0.04	0.611	-0.08	0.981	0.00	0.981	0.20	**0.014**
MIP-1β	0.14	0.14	0.163	-0.00	0.989	0.03	0.601	-0.08	0.434	0.13	0.085
GM-CSF	0.01	-0.09	0.304	-0.19	**0.001**	-0.01	0.926	-0.18	**0.040**	-0.07	0.251
G-CSF	0.06	0.03	0.844	-0.01	0.942	0.11	0.215	-0.07	0.626	0.17	0.117
Elafin	0.04	-0.02	0.678	-0.23	**0.001**	-0.13	**0.033**	0.10	0.349	0.02	0.773

SLPI	0.01	-0.20	**0.034**	0.01	0.899	0.07	0.288	0.08	0.489	0.14	0.103

Abbreviations: clin char= clinical characteristics; conc=concentration; diff=difference; epith=epithelial; G-CSF=granulocyte colony stimulating factor; GM-CSF=granulocyte macrophage colony stimulating factor; IL=interleukin; IP-10=interferon-inducible protein 10; MIP-1β= macrophage inflammatory protein 1β; PSA=prostate-specific antigen; SD=standard deviation; SLPI= secretory leukocyte protease inhibitor; VMB=vaginal microbiota; vs=versus.

1. Expressed in $\log_{10}$ pg/ml. The expected value for women with a menstrual cycle in the follicular phase of the cycle.
2. The between-women and within-women standard deviations for women in the model with presence and phase of the cycle as fixed effects.
3. From mixed effects linear regression models with each item in the first column as the outcome, individual women as random effects, and including presence and phase of the menstrual cycle as fixed effects.
4. From mixed effects linear regression models (separate model for each clinical characteristic and PSA) with each item in the first column as the outcome, individual women as random effects, and including the item in the first row as fixed effects, and controlled for presence and phase of the menstrual cycle. A model with recent vaginal cleansing (the evening or morning before the visit) as fixed effect controlled for presence and phase of the menstrual cycle was also fitted but the data are not shown because none of the findings were statistically significant. The clinical characteristics are clinician-observed during speculum examination.
5. The mean difference in concentration ($\log_{10}$ pg/ml) between luteal and follicular phase visits in women with a menstrual cycle.
6. The mean difference in concentration ($\log_{10}$ pg/ml) between women with amenorrhoea (all visits) and women with a cycle (all visits).
7. The mean difference in concentration ($\log_{10}$ pg/ml) between visits with PSA present versus absent, for visits with the same presence and phase of menstrual cycle.
8. The mean difference in concentration ($\log_{10}$ pg/ml) between visits at which the vaginal pH was 4.0-4.5, or >4.5, each compared to <4, for visits with the same presence and phase of menstrual cycle.
9. The mean difference in concentration ($\log_{10}$ pg/ml) between visits with vaginal discharge present versus absent, for visits with the same presence and phase of menstrual cycle.
10. The mean difference in concentration ($\log_{10}$ pg/ml) between visits with cervical mucus present versus absent for visits with the same presence and phase of menstrual cycle.
11. The mean difference in concentration ($\log_{10}$ pg/ml) between visits with cervical epithelial findings present versus absent, for visits with the same presence and phase of menstrual cycle. Cervical epithelial findings included abrasions, oedema, ecchymosis, petechiae, erythema, and ulcers.

6.3.3 VMB bacteria, Candida, and immune mediators over time (incident BV group)

All women in this cohort had a Nugent score of 0-3 at visit 1 (enrolment). The first visit during which BV was diagnosed was visit 2 in 16 women, visit 3 in seven women, visit 4 in 11 women, and visit 5 in six women (Figure 3). Two women had an intermediate VMB (Nugent score 4-6) prior to an incident BV visit. At the first visits during which BV was detected, the mean concentrations of *Lactobacillus* genus (-1.51 $\log_{10}$ geq/ml; p=0.005) and *L. vaginalis* (-1.35 $\log_{10}$ geq/ml; p=0.021) were statistically significantly lower, and the mean concentrations of *G. vaginalis* (2.84 $\log_{10}$ geq/ml; p<0.001), *A. vaginae* (3.92 $\log_{10}$ geq/ml; p<0.001), and *P. bivia* (1.38 $\log_{10}$ geq/ml; p=0.003) higher, than the mean concentrations at the preceding visit. (Table 4). The triple taxa qPCR vaginal health score was also statistically significantly lower at the first BV incident visit (Table 4). This vaginal health score was calculated as [$\log_{10}$ geq/ml (*Lactobacillus* genus) - $\log_{10}$ geq/ml (*G. vaginalis* + *A. vaginae*)] and a higher score therefore suggests better vaginal health (483). *C. albicans*, *L. jensenii* and *L. gasseri* were never present at any visits in 58%, 60% and 73% of the women, respectively. Mean concentrations of IL-1β (0.66 $\log_{10}$ pg/ml; p=0.003) and IL-12(p70) (0.22 $\log_{10}$ pg/ml; p=0.024) were significantly increased, and mean concentrations of IP-10 (-0.39 $\log_{10}$ pg/ml; p=0.046), elafin (-0.26 $\log_{10}$ pg/ml; p=0.010), and total protein (-0.17 $\log_{10}$ pg/ml; p=0.026) significantly decreased, at the first BV incident visit (Table 4).

Figure 3: Presence/absence and concentration of vaginal microbiota over the eight week study period in women with incident BV (Nugent 7-10). Each box depicts one visit for a particular woman. The shading of the box indicates the concentration level (in $\log_{10}$ geq/ml) of each taxon with darker colours depicting a higher concentration. If the species was absent the box is white. Boxes bordered by a red line are the first BV visit for that woman. Boxes in yellow denote visits with an intermediate Nugent score of 4-6 if present before development of BV.

Figure 3 Presence/absence and concentration of vaginal microbiota bacteria over the eight week study period in women with incident BV (Nugent 7-10)

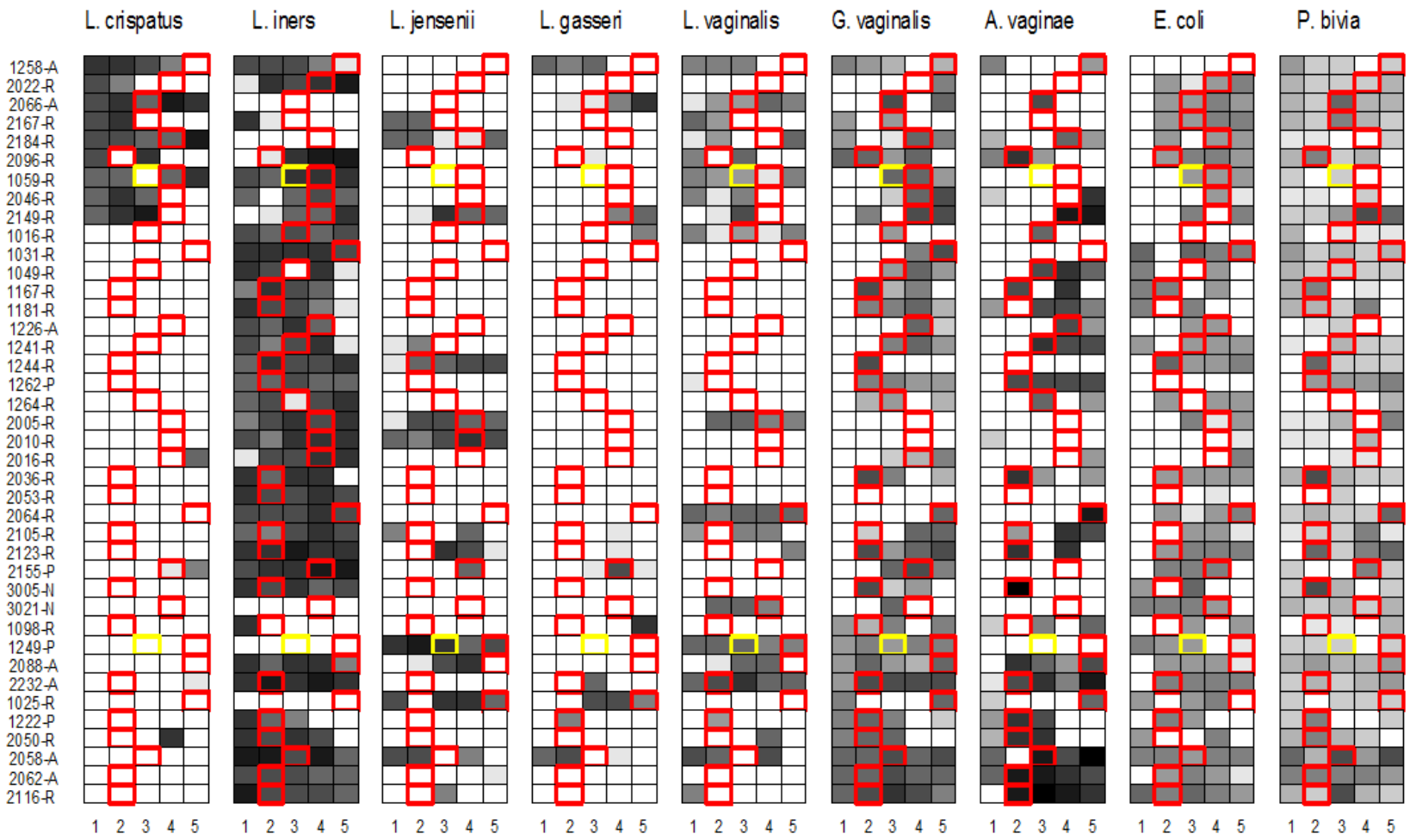


Table 4. Differences in VMB bacteria and immune mediator concentrations in 40 women with incident BV between the visit before the incident BV visit and the incident BV visit

	Visit before the first incident BV visit	First incident BV visit[1]	Mean concentration difference	P[3]
	Mean concentration[2]	Mean concentration[2]		
VMB bacteria				
Triple taxa qPCR vaginal health score[4]	5.44	0.20	-5.24	**<0.001**
Lactobacillus genus	7.60	6.09	-1.51	**0.005**
L. crispatus	1.91	0.86	-1.05	0.250
L. iners	5.84	4.85	-0.99	0.080
L. vaginalis	2.76	1.42	-1.35	**0.021**
G. vaginalis	2.11	4.95	2.84	**<0.001**
A. vaginae	0.20	4.12	3.92	**<0.001**
E. coli	3.04	2.75	-0.29	0.709
P. bivia	1.76	3.13	1.38	**0.003**
Immune mediators				
Total protein	8.29	8.12	-0.17	**0.026**
IL-1α	1.23	1.49	0.26	0.157
IL-1β	0.69	1.35	0.66	**0.003**
IL-6	0.77	1.06	0.28	0.081
IL-8	2.07	2.29	0.22	0.104
IL-12(p70)	-0.00	0.22	0.22	**0.024**
IL-1RA	4.71	4.99	0.28	0.250
IP-10	2.63	2.25	-0.39	**0.046**
MIP-1β	0.70	0.89	0.18	0.372
GM-CSF	0.34	0.28	-0.06	0.157
G-CSF	1.96	1.96	0.11	0.579
Elafin	5.07	4.81	-0.26	**0.010**
SLPI	4.80	4.62	-0.18	0.287

Abbreviations: BV=bacterial vaginosis; G-CSF=granulocyte colony stimulating factor; GM-CSF=granulocyte macrophage colony stimulating factor; IL=interleukin; IP-10=interferon-inducible protein 10; MIP-1β= macrophage inflammatory protein 1β; SLPI= secretory leukocyte protease inhibitor; VMB=vaginal microbiota.

1. The first incident BV visit was visit 2 for 16 women, visit 3 for 7 women, visit 4 for 11 women and visit 5 for 6 women.
2. Expressed in $\log_{10}$ genome equivalents per mL (geq/ml) for VMB bacteria and $\log_{10}$ pg/ml for immune mediators.
3. Wilcoxon signed rank tests. 4. $\log_{10}$ geq/ml (*Lactobacillus* genus) - $\log_{10}$ geq/ml (*G. vaginalis* + *A. vaginae*).

6.3.4 VMB bacteria and immune mediator associations over time (both groups)

In mixed effects linear regression models including all 80 women and controlled for presence and phase of menstrual cycle and PSA presence, a higher 'composite qPCR vaginal health score' was associated with a higher IP-10 concentration (2.53 $\log_{10}$ pg/ml; p<0.001) and lower IL-1α (-1.14 $\log_{10}$ pg/ml; p=0.005), IL-8 (-1.55 $\log_{10}$ pg/ml; p=0.002), and IL-12(p70) (-1.80 $\log_{10}$ pg/ml; p<0.001) concentrations (Table 5). This vaginal health score was calculated as [$\log_{10}$ geq/ml (*Lactobacillus* genus) - $\log_{10}$ geq/ml (*G. vaginalis* + *A. vaginae*)] and a higher score therefore suggests better vaginal health (483). The *Lactobacillus* genus concentration (which is one component of the vaginal health score) showed a similar pattern except that it was not significantly associated with the IL-1α concentration and the reduction in IL-12(p70) concentration did not reach statistical significance. The *L. crispatus* and *L. vaginalis* concentrations were not significantly associated with any immune mediator concentrations over time. The *L. iners* concentration was significantly positively associated with IP-10 and IL-8 concentrations and negatively associated with IL-1α concentration. The *P. bivia* concentration was positively associated with the IL-1α and IL-8 concentrations and negatively associated with the IP-10 concentration, and the *E. coli* concentration was positively associated with the IL-8 concentration.

Table 5. Longitudinal associations between VMB bacteria and immune mediator concentrations among all visits of all 80 women (with Nugent 0-3 throughout and with incident BV) over the eight week study period

	IL-1α		IL-8		IL-12(p70)		IP-10		Elafin		PSA		Amenorrhoea		Luteal phase	
	Est.[1]	p[1]	Est.[1]	p[1]	Est.[1]	p[1]	Est.[1]	p[1]	Est.[1]	p[1]	Est.[1]	p[1]	Est.[1]	p[1]	Est.[1]	p[1]
Triple taxa qPCR vaginal health score[2]	-1.14	**0.005**	-1.55	**0.002**	-1.80	**<0.001**	2.53	**<0.001**	0.34	0.383	-1.07	**0.002**	0.80	0.160	0.52	0.118
Lactobacillus genus[3]	0.02	0.939	-0.75	**0.003**	-0.42	0.105	0.92	**<0.001**	-0.21	0.301	-0.49	**0.005**	-0.37	0.135	0.34	0.054
L. crispatus[3]	0.49	0.353	-0.49	0.466	-0.05	0.921	0.79	0.098	-0.39	0.317	-0.55	0.218	-1.36	0.053	0.29	0.452
L. iners[3]	-0.58	**0.020**	0.77	**0.010**	-0.50	0.108	0.49	**0.011**	-0.42	0.078	-0.38	0.076	-0.26	0.280	-0.10	0.696
L. vaginalis[3]	0.24	0.563	0.60	0.289	-0.25	0.562	-0.43	0.276	-0.00	0.991	-0.14	0.688	0.18	0.722	-0.06	0.849
P. bivia[3]	0.52	**0.004**	0.78	**0.001**	0.03	0.917	-0.69	**<0.001**	0.20	0.296	-0.05	0.759	-0.29	0.214	-0.19	0.268
E. coli[3]	-0.00	0.995	0.55	**0.049**	-0.60	0.054	0.28	0.139	-0.05	0.832	0.29	0.160	-0.05	0.812	-0.14	0.491

Abbreviations: BV=bacterial vaginosis; Est=model estimate; IL=interleukin; IP-10=interferon-inducible protein 10; qPCR=quantitative polymerase chain reaction; PSA=prostate-specific antigen; VMB=vaginal microbiota; vs=versus.

1. From mixed effects multiple regression models with each item in the first column as the outcome, individual women as random effects, and fixed effects for all variables in the first row.
2. $\log_{10}$ geq/ml (*Lactobacillus* genus) - $\log_{10}$ geq/ml (*G. vaginalis* + *A. vaginae*).

For women with the bacteria present during at least 75% of visits and excluding the visits during which the bacteria was absent. We only included VMB bacteria that were consistently present (in at least 75% of the visits) in at least 25% of women.

6.4 Discussion

In this longitudinal study of young sub-Saharan African women, we confirmed that a Nugent score of 0-3 over an eight week period was associated with consistently high concentrations of *Lactobacillus* species (regularly accompanied by much lower concentrations of the BV-associated bacteria *G. vaginalis*, *A. vaginae*, and *P. bivia* and the pathobiont *E. coli*), whereas incident BV was associated with significantly reduced concentrations of lactobacilli and increased concentrations of *G. vaginalis*, *A. vaginae* and *P. bivia*, but not *E. coli*. In women with a normal VMB throughout the study, VMB variations were larger within women over time than between women, as has been seen in other studies (478). *L. iners* and *L. crispatus* were the dominant lactobacilli in our study, as has been seen in studies enrolling Caucasian European and American women (287, 471, 484). However, the following of our findings have not been reported in those studies: *L. crispatus* was often accompanied by *L. vaginalis*; *L. jensenii* and *L. gasseri* were never present in most women; and *E. coli* was regularly present in almost all women. Another important pathobiont in the vaginal niche is *Streptococcus agalactiae*, and unfortunately, we only have qPCR data for that organism at baseline (485). In a cross-sectional baseline analysis of all 430 women in the Vaginal Biomarkers Study using qPCRs, 16% had *S. agalactiae* (485) and 28% *E. coli* in their VMB (18). The limited number of other molecular VMB studies that reported on *S. agalactiae* and *E. coli* carriage showed varying results, with generally lower detection in studies that employed 16S sequencing compared to qPCR (394, 486, 487). Vaginal carriage of these pathobionts should be further investigated, preferably by qPCR in longitudinal studies, given their associations with vaginitis, reproductive health, and neonatal meningitis and sepsis (488).

In women with a normal VMB throughout the study, variations in concentrations of soluble immune mediators were greater within women over time than between women, which is in agreement with other studies (428, 452, 475). In the women developing BV, incident BV was associated with increased concentrations of proinflammatory cytokines and decreased concentrations of the antiprotease elafin and IP-10. Other studies have reported similar proinflammatory profiles associated with BV, as well as increased proteolytic activity (447, 450, 489, 490). IP-10 findings across studies are more difficult to interpret (see below). It should be noted that incident or recurrent urogenital infections other than BV could have been responsible for some of the variation in immune mediators seen. However, none of the 80 women in this study had symptomatic vaginal candidiasis throughout the study and *C. albicans*, detected only occasionally and never more than twice in the same women, was present in low concentrations in the majority of women. Furthermore, we screened all women for STIs at baseline and selected women without STIs (with the exception of chronic HSV-2 infection) for this sub-study. Only women with clinician-observed signs of urogenital infections during the eight-week follow-up period were retested for STIs, but such clinician-observed signs were rare (Table 1). We therefore believe that incident or recurrent STIs during the eight-week follow-up period were uncommon. However, these findings should be interpreted with caution since majority of STIs in women (such as CT and NG) are asymptomatic.

We assessed several other potential correlates of VMB bacteria as well as immune mediator concentration variations in women with a normal VMB throughout the study: the presence and phase of the menstrual cycle, the presence of PSA as a marker of recent sex, and recent vaginal cleansing. It is important to note that PSA would only be possible to measure in the proportion of women who did not use condoms during sexual intercourse. Amenorrhoeic women had a reduced concentration of lactobacilli, (notably *L. crispatus*), compared to women with a menstrual cycle even after controlling for PSA presence and recent vaginal cleansing, and this may be due to the induction of a hypo-oestrogenic state during injectable progestin use. Current evidence suggests that the VMB destabilising effect of hypo-oestrogenism in these women is larger than any potential protective effect associated with the absence of regular menstrual bleeding (486). Amenorrhoeic women also had increased concentrations of several proinflammatory immune mediators. This is in agreement with results from two African studies (Tanzania and South Africa/Kenya) (491, 492) but in contrast to the results of a recent study in Kenyan women that showed sustained decreases in IL-6, IL-8, and IL-1RA after initiation of depot medroxyprogesterone acetate (DMPA) injectable contraception (493). The comparison groups in these studies differed, with our study comparing amenorrhoeic injectable progestin users with all other women, the Tanzanian and South Africa/Kenya studies comparing current DMPA users with women not using hormonal contraception, and the Kenyan study comparing women before and after initiation of DMPA use. It is possible that DMPA use is immunosuppressive initially as it binds to the corticosteroid receptor with an affinity similar to that of cortisol (494), but becomes proinflammatory with prolonged use due to increasing hypo-oestrogenism which in turn can lead to VMB dysbiosis and vaginal wall atrophy.

We did find some differences in VMB bacteria and the concentration of immune mediators in samples collected during luteal phase visits compared to follicular phase visits, but these patterns were not consistent. While levels of both oestrogen and progesterone are higher in the luteal phase than the follicular phase of the menstrual cycle, we sampled women around days 9 and 23 of their cycles, and these time points do not correspond with peak hormone levels. Oestrogen in particular is known to associate with higher concentrations of lactobacilli (477, 495, 496). We did see increases in concentrations of *Lactobacillus* genus, *L. crispatus*, and *L. jensenii* at luteal phase visits, but these did not reach statistical significance. The differences in mean immune mediator concentrations between the luteal and follicular phases that we observed were not seen in the earlier mentioned Tanzanian study, and that study assessed menstrual cycle stage more carefully by urine pregnanediol 3-glucuronide testing (491).

Vaginal sex in the last 24-48 hours as measured by the presence of PSA in vaginal swab eluates was associated with concentrations of various lactobacilli, with *L. iners* showing the greatest reduction. PSA presence was also associated with higher concentrations of IL-6, IL-12(p70), and IP-10. Similar effects of recent vaginal sex on the VMB have been previously reported by us (428) and by others (408, 477, 497, 498). A direct effect was demonstrated *in vitro* when

seminal plasma was co-cultured with cervical epithelial cells (406). The VMB-destabilising and proinflammatory effects of sexual activity are likely due to the direct effect of seminal fluid as condom use seems to prevent the effects observed without condom use (408, 499). Studies showing concordance in vaginal microbiota and penile skin and urethral microbiota of the male sexual partner are suggestive that penile microbes may also destabilize the VMB (500, 501). Recent vaginal cleansing was not significantly associated with any changes in VMB bacteria or immune mediator concentrations in any of our analyses.

Using data from all 80 women, we investigated the direct associations between the concentrations of VMB bacteria and vaginal immune mediator concentrations over time while controlling for presence and phase of the menstrual cycle and PSA presence. Perhaps the most significant finding was that a higher 'composite qPCR vaginal health score' (suggesting better vaginal health) was associated with decreased concentrations of all three modelled proinflammatory cytokines (IL-1α, IL-8, and IL-12(p70)) and an increased concentration of IP-10. Unfortunately, the sample size of this sub-study was small; some statistically significant associations in the cross-sectional analyses of the Vaginal Biomarkers Study baseline data showed the same trends as this sub-study but did not reach statistical significance (480). When interpreting the cross-sectional (18, 480) and longitudinal data together, we conclude that *Lactobacillus* species are associated with an increase in IP-10 and reductions (*L. crispatus*, *L. vaginalis*) or no change in multiple proinflammatory cytokines; BV-associated bacteria are associated with a decrease in IP-10 and increases in multiple proinflammatory cytokines; and *E. coli* and *S. agalactiae* are associated with increases in IP-10 and multiple proinflammatory cytokines (the *S. agalactiae* data are unpublished). A cross-sectional Canadian study employing 16S sequencing to characterise the VMB reported very similar results: a decrease in IP-10 and increases in multiple proinflammatory cytokines in women with BV (community state type (CST)-4), and no inflammation but an increase in IP-10 in women with a *L. iners* dominated VMB (CST-3) (502). A longitudinal South African study also found significant increases in multiple proinflammatory cytokines at visits during which vaginal dysbiosis was detected, but no association with IP-10 (475). IP-10 (also known as CXCL10) is induced by type I and II interferons and TNF-α and is a ligand for the CXCR3 receptor (457-459). IP-10 levels are generally elevated in uncontrolled viral infection, but a reduction of IP-10 levels by pathogenic bacteria, and particularly combinations of bacteria, has been described before (460-463). The significance of this remains unclear. A recent study among women in South Africa by Masson *et al* found that increased IL-1β and reduced IP-10 concentrations in female genital secretions of HIV-negative women predicted the presence of BV and/or other treatable discharge-causing STIs (489). The combination of these two biomarkers identified a significantly higher proportion (77%) of women with BV and treatable STIs than clinical criteria (19%). Consequently, the authors suggested to explore the use of those biomarkers in the detection of BV and discharge causing STIs (490).

Our study had some limitations. Unfortunately, we could not afford to quantify all relevant bacteria and immune mediators in all longitudinal samples from all participants of the Vaginal

Biomarkers Study. The current sub-study design was considered a next best but feasible alternative. This design required us to select women based on longitudinal Nugent scores, which are a cruder way of classifying VMBs than the molecular methods we employed in the sub-study. However, multiple studies have shown a good correlation between the two methods in classifying woman as having a lactobacilli-dominated or dysbiotic VMB (340), with the molecular testing adding nuance. Our sub-study design also reduced our statistical power, especially related to the potential effects of VMB minority species. Due to the stringent selection criteria, our results may not be generalisable to all women.

In conclusion, our well-controlled longitudinal data confirm the inflammatory nature of anaerobic vaginal dysbiosis and *E. coli* colonisation, recent vaginal sex, and progestin-injectable use. While anaerobic vaginal dysbiosis or BV is by far the most common vaginal dysbiosis, high abundance of *E. coli*, *S. agalactiae*, and other pathobionts as a distinct inflammatory vaginal dysbiosis deserves further study. The roles of a selection of the vaginal mediators (IL-1α, IL-1β, IL-8, IL-12, IP-10) with or without the composite qPCR vaginal health score as predictive biomarkers for the above conditions warrant further investigation.

6.5 Methods

6.5.1 Ethical approvals

The study protocol was approved by the Ethical Review Committee, Kenyatta National Hospital, Kenya; the Human Research Ethics Committee (Medical), University of the Witwatersrand, South Africa; the Rwanda National Ethics Committee, Rwanda; the Institutional Review Board of the Institute of Tropical Medicine (ITM), Belgium; and the ethics committees of the Ghent University Hospital in Ghent and the Antwerp University Hospital in Antwerp, Belgium. In addition the study was approved by the National Council of Science and Technology, Kenya; and the National Health Research Committee, Rwanda. All methods described below were performed in accordance with the relevant guidelines and regulations.

6.5.2 Study participants and clinic visits

This paper describes a longitudinal analysis of 80 women who were part of the Vaginal Biomarkers Study, which enrolled a total of 430 women at three study sites: the International Centre of Reproductive Health Kenya in Mombasa, Kenya; the Wits Reproductive Health and HIV Institute in Johannesburg, South Africa and Rinda Ubuzima in Kigali, Rwanda. In the original study design, women were recruited into predefined groups: HIV-negative adult women (N=219), adolescents (N=60), and pregnant women (N=60) in Kenya and South Africa; HIV-positive women (N=30) and HIV-negative sex workers (N=30) in Rwanda; and women engaging in traditional vaginal practices in South Africa (N=31). For the current analysis, we selected 40 women who had a normal VMB (defined as a Nugent score of 0-3) at five consecutive visits over eight weeks and 40 women who had a normal VMB at baseline and incident BV (defined as a Nugent score of 7-10) at one of the four follow-up visits over eight weeks. Additional selection criteria were: none of the relevant samples were missing; women

tested negative for pregnancy, HIV, syphilis, *N. gonorrhoeae*, *C. trachomatis*, *T. vaginalis*), and vaginal candidiasis at baseline and did not become HIV-positive or pregnant (reference group) during follow-up; and reported not to engage in traditional vaginal practices (such as the use of cloth, lemon juice or detergents inside the vagina) at baseline. Women with positive HSV-2 serology at baseline were included due to the high prevalence of 34%. A total of 54 and 48 women qualified for the reference and incident BV groups, respectively, and 40 women were selected for each group from among the qualifying women at random. No matching was done.

Women were followed for five consecutive visits over eight weeks. The visits were tightly scheduled around the menstrual cycle with the enrolment visit (visit 1) scheduled shortly after the last day of the menstrual period on day 9 (±2 days) of the cycle; the absence of menses was verified during vaginal examination at this visit. The next four visits were scheduled with two week intervals over two menstrual cycles (visits 2-5). Thus, visits 3 and 5 coincided with day 9 (±2 days) of the menstrual cycle (the follicular phase) and visits 2 and 4 with day 23 (±2 days) or the luteal phase. The same visit schedule was followed for women using hormonal contraception, including those who were amenorrhoeic due to progestin-injectable use. At baseline, eligible women interested in participating provided written informed consent, were interviewed about sociodemographic and behavioural characteristics, underwent a physical and vaginal examination, and were tested for HIV and the above-mentioned reproductive tract infections. Interviews and vaginal examinations were also done at all subsequent visits.

6.5.3 Sample collection

At each of the five visits included in this sub-study, the following samples were collected before any other procedures in the following order: two sterile flocked swabs (Copan Diagnostics, Inc., Murrieta, CA) that were rotated against the mid-portion of the vaginal wall under visual inspection, dipped in the posterior fornix and carefully removed to prevent contamination; and a CVL that was obtained by gently flushing 10 ml normal saline through the speculum and aspirating the fluid from the posterior fornix. At each study site, one trained clinician performed all the examinations using one standard operating procedure to minimise inter-clinician variability.

6.5.4 Sample processing

CVLs were collected in 15 ml falcon tubes, kept on ice for transport (2-8°C), and processed within a maximum of one hour after collection. CVLs were centrifuged at 1,000 x g for 10 minutes at 4°C and supernatants (~9ml) were aliquoted into three fractions; two of approximately 4ml each and one of 1ml. The aliquots were stored at -80°C locally. CVLs and vaginal swabs, frozen at -80°C, were shipped in batches using a temperature-monitored dry shipper to the central laboratory at the ITM in Antwerp, Belgium, where they were stored at -80°C before analysis of soluble immune mediators and VMB bacteria.

6.5.5 Characterization of vaginal microbiota

Vaginal Gram-stained slides were examined and scored at the ITM using the Nugent method (346). qPCR was performed on extracted DNA from vaginal swab eluates for the following ten species and one genus: *Lactobacillus* genus, *L. crispatus*, *L. gasseri*, *L. iners*, *L. jensenii*, *L. vaginalis*, *A. vaginae*, *G. vaginalis*, *E. coli*, *P. bivia,* and *C. albicans* and in duplicate at the ITM and at the University of Ghent, Belgium, as previously described (18, 394). The number of organisms was expressed as genome equivalents per ml (geq/ml); the genomic concentration was calculated using the described genomic sizes of the type strains.

6.5.6 Quantification of soluble immune mediators in CVLs

Concentrations of the cytokines IL-1α, IL-1β, IL-6 and IL-12(p70), MIP-1β, IP-10 and IL-8, and growth factors GM-CSF and G-CSF in CVLs were measured at the ITM using the Bio-Plex™ human cytokine assay kit (Bio-Rad Laboratories NV-SA, Nazareth, Belgium) as previously described (428). Elafin, SLPI, IL-1RA and the total protein concentration in CVLs were measured in the Laboratory of Genital Tract Biology, Brigham and Women's Hospital, Boston, MA, USA. Elafin and SLPI were quantified using ELISA kits from R&D Systems (Minneapolis, MN) following manufacturers' instructions. IL-1RA was measured using the Meso Scale Discovery (MSD) multiplex platform and Sector Imager 2400 (MSD, Gaithersburg, MD). The MSD Discovery Workbench Software was used to convert relative luminescent units into protein concentrations (pg/ml) using interpolation from several log calibrator curves. Total protein in CVLs was determined by a bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL) using the Victor 2 counter. Optical densities were read at 450 nm with a second reference filter of 570 nm using a Victor2 multi-label reader and WorkOut Software (PerkinElmer, Waltham, MA).

6.5.7 Prostate-specific antigen detection

PSA was measured in vaginal swab eluates using the Seratec® PSA semiquant assay (Seratec Diagnostica, Göttingen, Germany). A volume of 150 µl of the eluate, in diluted phosphate buffered saline (1,200 µl; 1 part phosphate buffered saline and 9 parts saline, pH 7.4), was centrifuged for 10 min at 13,000 x g. After centrifugation, 120 µl of supernatant was used for testing according to the manufacturer's instructions.

6.5.8 Data analysis

Statistical analyses were performed using Stata 13 (StataCorp, College Station, TX), SAS 9.4 (SAS Institute Inc, Cary, NC) and R 3.0.1 (The R Foundation, Vienna, Austria). Over the five study visits, the detection of individual VMB bacteria by qPCR was classified as follows: never present; sporadically present (present at 25% or fewer visits); regularly present (present at 26 - 74% of visits) and consistently present (present at 75% or more visits). The concentrations of VMB bacteria (in geq/ml) and immune mediators (in pg/ml) were $\log_{10}$ transformed in all analyses.

For the women with a normal VMB throughout the study: Longitudinal variations in the concentrations of VMB bacteria were assessed in mixed effects linear regression models for those VMB bacteria consistently present in at least 25% of the women with a normal VMB throughout the study. All models included one VMB bacteria as the outcome and individual women as random effects. We added the following fixed effects: sampling in the luteal (visits 2 and 4) versus follicular phase (visits 1, 3 and 5) of the menstrual cycle, the absence (amenorrhoea due to progestin-injectable use) or presence of a menstrual cycle (either a natural cycle or regular withdrawal bleeds during combined contraceptive use), presence of PSA as a marker of sex within the last 24-48 hours (481, 482), and recent vaginal cleansing (the evening or morning just prior to the study visit).

For the women with a consistently normal VMB, mixed effects linear regression models were also fitted with each immune marker concentration as the outcome, individual women as random effects, and including presence and phase of the menstrual cycle as fixed effects. Further mixed effects linear regression models for each marker as outcome, controlled for presence and phase of the menstrual cycle, were fitted separately for the following covariates: vaginal pH category (<4.0, 4.0-4.5, >4.5), presence of clinician-observed abnormal vaginal discharge, cervical mucus, a cervical epithelial finding (abrasion, laceration, ecchymosis, petechiae, erythema, or ulcer), vaginal cleansing, and PSA. These covariates were selected based on previous cross-sectional analyses in the same study population (18, 431, 480) and based on published literature (473, 491).

In the women with incident BV, we assessed the mean change in concentrations of VMB bacteria and immune mediators between the visit preceding the first incident BV visit and the first incident BV visit using Wilcoxon signed rank tests. Furthermore, the direct associations between VMB bacteria concentrations (for VMB bacteria consistently present in at least 25% of the women) and immune marker concentrations (IL-1α, IL-8, IL-12, IP-10, and elafin) were determined in mixed effects linear regression models in all 80 women. All models included the concentration of an individual VMB bacterium as the outcome, individual women as random effects, and the following fixed effects: each immune mediator concentration, PSA presence, and presence and phase of menstrual cycle. We also considered a 'triple taxa qPCR vaginal health score' based on the concentrations of three key VMB bacteria [$\log_{10}$ (*Lactobacillus* genus) - $\log_{10}$ (*G. vaginalis* + *A. vaginae*)] as the outcome because this score was shown to be the best indicator of vaginal health in the Vaginal Biomarkers Study (483).

6.6 Data availability

According to the Institute of Tropical Medicine's policy, all data are available from the Institute of Tropical Medicine Institutional Data Access for researchers who meet the criteria for access to confidential data. Requests for data access can be made by emailing Mr. Jef Verellen, Quality Specialist at ITMresearchdataaccess@itg.be.

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Author contributions

Conceived and designed the study: V.J J.VDW G.V T.C. Performed the study & experiments: M.M S.D G.N J.K L.H T.C P.C S.J. Analysed the data: J.B V.J J.K. Wrote the original paper: V.J J.K J.VDW. Reviewed and contributed to the paper: all authors.

Competing financial interests

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

7 General Discussion

General Discussion

The work done in this PhD was part of a larger descriptive project whose primary purpose was to establish baseline ranges of immunological biomarkers related to the healthy vaginal environment in a group of healthy European women and groups of women targeted for vaginal microbicide development in Kenya, Rwanda and South Africa. These findings are relevant for microbicide safety but they are also useful for general HIV prevention and beyond. This is especially because of new developments since study inception that have shifted the focus to other prevention options such as oral PrEP and multipurpose technologies. My discussion will therefore look at the relevance of our study findings and research experience from a perspective that is broader than microbicide development.

7.1 Summary of findings

Starting from a small group of healthy Caucasian women based in Antwerp, Belgium and later to a larger and diverse group of women in Sub-Saharan Africa, we demonstrated a wide range in concentrations of selected soluble immune mediators in cervicovaginal lavages. In the Antwerp cohort of healthy women, variation in concentrations of most soluble mediators was larger between women than it was within women, contrasting our findings in the women from Sub-Saharan Africa. We described a number of physiological and behavioural factors that were associated with differences in concentrations of these immune mediators in the lower female genital tract. These include cervical ectopy, recent sexual activity as indicated by the presence of prostate-specific antigen in CVL, multiple sexual partners, abnormal vaginal discharge, cervical epithelial findings (abrasion, laceration, ecchymosis, petechiae, erythema or ulcer) and cervical mucus that were mostly associated with higher levels of soluble immune mediators indicative of inflammation such as IL-1 (α and β), IL-6, IL-8, IL-12(p70) or suppression of regulatory or protective immune mediators such as IL-1RA and SLPI. A cross-sectional analysis of these soluble markers in the women from Sub-Saharan Africa showed discrete profiles depending on their categorization i.e. pregnant, adolescent, engaging in vaginal practices (using substances other than water to dry, clean or tighten up their vagina) and HIV positivity. Our studies also demonstrated variation in soluble immune mediators depending on whether a woman was in the luteal or follicular phase of her menstrual cycle. These menstrual-cycle related variations were, however, inconsistent and as previously discussed, were not observed in a Tanzanian study with a more stringent assessment of the menstrual cycle stage (491). When we did a longitudinal analysis of these soluble markers, we found that amenorrhoeic women (due to progestin use) had a more pro-inflammatory female genital tract profile (increased IL-8, IL-12(p70) and MIP-1β) compared to women with a menstrual cycle.

In addition to assessing soluble immune mediators, we carried out molecular characterization (using qPCR) of the vaginal microbiota in those two cohorts and were among the first in the field to correlate these findings with levels of the soluble immune mediators. The Antwerp

cohort of a classical healthy population was limited by a small sample size (30 women) but even there, we reported a negative association between the presence of both *L. crispatus* and *L. jensenii* and cellular markers of inflammation – total T-cells, CD3+ HLA-DR+ and CD3+ CD4+ CCR5+ cells. Associations of bacterial species with soluble immune markers was limited in this cohort. Our findings were a lot more interesting during the cross-sectional and longitudinal analysis of microbiota data from the 430 sexually active women from Sub-Saharan Africa. Overall, the sub-cohort of 40 BV-free women followed longitudinally displayed relative stability of individual vaginal microbiota species – these were either consistently present or absent. *L. iners* was consistently present in majority (75%) of these African women but when its concentration was high, *L. crispatus* concentration was low and vice versa. *L. crispatus* was consistently present in 47% of these women without bacterial vaginosis and regularly accompanied by *L. vaginalis*. Surprisingly, *L. jensenii* and *L. gasseri* the dominant species in Antwerp were never present in most African women. In these healthy women, *E. coli* and *P. bivia* were present in at least 90% of the women but their levels were low just as it was with *G. vaginalis* and *A. vaginae*. As expected, bacteria that are generally linked with good vaginal health, *L. crispatus* and *L. vaginalis,* were associated with lower levels of pro-inflammatory cytokines in the cross-sectional analysis although this wasn't observed longitudinally. The protective antimicrobial proteins Elafin and SLPI were higher in women with specific lactobacilli species. In contrast, BV-associated pathobionts *A. vaginae*, *G. vaginalis*, *P. bivia* but also the pathobiont *E. coli* were associated with increased pro-inflammatory cytokines. Cervicovaginal lavage levels of the chemokine IP-10 were significantly higher in the presence of all lactobacilli (except *L. gasseri*) but were lower in women with high *P. bivia*, *A. vaginae* and *G. vaginalis*.

Beyond our assessment of individual bacterial species in vaginal samples, we also assessed for prevalent and incident bacterial vaginosis using Nugent scoring. Just as seen at individual bacterial level, vaginal dysbiosis (specifically BV) was characterized by a clear pro-inflammatory signature in terms of soluble immune mediators and reduced levels of IP-10, SLPI and total protein. When we assessed women with incident BV, mean concentrations of lactobacilli species were significantly lower but mean concentrations of *G. vaginalis*, *A. vaginae* and *P. bivia* were higher compared to their previous visits when they were BV negative.

Sexual activity and lack of a menstrual cycle due to progestin use were both found to be disruptive of the vaginal microbiota, mainly resulting in lower numbers of lactobacilli species (*L. iners* and *L. crispatus* respectively). Even though our sample size for the longitudinal sub-study was also small, a higher 'composite qPCR vaginal health score' (suggestive of better vaginal health) was associated with lower levels of the pro-inflammatory cytokines IL-1α, IL-8 and IL-12(p70) and higher IP-10 concentrations. Another surprising finding was the higher *in vitro* antiviral activity against HIV_{Bal} and HIV_{VI829} by CVL from women with BV compared to women without BV and with the highest concentrations of *L. crispatus* in our cohort.

Summary of important associations with soluble and cellular immunological markers in the female genital tract

	Lower levels	Higher levels
Bacterial species		
Lactobacilli		
Lactobacillus crispatus	IL-8, IL-1α, IL-1β, GM-CSF, IL-12, CD3+HLADR+, CD3+CD4+CCR5+, Total T-cells	IP-10
Lactobacillus vaginalis	IL-1α, MIP-1β, IL-12, IL-1β	SLPI, IP-10
Lactobacillus iners		IP-10, Elafin
Lactobacillus jensenii	CD3+HLADR+, CD3+CD4+CCR5+, Total T-cells	GM-CSF, SLPI, IP-10
Lactobacillus gasseri		IL-1RA, IL-12, GM-CSF
BV-associated bacteria		
Gardnerella vaginalis	IP-10	IL-8, IL-12, IL-1α, IL-1β
Atopobium vaginae	IP-10	IL-8, IL-12, IL-1α, IL-1β
Prevotella bivia		IL-1β, IL-8
Other		
Escherichia coli		IL-1RA, GM-CSF, IL-12, IL-12
		IL-6, IL-8, G-CSF, IP-10, MIP-1β
Age		IL-12, MIP-1β, IL-8
Lifetime no. of sexual partners		IL-1α, IL-1β, IL-6, IL-12, MIP-1β, IP-10, IL-8, G-CSF, Elafin, SLPI, Total protein
No. of sexual partners within 3 months		IL-1α, IL-1RA, MIP-1β, IL-8
Parity		IL-1β, IL-6, IL-12, MIP-1β, IL-8, G-CSF
Vaginal discharge		IL-1β, IL-6, IL-12, IL-1RA, MIP-1β, Il-8, G-CSF, Total protein
Cervical epithelial abnormalities		Il-1β, IL-6, IL-12, IL-1RA, MIP-1β, IL-8, G-CSF, Total protein
Colposcopic findings		IL-12, MIP-1β, IL-8
Ectopy	CD14+HLADR+	IL-1β, IL-6, IL-12, IL-1RA, MIP-1β, IP-10, IL-8, G-CSF, Total protein, Total monocytes, CD3+CD4+CCR5+
Reproductive tract infections		IL-1RA, Total protein
HSV-2		IL-8, Elafin
Recent vaginal sex	Elafin, Total protein	
Prostate-specific antigen		IL-1α, IL-12,
Product used to wash/clean/dry/tighten vagina		
BV by Nugent score	IP-10, Total protein	IL-1α, IL-1β, IL-6, IL-12, IL-8,
Vaginal pH	IP-10, GM-CSF, Elafin, Total protein	IL-1β, IL-12, IL-8,
Cervical mucus on speculum examination	IL-1RA	IL-1β, IL-6, MIP1β, IL-8, G-CSF
Cervical mucus colour	IL-1α, IL-1RA, Elafin, Total protein	IL-1β, IL-6, MIP-1β, IL-8, G-CSF

7.2 Importance of safety biomarkers

7.2.1 Cellular biomarkers

An early study by Stafford *et al* among female volunteers using 100mg of nonoxynol-9 daily reported an influx of HIV susceptible immune cells (lymphocytes and macrophages) in their genital mucosa raising suspicions about the safety of this virucidal surfactant (25). In our study of healthy Caucasian women in Antwerp, cervical ectopy and the presence of haemoglobin in cell supernatant were generally associated with higher percentages of cellular markers of inflammation. These cellular inflammation markers were reduced in the presence of specific lactobacilli species (*L. crispatus* and *L. jensenii*) known to be associated with good vaginal health. Depending on whether one was in the luteal or follicular phase of their menstrual cycle, we also observed differences in total monocytes and T-cells. Our results point to other physiological factors that would need to be considered as potential confounders in assessing the effects of candidate microbicides on immune cells in the female genital tract. A study by Cohen *et al* (503) that used cervical cytobrushes to collect cells from women in Kisumu, Kenya and San Francisco, USA reported a higher number and proportion of activated endocervical CD4+ T cells in the women from Kisumu compared to women from San Francisco. These researchers postulated that such differences could explain, at least in part, the increased risk in HIV acquisition among women from sub-Saharan Africa. In the context of microbicide safety, their results add to other epidemiological factors to be considered when looking at the female genital tract immunological milieu.

Unfortunately, we were unable to effectively characterize cellular immunity in the FGT samples of women from Sub-Saharan African in our study. Endocervical cell samples from the Caucasian women in Antwerp were processed within 30 minutes of collection with subsequent dipstick testing for leucocytes and haemoglobin and phenotypic characterization by flow cytometry. Because technical expertise and equipment for flow cytometry was lacking at our field sites in Rwanda, Kenya and South Africa, samples for cellular analysis were locally processed, frozen and shipped to Antwerp for analysis. Upon thawing, majority of the cells were found to be dead making it impossible for flow cytometric characterization of the few remaining cells. Herein lies the challenge with cellular characterization of FGT samples in microbicide trials many of which take place in resource-limited settings. In the study by Cohen *et al* (503), samples from the Kisumu site were also cryopreserved and shipped to a central laboratory in the US for analysis. Even though they were able to perform flow cytometry on these cells, the overall number of cervical cells were higher at the San Francisco site and they acknowledge that sampling (and possibly maybe processing and storage?) may contribute to the differences observed. Other studies report yields of between 0.01 to 1 x 10^6 viable cells from cytobrush cervicovaginal sampling (504, 505). Bere and colleagues (505, 506) attempted *in vitro* expansion of cytobrush-derived T cells and had the best success on day 7 with anti-CD3/28 beads and IL-2, IL-15 and IL-7. These methodological challenges limit analyses of cellular immune markers to phenotypic characterization but it would be even more interesting

to do functional characterization of endocervical cells. A standardized protocol should be developed for the assessment of fresh as well as frozen endocervical cells to be used in different clinical trials for comparability of results. A caveat is that cells may produce different cytokine and change expression of surface markers due to stress post-collection meaning that analysis of fresh cell samples will always be superior.

7.2.2 Soluble markers of inflammation

The presence of soluble immune markers in the female genital tract does not necessarily imply inflammation of the genital tract mucosa. Both pro- and anti-inflammatory soluble factors have been described in the vaginal secretions of healthy women (376, 382, 507) and various groups have generated data on these soluble markers in HIV-positive, high risk HIV-negative, low risk HIV-negative and microbicide trial participants. The balance between pro-inflammatory and anti-inflammatory immune markers is what determines whether they will be protective or harmful in the context of microbicide application and HIV acquisition (figure 18). Certain pro-inflammatory markers such as IL-1 and TNF-α are regulated by endogenous antagonists such as IL-1RA (376, 508) and the soluble receptors TNF-RI, II (376, 509) respectively resulting in protection and a balance of the pro-inflammatory effects. Based on work done largely with a cohort of HIV-exposed seronegative female sex workers in Nairobi but also confirmed in other cohorts, the importance of this innate immunity balance between serine proteases and serine protease inhibitors in the female genital mucosa has been established (370, 434, 510-513). Serine proteases such as myeloblastin aid HIV infection at the genital mucosa by causing endothelial cells to secrete chemoattractant chemokines such as IL-8 and MCP-1 with resultant increased inflammation and recruitment of HIV target cells such as T-cells to the site of infection. These proteases can also compromise female genital tract epithelial integrity by degradation of extracellular matrix proteins and basement membrane proteins such as collagen. This exposes underlying target cells to potential HIV infection. Such deleterious effects are countered by serine protease inhibitors such as those of the serpin family, elafin and SLPI through different mechanisms. These include the maintenance of epithelial integrity by regulation of host or pathogen proteases; anti-inflammatory activities such as the inhibition of cathepsin G that has been shown to enhance HIV replication *in vitro*; binding of membrane proteins in such a way that it inhibits the fusion of HIV and host cell membrane as done by SLPI and; direct inhibition of HIV replication by elafin and specific serpins. These serine protease inhibitors have been associated with HIV-esposed seronegative status in the studies mentioned above. One of those studies (370) importantly demonstrated both upregulation of serpin A5 and concommittant downregulation of myeloblastin confirming the fact that that balance is important in ensuring protection from HIV infection. Other markers such as IL-6 and IL-8 do not have these self-regulating mechanisms. For all of them, an excessive pro-inflammatory reaction in the female genital tract can result in a breach of the mucosal lining thereby recruiting and exposing underlying target cells to HIV infection. Selhorst *et al* (514) recently demonstrated an increase in HIV infection with genital inflammation and interestingly showed that genital inflammation (as defined by increase in

pro-inflammatory immune markers) allowed less fit or low infectivity viruses to establish HIV infection. It is also known that some of these pro-inflammatory cytokines such as IL-6 and IL-1 can cause HIV-1 replication in previously latently infected cells (515-517). Monitoring of these soluble markers during microbicide trials is therefore important to assess whether the microbicide candidate is safe or could potentially increase the risk of HIV acquisition by trial participants even in the absence of visible mucosal surface irritation.

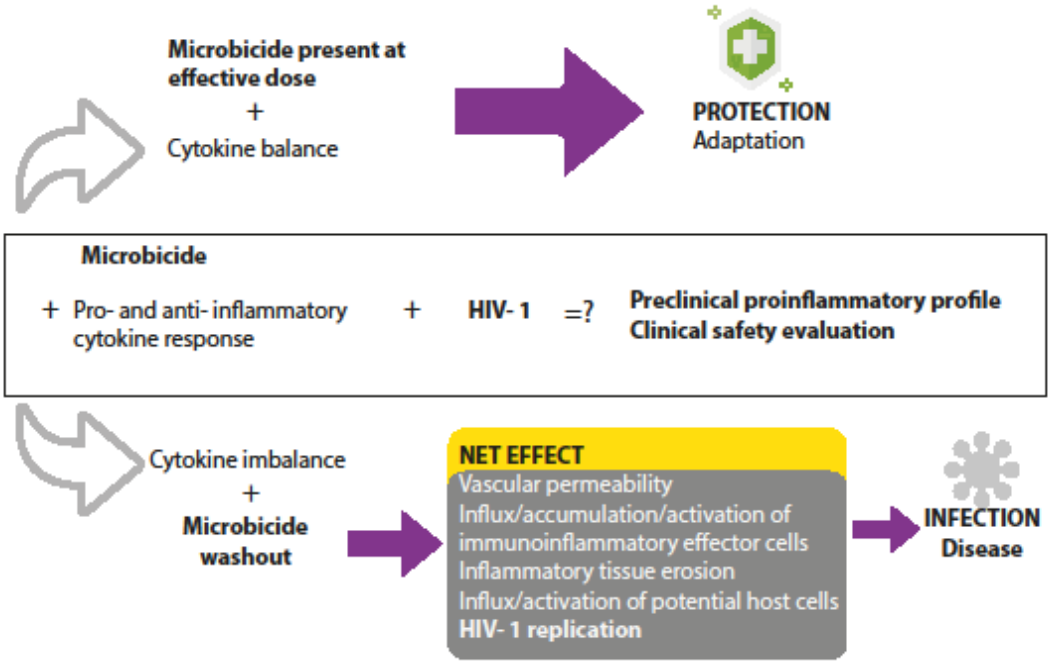

Figure 18 The net effect of anti-HIV-1 microbicide products depends on the balance between the proinflammatory and anti-inflammatory host factors and the effective anti-HIV-1 dose (adapted from (384))

7.2.3 Towards a more limited, representative panel for clinical use

We selected a panel of 12 proteins [inflammatory cytokines Interleukin-1α (IL-1α), IL-1β, IL-6 and IL-12(p70); anti-inflammatory cytokine IL-1 receptor antagonist (IL-1RA); CC chemokine macrophage inflammatory protein 1 beta (MIP-1β); CXC chemokines IFN-γ –induced protein (IP-10) and IL-8; growth factors granulocyte-macrophage colony-stimulating factor (GM-CSF); granulocyte colony-stimulating factor (G-CSF); elafin and β-defensin] for our studies based on an initial run with 27 analytes with results from two different populations in the USA and Rwanda (392). In our different study cohorts, we went ahead to describe how these analytes varied in concentration within and between women with different epidemiological, physiological, pathological and behavioural constructs. Some of our findings such as the inflammatory nature of vaginal dysbiosis (BV) were consistent with previous *in vitro* and *in vivo* observations while others such as increased antiviral activity of BV-infected CVL with high inflammatory markers were counter-intuitive.

Putting together all the data from both the Antwerp and Sub-Saharan African studies we conducted, we propose pro-inflammatory IL-1 (α and β), IL-6, IL-8, IL-12(p70); anti-inflammatory IL-1RA; the chemokine IP-10; and the antimicrobial protein SLPI as a smaller panel of soluble biomarkers for consideration for safety measurement of candidate vaginal microbicides. The pro-inflammatory markers we propose were repeatedly increased by the physiological, behavioural and pathological factors discussed above and a new microbicide candidate that causes a pro-inflammatory signature characterized by the same markers is likely to cause more harm than good. If, on the other hand, it suppressed the expression of IP-10 and SLPI as did BV, it would erode the protective effect these proteins have against HIV infection. As discussed by Fichorova *et al* (2004), some of these markers such as the IL-1 group are best assessed as ratios (*IL-1RA-to-IL-1(α+β)*) to eliminate the possibility of false positives. This would occur, for example, when a recorded increase in IL-1α and/or IL-1β is accompanied by concomitant secretion of IL-1RA that binds and regulates the pro-inflammatory cytokines.

During the present PhD process, some of the biomarkers we suggest (IL-1α, IL-1β, IL-8, SLPI and the IL-1RA-to- IL-1(α+β) ratio) were independently (with possible contribution from our earlier work) fronted as potential safety endpoints in a study conducted in 60 healthy premenopausal women by Mauck *et al* (2013) to explain the failure of nonoxynol-9 and CS. In an early study in HIV positive women, Fichorova *et al* (2004) also suggest IL-1, IL-1RA, IL-6 and IL-8 as good candidates because of their correlation with HIV-1 viral load or diseases status. Scott *et al* (385) measured a panel of 12 cytokines in cervicovaginal samples using the Luminex immunoassay in three different laboratories and demonstrated good interlaboratory agreement for absolute values of IL-1β, IL-6 and IL-8 but also reliable measurement of intra-subject variation for IL-1β and IL-6. This is an important finding in the context of comparison of biomarker values across different studies and study sites.

Outside of microbicide safety, it's interesting to note that Masson and colleagues (489) started with a panel of 42 markers and were able to narrow down on concomitant increased IL-1β and decreased IP-10 as biomarkers of genital inflammation. In their study, these biomarkers identified a large proportion (77%) of HIV-uninfected women who had an STI or BV while only 19% of them had been identified by syndromic screening. Other groups have worked with other markers and there will be need for consensus on which ones are the best to take forward. If we can agree on a smaller panel with four or so markers, that will be better especially in the context of clinical trials in limited resource settings.

7.3 Methodological aspects of soluble immune markers

There are several methodological considerations to keep in mind in the assessment of these soluble immune markers in the female genital tract. To begin with, there is sample collection which can be done in a variety of ways. i) Cervicovaginal lavages (CVL). This is the method we used in which the cervix and lateral vaginal walls are washed or flushed with a buffer such as normal saline and collected for analysis. ii) A small volume but less diluted sample can be collected using devices such as diaphragm cups that are placed over the cervix iii) Swabbing

of lateral vaginal walls with cytobrushes or swabs to collect fluid and iv) Absorption of cervicovaginal fluid using wicks such as sponges, tampons or strips. A good sample collection method has been described as one that is easy to perform, does not cause trauma in the female genital tract, is reproducible, does not over-dilute the sample, ensures collection of sufficient volumes to conduct multiple assays downstream and does not interfere with quantification of immune biomarkers in these assays (by for example, blood contamination) (518).

The few studies that have compared these sample collection methods demonstrated that cervicovaginal lavage gives better reproducibility in cytokine measurement compared to cervical wicks (518); though not directly related to soluble immune biomarkers, CVL was also superior to cervical swabs for HIV RNA assessment in the FGT (519); the limitation of diaphragm methods is that the sample collected is limited to secretions originating from the endo- and ectocervix missing out on vaginal secretions. A recent study that compared sample collection in pregnant women using a polyvinyl acetol sponge for cervical fluid and a menstrual cup for cervicovaginal fluid collection found both methods to be robust for cytokine measurement with the cup having the added advantage of ease of self-insertion and larger sample volume (520). Once samples have been collected, careful consideration has to be given to processing and storage to ensure sample integrity and the validity of downstream assays. It is advisable to process samples in low temperatures to prevent degradation of cytokines. In the same vein, one can end up with false negative or low cytokine measurements if samples are processed or stored in containers with high affinity for proteins. The presence of haemoglobin, mucus and albumin in the matrix utilized for sample collection and processing could also interfere with measurement of soluble immune markers using immunoassays.

Several commercially available bioassays or ELISAs are utilized in the actual measurement of soluble immune markers in female genital tract samples. Multiplex assays such as the Luminex and BioPlex offer the advantage of utilizing small sample volumes to assess several soluble markers. This is important when sample volumes are limited or required for performing other assays such as anti-viral activity measurement in the context of determining the safety of microbicide candidates. Most of these assays were developed for cytokine detection in serum or plasma and have only recently been modified for cytokine detection in CVL. In developing panels for multiplex assays, one has to be careful to avoid cross-reactivity of analytes and background interference from other proteins such as soluble receptors and other cytokine precursors. Efforts have been made to identify optimal sample dilutions for reproducible measurement of different soluble immunological mediators (384) but also to decrease CVL acidity as this can interfere with the measurement of some cytokines.

In our assays, we attempted to include IL-1RA in the BioPlex panel but 70% of the undiluted CVL samples had concentrations of this analyte that were above the highest standard used in this assay. This analyte was then analysed on its own using the single-spot Meso Scale Discovery electrochemiluminescence assay with appropriate sample dilutions (about 100x).

IL-8 is another of our proposed inflammation biomarker that would require a 10x screening dilution for a 10ml CVL sample. On the other end of the spectrum are cytokines whose concentrations are so low that they fall below the lower limit of detection of these immunoassays. In our initial assays among healthy, sexually active women in Antwerp, 47% of IL-12(p70) and 67% of GM-CSF were below the lower limit of quantification. In the cohort of 430 women from sub-Saharan Africa, these values were 14% and 7% for 12(p70) and GM-CSF respectively, using the same assay. This goes to further illustrate differences in concentrations of soluble immune markers between populations. Even though highly sensitive assays may be able to detect extremely low levels of these analytes in CVL samples, these measurements may not be reproducible and would require validation. Validation can, partly, be based on percentage coefficients of variation (CV) between duplicate measurements of the same sample. The lower the % CV, the better.

In an effort to assess the importance of technical and biological variables affecting the measurement of pro-inflammatory cytokines (IL-6 and IL-1β) standards in different laboratories, a seminal study conducted in 12 laboratories across 4 countries using 14 immunoassays and 4 detection platforms was performed (521). In summary, there was general reproducibility across laboratories for each immunoassay. Sources of variability were differences in sampling fluid (complex biological fluids such as CVL versus simple, protein-free matrix such as saline routinely used in sample collection and processing); assay type (inter-assay variability with electrochemiluminescence-based assays performing better in terms of detecting fold differences in analytes compared to Luminex-based assays) and; detection platforms (absorbance, chemiluminescence, electrochemiluminescence and fluorescence). Importantly, the authors bring out the need to agree on the biological significance of observed fold-differences in cytokine measurement. Clearly, if the relevant scientific community is to settle on a set of standard biomarkers of inflammation in the female genital tract for use in microbicide development and beyond, there will need to be a collaborative effort to validate and agree on the biomarkers themselves but also technical issues to do sample collection, processing, storage, the best assays to be utilized and interpretation of results from these assays.

7.4 A role for proteomics in CVL analysis?

Since the turn of the century, there has been a significant amount of work done toward proteomic characterization in the female genital tract in pregnant, HIV-exposed seronegative, health and BV-positive women with treatment outcomes (466, 522-528). In all, over 1,000 unique proteins have been identified in cervical mucus and cervicovaginal fluid (reviewed in (529). The largest category of these proteins were functionally classified as being involved in protein metabolism and modification (17%) followed by those involved in immunity and defense (10%). Twenty one percent of these proteins were localised in the cytoplasm and 21% were extracellular proteins.

A recent study by Borgdorff *et al* (525) compared relative protein abundance in human cervicovaginal samples assayed by mass spectrometry among women in four groups whose VMB was dominated by i) *Lactobacillus crispatus* ii) *Lactobacillus iners* iii) those with moderate vaginal dysbiosis and iv) those with severe dysbiosis. Their results supported previous findings of inflammation during dysbiosis but also disruption of the mucosal barrier as evidenced by mucus and cytoskeletal alterations, increased proteolytic activity and increased lactate dehydrogenase as a marker of cell death among others. Clearly, proteomics as a methodology brings rich data to the table in as far as characterization of the female genital tract is concerned going beyond what we did with the immunoassays approach.

That said, this method is not without its challenges. As an example, in the study by Borgdorff and colleagues, IL-1β, IL-6 and IL-8 all of which have been characterized in BV-positive women, including in our work, were not detected by mass spectrometry. This is probably a function of the dynamic range in proteomic studies – the difference between the highest and lowest protein concentrations in the sample under study reported to be between 6-12 orders of magnitude. High-abundance proteins tend to mask the detection of low-abundance proteins like cytokines and growth factors yet both classes could have biological/physiological significance. This can be overcome by initial fractionation to separate complex proteins and peptide mixtures into different constituents before further separation is done by liquid or gel chromatography for analysis. Reverse phase columns (most frequently used in proteomics) can then separate cytokines very well. It is rather the very low concentration that makes identification/quantification by liquid chromatography – mass spectrometry of cytokines in cervicovaginal fluid so difficult and why ELISA still remains the gold standard. Targeted mass spectrometry (MRM – mass reaction monitoring) approaches perform better and they have better specificity and sensitivity. As with the other procedures described above care is needed to ensure experimental reproducibility and careful handling of samples to reduce variability that could occur because of issues such as post-translational modifications of proteins because of tissue integrity, changes in oxygenation and differences in time before sample freezing or fixation. Here as well, there have been results that indicate the importance of physiological context and how protein synergism is necessary for biological function.

One of the earliest female genital tract proteomics study by Venkataraman and colleagues (466) demonstrated intrinsic anti-HIV-1 activity in human vaginal fluid. This antiviral activity could be attributed to the cationic polypeptide fraction of vaginal fluid from which 18 immunity and defense proteins were identified. Interestingly, majority of them (thirteen) did not have individual antiviral activity but worked together to provide protection from HIV-1 infection. These kinds of studies that demonstrate function are important because the mere presence of a peptide or protein in cervicovaginal fluid does not indicate functionality – and that would be the limit of the single cell genomic approaches. There is then a place for functional genomics approaches such as metagenomics, transcriptomics, proteomics and even metabolomics in the characterization of the female genital tract in the context of microbicide development and the prevention of HIV infection. These would enable

researchers to, for example, identify RNA sequences that are not translated into proteins or that end up as different proteins because of alternative splicing mechanisms, proteolysis and other post-translational modifications. Beyond the expansion of methodological approaches to characterize the female genital tract, researchers should possibly consider the protective or harmful role of other microbes in the female genital tract. This would include fungi, viruses and other parasites with overlapping or at least interactive causal pathways for inflammation and disease in the female genital tract.

7.5 The vaginal microbiome

Our study confirmed what was largely known from other studies, that lactobacilli species dominate the healthy vagina while these are reduced in vaginal dysbiosis and are replaced by multiple bacteria. Clearly though, it's not an all-or-nothing situation; BV-associated bacterial species do exist in healthy women but in low numbers and women with BV will also have low numbers of lactobacilli. Lactobacilli species are protective in the context of preventing HIV-acquisition partly through the different mechanisms previously discussed including the fact that they are non-inflammatory. When the female genital tract is dominated by non-lactobacilli species, it is considered deleterious to the woman's health even when the intermediate outcome is asymptomatic such as is the case with the majority of BV cases. Even then, the risk of mucosal inflammation and subsequent HIV infection still remains.

Different groups have gone ahead to rightly assess the effect of microbicide candidates on the vaginal microbiome. In an effort to further understand why some of the early microbicides failed, Ravel and colleagues (530) assessed the effect on the vaginal microbiome of 6% cellulose sulphate and 4% nonoxynol-9 applied twice daily over 13.5 days by healthy, sexually abstinent women with regular menses. Both products were shown to shift the vaginal microbiome to one dominated by strict anaerobes with reduced lactobacilli presence as assessed by 16S rRNA pyrosequencing. The third group treated with hydroxyethylcellulose (HEC)-based universal placebo demonstrated *Lactobacillus* (specifically *L. crispatus*)-dominance throughout the treatment period. This may partially explain product failure in the clinical trials. Other topical microbicide candidates tested for their effect on vaginal (and rectal) microbiota include the Griffithsin gel rectally applied in rhesus macaques with favourable proteome and microbiota outcomes (531); vaginally applied monoclonal antibodies 2F5, 4E10 and 2G12, all of which did not cause adverse change in vaginal microbiota as assessed by on-site microscopy as well as qPCR (532); vaginal rings containing dapivirine (25mg), maraviroc (100mg) or a combination of the two all used continuously for 28 days by HIV-negative women in the US with no significant change in Nugent Score, Lactobacillus species, Candida species or even quantity of biofilm (533) and; a recent study that described the association of rectally applied 1% TFV gel with mucosal proteome changes involving epidermal development (534).

This small selection of studies described above also illustrate another important consideration, that the assessment of the vaginal microbiome in the context of microbicide

safety can be done using microscopy, culture-dependent (not illustrated above) and by non-culture-based qualitative and (semi-) quantitative molecular methods such as sequencing and qPCR. Whatever the methodology, the effect of candidate microbicides on the vaginal microbiome is a critical endpoint that should be included in clinical trials.

7.6 Physiological context matters

Having discussed microbicide safety roles of vaginal cellular and soluble markers as well as the vaginal microbiome in isolation, it is important to look at considerations that reflect the fact that these factors operate in the context of a very complex and multifaceted female genital tract with both reproductive and immunological functions. On the whole, and considering the variety of internal and external exposures encountered, the female genital tract is quite robust and resistant to infection. Even HIV, the focus of our subject matter, has a low probability of infection per heterosexual encounter. This is testimony to the immunological resilience of the genital tract. That said, infections do occur and the global magnitude of the HIV/AIDS pandemic should continue to drive us to pursue prevention options that are both safe and effective – especially for the women that bear a disproportionate burden of infection. In our quest for safe vaginal microbicides, interpretation of *in vitro* experimental data should always be contextualized.

Our findings of higher CVL antiviral activity among women with BV compared to women without BV are a reminder of the importance of considering physiological context in interpreting these data. It is common knowledge that BV is associated with increased risk of HIV transmission and acquisition, most likely due to the after effects of genital inflammation and the bacteria associated with BV. Why then would CVL from BV-positive women have higher antiviral activity? Obviously, the limitation of this assay is that it only considers the effect of the soluble factors on an *in vitro* model of HIV infection in a cell line. *In vivo*, inflammation that results from infection with BV could result in a breach of the vaginal mucosa with subsequent recruitment and infection of susceptible cells in a way that overrides any protective effects of antimicrobial proteins present in vaginal fluids.

A study by Tan *et al* (535) calls attention to seminal factors in heterosexual HIV transmission by demonstrating that polyanions (a class of compounds to which the failed candidate microbicides cellulose sulphate, PRO200 and carrageenan belong) facilitate the formation of semen-derived enhancers of virus infection resulting in aggravated HIV infection *in vitro*. This is in agreement with another study by Zirafi and colleagues (536). Testing candidate compounds in the presence of semen, mimicking sexual activity, is therefore crucial in creating a viable physiological context similar to that in which heterosexual transmission of HIV occurs.

Lastly, we demonstrated a wide range in concentrations of soluble immune markers within and between women in our studies. Establishing 'normal' ranges is important in terms of identifying aberrations caused by candidate microbicides and other physiological, pathogenic or behavioural factors. But this can be difficult when, as we have discussed, there are

geographical differences in these markers in women targeted for microbicide trials. What works then is collection of baseline samples and comparing these soluble marker concentrations to those from samples collected during and after product use. While this is sufficient for purposes of demonstrating absolute changes, what can be challenging to determine is what these fold-changes in soluble markers mean *in vivo*. Does a 2-fold change in a cytokine with low (or high) baseline concentrations have biological significance? How can this be determined? It is important to remember that CVL samples, even with the advantages we have discussed are a significant dilution of markers found *in vivo*. Should we be expecting these samples to have, for example, significant antiviral activity as an undiluted samples would? Is this where undiluted samples such as those collected by menstrual cups have an advantage over CVL samples? Taken together these discussions highlight the importance of physiological context in the interpretation of results with cellular, soluble and microbiota safety biomarkers.

For the very same reason that we advocate for the consideration of physiological context, assessment of vaginal microbicide safety (or indeed HIV prevention as a whole) should also be done using the variety of ways we have discussed above and others. In summary, we are of the opinion that assessment of vaginal microbicide safety should include physical examination of mucosal irritation, an assessment of increase in cellular and soluble markers of inflammation, its effect on the vaginal microbiome and the correlation of all these components with each other and other factors discussed below. Early studies relied on physical examination to identify mucosal aberrations. Though insufficient on its own, this methodology has relevance and should continue to be used as recommended by the US FDA in its guidelines. Pelvic examination by visual inspection and speculum examination should be used to identify evidence of genital toxicity including epithelial disruption.

The effect of microbicide candidates on the upper female genital tract (fallopian tubes, ovaries and the uterus) should also be investigated. For obvious reasons, the FDA does not recommend biopsies unless with observed evidence of toxicity. Secondly phenotypic and functional characterization of soluble and cellular markers of inflammation should also be carried out where possible. Characterization of the cellular fraction could be as simple as utilizing strip tests to describe extravasation of leucocytes into the FGT or microscopic characterization of epithelial cells. To the extent to which it is feasible, the vaginal microbiome should also be described by a combination of microscopy, culture-dependent and culture-independent i.e. molecular methods. The utility of molecular methods for the characterization of the vaginal microbiome (in tandem with culture methods) cannot be understated. Its advent allowed for the description of bacterial species that were hitherto undiscovered or unexplored because they could not be cultured. It has been interesting to learn that certain bacteria such as *Gardnerella vaginalis* and *Lactobacillus iners* have genotypic variations (and probably even sub-species) (309, 537, 538) that may explain their differences in virulence since they are present in the vagina in both eubiosis and dysbiosis.

7.7 General implications of research findings

Our study was designed to characterize the female genital tract in the context of microbicide development; specifically, to identify different factors that result or are associated with sub-clinical inflammation that increases the chances of HIV acquisition in women. Our findings are relevant in that context but the implications are wider than that. We briefly discuss a few implications of our findings covering microbicide development and use but also other relevant contexts.

Development as diagnostic markers: BV and sexually transmitted infections are highly prevalent in resource-limited settings such as the continent of Africa. In the absence of sophisticated machinery for diagnosis of BV and STIs or sufficient, skilled human resources to carry out these tests, syndromic screening in which physicians rely on clinical symptoms is frequently used to identify women in need of treatment. Unfortunately, not all infected women show these symptoms, while even in the absence of symptoms, these women have been shown to have genital inflammation that potentially increases their risk of HIV infection. In fact, it is highly unlikely that a woman will come to a medical facility for consultation if they are not symptomatic. It would be most useful then for clinicians and ultimately for the women we all serve to have access to point-of-care tests that be used to detect biological markers associated with health and those with risk implications to allow for early intervention to reduce the risk of HIV infection. Jespers and colleagues (483) evaluated a qPCR tool based on molecular VMB data that performed well in the diagnosis of BV in a large and diverse cohort of African women. Its predictive value was based on a combination of log-transformed concentrations of *G. vaginalis*, *A. vaginae* and *Lactobacillus* genus with 93.4% sensitivity and 83.6% specificity. As previously discussed, a different group in South Africa described concurrent increase in IL-1β and decrease in IP-10 as being predictive of a treatable genital condition (489). These markers correctly identified 77% of HIV-uninfected women with BV or an STI while clinical symptoms only identified 19% of them. All these markers and the factors associated with their variation are described in our study and by other groups. If they could be developed into point-of-care tests that can be used by clinicians in resource-limited settings, they will be useful tools in the fight against sexually transmitted infections (including HIV) and in ensuring the general health of women at risk of infection with other sequelae. Serious consideration should be given to the development of a semiquantitative point-of-care test that combines assessment of increase in IL-1β, decrease in IP-10 and the composite qPCR vaginal health score.

"Bugs on drugs" – BV, general HIV prevention and beyond: A recent study by Klatt et al (539) demonstrated a three-fold reduction in the efficacy of the tenofovir microbicide gel in prevention of HIV infection among African women with a *Gardnerella vaginalis*-dominated vaginal microbiome compared to women with a *Lactobacillus*-dominated vaginal microbiome. In this study, the women with G. vaginalis dominance also had lower levels of genital tenofovir compared to women with Lactobacillus dominance and this was attributed to metabolism of

the drug by G. vaginalis. Thankfully, a different study showed no modulatory effect of the vaginal microbiome on the efficacy of oral PrEP among African women with high BV prevalence and high adherence to PrEP (540).

As reported by Hardy *et al* (541), BV has been associated with biofilm formation and this can happen even on intravaginal rings used as contraceptives or as multipurpose devices that also target prevention of HIV infection. Unfortunately, research on the effect of biofilm formation and drug release from these intravaginal devices is limited. More work needs to be done to understand this.

Lastly, a strong case for the utilization of probiotics for disease (BV, vulvovaginal candidiasis and other urinary tract infections) prevention and treatment was made by Gregor Reid in a recent review (542). It is common knowledge now (including from our study) that Lactobacilli species are protective. The idea of transferring these species into the vaginal tract, where they are naturally found, to offer protection is one that should be explored with sufficient checks to ensure that they are not deleterious in any way to recipients. Here as well, our study offers insight into the contexts in which these different vaginal microbiota are found, their immunomodulatory effect and the different factors that would need to be considered as potential confounders in studies that seek to understand safety in HIV prevention.

As recently reviewed by Unemo *et al* (543), the reproductive and obstetric sequelae of bacterial vaginosis are quite vast: increased risk of acquiring STIs (chlamydia, gonorrhoea, HSV-2 and HIV); transmitting HIV to male sexual partners; pelvic inflammatory disease; spontaneous abortion; pre-term birth; low birthweight and postpartum endometritis. Qualitatively, BV affects a woman's self-esteem, their sexual relationships and quality of life. To further compound this, the rates of recurrence post-treatment are quite high. Surely more work needs to be done to understand BV and how to mitigate its effect on the quality of life of the women affected by this condition.

Considering contraceptives: Our study demonstrated the pro-inflammatory and Lactobacillus-depleting nature of progestin use. The alteration of the vaginal microbiome and induction of inflammation in the female genital tract (that then promotes local HIV replication) are potential mechanisms that could explain current linkage of hormonal contraceptive use with increased risk of HIV infection. A study by Fichorova *et al* (544) demonstrated that this interactive effect could be modulated by genital tract infections that may or may not be diagnosed, possibly explaining the differences observed in studies on the link between hormonal contraceptive use and risk of HIV infection. An early study showed that combined oral contraceptives upregulated the expression of the HIV co-receptor CCR5 on CD4+ T cells in the FGT of healthy women (545). Because a lot of the studies that link the use of specific hormonal contraceptive use to increased risk of HIV acquisition were observational, there is need for stronger evidence to inform contraceptive policy and programming. There is a large ongoing open-label randomised clinical trial looking at DMPA, levonorgestrel and intra-uterine device (IUD) use with HIV infection as the primary endpoint that is designed to fill this

knowledge gap http://echo-consortium.com/ and results are expected next year (2019). This and other studies will need to consider the factors we have identified in our study as potential modifiers of the hormonal contraception-HIV infection relationship because of their effect on the VMB as well as cellular and soluble immune markers.

7.8 The broad perspective

7.8.1 Bringing it all together

We have described physiological, behavioural, cellular, soluble and vaginal microbiome markers that are directly or indirectly involved in protection or increasing the risk of infection of women with STIs including HIV. We have also discussed sampling, processing, storage, measurement, methodological and interpretation considerations that would allow for these factors to be usefully considered to offer protection for women, their sexual partners but also their dependents. All these things are like pieces of a puzzle – they need to be put together to make sense. We advocate for an increase in research consortia that carry out coordinated multi-dimensional studies that seek to further define each piece of the puzzle and then bring it next to the other pieces for holistic interpretation and application. This may actually call for coordination of consortia with different funding sources – especially in light of recent developments at the US National Institutes of Health that suggest a reduction in funding for large scale trials of topical microbicides such as gels and douches, focusing instead on systemic interventions [https://to.pbs.org/2ybxbLw and http://bit.ly/2yghxOG]. Better coordination will also allow for standardization of methodology with subsequent cross-comparison of study results moving the field of HIV prevention further and faster.

7.8.2 Beyond basic science

"PrEP works – if taken" That is an introductory statement that I have seen used over and over by many, including myself, in presentations to different audiences – both scientific and lay. Therein lies the tail end of HIV prevention efforts that determine whether they will bear fruit or not. It is the common thread that runs from the CAPRISA 004 vaginal microbicide gel study (130), the FEM-PrEP oral pre-exposure clinical trial (546) and more recently, The Ring Study that was assessing the efficacy of a monthly vaginal ring for HIV prevention (133). For these different products, efficacy was higher when adherence was good. Interestingly, it is the younger girls whom seem to struggle with adherence yet they are the group at highest risk of HIV infection even compared to young men the same age. Reasons for non-adherence to both topical and oral microbicides are varied; fear of or experience of side effects, societal stigma associated with PrEP use, lack of community support (sexual partner, peers and/or parents), fear of or experience of social harm such as intimate partner violence and, negative interactions with health service providers at health facilities among other reasons. I say this to show that for microbicides and other HIV prevention interventions to work, they must go beyond safety in the vaginal environment. These products must be comfortable and acceptable for the end users. For this to happen, these end users – women to be specific –

have to have their voices heard about which prevention products they prefer and how they prefer them. This has to happen early on in product development and not after resources have been spent on a product with excellent antiviral activity but that is poorly accepted by the women who need them. Indeed, it will not only be one type of product – just as it is with contraceptives, women may need options from which to choose depending on their stage in life and relationship circumstances. One woman may be happy with using condoms for HIV prevention because her partner supports her while another may require a more discreet option such as oral PrEP or the vaginal ring because she is in an abusive relationship. Even then, we have to have enabling societal, health systems and policy environments for effective use of these prevention options. How do you handle the risk of HIV resistance to ARVs because of sub-optimal doses of PrEP or use of PrEP when one is acutely infected? Will national governments be able to sustain these HIV prevention interventions in the absence of donor funding – a situation that seems ever more likely with reduction in HIV funding over the years? Is there enough local capacity in lower and middle income countries to conduct high quality basic and implementation science studies in HIV prevention? Do women (and other populations) actually want to use topical and oral PrEP or are we pushing it down their throats and genital tracts so to speak? - Think about the low PrEP uptake among eligible populations even in countries such as the US where PrEP has been available for the longest period. Effective HIV prevention will require a combination approach with biomedical, behavioural and structural strategies if we are to have a real impact on the epidemic.

7.8.3 More than just statistics

> To most physicians, my illness is a routine incident in the Rounds, while for me it's the crisis of my life.
>
> - Anatole Broyard

At a personal level, a few events over the course of my PhD study impacted me and my view of the work we are all doing towards HIV prevention. In 2011, during a EUROPRISE meeting in Stockholm, Sweden, a number of PhD students and I had the privilege of meeting Prof. Françoise Barré-Sinoussi, a French scientist who got a Nobel Prize in Physiology or Medicine for the discovery of HIV. That a person of her calibre would take time to mentor and inspire young, upcoming scientists was a major motivator towards keeping the work going in HIV prevention. During my stint in Prof. Raina Fichorova's lab in Boston, I will never forget the many times she talked of the CVL samples we had in the lab as 'gold'. Emphasizing the fact beyond them being just biological samples, they represented women who had given of their time and resources to support science and research aimed at improving the health of others. As such, we owe it to them to carry out our work with the highest level of dedication and integrity for the betterment of generations to come. In fact, it was outside a café on the street outside her lab that I saw this sign that said "The struggle of today, is not altogether for today – it is for a vast future also" President Abraham Lincoln – annual message to Congress

December 3, 1861. But the greatest impact was at a conference in Boston where a HIV positive woman was making a presentation on the impact of research and medicine on her life and she had a slide showing how her CD4 count had improved over time as a result of her access to ARVs! An ode to the fact that our work makes an impact – whether as basic scientists or public health experts. Those and other examples continue to inspire me (and others) in my current work in the public health sector doing HIV prevention implementation science work in Kenya and beyond.

8. References

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9. Summary

Majority of HIV transmission occurs through heterosexual intercourse. Even though the per-act probability of HIV transmission is low (happens in 3 to 50 out of every 10,000 heterosexual acts), it does occur and it has resulted in an epidemic that is over 30 years old and yet to be fully controlled. The HIV/AIDS epidemic has affected more women than men, especially in Sub-Saharan Africa where 60% of those living with HIV are women. Biologically, this could be explained by factors such as a higher surface area that is exposed to HIV in women compared to men during penetrative heterosexual intercourse, a higher viral load in men compared to women and developmental factors such as ectopy that exposes the monolayered endocervical mucosal surface to potential viral infection. Beyond the biological, other risk factors can be contextualized in existing gender inequity where relational or social power play increases the vulnerability of women to HIV infection. These risk factors include inability by women to negotiate condom use, intimate partner violence that has been linked with increased risk of HIV infection, exploitative relationships between young girls and much older men and economic disadvantage that drives young girls and women to engage in risky sexual behavior to provide for themselves and their dependents.

HIV prevention options exist but majority, including condoms and voluntary medical male circumcision are male-controlled. Vaginal microbicides that can be formulated as gels, rings, films, sponges, suppositories, implants and creams are a female-controlled HIV prevention option that could potentially tackle the barriers faced by the male-controlled options. Development of these vaginal microbicides has come a long way. First generation microbicides were non-specific surfactants whose mechanism of action involved the disruption of viral membranes. These functioned well *in vitro* but were found to be ineffective in subsequent clinical trials and one, nonoxynol-9, even increased the risk of HIV infection in participants who used it over time. It was postulated that this happened because nonoxynol-9 caused sub-clinical inflammation that breached the vaginal mucosal epithelia and exposed/attracted HIV target cells. This last finding necessitated the search for biomarkers that could be used to ensure the safety of candidate microbicides in future trials. This is the context in which this PhD project was carried out.

In order to identify these safety biomarkers in the female genital tract (FGT), it would be important to begin with a comprehensive characterization of the FGT. This is because as part of its reproductive function, there are changes in endogenous hormones and structure as part of the monthly menstrual cycle. These changes also occur when women use exogenous hormones for birth control. The lower female genital tract also has a robust soluble and cellular immune milieu of local and systemic origin. It is obvious that FGT immunity could be affected by the reproductive changes described above but also by sexual activity, vaginal practices as well as pathogenic and non-pathogenic organisms that find their way into the genital tract. In this context, one would need to know how all these factors affect the levels of

safety biomarkers so that any changes observed during the application of candidate microbicides can be correctly attributed to either the microbicide or these other factors. Beyond microbicide safety, this characterization would be useful for general HIV prevention.

We started out with a group of 30 healthy Caucasian women in Antwerp, Belgium aged 19 to 38 years and characterized cellular and soluble immunity in the lower FGT. Endocervical cells were collected using flocked swabs gently turned 360 degrees in the endocervical canal and analyzed using flow cytometry. Cervicovaginal lavage samples were collected and assayed for the concentration of ten cytokines/chemokines (inflammatory cytokines Interleukin-1α (IL-1α), IL-1β, IL-6 and IL-12(p70); anti-inflammatory cytokine IL-1 receptor antagonist (IL-1RA); CC chemokine macrophage inflammatory protein 1 beta (MIP-1β); CXC chemokines IFN-γ – induced protein (IP-10) and IL-8; growth factors granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF)), total protein and two antimicrobial proteins (SLPI, elafin and β-defensin) using ELISA and a multiplex immunoassay. This panel was selected based on an initial runs with 27 analytes with results from two different populations in Rwanda and the USA. Additionally, we did a qPCR characterization of their vaginal microbiome. These same methods (apart from cellular immune characterization) were applied to conduct both a cross-sectional and longitudinal characterization of the female genital tract in 430 sexually active women from Sub-Saharan Africa (Kenya, Rwanda and South Africa).

Briefly, we demonstrated a wide range in concentrations of selected soluble immune mediators in cervicovaginal lavages. We also described a number of physiological and behavioural factors that were associated with differences in concentrations of these immune mediators in the lower female genital tract. These include cervical ectopy, recent sexual activity as indicated by the presence of prostate-specific antigen in CVL, multiple sexual partners, abnormal vaginal discharge, cervical epithelial findings (abrasion, laceration, ecchymosis, petechiae, erythema or ulcer) and cervical mucus that were mostly associated with higher levels of soluble immune mediators indicative of inflammation such as IL-1 (α and β), IL-6, IL-8, IL-12(p70) or suppression of regulatory or protective immune mediators such as IL-1RA and SLPI. Our studies also demonstrated variations in soluble makers of inflammation associated with the stage in a woman's menstrual cycle. Importantly, longitudinal characterization showed that exogenous progestin use was associated with a pro-inflammatory female genital tract profile (increased IL-8, IL-12(p70) and MIP-1β).

Overall, the sub-cohort of 40 BV-free women followed longitudinally displayed relative stability of individual vaginal microbiota species – these were either consistently present or absent. *L. iners* was consistently present in majority (75%) of these African women but when its concentration was high, *L. crispatus* concentration was low and vice versa. *L. crispatus* was consistently present in 47% of these women without bacterial vaginosis and regularly accompanied by *L. vaginalis*. *L. jensenii* and *L. gasseri* the dominant species in Antwerp were never present in most African women. In these healthy women, *E. coli* and *P. bivia* were

present in at least 90% of the women but their levels were low just as it was with *G. vaginalis* and *A. vaginae*. As generally expected, bacteria that are generally linked with good vaginal health, *L. crispatus* and *L. vaginalis,* were associated with lower levels of pro-inflammatory cytokines in the cross-sectional analysis. The protective antimicrobial proteins Elafin and SLPI were higher in women with lactobacilli species. In contrast, BV-associated *A. vaginae*, *G. vaginalis* and *P. bivia* were associated with increased pro-inflammatory cytokines. Cervicovaginal lavage levels of the chemokine IP-10 were significantly higher in the presence of all lactobacilli (except *L. gasseri*) but were lower in women with high *P. bivia*, *A. vaginae* and *G. vaginalis*. Vaginal dysbiosis (specifically BV) was characterized by a clear pro-inflammatory signature in terms of soluble immune mediators and reduced levels of IP-10, SLPI and total protein. When we assessed women with incident BV, mean concentrations of lactobacilli species were significantly lower but mean concentrations of *G. vaginalis*, *A. vaginae* and *P. bivia* were higher compared to their previous visits when they were BV negative. Sexual activity and lack of a menstrual cycle due to progestin use were both found to be disruptive of the vaginal microbiota, mainly resulting in lower numbers of lactobacilli species (*L. iners* and *L. crispatus* respectively). A higher 'composite qPCR vaginal health score' (suggestive of better vaginal health) was associated with lower levels of the pro-inflammatory cytokines IL-1α, IL-8 and IL-12(p70) and higher IP-10 concentrations.

Based on our findings, we propose pro-inflammatory IL-1 (α and β), IL-6, IL-8, IL-12(p70); anti-inflammatory IL-1RA; the chemokine IP-10; and the antimicrobial protein SLPI as a smaller panel of soluble biomarkers for consideration for safety measurement of candidate vaginal microbicides. As discussed in detail in the thesis, these markers (with the exception of IL-12(p70) and SLPI) have been suggested by other groups as potential markers of safety because of good interlaboratory agreement in their measurement, low intra-subject variation, correlation with HIV-1 viral load or disease status and genital inflammation. Additionally, the assessment of microbicide safety should include clinical examination for visible signs of vaginal epithelia irritation and the effect of candidate microbicides on cellular markers of inflammation as well as the vaginal microbiome. To do this, there will be a need for large-scale coordination for harmonization of sample collection, processing, assays and the interpretation of results. An ideal outcome would be point-of-care tools that can be easily used in clinical setting in resource-limited settings to predict microbicide safety but more importantly, to identify women with increased susceptibility to HIV infection due to genital inflammation or vaginal dysbiosis. These women can then be targeted with combination sexually transmitted infections (including HIV) prevention and possible multiple prevention technology interventions for better sexual and reproductive health outcomes.

10. Samenvatting

HIV wordt vooral overgedragen via heteroseksuele betrekkingen. Ook al is de kans op transmissie klein per seksueel contact (ongeveer 3 tot 50 per 10,000), toch heeft deze vorm van besmetting geleid tot een pandemie, die nog steeds niet onder controle is. De HIV/aids epidemie treft vrouwen meer dan mannen, vooral in Sub-Saharisch Afrika, waar 60 % van de besmette personen vrouwen zijn. Dit kan gedeeltelijk door biologische factoren verklaard worden, zoals de grotere oppervlakte die in de vrouwelijke genitale tractus blootgesteld wordt aan het virus, de gemiddeld hogere virale lading bij mannen dan bij vrouwen en de ontwikkeling van “ectopie” die het éénlagig endovervicale slijmvlies blootstelt aan het virus. Behalve deze biologische factoren, is er ook genderongelijkheid, die via relationele en sociale factoren de kwetsbaarheid van vrouwen verhoogt. We vermelden hier de machteloosheid van vrouwen om condoomgebruik af te dwingen, het intieme partnergeweld dat leidt tot verhoogd risico op HIV besmetting, de ongelijke machtsverhouding in seksuele relaties tussen oudere mannen en jonge meisjes en de economische problemen, die ertoe kunnen leiden dat meisjes en vrouwen risicovel seksueel gedrag vertonen, om te voorzien in hun eigen levensonderhoud en dat van degenen, die van hen afhangen.

Er zijn heel wat mogelijkheden voor HIV preventie, maar de meeste zoals condooms en vrijwillige medisch begeleide circumcisie zijn onder controle van mannen. Vaginale microbiciden, die geformuleerd kunnen worden in gels, ringen, sponzen, zetpillen, implantaten en crèmes, worden door vrouwen gecontroleerd en kunnen daarom de bovengenoemde gender problemen oplossen. De ontwikkeling van deze producten neemt echter veel tijd. De eerste generatie microbiciden waren niet-specifieke surfactanten, die werkten via disruptie van virale membranen. Ze werkten goed in vitro, maar niet in klinische trials en het surfactant nonoxynol-9 verhoogde zelfs de besmettingskans over de tijd. Dit ongewenste effect werd verklaard door subklinische ontsteking, die het vaginale slijmvlies beschadigde en doelwitcellen voor HIV aantrok. Deze bevindingen tonen de noodzaak aan om biomerkers te zoeken, die de veiligheid van toekomstige kandidaat-microbiciden eerst moet aantonen vooraleer in fase 3 trial te gaan. De zoektocht naar deze biomerkers is precies de doelstelling van dit thesiswerk.

Om deze veiligheids-biomerkers te vinden in de vrouwelijke genitale tractus, moet het vaginale milieu eerst goed gekarakteriseerd worden. Als onderdeel van het voortplantingsfunctie en meer bepaald gedurende de menstruele cyclus, vinden er veranderingen in endogene hormoonspiegels en in morfologie plaats. Dit soort veranderingen kan ook het gevolg zijn van de exogene hormonen bij contraceptie. De lagere vrouwelijke genitale tractus is ook gekenmerkt door cellulaire en humorale immuun factoren, die zowel van lokale als systemische origine zijn. Dit lokale immuunsysteem wordt uiteraard beïnvloed door de hoger beschreven reproductieve veranderingen, maar ook door seksuele activiteit, vaginale praktijken en natuurlijk ook door pathogene en niet-pathogene micro-organismen,

die zich in de vagina bevinden. In deze context, willen we eerst weten hoe al deze factoren de veiligheids-biomerkers beïnvloeden, om te kunnen oordelen over veranderingen tijdens het gebruik van microbiciden. Daarenboven zal deze oefening ook nuttig zijn voor HIV preventie in het algemeen.

We begonnen met een groep van 30 gezonde blanke vrouwen in Antwerpen, België in de leeftijdscategorie van 19 tot 38 jaar en we karakteriseerden cellulaire en humorale immune merkers in de lagere genitale tractus. Endocervicale cellen werden verzameld met behulp van wieken die zachtjes 360 graden werden gedraaid in het endocervicale kanaal. De cellen werden dan vers geanalyseerd met behulp van flowcytometrie. Cervicovaginale lavage-monsters werden verzameld en getest op de concentratie van tien cytokinen / chemokinen (inflammatoire cytokinen Interleukin-la (IL-1α), IL-1β, IL-6 en IL-12 (p70); anti-inflammatoire cytokine IL-1 receptor antagonist (IL-1RA); CC chemokine macrofaag inflammatoire proteïne 1 bèta (MIP-1 ß); CXC chemokinen IFN-y-geïnduceerde proteïne (IP-10) en IL-8; groeifactoren granulocyt-macrofaag coloniestimulerende factor (GM- CSF) en granulocyt coloniestimulerende factor (G-CSF)); totaal eiwit en ook nog antimicrobiële eiwitten (SLPI, elafine en β-defensine) met behulp van ELISA en een multiplex immunoassay. Dit panel werd geselecteerd op basis van een eerste run met 27 analyten met resultaten van twee verschillende populaties in Rwanda en de VS. Daarnaast hebben we een qPCR-karakterisering van hun vaginaal microbioom gedaan.

Deze zelfde methoden (afgezien van cellulaire immunologische karakterisering) werden ook toegepast om zowel een transversale als longitudinale karakterisering van het vaginale milieu uit te voeren bij 430 seksueel actieve vrouwen uit Sub-Sahara Afrika (Kenia, Rwanda en Zuid-Afrika). Tijdens dit onderzoek hebben we een breed bereik aangetoond in de concentraties van geselecteerde oplosbare immuun-modulatoren in cervicovaginale lavages (CVL). We beschreven ook een aantal fysiologische en gedragsfactoren die verband hielden met verschillen in concentraties van deze merkers in de vagina. Dit waren cervicale ectopie, recente seksuele activiteit zoals aangetoond door de aanwezigheid van prostaatspecifiek antigeen in CVL, meerdere seksuele partners, abnormale vaginale secreties, cervicale epitheliale bevindingen (schaafwonden, scheuren, ecchymose, petechiën, erytheem of zweertjes) en verlies van cervicaal slijm. Deze factoren waren meestal geassocieerd met hogere niveaus van immuunmediatoren die indicatief zijn voor ontsteking, zoals IL-1 (α en β), IL-6, IL-8, IL-12 (p70) of verlaging van regulerende of beschermende modulators zoals IL-1RA en SLPI. Onze studies toonden ook variaties aan in oplosbare ontstekingsproducten die samenhangen met het stadium in de menstruatiecyclus van een vrouw. Belangrijk is dat longitudinale karakterisatie aantoonde dat exogeen progestageengebruik geassocieerd was met een pro-inflammatoir profiel van het vrouwelijke geslachtsorgaan (verhoogd IL-8, IL-12 (p70) en MIP-1β).

In een longitudinale sub-cohort van 40 aanvankelijk bacteriële vaginose (BV)-vrije vrouwen, werd een relatieve stabiliteit van individuele vaginale microbiota-soorten waargenomen- deze

waren ofwel consistent aanwezig of afwezig. L. iners was consistent aanwezig in de meerderheid (75%) van deze Afrikaanse vrouwen, maar als de concentratie hoog was, was de L. crispatus-concentratie laag en vice versa. L. crispatus was consequent aanwezig in 47% van deze vrouwen zonder bacteriële vaginose en werd regelmatig vergezeld door L. vaginalis. Daarentegen, waren L. jensenii en L. gasseri, de dominante soorten in Antwerpen, nooit aanwezig in de meeste Afrikaanse vrouwen. Bij deze gezonde vrouwen waren E. coli en P. bivia aanwezig bij minstens 90% van de vrouwen, maar hun niveaus waren laag, net als G. vaginalis en A. vaginae. Zoals te verwachten was, werden bacteriën die in het algemeen geassocieerd zijn met goede vaginale gezondheid, namelijk L. crispatus en L. vaginalis, tevens geassocieerd met lagere niveaus van pro-inflammatoire cytokinen uit de cross-sectionele analyse. De beschermende antimicrobiële eiwitten Elafin en SLPI waren hoger bij vrouwen met “goede” lactobacilli-soorten. Daarentegen waren BV-geassocieerde A. vaginae, G. vaginalis en P. bivia geassocieerd met verhoogde pro-inflammatoire cytokines. Cervicovaginale lavage niveaus van de chemokine IP-10 waren significant hoger in de aanwezigheid van alle lactobacilli (behalve L. gasseri) maar waren lager bij vrouwen met hoge P. bivia, A. vaginae en G. vaginalis.

Wanneer dan vaginale dysbiose (specifiek BV) optrad, was er ook een duidelijke verhoging van pro-inflammatoire merkers en verlaagde niveaus van IP-10, SLPI en totaal eiwit. Tijdens incidente BV waren de gemiddelde concentraties van lactobacilli soorten significant lager, maar de gemiddelde concentraties van G. vaginalis, A. vaginae en P. bivia waren hoger in vergelijking met hun vorige bezoeken toen ze BV negatief waren. Seksuele activiteit en het ontbreken van een menstruatiecyclus als gevolg van het gebruik van contraceptiva op basis van progestageen bleken allebei de vaginale microbiota te verstoren, voornamelijk resulterend in een lager aantal lactobacilli-soorten (L. iners en L. crispatus respectievelijk). Een hogere 'samengestelde qPCR-score voor vaginale gezondheid' (suggestief voor een betere vaginale gezondheid) was geassocieerd met lagere niveaus van de pro-inflammatoire cytokines IL-1α, IL-8 en IL-12 (p70) en hogere IP-10-concentraties.

Op basis van onze bevindingen stellen we beperkt panel voor van oplosbare biomerkers voor de evaluatie van de veiligheid van kandidaat vaginale microbiciden. Het bestaat uit de pro-inflammatoire IL-1 (α en β), IL-6, IL-8, IL-12 (p70); het ontstekingsremmende IL-1RA; het chemokine IP-10; en de antimicrobiële proteïne SLPI. Bovendien moet de beoordeling uiteraard ook een klinisch onderzoek omvatten naar zichtbare tekenen van vaginale epithelia-irritatie en het effect van kandidaat-microbiciden op cellulaire markers van ontsteking evenals op het vaginale microbioom. Om dit te bereiken, zal er behoefte zijn aan grootschalige coördinatie voor de harmonisatie van monsterverzameling en -verwerking; de standaardisatie van meetmethoden en de interpretatie van resultaten. Het zou ideaal zijn om point-of-care tools te ontwikkelen die gemakkelijk kunnen worden gebruikt in een klinische setting met beperkte middelen. Dit zou de veiligheidsmonitoring van microbiciden trials vergemakkelijken, maar wat nog belangrijker is: zo kunnen we ook vrouwen identificeren met een verhoogde vatbaarheid voor HIV-infectie als gevolg van genitale inflammatie of vaginale dysbiose. Deze vrouwen kunnen dan worden geörienteerd naar een preventie programma’s

van seksueel overdraagbare infecties (waaronder HIV) en mogelijke interventies met meervoudige preventie-technologie (inclusief contraceptie) voor betere resultaten op het gebied van seksuele en reproductieve gezondheid.

11. Curriculum Vitae

♦ ACADEMIC QUALIFICATIONS

Year: 2010 to present
University of Antwerp/Institute of Tropical Medicine, Antwerp.
PhD Biomedical Sciences

Year: 2008 to 2009
Hebrew University of Jerusalem: Master of Public Health (M.P.H)
Final Grade 90.72%

Year: 2005 – 2007
Vrije Universiteit Brussels: MSc. Molecular Biology
Magna cum Laude

Year: 1998 – 2003
Egerton University BSc. Biomedical Science & Technology
Second class honors upper division

Year: 1997 – 1998
Institute of Advanced
Technology (I.A.T) Windows/MS Dos ; certificate of participation
MS Word ; grade 'A'
MS Excel ; grade 'A'

Year: 1993 – 1996
Maseno School Kenya Certificate of Secondary Education (KCSE)
Grade B+

♦ THESIS LIST

MSc. THESIS
Title: Molecular and epidemiological characterization of HIV-1 transmission in a heterosexual cohort of discordant and concordant couples in Dakar, Senegal.
Promoter: Prof. Luc Kestens
Institute of Tropical Medicine, Antwerp
Co-promoters
Dr. Wim Jennes
Ing. Leo Heyndrickx
Grade Received: 17 out of 20.

Master of Public Health THESIS
Title: Intimate Partner Violence and HIV Infection among Married and Cohabiting Women in Kenya
Supervisor:
Dr. Moshe Hoshen
Grade Received: 90.67%

PUBLICATIONS:

Kababu M, Sakwa E, Karuga R, Ikahu A, Njeri I, **Kyongo J,** Khamali C, Mukoma W. *Use of a counsellor supported disclosure model to improve the uptake of couple HIV testing and counselling in Kenya: a quasi-experimental study.* BMC Public Health. 2018 May 18;18(1):638. doi: 10.1186/s12889-018-5495-5.

Jespers V, **Kyongo J,** Joseph S, Hardy L, Cools P, Crucitti T, Mwaura M, Ndayisaba G, Delany-Moretlwe S, Buyze J, Vanham G, van de Wijgert JHHM. *A longitudinal analysis of the vaginal microbiota and vaginal immune mediators in women from sub-Saharan Africa* Sci Rep. 2017 Sep 20;7(1):11974. doi: 10.1038/s41598-017-12198-6.

Kyongo JK, Crucitti T, Menten J, Hardy L, Cools P, Michiels J, Delany-Moretlwe S, Mwaura M, Ndayisaba G, Joseph S, Fichorova R, van de Wijgert J, Vanham G, Ariën KK, Jespers V. *A cross-sectional analysis of selected genital tract immunological markers and molecular vaginal microbiota in Sub-Saharan African women with relevance to HIV risk and prevention.* Clin Vaccine Immunol. 2015 May;22(5):526-38. doi: 10.1128/CVI.00762-14. Epub 2015 Mar 11. PMID: 25761460

Gautam R, Borgdorff H, Jespers V, Francis SC, Verhelst R, Mwaura M, Delany-Moretlwe S, Ndayisaba G, **Kyongo JK,** Hardy L, Menten J, Crucitti T, Tsivtsivadze E, Schuren F, van de Wijgert JH; Vaginal Biomarkers Study Group. Correlates of the molecular vaginal microbiota composition of African women. BMC Infect Dis. 2015 Feb 21;15:86. doi: 10.1186/s12879-015-0831-1. PMID: 25887567

Jespers V, Crucitti T, Menten J, Verhelst R, Mwaura M, Mandaliya K, Ndayisaba GF, Delany-Moretlwe S, Verstraelen H, Hardy L, Buvé A, van de Wijgert J; **Vaginal Biomarkers Study Group.** Prevalence and correlates of bacterial vaginosis in different sub-populations of women in sub-Saharan Africa: a cross-sectional study. PLoS One. 2014 Oct 7;9(10):e109670. doi: 10.1371/journal.pone.0109670. eCollection 2014

Arien, K.K., **J.K. Kyongo**, and G. Vanham, *Ex vivo models of HIV sexual transmission and microbicide development.* Curr HIV Res, 2012. **10**(22264048): p. 73-78.

Jennes W, **Kyongo JK**, Vanhommerig E, Camara M, Coppens S, Seydi M, Mboup S, Heyndrickx L, and Kestens L. (2012) *Molecular Epidemiology of HIV-1 Transmission in a Cohort of HIV-1 Concordant Heterosexual Couples from Dakar, Senegal.* PLoS ONE 7(5): e37402. doi:10.1371/journal.pone.0037402 Abstract accepted for oral presentation at the 2008 International Conference on AIDS and STIs in Africa (ICASA).

Kyongo JK, Jespers V, Goovaerts O, Michiels J, Menten J, Fichorova RN, Crucitti T, Vanham G, and Arien KK. (2012) *Searching for Lower Female Genital Tract Soluble and Cellular Biomarkers: Defining Levels and Predictors in a Cohort of Healthy Caucasian Women.* PLoS ONE 7(8): e43951. doi:10.1371/journal.pone.0043951 Presented in part at the 2012 International Microbicides Conference (Sydney, Australia).

♦ CONFERENCES ATTENDED/PARTCIPATION

23-27 July 2018 22nd International AIDS Conference (Amsterdam, the Netherlands) *Oral presentation*
28-31 May 2017 Maisha HIV and AIDS conference (Nairobi, Kenya) *Oral presentation*
17-21 October 2016 HIV research for prevention (Chicago, USA) *Poster presentation*
28-31 October 2014 HIV research for prevention (Cape Town, South Africa) *Poster presentation*
10-12 February 2014 International Master of Public Health Alumni East Africa Regional Meeting (Kampala, Uganda) *Health Systems in Global and Local Contexts*
29 May-1 June 2013 12th Congress of International Society for Immunology of Reproduction: ISIR (Boston, US) *Oral and poster presentations*
21-23 May 2013 30 Years of HIV Science (Paris, France) *Poster presentation*
12-20 February 2013 International Master of Public Health Alumni Conference (Jerusalem, Israel) *Oral presentation*
15-18 April 2012 International Microbicides Conference (Sydney, Australia) *Oral Presentation*
2-5 May 2011 EUROPRISE conference (Stockholm, Sweden) *Session discussant*
19-22 October 2009 AIDS Vaccine 2009 (Paris, France)
25-30 March 2007 Keystone Symposia (Whistler, Canada)
22-24 March 2005 3rd Annual Africa Genome Initiative (AGI) Conference (Nairobi, Kenya)

♦ AWARDS/SCHOLARSHIPS

2018 AIDS 2018 travel grant to attend and 22nd International AIDS Conference in Amsterdam, the Netherlands
2016 HIV research for prevention travel grant to attend and present poster at HIVR4P conference in Chicago, USA
2014 HIV research for prevention travel grant to attend and present poster at HIVR4P conference in Cape Town, South Africa
2014 Pears Family Charitable Foundation scholarship to attend the International Master of Public Health Alumni East Africa Regional Meeting in Kampala, Uganda.
2013 Clinical Science ESRI Young Investigator Award for presentation on "Identifying biomarkers of increased HIV transmission in African populations"
2013 ISIR/ASRI travel grant to attend ISIR conference in Boston, USA.
2013 Pears Family Charitable Foundation scholarship to attend the International Master of Public Health Alumni conference/continuing education workshop in Jerusalem, Israel.
2012 International Microbicides Conference scholarship to attend and do an oral presentation in Sydney, Australia
2010 The Legacy-Heritage Fund Ltd. and the Pears Family Charitable Foundation scholarship to attend Hebrew University alumni meeting/continuing education workshop.
2009 AIDS Vaccine conference scholarship in Paris, France
2008 The Legacy-Heritage Fund Ltd. and the Pears Family Charitable Foundation MPH scholarship at the Hebrew University of Jerusalem.
2007 Bill and Melinda Gates Foundation Global Travel Health Awards to attend Keystone Symposium in BC, Canada.
2005 Vlaamse Interuniversitaire Raad (VLIR), University Development Co-operation (UOS) Masters scholarship.

♦ PROFESSIONAL ASSOCIATIONS

- Member: International Society for Immunology of Reproduction (ISIR)
- Member: International AIDS Society (IAS)
- Secretary of the Research and Implementation Science Sub-Committee in the National PrEP Technical Working Group
- Member: Kenya HIV Research Coordinating Mechanism (KARSCOM)

♦ LANGUAGES

- Fluent in written and spoken English and Swahili
- Elementary German

♦ INTERESTS

- Communicable diseases and their Public Health implications
- Health behaviour and promotion