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**BIOLOGY OF HIV TRANSMISSION  
AND ANTIVIRAL  
MUCOSAL IMMUNITY  
IN THE FEMALE GENITAL TRACT**

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**IN AFRICA, AIDS HAS A WOMAN'S FACE**

*Kofi Annan*  
2002

*To my Family*

## ABSTRACT

Human immunodeficiency virus (HIV) is primarily transmitted through heterosexual intercourse and women in Sub-Saharan Africa are disproportionately affected. Correlates of protective immunity and mucosal factors influencing sexual HIV transmission need to be identified as they may serve as critical targets for HIV intervention. The aim of this thesis was to determine whether IgA antibodies and antimicrobial peptides contribute to mucosal immune defence in individuals who are at high risk of acquiring HIV.

Our understanding of the role of mucosal immunology in HIV transmission has been gained from studies of cohorts of highly HIV-exposed seronegative (HESN) individuals. The studies in the present thesis are based on clinical samples from well-characterized cohorts of Kenyan HESN individuals, including female sex workers and HIV serodiscordant couples (where one partner is HIV-positive and the other is HIV-negative).

Baseline cervicovaginal secretion (CVS) samples from HIV-negative female sex workers were analysed for levels of antimicrobial peptides with HIV neutralizing activity and the results were compared with subsequent HIV seroconversion. We found that the antimicrobial peptides HNP1-3 and LL-37 correlated with the *in vitro* HIV neutralizing activity of CVS. However, despite the *in vitro* HIV neutralizing properties of HNP1-3 and LL-37, higher levels of these peptides were associated with increased HIV acquisition. It is possible that elevated levels of these peptides were induced by sexually transmitted infections (STIs) and, even though the peptides neutralized HIV to some extent, the overall STI-associated inflammation resulted in susceptibility to HIV infection. In the same cohort, elevated levels of the antimicrobial peptide Trappin-2 were associated with reduced HIV acquisition.

In a prospective clinical study of HIV-discordant couples, CVS was obtained from three groups including HESN, HIV-positive and low-risk control women. Levels of selected antimicrobial peptides in CVS were comparable between the groups. However, elevated levels of HNP1-3 and LL-37 in the HESN women correlated with a higher viral load in the HIV-infected male partner. When analysing the humoral immune response we found that HESN women were five times more likely to have HIV neutralizing IgA detected in their CVS than low-risk controls. Unexpectedly, the presence of neutralizing IgA was inversely correlated to the male partner's viral load.

In conclusion, a significant proportion of HESN women displayed HIV neutralizing genital IgA compared to control women. Furthermore, the HIV neutralizing capacity of CVS correlated with subsets of specific antimicrobial peptides, although detection of high levels of antimicrobial molecules in clinical samples is not necessarily a marker of protection against HIV. Hence, the humoral immunity and the dual role of antimicrobial peptides, including their antiviral and proinflammatory properties, should be carefully evaluated in future clinical trials of preventive strategies against sexual HIV transmission.

## LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

- I. LEVINSON P, Kaul R, Kimani J, Ngugi E, Moses S, MacDonald KS, Broliden K, Hirbod T; Kibera HIV Study Group.  
**Levels of innate immune factors in genital fluids: association of alpha defensins and LL-37 with genital infections and increased HIV acquisition**  
*AIDS. 2009 Jan 28;23(3):309-17*
- II. Iqbal SM, Ball TB, LEVINSON P, Maranan L, Jaoko W, Wachihi C, Pak BJ, Podust VN, Broliden K, Hirbod T, Kaul R, Plummer FA.  
**Elevated elafin/trappin-2 in the female genital tract is associated with protection against HIV acquisition**  
*AIDS. 2009 Aug 24;23(13):1669-77*
- III. LEVINSON P, Choi R, Liu A, Hirbod T, Rhedin S, Payne B, Guthrie B, Bosire R, Cole AM, Farquhar C, Broliden K.  
**HIV neutralizing activity of cationic polypeptides in cervicovaginal secretions of women in HIV-serodiscordant relationships**  
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- IV. Choi R, LEVINSON P, Guthrie B, Payne B, Liu A, Bosire R, Kiarie J, Hirbod T, Broliden K, Farquhar C.  
**Cervicovaginal neutralizing IgA detected among highly exposed seronegative female partners in HIV-1-discordant Kenyan couples.**  
*Manuscript to be submitted*

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## LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
APC	Antigen presenting cell
ART	Anti-retroviral treatment
BV	Bacterial vaginosis
CAT	Couples Against Transmission
cDNA	Complimentary deoxyribonucleic acid
CCL5	Chemokine ligand 5
CCR5	Chemokine (C-C motif) receptor 5
CTL	Cytotoxic T Lymphocyte
CVS	Cervicovaginal secretion
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope
FGT	Female genital tract
HBD	Human $\beta$ -defensin
HIV	Human immunodeficiency virus
HESN	HIV exposed seronegative
HNP	Human neutrophil peptides
HSV-2	Herpes simplex virus type 2
Ig	Immunoglobulin
MS	Mass spectrometry
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PSA	Prostate specific antigen
RANTES	Regulated upon activation normal T cell expressed and secreted
RNA	Ribonucleic acid
RT	Reverse transcriptase
S-IgA	Secretory IgA
SLPI	Secretory leukocyte protease inhibitor
STI	Sexually transmitted infection



# 1 INTRODUCTION

## 1.1 THE GLOBAL HIV/AIDS EPIDEMIC

The human immunodeficiency virus (HIV) epidemic was silent until 30 years ago, when several reports of young US gay men with life threatening opportunistic infections were published in 1981, as evidence of a new immunodeficiency disorder (1), later defined acquired immune deficiency syndrome (AIDS). The role of HIV as a possible causative agent of AIDS was published in 1983 (2), a discovery that was awarded the Nobel Prize in 2008. Although the incidence of HIV infections has constantly decreased since the peak of the epidemic in the late 1990s, the total number of HIV-infected people continues to rise due to increased access to anti-retroviral therapy (ART), which has largely reduced AIDS-related deaths. Today there are more than 33 million people living with HIV worldwide, with 68% of HIV-infected people living in Sub-Saharan Africa (3). In this region, women aged 15–24 years are eight times more likely than men in the same age group to be HIV-positive and consequently the empowerment of women has been identified as an important goal for decreasing global HIV transmission (3).

## 1.2 THE HIV/AIDS EPIDEMIC IN KENYA

Kenya is a low-income country located in East Africa with a population of 40 million and an average life expectancy of around 60 years. The earliest case of HIV in Kenya was reported in 1984 (4) and HIV surveillance has been conducted since 1990, based on sampling of women attending antenatal clinics (5). The HIV epidemic has stabilized in the past few years and the HIV prevalence among adults (15–49 years) fell from about 10% in the late 1990s to 6.2% in 2009 (6). Kenya's HIV epidemic has been categorized both as generalized and as mixed, largely affecting the mainstream population but with the highest transmission rates among specific most-at-risk populations (Kenya country analysis 2009). Sex workers and their clients were early identified as the source of the general epidemic (7) and the risk groups have later been extended to include men having sex with men and prisoners (5). The geographical spread of HIV is high, with a prevalence ranging from 0.8% in North-Eastern Kenya, to 15% in Nyanza (5). Women are more commonly HIV-infected than men and young women have an HIV prevalence four times as high as men in the same age group (6). The general acceptance of concurrent/multiple partnerships and male circumcision are believed to be two major factors influencing the HIV epidemic in Kenya (5). Male circumcision rates vary substantially between different regions and a lower prevalence of male circumcision is associated with a higher HIV prevalence. Heterosexual transmission remains the major route of transmission in all provinces and occurs in various contexts: between steady sexual partners in HIV discordant couples, casual sexual partners and concurrent sexual partnerships (e.g. an individual with both a steady, long-term partner and a casual partner, or a person with more than one steady partner). A recent study reports that 62% of the HIV-infected in Kenya live in discordant relationships but only 20% of them know that they are HIV-positive (8).

### 1.3 THE HUMAN IMMUNODEFICIENCY VIRUS

The earliest documented HIV case originates from a plasma sample obtained from a Bantu male in the Democratic Republic of Congo in 1959 (9). However, humans are not the natural hosts of HIV and there is evidence that the virus was transmitted from primates to humans in Central Africa several times (10), the first time at the beginning of the 20<sup>th</sup> century (11). The exact routes of cross-species transmission are unknown, but it is believed that humans have been exposed to the virus through hunting primates infected with simian immunodeficiency virus (SIV) (10). Interestingly, the SIV infections do not cause clinical symptoms in their natural hosts, whereas other primates can develop AIDS-like symptoms upon infection (12). The virus presumably adapted to the human host over time and started to spread within the human population as two separate strains: HIV-1, which originates from SIV in chimpanzees (13) and HIV-2, which originates from sooty mangabeys (14, 15). The two strains are further subdivided into groups on the basis of evolutionary criteria. The vast majority of AIDS cases worldwide are due to infection with HIV-1 (hereafter referred to as HIV), which consists of four distinct groups denoted M, O, N and P (11, 16). The predominant M group is comprised of at least 11 different subtypes (clades) with differential distribution globally, designated by the letters A, B, C, D, F, G, H, J, K and L (17). Subtype C is the major cause of HIV infection worldwide, being the dominant subtype in Sub-Saharan Africa and India, followed by subtype A, which is the dominant subtype in Central Africa. Subtype B is the third most common subtype globally, dominating in the Americas, Western Europe and Australia.

#### 1.3.1 Viral structure of HIV and cellular tropism

HIV is an enveloped retrovirus that belongs to the Lentivirus genus. The virion is composed of two copies of an RNA genome and a vast number of enzymes required for infection and viral replication. The viral genome is reverse-transcribed into a complementary DNA (cDNA) in the infected host cell by the viral enzyme reverse transcriptase (RT). Another viral enzyme, integrase, facilitates integration of the viral DNA into the host cell chromosomes. The HIV genome consists of 9 genes of which Gag, pol and env are the three major genes. Gag encodes structural proteins, pol encodes enzymes used for viral replication and integration and env encodes the viral envelope glycoproteins. The smaller genes Tat and Rev have regulatory functions and the accessory genes Nef, Vif, Vpr and Vpu are important for efficient viral production (18). The HIV genome and viral proteins are enclosed by a conical capsid composed of gag p24 proteins. The capsid is surrounded by a matrix of the viral proteins p17, which in turn are surrounded by the viral envelope. The envelope is composed of a lipid-bilayer derived from the host cell membrane and proteins from the host cell, as well as the viral glycoproteins gp120 and gp41, which are embedded in the viral envelope (19). The envelope protein complex determines which particular cell types can be infected by HIV (cellular tropism) and facilitates the membrane fusion process that allows viral entry (20). Viral access to HIV target cells is mediated through the interaction of gp120 with the host receptor CD4, present on CD4<sup>+</sup> T cells, dendritic cells, monocytes and brain microglia (21). In addition, viral fusion and entry require binding of gp120 to one of the chemokine co-receptors on the surface of susceptible host cells (22, 23). One of the major HIV co-receptors is chemokine (C-C motif) receptor 5 (CCR5), present on memory CD4<sup>+</sup> T cells, dendritic cells and macrophages.

The other major co-receptor is CXCR4, which is expressed on activated T cells. Different HIV variants use different co-receptors and are divided into R5 and X4 strains, based on expression of CCR5 and CXCR4, respectively. HIV variants, called R5X4 HIV strains, are capable of using either co-receptor for viral entry (24). HIV infection is initiated by R5 strains and, in 50% of patients, X4 strains emerge in the course of infection (24), which correlates with a more severe clinical outcome and progression to AIDS (25, 26).

### **1.3.2 Clinical course of HIV infection**

HIV infection is typically transmitted through mucosal membranes, primarily during heterosexual intercourse. Established HIV infection causes progressive destruction of CD4+ T cells with subsequent loss of important host immune functions, leading to high susceptibility to opportunistic infections and malignancies, which are the hallmarks of AIDS (27). Some individuals develop AIDS within less than a year, whereas others sustain long-term HIV infection for more than 2 decades, and remain asymptomatic and without a substantial decrease in CD4 count (18).

After the initial mucosal transmission, the virus establishes a local infection in the submucosa during the first week. In the second or third week after HIV acquisition, the virus spreads to draining lymph nodes and is further disseminated through the blood to other lymphatic tissues. The lymphoid organs function as reservoirs of viral production (28) but the main site of CD4+ T cell depletion in all phases of HIV infection is the gastrointestinal tract (29). During the first few weeks, there is often a high viral titre present in the blood and most infected individuals experience a short illness with fever, rashes, tonsillitis or gastrointestinal symptoms, which are characteristic of primary HIV infection (30, 31). At this stage of infection, there is a significant drop in circulating CD4+ T cells, mainly due to extensive depletion of CD4+ T cells in the gastrointestinal tract, caused by viral cytopathic effects and cytotoxic T lymphocyte (CTL)-mediated killing of HIV-infected cells (29). The cellular immune response is usually activated in this phase and involves CD8+ T cells killing HIV-infected cells and restoring the levels of CD4+ T cell counts to subnormal levels, and also activation of the humoral immune response (18). The CTL response partially controls viral replication (32) and the viral load decreases to a viral set point, a prognostic marker for future disease progression (33). The next phase of HIV infection is asymptomatic but the progressive loss of CD4+ T cells continues. Subsequently the infected individual will develop clinical symptoms of AIDS if no ART drugs are administered.

### **1.3.3 Antiretroviral treatment**

The first anti-HIV drug, zidovudine (AZT), was licensed in 1987 and targets the viral enzyme RT. Since then, AZT has been followed by over 20 additional drugs in a remarkable development in the treatment of HIV-infected patients. The combination therapy of three or more ART drugs, also known as highly active antiretroviral therapy, was introduced in 1996 and has dramatically reduced HIV-related diseases and death among HIV-infected patients, even though eradication of the virus is not possible. Drugs available today are divided into four different classes based on their mechanism of action. Nucleoside or nucleotide RT inhibitors and non-nucleoside RT inhibitors prevent reverse transcription of the viral genome. Protease inhibitors interfere with viral assembly by blocking the activity of the protease enzyme. Entry inhibitors interfere with binding, fusion and entry of HIV to the host cell and include CCR5 receptor antagonists, which bind the HIV co-receptor on the surface of the target cell and block viral attachment. Integrase inhibitors prevent the viral genome from incorporating into the DNA of the host cell by blocking the viral enzyme integrase. The combination therapy of three or more drugs decreases the risk of drug resistance but compliance throughout the life-long treatment is of critical importance for clinical outcome and avoidance of drug resistance.

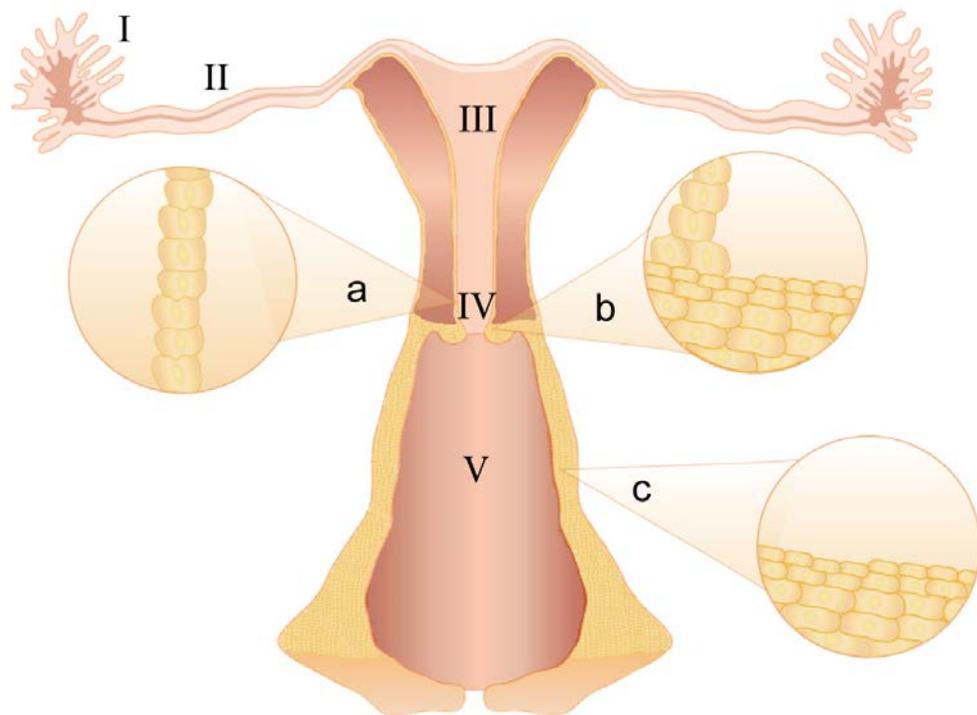
Cardiovascular disease is one of the most severe adverse effects of ART, although recent evidence suggests that cardiovascular complications are often caused by the HIV infection itself (34, 35). In recent years there has been a trend towards earlier treatment, based on randomized control trials indicating that both AIDS-related disease/death and non-AIDS-related disease increase when the CD4 count decreases (36). According to current international guidelines ART should be initiated in all patients with a CD4 count <350 and in all patients with AIDS-related conditions (37). One important benefit of early treatment is the reduction of infectivity (likely due to reduced viral load), which markedly decreases heterosexual HIV transmission risk (38, 39). Access to treatment has increased in recent years but still varies substantially between different regions. Approximately 41% of the HIV-infected in Sub-Saharan Africa in need of ART had access to treatment in the end of 2010 (3).

## **1.4 THE FEMALE GENITAL TRACT**

The female genital tract (FGT) consists of the ovaries, the uterine tubes, the uterus, the cervix and the vagina (Figure 1). The cavity of the cervix forms a connection between the vagina and the uterus. The intravaginal, outside part of the cervix is called the ectocervix and is lined by a non-keratinized, multilayered, squamous epithelium that continues into the vaginal epithelium. The cervical cavity is called the endocervix and is lined by a single layer of mucus-secreting columnar epithelium. The transformation zone is an area of transition, where the columnar epithelium present in the endocervix is replaced by squamous epithelium, characteristic of the ectocervix (40). The differentiation process is known as metaplasia and the location of the transformation zone fluctuates with hormonal changes and age. In adolescence, pregnancy and during oral contraceptive use, the transformation zone moves downwards onto the ectocervix, and endocervical mucosa may be exposed in the vagina, a condition known as cervical ectopy (40). Presence of cervical ectopy is a risk factor for various genital infections, including HIV (41).

The cervical transformation zone is believed to be a major site of cell-mediated immunity in the lower FGT, being the predominant site of T cells and antigen presenting cells (APCs) (42), and a site of inductive mucosa-associated lymphoid tissue (43). In contrast, the vaginal mucosa normally contains few T cells and APCs. Because of the high density of HIV target cells in the transformation zone it has been suggested that the cervix may be a primary site of HIV infection (42). In fact, vaginal challenges with SIV in non-human primates have shown that there is an early established infection in the endocervix and transformation zone (44, 45). However, mucosal sites throughout the FGT have been shown to serve as targets for HIV and the surface area of the vagina and ectocervix is approximately 15 times larger than that of the endocervix, potentiating the risk of HIV transmission through breaches in the epithelium (46).

The FGT forms an immunologic microenvironment that protects against invading pathogens and at the same time allows reproduction through tolerance of sperm. This is mediated through a balance between protective barriers and reproductive mechanisms and is influenced by hormonal changes (47). The protective barriers include the epithelium, the vaginal bacterial flora and the cervicovaginal secretion (CVS). The bacterial flora is dominated by Lactobacilli that provide a physical as well as chemical barrier through colonization of the epithelium and production of lactic acid, hydrogen peroxide and other antimicrobial compounds (48). The unique properties of the CVS are described in the following section.



**Figure 1**

*The female genital tract consists of (I) the ovaries, (II) the uterine tubes, (III) the uterus, (IV) the cervix and (V) the vagina. (a) The uterus and endocervix are lined with a single layer columnar epithelium. (b) Transformation zone. (c) The ectocervix and vagina are lined with a multilayered squamous epithelium. Copyright of Sam Hirbod, illustrator.*

### **1.4.1 Composition of the cervicovaginal secretion**

The CVS is a mixture of fluids originating from the endometrium, cervix, uterine tubes, vagina, various genital glands and plasma transudate. The major contributor to the CVS is the cervical mucus, which is a hydrogel that consists primarily of water (92–98%) (49). Other components of cervical mucus include electrolytes, glycoproteins, carbohydrates, amino sugars, immunoglobulins and antimicrobial polypeptides (47, 49-51). The composition of cervical mucus changes during menstrual cycle and in ovulation the mucus transforms from a viscous barrier to a watery fluid, which allows sperm transport (52).

The CVS hydrates the mucosa and creates a physiochemical barrier that prevents microbial invasion (50, 52). Interestingly, CVS has been shown to contain the heavy chain of secretory IgA, which binds type 1-piliated *Escherichia coli*, a common pathogen in the female genital tract.

The antimicrobial polypeptides of CVS comprise a major part of this thesis and will be further discussed in the next section.

## **1.5 MUCOSAL IMMUNITY IN THE FEMALE GENITAL TRACT**

The FGT is exposed to large numbers of pathogens and requires effective protection against invasion balanced by a high degree of tolerance for reproductive reasons. The epithelial surfaces function as physical barriers but can easily be breached; thus, innate and adaptive immune mechanisms are crucial for maintaining the genital mucosal immunity. Only the immunological aspects relevant to this thesis will be more thoroughly addressed below.

### **1.5.1 Innate immunity**

The innate immune system constitutes the first line of defence at mucosal sites and is characterized by a fast, non-specific response without immunological memory. Major functions include providing a physiochemical barrier to pathogens, direct clearance of pathogens, recruitment of immune cells to sites of infection and activation of the adaptive immune system (18). The innate immunity of the FGT involves macrophages, dendritic cells, neutrophils, natural killer cells and epithelial cells. These cells express pattern recognition receptors such as Toll-like receptors, C-type lectin receptors and mannose-binding lectin. The receptors recognize pathogen-associated molecular patterns on microorganisms, including lipopolysaccharide (from gram-negative bacteria), lipoteichoic acid (from gram positive bacteria), and bacterial and viral nucleic acids (18). Toll-like receptors are expressed on macrophages, dendritic cells, neutrophils, natural killer cells and also on epithelial cells throughout the FGT and mediate recruitment of immune cells, production of antimicrobial components that kill invading pathogens and provide a link between innate and acquired immunity (53, 54).

#### *1.5.1.1 Antimicrobial polypeptides in cervicovaginal secretions*

Numerous antimicrobial peptides and proteins are present in the FGT, including defensins, lactoferrin, secretory leukocyte protease inhibitor (SLPI), calprotectin, cathelicidins and chemokines (51, 55). The peptides are mainly produced by epithelial cells, cervical glands and neutrophils present in the cervicovaginal mucosa (51) and are influenced by hormonal changes (56, 57), inflammatory conditions (58-60) and genetic polymorphisms (61, 62). Most of the antimicrobial peptides are amphipathic and cationic, which facilitates binding to anionic lipid structures, and they typically exhibit their antimicrobial activity by disrupting bacterial or viral membranes (63, 64).

Despite the fact that many antimicrobial peptides demonstrate anti-HIV activity *in vitro* (65-70) their *in vivo* role in protection against HIV acquisition remains unclear. Since the antimicrobial peptides exhibit immunomodulatory effects on T cells, dendritic cells and neutrophils (71-75), they may recruit HIV target cells to the genital mucosa and potentially increase the risk of HIV infection (76).

#### 1.5.1.1.1 Defensins

Defensins are one of the main families of antimicrobial peptides, and are involved in a broad range of immunological mechanisms including chemotaxis, cell migration, opsonization, interaction with complement, histamine release and cytotoxicity (77). These cysteine-rich cationic peptides are further divided into the  $\alpha$ -defensin and  $\beta$ -defensin subfamilies and are present at mucosal sites throughout the body. The most well studied subset of  $\alpha$ -defensins are human neutrophil peptides (HNP) 1-3, which are mainly produced by neutrophils (78) but also by natural killer cells,  $\gamma\delta$  T cells, B cells and monocytes (79). Substantial amounts of HNP1-3 are present in the cervical mucus plug (80) and lower amounts are present in vaginal secretions (51). They display a broad range of antimicrobial activities and have been extensively investigated to determine their role in the inhibition of HIV (81-84). The human  $\beta$ -defensins (HBD) 2-3 are mainly produced by epithelial cells but also by monocytes, macrophages and dendritic cells (85) and their expression is induced by microorganisms and proinflammatory cytokines (77). Both HNP1-3 and HBD2-3 are able to inhibit HIV by a direct effect on the virion, as well as through target cell modification (68, 69, 86).

#### 1.5.1.1.2 Cathelicidins

The cathelicidins constitute the other main family of antimicrobial peptides and are similar to defensins in abundance and distribution but are structurally diverse (62). The only identified cathelicidin in humans is LL-37 (87) which is present at several mucosal sites including the cervix and vagina (88). LL-37 is highly constitutively expressed by neutrophils and epithelial cells, and also in response to inflammatory stimuli (85). In addition, the peptide is expressed by subsets of lymphocytes such as natural killer,  $\gamma\delta$  T cells, B cells and monocytes/macrophages (79). LL-37 has broad antimicrobial activity, a chemotactic effect on CD4+ T cells and has been reported to inhibit HIV replication *in vitro* (65, 79). In addition, LL-37 may induce the expression and release of  $\alpha$ -defensins from neutrophils (89).

#### 1.5.1.1.3 RANTES/CCL5

The chemokine ligand 5 (CCL5), also known as Regulated upon Activation Normal T cell Expressed and Secreted (RANTES), is produced by T cells and its expression is regulated by cell activation (90). RANTES is a chemoattractant for monocytes, eosinophils, basophils, and T cells, and activates both eosinophils and basophils to release their granule contents (91-93). RANTES has been identified as one of the major HIV suppressive factors produced by CD8+ T cells (66). It acts by blocking the HIV co-receptor CCR5 and its antiviral capacity has been confirmed in animal studies (94) and in the mucosa of female sex-workers (95, 96). RANTES and its derivatives have been successfully expressed and secreted by commensal *Lactobacillus* species, showing the potential of using live microbiocides (vaginally applied antimicrobial compounds) for treatment of infections in the FGT (97).

#### 1.5.1.1.4 SLPI and elafin/trappin-2

SLPI and elafin are serine protease inhibitors in the whey-acidic protein family and share 40% homology with each other, although they exhibit significant structural and functional differences (98). Elafin has a precursor molecule known as trappin-2, (an acronym for TRansglutaminase substrate and wAP domain containing ProteIN) (99); the terms “elafin” and “trappin-2” will be used interchangeably hereafter in this thesis.

SLPI and elafin are low molecular mass cationic peptides present at various mucosal sites (100, 101) and are synthesized by epithelial cells (102, 103), neutrophils (104, 105) and macrophages (106, 107). They are both constitutively expressed but their levels are influenced by hormonal changes and inflammation (108). Interestingly, secretion of SLPI but not elafin can be induced by  $\alpha$ -defensins (109).

Elafin is biologically active both as a cell-bound and secreted protein and exhibits broad antimicrobial activity (110). SLPI also has antimicrobial properties and inhibits an even greater variety of proteases (111). The anti-protease activity of SLPI and elafin has previously been regarded as their main physiological function (112). However, later studies have shown that SLPI and elafin also exert anti-inflammatory activity by other mechanisms, such as interfering with the NF- $\kappa$ B signalling pathway (113) and binding to bacterial lipopolysaccharide (114, 115). In addition, they are involved in immune regulatory functions (111).

SLPI and elafin have been shown to inhibit HIV infection *in vitro* (110, 116, 117). SLPI inhibits HIV infection by binding to annexin II and impairing annexin II-mediated stabilization of viral fusion (118). The mechanism by which elafin inhibits HIV activity is still unknown but may involve binding of trappin-2 to HIV binding sites at the surface of T cells (112). Elevated levels of elafin but not SLPI have been associated with reduced sexual HIV transmission (119). The role of SLPI in preventing sexual transmission of HIV remains unclear (120) although it has been associated with reduced HIV transmission through breast milk (121).

### 1.5.2 Adaptive immunity

Adaptive immunity is present in both the upper and lower FGT (122) and is of critical importance in understanding the mechanisms of HIV transmission and infection. Briefly, the adaptive immune system is characterized by specific recognition of pathogens generating a highly adaptive response and an immunological memory. Pathogen-derived antigens are processed by APCs, which subsequently activate different subsets of T cells. Activated CD4<sup>+</sup> T cells then stimulate synthesis of protective antibodies from B cells (humoral immunity) and activated cytotoxic CD8<sup>+</sup> T cells mediate the killing of infected cells (cell-mediated immunity) (18). The T cells comprise the major portion of all leukocytes in the FGT, with CD8<sup>+</sup> T cells predominating over CD4<sup>+</sup> T cells (123). The T cells are located mainly below the epithelium of the vagina and cervix and are also found amongst the epithelial cells (124). It was previously thought that the lymphoid cells in the FGT were not organized but there is now evidence of lymphoid aggregates differentially represented in the FGT.

Hormonally-regulated lymphoid aggregates, made up mainly of T cells lining a central B cell core and surrounded by macrophages, have been identified in the uterine endometrium (125, 126) but not in the uterine tubes, cervix or vagina (122). However, another group detected lymphoid aggregates consisting of numerous T cells, with an inner core of B cells, in the cervix (124). Furthermore, as previously described in this thesis, the cervix is an inductive site of mucosa-associated lymphoid tissue (43).

#### *1.5.2.1 Humoral immune responses*

Throughout the body, the humoral immune response is mediated by immunoglobulins (Ig) secreted by B cells. Antibodies are composed of pairings of two identical heavy chains and two identical light chains. The differential structure of the heavy chain defines the five major antibody classes - IgM, IgD, IgG, IgA and IgE (18). The basic monomer unit of all antibodies is composed of two identical Fab regions which bind antigen, linked through the hinge region to the Fc region, which mediates effector mechanisms (127).

IgA is the primary mucosal Ig, however, the ratio of IgA to IgG varies substantially depending on the mucosal site, with the predominant antibody in the FGT being IgG (128). Mucosal IgA antibodies are synthesized by plasma cells in the sub-mucosa and exist mainly as dimers, making them more stable than the monomeric serum IgA (18). There are two isotypes of IgA, IgA1 and IgA2, with IgA2 further subdivided into at least two allotypes, IgA2m(1) and IgA2m(2) (129). IgA1 and IgA2 differ in stability, mainly due to differences in the hinge region, making IgA1 more susceptible to cleavage by proteinases from pathogens, such as *Neisseria gonorrhoea* (129). The relative proportions of IgA1 and IgA2 varies greatly between different mucosal secretions (130), ranging from 80 - 90% IgA1 in male genital secretions to 60% IgA2 in female genital secretions (127).

Dimeric IgA is transported into mucosal secretions by the polymeric Ig receptor, which is expressed on the mucosal epithelial cells through a series of events. At the surface, external domains of the polymeric Ig receptor, termed the secretory component, forms a complex with dimeric IgA, which together with a J chain make up the secretory IgA (S-IgA), which is released into the mucosal secretions (129, 131).

Serum IgA and S-IgA have different immunological functions. Serum IgA is involved in antibody-dependent cell-mediated cytotoxicity, phagocytosis and release of cytokines through interaction with the cell receptor CD89, which is present on neutrophils, monocytes, eosinophils, some macrophages and dendritic cells (127). S-IgA is a hydrophilic, heavily glycosylated and negatively charged molecule that can inhibit adherence of microbes to the mucosa, neutralize bacterial toxins and activate basophil and eosinophils (132). Furthermore, S-IgA is capable of intracellular neutralization of viruses (133, 134), including HIV (135, 136).

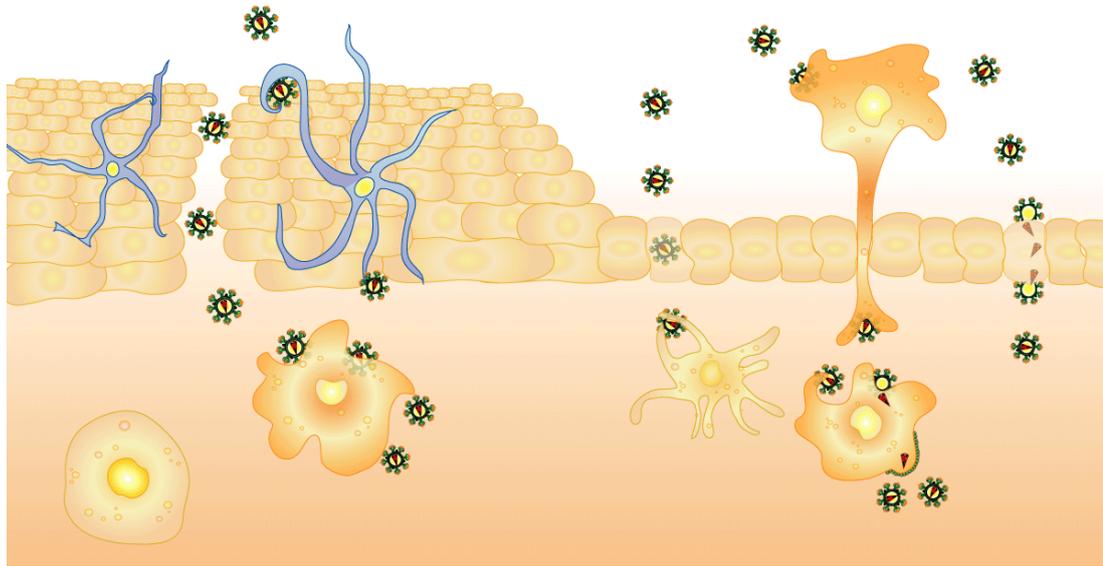
## 1.6 HIV TRANSMISSION IN THE FEMALE GENITAL TRACT

Heterosexual, intravenous and mother-to-child (pregnancy, delivery, breastfeeding) transmissions are the major routes of HIV spread globally, with the vast majority of HIV infections being acquired during heterosexual intercourse. The highest risk of sexual infection is attributed to anal intercourse (1:20-1:300) (46) followed by male-to-female vaginal intercourse (1:500) (137), male-to-female vaginal intercourse (1:1,000) (137) and oral intercourse (1:2,500) (46).

Women seem to be more susceptible to HIV acquisition during heterosexual intercourse than men and it is estimated that 30-40% of global HIV transmissions take place in the FGT (138). HIV is capable of infecting both the upper and lower FGT, including the cervix and the vagina, but as previously mentioned it remains unknown which specific site accounts for the majority of HIV transmissions (138).

Semen from HIV-infected men contains both free and cell-bound viruses (139), and both forms are capable of infection, as shown in macaques infected with SIV (140). The first-line of defence against HIV is the CVS (44), containing antimicrobial peptides previously described and other soluble antiviral factors that may confer HIV protection. Most virus particles remain trapped in the genital secretions as shown in *ex vivo* models of human cervical explants (141) but many virions can proceed to infect the mucosal cells. In fact, recent evidence indicates that a single virus causes close to 80% of heterosexual HIV transmissions (142).

A number of mechanisms of early transmission events have been suggested (Figure 2) and only a few will be described here. HIV may invade by direct infection (143), through abrasions in the epithelium (144) or by capture of intraepithelial Langerhans cells (145). The virions may also penetrate through gaps in the epithelium (46) or be rapidly transcytosed through epithelial cells to infect underlying CD4<sup>+</sup> T cells (146). *In vivo* studies of SIV infection in macaques have shown that the virus penetrates the cervicovaginal epithelium within 30-60 minutes of exposure (147) and establishes a founder population of infected cells within a couple of days, which subsequently propagate to a systemic infection through dissemination to lymphoid tissues (148). Studies using human explant models indicate that the primary target cells in early HIV infection include T cells, macrophages and subsets of dendritic cells (145, 149-151).



**Figure 2**

*Suggested routes of HIV transmission across multilayered and single-layered epithelium in the female genital tract including invasion through mucosal breaches, capture by Langerhans cells, transcytosis of virions, transmigration of infected donor cell and direct infection of epithelial cells.*

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#### *1.6.1.1 Determinants of increased HIV susceptibility in the female genital tract*

There are a number of factors that may influence HIV transmission risk, including protective host factors that have been extensively studied in highly HIV-exposed individuals. Here I will focus on determinants of increased HIV susceptibility.

A high plasma viral load in the HIV-infected partner seems to be the most important risk factor (137, 152, 153), but other viral properties and host factors associated with both the HIV-infected partner and the HIV-exposed partner may influence the risk of HIV-transmission. It is likely that any factor that increases the number of HIV target cells in the FGT may also increase HIV susceptibility (154). Genital inflammation and sexually transmitted infections (STIs), such as gonorrhoea, chlamydia and trichomoniasis, have been epidemiologically correlated to an increased risk of subsequent HIV acquisition (7, 137, 155). However, only one (156) of several randomized control trials of STI treatment has achieved reduced HIV acquisition (156-159).

Herpes simplex virus type 2 (HSV-2) is one of the most common STIs globally, with a prevalence of well over 60% among sexually active women in many African populations (160). Furthermore, infection with HSV-2 correlates with a substantial increase in HIV susceptibility (137, 158, 161, 162). HSV-2 infection not only causes genital ulcers (allowing HIV entry) but also causes a persistent increase in the number of HIV target cells in the genital mucosa, despite HSV-2 treatment (163, 164). This may explain why randomized trials of HSV-2 treatment have failed to reduce HIV transmission rates (165, 166).

The influence of endogenous and exogenous hormones on mucosal immunity in the FGT may be of critical importance. It has been suggested that the risk of HIV acquisition peaks during the week following ovulation due to hormonal suppression of genital immune responses (167). Several studies have reported a correlation between the use of hormonal contraception and increased risk of HIV acquisition (168-171). Suggested mechanisms for these observations include structural changes in the genital mucosa (thinning of the epithelium; induction of cervical ectopy), alterations in vaginal flora, increased risk of other STIs and changes in mucosal and systemic immunity (172).

## **1.7 HIV-EXPOSED SERONEGATIVE INDIVIDUALS**

There are numerous reports of individuals who have presumably been repeatedly exposed to HIV yet who remain uninfected when tested by both serology and polymerase chain reaction (PCR). These HIV-exposed seronegative (HESN) individuals are comprised of a small percentage of different high risk populations such as commercial sex workers, men who have sex with men, HIV serodiscordant couples, injection drug users and infants born to HIV-infected mothers. The proportion of HESN individuals within the groups and the risks of HIV acquisition among the groups vary greatly (173) depending on the route of transmission (174).

### **1.7.1 Commercial sex workers**

True HESN cohorts, including individuals that can be classified as resistant according to epidemiological models, comprise mostly of commercial sex workers in Africa and Asia (173). The Pumwani cohort in Nairobi, Kenya is one of the earliest and most extensively HESN cohorts studied, in which a subset of sex workers were identified as resistant to HIV epidemiologically (175).

There are several challenges associated with trials that investigate commercial sex workers, including maintenance and follow-up of these mobile cohorts, as well as many other potential confounders (173). Another obstacle is the lack of suitable negative control subjects, since low-risk controls do not allow control for the effects of genital infections or sex work (176). Nevertheless, commercial sex workers are a useful population to study because they undertake high-risk behaviours and are exposed to multiple viral isolates.

### **1.7.2 HIV serodiscordant couples**

HIV serodiscordant couples, in which one partner is HIV-positive and the other HIV-negative, represent a major population in mature HIV epidemics. In Sub-Saharan Africa, almost half of the HIV-infected population living in a couple have an uninfected partner (177), indicating that discordance is a major contributor to HIV transmission in Africa (177-179).

Recent data from Kenya suggest that 44% of people newly infected through heterosexual transmission are infected within marriage or cohabitation (5).

There are a number of challenges with trials investigating HIV-discordant couples. First, there is a substantial variation in yearly HIV exposures in discordant populations, ranging between 25 and 112 incidents per year (173), hence in many cases they should be regarded as a high-risk population rather than a HESN population. Second, a major obstacle is recruitment of both partners, which is illustrated by a large study in Uganda where only 25% of HIV-tested subjects were tested as a couple (180). Third, studies of HIV status disclosure have shown that there is a risk of separation or conflict if only one of the partners is diagnosed with HIV (181, 182). Finally, the HIV-negative partner is continuously exposed to the same HIV strain, whereas an effective HIV vaccine must presumably generate a cross-reactive immune response (173).

## **1.8 CORRELATES OF PROTECTION IN HIV-EXPOSED SERONEGATIVE INDIVIDUALS**

Homozygosity for the *CCR5D32* deletion mutation (183) is the most well defined mechanism of HIV resistance but accounts for only a minority of cases. Several other factors or combinations of factors may contribute to a reduced risk of HIV acquisition; importantly the protection may be lost over time (184). The identification and understanding of additional mechanisms affecting HIV susceptibility may be crucial for development of an effective vaccine or microbicide against HIV infection.

### **1.8.1 Innate immunity**

Apart from the chemical and mechanical barriers of mucus, antimicrobial peptides and epithelium, several other factors have been proposed to contribute to mucosal protection against HIV infection. Type I interferons are key mediators involved in response to viral infection and can activate cellular defence molecules such as APOBEC3G, which causes mutations in the viral genome, and TRIM5a, which targets the viral capsid (185). Both molecules have been associated with differential risk of HIV acquisition in HESN populations (186, 187).

Natural killer cells constitute around 10 to 15% of all circulating lymphocytes (188) and are important components of the innate immune system that harbour cytotoxic activity against virus-infected cells and tumour cells (18). Furthermore, natural killer cells may play an important role in linking innate and adaptive immunity (189). Increased natural killer cell activity has been associated with a reduced risk of HIV infection among HESN intravenous drug users (190).

## 1.8.2 Adaptive and humoral immunity

It has been suggested that HIV-specific cytotoxic T cells can mediate protection against HIV infection and there are several reports of HIV-specific CTL responses among HESN individuals in the absence of detectable HIV infection (191-193). A subset of commercial sex workers in one of these cohorts later seroconverted, despite previous CTL response, which was epidemiologically linked to reduced sex work (184). This may indicate that CTL response is a marker for HIV exposure, rather than involved in resistance to HIV acquisition (194) or that frequent exposure to HIV is needed to maintain a protective CTL response (184).

Several studies have detected HIV-specific IgA in mucosal samples of HESN individuals (195-198), although this has not been confirmed in other studies (199-201). Additional studies have reported presence of HIV neutralizing IgA responses in HESN populations (202-205), and thus demonstrated a functionality of the antibodies, which suggests that genital IgA may confer protection against HIV acquisition.

## 2 AIM OF THESIS

HIV is often defined as a mucosal infection but we still lack information about correlates of protective immunity against sexual HIV transmission. The overall aim of this thesis was to investigate the contribution of IgA antibodies and antimicrobial peptides to the mucosal immune defence in individuals at high risk of HIV acquisition. By performing studies of humoral and innate immunity in HESN individuals in cohorts of Kenyan female sex workers and HIV serodiscordant couples, the specific aims were:

- To determine the levels of antimicrobial peptides in CVS samples of HESN, HIV-positive and low-risk control women and assess their correlation with HIV inhibitory activity, high-risk sexual behaviour and genital infections (Paper I-III).
- To determine whether specific subsets of antimicrobial peptides are associated with HIV acquisition (Paper I-II).
- To investigate if the intrinsic HIV neutralizing capacity of CVS is dependent on the presence of cationic polypeptides by selectively depleting cationic peptides from neutralizing CVS and adding recombinant peptides to CVS lacking intrinsic activity (Paper III).
- To determine whether HIV neutralizing IgA antibodies in the CVS of HESN women is associated with protection against HIV acquisition (Paper IV).
- To define associations between co-factors for HIV exposure and presence of HIV neutralizing IgA in CVS (Paper IV).

## **3 MATERIAL AND METHODS**

### **3.1 STUDY POPULATIONS**

The protocol and patient-informed consent forms received approval by Institutional Review Boards or Independent Ethics Committees prior to the start of all studies. Written informed consent was obtained for all patients.

#### **3.1.1 Paper I**

An HIV prevention trial was performed in a cohort of 466 HIV-uninfected female sex workers from Kibera in Nairobi, Kenya (158). At enrolment all participants completed a detailed questionnaire regarding medical symptoms and sexual risk taking (such as client numbers, condom use, type of sex practiced, etc.). After trial completion, 113 participants were included in a prospective, nested case-control study on HIV and immunity (202), which was subsequently further extended (Paper I).

#### **3.1.2 Paper II**

The initial study was performed within the Pumwani sex worker cohort in Nairobi, Kenya. Three study groups (HIV-resistant, HIV-uninfected and HIV-infected women) were enrolled during ongoing biannual resurveys. Participants who had remained HIV-negative for more than 3 years of follow-up by both serology and real-time PCR, and who were clinically healthy and active in sex work, were defined as relatively HIV-resistant (175). HIV-uninfected sex workers were defined as HIV seronegative and active in sex work, but who had been followed for less than three years. These women came from similar socioeconomic and genetic backgrounds. Individuals were excluded from enrolment if they had a concomitant infection, were actively menstruating, or were post-menopausal. In total, 321 women were enrolled in the study, however 6 were later excluded from the study analysis due to possible menopause. The study was further extended to include a cohort of 113 HIV-uninfected female sex workers from Kibera in Nairobi, Kenya. Participants had been enrolled in an immune sub-study (202) nested within a randomized HIV prevention trial (158).

#### **3.1.3 Paper III-IV**

HIV serodiscordant couples (n=469) were enrolled in a prospective cohort study in Nairobi, Kenya and followed for up to 2 years with visits every 3 months (206). Eligible couples reported sexual intercourse with each other  $\geq 3$  times in the 3 months prior to screening and planned to remain together in Nairobi for the two-year duration of the study. Furthermore, female partners were not pregnant at enrolment, and the HIV-positive partners did not have clinical AIDS or a history of taking ART. HIV seropositive (Paper III) and HIV seronegative (Paper III-IV) women were selected from this larger cohort based on sample availability.

During the same time period, couples testing concordantly negative for HIV were recruited from the same voluntary counselling and testing centres as the discordant couples as a control population. Concordant negative couples also reported having sex with their partner  $\geq 3$  times in the 3 months prior to screening. A subset of women from these negative control couples was selected based on sample availability and assigned to the 'Low-risk' study group.

## **3.2 SAMPLE COLLECTION**

### **3.2.1 Paper I-II**

CVS from participants within the Kibera cohort (Paper I-II) were collected with a cotton-tipped swab that was rotated 360° in the cervical os; a second swab was used to collect secretions from the posterior vaginal fornix. Both swabs were transferred into a single vial containing 5 ml of phosphate buffered saline (PBS), transported to the laboratory on ice, centrifuged at 1500 rpm for 5 minutes to remove cellular debris and cryopreserved at -80°C.

Collection of cervicovaginal lavages in the Pumwani cohort (Paper II) was performed according to a separate protocol developed by the lab of lead investigator Dr. Plummer. Briefly, using sterile PBS, a 2 ml wash of the ectocervix was performed. Lavages were collected from the posterior fornix and placed in 15 ml tubes for transport to the laboratory on ice. Samples were aliquoted and frozen at -80 °C.

### **3.2.2 Paper III-IV**

CVS were collected by rotating a cotton swab 360° in the outer part of the endocervix and by rotating a different swab across the vaginal wall. Both swabs were transferred into a single vial containing 5 ml of PBS, transported to the laboratory on ice and centrifuged at 800 g for 10 minutes at 4°C. Supernatants were separated from the cell pellet, aliquoted and stored at -80°C.

For cationic polypeptide depletion experiments, a more concentrated CVS sample was required. One cotton swab soaked in mucus from the cervicovaginal tract was placed in 0.5 ml of saline and placed on ice. The vials were transported to the laboratory where the samples were stored at -80°C.

## **3.3 METHODS**

### **3.3.1 IgA1-depletion - Paper I, II, III**

The purification and depletion of IgA has been simplified by the use of jacalin, a jackfruit lectin that binds galactose residues present on the hinge region of serum IgA1 and sIgA1, but does not bind IgA2, IgG or IgM (207, 208). IgA1-depletion was performed by adding 800 µl of undiluted CVS to 400 µl of jacalin-agarose beads (Immunkemi, Stockholm, Sweden) and mixed for 2 h at 4°C followed by centrifugation at 2000 rpm for 5 minutes at 4°C. The unbound (IgA1-depleted) fraction was collected and stored at -80°C.

### **3.3.2 IgA1-purification - Paper IV**

To retrieve the bound IgA1 antibodies, the jacalin-agarose beads from 3.3.1 were thoroughly washed with PBS. Following this procedure, the bound IgA1 was eluted overnight at room temperature by adding 1 ml 0.8 M D-galactose. The supernatant (purified IgA1) was subsequently collected and stored at -80°C.

### **3.3.3 LL-37, HNP1-3, HBD2-3, SLPI, RANTES and IFN- $\alpha$ quantification – Paper I, II, III**

Commercial enzyme-linked immunosorbent assay (ELISA) kits were used, according to the manufacturers protocols, to quantify the  $\alpha$ -defensins HNP1-3, LL-37 (both Hycult biotechnology, Uden, the Netherlands), SLPI (R&D Systems Europe Ltd, Abingdon Oxon, UK), the  $\beta$ -defensins HBD2-3 (Phoenix Pharmaceuticals, INC., Burlingame, USA), RANTES (Biosource International, Camarillo, CA, USA) and IFN- $\alpha$  (PBL Biomedical Laboratories, Piscataway, USA).

### **3.3.4 Trappin-2 quantification – Paper II**

A commercial trappin-2 detection ELISA kit was used for measurement of the 9.9 kDa pre-elafin/trappin-2 form of the protein (HyCult Biotechnology, Uden, Netherlands), according to the manufacturers protocols. An in-house ELISA was established for measurement of both the 6 kDa elafin and 9.9 kDa forms of pre-elafin/trappin-2 in combination. Briefly, 96-well plates were coated overnight with a polyclonal antibody specific for both the 6 and 9.9 kDa forms of elafin/pre-elafin (R&D Systems, Minneapolis, MN, USA), blocked for one hour with 0.17% bovine serum albumin in PBS, then washed and incubated with CVS samples for 90 minutes at 37°C. Plates were washed and a monoclonal detection antibody (TRAB2F-HyCult Biotechnology, Netherlands) specific for both the 6 and 9.9 kDa form for elafin/pre-elafin was added and then incubated for 90 minutes. Plates were then washed and a secondary alkaline-phosphatase IgG mouse antibody (Sigma-Aldrich, Oakville, ON, USA) was added. Plates were read on an ELISA plate reader at 410 nm.

### **3.3.5 Measurement of prostate-specific antigen**

Prostate-specific antigen (PSA) levels in CVS were measured by an external accredited laboratory (Aleris Medilab, Täby, Sweden) using the ARCHITECT Total PSA assay (Abbott Ireland Diagnostics Division, Dublin, Ireland), a chemiluminescent microparticle immunoassay. The PSA levels in the CVS are reported as measured in the original 5 ml dilution. A level  $\geq 1$   $\mu\text{g}$  PSA/ml CVS was defined as a positive result (209).

### **3.3.6 Selective depletion of cationic polypeptides from CVS – Paper III**

Carboxymethyl weak cation exchange resin (Bio-Rad) was used to deplete cationic polypeptides from vaginal fluid (210). The carboxymethyl resin was pre-equilibrated by washing 6 times with a buffer resembling vaginal fluid in electrolyte composition (60 mM NaCl, 20 mM potassium phosphate, pH 6, (51). Equal volumes of CVS from four to six HIV seronegative donors were centrifuged and pooled before the extraction of cationic polypeptides. From each pool, 0.5 ml CVS was reserved and stored at -80°C as “unprocessed CVS.”

The remaining volume from each pool was carboxymethyl-extracted by mixing with equilibrated carboxymethyl resin at 4°C overnight in an end-over-end tumbler. Centrifugation (15,000 g, 4°C, 3 minutes) enabled collection of the carboxymethyl-depleted CVS supernatant, which was then cleared of residual resin by additional centrifugations and stored at -80°C. The carboxymethyl resin sediment was washed 5 times with 25 mM ammonium acetate, pH 7, and then cationic proteins were extracted by incubating the washed resin with 5% acetic acid in a tumbler at 4°C. Extracted proteins were collected and stored at -80°C after 2 h, and the resin was extracted a second time by incubation with more acetic acid overnight in a tumbler at 4°C. The first and second cationic extracts were pooled, clarified of residual resin, concentrated by vacuum centrifugation, and restored to the original volume. All samples were stored at -80°C until required for further experiments.

### **3.3.7 Recombinant innate factors and HIV neutralization – Paper III**

Recombinant SLPI (R&D Systems Europe Ltd, Abingdon Oxon, UK), LL-37 and HNP1-3 (both Hycult Biotechnology, Uden, the Netherlands) were added in serial dilutions to IgA-depleted CVS samples from low-risk HIV seronegative women who were selected based on lack of intrinsic neutralizing capacity. The recombinant peptides were incubated with the CVS samples for 1 h. Following this pre-incubation, the samples were run in the HIV neutralization assay, as described in section 3.3.11 of this thesis.

### **3.3.8 Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry - Paper II**

Protein concentrations of each sample were equalized prior to spotting onto a cation exchanger, CM10 array (Bio-Rad Laboratories, Mississauga, ON). All samples were thawed on ice and equalized for freeze/thaw cycles to control for protein degradation. Briefly, the CM10 array was prewashed with a binding buffer (50 mmol/L sodium acetate, pH 4, 0.1% triton), and samples were loaded at a final concentration of 2 mg of protein per spot. Chips were incubated on a microplate shaker at room temperature for 1 h and then washed with a wash buffer (50 mmol/L sodium acetate, pH 4) and HPLC water. The spots were allowed to dry, and then an energy-absorbing matrix of 50% sinapinic acid [in 0.5% trifluoroacetic acid/50% acetonitrile] was applied. Samples were acquired by PBS IIC SELDI-TOF mass spectrometry (MS), and univariate analysis for biomarkers was carried out using the Biomarker Wizard Software version 3.1 (Ciphergen Biosystems, Fremont, California, USA).

### **3.3.9 Tandem mass spectrometry identification of protein – Paper II**

Identification of the 6 kDa biomarker of HIV resistance was accomplished by purification of CVS samples using anion exchange chromatography (flow-through) followed by reverse phase chromatography (30% acetonitrile, 0.1% trifluoroacetic acid). The enriched preparation was reduced, alkylated and resolved by SDS-PAGE. The gel-purified protein was reconfirmed to be the 6 kDa peak of interest by running on the SELDI-TOF MS, then digested with trypsin and unique fragments analysed by tandem MS. Ions were fragmented by collision-induced dissociation, and the MS/MS spectrum was submitted to the Mascot search tool for identification (<http://www.matrix-science.com/>). In total, four ions from the tryptic digest of the 6 kDa protein were submitted for identification, and each were found to have extensive homology to elafin, confirming the identity of this protein.

### 3.3.10 HIV neutralization assay – Paper I, III and IV

HIV neutralization assays were performed according to a predefined protocol and neutralization cut off (202). Primary isolates (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) were collected from phytohemagglutinin and interleukin-2 stimulated peripheral blood mononuclear cells (PBMCs). The TCID<sub>50</sub> value was determined and supernatants were aliquoted and stored at -80°C. Since the TCID<sub>50</sub> may differ between PBMC donors, at least two viral dilutions were used in each assay. The CVS were not further diluted or concentrated when tested for neutralizing capacity. Duplicate wells of 75 µl of each virus dilution and 75 µl of each sample fraction were incubated for 1 h at 37°C followed by addition of a mixture of 1 x 10<sup>5</sup> phytohemagglutinin-stimulated PBMCs from 2-3 donors. After 24 h incubation at 37°C, the cells were centrifuged, unbound virus was washed away and 200 µl of fresh RPMI 1640 medium with bovine serum albumin and interleukin-2 were added to each well. On day 3, 120 µl of medium was discarded and replaced with new medium, and on day 6 supernatants were collected for analysis of virus production with a p24 antigen ELISA (Vironostika HIV-1 Antigen; Electra-Box Diagnostica AB, Stockholm, Sweden). As previously described, neutralization was defined as a ≥67% reduction of p24 antigen level in the supernatant compared to p24 antigen level when the virus isolate was incubated in the presence of low-risk HIV seronegative samples. Positive control samples (HIV IgG positive serum) were included in each assay.

### 3.3.11 STI screening - Paper I

At enrolment all participants underwent a complete physical examination and STI testing and treatment (158). Cervical swabs were obtained for *Neisseria gonorrhoea* and *Chlamydia trachomatis* PCR assays (Amplicor PCR Diagnostics, Roche Diagnostic Systems, Ontario, Canada) and for *N. gonorrhoea* culture. *Trichomonas vaginalis* cultures were performed using In Pouch TV (Biomed Diagnostics, San Jose, CA). A vaginal gram test was performed; BV was defined as a Nugent score of 7-10, and vaginal candidiasis as the finding of yeast on gram stain. If a genital ulcer was present, a swab of the ulcer base was taken for *H. ducreyia* culture on an activated charcoal medium. Blood samples were obtained for HIV and syphilis serology. The rapid plasma reagin test (RPR test, Becton Dickinson, Groot-Bijgaarden, Belgium) was performed to diagnose syphilis, and positive samples were confirmed by *Treponema pallidum* haemagglutination assay (Randox Laboratories, UK). HIV screening ELISA was performed using the Detect-HIV kit (BioChem ImmunoSystems Inc, Montreal, Canada) and positive tests were confirmed with the Recombigen HIV-1/HIV-2 enzyme immunoassay (Cambridge Biotech Corporation, Galway, Ireland).

### 3.3.12 STI screening - Paper III-IV

HIV rapid testing was conducted in the Study Clinic using two commercial kits (Determine® HIV-1/2 Rapid Test, Abbott Laboratories, Abbott Park, IL, USA; Bioline™ Recombigen HIV Test, Standard Diagnostic Inc., Korea). Positive or indeterminate results were confirmed with HIV-1 ELISA using the Vironostika® HIV Uni-Form II Ag/Ab ELISA kit (Biomerieux Laboratories, the Netherlands) in the University of Nairobi's Obstetrics/Gynecology laboratory. Plasma HIV-1 RNA viral load in HIV seropositive partners was quantified using the Gen-Probe Transcription Mediated Amplification assay (211). Herpes simplex virus type 2 (HSV-2) serostatus was determined using the HerpeSelect IgG ELISA kit (Focus Technologies, Cypress, CA, USA) using a cut off of 3.5 to improve specificity.

Syphilis testing was performed using the rapid plasma regain Card Test Macro-Vue kit (Becton Dickinson, Franklin Lakes, NJ, USA), with reactive rapid plasma regain tests confirmed with *Treponema pallidum* haemagglutination assay (RANDOX Laboratories, Crumlin, UK). Vaginal gram tests were performed and BV was defined as a Nugent score of 7-10.

### **3.4 STATISTICAL ANALYSIS**

#### **3.4.1 Paper I**

All analyses were performed with SPSS version 16.0 software (Chicago, Illinois, USA). Samples with undetectable levels of innate factors were assigned a value at the assay cut-off. Levels were log<sub>10</sub> transformed, and baseline (cross-sectional) associations of innate immune factors were examined: continuous variables were compared between groups using the independent samples t-test (after Levene's test for equality of variances) and categorical variables using Fisher's exact test. Commercial sex workers acquiring HIV during subsequent clinical follow-up (cases) were matched by an external biostatistician to controls who had remained HIV-uninfected, as previously described (202). Matching was based on time of study enrolment, study arm and duration of HIV seronegative follow-up. Univariate associations of HIV acquisition were assessed in a matched case-control format, using Mantel-Haenszel analysis (dichotomous variables) or conditional logistic regression (continuous variables). Stratified multivariable analyses of the associations of HIV acquisition were performed by conditional logistic regression using a Cox proportional hazards model, with the inclusion of variables associated with HIV acquisition in univariate analysis ( $p \leq 0.10$ ). SPSS does not offer conditional logistic regression per se, and so these analyses were performed using an established adaptation of Cox regression to obtain equivalent results.

#### **3.4.2 Paper II**

Statistical analysis of MS data to identify biomarkers was performed using the Ciphergen Biomarker Wizard Software version 3.1 as part of the biomarker discovery platform. Confirmation of the differences between elafin/trappin-2 levels by ELISA was performed using Mann-Whitney U tests. Although a total of 579 CVS samples were assayed, some samples came from the same individual over multiple time points during the 2-year course of sample collection. The biomarker discovery data represent the aggregate mean of each individual's MS intensity for the elafin/trappin-2 peak. ELISA data also represent the aggregate mean of the elafin/trappin-2 measurements per individual. It should be noted that over multiple time points, CVS levels of elafin for each individual remained relatively constant. Comparison between the MS intensity values and ELISA data for elafin/trappin-2 levels was done by Pearson's correlation test, and confounding variables were analysed by multivariate regression analysis. The potential protective role of elafin was then tested within the independent Kibera cohort, with elafin levels assayed by investigators blind to clinical outcome. All analyses were performed with SPSS version 16.0 software as described above for Paper I.

### **3.4.3 Paper III**

Statistical comparisons of the immune parameters between groups were performed using the Mann-Whitney U test, and calculations were performed using the GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). A p-value of <0.05 was considered statistically significant. Due to lack of sufficient volume of some CVS samples, not all samples were assessed for all parameters in the following sections. Since a partner's viral load of 10,000 or greater has been shown as an epidemiologic determinant for higher transmission risk in this geographical setting (212), this level was selected as a cut off for dividing the HESN women into two groups: HESN women whose partner's viral load was higher than 10,000 versus HESN women whose partner's viral load was lower than 10,000.

### **3.4.4 Paper IV**

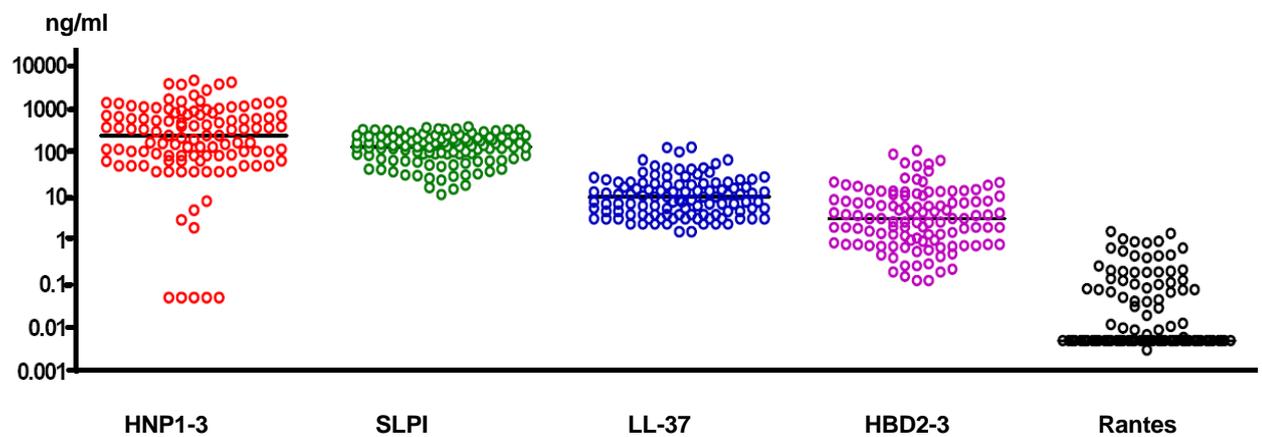
All multivariate analyses were adjusted for the male partner's CD4 count. Independent t-tests for continuous variables and Pearson's chi-squared test for dichotomous variables were used for univariate comparisons between neutralizing IgA responses and correlates. Logistic regression was used to assess association in a multivariate model. All analyses were done both with and without the exclusion of samples with PSA  $\geq 1$   $\mu\text{g/ml}$ . PSA measurements were performed to ensure that the presence of neutralizing IgA in the cervicovaginal sample was not from the male partner.

## 4 RESULTS AND DISCUSSION

### 4.1 MUCOSAL INNATE IMMUNE RESPONSES IN HIGH-RISK INDIVIDUALS

#### 4.1.1 Levels of antimicrobial peptides in genital fluids of HESN

One of the major aims of this thesis was to study antimicrobial peptides in relation to HIV. In Paper I, we quantified levels of peptides previously shown to have anti-HIV activity *in vitro* in a cohort of 113 commercial sex workers in Kibera area of Nairobi, Kenya. HNP1-3 and SLPI were most abundant and HBD2-3 and LL-37 levels were lower but easily detected. RANTES was only found in picogram levels, and IFN- $\alpha$  was undetectable (Figure 3).



**Figure 3**

The CVS from 113 sex working Kenyan women were measured for levels of HNP1-3, SLPI, LL-37, HBD2-3 and RANTES. Each circle denotes one individual. IFN- $\alpha$  levels did not reach the detection limit of the assay. The Y-axis shows the concentration (ng/ml) of each molecule as measured in the original 5 ml CVS dilution. Solid line indicates median concentration.

In a cohort study of Kenyan HIV serodiscordant couples, named Couples Against Transmission (CAT), CVS samples from 296 women were assessed for levels of cationic polypeptides (Paper III). The levels of HNP1-3 were comparable between the Kibera sex worker cohort and the serodiscordant CAT cohort (median values 175 and 185 ng/ml, respectively). However, less LL-37 was present in the CAT cohort than in the Kibera sex worker cohort (median values 7 and 12 ng/ml, respectively) (CAT vs. Kibera:  $p < 0.0001$ ). Likewise, significantly lower levels of SLPI were found in the CAT cohort compared to the Kibera cohort (median values 60 and 204 ng/ml, respectively).

#### 4.1.2 Expression of antimicrobial peptides and genital infections

A number of antimicrobial peptides have been demonstrated to have anti-HIV activity *in vitro* (65-70) and several studies have reported that antimicrobial peptides mediate protection in HIV-exposed individuals (96, 119, 121, 213). The antimicrobial peptides HNP1-3 have been suggested to be involved in recruitment of potential HIV target cells in inflammation (71) and elevated levels of HNP1-3 have been observed in inflammatory conditions of the female genital tract (76, 214). In Paper I and II we measured levels of cervicovaginal polypeptides and assessed their correlation with STIs and genital inflammation. In the Kibera sex worker cohort genital infections were common at enrolment and the prevalence of *C. trachomatis*, *N. gonorrhoea* and *T. vaginalis* was high (15%, 12% and 13%, respectively). In addition, 8% of sex workers were infected with syphilis and 71% were HSV-2 seropositive. Several genital infections were individually correlated with increased levels of antimicrobial peptides, including defensins and LL-37 (Table 1). Study subjects with several simultaneous co-infections had increased levels of both defensins and LL-37.

Genital infection		HNP1-3	LL-37	HBD-2
		Median (ng/ml)	Median (ng/ml)	Median (ng/ml)
C. trachomatis	Yes	1000	25**	12*
	No	215	10	4
N. gonorrhoea	Yes	1000*	19*	18*
	No	240	10	4
T. vaginalis	Yes	319	15	12*
	No	284	11	4
Candidiasis	Yes	751	35**	7
	No	175	9	4

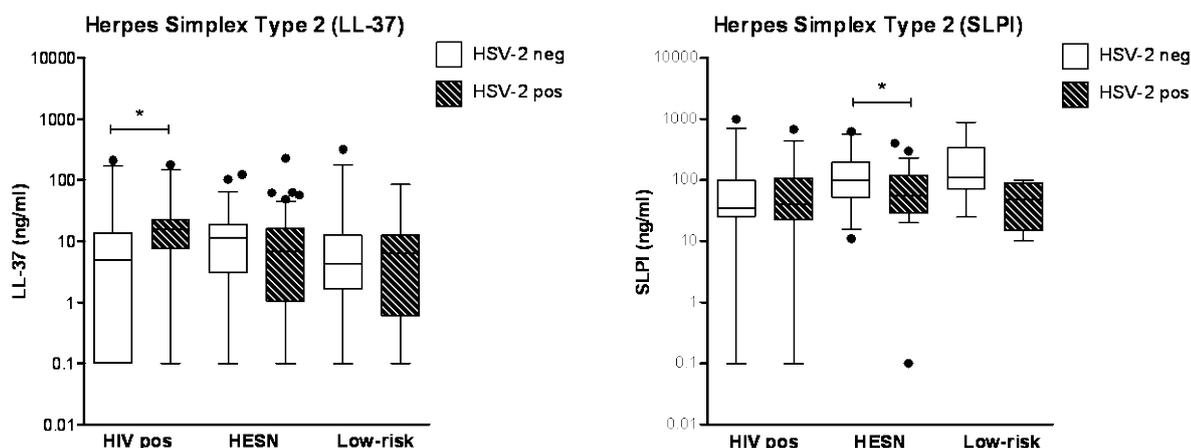
**Table 1**

*Several genital infections were individually or collectively correlated with increased levels of antimicrobial peptides. All concentrations are given in ng/ml. Only genital infections with a significant correlation to peptide levels are shown. Peptide levels were measured in the original 5 ml CVS dilution.*

\*p < 0.05; \*\*p < 0.01

Some studies have reported BV to be associated with decreased genital levels of SLPI and defensins (59, 213, 215). BV was present in 59% of participants in the Kibera sex worker cohort, but we did not observe any differences in levels of any antimicrobial peptides. Likewise, neither HSV-2 nor syphilis seropositivity were associated with alterations of peptide levels.

In the discordant CAT cohort, the prevalence of genital infections was very low, with only 3% infected by *T. vaginalis* and only one subject with syphilis (Paper III). Among the HESN women in the CAT cohort, 67% were HSV-2 infected, which is comparable to the percentage of HSV-2 infected participants in the Kibera sex worker cohort. In contrast, only 13% of HESN women in the CAT cohort were diagnosed with BV. In concordance with the findings in Paper I, BV was not associated with the levels of any of the peptides measured for HIV-negative CAT subjects (HESN and Low-risk). Presence of BV was however associated with higher levels of SLPI in HIV-positive women (median levels of SLPI in BV positive vs. BV negative women: 42 vs. 24 ng/ml, respectively,  $p=0.035$ ) In contrast to the sex worker cohort, HSV-2 infection was correlated with altered levels of peptides in the CAT cohort (Paper III). HIV-positive women who were co-infected with HSV-2 had significantly higher levels of LL-37 than those who were HSV-2 seronegative (16 vs. 5 ng/ml, respectively,  $p=0.016$ ). In the HESN group, lower levels of SLPI were observed in HSV-2 seropositive women compared to HSV-2 seronegative women (55 vs. 99 ng/ml, respectively,  $p=0.030$ ) (Figure 4).



**Figure 4**

*In the CAT cohort, CVS were collected from HIV-positive women (HIV pos) as well as from HIV-negative women with either a HIV-positive partner (HESN) or a HIV-negative partner (Low-risk). Comparison of women with or without HSV-2 infection (HSV+/-) and levels of LL-37 (left) [HIV pos HSV- (n=24); HIV pos HSV+ (n=28); HESN HSV- (n=52); HESN HSV+ (n=109), Low-risk HSV- (n=36), Low-risk HSV+ (n=22)] and SLPI (right) [(HIV pos HSV- (n=28); HIV pos HSV+ (n=32); HESN HSV- (n=28); HESN HSV+ (n=58); Low-risk HSV- (n=28); Low-risk HSV+ (n=9)].*

#### 4.1.3 Antimicrobial peptides and HIV neutralization

Analysis of CVS samples from the HESN women in the Kibera sex worker cohort revealed that 23 of 113 (20%) of IgA1-depleted samples could neutralize a clade A primary isolate and 13 (12%) could neutralize both a clade A isolate and a clade C isolate (Paper I). Neutralizing capacity against one single clade did not correlate with the levels of any of the antimicrobials measured. However, those samples that could neutralize both clades had higher concentrations of HNP1-3 ( $p=0.007$ ) and LL-37 ( $p=0.002$ ).

The IgA1-depleted CVS samples in the serodiscordant CAT cohort were also assessed for HIV neutralizing capacity (Paper III). In 27 of 152 (18%) genital samples from the HESN women, HIV was neutralized. In the Low-risk group and in the HIV-positive group, the corresponding proportion was 17 of 63 (27%) and 17 of 46 (37%), respectively. In the HESN group, samples that could neutralize HIV had significantly lower levels of SLPI (31 ng/ml vs. 76 ng/ml, respectively,  $p=0.02$ ). No differences were seen between neutralizing and non-neutralizing samples for levels of HNP1-3 or LL-37 in any of the study groups.

Comparison of the HIV neutralizing capacity of the HESN women in these two cohorts revealed that the relative ability of HESN women to inhibit HIV in the sex worker cohort (Paper I) was in the same range as in the serodiscordant cohort (Paper III), which validated the PBMC assay for measuring HIV inhibitory capacity. An unexpected finding in paper III was that a higher percentage of women in the Low-risk group (27%) compared to the HESN group (18%) was able to neutralize HIV. One explanation for this observation could be that differences in sexual behaviour between control women and HESN women, including more frequent unprotected sex among controls, may have induced an elevated innate immune response in the genital tract that increased the neutralizing capacity of these women.

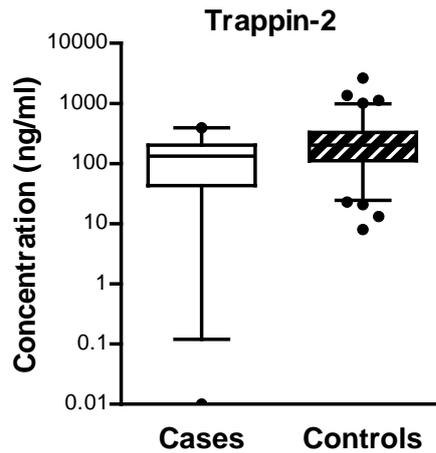
#### **4.1.4 Antimicrobial peptides and HIV acquisition**

In Paper I, the association of peptide levels with subsequent HIV acquisition was assessed in a case-control format by comparing peptide levels in women who subsequently acquired HIV with matched controls who remained uninfected with HIV. Despite the association of HNP1-3 and LL-37 with an increased HIV neutralizing ability of CVS, when a stratified multivariable model was applied, HNP1-3 and LL-37 were independently associated with increased HIV acquisition (adjusted OR 8.2 ( $p=0.02$ ) and 6.9 ( $p=0.05$ ), respectively). No associations were observed between the levels of any of the other peptides measured. Furthermore, presence of a bacterial STI at baseline was not associated with subsequent HIV acquisition.

The finding that HNP1-3 and LL-37 correlated with HIV susceptibility, despite their HIV-inhibitory activity *in vitro*, was unexpected. Concomitant STIs influence the levels of innate factors, which confound the ability to assess their *in vivo* role in protection against HIV acquisition, which will be further discussed in the Conclusions.

##### *4.1.4.1 Genital expression of trappin-2 and HIV acquisition*

In Paper II, a cross-sectional study on peptides associated with HIV resistance was performed on the Kenyan Pumwani sex worker cohort. Mass spectrometry of genital samples from the initial study participants identified trappin-2 as the most significant peptide associated with HIV resistance, keeping in mind that the protein chip used exclusively captures molecules with a positive surface charge, limiting the detectable subset of peptides. The study was subsequently extended to include the 113 sex workers from the Kibera-cohort in Paper I. The association of baseline trappin-2 levels with subsequent HIV acquisition was assessed in a nested, stratified case-control format, as described in Paper I. Univariate analysis revealed that elafin/trappin-2 levels were significantly higher in the women who remained HIV-negative (mean 263 ng/ml,  $n=89$ ) compared to those who acquired HIV (mean 160 ng/ml,  $n=23$ ) (Figure 5). There was no association of trappin-2 levels with any STI or BV.



**Figure 5**

*Trappin-2 is elevated in women who did not seroconvert in a case–control study of HIV susceptibility. Cases are defined as the female sex workers who became HIV-positive during the trial. Controls are individuals who did not acquire HIV. Trappin-2 was measured in CVS samples obtained upon enrolment and showed elevated levels of trappin-2 in the control individuals who did not acquire HIV during the trial ( $p=0.044$ ).*

#### **4.1.5 Levels of antimicrobial peptides in relation to partner’s viral load**

Among the serodiscordant couples in the CAT cohort, none of the HIV-infected partners of the HESN women were on ART at enrolment. About two-thirds of the partners had a plasma viral load above 10,000 HIV RNA copies per ml, a threshold shown to correlate with a high risk of HIV transmission in this setting (212). Since the partner’s viral load could potentially influence the levels of cationic polypeptides measured in the CVS samples, viral load was compared to levels of the individual peptides. Indeed, when the HIV-infected partners were stratified into two groups according to viral load (more or less than 10,000), significant associations were seen with cationic polypeptide levels in the CVS of the corresponding HIV-uninfected partner. HESN women whose partner’s viral loads were higher than 10,000 ( $n=127$ ) had significantly higher levels of HNP1-3 and LL-37 than HESN women whose partner’s viral loads were less than 10,000 ( $n=37$ ) (HNP1-3: 191 vs. 109 ng/ml, respectively,  $p=0.036$ ; LL-37: 9 vs. 4 ng/ml, respectively,  $p=0.028$ ). Levels of SLPI were however not affected by partner’s viral load. Thus, although seminal fluid and plasma viral load may not be directly correlated, sexual exposure to a high viral load may have provoked a mucosal inflammatory response, including the release of these peptides into the genital secretions.

#### **4.1.6 Antimicrobial peptides and presence of mucosal PSA**

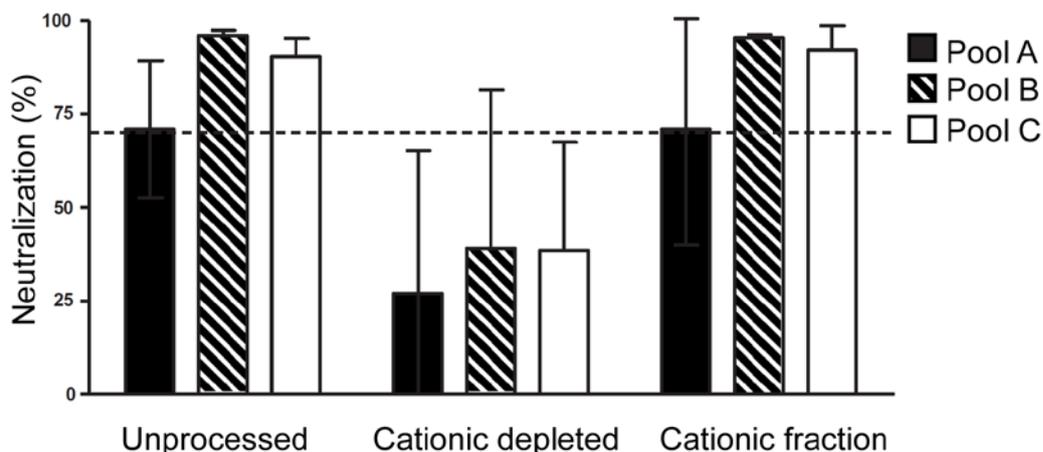
It is difficult to control for direct effects of sexual intercourse on the composition of the CVS but in order to evaluate the influence of seminal contamination, the CVS samples in the CAT cohort were assayed for the presence of PSA ( $\geq 1 \mu\text{g/ml}$ ) and then compared for levels of cationic polypeptides. PSA was found in 33% of the CVS samples in the Low-risk group, followed by 12% of the CVS samples in the HIV-positive group and 10% of the CVS samples in the HESN group.

Assessing all study groups together, no statistically significant differences were seen between PSA-positive and PSA-negative CVS samples for levels of HNP1-3 (median levels: 160 vs. 205 ng/ml, respectively,  $p=0.19$ ). Levels of LL-37 were however significantly lower among the PSA-positive vs. the PSA-negative CVS samples (median levels 3 vs. 7 ng/ml, respectively,  $p=0.015$ ). In contrast, the levels of SLPI were significantly higher among the PSA-positive vs. the PSA-negative CVS samples (median levels: 101 vs. 59 ng/ml, respectively,  $p=0.029$ ). Reanalysing the data by excluding the PSA-positive samples did not significantly affect the median values of the peptide levels. However, for LL-37, exclusion of PSA-positive samples resulted in a significant difference between the HIV-positive and Low-risk groups (13 vs. 5 ng/ml, respectively,  $p=0.04$ ).

#### 4.1.7 *In vitro* experiments of antimicrobial peptides and HIV neutralization

##### 4.1.7.1 Innate peptides contribute to the anti-HIV activity of CVS

Cationic polypeptides were selectively removed from three groups (A, B, C) of pooled HIV neutralizing CVS samples, and removal was confirmed by acid urea-PAGE. The cationic polypeptide fractions were then tested for HIV neutralization activity, which revealed that each fraction had activity equivalent to that of the whole pool. The remaining peptide-depleted CVS samples had no neutralizing activity, indicating that cationic polypeptides significantly contribute to the functional anti-HIV activity of CVS (Figure 6).

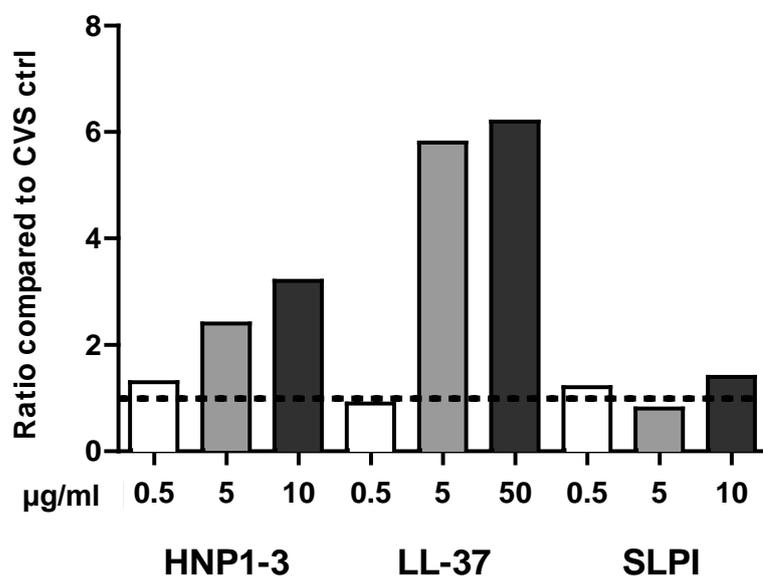


**Figure 6**

*The HIV neutralizing activities of unprocessed CVS, cationic-depleted CVS and cationic polypeptides extracted from the carboxymethyl resin (the cationic fraction); all were tested individually for each pool of CVS samples. The concentrations of LL-37 and HNP1-3, respectively, in the different pools were: A: 4 and 30 ng/ml, B: 2 and 10 ng/ml, C: 2 and 10 ng/ml. The results shown are from one representative experiment using duplicate wells and two different virus dilutions (median values  $\pm$ SEM).*

#### 4.1.7.2 Recombinant peptides can increase HIV neutralizing activity of CVS

In a previous study by a collaborator (210), depletion of cationic polypeptides in CVS from healthy low-risk women reduced the intrinsic HIV neutralizing capacity of CVS. Adding back the whole cationic polypeptide fraction to the CVS subsequently restored the anti-HIV activity. We performed a similar *in vitro* study with CVS samples from Kenyan HIV seronegative women at risk of HIV infection, using CVS samples lacking anti-HIV activity, which were pooled into groups for repeated experiments. Recombinant SLPI, HNP1-3 and LL-37 were evaluated in three independent experiments. HNP1-3 and LL-37, but not SLPI, induced a two to six-fold increase in HIV inhibiting activity when assessed at about 10-50 times the physiological concentrations. No effect for any of the peptides was seen at lower concentrations (close to physiological levels). These *in vitro* experiments suggest that the cationic polypeptide components strongly contribute to the intrinsic HIV neutralizing capacity of CVS (Figure 7).



**Figure 7**

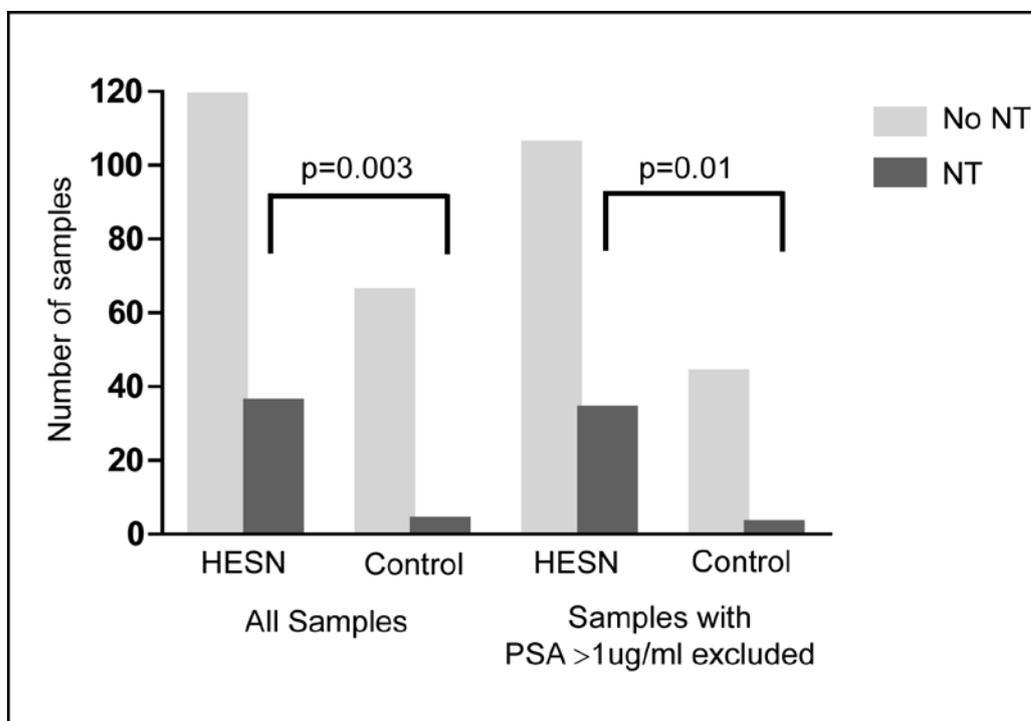
*Recombinant forms of HNP1-3, LL-37 and SLPI were assessed for HIV neutralizing activity by pre-incubating them individually in serial dilutions with CVS. The CVS samples were selected based on lack of intrinsic neutralizing activity. The ratio of HIV neutralizing activity was calculated as: CVS plus peptide divided by CVS alone. One representative experiment from at least three independent experiments is shown.*

## 4.2 MUCOSAL HUMORAL IMMUNE RESPONSES IN HIGH-RISK INDIVIDUALS

### 4.2.1 Prevalence and durability of HIV neutralizing cervicovaginal IgA

Several studies have identified the presence of neutralizing IgA in such HESN individuals (202-205). In one of our previous studies of the Kibera cohort, HIV neutralizing IgA was detected among 81% of HIV-negative sex workers who remained HIV-uninfected and was strongly associated with reduced acquisition of HIV (202). We wanted to further investigate this finding in a highly relevant cohort for studying heterosexual HIV transmission.

A subsequent study (Paper IV) was thus performed in the serodiscordant CAT cohort, which revealed that 36 of 155 (23%) HESN women and 4 of 70 (6%) control women had HIV neutralizing cervicovaginal IgA (OR=5.0;  $p=0.003$ ) (Figure 8). When excluding cervicovaginal samples with detectable PSA, HIV neutralizing IgA was detected in 34 of 140 (24%) HESN women and 3 of 47 (6%) control women ( $p=0.01$ ).



**Figure 8**

*HIV neutralizing capacity of IgA. The study samples were sorted according to if neutralization occurred (NT) or not (no NT). HIV neutralizing IgA was detected in 36 of 155 (23%) HESN women and 4 of 70 (6%) control women in the CAT cohort (OR=5.0;  $p=0.003$ ). When CVS samples with detectable PSA were excluded, HIV neutralizing IgA was detected in 34 of 140 (24%) HESN women and 3 of 47 (6%) control women ( $p=0.01$ ).*

To determine the durability of IgA responses, follow-up CVS samples were collected every 6 months after enrolment. Thirty of the 36 women who had neutralizing IgA in their CVS samples at baseline had subsequent visit samples for a varying time period up to 1 year. HIV neutralizing activity was persistently found in all follow-up visits among those who had neutralizing IgA at baseline. This finding indicates that the humoral response in the cervicovaginal mucosa of HESN women is durable.

At enrolment in the CAT study, presence of PSA was detected in 10% of the HESN CVS samples and 20% of the HESN women reported non-perfect condom use during the last month. Despite this evidence of unprotected sex, only five HESN women acquired HIV during the 2-year follow-up study period. None of these women had detectable HIV neutralizing IgA in their CVS samples but with such a small number of HIV acquisitions, our study was not powered to determine if the presence of neutralizing IgA decreases the acquisition of HIV.

#### **4.2.2 Correlates of HIV neutralizing cervicovaginal IgA**

In the HESN women of the serodiscordant CAT cohort, the presence of genital IgA with anti-HIV activity was associated with lower partner's plasma viral load (OR 0.70;  $p=0.02$ ) (Paper IV). Concordantly, there was also a trend for an association of HIV neutralizing IgA response and lower partner's seminal viral load (OR 0.57;  $p=0.06$ ), which was significant when samples with detectable PSA were excluded (OR 0.51,  $p=0.04$ ).

The inverse association between partner's viral load and HIV neutralizing genital IgA response was unexpected, as our original hypothesis was that exposure to higher partner's viral load would elicit more neutralizing IgA response from HESN women. One explanation for this association could be that the specific HIV strains that elicit neutralizing IgA responses in HESN women also mediate neutralizing humoral responses in their HIV-infected partners. It is not unlikely that such neutralizing capacity may lower viral replication and shedding at mucosal surfaces in the HIV-positive individual.

HIV neutralizing IgA was associated with neither HSV-2, BV, previous STIs nor male partner's factors such as HSV-2 positivity, circumcision status or CD4 count.

## 5 CONCLUSIONS

As previously stated in this thesis, there is a lack of information about correlates of protective immunity against sexual HIV transmission. In order to develop preventive measures against HIV, the vaccine field requires more studies focusing on the mechanisms underlying mucosal HIV transmission. It is generally accepted that an optimal HIV vaccine should ideally induce a broadly neutralizing antibody response that is persistent over time, as well as effective cell-mediated immunity in order to defeat the challenging features of HIV (216).

Real time studies of *in vivo* HIV transmission in humans are not possible for obvious ethical reasons. Animal studies, especially in non-human primates, can provide useful knowledge about the initial transmission events in human HIV infection but the research field is largely destined to perform *in vitro* assays and epidemiological studies of correlates to HIV transmission and protection in HESN populations. The role of mucosal immunology in genital HIV-transmission has indeed gained knowledge from studies on cohorts of such highly HIV-exposed individuals. While other studies have focused on cellular immune responses we have here assessed the contribution of IgA antibodies and selected innate immune molecules to HIV resistance. In the present thesis we have had access to clinical samples from well-characterized cohorts of Kenyan HESN individuals including female sex workers and HIV-serodiscordant couples.

An important conclusion I have learned from our studies of HESN populations is that human biology, HIV and their combined interactions are exceedingly complicated and that the outcome of the studies may put the initial hypothesis in doubt.

### 5.1 CORRELATES OF PROTECTION IN HIV-EXPOSED SERONEGATIVE INDIVIDUALS

There are several caveats associated with studying cohorts of HESN populations in different risk settings, including ethical considerations, recruitment of a representative target population, identification of relevant socio-demographic factors and retaining the cohort during follow-up. Moreover, performing experimental studies on these study subjects is also linked to obstacles such as selection of relevant immune parameters to measure, proper sample collection, development of validated methods and interpretation of laboratory data.

Our group has previously reported several correlates of mucosal protection against HIV transmission in different high-risk cohorts (119, 202, 205, 217). The high-risk populations include a Swedish cohort of discordant couples of men who have sex with men, Kenyan females with HIV-infected partners and commercial sex workers in high-endemic areas in Kenya. We have also studied different mucosal compartments in relation to HIV transmission, including the oral cavity, female genital tract and foreskin (202, 205, 218).

This may provide significant knowledge about the biological reasons for the highly variable HIV transmission risk depending on mucosal site exposed. We have further identified the presence of likely HIV target cells at different locations in the female genital tract (ectocervix, endometrium and endocervix) (126, 219, 220) as well as in the foreskin of the male genitalia (218).

### 5.1.1 HIV neutralizing mucosal IgA

The reason we focused on IgA in mucosal infections is that IgA is the major mucosal antibody (although not in the female genital tract) and S-IgA has a documented role in inhibition of viral infection (197). Several human broadly neutralizing HIV-specific antibodies have been isolated from HIV-positive individuals (221) but they are all of the IgG subclass. A major challenge in vaccine design is to elicit a persistent antibody concentration that is high enough for protective immunity. In most cases, there is a high correlation between the *in vitro* neutralizing capacity of a specific antibody and the antibody-mediated protection *in vivo*, which has been shown in non-human primate models (222). Interestingly, a recent study showed that vaccine-induced HIV neutralizing vaginal IgA protected macaques against vaginal challenge with simian-HIV (223).

Detection of genital HIV-specific IgA and HIV neutralizing mucosal IgA responses in HESN populations has been previously discussed in this thesis. However, it is important to make the distinction between HIV *neutralizing* IgA and HIV-*specific* IgA, since HIV neutralization may be achieved without specificity to HIV antigens through different mechanisms, including antibody-mediated blocking of CCR5 on target cells (224, 225) or through alloimmunisation directed against human leukocyte antigens in seminal or cervicovaginal fluid (226). In the studies performed by our group, we have not been able to determine the specificity of the HIV neutralizing IgA in any of the different mucosal compartments examined (202, 204, 205), except in one study (227). However, important findings for the role of IgA include one study (202) in which we found that the genital IgA response in HESN sex workers was correlated with subsequent protection from HIV acquisition. Another group identified HIV-specific genital IgA in HESN sex workers that was not associated with neutralizing capacity but was associated with the number of HIV exposures (176).

An important evidence of the presence of functional IgA antibodies in HESN individuals was recently revealed. In an elegant study, Tudor et al.(197) generated a mucosal Fab IgA library from cervical B cells of HESN women and found that gp41-specific IgA blocked HIV transcytosis *in vitro* and mediated HIV neutralization in CD4+ T cells. Furthermore, they characterized the Fab genes at a molecular level, linking the functional HIV-resistance to a specific origin in the antibody gene.

Suggested mechanisms for induction of antibody-mediated immune responses without a productive infection include the hypothesis that HESN women may indeed be locally and transiently infected but somehow manage to clear the infection before it reaches the local lymph nodes. Indeed, one study has reported detection of low levels of viral replication in the genital mucosa of HESN individuals that remained HIV seronegative (228).

In addition, there are mechanisms described for T cell-independent IgA class switching (229), which could potentially explain the detection of mucosal IgA response against HIV in the absence of HIV-specific IgG in HIV-negative subjects.

In summary, in the discordant couple study presented in this thesis we found that HESN women were five times more likely to have HIV neutralizing IgA detected in CVS as compared to low-risk controls. Moreover, we have previously reported a significant association between genital IgA response and protection against HIV acquisition in HESN women (202). The lack of correlation reported in other studies (176) may in part be related to variations between the HESN cohorts and the different methods used for analysing the mucosal samples.

### **5.1.2 Innate immune responses**

There is lack of knowledge about the repertoire and levels of antimicrobial peptides that are expressed in the female genital tract during normal and inflammatory conditions. Furthermore, the individual biochemical properties of many identified peptides are unknown, as well as their collective synergistic effects. In that context, it is a challenge to identify appropriate peptides for investigating antimicrobial anti-HIV activity in the female genital mucosa and to interpret the findings.

The high-risk sexual behaviour of sex workers puts them at increased risk of contracting both HIV and other STIs. Genital infections may in turn induce inflammation and increased host production of antimicrobial peptides (60, 76, 214, 230). In order to address these aspects, we measured genital levels of a subset of cationic polypeptides in different HESN populations and investigated their anti-HIV activity as well as correlations to genital infections and future HIV acquisition (Paper I-III).

Kenyan female sex workers were included in a study in which baseline CVS samples were collected and their HIV serostatus was assessed about two years later (Paper I). These samples were analysed for levels of antimicrobial peptides with HIV neutralizing activity and the results were subsequently correlated to HIV seroconversion (cases) or HIV seronegativity (controls). STIs were common at enrolment and correlated with increased levels of antimicrobial peptides in CVS, including defensins and LL-37. In addition, the presence of several simultaneous co-infections was associated with elevated levels of defensins and LL-37. Furthermore, we found that elevated levels of trappin-2 correlated with protection against HIV acquisition among female sex-workers (Paper II). However, despite significant HIV-inhibitory activity, high levels of HNP1-3 and LL-37 were associated with increased HIV acquisition in the same cohort (Paper I).

Even though there was no association between subsequent HIV seroconversion and genital infections at baseline, HIV acquisition at follow-up was strongly correlated to recent bacterial STI (158). Thus, it is possible that individuals with higher levels of LL-37 and HNP1-3 at baseline may have comprised a subgroup of sex-workers with increased rates of STIs that were the cause of their increased HIV susceptibility.

However, the possibility remains that these antimicrobial peptides may have directly caused increased HIV susceptibility through local enhancement of HIV replication (76) or through recruitment of HIV-susceptible target cells (71).

It may seem logical that a strong mucosal innate immune response has a better chance to inhibit HIV infection by clearing the mucosal surface from HIV particles. However, as illustrated in paper I, upregulation of innate immune factors may instead have a chemotactic function and result in recruitment of more HIV target cells. In fact, it has been suggested that the mechanism of HIV resistance in HESN is related to immune quiescence, based on the finding that Kenyan HESN sex workers produced lower levels of proinflammatory cytokines at baseline than HIV-negative controls (231). The term immune quiescence thus refers to a non-inflammatory status with a low specific HIV immune response and low numbers of HIV target cells in the mucosal tissue.

Measurement of peptide levels in highly diluted CVS samples using different commercial ELISA kits revealed that peptide levels do not necessarily correspond to the true physiological levels of all biologically active forms of specific peptides. In addition, several of the small differences in peptide concentrations observed in our study groups may not be of biological relevance.

In conclusion, the correlation between levels of antimicrobial peptides (individual or combinations of) and susceptibility or resistance to HIV remains unclear due to their numerous immunomodulatory functions and their likely synergistic effects. The dual role of antimicrobial peptides including antiviral and proinflammatory properties should be carefully evaluated in the context of future clinical trials of vaccines and other preventive strategies against sexual HIV transmission.

## **5.2 RELEVANCE OF *IN VITRO* HIV NEUTRALIZATION**

There are several different HIV neutralization assays available for studying HIV-inhibitory activity. The studies in this thesis are all based on well-established primary target cell assays, using HIV isolates derived from patients. The primary HIV isolates are used to infect freshly prepared PBMCs from HIV-negative healthy blood donors and productive HIV infection (single or multiple rounds) is detected by measuring expression of the viral p24 antigen. The PBMC assay is designed to resemble the *in vivo* infection, using relevant HIV target cells and virus isolates from HIV-infected patients. The disadvantages of this approach are that the assay is time-consuming and affected by donor variability, since immune cells from different donors may differ in HIV-susceptibility due to host genetics. In our studies we have tried to decrease this variation by pooling PBMCs from at least two donors for each experiment and by using triplicates of each experiment with different viral concentrations.

A more recently developed neutralization assay is based on pseudoviruses, which are molecularly constructed defective virus particles carrying the Env proteins of choice. The pseudoviruses can infect cell lines, such as TZM-bl cells, which are engineered to express high levels of the HIV co-receptors CD4 and CCR5. The pseudoviruses are only capable of one single round infection and infection is monitored by measuring expression of reporter genes.

The pseudoviral assay is safe (not capable of infecting humans), relatively rapid and considered to be highly reproducible. However the only parameters measured by the pseudovirus assay are inhibition of viral attachment/entry. In contrast, the PBMC assay measures the functional anti-HIV activity, which may be mediated through many different mechanisms and theoretically detects a broader array of antibodies and other mucosal factors exhibiting HIV-inhibitory capacity. Several groups have compared these two assays and found large differences in neutralizing activity (232-234), resulting in neutralizing activity that is either more easily detected or absent depending on the assay used.

The neutralizing capacity of mucosal samples also varies depending on which HIV isolate is used. The most prevalent subtype of HIV in Nairobi is a highly divergent clade A subtype, followed by clade D and C (235). Due to the high inter- and intra-variability between different HIV strains, the neutralizing capacity of a mucosal sample is likely to vary, depending on the nature of previous HIV-exposure. It has been previously demonstrated that some sex workers had a broad cross-clade neutralizing activity (203) and in Paper I we observed a cross-clade neutralization among 12% of the mucosal samples from the sex workers in the Kibera cohort (Paper I). It is likely that the women in the discordant CAT cohort would exhibit a more narrow neutralizing capacity, being exposed to only one partner's virus (unless existence of concurrent partnerships). Due to small sample volume in the CAT cohort, we were not able to address this aspect and could only investigate neutralizing response to a clade A virus (Papers III-IV).

### **5.3 FUTURE DIRECTIONS**

One of our planned endpoints in the CAT cohort was to investigate if presence of HIV neutralizing IgA at baseline was correlated with HIV acquisition during 2 years of follow-up. In the power calculations we assumed a HIV transmission rate much lower than the mean HIV transmission rate among discordant couples in previous studies and we expected to observe approximately 24 transmission events during the 2-year follow-up period among the HIV-negative women. Due to counselling, surveillance, subsequent antiretroviral treatment of HIV-infected partners, and possibly additional unknown factors, the number of seroconversions were limited to five and thus this endpoint had to be abandoned.

The nature of the epidemic and the scaling-up of ART in many areas have lead to a significant drop in HIV incidence in the general population as well as in the 'high-risk' cohorts, which renders a need of larger study cohorts and longer follow-up time when conducting HIV research. This should not be regarded as a complicating matter for research, but as a highly anticipated development for an epidemic that still has a devastating effect on entire communities in many areas worldwide, especially in Sub-Saharan Africa. Despite the promising trend for decreased HIV incidence and increasing availability of ART for HIV-infected people globally, the need for protective strategies for HIV-prevention remains.

## 6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Humant immunbristvirus (HIV) har sitt ursprung i Västafrika, där det överfördes från schimpans till människa i början av 1900-talet, troligtvis i samband med jakt. Så småningom spreds HIV vidare till USA och blev allmänt känt 1981, då amerikanska läkare rapporterade flera fall av ovanliga immunbristrelaterade sjukdomar hos unga homosexuella män. Man förstod snart att det rörde sig om en infektionssjukdom och två år senare kunde franska forskare med hjälp av patientprover isolera det virus, som orsakar förvärvat immunbristsyndrom (AIDS). Efter identifieringen av HIV utvecklades metoder för diagnostik och kontroll av blodprodukter som begränsade smittspridningen men HIV hade redan då spritt sig som en löpeld över världen. Inom ett par år efter upptäckten av HIV lanserades det första läkemedlet mot HIV men inte förrän mitten av 1990-talet kom effektiva virushämmande mediciner som påtagligt kunde bromsa sjukdomsförloppet. Nu har en HIV-infekterad person med framgångsrik medicinsk behandling i det närmaste normal livslängd och mycket låg smittsamhet. Idag lever uppskattningsvis 33 miljoner människor med HIV och 25 miljoner människor har redan dött av sjukdomen. De flesta HIV-infekterade bor i södra Afrika, där unga kvinnor är en särskilt drabbad grupp. En viktig faktor för att minska spridningen av HIV i världen anses därför vara att stärka kvinnors rättigheter.

HIV smittar via infekterat blod och barn kan få viruset av sina mödrar under graviditet, förlossning eller amning. Majoriteten av de HIV-infekterade i världen har dock smittats via heterosexuellt samlag och därför är det viktigt att förstå vilka faktorer som har betydelse för sexuell smittoöverföring och att studera det lokala immunförsvaret i underlivets slemhinnor.

Den statistiska risken för en kvinna att smittas med HIV vid vaginalt samlag med en HIV-infekterad man är cirka 1:500 och något lägre för en man i motsvarande situation. Orsaken till att risken inte är större beror sannolikt både på fysiska barriärer i slemhinnorna och ett naturligt förekommande immunförsvaret mot sjukdomsframkallande organismer. Slemhinnan i slidan har ett robust ytskikt med flera lager av celler, som tål mekanisk nötning. Dessutom fastnar många organismer i det sekret som täcker ytan i slidan. I slidsekretet finns även små proteinmolekyler som har visat sig kunna bekämpa olika bakterier och virus, inklusive HIV. Ett ytterligare skydd mot infektioner som finns på ytan av kroppens olika slemhinnor är antikroppar, särskilt en typ som kallas för IgA-antikroppar.

Det finns många exempel på människor som utsatts för sexuell HIV-smitta under flera års tid utan att bli smittade och som tycks ha ett naturligt förekommande skydd mot HIV. Detta skydd kan antingen vara medfött eller utvecklas över tid genom att utsättas för HIV upprepade gånger. Denna avhandling bygger på studier av sådana HIV-exponerade men icke infekterade individer i Nairobi, Kenya. Individerna i studierna utgjordes dels av prostituerade kvinnor och dels av så kallade HIV-diskordanta par (en partner är HIV-positiv och den andra HIV-negativ). Målet med studierna var att kartlägga förekomsten av lokalt skyddande faktorer mot sexuell HIV-smitta hos kvinnor,

vilket kan bidra med viktig kunskap för utvecklingen av HIV-vacciner eller mikrobicider (lokalt applicerade ämnen som skydd innan samlag).

Den första studien syftade till att kartlägga vilka proteinmolekyler med anti-HIV-effekt som finns i slidsekret från en grupp HIV-exponerade men icke infekterade prostituerade kvinnor. Sekret från underlivets slemhinnor samlades in från kvinnorna vid studiens början och vi mätte nivåer av olika molekyler med tidigare känd anti-HIV-effekt. Efter cirka två år kontrollerade man vilka av kvinnorna som hade blivit HIV-infekterade under studiens gång. Det visade sig oväntat nog, att de kvinnor som blivit HIV-smittade hade högre nivåer av två molekyler som heter HNP1-3 och LL-37, trots att dessa molekyler har en känd anti-HIV-effekt. Nivåerna av HNP1-3 och LL-37 var även högre hos de kvinnor som hade klamydia och gonorré. Man vet att sexuellt överförbara infektioner ökar risken att smittas av HIV och vi drog därför slutsatsen att även om de här molekylerna har en viss effekt mot HIV, så kan de också vara ett tecken på en infektion i slemhinnan, som i sig gör individen mer mottaglig för HIV. De kvinnor som förblev HIV-negativa hade högre nivåer av en annan molekyl, Trappin-2, som också har anti-HIV-effekt och som därför skulle kunna bidra till det naturliga skyddet mot HIV i underlivets slemhinnor.

I en annan studie baserad på HIV-diskordanta par, samlade vi in prov från slemhinna (slidsekret och sperma) samt blodprover från individerna. Vi undersökte förekomsten av molekyler med anti-HIV-effekt i slidsekret hos tre olika grupper av kvinnor: 1) HIV-positiva kvinnor 2) HIV-negativa kvinnor med en HIV-positiv partner 3) HIV-negativa kvinnor med en HIV negativ partner (kontrollgrupp). Det var ingen skillnad i nivåerna av de uppmätta molekylerna mellan grupperna. Däremot fanns det skillnader mellan kvinnorna inom grupp 2. De kvinnor vars partner hade höga HIV-virusnivåer, hade större mängd HNP1-3 och LL-37 i sitt slidsekret än övriga kvinnor i gruppen. En förklaring kan vara att den höga HIV-exponeringen stimulerade produktionen av dessa molekyler i slemhinnan.

Flera tidigare studier har visat att det finns HIV-exponerade men oinfekterade kvinnor som har IgA-antikroppar med funktionell effekt mot HIV. Detta kan vara en orsak till att de har minskad risk att bli HIV-smittade jämfört med andra. Vi undersökte därför om kvinnorna i de HIV-diskordanta paren hade sådana IgA-antikroppar i sitt slidsekret. Det visade sig att kvinnorna i grupp 2, (som var oinfekterade men hade en HIV-infekterad man) hade IgA med anti-HIV-effekt i fem gånger högre utsträckning än kvinnorna i kontrollgruppen. Därför drog vi slutsatsen att de hade producerat dessa antikroppar som svar på HIV-exponering genom oskyddat sex med sin HIV-infekterade partner.

Sammanfattningsvis kan en förklaring till att en betydande andel HIV-exponerade kvinnor förblir oinfekterade, bero på förekomst av skyddande IgA-antikroppar i slidsekretet. Däremot är effekten av olika proteinmolekyler såsom HNP1-3 och LL-37 mer osäker, eftersom de också är förknippade med infektion/inflammation i slidan. Antikroppsskyddet och den tudelade effekten av dessa proteinmolekyler måste utvärderas noggrant i framtida forskningsstudier när man ska utveckla ett HIV-vaccin eller annan typ av skydd mot sexuellt överförd HIV-infektion.

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