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FACTORS ASSOCIATED WITH HIV SUSCEPTIBILITY IN THE FEMALE GENITAL TRACT

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Factors associated with HIV susceptibility in the female genital tract

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

ABSTRACT

The majority of new HIV infections in women are transmitted through vaginal intercourse where the female genital tract (FGT) functions as the portal of viral entry. The aim of this thesis was to characterize mucosal factors within the FGT to better understand potential molecular mechanisms associated with altered HIV-susceptibility.

The vaginal microbiome and the menstrual cycle are associated with altered HIV-susceptibility, but their collective impact on the cervicovaginal milieu remains largely unknown. In **Paper 1**, we studied healthy, Swedish women and observed the largest changes of the genital proteome during the estradiol-dominated ovulatory phase. This phase was characterized by a decrease in neutrophil-associated proteins and pathways and an increase in epithelial barrier-promoting proteins, as compared to the progesterone-dominated luteal phase. Menstrual cycle-related changes in epithelial barrier proteins were enhanced in women with a non-*Lactobacillus* dominated vaginal microbiome. This study showed that female sex hormones modulated genital inflammation and epithelial barrier function and that these changes were further impacted by the vaginal microbiome.

Semen can induce an inflammatory response in the FGT, but its role in HIV-transmission is largely unknown and limited due to a lack of adequate experimental models. In **Paper 2,** we used a genital tissue explant model to show that seminal plasma induced genital inflammation and increased HIV-infectivity, highlighting the importance of including seminal plasma as a factor in HIV-transmission studies. The genital tissue explant model used in this study could also be suitable for studying HIV-transmission and evaluation of microbicides.

Studies in female sex workers (FSWs) at high risk of HIV-infection reveal alterations in the genital mucosal milieu. In **Paper 3**, we used a high-throughput bead-based affinity set-up to evaluate protein expression in genital secretions of another cohort at risk of HIV-infection, namely Kenyan HIV-seronegative women living in HIV-serodiscordant relationships. As compared to HIV-seronegative women in HIV-seroconcordant relationships, we found alterations in genital proteins involved in inflammation and epithelial barrier remodeling pathways. Such phenotype was observed despite low levels of clinical inflammation and high levels of safe sex practices in this cohort. These results suggest that women in HIV-serodiscordant relationships have a unique cervicovaginal environment, and that this may be associated with an altered susceptibility to HIV infection. However further studies would be required to fully elucidate the relationship between the observed phenotype and HIV infection risk.

Observational and experimental studies indicate that use of the injectable progestin-based contraceptive depot medroxyprogesterone acetate (DMPA) is associated with increased risk of HIV. In **Paper 4,** we revealed a transcriptional profile consistent with impaired epithelial integrity and increased immune activation in ectocervical tissues from Kenyan FSWs using DMPA. In situ-based imaging analysis revealed a thinner superficial epithelial layer and an altered distribution of potential HIV-target cells. Collectively, these results suggest that DMPA may weaken the epithelial barrier and contribute to increased HIV-susceptibility.

In summary, female sex hormones, living in a HIV-serodiscordant relationship and seminal factors induce changes in the FGT that may alter HIV-susceptibility. Endogenous and exogenous progesterone/progestins reduce epithelial barrier integrity and induce cervicovaginal inflammation. The knowledge gained from these studies can help guide the development of safe contraceptive methods. The aforementioned factors must be taken into consideration when designing and interpreting results from clinical studies in the HIV-prevention field, and can also help guide the development of prophylactic compounds aimed at reducing HIV-transmission.

LIST OF SCIENTIFIC PAPERS

I. **The vaginal microbiome amplifies sex hormone-associated cyclic changes in cervicovaginal inflammation and epithelial barrier disruption**
FRIDEBORG BRADLEY*, Kenzie Birse*, Klara Hasselrot, Laura Noël-Romas, Andrea Introini, Hugo Wefer, Maike Seifert, Lars Engstrand, Annelie Tjernlund, Kristina Broliden[#], Adam D. Burgener[#].
*Equal contribution. [#]Shared senior authors
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II. **Seminal plasma induces inflammation and enhances HIV-1 replication in human cervical tissue explants**
Andrea Introini, Stephanie Boström, FRIDEBORG BRADLEY, Anna Gibbs, Annelie Tjernlund, Kristina Broliden
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III. **A high through-put bead-based affinity assay enables analysis of genital protein signatures in women at risk of HIV infection**
Anna Månberg*, FRIDEBORG BRADLEY*, Ulrika Qundos, Brandon L. Guthrie, Kenzie Birse, Laura Noël-Romas, Cecilia Lindskog, Rose Bosire, James Kiarie, Carey Farquhar, Adam D. Burgener, Peter Nilsson[#], Kristina Broliden[#]
*Equal contribution. [#]Shared senior authors
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IV. **Transcriptional profiling and imaging analysis reveal impaired epithelial barrier structure in ectocervical tissues from Kenyan women using depot medroxyprogesterone acetate**
FRIDEBORG BRADLEY, Gabriella Edfeldt, Julie Lajoie, Alexandra Åhlberg, Kenneth Omollo, Anastasios Damdimopoulos, Julius Oyugi, Joshua Kimani, Keith Fowke, Annelie Tjernlund, Kristina Broliden
Manuscript.

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
AMP	Antimicrobial peptide
ART	Antiretroviral therapy
BH	Benjamini-Hochberg
BV	Bacterial vaginosis
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CVL	Cervicovaginal lavage
CVS	Cervicovaginal secretions
CXCL	CXC motif chemokine
DC	Dendritic cells
DMPA	Depot medroxyprogesterone acetate
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
FDR	False discovery rate
FGT	Female genital tract
FSH	Follicle-stimulating hormone
FSW	Female sex worker
GnRH	Gonadontropin releasing hormone
gp	glycoprotein
GR	Glucocorticoid receptor
HC	Hormonal contraceptives
HESN	HIV-exposed seronegative
HIV	Human Immunodeficiency Virus
HNP	Human neutrophil peptide
HPA	Human Protein Atlas
HPV	Human papilloma virus
Ig	Immunoglobulin

IL	Interleukin
LC	Langerhans cells
LH	Luteinizing hormone
LME	Linear mixed effect
LMP	Last menstrual period
MFI	Median fluorescence intensity
MPA	Medroxyprogesterone acetate
MS	Mass spectrometry
MSM	Men who have sex with men
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer solution
PEP	Post-exposure prophylaxis
PR	Progesterone receptor
PrEP	Pre-exposure prophylaxis
PSA	Prostate specific antigen
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
SIV	Simian immunodeficiency virus
STI	Sexually transmitted infection
T/F	Transmitter/founder
Th17	T-helper 17 cells
UNAIDS	United Nations Programme on HIV/AIDS

1 BACKGROUND

1.1 INTRODUCTION TO HIV/AIDS

1.1.1 Discovery of HIV/AIDS

In 1981, the first case report of five previously healthy, homosexual young men presenting with *Pneumocystis carinii* pneumonia was published (1). The report concluded that "The above observations suggest the possibility of a cellular-immune dysfunction related to a common exposure that predisposes individuals to opportunistic infections." In the following year, additional cases of young, homosexual men and intravenous drug users with opportunistic infections and Kaposi's sarcoma were identified, and this group of symptoms were called acquired immunodeficiency syndrome (AIDS) (2). The etiology of AIDS was in 1983 discovered to be a virus that would later be named Human Immunodeficiency Virus (HIV)-1 (3, 4), a discovery that resulted in the Nobel Prize in Medicine and Physiology in 2008. In the beginning of the epidemic, HIV was primarily spread in men who have sex with men (MSM) populations, but today the global epidemic is predominately heterosexual in nature, even though certain populations, including MSM, counts for a significant part of the burden in some countries (5). There are two types of the virus, HIV-1 and HIV-2, but the vast majority of cases worldwide are caused by HIV-1, which will here be referred to as HIV.

1.1.2 The HIV epidemic

Since 1980's, HIV has spread across the world, and WHO estimates that 39.5 million people were infected with HIV in 2018 (6). Sub-Saharan Africa bears a disproportionate HIV-burden, and an estimated 65% (25.7 million) of HIV-infected individuals live in Africa (6). Global incidence rates have been declining since the late 1990's, but despite this, a staggering 1.7 million people were newly infected with HIV in 2018, 61% of these in sub-Saharan Africa (6). In 2014, the United Nations Programme on HIV/AIDS (UNAIDS) launched ambitious treatment targets called "90-90-90", stating that by 2020, 90% of HIV-infected individuals will know their HIV status, 90% of all HIV-diagnosed individuals will be on sustained antiretroviral therapy (ART), and that 90% of individuals receiving ART will have viral suppression (7). However, only 60 of the 170 countries included in the annual report by UNAIDS reached all three targets in 2018, and many countries are considered unlikely to reach the targets by 2020 (8). Globally so far, 79% of HIV-infected individuals know their HIV-status and of these, 78% are on ART, and 86% of these show viral suppression (8).

1.1.2.1 HIV in Kenya

The East African nation of Kenya is one of the most affected countries by the HIV epidemic, and will be discussed here since two cohorts in this thesis are Kenyan. The adult HIV-prevalence in Kenya peaked at 10-11% in the mid 1990's (9), and has now declined to

an estimated 4.7% in 2018 as compared to a global prevalence of 0.8% (6). The HIV incidence has declined from 6.2 to 1.02 per 1000 in year 2000 and 2018, respectively (6, 9). Despite such advances of decreased prevalence and incidence rates, 46, 000 individuals are newly infected each year (6). UNAIDS has identified key populations at especially high risk for HIV-infection, including sex workers, MSM, transgender women and intravenous drug users (6). In Eastern and Southern Africa, key populations and their sexual partners account for an estimated 25% of new HIV-infections. In Kenya, the HIV burden among female sex workers (FSWs) remains very high with an estimated HIV-prevalence of 29% in 2011 (10), with no more recent estimates available. Reasons for such high prevalence in FSWs include work-related, including sexual violence, inability to negotiate safe sex practices, criminalization of sex work as well as stigma and discrimination (5). By focusing on reducing such factors, HIV-transmission within this group, and subsequently within the general population, may decrease.

1.1.3 Transmission of HIV

HIV is spread by three main modes of transmission: sexual, parenteral and vertical (mother-to-child), and heterosexual transmission is the most common mode globally (5). Estimated transmission rates vary widely between the different modes of transmission and also between different studies. A meta-analysis estimate the risk of HIV-acquisition for an HIV-negative woman during vaginal intercourse with an HIV-positive man without ART to 0.08-0.30% per sexual act (11). The risk of HIV-acquisition during receptive anal intercourse is substantially higher, estimated at 1.7% per sexual act (11). However, the risk of sexual transmission depends on many other factors in addition to type of sexual practice, such as plasma viral load (12, 13), concordant or recent sexually transmitted infections (STIs) and/or genital inflammation (14-18), genital ulcering (12, 17), cervicovaginal microbiota composition (19), male circumcision (20, 21) and use of hormonal contraceptives (HC) (22-24).

1.2 DEFENSES AGAINST HIV IN THE FEMALE GENITAL TRACT

The female genital tract (FGT) can be divided into the lower (ectocervix and vagina) and upper (ovaries, fallopian tubes, uterus, endocervix) FGT. The lower FGT is lined by a multilayer squamous epithelium, whereas the endometrium and endocervix of the upper FGT is covered by a single-layered columnar epithelium. The lower FGT is the first site of contact for HIV transmitted through vaginal intercourse, and the complex defense mechanism of the FGT against HIV include both anatomical (the mucus layer and epithelium) and biological (antimicrobial factors and immune cells) barriers (Figure 1).

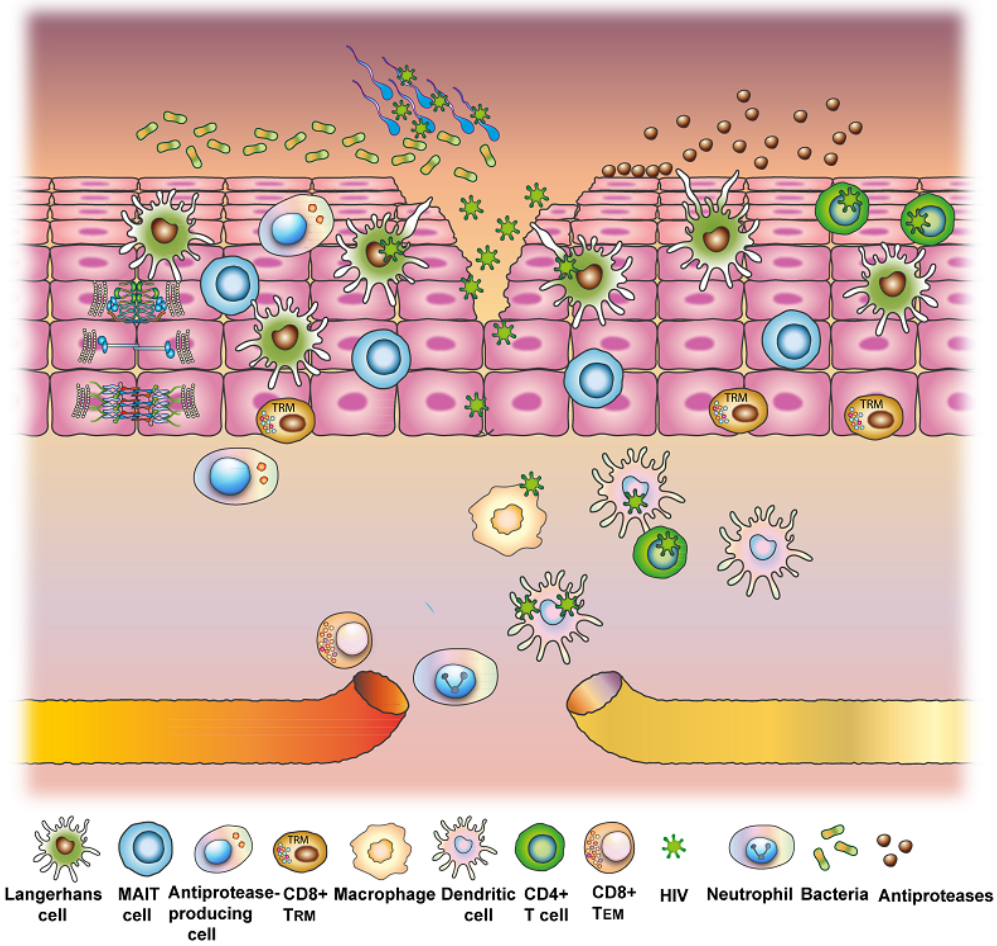

Figure 1. Schematic representation of the epithelium in the female genital tract and suggested mechanisms for heterosexual transmission of HIV. The ectocervix and vagina of the lower female genital tract are lined by a multilayer, stratified squamous epithelium, providing a physical barrier preventing the virus from entering the body. Proposed mechanisms for how the virus can traverse the epithelium is through microabrasions in the epithelium or by uptake by intraepithelial Langerhans cells.

1.2.1 Anatomical barriers to HIV: mucus and the epithelium

In order to establish an infection, the virus must traverse the epithelium of the FGT to reach susceptible HIV-target cells in the epithelium and/or submucosa. Once a small population of HIV-infected cells has been established in the submucosa, the virus is eventually spread to the draining lymph nodes through the lymphatic system (25). In the lymph nodes, massive viral replication occurs and the infection is subsequently disseminated in the body.

However, first the virus and/or virally infected cells must overcome several anatomical barriers. A layer of mucus, produced primarily by endocervical cells, line the epithelium of the cervix and vagina and can provide protecting against HIV by physically trapping virions and seminal cells (26, 27). The mucus lining the endocervix is thicker and more viscous than the mucus lining the ectocervix and vagina, where the mucus from the endocervix is mixed with vaginal fluid. Additionally, commensal bacteria, usually dominated by *Lactobacillus*, line the epithelial cells and contribute to an acidic and anti-inflammatory genital environment which is unfavorable for HIV (19, 28, 29) (see section 1.3.3).

The epithelium underneath the mucus layer forms an additional physical barrier that hinders the virus from reaching target cells and submucosa. The epithelium of the lower FGT is a non-keratinized stratified epithelium that consists of several layers of keratinocytes that undergo programmed differentiation from the basal membrane to the most apical layer before being exfoliated into the vaginal lumen. The nomenclature of the layers within the epithelium varies between studies, but it is usually considered to consist of three layer: the stratum basale/parabasal layer, the suprabasal/intermediate layer and the stratum corneum/superficial layer (30). The thickness of the vaginal and ectocervical epithelium varies but is approximately 150-500 μm and consists of 25-30 cell layers (31-34). The keratinocytes of the stratified epithelium are held together by cell junctions such as tight junctions, adherence junctions and desmosomes, contributing to the barrier function of the epithelium (33, 35-37). The intact squamous epithelium of the vagina/ectocervix is more robust against HIV-invasion than the columnar epithelium of the endocervix, but the surface area of the vagina/ectocervix is more than 15 times that of the endocervix, and the relative contribution of the different sites in establishing initial infection is not known (38). The transformation zone, where the columnar epithelium of the upper FGT meets the squamous epithelium of the lower FGT, is rich in immune cells (including HIV target cells) (39), indicating that this area could be especially susceptible to HIV.

Several mechanisms of how HIV crosses the epithelium have been proposed, such as for the single columnar epithelium of the upper FGT: i) transcytosis/endocytosis of the virus across the epithelium, ii) transmigration of the virus or iii) translocation by dendritic cells (40, 41). To cross the multilayer squamous epithelium of the lower FGT, the virus and/or virally infected cells have been suggested to transmigrate through micro-abrasion in the epithelium and/or by uptake of the virus by intraepithelial Langerhans cells (LC) (see section on HIV target cells below). Furthermore, Carias et al. demonstrated in cervical explants that HIV virions could penetrate intact squamous epithelium and passively diffuse up to 50 μm, but most virions did not diffuse past 10 μm in both ectocervical and endocervical tissues (36). Interestingly, when disrupting cellular junctions, more virus penetrated the tissues and to greater depths, indicating that intact cellular junctions hinder passive diffusion of the virus. In addition, in an in vitro model of monolayer genital epithelial cells, exposure to HIV disrupted tight junctions and increased permeability of the epithelium, allowing virus entry (42). The importance of epithelial thickness has been clearly demonstrated in non-human primate models, where a thin epithelial lining (following progestin treatment) greatly enhances simian immunodeficiency virus (SIV) transmission (43). However, the exact mechanisms of HIV transmission in humans and the effect of the thickness and integrity of the epithelium as such remain largely unknown.

1.2.2 Biological barriers to HIV: antimicrobial factors and immune cells

Cervicovaginal secretions (CVS) bathes the lining of the lower FGT and is composed of a mixture of plasma transudate, fluid originating from the upper FGT, cervical mucus, secretion from serous cells in submucosal glands, and secretions from immune and epithelial cells (44). The composition of CVS is dependent on several factors such as the menstrual cycle (45-48), use of HC (49, 50), genital inflammation (51) and the cervicovaginal microbiome (52).

Antimicrobial peptides (AMPs) are small proteins of the innate immune system that have broad anti-microbial effects against many pathogens, including HIV. CVS contain several AMPs secreted from immune and epithelial cells, such as human α and β-defensins and the cathelicidin LL-37 (53-57) with anti-HIV activity in vitro (58). A study by Venkataraman et al. demonstrated anti-HIV activity only when combining several AMPs at physiological concentrations, suggesting AMPs have a synergetic effect on HIV neutralization (59). However, despite such anti-HIV activity in vitro, the protective role of AMPs against HIV in vivo remains unclear (58). Some AMPs are even associated with increased risk for HIV-acquisition (60, 61), presumably by recruiting HIV target cells to the mucosa. Additional proteins in genital secretions are emerging as having potential protective roles against HIV, such as the proteins SLPI and PI3/elafin as well as other anti-proteases, especially serine protease inhibitors (serpins) (57, 59, 62-64). However, it seems as if it is the combined effect of proteins/AMPs on the genital mucosa, rather than individual components, that modulate HIV-susceptibility (58, 65, 66).

There is an abundance of innate immune cells in the FGT that contribute to the defense against HIV. They recognize pattern associated molecular patterns, such as HIV single-stranded ribonucleic acid (RNA) or the surface glycoprotein (gp) 120 on the HIV viral particle (67, 68), triggering the release of pro-inflammatory cytokines and the promotion of an antiviral environment (69). Importantly, both immune and epithelial cells produce numerous cytokines/chemokines and AMPs with important immunomodulatory roles (70, 71), such as recruitment of additional immune cells (72, 73). However, the presence of immune cells in the FGT is a double-edged sword since some immune cells can also function as early target cells for HIV (74).

1.2.2.1 HIV target cells in the FGT

In order to enter a target cells, the surface molecule gp120 on the HIV-virus binds to a cluster of differentiation (CD) 4 receptor on the target cell, inducing a conformational change allowing the binding to one of the co-receptors for HIV, mainly CC chemokine receptor (CCR) 5 and CXC chemokine receptor 4. This causes the gp41 protein on the viral surface to penetrate the plasma membrane, allowing viral fusion and penetration of the host cell. HIV-strains that utilize the CCR5 and CXC chemokine receptor 4 coreceptors are referred to as "R5" or "X4" tropic strains, respectively. Despite the fact that blood, semen, CVS and rectal secretions contain both R5 and X4 tropic strains, in general only R5 strains are responsible for primary transmission events (75).

$CD4^+$ T-cells in the FGT are considered the main target cells for the HIV virus (76, 77), and recent studies have identified subsets of $CD4^+$ T-cells, namely T-helper 17 cells (Th17) and cells expressing the integrins $\alpha_4\beta_7$ or $\alpha_4\beta_1$, that seem especially susceptible to the virus (78, 79). $CD4^+$ T-cells in the FGT are mainly infected through the CD4/CCR5 receptors. $CD4^+$ T-cells are located in the epithelium and in the underlying submucosa in the FGT (80, 81).

Other HIV-target cells in the FGT include macrophages and dendritic cells (DC), including a subset of DC called LC (82-84). These cells express CD4 and CCR5, and also, to varying

degrees depending on subtype, C-type lectin receptors such as DC-SIGN, Langerin and Mannose-receptor, that can functions as receptors for the virus (85-87). However, the consequences of HIV uptake via the different receptors may differ (85, 88). Intra-epithelial LC reside within the female genital epithelium (82), and are considered to be one of the first cells that encounter the virus and can pass on the virus to $CD4^+$ T-cells (89). HIV can also be degraded in Birbeck granules in LC, but a recent study demonstrated that LC in the FGT do not harbor Birbeck granules and that infection of vaginal LC sustained productive HIV-infection (90). Other subtypes of DC and macrophages reside mainly in the submucosa, and can facilitate $CD4^+$ T-cell infection (85) and be productively infected by HIV *ex vivo* (91), respectively.

1.3 FACTORS THAT INFLUENCE HIV SUSCEPTIBILITY IN THE FGT

Mucosal inflammation, female sex hormones and the cervicovaginal microbiome can influence HIV susceptibility in the FGT, as discussed below.

1.3.1 Mucosal inflammation

Inflammation is the body's response to noxious stimuli, such as foreign pathogens, and hallmarks of inflammation include secretion of cytokines and accumulation of immune cells. However, genital inflammation, as defined by elevated mucosal cytokines/chemokines, has been associated with increased HIV acquisition (14, 92-94). Several cytokines are chemotactic to HIV target cells (71, 95, 96), and one potential mechanism for increased HIV susceptibility is an accumulation of such cells in genital tissues (51, 97). Furthermore, the pro-inflammatory cytokine TNFα can disrupt tight junction proteins in the epithelial barrier, contributing to decreased epithelial barrier integrity and increased access of the virus to target cells (42). Elevated mucosal pro-inflammatory cytokines have also been shown to be associated with a proteomic signature of neutrophil accumulation, a weakened epithelial barrier and elevated levels of genital HIV-target cells (51). In addition to the evidence that increased inflammation enhances HIV susceptibility, reduced genital inflammation has been associated with protection to HIV (see section 1.4.1), further emphasizing the role of inflammation in HIV-transmission. However, the exact mechanisms of how genital inflammation affects the genital epithelium and/or HIV susceptibility are far from well characterized.

1.3.1.1 Semen-induced mucosal inflammation

As mentioned previously, the vast majority of new HIV-infections are transmitted through heterosexual intercourse, where the virus and/or infected cells in semen are deposited in the vagina. Exposure to semen in the FGT is associated with a so called "leukocytic reaction", characterized by a pro-inflammatory response with an influx of immune cells, most notably neutrophils, in the cervix (98, 99). This was originally characterized in cervical smears after artificial insemination, but unprotected coitus also leads to increased expression in ectocervical biopsies of several pro-inflammatory genes as well as influx of leukocytes to the mucosa (100). Interestingly, the observed leukocyte influx was not seen with protected coitus, indicating that it is the mucosal reaction to the semen itself and not the mechanical

effect of coitus that causes such phenotype. However, another study assessing genital secretions after unprotected intercourse did not observe a general increase in cytokines (101). In addition, studies on the effect of semen in the FGT are complicated since incubation with semen and/or seminal plasma can induce toxicity in isolated cells (102, 103). Recent studies have investigated the role of semen in HIV-transmission (reviewed in (104)), suggesting that, although somewhat contradictory results, the effect of semen can modulate the genital micro-environment as to cause enhanced susceptibility to HIV. However, the effect of semen of the female genital mucosa needs further studies, especially on how semen-induced inflammation affects HIV-transmission.

1.3.2 Female sex hormones

It has been suggested that the menstrual cycle and HC affect HIV susceptibility and that this might be a result of alterations in endogenous and exogenous female sex hormones (45, 105-113). This effect is therefore described in further detail below.

1.3.2.1 The menstrual cycle

The cyclic changes in the FGT over the menstrual cycle are essential for reproduction and are governed by the fluctuations of the ovarian sex hormones estradiol and progesterone. These fluctuations are under the control of the complex hypothalamic-pituitary-gonadal axis. Very briefly, the pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus regulates the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gland (114). LH and FSH affects the production of the female sex hormones estradiol and progesterone in the ovaries, which in turn exert feedback loops, both positive and negative, at the pituitary and hypothalamic level in a complex feedback system.

The menstrual cycle can be divided into different phases/stages depending on if referring to the ovarian or the uterine cycle (Figure 2). The uterine cycle can be divided into menses, the proliferative and the secretory phase, whereas the ovarian cycle consists of the follicular, mid-cycle/periovulatory and the luteal phase. In this thesis, the terms follicular, ovulatory and luteal phase will be used.

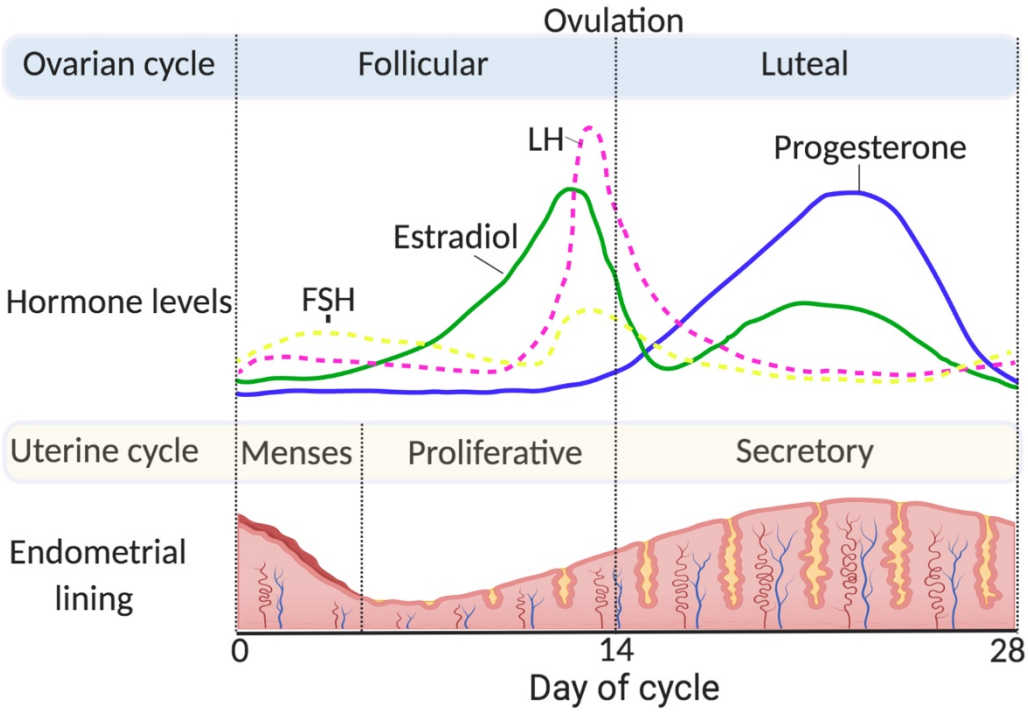

Figure 2. Hormonal fluctuations over the menstrual cycle. The cyclic changes in female sex hormones levels in serum over a classical 28-day menstrual cycle. FSH: follicle stimulating hormone, LH: luteinizing hormone. Figure created using Biorender.com.

1.3.2.2 The phases of the menstrual cycle

The follicular phase is the period between the initiation of bleeding/menses until ovulation. During menses, the functional layer of the endometrium is sloughed off and the levels of estradiol and progesterone are low, stimulating the increased secretion of GnRH, LH and FSH (114). FSH from the anterior pituitary gland stimulates the follicular cells in the ovaries to produce estradiol. Estradiol stimulates the proliferation of the endometrium, including growth of all tissue components such as glands, stromal and endothelial cells. The spiral arteries lengthen as the endometrium is re-established and at the end of the follicular phase, these arteries are slightly coiled. The high levels of estradiol stimulate a surge in LH secretion, and this LH-surge triggers ovulation in the ovaries. The luteal phase is the period between ovulation until the start of next menstrual bleeding. The remaining follicle after ovulation is transformed into the corpus luteum that produces progesterone and estradiol. Peak progesterone and estradiol levels are reached at approximately 8 days after ovulation (115). Progesterone stimulates the endometrium to develop spiral vessels that lengthen and become more coiled, enlargement of glands that become corkscrew shaped as well as tissue edema (116). In the late luteal phase, leukocytes infiltrate the endometrium (116, 117). If no fertilization occurs, the corpus luteum degenerates and levels of progesterone and estradiol decrease rapidly, initiating menstruation.

1.3.2.3 Variation in length of the menstrual cycle

Menstrual cycle length is the interval from the first day of menses up to and including the day before subsequent menses. There is large inter- and intra-individual variation in

menstrual cycle length between women (118, 119), but a menstrual cycle between 21 and 35 days in length is usually considered normal (reviewed in (120)). A large study in American women from 1967 demonstrated an average menstrual cycle length of 28 days in women between the ages of 20-40, and that menstrual cycles successively shorten after 20 years of age (121). Similar results have been found in more recent studies (118, 122). However, only 10-15% of cycles in reproductive aged women are actually 28 days in length (115, 119). A recent study of >600, 000 ovulatory menstrual cycles based on data from a digital fertility awareness application showed that 13% of cycles were 28 days in length, and that 26% of cycles were 31 days or longer (123). In general, the greatest variability in length is in the follicular phase (115, 119). Traditionally it was believed to be little variation in length of the luteal phase (approx. 14 days) because of the set duration of the survival of the corpus luteum, but studies have observed luteal phase lengths between 7-19 days (119, 120, 124).

1.3.2.4 The menstrual cycle and HIV susceptibility

An increased susceptibility to simian-HIV/SIV during the late luteal/menstrual phase has been observed in non-human primates (105, 106, 125). Furthermore, in cervical explants, productive HIV-infection was only observed in tissues donated from women in the luteal phase of the menstrual cycle (113), but this has not been seen by others (126). Wira et al. observed that several aspects of the innate and adaptive immune system in the FGT are suppressed 7-10 days post ovulation in order to facilitate procreation, and they hypothesize that this creates a so called "Window of vulnerability" during which women are more susceptible to HIV and other STIs (108, 109). Birse et al. found an increase in proteins involved in epithelial barrier remodelling and immune activation in the luteal phase, suggesting this represents a period of decreased barrier integrity and active immune cell recruitment to the mucosa, which might enhance HIV-susceptibility (45). An inflammatory profile has been suggested by gene expression analysis in the luteal (32, 127, 128) and late luteal (129) phase in vaginal (32), endocervical (127, 128) and endometrial (129) tissues. Such pro-inflammatory state in the FGT in the luteal phase could theoretically be associated with an increase in HIV-target cells in the luteal phase, which has been observed in some (126, 130), but not all studies (31, 32, 47, 131). A general increase in inflammatory cytokines in the luteal phase could potentially confirm an inflammatory state, but studies have shown inconsistent results, including both increase and decrease of individual inflammatory cytokines in the luteal phase, as well as no change (47, 50, 55, 132, 133). In addition, in a comprehensive study of mucosal markers of HIV susceptibility, Thurman et al. found no difference between the follicular and luteal phase (32), and the full impact of the menstrual cycle on HIV susceptibility in women remains to be elucidated.

1.3.2.5 Progesterone and estradiol

Estradiol and progesterone are steroid sex hormones that are synthesized from cholesterol, in the non-pregnant female the main production occurs in the ovaries. Prior to ovulation, such steroidogenesis occurs in the follicle, and after ovulation in the corpus luteum (134, 135). Progesterone and estradiol can diffuse through the outer cell membrane and exert

their function by binding to intracellular ligand-activated steroid receptors, mainly the estrogen receptor (ER) and progesterone receptor (PR) for estradiol and progesterone, respectively. The hormone/receptor complex bind to the DNA strand at specific sites called hormone-responsive elements and thereby influencing gene transcription and protein synthesis (134). In addition to the genomic effects mediated by the nuclear receptors, progesterone and estradiol can bind to receptors on the cell-surface, which can induce direct, non-genomic effects (134, 135). There are subtypes/isoforms of the ER and PR and the expression of the different receptors varies throughout the FGT (135). However, the biological response is not only dependent on the type of receptors expressed but also on the tissue expression of intracellular proteins, such as coactivators or corepressors, that can modify the effect (134). Progesterone and estradiol can also modulate the effect of the other- in uterine cells, estradiol stimulates the expression of PRs, and progesterone can decrease the expression of ER (136). This highlights the complex interplay between estradiol and progesterone as well as the importance of the "priming" of the endometrium by estradiol during the follicular phase to prepare for progesterone-induced changes during the luteal phase. Progesterone can bind and exert its action by the PR, but it can also, to varying degrees, signal through other nuclear receptors (110).

1.3.2.6 Hormonal contraceptives

The development of HC has revolutionized women's sexual freedom and the use of HC is now widespread worldwide. There are different formulations of HC such as per oral pill, injectable, intrauterine device, skin patch, vaginal ring and subdermal implant. Progestins are synthetic progestogenic steroids designed to act with progesterone-like effects to the PR, some HC contain only progestin whereas others contain progestin and a synthetic estrogen component. They are several different progestins used in HC and importantly, they exert their effect not only via the PR receptor but the different progestins show differential affinities and activities via other steroid receptors, such as glucocorticoid receptor (GR) and the mineralocorticoid and androgen receptors (reviewed in (110)).

The most common type of HC in sub-Saharan Africa are long-lasting injectables, most often depot medroxyprogesterone acetate (DMPA). DMPA is a progestin-only HC that is provided intramuscularly every 12th weeks and prevents pregnancy by blocking the LH-surge (resulting in anovulation) as well as thickening of the cervical mucus and endometrial thinning. However, meta analyses of high-quality observational studies have estimated that DMPA usage is associated with an up to 50% increased risk of HIV acquisition relative to no use of HC (22-24). Yet, it remains unknown if such increased risk is due to the biological effects of DMPA or because of limitations of the observational studies, such as confounding or bias. To reduce the limitations of observational studies, a large randomized, multicenter study compared DMPA with two other types of long-lasting contraceptives—levonorgestrel implant and copper-intrauterine device (137). Here, DMPA usage was not found to be associated with an increased risk for HIV acquisition compared to these compounds, although critically, this study lacked a comparison between DMPA and no contraception or infrequent condom use. In addition, this study was designed to detect a "substantial" or "meaningful" increase in HIV risk between study arm, such risk defined as high as at least 50% difference. Thus, a clear epidemiological link between HIV infection

risk and DMPA use remains to be established (138). Interestingly, no increased HIV-acquisition risk has been observed for NET-EN (22), another progestin-only injectable contraceptive, although data are somewhat limited. A proposed mechanism of the varying effects of DMPA and NET-EN on HIV-acquisition is via the GR, since the progestin medroxyprogesterone acetate (MPA) in DMPA but not NET (the progestin in NET-EN) show strong effect on the GR (139).

Most studies show no association between combined oral contraceptives, i.e. contraceptives with both an estrogen and a progestin component, and HIV-susceptibility (22, 23). Moreover, in the non-human primate model, vaginal and systemic estrogen treatment is protective against vaginal SIV transmission (140, 141). In addition, in post-menopausal women, local estrogen treatment is associated with a thicker vaginal epithelium (131). The use of DMPA suppresses endogenous estradiol production and is associated with hypoestrogenism (142, 143). Taken together, this once again raises the suspicion whether high levels of progesterone and/or progestins is associated with increased HIV acquisition, and that perhaps estradiol exerts a protective effect on the female genital mucosa.

One theory of how MPA and/or other progestins might contribute to increased HIV-susceptibility is by causing a thinner vaginal/ectocervical epithelium. This has been clearly demonstrated in non-human primates (43, 144), but studies in humans are inconclusive and most studies do not show a substantial effect on epithelial thickness in response to exogenous progestins (31, 34, 143, 145). It has been hypothesized that MPA/progesterone increases HIV susceptibility by modulating the inflammatory/immune activating milieu in the FGT and recruitment of HIV target cells (31, 130, 146, 147). Some previous studies show an increase in genital inflammation (as measured by cervicovaginal cytokines) in response to DMPA treatment (50, 148) while others instead observe a general decrease (149, 150). The data on HIV-target cells is also rather contradictory, DMPA use has been associated with increased genital target cells in some (31, 130, 147) but not all (151) studies.

1.3.3 The cervicovaginal microbiome

The cervicovaginal microbiome is critical for maintaining vaginal health and mucosal barrier function. Recent advances in molecular technologies have allowed the identification of clusters, called “community state types” or “cervicotypes”, based on the composition of the cervicovaginal microbiome (152-156). Although heterogeneity exists between studies, all studies identify clusters dominated by different species of lactic-acid producing *Lactobacillus* as well as high diversity clusters usually dominated by *Gardnerella* and other facultative anaerobic or anaerobic bacteria. Such high diversity states, characterized by a loss of *Lactobacillus* with subsequent overgrowth of anaerobic bacteria, is often associated with the clinical diagnosis of bacterial vaginosis (BV) (155, 157). Additionally, high diversity states are more prevalent in women in sub-Saharan Africa than in Caucasian women from the developed world (19, 152, 153). In addition, black North American women show higher diversity and less *Lactobacillus* dominance as compared to Caucasian women from the same geographical area (152). The reasons for such ethnic differences in cericovaginal microbiome composition are not known but could be related to genetic

variations such as polymorphisms in genes associated with immune and hormone-response (158).

Increasing bacterial diversity is associated with elevated genital inflammation (19, 52, 153, 159) and increased risk of HIV-acquisition (19, 160) suggesting a link between cervicovaginal bacteria and the local immunological milieu in the FGT mucosa. Significant alterations in the cervicovaginal proteome, especially relating to epithelial barrier function, has also been observed with increasing diversity (52) and *Gardnerella vaginalis*-dominated communities (161). Also, cervicovaginal microbiome composition can alter the genital immune response to DMPA (162) and non-steroidal anti-inflammatory drugs (163). In addition, a recent study by Klatt et al. demonstrated that a cervicovaginal microbiome dominated by *Gardnerella vaginalis* and other anaerobs decreased the efficacy of a microbicide containing the antiviral agent tenofovir (164). This highlights the importance of considering the cervicovaginal microbiome in the HIV prevention field. Interestingly, cervicovaginal microbiome clusters dominated by the different species of *Lactobacillus*, mainly *L.crispatus* and *L.iners*, seem to have distinct effects of genital inflammation (52, 153) and HIV-susceptibility (19), with *L. crispatus* dominated cervicovaginal microbiomes associated with the lowest risk of HIV-acquisition as compared to high-diverse states. Collectively, the above mentioned studies indicate that the cervicovaginal microbiome play an important role in genital inflammation and mucosal barrier function, which may have implications for HIV-susceptibility.

The interplay between the vaginal microbiome, female sex hormones including HC, and genital inflammation is an area of great interest within the field. A systematic review suggested that DMPA is associated with a decrease in BV (i.e. an increase in *Lactobacillus*-dominated microflora) (165), whereas other studies have shown either no change (130, 153), or a decrease (142, 151) in the prevalence of *Lactobacillus* species in users of injectable progestin-contraceptives, mostly DMPA. However, the studies differed in study design (longitudinal vs. cross-sectional), as well as which methods were used to determine vaginal microbiota composition (culture based vs. 16S rRNA gene sequencing). In addition, Miller et al. (142) and Mitchell et al. (151) only observed a decreased in H_20_2-producing *Lactobacillus*. Such results of decreased *Lactobacillus* in DMPA users are however supported by a recent study in Kenyan FSWs showing that MPA correlated with increased diversity of the cervicovaginal microbiome (166). Nevertheless, there remains a substantial lack of knowledge within the field about the effect of DMPA on the vaginal microbiome, and especially on underlying mechanisms. Few studies have examined the relationship of vaginal microbiota to the menstrual cycle in detail but observable changes have included decreased stability (167) and increase in *Gardnerella vaginalis* (168) or non-*Lactobacillus* (169) taxa during menstruation. Furthermore, while the functional properties of the microbiome are implicated in proper mucosal functioning and inflammation (reviewed in (29, 170)), this has not been studied in the context of the menstrual cycle.

1.4 PROPOSED CORRELATES OF PROTECTION AGAINST HIV IN HIV-EXPOSED SERONEGATIVE INDIVIDUALS

Some individuals remain HIV seronegative, defined by lack of HIV Immunoglobulin (Ig) G in plasma or serum, despite continuous exposure to HIV, referred to as HIV-exposed seronegative (HESN) individuals in the scientific literature (171). However, there is no consensus of the amount of HIV-exposure needed to be classified as a HESN individual, and definitions vary widely between studies. Nevertheless, studying mucosal samples from HESN women can help elucidate natural protection to HIV and identify signatures associated with continuous HIV-exposure.

The one confirmed resistance mechanism towards HIV is a homozygous, 32-base pair deletion in the gene coding for the HIV coreceptor CCR5 (172). However, this deletion is uncommon in black Sub-Saharan populations (173) and accounts for only very few cases of resistant individuals. This has led to the speculation that other factors contribute to decreased susceptibility to the virus in HESN individuals. Several proposed correlates of protection to HIV in HESN individuals have been identified (reviewed in (174-177). Earlier work focused on plasma/blood, but more recent studies have focused on the FGT mucosa. However, following many years of research in the field, it may be concluded that the mechanisms for a relative protective phenotype against HIV infection is most likely a combination of multiple factors.

1.4.1 FSW cohort studies of mucosal protection against HIV

Many studies investigating proposed mucosal correlates of protection to HIV have been performed in FSW cohorts (171). FSWs are often engaged in high-risk sexual behavior and have high levels of concordant STIs and are therefore at substantial risk of HIV infection. FSW cohorts can therefore be useful to study to decipher factors associated with HIV-susceptibility (171).

Several chemokines and cytokines have been identified as overexpressed in genital samples of HESN FSWs, such as RANTES/Chemokine (C-C motif) ligand (CCL) 5 (178, 179) and interferon-α (179). These cytokines have known anti-HIV activity in vitro; RANTES is secreted by $CD8^+$ T cells and other cells, and inhibits HIV infection by competing with the virus binding to the CCR5 receptor (180, 181), and interferon-α can inhibit HIV-replication (182). However, the presence of RANTES is truly a double-edged sword since RANTES is also a potent chemoattractant for T-cells (72), that may function as HIV-target cells. In contrast to the above mentioned increase in cytokines, a decrease in cytokines has also been observed in genital secretions of HESN FSWs (183, 184). Other proteins that have been elevated in the FGT of HESN FSWs are several with anti-inflammatory properties such as serine protease inhibitors (57, 62, 63, 185) and cysteine protease inhibitors (62), known as serpins and cystatins, respectively. Such decrease in cytokines and upregulation of anti-inflammatory proteins supports the hypothesis proposed by Card et al that HESN may be protected against HIV based on a phenotype of low baseline immune activation in the periphery and genital mucosa, called “immune quiescence” (174). The Immune quiescence theory was based primarily on low T-cell activation, including lower baseline cytokine

production in peripheral blood mononuclear cells (PBMCs) (186), and an increase in regulatory T-cells in the periphery of HESN individuals (174, 187). In the FGT, in addition to the increased levels of antiproteases and decreased cytokines mentioned above, cervical mononuclear cells show reduced gene-expression for the Th17 associated cytokines IL-22 and IL-17 (188). This is especially interesting since Th17 cells have been implicated as especially susceptible to HIV-infection (79, 189), and a decreased presence or activation of these cells could indicate a protective phenotype.

1.5 HIV-SERODISCORDANT COUPLES

HIV-serodiscordant couples are couples with one HIV-seronegative and one HIV-seropositive partner. HIV-serodiscordance is common in sub-Saharan Africa, and it is estimated that 44% of new HIV-infections in Kenya occur within stable relationships (190). The infectious burden of the HIV-seronegative partner varies greatly between cohorts and is dependent on many factors, such as type of sex, frequency of sexual intercourse, male circumcision, partner's viral load and if the partner is taking ART. However, studies of HIV-serodiscordant couples can still aid our understanding of natural protection in high-risk groups as well as the immune response involved in repeated HIV exposure. In addition, since HIV-serodiscordant couples are an important target group for preventative HIV measures, it is of importance to characterize their mucosal phenotype to correctly design and interpret clinical trials in this risk group.

A small study in HIV-serodiscordant couples identified the innate molecules α-defensins human neutrophil peptide (HNP)1-3 to be overexpressed in genital tissues from HIV-seronegative women in HIV-serodiscordant couples (191). However, the authors conclude that it cannot be determined if the α-defensins contribute to resistance to HIV in these women or if it is an effect of chronic immune stimulation. In contrast, Levinson et al. did not observe a difference in HNP1-3 in a cohort of Kenyan HIV-serodiscordant couples as compared to low-risk controls, but they did observe that high viral load of the HIV-positive partner correlated with higher levels of HNP1-3 (192). This could indicate that exposure to high viral load induced a local inflammatory response with release of HNP1-3. Interestingly, HNP1-3 were associated with increased HIV acquisition in a FSW cohort (61). However, increased levels of HNP1-3 were associated with bacterial STIs, and perhaps the genital inflammatory response to STIs with a potential increase in HIV target cells, overrode the antiviral activities of the α-defensins.

In addition to molecules of the innate immune systems, studies indicate that components of the adaptive immune system might be altered in HIV-serodiscordant couples. Choi et al. demonstrated that HIV-1 neutralizing IgA antibodies were five times more common in genital secretions of women in HIV-serodiscordant relationships as compared to low-risk controls (193). However, in a smaller study, HIV specific IgA antibodies were not found in genital secretions of the HIV-seronegative partner (194) and the presence and effect of mucosal HIV-specific IgA antibodies in HESN individuals remains controversial (195). HIV-specific T-cell responses have been observed in PBMCs from the HIV-negative partner in serodiscordant couples (196, 197), but the T-cell response of genital lymphocytes in HIV-serodiscordant couples remain largely unknown. Thus, the genital mucosal

environment in HIV-serodiscordant couples remain largely unexplored, and it is essential to characterize this as these individuals represent an important target group for preventative HIV measures.

1.6 HIV PREVENTION STRATEGIES

Despite significant reduction in HIV-infection rates in many countries, there is still a great need for effective HIV-prevention strategies against HIV-transmission including ART, vaccines, microbicides, male circumcision and pre- and post-exposure prophylaxis (PrEP and PEP, respectively). Furthermore, women may not be able to negotiate condom usage, and it is therefore especially important to develop female controlled HIV-prevention strategies.

1.6.1 Antiretroviral therapy

One of the greatest scientific advances in the HIV field is the discovery and wide-spread use of ART. Standard ART consists of the combination of at least three antiretroviral drugs, and the WHO recommends ART initiation in all HIV-positive individuals regardless of CD4 T-cell count (198). ART not only reduces morbidity and mortality, but it can reduce viral loads to undetectable levels, and the Public Health Agency of Sweden concludes that there is no risk of transmission of HIV during sexual intercourse if the HIV positive partner fulfill the criteria for effective treatment with ART, including long-lasting viral suppression (199). Thus, widely accessible ART is a very important HIV prevention strategy and ART accessibility is one of the cornerstones of the UNAIDS strategies to combat the HIV epidemic. Despite recent advances in global ART coverage and viral suppression, only 53% of all people living with HIV were estimated to be virally suppressed in 2018 (8). In addition to ART in HIV-positive patients, PrEP are antiretroviral drugs that can be taken orally or administered locally as a microbicide (see section below) in HIV-seronegative persons with high risk of HIV-infection in order to decrease the risk of HIV-acquisition.

1.6.2 Vaccines

It has proven difficult to develop an effective preventative vaccine against HIV, partly because HIV is able to form latent proviral deoxyribonucleic acid (DNA) and targets for neutralizing antibodies are highly variable and camouflaged by heavy glycosylation (200). Most recently, a phase IIb/III follow-up trial to the moderately successful RV144 (201) was ended early because the regimen did not prevent HIV infections (202), being a significant setback for the vaccine field. However, there are other vaccine efficacy trials underway in sub-Saharan Africa as well trials testing passive antibody immunity (Bekker Lancet 2020), giving hope to finding an effective vaccine.

1.6.3 Microbicides

Microbicides are topical compounds that are inserted vaginally or rectally to prevent HIV-transmission. There are different microbicide formulation, such as gels/creams, enemas, vaginal films or vaginal rings, and they contain medications that exert their action by either inhibiting HIV-entry into immune cells or preventing viral replication (203). As of today,

microbicides against HIV are only at the research stage, but there are some promising findings. In the CAPRISA 004 trial, a tenofovir-based gel inserted vaginally prior to and after coitus showed an overall reduction of 39% in South African women (204). Interestingly, genital inflammation (94) and *Gardnerella*-dominated vaginal microflora undermined efficacy of the gel (164). Such findings highlight the importance of the mucosal milieu in microbicide efficacy and indicate that reducing genital inflammation and/or modifying vaginal microbiota composition in women may augment HIV prevention efforts. However, a follow-up phase 3 trial (FACTS 001) did not demonstrate efficacy in reducing HIV-transmission, and the authors concluded that alternative "products that are less user dependent… or do not need high adherence are needed" (205). Indeed, one of the disadvantages with gels is that is it considered "messy", which might decrease adherence. In contrast, vaginal rings are more user friendly, and a monthly vaginal ring system containing the antiretroviral dapivirine decreased HIV incidence by approximately 30% in two phase III trials (206, 207). Results from an open-label extension trial (DREAM trial) for the dapivirine ring indicate higher adherence as compared to the previously mentioned trials and a statistically simulated risk reduction rate of 63% (208).

Important lessons for the field can be learned from the failure of the Nonoxynol-9 gel-microbicide in FSWs, where frequent use *increased* HIV-acquisition (209). Subsequent studies showed that Nonoxynol-9 induce genital inflammation (210) and a thinned vaginal epithelium in a murine model (211). The Nonoxynol-9 study highlights the importance of rigorous pre-clinical safety studies of the genital mucosa and evaluation of markers of genital inflammation prior to clinical trials, especially evaluating the effect of multiple exposures.

2 AIMS

The overall aim of this thesis was to investigate factors associated with HIV susceptibility in the FGT. The specific aims were as follows:

I. To study the effect of endogenous and exogenous female sex hormones on the mucosa in the FGT by assessing genital samples from:
   a. longitudinally followed Swedish healthy women over one menstrual cycle (Paper 1)
   b. HIV seronegative FSWs using injectable progestin-based HC (DMPA) vs. those who use no HC (Paper 4)

II. To study the effect of seminal plasma on the immune response and HIV-transmission in the FGT using a human ectocervical explant model (Paper 2)

III. To characterize the cervicovaginal protein signature in women living in HIV-serodiscordant relationships (Paper 3)

IV. To evaluate the feasibility of using a bead-based affinity set-up for high-throughput protein profiling of genital secretions (Paper 3)

3 MATERIAL AND METHODS

3.1 STUDY COHORTS AND SAMPLE COLLECTION

All study participants for Paper 1-4 were over the age of 18 and answered questionnaires about medical history and sexual practices at enrollment.

3.1.1 Paper 1: The Immunology of Menses (IMMENSE) cohort

Healthy, Swedish volunteers were recruited and sampled at three time points during one menstrual cycle, aiming for day 7, 14 and 21 since initiation of last menses. Inclusion criteria included age 18-35, regularly cycling with menstrual cycle length of 24-35 days and no use of HC for the past 3 months. Exclusion criteria were frequent recurrent episodes of BV or vaginal candidiasis. Participants were instructed not to have unprotected sex 48 hours prior to sampling. All participants were tested, and excluded if positive, for the following at enrollment: HIV-1/2, *Chlamydia trachomatis, Neisseria gonorrhoeae*, cervical cellular dysplasia, and genital herpes simplex-like lesions. Participants were also screened for BV by clinical examination. Human papilloma virus (HPV) testing was also performed, but asymptomatic HPV was not exclusionary. At each visit, venous blood was collected for measurement of female sex hormones. CVS were collected by rotating one cotton tip in the cervical os, and another cotton tip was used for collecting secretions in the posterior fornix. Both cotton tips were transferred into the same vial containing 5 ml phosphate buffer solution (PBS), kept on ice and transported to the lab where it was centrifuged to remove cellular debris and subsequently stored at -80^{o}C. The cellular debris is here referred to as the "cell pellet", and was suspended in RNA-later before stored at -80^{o}C until use.

3.1.2 Paper 2: The St Göran hysterectomy cohort

Pre-menopausal women undergoing hysterectomy for non-malignant and non-inflammatory causes at St Görans Hospital (Cevita Care GynStockholm St Göran), Stockholm were included in this study. Exclusion criteria included systemic immunosuppressive therapy, clinical symptom of an STI in the three months prior to surgery and/or HPV positivity. Immediately after the hysterectomy, 4-9cm^2 sample of mucosa was dissected from the ectocervix by a pathologist, put in 4^{o}C culture medium and kept on ice until processed. HPV genotyping of the cervix was performed on cervical swabs. After dissection of the ectocervical tissue into 8 mm^3 tissue blocks, one tissue block was immediately snap-frozen and cryopreserved at -80^{o}C, and the other tissue blocks were utilized for the fresh explant model. Semen was collected from HIV-negative adult volunteers recruited at the Venhälsan clinic of the Södersjukhuset. Exclusion criteria included clinical symptoms of STIs within 3 months prior to donation, infertility (if known) and systemic immunosuppressive therapy. The semen was collected by masturbation after 48 hours of abstinence and collected in a sterile container.

3.1.3 Paper 3: Couples Against Transmission cohort

The study participants included in this study were part of a larger cohort that has been described previously (212). Women in HIV-serodiscordant relationships, where the male partner was HIV-seropositive and the female partner was HIV-seronegative, were recruited at voluntary counseling and testing centers in Nairobi, Kenya between 2007 and 2009. This group is referred to as the "serodiscordant group". HIV-seronegative women in seroconcordant couples (i.e. with a HIV-seronegative sexual partner) were recruited from the same testing centers and included as a control group. Eligible participants were not pregnant, reported sex ≥ 3 times with their study partner in the past three months and planned to remain together with their study partner for the duration of the study. Participants with a study partner currently on ART or with a history of clinical AIDS were excluded. The study participants were counselled about safe sex practices and had access to free STI testing and treatment. Women in serodiscordant couples reported high level of safe sex practices, and only18% reported unprotected sex in the past month. The HIV incidence for male-to-female in the larger parent study cohort is low at 1.1 per 100 person years (212). Questions on antiviral use were asked at study exit, and the use of PrEP and PEP in this cohort can be assumed to be low.

The serodiscordant women were sampled with CVS (same procedure as in Paper 1) at enrollment ('0'), 6 and 12 months later. Participants in the control group were only sampled once. All participants were tested for BV, *Trichomonas vaginalis*, syphilis, herpes simplex virus (HSV)-2 serostatus and HIV at enrollment. HIV and HSV-2 was tested at all visits included in this study and BV was assessed by Nugent Score. HIV RNA levels and CD4 cell counts were measured at every study visit for HIV-seropositive male partners. Prostate specific antigen (PSA), as a measure of recent unprotected sex (213), was measured in CVS at enrollment. For the part of this study where comparing the protein profile of serodiscordant vs control women (more details below), women using HC were excluded. In the partially overlapping cohort that was used to compare two proteomic techniques, women using HC were included.

3.1.4 Paper 4: The Pumwani cohort

The samples used in this study were part of a larger, longitudinal cohort within the Pumwani FSW cohort, this cohort has been described in detail previously (147, 214). Briefly, this community-based cohort of FSWs in the slum of Kibera, Nairobi, Kenya was established in 1984 to study the epidemiology and immunobiology of STIs including HIV (215). Study participants and other inhabitants in the slum have access to basic health care, STI-testing and treatment as well as HIV-counseling at the study driven health centers. The women included in the study are self-defined FSWs, and there is no formal criteria that need to be fulfilled in order to be classified as a FSW. The mean number of clients per day in this cohort is 4 (216), and the majority of women also report regular sexual intercourse with at least one so called "regular partner", such as boyfriend(s) or husband. The reported condom use is much lower with the regular partner than with clients. Data collected from the Pumwani cohort between

1985-1994 showed a high HIV incidence of 42 per 100 person-years (215). However, since then safe sex practices have increased substantially (147) and STIs decreased in this cohort, and the annual HIV-incidence rate was estimated to 2.2% between 2008 and 2011 (16).

Inclusion criteria at enrollment included: actively involved in sex work, age 18-50, not pregnant/breastfeeding, no prior hysterectomy, not being menopausal, negative for *Chlamydia trachomatis, Neisseria gonorrhoeae,* HIV and syphilis. HIV- serology was performed at enrollment, approximately 6 weeks after the study visit and 3-6 months after study completion. For this sub-study, only HIV seronegative women were included. Study participants were either regularly cycling and not using any type of HC (control group) or using DMPA since at least 6 months (DMPA group). The regularly cycling controls were aimed at sampling in the follicular phase of the menstrual cycle based on self-reported days since last menses, and sampling in the DMPA group was aimed at 4-8 weeks since last DMPA injection.

At the study visit, cervicovaginal lavage (CVL) was collected by washing the endocervix with 2 ml sterile PBS, and the lavage was aspirated from the posterior fornix (217). Samples were kept on ice until processed in the lab, where they were centrifuged and aliquoted. The cellular debris ("cell pellet") was resuspended in RNA-later, and the CVL and cell pellet were stored at -80^{o}C until analysis. Two ectocervical biopsies were collected at the study visit, one was immediately snap frozen and the other placed in RNA-later. Both biopsies were stored at -80^{o}C until further analysis. The study participants were instructed not to have unprotected sex for 14 days post biopsy to enable healing, and they were compensated for loss of income during this time (214). To ensure healing and abstinence, the study participants were seen at the clinic 2-5 days post biopsy.

3.2 ETHICAL CONSIDERATIONS

Ethical permission from corresponding review boards were obtained for all studies presented in this thesis.

Gynecological exams and the collection of genital samples can be associated with a feeling of discomfort. To minimize discomfort, all genital samples were collected by an experienced gynecologist. Ectocervical biopsies can cause light pain and bleeding, but are standard procedures that are part of routine screening for cellular atypia in Sweden and are generally well tolerated.

The collection of ectocervical biopsies in a cohort of high HIV-risk women (Paper 4) may raise concerns of increased HIV susceptibility. To decrease the risk of HIV-acquisition post sampling, all women were asked to abstain from vaginal sex for two weeks. A rigorous study protocol was implemented to support post-biopsy abstinence, including counseling, text message reminders and PSA-testing 3-5 days post biopsy (214). The participants were also compensated for the loss of income during these weeks. The monetary compensation had been agreed upon together with representatives from the sex worker community and the local

ethics committee. In addition, ectocervical biopsies in high risk women have previously been considered safe and well tolerated (218). Study participants were tested for HIV 3-6 months after study completion, and none of the study participants seroconverted.

None of the study participants received direct personal gain by being in the study, but we believe that the benefit of increased knowledge about HIV infection and prevention overweighed the discomfort of sampling.

3.3 METHODOLOGICAL CONSIDERATIONS

Described below are the most important methodological considerations, detailed descriptions of the methods used are presented in the corresponding papers or manuscript.

3.3.1 Analysis of proteins in genital secretions

The identification of proteins in genital secretions, defined here as both CVS and CVL, is commonly used to study mucosal immune responses in the FGT. Several techniques, used separately or in combination, that have been used to study proteins in genital secretions include enzyme-linked immunosorbent assay (ELISA), bead-based antibody techniques, Western blot and mass spectrometry (MS). Earlier research within the field has focused on a few individual proteins, but in the last decades, the field of proteomics has evolved. Proteomics is the large-scale study of proteins and is used to study the proteome, or all the proteins within a biological group, such as a cell, tissue or organ. Proteomics can be utilized to characterize genital secretions using a systems biology based-approach, which may provide a better understanding of the complex mucosal immune system since it allows examination of hundreds of proteins at the same time (219). Several proteomic techniques have been developed, of which quantitative MS and a high-throughput bead-based single-binder affinity assay will be discussed here because of their relevance to this thesis.

It is important to keep in mind when interpreting results from protein profiling studies that it is a snapshot of a highly dynamic environment. In addition, the protein content of genital secretions tell us the amount (relative or absolute) of each protein, but not the biological activity, since it is dependent on the presence of other proteins, activation status of the proteins as well as localization in the tissue. Another issue is the lack of standardization for sampling of genital secretions, common sampling methods used include lavage (CVL), swabs, swabs diluted in PBS (CVS), cervical sponges, cervical cups and more. Not only does the different methods primarily sample different parts of the FGT, but protein yield varies between methods (220).

3.3.1.1 Tandem mass spectrometry technique

In paper 1 and 3, CVS samples were analyzed using a shotgun tandem MS technique (Figure 3).

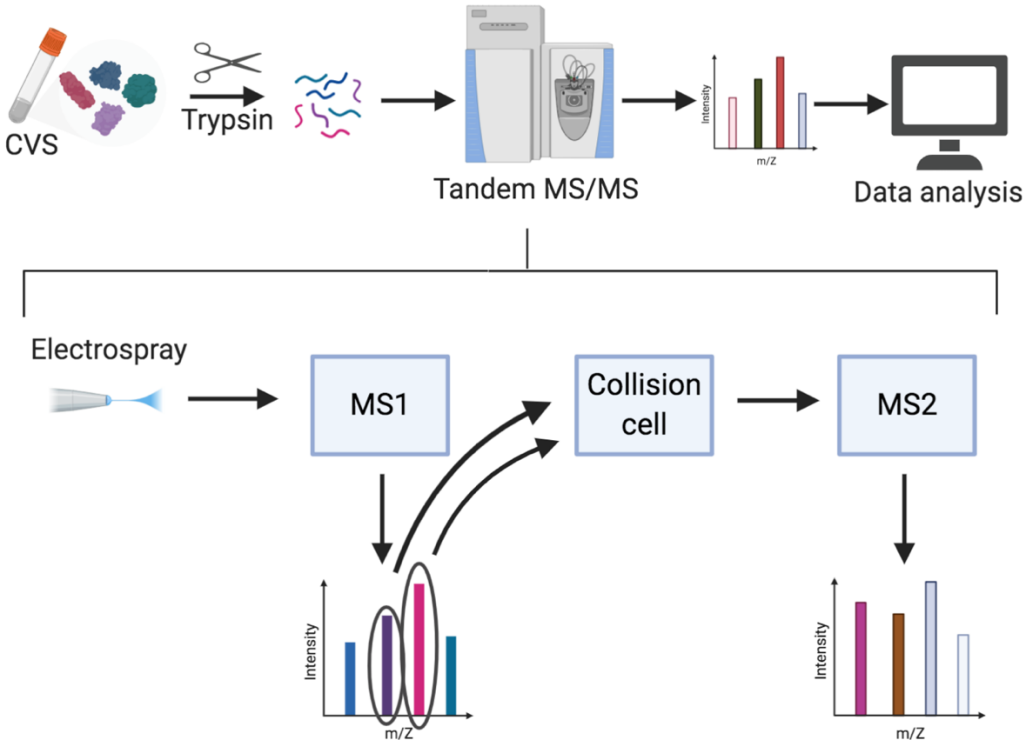

Figure 3. Schematic illustration of tandem mass-spectrometry. Proteins in CVS are digested by trypsin into peptides. These peptides are separated by liquid chromatography and injected one by one into the tandem mass spectrometer. The peptides are ionized using electrospray and separated by their mass to charge ratio (m/z) in the first mass spectrometer (MS1), resulting in a mass spectrum. Each peak/ionized peptide from the mass spectrum is then chosen for further fragmented into smaller peptide fragments in a collision cell, and analyzed again by a second mass spectrometer (MS2) in order to determine the amino acid sequence of the peptide. The data from the resulting mass spectrum is then compared against protein databases for protein identification and relative quantification. CVS: cervicovaginal secretions. Figure created using BioRender.com.

MS proteomic techniques have been used extensively to study secretions in the FGT (219). An advantage of shotgun MS based techniques is that, in contrast to antibody based methods, no selection of proteins is needed prior to analysis, thereby allowing for a truly unbiased approach (Table 1). In addition, MS based techniques can identify different isoforms of proteins that might not be distinguished by antibody-based techniques because of similarity of amino acid sequences, such as the α-defensins HNP1-3 (221). Also, since it does not require antibodies directed towards the proteins of interest, MS-based techniques can also identify unknown proteins isoforms, as well as different post-translational modifications.

	Tandem shotgun mass-spectrometry	High-throughput bead-based single-binder affinity assay
Advantages	Unbiased approach Not dependent on availability of high quality antibodies Can detect post-translational modifications and previously unknown protein isoforms	Hundreds of samples can be analyzed at the same time Small amount of sample needed for analysis No depletion of high abundant proteins needed prior to analysis
Limitations	Time and labor intensive Extensive pre-sample treatment	Dependent on the availability of high quality antibodies Antibody performance is context dependent and antibody validation needed for each sample type

Table 1. Advantages and limitations of a tandem mass spectrometry based technique vs. a high-throughput bead-based single binder affinity assay.

However, as with all methods, there are limitations. There is a large dynamic range of the proteome in genital secretions, and the presence of high abundant proteins can mask the detection of low abundant proteins. However, this can be compensated for by depletion/removal of high abundance proteins prior to analysis. Other limitations include extensive pre-sample treatment, the need for sophisticated software and that some MS techniques are labor and time intensive.

3.3.1.2 Bead-based single-binder affinity assay

In paper 3, CVS samples were analyzed using a high-throughput bead-based affinity assay (Figure 4). The antibody panel was designed using proteins selected based on previous MS analysis of genital secretions as well as proteins described as related to HIV resistance or inflammation in the FGT.

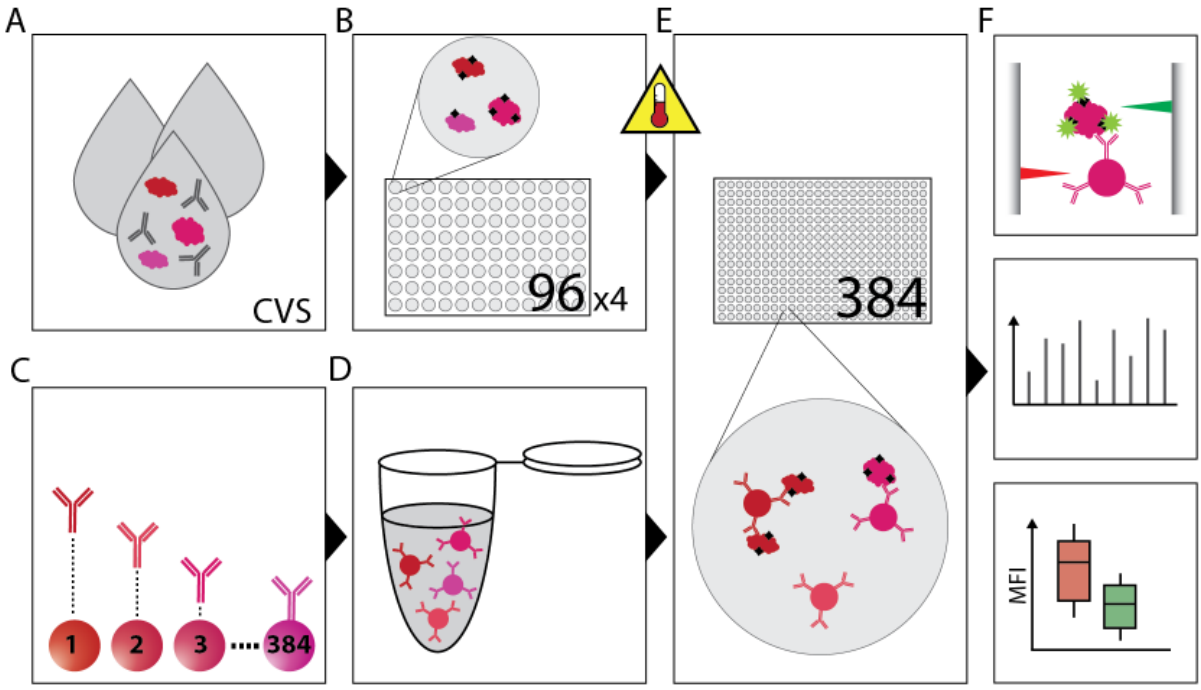

Figure 4. Schematic overview of the bead-based set-up. A. CVS containing a plethora of proteins and antibodies. B. Samples are diluted and biotinylated. The black dots represent biotin attached to the proteins. C, D. In parallel to the steps described in A + B, antibodies selected for detection of proteins of biological relevance are immobilized to color-coded magnetic beads and mixed together to form a suspension bead array. E. The biotinylated samples are further diluted, heat treated and mixed with the suspension bead array. Unbound proteins are washed away after an overnight incubation and a streptavidin-conjugated fluorophore is added for detection. F. Read-out is performed using a FlexMap 3D instrument (Luminex Corp., Austin, United States), and binding events are displayed as MFI. CVS: cervicovaginal secretions. MFI: median fluorescence intensity. Adapted and reprinted with permission (222).

High-throughput antibody based microarray techniques have emerged as a complement to MS based techniques (223). A large portion of the work can be automated and performed in microarray plates, thereby allowing many samples to be processed at the same time (224). One important aspect to achieve such high throughput is that the same amount of sample, regardless of initial protein content, is used for analysis. Another advantage is that no depletion of high abundant proteins is needed prior to analysis.

A limitation is that proteins of interest need to be identified prior to analysis, and the method is thereby more suitable for hypothesis validating studies. The assay is also dependent on the availability of high-quality antibodies directed to the proteins of interest. The antibodies used in Paper 3 are generated via the Human Protein Atlas (HPA) project, a Swedish-based project aimed at mapping and producing antibodies against all human proteins (225). The HPA antibodies used in this thesis are created by immunizing rabbits with antigen, and the produced polyclonal antibodies are validated on protein microarray plates, but not in solution (226, 227). We can therefore not be certain of antibody specificity in complex environments such as genital secretions. One of the major challenges to antibody-based techniques such as this one is evaluating antibody selectivity- are we detecting the protein that we intend to? (228) However, there are several ways to evaluate antibody selectivity, such as sandwich assays and including several antibodies targeting different parts of the same protein.

Concordant results between these antibodies would support that the intended target was captured.

3.3.1.3 Cytokine bead array immunoassay

The concentration of selected cytokines in CVS (Paper 1) and explant conditioned medium (Paper 2) were measured using a multiplex bead array immunoassay. The cytokines chosen for study in both of these studies were pre-selected based on their function as inflammation regulators and abundance in genital secretions (50, 51, 133, 229, 230). The multiplex bead array immunoassay is well established and commonly used in immunological studies to identify and quantify the levels of cytokines/proteins. Using this bead-based technique, several cytokines can be measured simultaneously. A standard curve is used allowing absolute quantification of cytokines concentrations, in contrast to the other methods mentioned above. However, as for all antibody-based techniques, it is dependent on the availability of high quality antibodies. It is also more time and labor consuming than the high-throughput bead-based affinity assay described above.

3.3.2 Tissue based techniques

3.3.2.1 In situ based imaging analysis

In Paper 3, ectocervical and vaginal tissues were stained by immunohistochemistry for the visualization of six proteins of interest, the proteins were selected based on their association with the HIV-serodiscordant phenotype in the antibody-based affinity array analysis. In Paper 4, ectocervical biopsies were stained by immunofluorescence for the immune cell marker CD4 and the adherence junction protein E-cadherin.

The greatest advantage of in situ based imaging of genital tissues is the ability to visualize proteins and cells in intact tissue, thereby allowing assessment of their 'natural' abundance and distribution. A limitation is a dependency on high-quality antibodies and cross reactivity between antibodies limits the numbers of markers that can be assessed at the same time. Another limitation is that it is time and labor intensive and that it usually only allows semi-quantitative analysis of cells/structures, making it difficult to compare results between studies. However, computerized image analysis allowing higher throughput and reproducibility have expanded over the years. A newly developed, quantitative image analysis workflow set up in our lab was used in Paper 4 to enumerate $CD4^+$ cells and E-cadherin (see Paper 4 for details).

3.3.2.2 Transcriptomics

In Paper 4, the ectocervical biopsies in RNA-later were analyzed by RNA sequencing (RNA-seq). All RNA molecules in a cell, population of cells, or tissue is referred to as the "transcriptome", and transcriptomics is the study of the transcriptome. In contrast to the genome that is very stable, the transcriptome varies with external environmental conditions and can therefore be highly variable, even within the same cell or tissue type.

There are several important advantages of using transcriptomics to study tissues in the FGT. First of all, this unbiased approach can identity factors that might not have been uncovered using a hypothesis driven approach. Also, by focusing on pathways and group of genes rather than individual genes/proteins, it is possible to get a more comprehensive understanding of underlying biological mechanisms. To analyze the expression of only a few genes (i.e. not the entire transcriptome), reverse transcriptase based quantitative real-time polymerase chain reaction is the gold standard and widely used in research and clinical settings. For transcriptome profiling, there are two main methods used today: microarray and RNA-seq (231). RNA-seq have several advantages over microarrays, such as ability to detect wider dynamic range, higher specificity, less amount of RNA required for analysis and that is does not require transcript specific probes (231-233). However, a major challenge with RNA-seq is the amount and complexity of data generated, and that it does not yet exist a gold standard for analysis of this type of data. Another limitation when performing RNA-seq on entire tissues, as in our study, is that we do not know from which cells the transcripts originate, this however would be possible with single-cell RNA-seq.

In the biological field, we are obviously interested in biological processes and the mechanisms that govern these. In cells, this is represented by proteins and the derivatives thereof. Gene expression is a proxy for protein expression, but the transcriptome is not directly corresponding to the proteome because of varying degrees of mRNA and protein degradation (234) as well as other post-transcriptional and post-translational events. On the other hand, detection of proteins also has limitations as they can easily be degraded and/or post-translationally modified. Therefore, the gene expression could be more representative of the activation state of the cell. Another advantage of transcriptomics is that nucleic acids such as RNA, in contrast to proteins, can be amplified which facilitates detection.

3.3.2.3 The ectocervical tissue explant model

The ectocervical explant model offers several key advantages for studying the mucosal immune response and mechanisms of HIV-transmission (235). For ethical reasons, the mechanisms of HIV-transmission in the female genital mucosa cannot be studied in vivo in humans, and the non-human primate field is restricted by anatomical differences in the genital tract between the species (235, 236). Cell lines, isolated primary cells and PBMCs are useful in studying many aspects of HIV, but because of lack of tissue architecture and the native communication between cells, these models do not accurately mimic mucosal events. Human cervical explant models can therefore serve as a bridge between cell culture models and in vivo studies.

Among other key features of explant-based models of HIV infection, conserved tissue architecture, including the physiological spatial and functional relationship between multiple cell types, allow for a faithful representation of mucosal events, although for a restricted time window. Another important advantage is that explants can support HIV replication without exogenous stimulation as opposed to PBMCs (235, 237). In addition, it is a very versatile

model that can be used for microbicide testing (238). Explants can also be studied together with genital co-pathogens that are important for HIV-transmission (235).

However, there are certain caveats and challenges associated with using human cervical tissue explants for HIV transmission research (239). A limitation of the explant system is that the tissues are disconnected from the body and blood supply, including immune cells, and it therefore does not fully mimic an in vivo system. Other challenges include donor-to-donor variability between explants, problems with reliable tissue polarization and progressive decay of tissue structure with culture time (235, 238). As compared to using cell lines, tissue explants are often more challenging to acquire which, together with high protocol complexity, make it a low-throughput method (238). Most tissues used for research originate from anonymous donors, and there is often limited clinical data available on tissue donors, including pre-surgical medications and hormone therapy. However, in our study 2, all study participants answered a questionnaire about medical history and exogenous hormone usage, and therefore this is not an issue in our study.

3.3.3 Menstrual cycle phasing

One of the reasons for the discrepancies in studies of the menstrual cycle might be the wide variety of ways of determining menstrual cycle phase in the scientific literature. The gold standard is the use of serial vaginal ultrasounds to determine when ovulation occurs (240), thereby identifying the follicular and luteal phase as prior to and after ovulation, respectively. However, this method is invasive, expensive and time consuming, and not feasible for most studies. Therefore, several other strategies to predict ovulation and/or menstrual cycle phase are used, alone or in combination, such as i) days since the first day of the last menstrual period (LMP) (45), ii) luteal phase serum/urinary progesterone cutoff (32, 241), ii) ratio of urinary estrogen and progesterone metabolites (242, 243), iii) ratio of serum progesterone >2 in the luteal/follicular phase (47), iii) endometrial histology/dating (116), iv) identification of urinary/serum LH surge (124) and v) shifts in basal body temperature (244). Since the LH surge accurately predicts ovulation (240, 245), one of the best indirect method of estimating ovulation is serial sampling of LH in serum or urine (246). Prospective days since LMPs, counting days since last menses, is easy, inexpensive and non-invasive, yet considering the large variation in length of the follicular and luteal phase, this method cannot reliably identify menstrual cycle phase (119, 123).

In order to as correctly as possible define the different phases of the menstrual cycle in Paper 1, we used a combination of days since LMP and female sex hormones. The serum levels of progesterone, estradiol, LH and FSH were measured at every visit. For Paper 2, the majority of study participants had irregular bleeding patterns because of myomas and in some cases treatment with progestin-based compounds, and therefore menstrual cycle phase was not estimated. For Paper 3, no data on menstrual cycle phase was available. For Paper 4, the sampling was aimed for the follicular phase of the menstrual cycle based on LMP.

3.4 STATISTICAL ANALYSIS

3.4.1 Paper 1

To determine the effect of menstrual cycle phase on relative protein abundance (assessed by MS) and absolute cytokine abundance (in pg/ml, assessed by cytokine bead array immunoassay), we used a random intercept linear mixed effect (LME) model. The LME model was chosen primarily for its ability to handle missing data points as well as possibility to combine fixed and random effects. Fixed effects in the model were menstrual cycle phase and cervicovaginal microbiome composition (as defined by 16S rRNA gene sequencing) with an interaction term between fixed effects. Each woman (subject) was included as a random effect. Correlation analysis between serum hormone levels and protein/cytokine levels were performed using Spearman's correlation. To adjust for multiple comparisons testing, Benjamini-Hochberg (BH) (247) adjustments were performed. Proteins with BH adj $p < 0.05$ and Log_2 fold change >1 between menstrual cycle phases were considered significant.

3.4.2 Paper 2

In order to account for inter-individual differences between tissue donors and seminal-plasma pools, the results of the treated explants were normalized to donor-matched untreated explants, and this ratio was termed n-fold. The difference in n-fold change between culture conditions were compared against 1 using the Wilcoxon signed rank test. When groups of mixed paired/non-paired data were evaluated, n-fold differences were evaluated using the Mann-Whitney U. For groups containing only paired data, the Wilcoxon matched-pairs signed rank test was used to evaluate difference in n-fold. When the difference in n-fold change of cytokines between multiple groups were compared, Kruskal-Wallis test was used for groups with mixed paired and unpaired data, and the Friedman test for only paired data. Dunn's post hoc test was applied, $p < 0.05$ indicated statistical significance.

3.4.3 Paper 3

To assess differences in demographic parameters between the serodicordant and the control group, a logistic regression model was used. All p values were subjected to multiple comparisons testing using BH, and BH adj. $p < 0.05$ were considered significant.

3.4.3.1 Univariate analysis

The relative abundance protein data from the affinity set-up were compared between the serodiscordant group and the control group using the Mann-Whitney U test because of non-normality of the data. Two-group comparisons were evaluated between the control group and all three time points for the serodiscordant group, the presented numbers are the least significant (over all three time points).

3.4.3.2 Adjustment for potential confounders

To adjust for potential confounders, we used two statistical models: a bivariate linear regression model and a LME model. Potential confounders were demographic parameters that varied ($p<0.1$) between study groups. The bivariate linear regression model was assessed for the proteins with BH adj. $p<0.05$. The log-transformed intensity of each protein was included as the dependent variable, and potential confounders including serodiscordant/control variables as independent variables. The bivariate linear regression models (one for each potential confounder) was compared to the crude model, and confounding was present if the adjusted model differed by greater than +/- 10%. In order to further evaluate the effect of potential confounders, the data was adjusted using a LME model. The above mentioned potential confounders were included as fixed effects, and visit code as an estimate of sample time was included as a random effect. This LME-adjusted data was subjected to univariate analysis (as described above) as well as a multivariate, discriminant data analysis approach. For this, a least absolute shrinkage and selection operator algorithm was applied to determine the minimum set of proteins necessary to distinguish the two study groups.

3.4.3.3 Comparative analysis between MS and antibody-based data

In order to validate the findings using a separate proteomic technique, a subset of samples were analyzed both by MS and the bead-based affinity technique. Median fluorescence intensity (MFI) values from the bead-based affinity technique were compared with abundance data from the MS analysis using Spearman's correlation.

3.4.4 Paper 4

For the demographic parameters, Mann-Whitney U was performed to assess continuous variables and Pearson's Chi-squared test was used to assess categorical variables between the DMPA and the control group. For the RNA-seq data, differences in gene expression between study groups (DMPA vs controls) were evaluated using linear regression and subjected to multiple comparison testing using false discovery rate (FDR). Differentially expressed genes were defined as FDR adj. $p<0.05$. For the imaging data, a Mann-Whitney U test followed by BH correction was used for comparisons between groups for the (in total) 18 measurements acquired by image analysis and BH adj $p < 0.05$ was considered significant.

4 RESULTS AND DISCUSSION

4.1 FEMALE SEX HORMONES AND HIV SUSCEPTIBILITY

There is a plethora of evidence suggesting that female sex hormones, both endogenous and exogenous, influence HIV-susceptibility in the FGT. Some of the most important factors affected by female sex hormones include genital inflammation, epithelial barrier integrity and cervicovaginal microbiome composition, as discussed below.

4.1.1 Endogenous female sex hormones

In paper 1, our aim was to characterize how the fluctuations in female sex hormones over the menstrual cycle influence the local immunological milieu and cervicovaginal microbiome in the FGT. We also aimed to collectively evaluate the immunological and microbial changes associated with the menstrual cycle to reveal potential interactions.

4.1.1.1 Proteins and cytokines in genital secretions

In order to characterize the local immunological milieu, CVS were analyzed by MS and selected cytokines analyzed using a multiplex bead array immunoassay. Samples were classified as either follicular (n=13), ovulatory (n=5) or luteal (n= 14) based on the stringent criteria using days since LMP and serum hormone measurements. MS analysis revealed 406 unique proteins in the cervical secretions. Using a LME model to adjust for cervicovaginal microbiome composition and baseline difference between women, the largest change in proteins composition was seen for the ovulatory phase as compared to both the follicular and luteal phases. The difference between the follicular and luteal phase was much smaller, and there was a large overlap in protein composition between these phases.

In order to comprehensively characterize the biological pathways of the different phases, proteins including cytokine expression profiles were used for pathway analysis. In the follicular and luteal phases as compared to the ovulatory phase, pathways relating to immune response and cellular inflammation were predicted to be upregulated. There was a large overlap in pathways upregulated in these two phases, but the magnitude was in general larger for the luteal vs. ovulatory phase than for the follicular vs. ovulatory phase. Several proteins common for these pathways include neutrophil proteases, integrins, S100 proteins, antiproteases and leukocyte factors. In the ovulatory phase, pathways predicted to be upregulated included epithelial cell differentiation, tissue homeostasis and cell-to-cell adhesion. Inflammatory pathways, including leukocyte recruitment, were downregulated in the ovulatory phase. Proteins associated with the ovulatory phase included antiproteases important for regulating inflammation, mucins and proteins involved in epithelial barrier integrity and repair. Despite the high estradiol levels in the ovulatory phase, only one protein (KRT6C) was positively correlated with estradiol concentrations. To further characterize the inflammatory status of the different phases, we used a multiplex bead array immunoassay to measure selected cytokines. Several of these cytokines are present at low concentrations in

genital secretions and might not be identified by MS. The cytokine analysis revealed that three pro-inflammatory cytokines, namely TNF, CCL20 and interleukin(IL) 6, were reduced in the ovulatory phase as compared to the luteal phase ($p<0.05$), although none passed multiple comparison corrections.

To conclude, the progesterone-high luteal phase was characterized by leukocyte and neutrophil recruitment pathways and pro-inflammatory cytokines. In contrast, the progesterone low, estradiol-high ovulatory phase was characterized by upregulation of anti-inflammatory, wound-healing and antimicrobial pathways, as well as by a decrease in pro-inflammatory cytokines. The follicular phase was a mixture between the other two, with inflammatory pathways upregulated, but to a lesser extent than in the luteal phase, and with elevated anti-proteases (Figure 5).

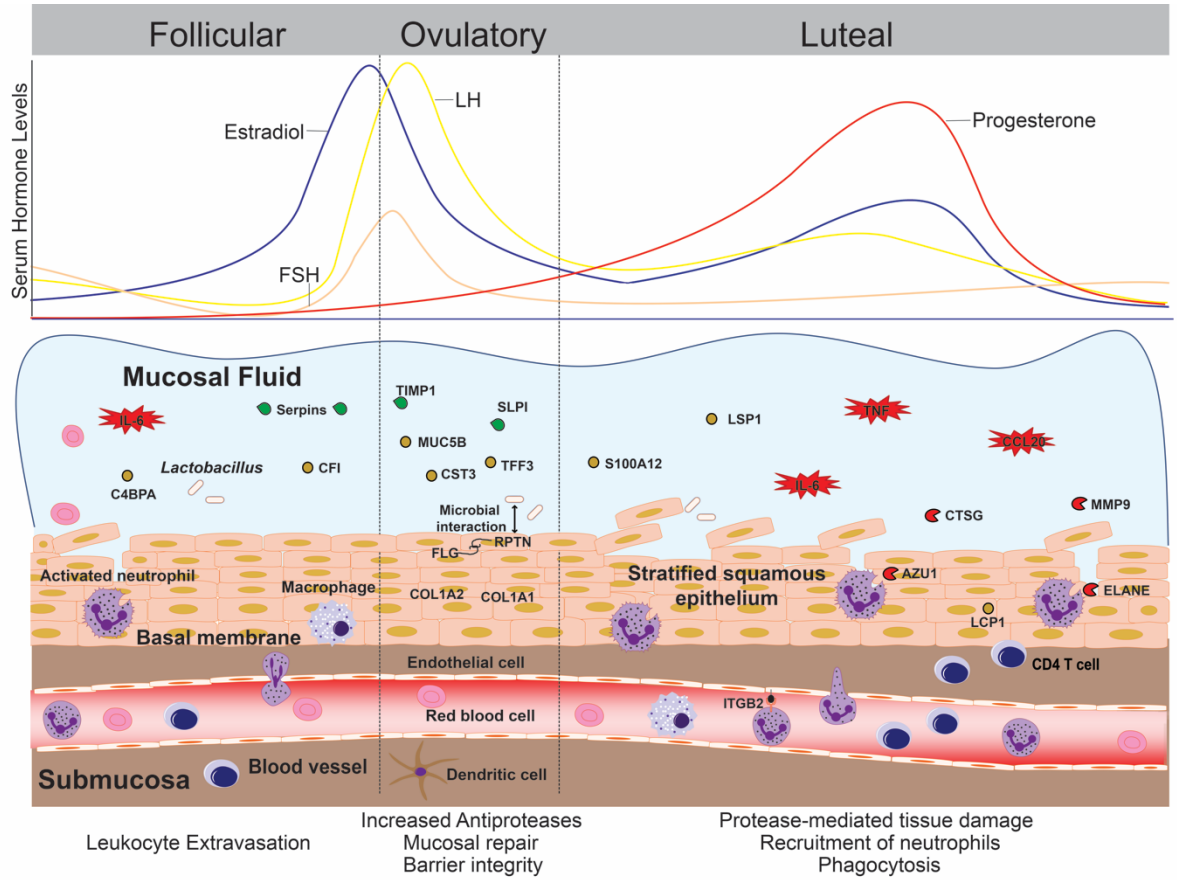

*Figure 5. Illustration of proposed mechanisms during the phases of the menstrual cycle***.** Figure originally published in (248), reprinted with permission.

Several other studies have also reported an inflammatory profile in the FGT in the luteal phase (32, 45, 127, 128). Such pro-inflammatory state in the FGT in the luteal phase could be associated with an increase in HIV-target cells, which has been observed in some (126, 130), but not all studies (31, 32, 47, 131). Furthermore, a general increase in inflammatory cytokines in the luteal phase could potentially confirm an inflammatory state. We observed a slight increase in the inflammatory cytokines IL6, TNF and CCL20 in the luteal phase, but these results should be interpreted cautiously because they do not pass multiple comparison

testing. Previous studies of cytokine expression in genital secretions have shown inconsistent results, including both increase and decrease of individual inflammatory cytokines in the luteal phase, as well as no change (47, 50, 55, 132, 133). Other proteins with implications for decreased HIV-susceptibility, such as antiproteases including serpins, have been associated with the follicular phase (45, 46). In concordance with this data, we observed an increase in antiproteases in the follicular phase, but also in the ovulatory phase. We also showed a distinct increase in the antiprotease SLPI, with known anti-HIV-activity, in the ovulatory phase, whereas previous studies have shown a decrease (55), an increase (249) or no change (48, 53) in the ovulatory/midcycle phase.

These inconsistent results warrant some discussion. Firstly, it should be noted that an inflammatory response will be followed by a counteractive anti-inflammatory response in the body, and this process is a continuum. Therefore, at a given timepoint, there will be both "inflammatory" and "anti-inflammatory" processes ongoing, and inflammation mediators from both such processes will be present at the same time. In addition, since the immune system is very complex and the function of each protein/cytokine is dependent on other proteins, it is precarious and can be misleading to focus on individual proteins/cytokines. Instead, the data must be interpreted collectively, such as by pathway analysis, to give a representative picture of the inflammatory state of the mucosa.

In addition, there are several other possible explanations for the discrepancies between studies. Other factors likely to affect the results include the varying model systems and methods used, there is a the wide variety of methods used to estimate menstrual cycle phase as well as cross-sectional vs longitudinal study designs. We observed the largest difference in the short ovulatory phase, whereas many studies are lacking data on this phase. Also, there is a large variation in sex hormone levels *within* each menstrual cycle phase, and timing of sampling within each phase might vary between studies. A limitation of our own study is that we only assessed serum hormone measurements once a week, which could have contributed to a lack of detection of important hormonal fluctuations essential for menstrual cycle classification, such as the LH-peak. The progesterone cut-off level used to defined the luteal phase (ratio >2 as compared to the follicular phase) in our study is comparable to the cutoff used by others (47, 130). However, higher progesterone reference values levels are used in the clinical setting to define the mid-luteal phase, and our more lenient definition may affect protein expression.

Although discrepancies between studies exists, the combined results of the above mentioned studies indicate that the luteal phase is associated with increased inflammation. Such increased inflammation and increase in HIV target cells could potentially explain an increase in HIV-susceptibility in the luteal phase (106, 113) that is observed in some, but not all (32, 126) human genital explant studies, as well as non-human primate models (105, 106). It is also possible that the reduced mucosal levels of antimicrobials proteins, such as SLPI, observed in the luteal phase may facilitate HIV-transmission. In addition, proteins involved in epithelial barrier remodeling and tissue homeostasis were decreased, indicating that the epithelial barrier is weakened during this phase. As mentioned above, the largest change of

the luteal phase was in comparison to the ovulatory phase, which was associated with an increase in epithelial barrier proteins and decreased inflammation.

Combined, an inflammatory profile, which could be associated with increased target cells, a decrease in antimicrobial proteins and a weakened epithelial barrier in the luteal phase may be associated with an increased HIV-susceptibility. In contrast, the ovulatory phase profile of decreased inflammation and target cells, an increase in antimicrobial proteins and a robust epithelial barrier may confer protection against HIV infection.

4.1.1.2 The cervicovaginal microbiome and the menstrual cycle

Although it is well established that the cervicovaginal microbiome influences the immunological milieu including epithelial barrier integrity in the FGT (19, 52, 153, 161), less is known about the interplay between the menstrual cycle, the cervicovaginal microbiome and the immune system. We wanted to characterize how the cervicovaginal microbiome fluctuates over the menstrual cycle as well as any potential interactions between the menstrual cycle and the cervicovaginal microbiome.

To characterize the cervicovaginal microbiome , the V3-V4 region of 16S rRNA gene was sequenced, and *Lactobacillus*-dominance was defined as >50% of reads belonging to the *Lactobacillus* genus. The majority of study participants showed a stable microbiome composition over all visits: 63% (n=10) were consistently *Lactobacillus*-dominant, 25% (n=4) consistently non-*Lactobacillus* dominant and 13% (n=2) transitioned from one state to the other over the menstrual cycle. The 16S rRNA gene sequencing data was compared to data at the metaproteome level, and sample classification was concordant in 91% (43/47) of samples. Our findings of *Lactobacillus*-dominance are similar to other studies in Caucasian women (152, 154, 156). In concordance with several other (156, 167), but not all (250) studies, we observed a relatively stable microbiome composition across the menstrual cycle. Previous studies have observed an increased diversity (167), increase in *Gardnerella (168)* and/or other non-*Lactobacillus* taxa (169) around menses, but we did not assess menses-related changes in our study.

To further characterize potential interactions between the menstrual cycle and the cervicovaginal microbiome, an interaction term between the two fixed effects (menstrual cycle phase and cervicovaginal microbiome composition) was added to the LME model. Several proteins important for the epithelial barrier demonstrated an interaction between these two effect. These proteins showed a significant decrease from the ovulatory to the luteal phase, but non-*Lactobacillus*-dominant women showed a greater decline in expression relative to *Lactobacillus*-dominant women. Of these proteins, the epithelial protein RPTN was the most significant and the only protein that passed multiple comparison corrections. Such findings could indicate that menstrual-cycle induced changes to HIV-susceptibility in the luteal phase could be reduced by *Lactobacillus*. However, because of the small sample size and only one non- *Lactobacillus* dominant woman in the ovulatory phase, these findings should be interpreted cautiously. In addition to the limitation of the small sample size

mentioned above, another limitation is that the 16S rRNA gene sequencing method used could not consistently distinguish between different species of *Lactobacillus,* and therefore no sub-analysis for different species of *Lactobacillus* was performed. In addition, it would have been very interesting to assess the effect of the cervicovaginal microbiome within each menstrual cycle phase, however, the study was not powered for this. To conclude, our results indicate that the cervicovaginal microbiome might influence cycle-related changes to epithelial barrier integrity.

4.1.2 Exogenous female sex hormones

Observational studies in sub-Saharan Africa suggest that the use of the injectable progestin-containing contraceptives DMPA is associated with an up to 50% increased risk of HIV acquisition (22-24), although the results may be confounded by behavioral factors. Thus, as suggested, an epidemiological link remains to be established as defined by prospective studies comparing use of DMPA to relevant control groups (138). Suggested mechanisms for increased HIV-risk in women using DMPA in the FGT include an altered genital immune environment, including increased number and infectivity of HIV-target cells, and decreased epithelial barrier integrity, as discussed below. In Paper 4, our aim was to characterize how DMPA affects the ectocervical epithelium using RNA-seq and in situ based imaging.

RNA-seq was performed on ectocervical tissue from Kenyan FSW using DMPA (n= 32) and control women without HC usage (n= 65), aimed at collection in the follicular phase of the menstrual cycle. The DMPA group was significantly younger and reported shorter duration of sex work, but comparable for the remaining demographic parameters. Of the 15,326 identified genes, 1,279 (8%) and 1,244 (8%) were upregulated (FDR $p < 0.05$) in the DMPA and the control group, respectively. Of these top upregulated genes in the DMPA group, several (*OLFM4, PCDH8, DUSP4*) are regulated by estrogens (251-253). We also observed an increase in the gene coding for the neutrophil-attracting chemokine CXC motif chemokine (CXCL)6 (254). In the DMPA group, genes associated with epithelial barrier structure and function were downregulated (Figure 6). Interestingly, desmosomal cadherins were also downregulated in the DMPA group, and the protease *CAPN14*, that degrades the desmosomal cadherin *DSG1* (255), was upregulated.

To further characterize the effect of DMPA on the epithelium, the differentially expressed genes were used to identify gene sets and biological processes using a gene ontology software. The most upregulated and downregulated biological processes in the DMPA group were related to immune activation and development and maintenance of the epithelium, respectively. These results are concordant with the results of the individual gene analysis, indicating that both epithelial barrier genes and pathways were downregulated in the DMPA group. In contrast, genes and biological pathways related to immune activation were upregulated in the DMPA group.

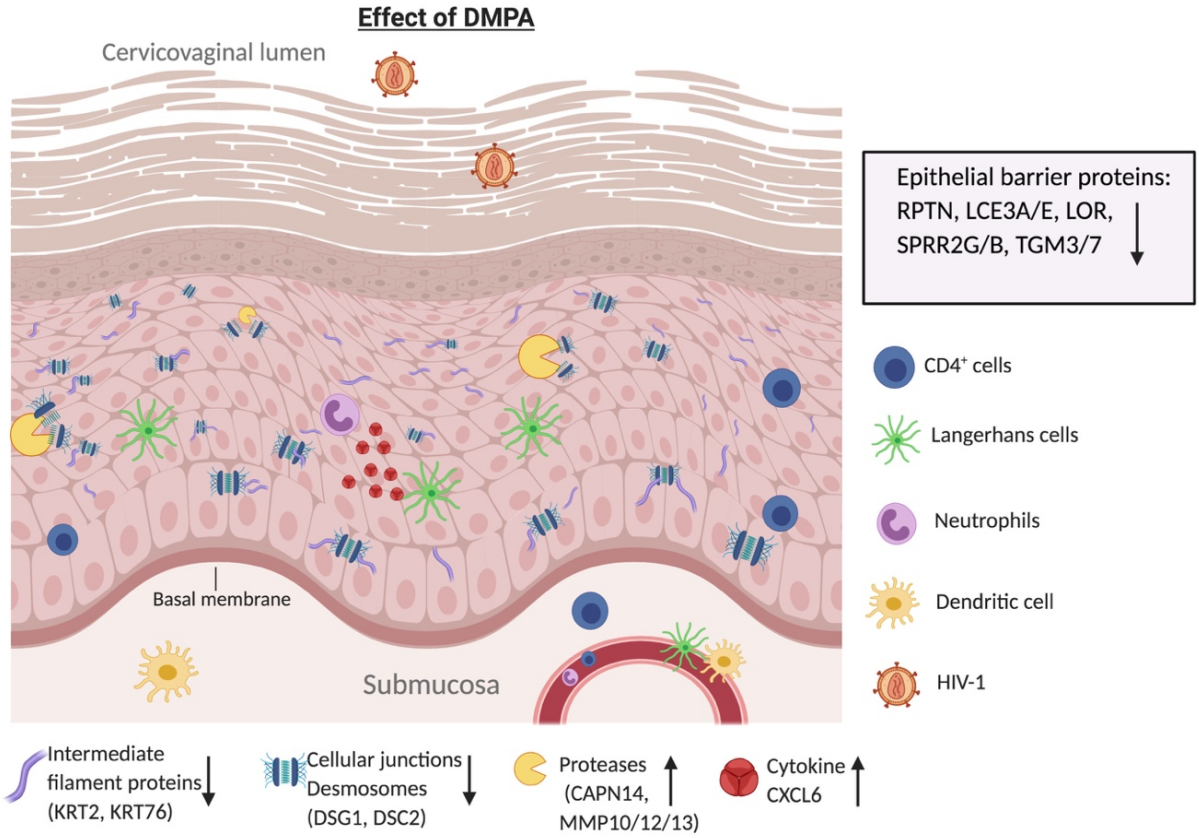

Figure 6. Schematic illustration of the proposed effects of DMPA use on the ectocervical epithelium. Created using Biorender.com.

To complement the gene expression analysis at the structural and protein level, the ectocervical tissue biopsies were evaluated by in situ staining combined with digital tissue analysis to measure epithelial thickness, evaluate integrity and assess HIV-target cell numbers and localization. Our results show that the DMPA group have thinner superficial layer as compared to the control group, but there was no difference in total thickness of the epithelium. The DMPA group also had a lower E-cadherin coverage in the epithelium and a higher proportion of $CD4^+$ cells located in the upper intermediate layer (the layer just below the most superficial layer).

Previous studies indicate that desmosomal cadherins are affected by DMPA use (35, 256, 257) and a downregulation of desmosomal cadherins was observed in our gene expression analysis. We therefore wanted to look specifically at the expression of the desmosomal cadherin Desmoglein-1 in tissues, and immunofluorescent in situ staining of Desmoglein-1 was performed on three representative samples from each study group. Visual inspection in this limited sample set revealed that Desmoglein-1 was mostly present in the lower intermediate layer, and that the area coverage of Desmoglein-1 was lower in the DMPA group as compared to the control group.

To summarize, in Paper 4 we demonstrated that the use of DMPA is associated with an increase in immune activation pathways. We also observed a downregulation of genes coding for epithelial barrier proteins and pathways involved in epithelial barrier development and

differentiation in the DMPA group. Desmosomal genes were downregulated, which was also visualized at the protein level using in situ based imaging. Confirming the results of a weaker epithelial barrier obtained by gene expression analysis, we observed a thinner superficial layer and a more leaky epithelium, as assessed by E-cadherin expression, in women using DMPA.

Our results indicate an enhanced immune activation in the FGT. Previous studies from in vivo studies on the effect of DMPA on soluble immune modulators in the FGT, such as cytokines, are however contradictory - studies show a general increase (50, 148), decrease (149, 150) or selective but not broad increase (258) in inflammatory cytokines. There are several plausible explanations for such discrepancies between studies, including differences in study design (size of study population, cross-sectional vs longitudinal, time span between measurements) as well as population differences in cervicovaginal microbiome composition and/or STIs and variability in expression of factors depending on site of sampling within the FGT. Indeed, a microarray based transcriptomics study observed a pro-inflammatory profile in DMPA-users in endometrial tissues, but not in tissues from the transformation zone (259). This is in contrast to our study, where we observed such signature of immune activation in the ectocervix. Our results are also in contrast with those of Zalenskaya et al., that observed a downregulation of genes involved in immune activation in DMPA users in ectocervical tissues (35). However, in a subgroup of DMPA-users in their study, molecular functions associated with immune cell movement categories were activated, indicating that DMPA may have varying effect in different individuals/subpopulations. Immune activation can also be evaluated by numbers of HIV-target cells in the FGT, and DMPA has been associated with increased target cells in some (31, 130, 147) but not all (151) studies. Although we did not find an increase in total $CD4^+$ cells, used as a proxy for HIV-target cells, in DMPA users, we did observe a more apical distribution, suggesting that the $CD4^+$ cells are more accessible to virus particles at the vaginal lumen.

DMPA is associated with a dramatic thinning of the cervicovaginal epithelium in non-human primates (144), but the majority of studies in humans do not demonstrate a significant effect (31, 34, 143, 145). However, these studies evaluate total epithelial thickness, whereas our imaging technique allowed specific measurements of the layers within the epithelium. In concordance to the above mentioned studies, we did not observe a difference in total epithelial thickness in DMPA users, but, as stated above, we noted a thinner superficial layer. A thinner superficial layer in response to DMPA was also demonstrated in non-human primates (260) as well as in progestin-containing intrauterine device users (33). In the latter study, the progestin intrauterine device was also associated with a decrease in the expression of the tight junction protein ZO-1, indicating a weakened epithelial barrier. Although we did not specifically assess ZO-1 by in situ staining, we observed lower coverage of the adherence junction protein E-cadherin coverage in the DMPA group. This, in combination with a thinner superficial layer, indicate a weaker epithelial barrier and easier access for the virus to HIV target cells.

In additional to potential effects of DMPA on ZO-1 (expressed in epithelial tight junctions) and E-cadherin (expressed in adherence junctions), recent studies indicate a DMPA-induced effect on desmosomes in mice (256, 257) and humans (35, 256). Desmosomes contribute to

cell-cell adhesion between keratinocytes in epithelia and, together with tight junctions and adherence junctions, strengthen epithelial integrity (37). We observed a downregulation of the desmosomal proteins at the gene level in DMPA users, and protein expression of the desmosomal protein Desmoglein-1 was confirmed by in situ based imaging. In addition, the protease CAPN14, that degrades Desmoglein-1 (255), was upregulated with DMPA use. Zalenskaya (35) and Quispe Calla et al. (256, 257) also reported a DMPA-associated downregulation of Desmoglein-1, both at the gene and protein level, in human (35, 256) and mice (256, 257) tissues, and Zalenskaya et al. also observed an upregulation of CAPN14. In mice, a DMPA-associated decrease in expression of Desmoglein-1 promoted increased susceptibility to intravaginal HSV-2 infection and induced enhanced genital mucosal permeability in their experimental mouse model, indicating that decreased desmosomal function influences permeability of the epithelium.

There are several other striking similarities in the results of the study by Zalenskaya et al. and ours (35). They also observed DMPA-associated regulation of epithelial barrier genes and corresponding biological processes involved in epithelial barrier development. The similar results are even more interesting considering the large differences between their study and ours including method (micro-array whole-genome transcriptome profiling vs. RNA-seq), study population (North American non-sex workers vs. FSWs in a HIV-endemic area) and study design (longitudinal vs. cross-sectional). Also, they studied short term (6 weeks) use of DMPA, whereas the study population in our study had been using DMPA for at least 6 months.

Limitations include that we are lacking data on time since last injection of DMPA, as well as of serum measurements of MPA. Several recent studies have demonstrated a dose-dependent effect of MPA on the genital epithelium (162, 257), further highlighting the benefit of evaluating MPA concentrations. Another limitation in the study is the differences in “years in sex work” and “age” between the DMPA vs. control group, which may influence the results. In addition, the imaging data on Desmoglein-1 needs further analysis. We also used $CD4^+$ cells as a proxy for HIV target cells, but would further like to phenotype such cells using additional markers and co-receptors for HIV, such as CCR5 and C-type lectin rectors. In addition, mRNA from the entire ectocervical biopsy was isolated and sequenced and the varying proportions of epithelium/submucosa in the biopsies could potentially bias the results. Also, we cannot know exactly from which cells the transcripts originate. This would however be possible using single cells RNA-seq.

In conclusion, our transcriptional profiling and imaging data reveal decreased epithelial barrier integrity and higher immune activation in the ectocervical mucosa of women using DMPA. Our results further suggest that the degradation of proteins essential for desmosomal function is one possible mechanism that contributes to this decreased ectocervical tissue integrity. Critically, these combined mechanisms may lead to increased HIV susceptibility in women using DMPA.

4.1.3 Drivers of sex hormone related changes to HIV susceptibility factors

Combined, the results from Paper 1 and 4 help elucidate potential mechanisms of how endogenous and exogenous female sex hormones influence the lower FGT with focus on factors important for HIV-susceptibility. The studies differ in design and explore different aspects of how endogenous and exogenous sex hormones act in a complex context. Important factors that need to be taken into account when interpreting data on hormonal influence include presence of plasma serum proteins, pharmacokinetic aspects and affinity of the respective compound to different steroid receptors (reviewed in (110)). Other aspects that will influence the effect of sex hormones are local tissue concentration of the hormone, which can vary from serum concentrations (261), distribution of receptors in different tissues, cross-talk between receptors and presence of other receptor-ligands. In addition, the endogenous production of female sex hormones is dependent of the intertwined hypothalamic-pituitary-adrenal and hypothalamic-pituitary-gonadal axis, that is regulated at each of the anatomical levels (hypothalamus, pituitary gland, gonads, adrenal glands) with various feed-back loops. In short, it is an extremely complex system and it is outside the scope of this thesis to go into details of each of the above mentioned aspects. Here, I will give a brief overview of the possible mechanisms that drive the phenotypes observed in Paper 1 and Paper 4.

In Paper 1, we studied the fluctuations in exogenous hormones over the menstrual cycle. The largest effects were seen in the ovulatory phase (high estradiol, low progesterone) as compared to the luteal phase (medium/high estradiol, high progesterone). It is important to keep in mind that the observed effect is the combined effect of these hormones, and most likely several other factors. The current study design does not allow determination of which of these hormones is the strongest influencer or "driver". However, it can be speculated whether high levels of estradiol exert a protective effect on the genital mucosa against HIV-acquisition, as outlined below. Studies in non-human primates indicate that local and systemic estrogen treatment is associated with a thicker vaginal epithelium and decreased susceptibility to intravaginal SIV-challenge (140, 141). Post-menopausal women, with low levels of estradiol but also progesterone, show a thinner genital epithelium and increased HIV-replication in ectocervical tissues as compared to pre-menopausal women (131). However, epithelial thickness and HIV-susceptibility were increased by a vaginal estradiol-containing cream. Another potential protective mechanism of estradiol is that estradiol is believed to be the main driver for shifting the cervicovaginal microbiome composition to beneficial *Lactobacillus*-dominance (262), at least in part by promoting glycogen in the genital epithelium, which in turn can support *Lactobacillus* (263). In additional, both estradiol and progesterone can have profound effects on immune functions in both the upper and lower FGT (reviewed in (109)), which might affect HIV-susceptibility.

It has also been proposed that the hypoestrogenism induced by DMPA may contribute to the increased HIV-transmission in DMPA-users (reviewed in (264)). Importantly, progestins exert varying effects on endogenous estradiol production, with DMPA inducing the most pronounced suppression of estradiol among several progestins. Interestingly in our Paper 4, several of the most significant genes upregulated in DMPA users have previously been shown

to be regulated by estrogen, and it can be speculated whether estrogen may drive the effects observed in the genital epithelium. However, this needs to be further explored, and serum estradiol concentrations would be useful for this.

In addition to the proposed effect of hypoestrogenism, the effect of DMPA on HIV-susceptibility can also be mediated by the active progestin MPA. As mentioned previously, MPA show high affinity not only to the PR but also to the GR, and act as a partial to full GR-agonist (265). This is in contrast to other progestins and endogenous progesterone. It has been suggested that such GR-activity modulate HIV-acquisition and pathogenesis (139). The GR-mediated effect on HIV-susceptibility is supported by several studies (139, 266, 267), including a recent study showing that the progestin MPA, but not NET (used in the injectable contraceptive NET-EN) enhanced R5 tropic HIV-replication in a genital explant model, and that this effect was likely mediated by the GR (267). In summary, the effect of DMPA on the FGT mucosa is most likely mediated by a combination of DMPA-induced hypoestrogenism and the effect of MPA, acting via the PR and/or GR to varying degrees.

4.2 THE USE OF A GENITAL EXPLANT MODEL TO EVALUATE THE EFFECT OF SEMINAL PLASMA ON INFLAMMATION AND HIV-TRANSMISSION IN THE FGT

The majority of new HIV infections in women is due to deposition of semen from HIV-infected men in the vagina during sexual intercourse (5), but the initial events of HIV-transmission in the FGT remain largely unknown, partly because of lack of adequate model systems (268). Previous studies indicate that semen deposition induce an inflammatory response and neutrophil accumulation in the FGT (99, 100), but it remains largely unknown how this affects HIV-susceptibility. Therefore, in Paper 2 we used a genital explant model to evaluate the effect of seminal plasma on the immunological milieu and HIV-replication in the FGT.

In order to evaluate how seminal plasma affects the immunological milieu in the genital mucosa, ectocervical explants from healthy donors were exposed to seminal plasma. When compared to donor-matched untreated explants, incubation with seminal plasma induced an increase for all cytokines (IL-6, TNF, CCL5, CCL20, CXCL1, CXCL8) except for IL-1α and IL-10, as measured by bead-based immunoassay in culture medium. When examining the transcription of genes corresponding to the measured cytokines by quantitative real-time polymerase chain reaction, we observed matching transcription and protein expression for 5 of the 8 cytokines assessed by both methods. For the majority of cytokines and conditions, the addition of the cyclooxygenase-inhibitor indomethacin did not significantly alter the cytokine response, suggesting that the seminal plasma-induced response is not affected by endogenous prostaglandin production.

To test if we could recapitulate the influx of leukocytes to the mucosa after coitus, peripheral blood leukocytes were incubated with culture medium from donor-matched untreated and seminal plasma-treated explants in a transwell system. In comparison to culture medium,

seminal plasma-treatment increased transmigration of total leukocytes, neutrophils and monocytes, but not lymphocytes. Further analysis of receptors on transmigrated monocytes show a downregulation of the chemokine receptor CCR5, indicating that the production of the CCR5 ligand CCL5 (RANTES) in seminal plasma-treated explants could account for recruitment of monocytes.

Previous studies indicate that incubation of isolated cells together with semen and/or seminal plasma is toxic (102, 103), and we therefore wanted to evaluate cell viability in our model. Apoptosis and necrosis was determined by measuring the expression of Annexin V and the binding of an amine-reactive dye in individual cells isolated from explants after incubation with either seminal plasma or culture medium only, using flow cytometry. There was no difference in the expression of these markers after treatment with seminal plasma in both leukocytes ($CD45^+$) and non-immune cells ($CD45^-$), whereas cells from explants incubated with the apoptosis-inducing drug camptothecin showed a distinct staining pattern. To further evaluate the effect on seminal plasma on tissue viability, an in situ analysis of chromatin fragmentation was performed, revealing a few apoptotic cells in the most apical epithelial layers of the epithelium as well as shedding of epithelial layers. There was no difference in explants treated with seminal plasma vs. controls, indicating that seminal plasma does not induce apoptosis in our model.

Since genital inflammation is associated with increased HIV-susceptibility in vivo (14, 92) and it has been suggested that semen can impact HIV-transmission (269), we wanted to evaluate how seminal plasma induced inflammation affected HIV transmission in our model. Donor-matched untreated and seminal plasma-treated explants were infected with CCR5-tropic laboratory-adapted virus HIV-1_{BaL} and selected transmitter/founder (T/F) HIV-1 molecular clones. Productive HIV-infection was determined by the release of HIV core protein $p24_{gag}$ measured over time in culture medium supernatant as well as HIV-1 DNA in explants harvested at the end of culture. For the T/F strains, productive HIV-1 infection was not achieved, regardless of culture conditions. For HIV 1_{BaL}, treatment with seminal plasma significantly increased the cumulative production of $p24_{gag}$ in culture medium. This was observed both when first incubating explants with seminal plasma and then adding the virus-inoculum, as well as when mixing seminal plasma and virus. We also observed increased levels of HIV-1 DNA in seminal plasma-treated vs donor-matched untreated explants. Treatment of explants with the anti-retroviral drug lamivudine as a negative control showed constant decline of $p24_{gag}$ production in culture medium supernatant and very low levels of HIV-1 DNA in explants.

To summarize, we observed a pro-inflammatory reaction as well as increased HIV-1 infectivity in response to seminal plasma in an ectocervical explant model. Our results of elevated pro-inflammatory cytokines in response to seminal plasma/semen are in concordance with previous studies in cell lines and primary genital cells (270, 271) and a genital explant study (272)). The in vivo human studies are limited, but Sharkey et al. demonstrated an increased mRNA expression in human ectocervical biopsies before and after unprotected vaginal coitus for several pro-inflammatory cytokines (100). Interestingly, such increase was

not observed after protected coitus, supporting our results that it is the semen or derivates thereof (such as seminal plasma) that mediate such increase in cytokines. In contrast, another study revealed an overall lack of increase in pro-inflammatory cytokines in genital secretions after unprotected coitus (101).

An important limitation is that due to the lack of explant polarization, the model does not mimic the exact route of HIV-transmission in vivo. However, the detection of cells harboring HIV RNA in explants harvested at day 18 post-infection suggests that this system could be used to study the pool of HIV-founder cells and their implication in the local response to biological as well as pharmacological treatment. Interestingly, productive HIV 1-infection was only observed for HIV_{BaL} but not T/F HIV-1 clones, highlighting differences between strains and experimental models of HIV infection. Importantly, incubation of explants with seminal plasma as evaluated after 24 hour culture, did not seem to induce cell toxicity in our model, in contrast to studies in isolated cells (102, 103). In addition, the genital explant model described above is flexible and open to modifications. Partially intact tissue architecture, absence of seminal plasma-induced toxicity and flexibility of the model support the suitability of using genital explants to evaluate the safety and efficacy of microbicides and other local applications in the FGT in the context of unprotected sex beyond HIV infection.

4.3 THE GENITAL PROTEOME OF HIV-SERONEGATIVE WOMEN LIVING IN HIV-SERODISCORDANT RELATIONSHIPS

Studies of HIV-serodiscordant couples can aid our understanding of natural relative resistance noted in some high-risk groups as well as the immune response evoked by continuous HIV exposure. Previous studies have identified both elevated (57, 62, 63, 178, 179) and decreased (183, 192) levels of inflammatory proteins (such as several cytokines) and anti-inflammatory anti-proteases in the FGT. A low baseline immune activation (so called “immune quiescence”) has also been proposed as a mechanism of protection against HIV-infection (174). However, these findings are primarily based on FSWs and other cohorts with high levels of unprotected sex and STIs/clinical inflammation. In contrast, in the HIV-serodiscordant cohort in Paper 3, we observed low levels of STIs/clinical inflammation and high levels of protected sex. We therefore hypothesized that their genital protein composition would be similar to that of the general population in the same geographical area. To characterize the genital proteome of women living in HIV-serodiscordant relationships, CVS from these women were assessed by a high-throughput bead based protein profiling technique (See Figure 7 for overview of the study setup).

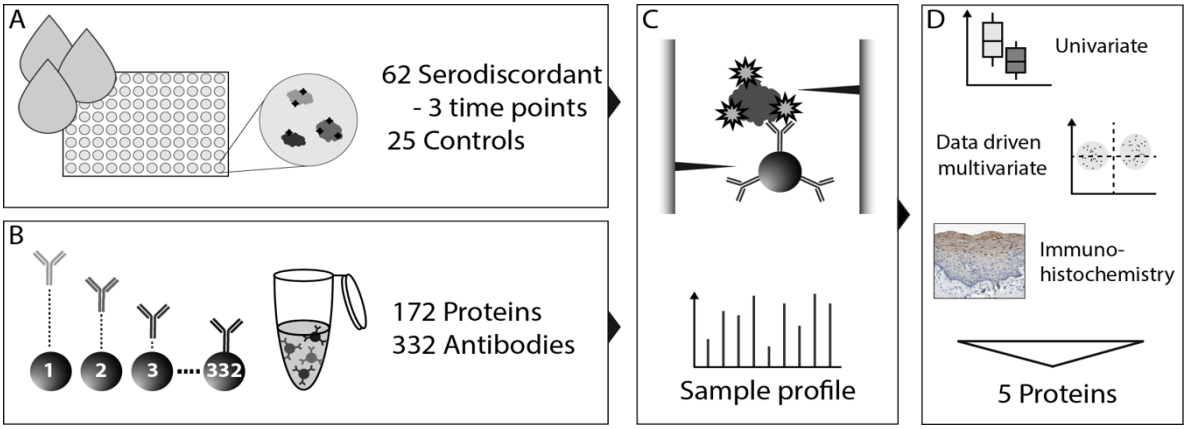

Figure 7. Overview of study setup. A. Cervicovaginal secretions from women in HIV-serodiscordant relationships were collected at three time points, and from controls (women in HIV-seroconcordant negative relationships) at one time point. B. A total of 332 antibodies directed towards 172 unique proteins were coupled to color-coded magnetic beads and mixed in a suspension bead array. C. Beads and samples were incubated and levels of proteins bound to the beads were measured using Luminex. D. Protein levels were subjected to univariate as well as data driven multivariate statistical analysis. The most interesting proteins from the univariate and multivariate analysis were stained for in genital tissues, resulting in a total of five proteins consistently associated with the HIV-serodiscordant phenotype that were also visualized in genital tissues. Original version of figure was originally published in (273), adapted and reprinted with permission.

To identify proteins associated with the serodiscordant phenotype, the protein composition in CVS was compared to the control group, revealing 24 proteins found at higher levels ($p<0.05$) in the serodiscordant group. These included serine proteases (KLK10, PLG), protease inhibitors (SPINK5, SERPINB1/5), proteins involved in epithelial barrier function (SPRR3, SPINK5) and immune activation/inflammation (CAPG, KLK10, S100A9, IL18, CXCL9, CCL21). Of these, 4 (CAPG, KLK10, TTR, SPRR3) passed multiple comparison corrections. The data was adjusted to account for differences between study groups for several demographic parameters, revealing even stronger associations with the HIV-serodiscordant phenotype for the most significant proteins. Data-driven multivariate analysis was performed in order to reveal potentially intersecting biological relationships, identifying two additional proteins (the antiproteases PI3/elafin and CSTB) as associated with the serodiscordant phenotype.

Next, we wanted to evaluate the expression of the most interesting proteins in genital tissues in order to confirm their presence in the FGT. The proteins chosen for immunohistological staining were the most significant proteins identified in the univariate analysis (CAPG, TTR, KLK10, SPRR3), and two additional proteins (PI3 and CSTB) identified using data driven modeling. All proteins except TTR were positively stained for in genital tissues; SPRR3, KLK10, CSTB and PI3 were expressed in the epithelium and CAPG in the submucosa of the cervix and vagina.

In conclusion, by using a combination of different methods, we identified five proteins (CAPG, KLK10, SPRR3, CSTB and PI3) that were associated with the serodiscordant phenotype with confirmed expression in genital tissues.

In contrast to our original hypothesis that the cervicovaginal proteome of HIV-serodiscordant women would be similar to that of control women, we observed a distinct genital proteome profile of altered levels of proteins involved in inflammation and epithelial barrier function. In the HIV-serodiscordant group, both serine proteases (KLK10, PLG) and serine/cystein protease inhibitors (PI3, SPINK5, CSTB, SERPINB1/5) were elevated. PI3 (185, 274) and CSTB (275) have anti-HIV in vitro, and increased levels of several protease inhibitors, including PI3 and CSTB, have been identified in FSW cohorts previously as potential correlates of protection (57, 62, 63, 185). Serine protease inhibitors play important roles in regulating inflammation and tissue development and can contribute to an anti-inflammatory local environment (64, 276). However, there is a constant balance in the body between proteases and anti-proteases, and it is the net effect of these opposing forces as well as other pro/anti-inflammatory proteins and molecules that will determine the local immunological effect and might contribute to altered HIV-susceptibility. In addition, as mentioned previously, an inflammatory response will be followed and counteracted by an anti-inflammatory response, and this is a continuum. Indeed, in the serodiscordant group we observed altered levels of both "pro" and "anti"-inflammatory proteins, once again highlighting the difficulties on determining the net effect on the mucosa based on protein expression. In conclusion, we can conclude that proteins involved in genital inflammation and immunity are altered in the HIV-serodiscordant group, but the current study design does not allow more detailed examination of the combined effect of these protein on the female genital mucosa.

In addition to their above mentioned function in inflammation, several serpins and other anti-proteases are involved in maintenance of the epithelial barrier (276-279). We also observed SPRR3, a structural protein in the cornified envelope of the epidermis (280), to be elevated in the serodiscordant group. In addition, these proteins showed positive staining in genital tissues, indicating that they are present and possibly have a function in the FGT. Alterations to epithelial barrier integrity and function are suggested to be associated with altered susceptibility to HIV-infection (36, 40, 42, 51, 65), at least in part because of easier access of the virus to potential HIV-target cells in the epithelium and submucosa. Collectively, these data suggest that epithelial barrier integrity and/or function is altered in the HIV-serodiscordant group, but our study design does not allow further exploration of such epithelial barrier-associated alteration in HIV susceptibility.

Several limitations exist, including the low sample size and lack of longitudinal samples for control women. Another limitation is the difference between groups for several demographic parameters, which could potentially bias the results. However, these potential confounders were thoroughly adjusted for using different statistical methods, revealing that the association between the HIV-serodiscordant phenotype and the most statistically significant proteins remained. Yet, it is possible that these and other unknown confounders could affect the results. The menstrual cycle (45, 248) and the cervicovaginal microbiome (52) can influence the protein composition in the FGT, but unfortunately we are lacking data on these factors. Further, a limitation of ours and other proteomic studies is that, since no functional analyses were performed, our results are purely observational and we cannot decipher biological

activity in the adjacent genital tissue. Not only are the functions of proteins complex and should be interpreted together with other proteins, including proteins with opposing effect, but depending on if proteins are secreted basolaterally or apically, they can exert varying effect on the underlying tissues (281). However, we confirmed the presence of the most interesting proteins in genital tissues, indicating that they perform a function in the FGT.

In conclusion, we observed a distinct proteomic profile of altered levels of proteins involved in inflammation and epithelial barrier function in a cohort of HIV-serodiscordant women with low clinical inflammation and high frequencies of safe sex practices. Our findings complement studies in FSW cohorts and cohorts with high levels of clinical inflammation, and our finding need to be taken into consideration when designing and interpreting results from clinical studies in settings with high HIV-prevalence.

4.4 EVALUATING THE FEASIBILITY OF A HIGH-THROUGHPUT BEAD BASED ASSAY IN IDENTIFYING PROTEINS IN GENITAL SECRETIONS

A secondary aim of Paper 3 was to evaluate the feasibility of using a high-throughput bead-based protein profiling method when assessing genital secretions. The majority of proteomic studies in genital secretions have been conducted using MS-based methods, and although the use of antibody-based methods such as ELISA is common in studying a limited numbers of proteins simultaneously, the use of high-throughput antibody-based affinity methods are rare.

To evaluate antibody selectivity, more than one antibody directed to the same protein ("paired antibodies") were used for several proteins. For the 24 proteins identified to be associated with HIV-serodiscordant status in the univariate analysis, 5 were evaluated using paired antibodies. For paired antibodies generated using the same immunogen, we observed a spearman's rho of 0.87-0.99 (range), whereas for antibodies generated towards different immunogens (non-overlapping amino-acid sequence), the spearman's rho was slightly lower at 0.73-0.84 (range).

To validate the findings of the affinity bead based analysis using another protein identification technique, CVS samples were analyzed by both affinity-based protein profiling and a tandem MS-based method. 79 antibody/proteins pairs were identified in 85 matching samples, and the median Spearman's rho for these was 0.46 (range -0.35– 0.77). For the five proteins consistently identified in the first part of the manuscript as being associated with the HIV-serodiscordant phenotype, clear positive correlations were observed for SPRR3, KLK10 and PI3 (range rho 0.47-0.53, BH adj. p 0.0003), whereas CAPG and CSTB exhibited weak correlations (range rho 0.06-0.24, BH adj $p>0.05$).

In general, we observed a medium high correlation between the bead-based protein profiling method and a MS based method. The strength of the correlation varied for different proteins and also for antibodies directed towards the same protein, which is not surprising since the antibodies are polyclonal and therefore bind with varying affinities to epitopes with likely various degree of exposure. There are several possible explanations to the discrepancies

between the two methods, including that both methods measure relative, as compared to absolute, quantification of protein content. In addition, the HPA antibodies used have been validated in microarray plates but not in aqueous solutions, and since the function of antibodies is context dependent (282), we cannot be certain of antibody specificity in complex environments such as CVS. Furthermore, the natural configuration of the protein and post-translational modifications may hide epitopes, which also could affect antibody affinity. However, we observed high correlations between antibodies directed towards non-overlapping sequencing of the same protein, indicating that the intended target protein was identified.

To conclude, the high correlation between paired antibodies as well as a medium high correlation between separate proteomic techniques indicate this it is feasible and appropriate to use a bead-based affinity technique to analyze genital samples in a high-throughput fashion.

5 CONCLUDING REMARKS

The studies presented in this thesis show that female sex hormones, HIV-serodiscordant status and seminal plasma induce changes to the female genital mucosa that may alter the susceptibility to HIV.

- The fluctuations of female sex hormones over the course of the menstrual cycle exert a profound effect on the cervicovaginal proteome. The largest change is observed during the short ovulatory phase, which is characterized by a decrease in inflammatory markers and increase is markers of epithelial barrier integrity (Paper 1).
- The cervicovaginal microbiome in healthy Swedish women is dominated by *Lactobacillus* and is not affected by the cycling of endogenous sex hormones over the course of the menstrual cycle (Paper 1).
- Sex-hormone induced fluctuations in the cervicovaginal proteome over the course of the menstrual cycle might be exacerbated in women with non-*Lactobacillus* dominant microflora (Paper 1).
- The progesterone-high luteal phase and use of the progestin-containing injectable contraceptive DMPA enhance immune activation in the FGT and decrease epithelial barrier integrity (Paper 1 and 4).
- The use of DMPA is associated with degradation of proteins essential for desmosomal function and a thinner superficial epithelial layer in the ectocervical epithelium (Paper 4).
- Seminal plasma induces genital inflammation and increases HIV-infectivity as demonstrated in an ectocervical genital explant model (Paper 2).
- The ectocervical genital explant model is a useful platform to decipher the intial events of HIV-transmission and the effect of seminal plasma on such intial events (Paper 2).
- HIV-seronegative women living in HIV-serodiscordant relationships show alterations in the genital expression of proteins involved in inflammation and epithelial barrier integrity (Paper 3).
- A high-throughput bead-based set up is a suitable method for evaluating proteins in genital secretions, and there is a medium high correlation with a mass spectrometry-based method (Paper 3).

6 FUTURE PERSPECTIVES

In 2002 the United Nations Secretary General Kofi Annan said “In Africa, AIDS has a woman’s face”. Almost 20 years later, this still holds true. The scientific community owe is to the millions of women living with and at risk of HIV to use funds wisely to develop HIV prevention strategies accessible to all. Despite almost 40 years of research, there is still no HIV-vaccine in clinical use. In wait for an effective HIV-vaccine, we might have to put our hopes to other preventive measures, especially female-controlled preventive measures, such as for example microbicides.

With this thesis, I aimed to characterize mucosal factors within the FGT to better understand potential molecular mechanisms associated with altered HIV-susceptibility. This knowledge could aid in identifying genital mucosal signatures associated either with protection or increased risk of HIV-infection. Such mucosal signatures could help guide the preventative field, perhaps women with a certain signature benefit from one type of preventative measures as compared to other women. If we could stratify women based on mucosal markers, it might be possible to personalize preventative interventions aimed at reducing HIV-transmission. In addition, by identifying a protective mucosal signature, the scientific field may find solutions how to pharmacologically induce such mucosal state.

Effective, safe and widely available contraceptives are a global health priority, and reports about an increased risk of HIV-acquisition associated with some HC must be taken very seriously. In order to aid in the development of safe contraceptives, it is essential to understand potential underlying mechanisms of HC as well as endogenous female sex hormones. Ideally, HC and HIV-prevention compounds could be combined in one formulation to facility use. Therefore, it is especially important to investigate the effect of HC in populations that would be targets for HIV-prevention methods, such as FSW, as we have done in Paper 4. Studies of the populations intended for preventative measures can reduce geographical, ethnical and cultural differences that might otherwise have influenced the results.

The -omics field has exploded in the past years, and it offers great advantages in trying to achieve a comprehensive picture of the complex mucosal milieu in the FGT. In this thesis, we used proteomics, transcriptomics and gene sequencing techniques to study the effect of sex hormones and HIV-serodiscordant status on the FGT. There are advantages and limitations with each of these -omics, and I believe that the future lies in the combination of data from several -omics, so called multi-omics. Multi-omics has the potential to identify novel interactions that are not identified using single -omics, and is especially suitable for studying very complex systems, such as the immune system in the FGT. In a continuation of Paper 4, we have performed protein profiling on matching genital samples with the aim of identifying potential intersecting relationships between the transcriptome and proteome in order to more comprehensively characterize the effect of DMPA on the mucosa in the FGT.

The studies in this thesis, except for Paper 3, are of an observational nature. In my thesis, I have discussed the challenges of interpreting observational studies to decipher the net effect on the mucosa as well as the need for follow-up functional studies. The genital explant model presented in Paper 2 can aid in such studies, for example by studying the effect of female sex hormones or different microbial species on HIV-transmission. This explant model is also an excellent tool in evaluation of microbicides. In the future, I hope to experimentally assess the role of endogenous and exogenous female sex hormones in an explant model.

There is still a long way to go to combat HIV/AIDS, but effective prevention strategies in women play a key role and should be considered a global health priority. In the foreseeable future, hopefully the United Nations Secretary General can say “In Africa, AIDS has a face of the past”.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

7.1 BAKGRUND

HIV är ett virus som attackerar kroppens immunförsvar och om viruset förblir obehandlat så kan det leda till den dödliga sjukdomen AIDS, då immunförsvaret helt slås ut. Det finns mediciner som bromsar utvecklingen av AIDS men det finns inget botemedel och inte heller något vaccin. Sedan HIV viruset identifierades på 1980-talet så har det spridit sig över världen, och Världshälsoorganisationen uppskattar att det idag finns 38 miljoner smittade, majoriteten i södra Afrika.

HIV kan smittas via blodet och över slemhinnor, såsom vid sexuell kontakt. Det finns många faktorer som påverkar hur stor risk det är att smittas, såsom typ av samlag, virusnivåer hos den smittande individen och samtidiga andra könssjukdomar. Den största delen av nya HIV-infektioner globalt sker vid oskyddat vaginalt samlag. Det finns mekaniska och immunologiska faktorer i genitala slemhinnan som skyddar kroppen mot HIV. Mekaniska barriärer är t.ex. det skyddande sekret som täcker slemhinnan samt epitelet, som är den yttersta delen av slemhinnan. I sekretet finns det utsöndrade proteiner (äggviteämnen), bland annat ifrån immunförsvaret, som kan motverka HIV. Ibland kan viruset, trots dessa skyddsbarriärer, ta sig igenom och infektera immunceller i slemhinnan och så spridas vidare i kroppen.

I denna avhandling har vi studerat faktorer som påverkar den genitala slemhinnan, samt hur dessa kan ha betydelse för smittoöverföring av HIV.

7.2 DELSTUDIE 1-4

Tidigare studier talar för att hormonvariationer i menscykel kan påverka mottagligheten för HIV och att det kan finnas en ökad risk för smitta under den senare delen av menscykeln då nivåerna av könshormonet progesteron är som högst. I **delstudie 1** samlade vi in slidsekret från kvinnor under olika faser i deras menscykel och mätte proteinnivåer i sekreten. Vi visade att proteiner som tros ha en skyddade effekt på slemhinnan var allra högst runt ägglossning och som lägst före menstruation, dvs. i den progesteron-dominerade fasen. Vi såg även att proteiner och molekyler som tyder på inflammation var högre i den fasen. Ökad genital inflammation talar också för att risken att smittas av HIV-infektion kan öka, bland annat då det kan leda till en ansamling av målceller för HIV. Våra resultat stödjer tidigare studier som visar att risken för HIV-smitta kan variera över menscykeln.

Trots många år av forskning kring HIV så är relativt okänt vad som händer initialt i genitalslemhinnan vid HIV-smitta, och det beror till viss del på att det saknas bra experimentella modeller för att studera detta. Det är heller inte känt hur sädesvätska påverkar den genitala slemhinnan och smittoöverföringen av HIV. I **delstudie 2** använde vi ett modell-system där vävnad från livmodertappen exponerades för sädesvätska. Vi kunde påvisa att

denna vävnad svarade med genital inflammation och blev lättare infekterad av HIV än vävnad som ej exponerats för sädesvätska. Den här studien visar att det är viktigt att inkludera sädesvätska som en faktor vid studier av HIV-överföringen. Vi hoppas också att detta modellsystem kan användas för att utvärdera skyddande ämnen som kan appliceras på slemhinnan för att minska smittoöverföringen, t.ex. mikrobicider.

Runtom i världen finns det individer och grupper som trots exponering för HIV undgått att bli smittade, t.ex. vissa kvinnliga prostituerade. En del studier talar för att deras genitala immunförsvar skiljer sig från andra kvinnors och kan utgöra ett skydd mot viruset. I **delstudie 3** så undersökte vi en annan grupp kvinnor som är utsatta för HIV-smitta, nämligen kenyanska HIV-negativa kvinnor med en HIV-positiv manlig partner. Sådana par kallas serodiskordanta. Till skillnad från många grupper av prostituerade som tidigare studerats så har den här gruppen låga nivåer av andra riskfaktorer som samtidiga könssjukdomar och de har också en hög grad av kondomanvändning. Vi jämförde slidsekretet hos dessa kvinnor med slidsekret från HIV-negativa kvinnor med en HIV-negativ partner (kontrollgrupp). Vår hypotes var att slidsekretet från kvinnorna i diskordanta par inte skulle skilja sig nämnvärt från kontrollerna. Vi kunde dock påvisa skillnader av proteiner involverade i barriärfunktionen av slemhinnan samt immunförsvaret, vilket talar för att HIV-negativa kvinnor i serodiskordanta förhållanden har en distinkt ”genital profil” som skiljer sig från kvinnor med en HIV-negativ partner. Ytterligare studier krävs för att förstå om och hur dessa skillnaderna i dessa proteiner kan påverka risk för HIV-infektion.

En del studier tyder på att användningen av vissa typer av hormonella preventivmedel, framförallt så kallad p-spruta, är kopplat till ökad risk för att bli smittad av HIV. Orsaken till detta är dock okänd och skulle kunna bero på olika riskbeteende vid p-spruta användning alternativt mer biologiska effekter som ökar mottagligheten för HIV. I **delstudie 4** jämförde vi den genitala slemhinnan hos kenyanska kvinnor som använder p-spruta med kvinnor som inte använder några hormonella preventivmedel. Hos kvinnorna som använder p-spruta såg vi en nedreglering av gener som är viktiga för barriärfunktion, bland annat gener som kodar för ”klister-proteiner” som normalt sett bidrar till en intakt slemhinna. Dessa kvinnor hade även ett tunnare övre lager av slemhinnan jämfört med kontroller. Detta kan tala för att användning av p-spruta bidrar till en mer lucker/genomsläpplig slemhinna, och att detta eventuellt kan bidra till ökad mottaglighet för HIV-smitta hos dessa kvinnor. Denna kunskap är viktigt dels för att förstå hur preventivmedel påverkar slemhinnan och dels för utveckling och rådgivning mot säkra hormonella preventivmedel.

7.3 SAMMANFATTNING

Dessa studier bidrar till att kartlägga faktorer som påverkar den genitala slemhinnan och som kan ha betydelse för HIV-smitta hos kvinnor. Den kunskapen kan förhoppningsvis bidra till att utveckla förebyggande åtgärder för att minska smittspridningen av HIV, såsom t.ex. vacciner eller mikrobicider.

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