Measurement and characterization of HIV inhibitory Clade A Serpins in the cervical mucosa of highly HIV-1 exposed seronegative individuals

By

Syeda Sharmin Rahman

A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Medical Microbiology and Infectious Diseases

University of Manitoba

Winnipeg, Manitoba

Abstract

Objective: Serpins are serine protease inhibitors that are involved in a wide variety of biological functions in nature. They are known to regulate inflammation processes as well as provide host defense against microorganisms. Recent evidence has associated many types of mucosal serpins with a protective phenotype against HIV infection in women. Our hypothesis is that serpins with known antiviral activity against HIV-1 are correlated with protection in a group of HIV exposed seronegative individuals (HIV-resistant) from the Pumwani sex worker cohort. **Study design:** Cervico-vaginal lavage (CVL) fluid was collected from 66 HIV-positive, 82 HIV-negative and 84 HIV-resistant sex workers from the cohort. Clinical and epidemiological information was recorded at the time of sample collection. CVL protein levels were determined by BCA assay and serpin (A1 and A3) concentrations by a commercially available ELISA kit. Mucosal serpin concentrations were compared against clinical and epidemiological factors as well as sexual practices. **Results:** Serpin A1 was significantly higher in the HIV-resistant group compared to the HIV-negative controls (Anova: p=0.0470*). Total concentration of serpin A3 did not reach statistical significance between groups. Serpins did not correlate with age, sexual practices, contraceptive use or number of pregnancies. Serpins were differentially abundant during different stages of the menstrual cycle whereas serpin A1 was elevated during the proliferation phase but not in secretory phase (p=0.0275*).

Conclusion: Serpin A1 was correlated with HIV-protection in this group of HESN women. This work will contribute to a more complete understanding of mechanisms of resistance and susceptibility to HIV infection.

Acknowledgements

I take this opportunity to convey my sincere thanks to my supervisor, Dr. Blake Ball for providing me this incredible opportunity to be a part of his laboratory and to contribute to his research. He is very kind, supportive and understanding and he has always been there from day one. He has provided me necessary advice to analyze all the various troublesome aspects of my project. I could not have imagined having a better advisor and mentor for my MSc. study.

I would also like to thank my co-supervisor Dr. Frank Plummer for his guidance, encouragement, and for providing scientific knowledge that have taught me to think critically and has truly benefited me in this field.

I would also like to thank my Committee members, Drs. Xiaojian Yao, Michael Carpenter, and Emmanuel Ho, for providing me with excellent guidance, criticisms, insightful comments, and for encouraging me to go ahead with my thesis.

I am deeply indebted to my mentor, Dr. Adam Burgener whose help, suggestions and encouragement have helped me in all the time of research and writing of this thesis. With his enthusiasm, his inspiration, and his great efforts to explain things clearly and simply, he made this research effortless for me. Thank you so much for being there in my time of need.

My sincere thanks also go to Christine Mesa, Margot Plews, Leslie Slaney, Ian MacLean, Kenzie Mogk and Sue Ramdahin for their technical expertise and guidance and to Rasheda Rabbani, biostatistician from Manitoba Institute of Child Health (MICH), for helping me with scientific data analysis.

I would like to thank Drs. Adrienne Meyers, Ruey Chi-Su, Nyla Dil, and Sandy Kiazyk for their friendship and for inspiring me in research and life through our interactions during the long hours in the lab. I would also like to thank everyone from the Plummer/Ball/Fowke HIV group, in particular Aida, Nadine, Jill, Melissa, Derek, Meika, Winnie, Elnaz, Were, Caitlin, Lindsay, Sujata, Jen, Catherine and Courtney for their help, support and advice at one or more points through my degree.

I would also like to show my gratitude to Angela Nelson, from the Department of Medical Microbiology and Infectious Diseases, for providing administrative assistance.

Finally, I would like to thank all the women of the Pumwani CSW cohort and all the staff members. Without their participation and dedication, this research work would not be possible.

Last (but not the least) I would like to thank my family for their love, support, caring. This dissertation is simply impossible without them.

Dedication

I would like to dedicate this thesis to my husband, Rafiqul and my sons, Farhan and Fardin for their unconditional love, support, encouragement and great patience at all times. You have always inspired me and made the completion of this thesis effortless for me. Without your understanding and encouragement, I would not have finished the degree.

This thesis is also dedicated to my parents who have always been patient, and supportive to me through this incredibly long and painful journey. Thanks for your faith in me and for teaching me that even the largest task can be accomplished if it is done one step at a time.

Table of contents

List of Abbreviations

AIDS- Acquired Immune Deficiency Syndrome

CVL-Cervico-vaginal Lavage

CTL-Cytotoxic T lymphocyte

DNA-Deoxyribonucleic Acid

ELISA-Enzyme Linked Immunosorbent Assay

FGT-Female Genital Tract

HAART- Highly Active Anti Retroviral Therapy

HESN- Highly HIV-1 Exposed Sero Negative

HIV- Human Immunodeficiency Virus

HSV-Herpes Simplex Virus

IFN-Interferon

IL-Interleukin

KIR-Killer Immunoglobulin-like Receptor

LC-Langerhans cell

LTNP-Long Term Non-Progressor

LTR- Long Terminal Repeats

MIP-Macrophage Inflammatory Protein

MSM-Men have Sex with Men

NKc-Natural Killer cell

PBMC-Peripheral Blood Mononuclear Cell

RANTES-Regulated upon Activation Normal T cell Expressed and Secreted

RNA-Ribonucleic Acid

SDF-1-Stromal Differentiation Factor-1

SELDI-TOF-Surface Enhanced Laser Desorption Ionization- Time of Flight

SLPI-Secretory Leukocyte Protease Inhibitor

STI-Sexually Transmitted Infection

TER-Trans-Epithelial Resistance

TLR-Toll like Receptor

TNF-Tumor Necrosis Factor

UTI-Urinary Tract Infection

List of Figures

1. Introduction

The Human immunodeficiency virus (HIV) pandemic has infected over 33.3 million people resulting in 1.8 million AIDS related deaths by the end of 2009 (1). Despite improvements in treatment using highly active antiretroviral therapy (HAART), large scale use of intervention strategies like condom use, and male circumcision, every year more than two million people are infected with HIV. In the worst affected region, Sub-Saharan Africa, approximately 22.5 million people are living with HIV-1 and women account for 76% of all HIV-1 infected individuals (1). The exponential spread of HIV in Africa has been occurring due to poverty, sex with multiple partners, ignorance, war, stigma and lower position of women in society (2-3). Young women aged 15-24 years are eight times more likely to get HIV-1 infection than men in Sub-Saharan Africa(1). Women are considered to be the most vulnerable as they are not protected by the law following sexual abuse, cannot refuse sex or request condom use even if the husband is infected with HIV-1, because of their culture (2, 4). However, men are also at risk for HIV infection. The significant risk factors associated with HIV infection for men are older age, alcohol consumption and circumcision status, whereas for women, the factors associated with increased risk are younger age, prostitution, co-infection with classic sexually transmitted infections (STIs) such as HSV-2 and/or syphilis, being married, and a high number of sex partners (5). As women in this region are more vulnerable than men to HIV-1 infection, there is an urgent need to develop a successful vaccine or female controlled microbicide (a product that can be used in the vagina or rectum to prevent STIs including HIV-1).

It is known that heterosexual intercourse is one of the modes of transmission of HIV-1, but only 1% of genital exposure to HIV-1 results in an infection, implying that not all individuals who are exposed to HIV-1 become infected or that the female genital tract (FGT) is capable of stopping HIV-1 (6). Evidence suggests that the micro and macro environments of the FGT play an important role as doorkeeper and thus determines whether HIV introduced to the vaginal environment after sexual exposure will be able to establish a productive infection. Indeed, mucosal secretions like SLPI, defensin, elafin/trappin2, lysozyme and lactoferrin present within the FGT, either increase or decrease susceptibility to HIV-infection and it is likely that their antimicrobial activity depends on their physiological concentrations (7). In view of the fact that all individuals exposed to HIV do not become infected, it is necessary to comprehend the factors mediating HIV protection.

Clues to potential protective mechanisms in women may come from studying highly HIV-1 exposed seronegative (HESN) individuals who can be epidemiologically defined as HIV-resistant in the Pumwani sex worker cohort. This small subset of women has been identified as HIV-resistant as they remain uninfected despite having been exposed to HIV during sex and remains HIV-negative during follow up of this cohort for more than 7 years (8). Research conducted within this group has given us a unique opportunity to study the natural resistance to HIV infection. These women have novel innate and adaptive immune responses against HIV-1 in the cervicovaginal compartment that includes HIV specific cellular CD4 and CD8 immune responses (9), expression of anti-HIV factors like RANTES (10), CC-chemokines SLPI (11), Elafin/trappin-2 (12) and MIP-α/β (13). Previous studies by our group have shown these women have

differences in protein expression of other innate factors at the mucosal level. Antiproteases that play important roles in regulating inflammation and in defence mechanism against pathogens were found to be over expressed in HIV-resistant women. These include many proteins from serpin family, cystatins, elafin, and other protease inhibitor like A2ML1 (14). Therefore, exploring the mucosal immune correlates of natural protection against HIV epidemic is one of the important ways to develop preventive strategies, topical microbicides, and an HIV-1 vaccine.

2. HIV Virus

HIV is a Lentivirus belonging to the family Retroviridae. It is the virus that is responsible for Acquired Immune Deficiency Syndrome (AIDS) by causing the functional impairment of the immune system mainly in CD4+ T cells. Being a retrovirus, it has two copies of a single-stranded RNA genome and upon entry into the target cell, the viral RNA genome is converted into double-stranded DNA by the enzyme reverse transcriptase. The resulting viral DNA is then integrated into the host's genome by a virally encoded integrase and host co-factors. As HIV encodes only 15 proteins, it mainly depends on host cell proteins for successful infection (15-16). The diseases caused by lentivirus genus viruses are characterized by having a long incubation period. Indeed the HIV-1infected patient may remain asymptomatic for 2 to 10 years by maintaining stable viral loads with the CD4 counts generally more than 500/μl (17).

2.1. HIV classification

There are two types of HIV virus: HIV-1 and HIV-2. HIV-1 is the most virulent and infectious and it is responsible for the majority of worldwide HIV infections. So, HIV-1 will be the focus of this thesis. HIV-2 is less infective compared to HIV -1 and is mainly confined to West Africa (18).

Phylogenic analysis of HIV shows there are three groups of HIV-1 namely M (major), N (new) and O (outer). M-group consists of nine clades: A, B, C, D, F, G, H, J and K and varied number of 48 circulating recombinant form (CRF) derived from these pure subtypes. M is primarily responsible for the global HIV epidemic and the diverse distribution of clades has been seen around the world. Clades A and D predominate in Kenya while in Canada, the majority of the HIV-1 is clade B (19-20). However, approximately 56% of all cases of worldwide HIV/AIDS are due to HIV clade C (HIV-C) and in sub-Saharan Africa, India and China, the majority of them are found as B/C recombinant virus (21). Clade B infection is seen mainly among white men who have sex with men and injection drug users. Due to many structural and functional differences, the HIV-1 subtype may influence HIV transmission, cellular tropism, virus replication, anti-retroviral (ARV) susceptibility, organ involvement, and disease progression (22).

2.2. HIV genome

The HIV-1 genome encodes some important structural and non-structural (accessory) proteins which are unique for HIV-1. The proteins are separated into three groups: Structural proteins (*Env, Gag, and Pol*), the regulatory proteins (*Tat* and *Rev*) and the accessory proteins, (*Vpu, Vpr, Vif,* and *Nef*) (23).

Env (Envelope) protein is synthesized as a polyprotein precursor "gp160" which is cleaved by the host protein furin to form Env glycoprotein gp120 and the glycoprotein gp41. The *gag* (group-specific antigen) *and pol* (polymerase) are encoded by the gag-pol gene and are synthesized as a gag-pol polyprotein precursor which is cleaved by the viral protease (PR). *Gag* generates the mature Gag proteins matrix (also known as MA or p17), capsid (CA or p24), nucleocapsid (NC or p7), and p6 which comprise the viral core. The *pol*-encoded enzymes PR, reverse transcriptase (RT), and integrase (IN), are important for host cell infection (24).

Tat (transcriptional transactivator) gene encodes two proteins that bind with a RNA element, known as the transactivation response region and enhances the transcriptional activity from the HIV LTR. The *Rev* (regulator of virion) gene encodes a protein that helps to export un-spliced mRNA from the nucleus to the cell cytoplasm by binding with the rev-response element (RRE) (24).

Vif (virion infectivity factor) protein interferes with host antiviral protein apolipoprotein B mRNA editing enzyme catalytic polypeptide like 3G (APOBEC3G) and enhances HIV-1 infectivity (25). Proteins encoded by *Vpr* (viral protein r) gene accelerate HIV-1 protein production and by mimicking the importin-β, cause nuclear import of the viral pre-integration complex (PIC). Expressing *Vpr* has also been shown to stop cells in the G2 phase of the cell cycle, to impair the dendritic cell (DC) maturation, deregulate co-stimulatory molecules and cytokine production in T cells (26). Some evidences also suggest that Vpr in infected DC cells down regulates CD8+ T cell proliferation and induces apoptosis by producing tumor necrosis factor alpha (TNF-α) (26). The *Vpu* (viral protein u) encoded protein causes virus particles assembly and helps budding out of the

cell (27). *Nef* (negative replication factor) gene encodes a protein that is found to down regulate CD4 and Major histocompatibility class I (MHC class I) molecules from the cell surface. This action may enhance cell survival as the ability of cytotoxic T lymphocytes (CTLs) to detect and eliminate virus-expressing cells is impaired (28). The functions of these proteins will be discussed more in detail in the HIV life cycle.

2.3. HIV life cycle

After gaining entry into the host cell through interaction of the viral envelope glycoprotein gp120 with the CD4 molecule, dendritic cells (DC) that express the CD4 molecule on their surface, and langerhans cells (LC) are the main targets for HIV-1 infection (29-30). Binding of gp120 and CD4 molecules also causes gp120 to undergo a structural change and expose a co-receptor binding site, the "V3 loop region" that allows them to bind with a 7-transmembrane-domain chemokine receptor such as CCR5 or CXCR4. It has been shown that the HIV-1 co-receptor usage is determined by the viral envelope, primarily the V1, V2 and V3 regions of the external HIV-1 envelope glycoprotein, gp120 (31-32).

There are several chemokine receptors utilized by HIV-1 in in vitro studies but CCR5 and CXCR4 are the primary HIV-1 co-receptors utilized in addition to the CD4 receptor (33). Based on the ability to bind with these co-receptors, there are three HIV-1 variants: CXCR4-tropic (X4), CCR5-tropic (R5), or dual-mixed tropic (X4-R5) (34). CXCR4 tropic HIV-1 is considered T-tropic virus because it primarily infects naïve and memory CD4+ T cells and it is more cytopathic than R5 virus as it is associated with accelerated disease progression and decreased CD4 T-cell count. CCR5 tropic HIV is

called M-tropic virus because it mainly infects monocyte/macrophages and memory CD4+ T cells and it is more infectious than CXCR4 tropic virus. The dual tropic virus infects both macrophages and T-lymphocytes (35-38). R5 tropic virus is predominant in initial and chronic phase of HIV-1 infection and after establishment of infection and subsequent disease progression; R5 tropic virus switches to CXCR4 tropism and causes rapid CD4 T cell depletion and a rapid progression to AIDS. However, R5- tropic HIV viral mutations can increase their pathogenicity in the course of HIV disease without switching to a CXCR4 tropic variant (39-40).

The role of CCR5 in initiation and persistence of HIV infection is evidenced by subjects with a CCR5Δ32 allele polymorphism that are resistant to HIV-1 infection (41). A numbers of studies have suggested a higher prevalence of R5 HIV-1 variants in the acute phase of infection of genital mucosa because of higher expression of R5 on macrophage, DC cell and langerhans cell (LC) than the CXCR4 variant. Additionally it was found that CXCR4-using virus are trapped and inactivated by mucin and innate antiviral proteins in the genital mucosa (42-43).

The binding of CD4 bound gp120 with the co-receptor also triggers a conformational change that exposes the N-terminus of the transmembrane glycoprotein of Env, gp41, and facilitates the fusion of viral and hosts cell membrane (39). Once HIV binds to the host cell, the core which includes HIV RNA, viral capsid, reverse transcriptase (RT), integrase (INT), ribonuclease, and protease (PR), and a number of other viral accessory proteins is injected into the cell cytoplasm. Following this, the reverse transcriptase causes uncoating and reverse transcription of the single-stranded RNA genome into a double stranded (ds) viral DNA. The double stranded viral DNA is

then transported to the host cell nucleus via microtubules through a nuclear pore and the integration of the ds viral DNA into transcriptionally active sites of the host chromosome is carried out by another viral enzyme called integrase (44). At this stage the integrated viral DNA is referred to as a provirus and may remain dormant in the latent stage of HIV infection. Certain cellular transcription factors mainly NF-κB which is up regulated during T-cell activation are required to produce active virus (45).

During viral replication, initiation of transcription of provirus to mRNA occurs at the HIV-1 Long terminal repeats (LTR) site and is modulated by a small HIV-1 encoded RNA-binding protein Tat. It is well documented that RNA synthesis is increased two log fold when Tat binds with the RNA element, known as the transactivation response region (TAR) (46). Transcription of the provirus leads to the generation of three major classes of mRNA: 1) unspliced mRNAs, which function as Gag and GagPol polyprotein precursors, 2) partially spliced mRNAs, which encode the Env, Vif, Vpu, and Vpr proteins, and 3) multiple spliced mRNAs, which are translated into Rev, Tat, and Nef. mRNA transport out of the nucleus to the cytoplasm is mediated through a novel viral protein, Rev and Rev responsive element (RRE) (47).

Once in the cytoplasm the assembly process begins and Gag precursor polyprotein, Pr55Gag acts as the major player in virus assembly. This protein binds to the membrane, promotes Gag-Gag interactions, encapsulates the viral RNA genome, and stimulates budding out of the host cell (16, 24). After virus release from the plasma membrane, the viral Gag and GagPol polyprotein precursors are cleaved by viral PR protein to generate the mature Gag and Pol proteins and initiates a series of structural

rearrangements that ultimately leads to virion maturation (24). This mature virus is then able to infect another cell.

When the mature virus comes in contact with the mucosal surface it invades the epithelium by transcytosis or binding with DC cells or through breached epithelium (48). By binding with DC cells on epithelial surface called langerhans cell (LC) and submucosal DC, it is transported to the lymph node where it gain access to CD4 T cells and infects them (49-50). Once HIV reaches the lymph node and begins replicating, it spreads throughout the body and organ system via the lymphatic and circulatory system.

3. HIV transmission

Globally, sexual intercourse is the predominate route of HIV transmission but it can be transmitted by intravenous drug use (IVD), through infected blood transfusion and from mother to child during child birth or breast feeding (51).

HIV-1 transmission through the sexual route occurs in three ways: penile-vaginal intercourse, penile-anal intercourse and oral-genital sex. HIV-1 transmission through penile-vaginal or heterosexual coitus depends on the viral load in the female and male genital tracts, the presence of other sexually transmitted diseases as well as the number and types of target cells utilized by HIV-1 (52). The vagina and ecto-cervix, both with a multi-layered, stratified, squamous epithelium have a marked physical barrier and can resist viral mucosal transmission. However, a recent study by Nazli A. et al showed that TNF-α which is released after interaction of HIV-1 Env protein with genital epithelial cells, can reduce the epithelial integrity measured by trans-epithelial resistance (TER) mechanism, and causes breaching of the epithelial barrier thus increasing HIV-1

transmission (53). The male genital tract is also found to be infected during primary HIV-1 infection and viral load in semen correlates with plasma viral load (54). The penile fore skin (inner and outer) is shown to have HIV-1 target cells like LC and T-cells that expresses CD4, and CCR5. Seminal fluid contains prostatic acidic phosphatase (PAP) which forms amyloid fibril can capture HIV particles and increase HIV-1 transmission (52, 55). However, some randomized clinical trail in Africa concluded that there is 60% reduction in HIV-1 transmission from infected women to men due to circumcision (55).

Penile-rectal HIV-1 transmission can be seen everywhere in the world and mainly among men who have sex with men (MSM) (56). Unprotected anal intercourse is found to be 10–20 times riskier than unprotected vaginal intercourse per act for risk of HIV acquisition (57). This may be due to the fact that the epithelial lining of rectal mucosa is a thin simple columnar epithelium which can be easily transcytosed by HIV-1(58). Also using saliva as a lubricant in an insertive and/or receptive anal sexual practice increases HIV-1 acquisition (59).

The risk of HIV-1 transmission via oral sex is relatively lower than for vaginal or anal sex. Oro-genital sex is mainly practised by HIV+ discordant heterosexual couples who believe it safer than anal and vaginal sex. The thick intact epithelial layers, presence of mucins which trap the virus, the low CD4+ target cells and anti-HIV peptides like Secretory Leukocyte Protease Inhibitor (SLPI) make this site unavailable to HIV-transmission (60-61). But oral or genital ulcer, gingivitis, periodontitis, and gum bleeding are some factors that may increase the risk of transmission (62). Also Chen et al suggested that alcohol can increase HIV transmission in the oral cavity by altering the compartmentalization of CCR4 in epithelial keratinocytes of the oral cavity (63).

Vertical transmission from mother to child occurs in gestation, during labor and delivery and during postpartum breast-feeding. According to Pisani E. et al, 90% of the world's HIV infected children got their virus through vertical transmission from a HIV-infected mother and live in Sub-Saharan Africa (64). In utero HIV transmission depends on maternal immune status, placental membrane and placental villous inflammation (65). The trophoblast cells that form the placenta are not susceptible to HIV but by transcytosis HIV can travel through these cells and TNF-α is an inducer of this process (66). The majority of vertical transmission occurs during vaginal child- birth and the risk of transmission depends on maternal plasma viral load, chorioamnionitis, prolonged rupture of membranes and increased bleeding due to episiotomy, perineal laceration, and intrapartum hemorrhage. Different meta-analysis showed that an elective caesarean section delivery can cut the risk of HIV-1 transmission in half (64, 67).

Without any anti-retro viral treatment, the rate of mother to child infection through breast milk is about 16% and it increases with prolonged breast-feeding. One of the recent studies in Nairobi found a significant association of maternal plasma virus load, lower $CD4^+$ cell counts and rate of HIV-1 DNA in genital secretions with higher breast milk HIV-1 RNA levels (68). HIV-1 can replicate in ductal and alveolar mammary cells and is found in both cell-associated and cell-free components of breast milk. It may transmit through breaches of mucosal surfaces, transport via M cells, or directly infect the enterocytes. It can be aggravated by breast pathologies such as clinical and subclinical mastitis, nipple bleeding, and abscesses common in both the general and HIV-infected population (64, 69).

Although transmission of HIV-1 is attributed to four different routes, sexual intercourse is considered the major player in HIV-1 transmission evidenced by many ecological observations and modeling studies that are published in the annual report of World Health Organization (WHO) and UNAIDS (70).

4. Innate immune system against HIV

Innate immunity is the first line of defence against invading pathogens and it provides rapid, non-specific immune response to limit microbial replication and dissemination. Innate immune system comprises 'cellular constituents' and 'soluble factors'. LCs, DCs, and natural killer cells (NKs) are the examples of some innate immune cells that function at early phase of HIV-1 infection and later induce adaptive immunity.

4.1. Cellular components of the innate immune system

Cellular components against HIV-1 can also be divided into three groups: 1) cellular types consist of LCs, DCs, NKs, macrophage and γδ T cells and also Pattern recognition receptors (PRRs) such as Nod-like receptor, Retinoic acid inducible Gene-1, and Toll- like receptors (TLRs), 2) extra cellular responses consists of complement and cytokines and 3) intra cellular mechanism such as APOBEC3G, tetherin/BST-2 and TRIM5α. Cellular innate immunity provides protection against HIV-1 infection at different sites by using different cells. DC and LC provide protection in vaginal and penile epithelium, macrophage, DC, and NK cells are found in the submucosal layer whereas γδ cells are present in vaginal and rectal mucosa (71). Although the main

function of these innate immune factors is to fight HIV-1 infection, sometimes they have been shown to advance the infection by facilitating transmission or replication of virus.

DC cells are the sentinels of the immune system and the first subset of cell that come in contact with invading pathogens. Being a potent antigen presenting cell (APC), they capture pathogens and present them to T cells to trigger innate and adaptive responses. There are two kinds of DC cells in humans: CD11c+ myeloid dendritic cells (cDCs) and plasmacytoid dendritic cells (pDC). Langerhans cells (LCs) and dermal dendritic cells are examples of cDCs and they are located in skin, genital/gut mucosa and in blood. pDCs are found in blood, mucosa and lymph nodes, and may first come across HIV during early local viral replication/spread (72). Activation of DCs by HIV is mainly thought to involve conserved Pattern recognition receptors (PRRs) such as Nod-like receptor, RIG-1 like receptor, and Toll- like receptors (TLRs). They recognize pathogen-associated molecular patterns (PAMPs) and trigger signalling pathways to activate host innate immunity and function to create a bridge between innate and adaptive immunity (72). TLRs were first discovered in the fruit fly Drosophila (73) and are the best characterized receptors that recognize PAMPS. Currently there are ten well- described TLRs that can be found in epithelial cells, monocytes, macrophage, mature and immature DC, NK cells, and human genital tract cells express all ten major TLRs (74). cDCs express TLRs, 2–9, so they can respond to a wide range of pathogens, whereas pDCs express TLR7 and TLR9 and they recognize ssRNA viruses via TLR7 or CpG ODN motifs via TLR9 (72).

Generally, DC cells are resistant to HIV-1 infection but in vivo studies showed that freshly isolated DCs can be infected by the macrophage tropic HIV-1 and transport

HIV to CD4 T cells by forming conjugates with T cells (75). However, when HIV-1 binds with C-type lectin receptor 'langerin' which is expressed by Langerhans cells, langerin can internalised the HIV virus to birbeck granules, where the virus is rapidly degraded. However, this function is abrogated at high viral concentrations (76). A functional inhibition of cDC and pDC is also found during acute HIV-1 infection with the highest viral load. The same study also revealed that the decline of antigen-presenting property of cDC is transient and reversible during primary HIV-1 infection whereas inhibition of IFN-α secretion in pDCs is observed during acute infection but continuously persists during the subsequent disease progression (77). In rectal mucosa DC-SIGN+ DC cells have been shown to bind with HIV and transfer those to peripheral blood CD4+ T cell (78).

γδ T cells are a small subset of T cells that are located in genital and rectal mucosa and they are another important innate immune component that act against HIV-1. They prevent HIV transmission by generating antiviral suppressor factors MIP-1α (CC-L-3), MIP-1β (CCL-4), and RANTES (CCL-5), which can suppress cell surface expression of CCR5 co-receptors (79). NKs are innate immune cells that interact with DC and T cells to differentiate the adaptive immune system. NK cell's anti HIV action is supported by an elevated level of NK cell number and function in HIV exposed seronegative individuals (80). In the early phase of acute HIV infection, NK cell numbers are found to be inversely correlated with virus-specific $CD8^+$ T cell responses indicating that NK cells are affective against HIV in early phases of infection whereas CD8+T cells respond at the progressive stage (81). NK cells can control HIV-1 infection through recognition of virally infected cells by both activating and inhibitory killer immunoglobulin-like

receptors (KIRs) (82). An epidemiological study showed that the (KIR) 3DS1 and (KIR) 3DL1 (the activating KIR allele) in conjunction with human leukocyte antigen (HLA)–B Bw4-80I was associated with slower disease progression (83). NK cells can also kill HIV-1 infected CD4+ T cells in which HLA-A and -B molecules are down regulated (84). This suggests that NK cells have an important role in mediating resistance to HIV but the mechanism of inhibition is still poorly understood.

The extra cellular components of innate immune cells consist of cytokines and chemokines that are secreted by cDCs and pDCs. The immuno regulatory cytokines are essential for lymphocyte activation, humoral and CTL response. IL-12, and IL-15 are secreted by cDC and IL-12 is necessary to induce T helper 1 (Th1) cell responses, which leads to cytotoxic T lymphocyte (CTL) activation and killing of viral infected cells. IL-15 is also necessary for maintenance of memory $CD8^+$ T-cell responses (85). In vitro studies showed pDC up regulates the expression of MHC class 1 and co-stimulatory molecules, and by producing high levels of type 1 IFNs (IFN-α and IFN-β) and other cytokines, it blocks HIV replication in CD4+ T cells and macrophages at multiple stages in the viral life-cycle. IFN- α also maintains the activated T cell population by preventing apoptosis (71, 86). A study of cytokine responses of PBMC from HIV infected individuals shown that, some of them act to enhance HIV replication by increasing the inflammation and tissue damage. The same study also revealed no significant difference of spontaneous and LPS-induced TNF-α, IL-1, and IL-6 secretion between HIV uninfected and AIDS patients (87).

TNF- α is produced by NK cells, macrophage and T cells and is found to enhance HIV-1 activation by inducing NFκB which results in transcriptional activation

and HIV replication (88). The IL-1 cytokine which is produced by activated macrophages and fibroblasts enhances HIV replication by inducing NFκB activity in T cells whereas IL-2 is thought to stimulate HIV replication via indirect induction of TNF- α and IL-6 production. IL-6 is an acute phase protein and evidence has shown that IL-6 induces HIV activation and replication in monocyte derived macrophages but not in T cells. It induces posttranscriptional HIV expression, apparently by enhancing viral protein translation (89). Stromal cell-derived factor-1 (SDF-1) is a chemokine that is a natural ligand for CXCR4 co-receptor which is used by HIV at later stage of disease progression (90). Although over-activation of cytokines and chemokines of the innate immune system leads to cell and tissue damage, they are still important for the primary defence against invading pathogens and they induces activation of signals that inform the adaptive immune system to respond.

The intra cellular part of the innate immune mechanism against HIV-1 is mediated by APOBEC3G (apolipoprotein B mRNA-editing, enzymecatalytic polypeptide like-3G). It is found in human T lymphocytes, monocytes, and macrophages and is regulated by IL-2 and IL-15, and IFNα and IFNγ (71). APOBEC3G has shown anti-HIV activity by inhibiting the viral replication. This cellular enzyme is packed in the newly formed virion and converts the viral cytidine to uridine in viral RNA making them non-functional (91). However, in the presence of Vif APOBEC3G can not integrate into virions and Vif causes ubiquitination and proteasomal degradation of this enzyme (92).

Recently tetherin/BST-2 and TRIM5α were identified as a retroviral restriction factors (93-94). Tetherin, also known as bone marrow stromal cell antigen-2 (BST-2) or CD317, is an interferon inducible transmembrane protein that causes retention of virions

on the cell surface or within the cells that produce them and limits the spread of enveloped virus. Studies using an artificial 'tetherin' showed that tetherin is incorporated into HIV-1 particles as a parallel homodimer using either of its two membrane anchors: putative glycophosphatidylinositol (GPI) modified C-terminus or the transmembrane domain and prevent the virion budding through the plasma membrane. The functions of tetherin are antagonized by HIV-1 encoded accessory protein, Vpu (95).

The host cellular restriction factor TRIM5α is a member of the tripartite motif (TRIM) family of proteins. It contains RING domains, B-boxes, coiled coil domains and a B30.2 (SPRY) domain. It is effective in inhibition of human immunodeficiency virus (HIV-1) in rhesus monkeys, whereas in human, TRIM5α only modestly blocks HIV-1 infection but efficiently restricts N-tropic murine leukemia virus (N-MLV). In rhesus monkeys, TRIM5α disrupts viral cDNA synthesis directly or indirectly and prevents HIV-1 infection before or during early reverse transcription by causing premature un-coating of the retroviral capsid (94). It also blocks HIV-1 production through rapid degradation of HIV-1 Gag polyproteins (96). Javanbakht et al showed that trimerization by the coiled-coil domain and B30.2 domain (SPRY) domain is necessary for TRIM5α to bind HIV-1 capsid complexes and control viral infection (97).

In brief, all these cellular components can recognize a wide array of pathogens including HIV by TLRs and the production of cytokines and chemokines provide a first line of defence and attracts other immune cells to the site of inflammation to initiate adaptive immune responses.

4.2. Soluble factors of the innate immune system

The low efficiency of heterosexual HIV transmission is not only due to the presence of cellular factors but also the fact that the intact vaginal epithelium acts as a physical barrier to HIV and the environment of the vaginal lumen (mucus, low pH, increased H_2O_2) does not provide the optimal milieu for viral replication. In addition, there are numerous secreted soluble proteins, previously described as anti-HIV factors that are found in cervicovaginal compartment of the FGT. Some examples are Secretory Leukocyte Protease Inhibitor (SLPI), lactoferrin, antimicrobial peptide defensins and trappin-2/elafin (11).

SLPI and trappin-2/elafin are protease inhibitors and members of the Whey acidic proteins. SLPI is present not only in vaginal mucosa but also in oral mucosa where it is isolated in large quantities from parotid secretions. It is thought that the presence of SLPI decreases the risk of oral HIV transmission (61). The anti-HIV function of SLPI is mediated by binding with annexin II and promotes HIV entry by stabilising virus fusion. SLPI impairs annexin II mediated viral fusion (11).

Trappin-2/elafin is a serine protease inhibitor produced by epithelial cells, macrophages and neutrophils. Its primary function is to protect host tissue from non-specific protease secreted by the host during inflammation. Trappin-2/elafin was found to be elevated in HIV exposed resistant women (12). Lactoferrin is an iron-binding glycoprotein secreted from serous cells in submucosal glands and is also found in milk, tears, saliva. It is released by neutrophils during infection. Previous studies showed that presence of lactoferrin in mothers milk decrease the vertical HIV transmission from HIV+ mother to their children (98). Lactoferrin can inhibit HIV-1 replication in T cells

by preventing adsorption and penetration of the virus. Groot et al showed that native lactoferrin can inhibit the binding of the DC-SIGN receptor of DC cells with gp120 and block DC mediated HIV transmission to T cells (99).

Defensins are small antimicrobial cationic peptides secreted primarily by neutrophil but also expressed by epithelial cells. There are three subtypes of defensin: α-defensins, β-defensins, and θ-defensins. α, β-defensins have diverse anti-HIV mechanism. α-defensins can inactivate the HIV virion directly or inhibit HIV-1 replication through alteration of protein kinase C (PKC) activity, whereas β-defensins down regulate the expression of co-receptor CXCR4 (100-101).

Recent studies also showed anti-proteases like lysozymes, cystatin and serpins are elevated in the cervico-vaginal compartment of HIV-1 resistant women (14, 102). All these antimicrobial peptides work together to create an environment in the FGT that is unsuitable for HIV-1 viral transmission and subsequent infection.

5. Adaptive immune system against HIV

The adaptive immune system provides the more specific immune response tailored against foreign antigen. It can distinguish non-self antigen from self- antigen. This immune system is MHC restricted and has a memory component that triggers a greater and faster immune response if the same pathogen infects again. During HIV-1 infection, dendritic cells (DCs) are the major cells that come in contact with the virus and link the innate immune system to the adaptive immune system (103). Pro-inflammatory cytokines like IL-1β, TNFα, and type 1 IFNs stimulate immature DC cells to produce cytokines and chemokines and later, mature DCs migrate to the lymph node, with

different antigens coupled to a MHC receptor to prime naïve T cells. As well, activated DCs also express co-stimulatory molecules such as CD80, CD86 and a number of molecules of the TNF super family which activate the adaptive immune system (103). Experimental evidence shows that a second signal from these costimulatory molecules, cytokines IL-12, IL-18, and IL-15 and also some chemokines are required to boost a proper activation of CD4+ Th cell and CD8+ CTL responses (104). The antigens from bacteria or parasites are usually presented on the MHC Class II molecules and activates CD4+ T cells while viral antigen is MHC class I restricted and usually activate CD8+ T cell (105). The adaptive immune system comprises two broad arms: humoral immunity and cell-mediated immunity.

5.1. The humoral immune response in HIV-1 infection

In the acute phase of HIV infection, antibody production by B cells against Env, Gag, Pol protein, and p24 protein can be used to monitor and determine the phases of HIV disease progression (106). The first B cell response against HIV can be noticed in plasma as virion antibody immune complex eight days after detection of viral RNA. IgM antibodies to gp41 are identified after 5 days (107). Most of the protective roles of antibody are demonstrated in a macaque SIV model and systemic or intravaginally administered HIV-1 specific monoclonal neutralizing IgG antibodies showed that there is a protective effect in the vagina of the monkey (108). IgA antibodies induced by mucosal immunization with HIV-1 gp41 envelope protein showed blocking of transcytosis of HIV-1 in mice (109). Furthermore, HIV specific IgA was found to neutralize the cross clade HIV antigen in the HIV-1 resistant women and also to block the transcytosis across

a human cervical epithelium model (110-111). However a recent study by Mestecky et al demonstrated that in plasma and cervicovaginal secretions of HIV-1 resistant sex workers there are no detectable IgG and IgA levels but in HIV-infected individual there is increased activation of IgG antibody without any detectable or low IgA. They concluded that HIV-1 resistant women do not have a detectable mucosal or systemic humoral immune response to HIV (112).

To prevent HIV infection, broadly neutralizing antibodies are needed. Several of these antibodies have been isolated from the B cells of HIV positive patients (113). These neutralizing antibodies (NAb) develop slowly, appearing 8-12 weeks after HIV infection and have reactivity against Env and other viral proteins (114). Recently two new NAb have been isolated from a patient infected by a clade A virus that bind the native form of gp160 at a relatively low titre and can neutralize 120 viral variants (115). A non-human-primates (NHP) model showed infection with chimeric SIV can be prevented by transferring passively broadly neutralizing anti-HIV-1 mAb before or at the time of challenge (116). Therefore, if a vaccine can be developed that is capable of eliciting a high titre of Nab to viral particles, then those antibodies will be effective in preventing HIV transmission.

Although neutralizing antibodies can protect cells from susceptible pathogens they often do not have any effect on already infected cells. Therefore, antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell-mediated viral inhibition (ADCVI) antibodies can work together with innate factors and destroy virus infected cells. The infected cell binds with specific antibody binding site of ADCC

or ADCIV antibodies and Fc receptor of these antibodies bind with receptors on NK cells, monocyte and neutrophil enabling them to kill the virus infected cells (117).

5.2. The cell mediated adaptive immune system in HIV-1 infection

Cell-mediated immunity is mediated by T helper cells (CD4+ T cells) and cytotoxic T cells (CD8+ T cells). The functions of T helper cells (Th) are to prime B cell responses and activate cytotoxic T lymphocytes (CTL). Activation of T helper cells starts when APCs present peptides through MHC class I and II and activate these cells. Following this, cytokines released by Th cells promote differentiation of Th cells into Th1, Th2, Th17 and T regulatory cells (Treg) types. Without proper co-stimulatory signals the Th cell may experience programmed cell death (apoptosis) or may go into an anergy or non-responsive state (118).

T helper cells differentiation into Th1 cells is mediated by INF-γ which is mainly produced by activated NK cells (119). The development of Th1 cells from activated Th cells is regulated by IL-12 and the presence of IL-4 can inhibit this process. Th1 cells produce interleukin-2 (IL-2), IFN- γ and TNF α and β and these are necessary for cell mediate immune response and development of CTL (118). HIV specific CTL responses are noticed in GIT and the reproductive tract (RT) after establishment of viral infection. In the rhesus macaque model, vaginal infection following SIVmac exposure showed that a detectable CTL response was low and observed in the reproductive tract after several days and in the GIT after 20 days post-infection. The investigators termed this CTL response as “too little and too late" (120). In contrast, Kaul et al., found an increased CTL response in HIV-resistant sex worker and suggested this response has a

protective role in the cervical mucosa (9). In the gastrointestinal tract, HIV infects and proliferates most rapidly in the memory CD4+ T cells as majority of CD4+ T-cells are memory sub-type. It has been demonstrated that after HAART therapy, memory CD4+ T cell counts become normal in PBMC but not in GI mucosa (121).

When IFN- γ production is limited then IL-4 induces Th2 production. Th2 cells produce IL-4, IL-5, IL-10, and IL-13 and steer the humoral arm of the immune response. These cells also promote production of IgE by B cells via the secretion of IL-4 and activation of eosinophils by IL-5 and mast cells via IL-3, IL-4, and IL-10 (118). During the initial stage of HIV infection, the CTL response is predominant but gradually this response declines and as the disease progresses Th2 cells take control by producing IgE. Increased IgE levels are found in HIV-infected children and it correlates with disease progression and its may be due to the shift from Th1 to Th2 cytokine production (122). The cause of Th1 to Th2 shift is induction of CXCR4 HIV co-receptor synthesis in T cells by IL-4 (123) or may be the stimulation of basophil by HIV gp120 to produce IL-4 (124), which is a key inducer of Th2 type immunity.

Th 17 cells are an effector T cell subset and characterized by IL-17 production. By releasing inflammatory cytokines and chemokines and also by recruiting neutrophils, Th 17 cells can promote inflammation. Although Th17 cells provide protection against a variety of bacterial and fungal pathogens in mucosa and skin, an in vitro study showed Th17 cells can be infected with SIV and HIV as these cells express the HIV co-receptor CCR5 and produce very low levels of CCR5 ligands MIP-1a and MIP-1b (125). On the other hand Th 17 has shown a protective role against HIV by inducing innate immune response via increase expression of antimicrobial peptides (126). The role of these cells

either in promoting or in suppressing HIV replication in target CD4+T cells is still not fully characterized.

There are two types of Treg cells, natural Treg cells which are produced in the thymus and induced T reg cells that are produced peripherally by CD4+ T cells after antigenic stimulation. The function of Treg cells is to limit inappropriate or excessive responses of T cells to pathogens (127). These cells are characterized by the expression of the forkhead family transcription factor (FOXP3) and interleukin-2 (IL-2) receptor α chain (CD25). In chronic HIV infection T regs are thought to suppress HIV-induced immune hyper activation, which results in a reduced availability of target cells for HIV replication. However, T regs are found to susceptible to HIV infection as these cells also express CXCR4 and CCR5 (128).

The role of cytotoxic T lymphocytes or CD8+ T cells in HIV infection is well established. It is already proven that in HIV positive individuals, CD8+ T cells can inhibit HIV replication in CD4+ T cells. In addition, removal of CD8+ T cells from PBMCs of HIV-negative individuals results in increased HIV infection (129-130). The control of viral replication by CD8+ T cells is mediated by an antigen specific cytolytic pathway or by soluble antiviral factors. The cytolytic mediated killing by CD8+ T cells occur via the recognition of MHC class I bound viral antigen on the cell surface and the direct killing of the infected host cells by antigen-specific CTL (131). On the other hand, non-cytolytic killing by CD8+ T cells soluble factor is mediated by induction of STAT1 activation and IRF-1 gene expression that may lead to inhibition of the HIV-1 LTR mediated RNA transcription (132).

Although CD8+ T cells are critically important for the control of HIV infection, some studies showed that HIV can infect these cells and that CD8+ T cells harbour large number of provirus (131). HIV can infect the CD8+ T cells via both CD4 dependent and independent pathways and the thymus is a source of CD4 dependent CD8+ T cell infection. As such, infection of CD4+ T cells by HIV virus and loss of CD4+ T cell helper function may lead to impaired clonal expansion and function of CD8+ T lymphocytes. Furthermore, destruction of the thymus by chronic HIV infection has been suggested as a cause of decreasing number of circulatory naïve and memory CD8+ T cells observed in disease progression (131).

6. HIV-1 transmission in the female genital tract (FGT)

Globally HIV-transmission mainly occurs via heterosexual intercourse from male to female. While, the infection rate of only 1 per 1000 sexual acts in the study of monogamous discordant couples from the Rakai cohort suggests that the 99% protection is likely due to natural and innate factors in the FGT (6). The normal flora such as Lactobacillus maintains a low vaginal pH and secretes molecule like hydrogen peroxide (H_2O_2) that inhibits HIV infection (133). Also, the lining epitheliums of the FGT and mucus rich luminal secretions that trap bacteria and virus and promote ciliary cleaning make the FGT relatively protected against HIV transmission.

Even though there are several protective features present in the FGT, there is an immense amount of HIV-transmission across the epithelial barrier, infection of lymphatic cells, mucosal and systemic dissemination (7). The polarized single layer columnar epithelium of endocervix influences HIV-1 entry by transcytosis, whereas the leakiness

of multilayered non-keratinized squamous epithelium of the ectocervix and vagina due to micro trauma occurring during intercourse or due to inflammation enhances HIV-1 transmission by making the CD4+ T calls and DC cells available for the virus (134). In addition, the squamo-columner junction of the ecto and endocervix known as the "Transformation zone" or T- zone is found to be filled with an abundance of lymphocytes and antigen presenting cells (APCs) that make this site susceptible to HIV-1 transmission (135).

In vitro studies demonstrated that HIV can invade the intact epithelium through the binding of gp120 to genital epithelial cells which leads to TNF-α production and then this can cause breaching of intact epithelial barrier thus enhances HIV-1 transmission (53). Moreover, the presence of sexually transmitted diseases (ulcerative STIs like Herpes simplex virus type-2 (HSV-2) or non-ulcerative STI like Chlamydia) in the FGT can affect commensal micro flora and decrease the vaginal pH making it a hospitable milieu for HIV infection or increasing the susceptibility of CD4+T cells and DCs to HIV-1 infection (136-137). In fact HSV-2 is found to be a synergistic co-pathogen for HIV and enhances HIV-1 transmission by causing ulcerative lesions or recruitment of HIV-1 target cells at the mucosal site (138-139).

HIV transmission in the FGT also depends on HIV infectivity and viral load of male genital fluid (140). HIV infected cells from a sexual partner can be deposited into the vaginal lumen and semen itself makes the environment susceptible to HIV-1 by neutralizing the acidic pH and inactivating H_2O_2 (141). Münch *et al.* showed that human semen contains amyloid fibrils formed from prostatic acid phosphatase (PAP), termed "semen-derived enhancer of virus infection" (SEVI), which can enhance HIV infection

even in the acidic pH of the vagina. SEVI can enhance virion–cell attachment and fusion by decreasing the electrostatic repulsion between the negatively charged surface of the virion and the target cell and also by enhancing receptor-mediated viral entry (52, 142).

HIV can establish productive infection after coming in contact with vaginal mucosa. Most of the evidence comes from mechanistic studies in Rhesus macaques and Simian Immunodeficiency virus (SIV) as they reasonably mimic the anatomy, target-cell populations and immunology of a human host (143). Following genital mucosal exposure to HIV, there is no detectable viral mRNA in the circulation for ten days as the virus uses this period to produce new mature virions which further infect susceptible target cells at the portal of entry (144). When the population of infected cells is increased, free and cell bound HIV crosses the epithelial barriers by a number of pathways including direct passage through epithelial breaches, transcytosis, and binding and/or uptake by mucosal dendritic cells (48). Within the epithelium HIV first comes in contact with the intra-epithelium dendritic cells called Langerhans cell (LC) which express CD1a and the C-type lectin receptor langerin (CD207), CD4 and CCR5, but not CXCR4 or DC-SIGN.

The sub-epithelial DC expresses DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN), CD4 and CCR5 (145). It has been shown that HIV-1 binds with subepithelial DC and monocyte derived DC by DC-SIGN and other C-type lectins but binding with LC occurs in a CD4, CCR5- dependent manner. Following HIV antigen exposure, LCs migrate to the nearest lymph node via afferent lymphatics to prime the immune response against the antigen which may also facilitate the transportation of protected intracellular HIV to subepithelial DC and CD4 lymphocytes. Binding with DC-SIGN+ DC in the sub mucosa also promotes capture and uptake of virus. By using this

DC cell as a shuttle to the lymph node, HIV-1 subsequently transfers to CD4+ T cells and macrophages where it replicates (49-50).

To increase the founder population of infected cells, in addition to DC and LC cells, CD4+ T cells are also infected by HIV-1 at the entry to the vaginal mucosa. Resting and activated memory CD4+ T cells are immune cells that can be seen at the front line of the mucosal surface to guard it from invading pathogens. Activated CD4+ T cells are potent host cells in which HIV-1 can replicate. Recent studies with SIV showed that resting CD4+ T cells have some CCR5 co-receptor expression and can be infected with HIV. Although virion production in resting CD4+ T cells is low, they are in higher abundance than DCs and activated CD4+ T cells. Therefore, any inflammatory process or presence of STI can activate these resting cells and increase the additional target cells for HIV (143).

While many methods can increase the susceptibility of HIV-1 infection in the female genital tract, the need for the development of a vaccine or some topical microbicides with anti-HIV activity that could be applied directly to the vaginal surface are crucial.

7. HIV vaccine status

Since HIV-1 was discovered, the development of vaccine to halt the spread of disease has been a major goal. An ideal vaccine against HIV-1 should be safe, affordable, long lasting and should induce a general immune response by producing antibodies or cytotoxic T cells (CTL) to fight various strain of virus at the site of infection. A vaccine would be successful when it will have a prophylactic effect to

prevent infection and a therapeutic effect to slow down the disease progression and maintain the viral load and CD4 count in a post-infected cases (106). There are three kinds of vaccines that have been studied to prevent HIV infection: 1) A subunit/protein vaccine contains parts of the virus produced in the laboratory by genetic engineering technique and can elicit an anti-HIV immune response 2) A recombinant vector vaccine in which attenuated viruses like ALVAC (a canarypox virus), MVA (a type of cowpox virus), VEE (a virus that normally infects horses), and adenovirus-5 are used as vectors or carriers to deliver copies of HIV genes into cells, which produce HIV protein which in turn generate a strong anti-HIV immune response and 3) A DNA plasmid vaccine that delivers genes encoding HIV epitopes. In some clinical trials, two or more types of vaccines are used in combination and referred to as a 'Prime-boost strategy' to get a synergistic effect (113). Currently, 163 HIV vaccines are in different phases of clinical trials: 3 in phase III, 31 in phase II and the rest are in phase I (106).

The RV144 vaccine study that had reached phase III trial was performed in Thailand and evaluated the prime-boost strategy of the gp120 subunit vaccine and ALVAC (a canarypox virus) vector vaccine. The result revealed a 31.2% reduction in HIV-1 infection in the modified intention-to-treat analysis. However, the vaccine did not show any efficacy against viral load or CD4+ T cell counts in individuals with HIV-1 infection (106). Another vaccine candidate was a recombinant adenovirus serotypes 5 (rAd5) vectors encoding the HIV proteins Gag, Pol and Nef. Also known as the Step and Phambili Studies, these were not found to be effective against HIV-1 acquisition or reducing the viral load after HIV infection in uncircumcised Ad5 sero-positive MSM (146).

A DNA/Ad5 vaccine trial known as HVTN505 is now in phase III trial and currently is in progress in the USA. Multiple DNA priming and an Ad5 vector with Gag, Pol, Env and Nef boost regimens showed an effective CD8+ T cell mediated anti-viral activity against different HIV strain in phase I and II. Indeed, the DNA/Ad5 prime-boost regimen can perk up HIV-Env specific T cell response seven times more than the Ad5 recombinant vaccine alone. This study used men having sex with men (MSM) who were circumcised and did not have any pre-existing antibodies against Ad5 (146).

8. HIV microbicide status

Microbicides are self-administered reagents, topically applied to the mucosal surface of the vagina or rectum as a pre-prophylaxis to prevent HIV infection. The high risk of HIV infection in women due to biological and socio-economical causes indicates the urgency to develop these female control microbicides. Vulnerable women, particularly sex workers who get more money by agreeing not to use condoms or married women who cannot ask their husband to use condoms even he is HIV infected, will be protected from infection by using these microbicides.

A microbicide can come in different forms: gel form, oral form or as a vaginal ring. An ideal microbicide should be active against HIV and other sexually transmitted diseases but not disturb the normal vaginal flora; additionally it needs to be simple to use and the cost should be low, as it would be applied before each sexual act. Currently about fifty candidates are being used to develop active microbicides (147). There are different classes of microbicides that are divided according to their mechanism of action.

Non- HIV-specific microbicides: these are also called surfactants and they mainly work by disrupting the microbial lipid membrane. Nonoxynol-9 (N-9) was the first microbicide used for a HIV trail and it was an effective and inexpensive spermicide. However, the trial was stopped when it became evident that N-9 was toxic and caused abrasion of the vaginal mucosa which increased susceptibility to HIV infection (148). Trials of C31G or Savvy and Sodium lauryl sulfate (SLS), two other non-HIV specific microbicides were also halted because of the higher risk of contracting HIV-1 (149).

Vaginal environment protectors: Maintaining the normal vaginal environment can be an effective means to prevent HIV transmission. Lactobacilli, which are normal flora of the vagina, create an inhospitable environment for HIV by secreting H_2O_2 and reducing vaginal pH. Therefore, in theory, microbicides from this category would maintain and restore the vaginal pH in the presence of semen, which is alkaline. In clinical trails it did not show efficacy to women during vaginal intercourse (150). Acidform is an acid-buffering system that is a well tolerated gel but caused irritation in some studies and showed little effectiveness (150).

Poly anionic entry inhibitors: This negatively charged microbicide binds with the positively charged viral envelop and disrupts the attachment of gp120 protein to CD4+ T cells (151). PRO2000 is a sulfonated polyanionic compound that also interferes with the viral transmission in the vaginal epithelium. The trial was halted as there were higher rates of HIV infections in treatment group than in the placebo group (152). 3% SPL7013 Gel (VivaGel), is a Dendrimer compound that also has a highly charged surface

and binds with gp120 on HIV envelop surface and prevents binding of HIV with host cells. In in vitro studies and in an animal model, this microbicide inhibits not only HIV but also HSV-2 and Human Papilloma virus (HPV). Currently it is in a phase 1 randomized trial (153).

Entry inhibitory microbicides: Blocking of the CCR5 or CXCR4 co-receptors can decrease HIV transmission. 5P12-RANTES is one of the promising microbicides that can bind with CCR5 co-receptor without activating pro-inflammatory signal transduction pathways. In Rhesus macaques it showed 100% protection after SHIV challenge (154). Currently only two HIV-1 entry inhibitors are approved for clinical use. Maraviroc (Celsentri), blocks infection by R5-tropic HIV-1 strains by binding to the HIV-1 co-receptor CCR5. The second inhibitor T-20 enfuvirtide (Fuzeon), is derived from the HIV-1 glycoprotein transmembrane (gp41). It prevents the fusion of viral and target cell membranes by inhibiting the formation of the six-helix bundle between gp41 N-terminal and C-terminal heptad repeat regions (155).

Reverse transcriptase inhibitors: The Centre for the AIDS Program of Research in South Africa (CAPRISA) 004 trial, a double-blind, randomized controlled trial, assessed effectiveness and safety of Tenofovir, a 1% nucleotide reverse transcriptase inhibitor (NRTI) gel based compound, for the prevention of HIV acquisition in women. It is the first vaginal microbicides proven to be safe and protect women by blocking HIV-1 transmission. This study revealed that Tenofovir gel can cause a 39% reduction in HIV-1 infection and that the rate increases to 54% in high adherers. Gel adherence was estimated by dividing in half the number of returned used applicators each month by the

number of reported sex acts that month. This gel also gives about 50% protection against HSV-2 which is beneficial for women as HSV-2 increase the risk of HIV transmission (156).

Protease inhibitors (PI): PIs work by preventing the post-integration step of HIV replication cycle which means these microbicides can be used if surface-active components fail. Lopinavir, ritonavir and darunavir are example of some PIs that show promising efficacy (157).

As the female control barrier method is not as effective as expected (158), using prophylactic microbicides along with ART may increase the synergistic effect against primary HIV-1 infection and prevent systemic dissemination. There are some promising candidates for HIV microbicides but none of them have shown complete success at preventing HIV infection. This is primarily due to our lack of understanding of mucosal immunity and what protects against HIV infection.

9. HIV resistance and correlates of protection

There are individuals around the world that despite being exposed to HIV do not become infected, or if they do become infected do not progress to AIDS. One of the best characterized HESN group are a subset of women from the Pumani sex worker cohort where approximately 10% of commercial sex workers are found to be resistant to HIV infection (8). This phenomenon is also documented in MSM (159), injection drug users (160), and discordant couples (one partner is HIV infected) (161). Within HIV-1-infected individuals some are able to maintain high CD4 counts (above 400) for over 10 years and

are termed long-term non-progressors (LTNP) (162). The rate of disease progression is very slow in this group and shows a natural resistance to HIV (162).

Some infected people are able to maintain an undetectable viral load in plasma (even though RNA is detected) and are known as elite controllers (163). These elite controllers sometimes share immunological features with the HIV resistant group. The only validated mechanism of HIV-1 resistance is found in those who are homozygous for a deletion of 32 base pair in their CCR5 gene. This group has a non-functional CCR5 receptor and is resistant to R5 tropic HIV infection but remains susceptible to X4 tropic HIV. Heterozygous carriers of this mutant allele also have slower diseases progression after HIV infection (164). By unrevealing the mechanisms of natural resistance in these groups we may gain a better understanding of how to design a successful vaccine and/or preventative treatment.

The HIV-resistant individuals from the Pumwani sex worker cohort have provided a plethora of information of natural correlates of protection against HIV. HIV resistance has been associated with different HLA alleles where the super type A2/6802 and HLA DRB1*01 is found to be associated with a reduced risk of infection (165). Genetic correlates of protection have included a polymorphism in the Interferon regulatory factor-1 (IRF-1) gene. Lower levels of IRF-1 protein expression are thought to limit HIV infection during early infection events (166). Resistant women also express high level of HIV-1-gag specific T-helper and CTL responses in blood (167). Systemic levels of MIP-1α, MIP- 1β and RANTES, which are ligands for the CCR5 co-receptor and inhibitors for HIV, have not been associated with HIV-1 resistance (13). McLaren et al demonstrated a dormant immune phenotype in the peripheral blood mononuclear cells

(PBMCs) of these individuals which correlated with lower levels of IL-1β, IL-6, and TNF. Lower levels of these cytokines are associated with decrease viral replication and have also been shown to reduce the number of susceptible cells in vivo. There is also lower gene expression of the T cell receptor (TCR) which leads to less T cell activation and decrease HIV infection (168). Lower expression of CD69 and higher levels of CD4+CD25+FOXP3+ regulatory T cells are also observed in resistant women suggesting lower immune activation in peripheral blood against HIV infection (169).

The HIV resistant group also demonstrated a fascinating immune regulation by maintaining an active innate and adaptive response at the genital mucosa which is the primary site of infection in heterosexual intercourse. Different soluble factors/ antimicrobial peptides and immune cells in the FGT work together to fight the early HIV-infection and to keep a lower immune activation at the systemic level. By doing so, the FGT makes fewer cells available for further infection. In the HESN population, the genital mucosa showed higher level of HIV-specific neutralizing IgA and a higher frequency CTL response than did blood (9, 110). Furthermore, increased HIV-specific CD8+ T cells have been observed in both systemic and genital mucosa which correlates with HIV-resistance, but elevated CD4+ T cells is only observed in the genital tract of HIV resistant individuals. This study also showed elevated RANTES expression in the genital mucosa of HIV resistant women (10).

The elevated levels of antimicrobial peptides in the female genital tract have been found to be correlated with HIV-1 resistance. Antimicrobials and antiproteases like SLPI, elafin/trapin2, serpins, and cystatins are found to be increased in the cervicovaginal compartment of HIV-resistant women (7, 12, 14). Furthermore, proteomic studies of

cervico-vaginal secretions of sex workers from the Pumwani sex worker cohort revealed that HIV-resistant women over expressed anti-proteases (14). In this pilot study, using a gel-based approach, 2 dimensional- difference gel electrophoresis (2D-DIGE), many differentially expressed proteins were identified in HIV-resistant women in comparison to other groups. These included serpin B1, serpin B3, serpin B4, alpha2 macroglobulin-like 1protein, cystatin A and many more (14). Additionally in-depth analysis on a larger number of individuals using a mass spectrometry approach (2-D-LC-FTMS and SELDI-TOF MS) of CVL samples from 532 women (128 HIV-resistant (HIV-R), 220 HIV-uninfected (HIV-U) and 184 HIV-infected (HIV-I) sex workers) found that 50% of the over expressed proteins were anti-proteases. The majority of these antiproteases were serpins, including serpin A1, A3, B1, B3, B4, B13 and C1. This data demonstrated that HIV-R sex workers over expressed anti-proteases mainly from the serpin family and that these serpins may be playing a role in a resistance mechanism in the cervical mucosa of the FGT.

All together these studies showed that the HIV resistant group has a unique innate and adaptive immune response and that this information may be useful for vaccine/microbicide design.

10. Serine Protease Inhibitors or Serpins and HIV infection

Serpins or serine protease inhibitors are members of the largest and most diverse super family of protease inhibitors. More than 1000 serpins have been identified in plants, viruses and fungii and to this point 36 of them are found in human. There are 9 clades of serpins (A-I) based on shared phylogeny. Among them 8 clades are extra-cellular and

only clade B has intracellular proteins. These are medium sized molecules containing about 330-500 amino acids and consist of eight well-defined helices (A-H), three β sheets (A-C) and a reactive central loop that is mobile, exposed and causes the proteolysis by an irreversible “bait- and- trap” mechanism (170). Serpins not only inhibit serine proteases but also caspases and papain-like cystine proteases. Additionally, they function as non-inhibitory hormone transporters for corticosteroid and thyroxin binding globulin and tumor suppressor gene. Serpins are synthesized in hepatic cells and can be found in epithelial cells, endothelial cells and immune cells (171).

Serine proteases secreted by neutrophil and CTLs are known to play an important role in the immune system by killing the invading pathogens, by remodeling tissue and also by activating pro inflammatory cytokines and receptors (172). But a balance between proteases and anti-proteases is essential, because excessive or prolonged action of these proteases leads to severe inflammation, premature cell death and tissue damage and serpins are the known regulators of these proteases. Indeed they are potent immune regulator by inhibiting complement activation, apoptosis, and the secretion of inflammatory mediators (173-176). Benarafa C et al. in their knock-out mice study showed that serpins are important effectors of the mucosal immune system against protease mediated inflammatory damage during bacterial infection (177).

Some serpins inhibit elastase and cathepsin G and this can have many adverse effects if not controlled. In lung emphysema and cystic fibrosis, severe pulmonary impairment is associated with an elastase-antielastase imbalance. It is now a well established fact that excess elastase level due to deficiency of serpin A1 leads to this imbalance that results in extensive lung alveolar damage and patient developed

pulmonary symptoms (178). Elastase also impairs the wound healing procedure by degrading the elastin and fibronectin. Serpin A1 and B1 have shown to counteract this process (179). Serpin C1 is very important for regulation of the coagulation pathway and fibrinolytic pathway (180). Also, serpin C1 interferes with signal transduction that leads to NF-kB activation in monocytes and endothelial cells (181). A recent study has shown that serpin C1 can inhibit HIV replication by activating the prostaglandin synthetase-2 that impairs the NFκB pathway (182).

The prospects of serpins as an anti-HIV factor arose when Hattori et al first discovered that an extracellular membrane bound protease on the T cell surface mediated HIV-1 infection and trypstatin a protease inhibitor inhibited the syncytium formation (183). Later it was revealed that the V3 domain of gp120 contains aspartic acid protein like, trypsin and chymotrypsin like cleavage sites and that membrane proteases bind with HIV-1 via gp120 ligand. Further experiments showed that cathepsin G is the membrane proteinase for U-937 cells and serine protease TL2 is for T4 lymphocytes; both facilitate HIV-1 infection (184). Elastase was found as an membrane bound protein in phagocytes and positively correlated with HIV infection (185). It also serves as a secondary receptor for HIV and destroys the chemokine SDF-1, a natural ligand for HIV CXCR4 co-receptor. Elastase has been shown to increase HIV-1 gp 120 binding with CD4 and the CXCR4 co-receptor in murine cell line (184). Thrombin, another serine protease, propagates HIV infection in brain by changing the blood-brain permeability (186).

Serine proteases mediate increased HIV-1 infection, and as serrpins are the regulators of these proteins, it can be summarized that serpins are playing a novel role in inhibiting HIV-1 infection. Recently several studies have been focusing on these serpins

and most of these in vitro studies revealed that several serpins (serpin A1, A3, C1) have multiple mechanisms to inhibit HIV directly or by making the environment unsuitable for HIV infection**.**

10.1. Serpin A1 or α1-antitrypsin (α1-AT) is an acute phase protein and potent inhibitor of nutrophil elastase, proteinase 3, kallikreins 7 and intracellular and cell surface proteases. This is a 394 amino acid, 52kDa plasma glycorotein mainly synthesized by liver but also produced by monocyte, macrophages and lung alveolar cells and intestinal eithelium. In liver, IL-6 and oncostatin M are potent inducers for A1 synthesis, whereas in monocytes IL-1β and TNF-α and LPS are the inducers. The plasma concentration of serpin A1 is 0.9-1.75 g/L and the half life is 3-5 days. But the concentration may rise to 3-5 fold during inflammation or infection in response to inflammatory cytokines. Oral contraception and pregnancy also increase the plasma concentration of serpin A1 (178, 184).

Serpin A1 mainly inhibits protease mediated inflammation but it has several immunoregulatory properties independent of protease inhibition. It induces the release of IL-1 receptor antagonist (187), inhibits LPS/endotoxin induced TNF-α expression and enhances IL-10 expression in monocytes, neutralizes LPS mediated over-activation of monocytes via regulation of CD14 expression (188), inhibits pro-inflammatory cytokines IL-8, IL-6, IL-1β and TNF-α production in whole blood (189), and also inhibits IL-8 and soluble immune complex receptor–mediated nutrophil chemotaxis (190).

Serpin A1 seems to have several potential mechanisms of HIV inhibition from viral entry to the replication cycle of HIV-1. The C- terminal fragment of serpin A1

interacts with the gp41fusion peptide and blocks different strains of HIV-1 entry as well as ART resistant HIV-1 entry (191). It also blocks the host cellular protein furin that cleaves gp160 into gp120 and gp41(192). The C-terminal 26-residue of serpinA1 also inhibits HIV LTR driven transcription (193). HIV protein PR (an aspartyl protease) that is necessary for gag-pol polyprotein processing is inhibited by serpinA1(192). In PBMCs and monocytic cells, serpin A1 decrease HIV replication by decreasing NFκB activity (194). A recent study by Zhou et al suggested that serpin A1 also inhibits NFκB activity in follicular DCs. The nuclear translocation of NFκB factor from cytoplasm to nucleus is necessary for HIV transcription that occurs after degration of IκBα protein in cytoplasm. Serpin A1 is able to block this IκBα degradation by altered polyubiquitination and prevent NFκB translocation (195).

Breaches of the surface epithelium increase HIV transmission and in this case a rapid wound healing process is desirable. While, elastase impairs wound healing by degrading fibronectin, serpin A1 can inhibits this action (179). A recent cross-sectional study confirmed the significantly lower serum serpin A1 level in HIV infected patients compared to healthy individuals and that the levels were lower when the CD4 count is below 200 cells/μL (196).

Taken all together it is clear that serpin A1 has a potential role in the inhibition of protease mediated HIV-1 infection.

10.2. Serpin A3 or α1 antichymotrypsin (ACT) also belongs to the serpin superfamily. It is present in blood and contains 423 amino acids and has a molecular mass of 55-66kDa. It acts as an inhibitor of cathepsin G, mast cell chymase, pancreatic

chymotrypsin, and Kallikrein 2,3. It is also an acute phase protein and ACT gene expression in hepatic cells is modulated by IL-1, IL-6 and in astrocytes by oncostatin M. The plasma level of SerpinA3 is 220±40ug/ml and half life is 4-5 days (197).

Cathepsin G is present in neutrophil granules and is found strongly associated with serpin A3. Cathepsin G is also present in cervical secretion. At the site of inflammation, it is released by neutrophils and kills the pathogen and activates proinflammatory cytokines IL-1β and TNF-α. If it is not controlled by serpins it may lead to tissue damage (173). Cathepsin G also acts as a chemo attractant for neutrophils and stimulates T-cells, which are targets for HIV-1-infection (198). In addition, cathepsins can enhance HIV-1 infection by cleaving anti-viral factors such as RANTES, and MIP-3α (199). Inhibition of this cathepsin G by serpin A3 is one vital mode to inhibit HIV-1 infection.

11. Rationale for this project

Recent evidence has shown that HIV-resistant women over-express specific serpin antiproteases in their cervical mucosa. Due to the known antiviral function of both serpin A1, and serpin A3 against HIV-1 infection, and their known roles in inflammation, an in-depth examination (quantification of serpins, relationship with plasma serpin levels, association of serpins with biological/epidemiological confounders) of these proteins in HIV-R women was conducted.

12. Hypothesis

The global hypothesis of my Master's project is that the levels of clade A serpins are elevated in the cervical mucosa of highly HIV-1 exposed seronegative (HESN) women and that these levels correlate to HIV-1 resistance. These mucosal serpins also correlate to systemic levels and their expression levels in the mucosal compartment vary due to epidemiological confounders like sexual practices, contraceptive use, menstrual cycle or concurrent infections.

To test this hypothesis, this thesis attempts to fulfill three specific objectives:

Objective 1: To measure the serpin A1 and A3 levels in CVL fluid from HIV-1-resistant/HESN and susceptible individuals and determine if there is any statistical difference between the groups.

Objective 2: To determine if mucosal serpin expression is correlated to systemic serpin expression.

Objective 3: To determine the impact of epidemiological and clinical factors upon mucosal serpin expression.

13. Materials and methods

13.1. Study population

The Pumwani Sex Worker Cohort was established in 1985 in the Pumwani district of Nairobi to study the immunobiology and epidemiology of STIs including HIV. This is an open cohort that currently comprises more than 3000 commercial sex workers (CSW), of which approximately 1100 individuals are in active follow-up. In addition to research, this cohort is provided with free medical care, STI and HIV prevention services like counseling on safe sex, condom use and anti-retroviral treatment for infected individuals. The enrollees in this cohort are recalled biannually to participate in a resurvey to collect biological samples such as blood, cervico vaginal lavage (CVL) fluid, cervical mononuclear cells (CMC) for HIV research and STI testing. A resurvey form is filled out which gives the information about patient's detailed medical background, gestational and menstrual history and also provides the chronicles of sexual practices of these women. The women in this cohort are divided into three study groups based on their HIV status and follow-up period by this cohort.

HIV-1 Resistant women (HIV-R): these women have been followed for more than 7 years, continuing sex work and remain HIV negative by both serology and Reverse transcription polymerase chain reaction (RT-PCR). Currently 150 such individuals are identified in this cohort.

HIV-1 Susceptible Non-resistant/ HIV-negative Sex Workers (HIV-N): this group is the HIV uninfected individuals who recently enrolled in this cohort and have not been followed for a long enough period to be considered resistant. We expect that the

majority of these women are susceptible to HIV-1 as 85% will eventually seroconverted in 7 years.

HIV-Positive women (HIV-P): These women are all HIV-positive, some of them were positive when enrolled in this cohort and some seroconverted either from HIV-R or HIV-N status over the year. They are at various stages of HIV disease and some of them have already progressed to AIDS.

13.2. Study subjects for this project

For this project, CVL samples were collected from **84** resistant/HIV-1 highly exposed seronegative (HESN), **66** HIV-1 positive and **82** HIV-1 negative sex workers from the Pumwani cohort. The negative group was further divided into two groups. According to definition, HIV-negative individual are those who have been in the cohort for less than 7 years. However, some sex workers were found in the cohort for less than 7 years but their exposure time to HIV-1 due to sex work was more than 7 years. So this HIV-N group is categorized as:

1) In the cohort <7 years and engaged in sex work <7 years=52 sex workers

2) In the cohort <7 years but engaged in sex work >7years=30 sex workers

Plasma samples were collected for the 2nd part of the project. A total of 27 plasma samples were collected from the following groups: 9 plasma samples from HIV-R, 7 from HIV-P, 8 from HIV-N<7 years exposure and 3 from the HIV-N>7 years exposure group. The number of plasma samples was low because to see the correlation of mucosal and systemic serpin levels, it was necessary to find plasma samples that were collected at the same time as

the CVL samples. As it is a large cohort, blood and CVL collections from every individual at the same time were not possible and 27 samples were found to be collected along with CVL samples for this study.

13.3. Collection of cervico-vaginal lavage (CVL) samples

Consent was obtained from each participant and the physician or nurse explained the procedure to her before collecting the samples. Women who were menstruating at that time were asked to return at a later date for the examination and sample collection. CVL samples were collected by washing the ectocervix with 2ml of sterile 1x phosphate buffered saline (PBS) and the lavage was collected from the posterior fornix by the syringe. Samples were placed into a 15ml conical tube, centrifuged to remove cellular debris, and the supernatant was stored at -80°C until shipment back to Winnipeg in liquid nitrogen. Samples were frozen at -80°C until further analysis.

13.4. BCA protein assay (bicinchoninic acid)

Protein concentration of each sample was measured by BCA protein assay (catalog no. 71285, Cedar lane) according to the manufacturer's protocol. In brief, BSA was prepared over the range of 0-1000 µg/mL in sterile eppendorf tubes. The working reagent was prepared by combining 200 µl of BCA solution with 4 µl of 4% cupric sulfate per sample.

Twenty-five µl of diluted samples (1:2) and standard were transferred in duplicate to an ELISA plate. Two hundred µl of working reagent was added to each sample, shaken briefly on a rocker (Bellco: Rocker Platform 7740-10010) and incubated

at 37^{o}C for 30 minutes. After incubation, samples and standards were read at 562 nm on a microplate reader (Molecular Devices 02500: SPECTRA max PLUS 384).

13.5. Silver stain

Silver staining of each sample was done by using the SilverQuest staining kit (catalogue number LC6070, Invitrogen) according to manufacturer's protocol. In brief, one μg of each protein sample was loaded onto a 4-12 % NuPAGE® SDS-PAGE gradient gel and electrophoresed at 200 V for approximately 40 minutes using a gel electrophoresis unit (XCell4 SureLock™ Midi-Cell Runner oe: Invitrogen). After electrophoresis, the polyacrylamide gel was rinsed with ultra pure water and fixed with 100 ml of fixative for 20 minutes with gentle rotation. Then the gel was washed with 30% ethanol for 10 minutes and incubated with 100 ml of Sensitizing solution for another 10 minutes. Following the wash of gel again with 30% ethanol and then ultra pure water, the gel was incubated in 100 ml of staining solution. After washing the gel again, the Developer was added to the gel for 4-8 minutes and once the appropriate staining intensity was achieved, 10 ml of Stopper solution was added directly to the gel while still immersed in developing solution. Then finally the gel was washed with ultra pure water and the image was taken.

13.6. Serpin A1 ELISA reagents and protocol

Serpin A1 ELISA was performed using a commercially available human A1AT ELISA kit (Immunology Consultants Laboratory, Inc. Newberg, OR), according to the manufacturers' protocol. The serpin A1 ELISA kit consisted of a flat-bottomed 96 wells plate coated with anti-Alpha 1-Antitrypsin antibody, diluent concentrate/running buffer,

wash solution, enzyme- antibody conjugate-Human Alpha 1-Antitrypsin antibody conjugated with horseradish peroxidase in a stabilizing buffer, Chromogen-substrate solution-3, 3', 5, 5'-Tetramethybenzidine (TMB) and hydrogen peroxide in citric acid buffer, Stop solution containing 0.3 M sulfuric acid and Lyophilized Human Alpha 1-Antitrypsin calibrator for standard.

This is a double antibody sandwich ELISA. The anti-Alpha1-Antitrypsin antibodies were adsorbed to the surface of polystyrene 96– microtitre wells. To prepare the standard, 3µl of Human Alpha-1 Antitrypsin Calibrator was mixed with 932µl of 1X Diluent concentrate in sterile eppendorf tubes and then added in duplicate and serially diluted from 500ng/ml to 7.81ng/ml to the ELISA plate. Next, 100µl of each CVL samples were added in duplicate and in a 1:2 dilution into the pre designated well and incubated at 37^0C for 60 min. Following incubation, plates were washed for four times with diluted wash solution to remove unbound proteins and 100µl of Anti-A1AT-HRP Conjugate was added and then incubated for 30 minutes in the dark. Following another washing step, 100µl of Chromogenic substrate TMB was added into each well and after 10 minutes incubation, 100µl of Stop Solution was added and the plates was read on an ELISA plate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA) at 450 nm. The quantity of serpin A1 was measured based on the standard curve and all samples were run blinded.

After obtaining total (ng/ml) serpin A1 levels, they were normalized to the total µg of protein (ng/µg) measured by the BCA assay. Only total levels of serpin A1 were determined in plasma samples and normalized by volume.

13.7. Serpin A3 ELISA protocol

Serpin A3 ELISA was done by Alpha 1-Antichymotrypsin ELISA kit ((Immunology Consultants Laboratory, Inc. Newberg, OR), following the same protocol as serpin A1. The 96 wells plate was coated with anti Human Alpha-1 Antichymotrypsin antibodies. To prepare the standard, 80µl of Human Alpha-1 Antichymotrypsin Calibrator was mixed with 592µl of 1X Diluent concentrate in sterile eppendorf tubes and added in duplicate and serially diluted from 400ng/ml to 6.25ng/ml to the ELISA plate. Next, 100µl of each CVL samples were added in duplicate and in a 1:2 dilution into the pre designated well and incubated at 37^0C for 60 min. Following incubation, plates were washed for four times with diluted wash solution to remove unbound proteins and 100µl of Anti-A1AT-HRP Conjugate was added and then incubated for 15 minutes covered in dark. Following another washing step, 100µl Chromogenic substrate TMB was added into each well and after 5 minutes 100µl of Stop Solution was added and the plates was read on an ELISA plate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA) at 450 nm. The quantity of serpin A3 was measured based on the standard curve and all samples were run blinded.

Total (ng/ml) serpin A3 levels were normalized to the total µg of protein (ng/µg) measured by the BCA assay. Also, total levels of serpin A3 were determined in plasma samples and normalized by volume.

13.8. Western Immunoblot

One µg of protein from each sample was loaded onto a 4-12 % NuPAGE® SDS-PAGE gel and electrophoresed at 200 V for approximately 40 minutes using a gel

electrophoresis unit (XCell4 SureLock™ Midi-Cell Runner oe: Invitrogen). The gel was rinsed with ddH2O, and proteins were transferred onto a nitrocellulose membrane using the Invitrogen iBlot® gel transfer device according to the manufacturer's instructions. The blots were blocked with 5% non-fat milk for 1 hour at room temperature, incubated overnight at 4^{o}C with the appropriate dilution (1:1000) of primary antibody. The blots were then washed 3-times with TBST, and incubated with the appropriate horseradish peroxidase (HRP-) conjugated secondary antibody (1:5000 dilution). The bands were developed with Immobilon detection reagent (Millipore). The primary antibodies used and catalogue numbers were as follows: serpin A1 (alpha 1 antitrypsin) (Abnova: PAB11182); serpin A3 (alpha 1 antichymotrypsin) (Abcam: ab54693).

13.9. Statistical analysis

Statistical analysis was performed using Prism Statistical software (version 5). The ELISA data show the mean and standard error of aggregate values. Across group variations for protein expression data, clinical information was calculated by non-parametric Kruskal-Wallis statistical test (1-way Anova) and inter-group variations were analyzed using unpaired two-tailed Mann-Whitney t- tests. These non-parametric tests were used because the data were not assumed to follow a normal/Gaussian distribution. Significant differences were those that had p values of <0.05.

Associations between protein expression information and clinical data sets were calculated using nonparametric Spearman correlation tests (at 95% confidence intervals), which generated both p and R values, where p values <0.05 were deemed significant.

14. Results

The results section of this thesis is divided into four sections:

14.1. Section I: Protein assays of CVL samples

14.1.1. Analysis of protein levels in CVL samples by BCA assay

14.1.2. Silver staining gels were used to confirm results in selected CVL samples

14.2. Section II: Measurement of serpin A1 and serpin A3 levels in CVLs of commercial sex workers from Kenya

14.2.1. Total serpin A1 measurement (ng/ml) by ELISA kit and normalized them per μg of sample protein concentration (ng/μg)

14.2.2. Total serpin A3 measurement (ng/ml) by ELISA kit and normalized them per μg of sample protein concentration (ng/μg)

14.2.3. Western blots to confirm the ELISA result

14.2.4. Correlation of CVL serpin A1 and A3

14.3. Section III: Measurement and characterization of serpin A1 and serpin A3 levels in Plasma samples of commercial sex workers from Kenya

14.3.1. Correlation of CVL and plasma concentration of serpin A1 and A3

14.3.2. Correlation of plasma serpin A1 and A3

14.4. Section IV: Association of serpin A1 and serpin A3 with Epidemiological Variables

Section I

Protein assays of cervico-vaginal lavage samples

14.1.1. Analysis of protein levels in CVL samples by BCA assay:

Protein concentrations of 232 cervico-vaginal lavage (CVL) samples collected from women of the Pumwani sex worker cohort were measured by the BCA protein assay before performing the ELISA. The result did not show any significant difference between four groups (p=0.4083): 84 HIV-resistant (mean± SEM=371.2±27.70μg/ml), 66 HIV-positive (mean± SEM=355.0 ±29.12 μg/ml), 30 HIV-negative and >7years exposure (HIV-N>7years) (mean± SEM=455.0±51.79 μg/ml) and 52 HIV-negative and <7 years exposure (HIV-N<7 years) (mean± SEM=396.1±37.95 μg/ml) (Figure 1).

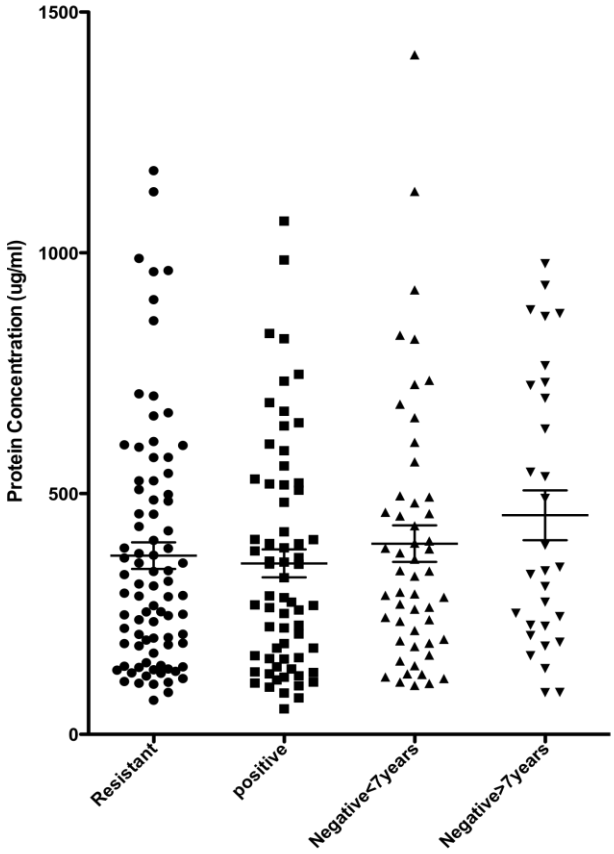


Figure1: Protein expression in CVL samples. Total protein concentrations of 232 CVL samples (HIV-R=84, HIV-P=66, HIV-N<7 years exposure=52 and HIV-N>7years exposure=30) were measured by BCA protein assay. Horizontal bars represent the mean +/- the standard error of the mean. Both Kruskal-Wallis (1-way Anova) and unpaired two-tailed Mann-Whitney statistical tests were performed to assess expression differences between groups (95% confidence level, or $p < 0.05$).

14.1.2. Silver staining of selected CVL samples to confirm the results of protein assay:

After measuring the protein concentration of all CVL samples by BCA protein assay, silver staining was performed to observe protein band variation and confirm the BCA protein assay. Five CVL samples with high protein concentration (400-1000µg/ml) and four CVL samples with low protein concentration (<400µg/ml) measured by BCA assay were selected for silver stain. For positive control, BSA protein at different concentration (1µl, 2µl and 4µl) was established and loaded onto the 4-12% SDS-PAGE gradient gel (lane 10-12). The lane-13 was used for the molecular weight marker. Based on figure 2, the silver stained SDS polyacrylamide gels showed high protein concentrated CVL samples with strong visible bands ranging from 10-110kDa (lane 5-9), whereas band intensity was not so strong for CVL samples with low protein concentration (lane 1-4). These results confirmed the BCA assay results by showing a greater number of bands and intensity in high protein samples.

Serpin A1 and serpin A3 have known molecular weights of 55-65 kDa and the main reason of doing this silver stain was to confirm the presence of proteins in this molecular weight range. This result is consistent with the study by Burgener et al. that also found different molecular weight proteins in CVL fluid (14).

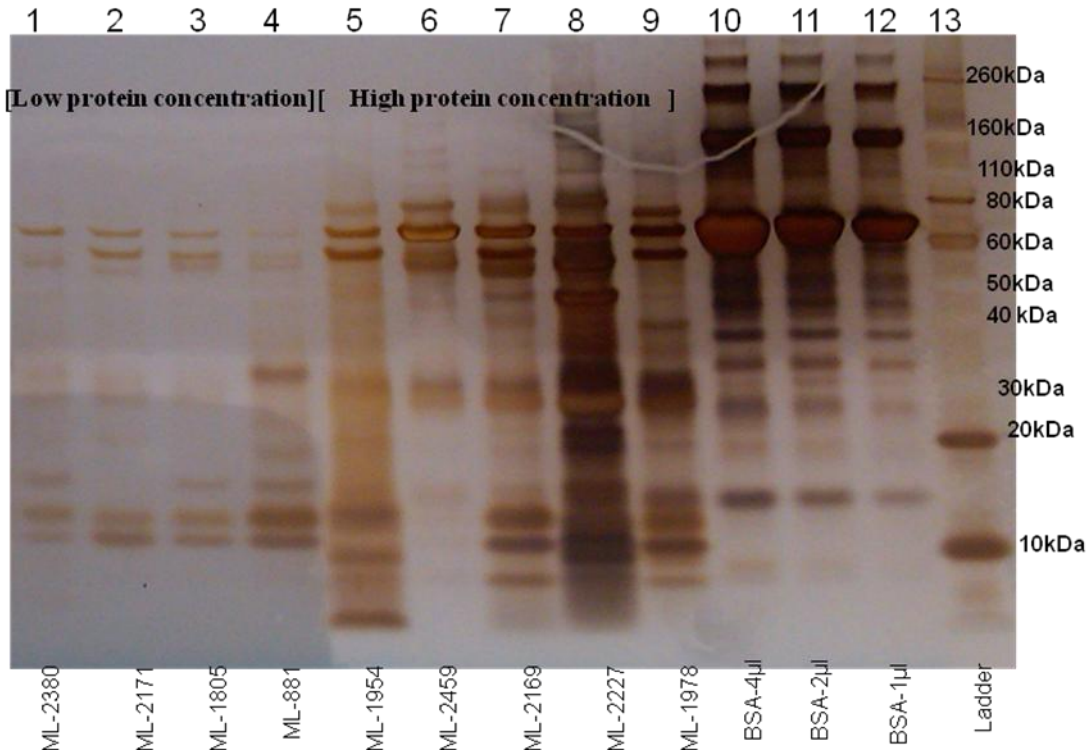


Figure 2: Silver staining of SDS-PAGE separated CVL proteins. Patient samples determined to be low (lane 1-4) or high (lane 5-9) protein concentration samples by the BCA assay were subjected to SDS-PAGE and silver staining. ML-xxxx corresponds to patient sample codes. Lane 10 to12, positive control (BSA protein, 4µl, 2µl and 1µL respectively) and lane 13, molecular size marker.

Section II

Measurement of serpin A1 and serpin A3 levels in cervico-vaginal lavage of commercial sex workers from Kenya

14.2.1. Characterization of serpin A1 levels in CVL samples:

In order to determine the level of serpin A1 in CVL samples and their association with HIV resistance, total serpin protein levels for 232 CVL samples collected from 2008-2010 resurveys were measured by a commercially available human A1AT ELISA kit. As seen in figure 3(a), detectable levels of total serpin A1 were found in 84 HIV resistant (HIV-R; mean ±SEM=3.0±0.065 ng/ml, median=3.0 ng/ml), 66 HIV positive (HIV-P; mean± SEM=2.9±0.071ng/ml; median=2.90 ng/ml), 30 HIV negative and exposed to HIV >7years (HIV-N>7years exposure; mean± SEM=2.9±0.149ng/ml; median=3.0 ng/ml) and 52 HIV-negative and exposed to HIV<7 years (HIV-N<7 years exposure; mean± SEM=2.8±0.083ng/ml, median=2.9 ng/ml) samples. There was no significant increase in total (not normalized) serpin A1 level in HIV R women as compared to the other three groups.

Serpin A1 levels were then normalized to protein content. Here a significant difference was found between HIV- resistant (HIV-R; mean± SEM=0.48±0.05ng/ug, median=0.44 ng/ug) and HIV-N<7 years exposure group (mean± SEM=0.29±0.07ng/ug, median=0.31ng/ug) (p value= 0.0470*) (figure 3b). The normalized serpin A1 level/total protein concentration was also found to be different between HIV-positive (mean± SEM=0.44±0.05ng/ug, median=0.50ng/ug) and HIV-N<7 years exposure group but did not reach the significance level (p value=0.0526). There was no difference of normalized serpin A1 level between HIV resistant and HIV-N >7years groups (mean± SEM=0.33±0.10ng/ug, median=0.41ng/ug).

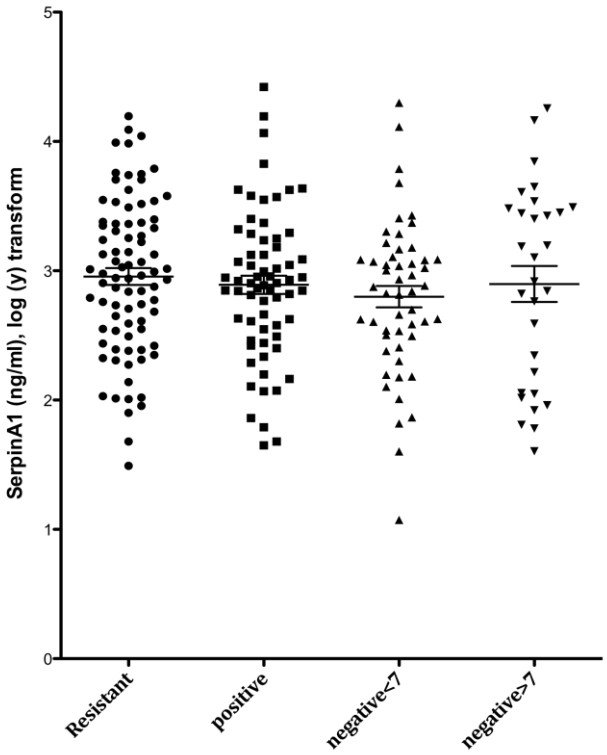


Figure 3a: Serpin A1 per volume of CVL samples measured by ELISA. Serpin A1 levels were measured in 232 CVL samples (HIV-R=84; HIV-P=66; HIV-Negative<7 years exposure=52; and HIV-N>7years exposure=30) by using a commercially available human A1AT ELISA kit. This figure shows total (ng/ml) concentration of serpin A1 level. Both Kruskal-Wallis (1-way Anova) and unpaired two-tailed Mann-Whitney statistical tests were performed to assess expression differences between groups (95% confidence level, or $p < 0.05$). Horizontal bars represent the mean +/- the standard error of the mean.

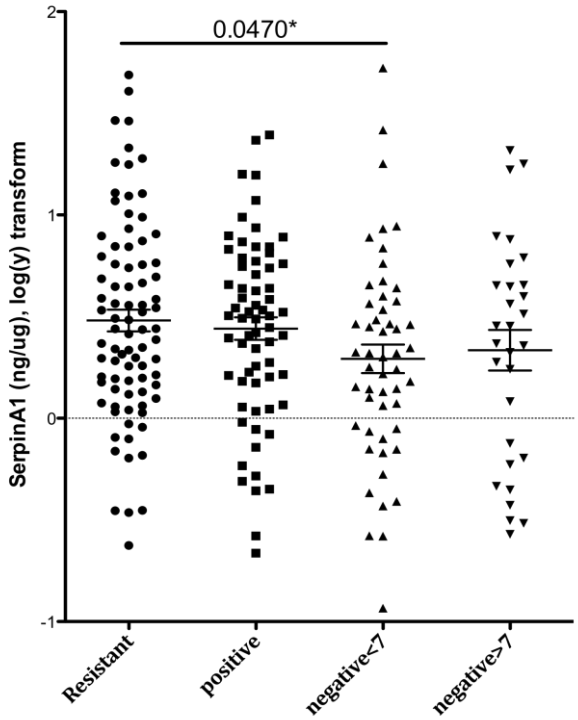


Figure 3b: Normalized serpin A1 level in CVL samples. This figure shows normalized serpin A1 levels to the total protein concentration (ng/µg of protein) of CVL samples. the CVL samples were grouped based on HIV-status: HIV-R,n=84; HIV-P, n=66; HIV-N<7years exposure, n=52 and HIV-N>7years exposure, n=30. Horizontal bars represent mean levels +/- the standard error of the mean. All samples were run in duplicate. Both Kruskal-Wallis (1-way Anova) and unpaired two-tailed Mann-Whitney statistical tests were performed to assess expression differences between groups (95% confidence level, or $p < 0.05$).

14.2.2. Measurement of serpin A3 levels in cervico-vaginal lavage (CVL) samples:

In order to determine serpin A3 levels in CVL and their association with HIV resistance, serpin A3 protein levels were measured using the same 232 CVL samples collected from 2008-2010 resurveys by a commercially available human Alpha 1-Antichymotrypsin ELISA kits. As seen in figure 4a, detectable levels of total serpin A3 were found in HIV-R (n=84; mean± SEM=1.99±0.07ng/ml, median=2.10 ng/ml), HIV-P (n=66; mean± SEM=1.89±0.06ng/ml, median=1.94ng/ml), HIV-N>7years exposure (n=30; mean ±SEM=2.02±0.14ng/ml, median=2.11ng/ml) and HIV-N<7 years exposure (n=52; mean ±SEM=2.02±0.08ng/ml, median=2.14ng/ml) individuals. There was no significant difference in the serpin A3 levels in HIV R women as compared to other three groups.

Additionally, normalized serpin A3 levels to the total protein concentration in four groups did not show any significant difference (figure 4b): HIV Resistant (mean± SEM=-0.47±0.05ng/ug, median=-0.40ng/ug), HIV positive (mean± SEM=-0.55±0.05ng/ug, median=-0.54ng/ug), HIV Negative<7 years exposure (mean± SEM=-0.49±0.07ng/ug, median=-0.36ng/ug) and HIV negative>7years exposure (mean± SEM=-0.58±0.12ng/ug, median=-0.45ng/ug).

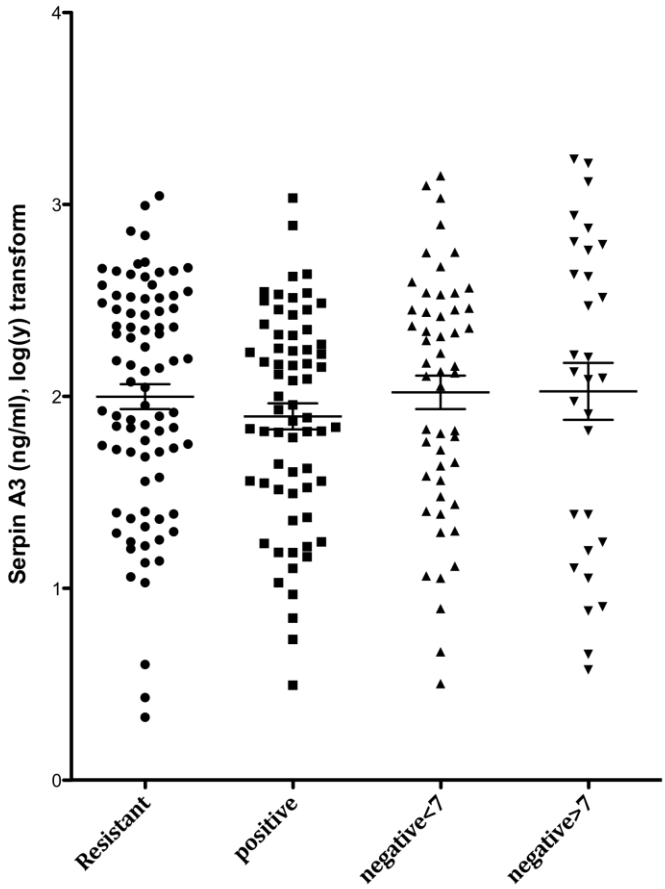


Figure 4a: ELISA of serpin A3 in the CVL samples of commercial sex workers. Serpin A3 levels per volume of CVL samples (ng/ml) were measured in duplicate by Alpha 1-Antichymotrypsin ELISA kit. The CVL samples were grouped based on HIV-status: HIV-R,n=84; HIV-P, n=66; HIV-N<7years exposure, n=52 and HIV-N>7years exposure, n=30. Both the Kruskal-Wallis (1-way Anova) and unpaired two-tailed Mann-Whitney statistical tests were performed to assess expression differences between groups (95% confidence level, or $p < 0.05$). Horizontal bars represent mean levels +/- the standard error of the mean.

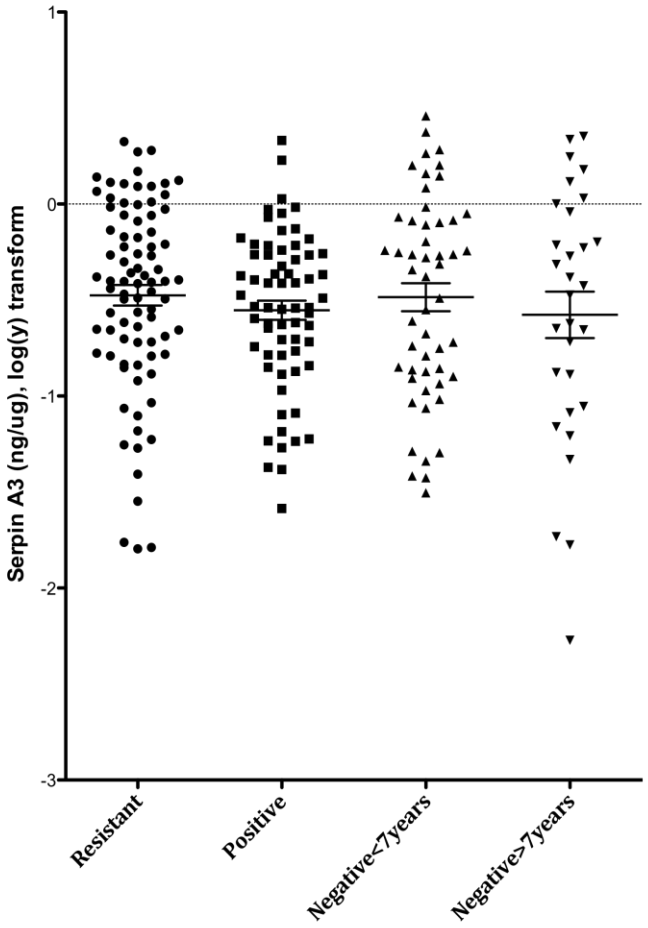


Figure 4b: Normalized serpin A3 level to the total protein concentration in CVL samples. Serpin A3 levels measured by ELISA were normalized to the total protein concentration (ng/µg) of CVL samples of 84 HIV-R; 66 HIV-P; 52 HIV-N<7years exposure, and 30 HIV-N>7years exposure individuals. Both the Kruskal-Wallis (1-way Anova) and unpaired two-tailed Mann-Whitney statistical tests were performed to assess expression differences between groups (95% confidence level, or $p < 0.05$). Horizontal bars represent mean levels +/- the standard error of the mean.

14.2.3. Confirmation of ELISA kit results by Western blot:

Western blots were performed to confirm the specificity and accuracy of the serpin A1 and A3 ELISA kits. Six individuals (HIV-R, ML-1978, 2001; HIV-P, ML-2269, 1941; HIV-N, ML-2296, 2171) with high (3000 ng/ml -10,000 ng/ml) and six individuals (HIV-R, ML-881, 1668; HIV-P, ML-1862, 1248, HIV-N, ML-2104, 2357) with low (1ng/ml-50ng/ml) serpin A1 levels were chosen to confirm serpin A1 ELISA result. 1 μg of serpin A1 from each sample was resolved by 4-12% Bis-tris SDS-PAGE gradient gel, transferred to nitrocellulose membranes (lanes 2-13). Lyophilized Human Alpha 1-Antitrypsin calibrator from serpin A1 ELISA kit, used as a positive control (lane-14). Molecular weight marker (MWM) was loaded in lane 1. The blots were blocked with 5% non-fat milk for 1 hour at room temperature, incubated overnight at 4^{o}C with the appropriate dilution (1:1000) of rabbit polyclonal antibody raised against native serpin A1 (Abnova; PAB11182). The blots were then washed 3-times with TBST, and incubated with the appropriate horseradish peroxidase (HRP-) conjugated secondary antibody (1:5000 dilution). After following rest of the procedure, the result showed a number of visible bands ranging from 40-70 kDa (lanes 2-7) but there were no visible bands in lanes 8-13 (Figure 5a) and confirmed the expression of serpin A1 in CVLs.

For serpin A3, six individuals (HIV-R, ML-1954, 1978; HIV-P, ML-2227, 2169; HIV-N, ML- 2459, 2330) with high (400 ng/ml -1700 ng/ml) and six individuals (HIV-R, ML-2012, 881; HIV-P, ML-1947, 1805; HIV-N, ML- 2171, 2380) with low (2ng/ml-75ng/ml) serpin A3 were chosen to confirm serpin A3 ELISA result. For this experiment, two separate 4-12% Bis-tris SDS-PAGE gradient gels were used. In one gel, 1μg of protein from each samples with high serpin A3 level was loaded (lanes 3-8) along with

Human Alpha-1 Antichymotrypsin Calibrator from serpin A3 ELISA kit as a positive control (lane-2). MWM was loaded in lane-1. 1μg of protein from samples with low sepin A3 level was loaded onto a separate gel (lanes 9-14). Lane 15 was used for positive control (Human Alpha-1 Antichymotrypsin Calibrator) and lane-16 for MWM. Following the same procedure as serpin A1, the blots were incubated overnight at $4^{o}C$ with the appropriate dilution (1:1000) of mouse monoclonal antibody raised against native serpin A3 (Abcam: ab54693). After washing the blots 3-times with TBST, they were incubated with the appropriate horseradish peroxidase (HRP-) conjugated secondary antibody (1:5000 dilution). Based on figure 5b, proteins from samples with high serpin A3 levels illustrated appropriate bands ranging from 35kDa to 60kDa markers (lanes 3-8), but we did not observe any expression of proteins with low serpin A3 levels (lanes 9-14).

To confirm the concentration of serpins obtained by ELISA, volumetric analysis was performed to quantify the band intensities for serpin A1 and A3 immunoblots. The relative intensity patterns of the immunoblots did not correlate with the ELISA results of serpin A1 ($p=0.1874$) and A3 ($p=0.4075$). However, one caveat of the WB and the ELISA was that they used different antibodies (1 antibody for WB; 2 different antibodies for ELISA capture and detection) and there was a possibility that each assay might be measuring different/specific isoforms or cleaved forms of serpins. Additionally, with only 12 samples (6 per group), we might not have statistical power to prove it. More works need to be done to be able to generate reagents or antibodies for WB and ELISA, or assays to more precisely detect serpin A1/A3 and all their potential isoforms found in CVL samples. Overall, these results validate the serpin expressions obtained by using commercial ELISA kit.

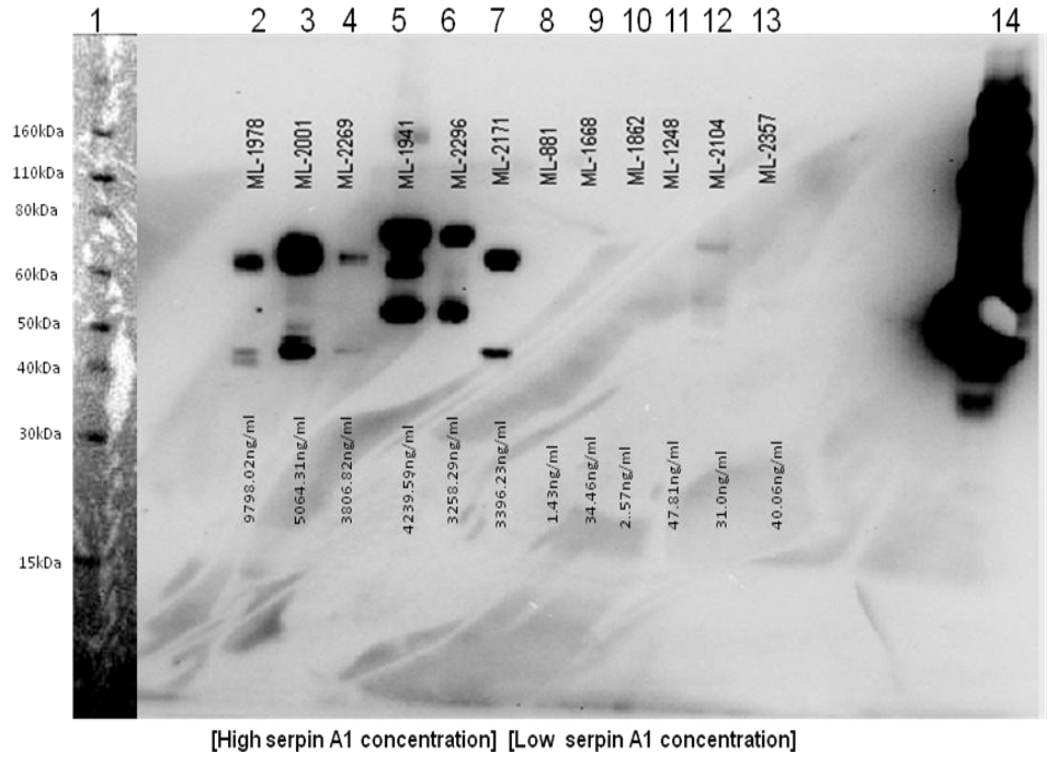


Figure 5a: Immunoblot analysis of serpin A1 levels: 1µg of protein from six individuals with high serpin A1 levels (lanes 2-7) and six individuals with low serpin A1 levels (lanes 8-13) was resolved by a 4-12% SDS-PAGE gradient gel, probed by specific antibody and immunoblotted for detection of serpin A1. Positive control shown in lane 14 illustrating appropriate antibody was able to detect proteins of interest. Molecular weight marker was in lane-1.

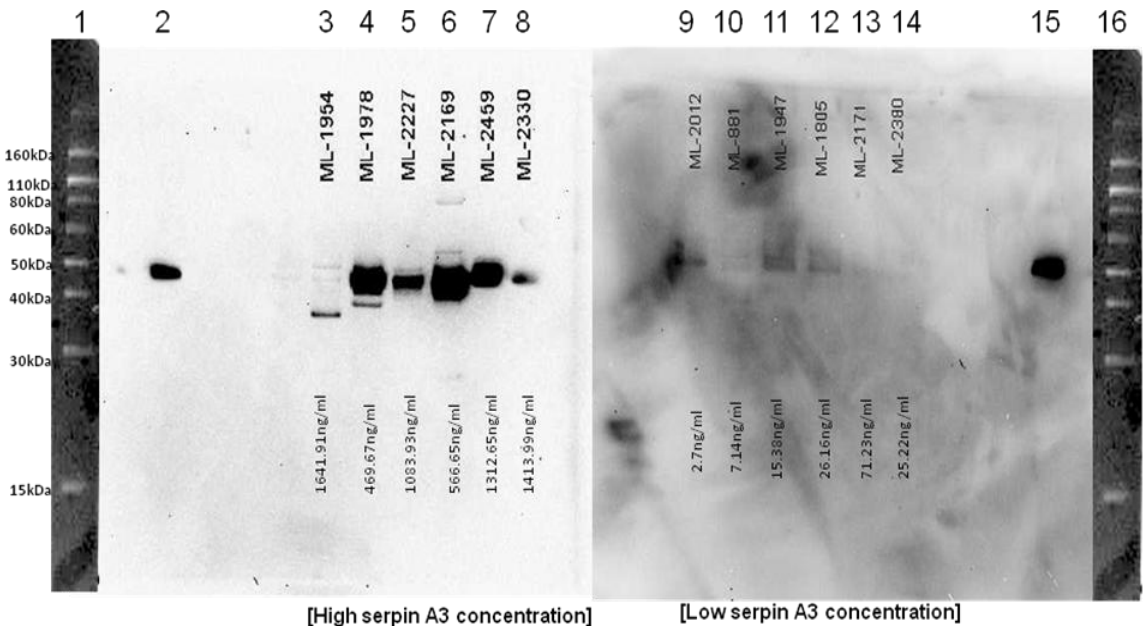


Figure 5b: Immunoblot analysis to confirm serpin A3 levels determined by ELISA. Lanes 3-8 were used for CVL protein (1µg) from six individuals with high serpin A3 levels and lanes 9-14 were for six individuals with low serpin A3 levels. All samples were subjected to reducing on a 4-12% SDS-PAGE gradient gel and immunoblotted for detection of serpin A3 by using mouse monoclonal antibody raised against native serpin A3. Positive control shown in lanes 2 and 15 illustrating appropriate antibody was able to detect proteins of interest. Lanes 1 and 16 were used for molecular weight marker.

14.2.4. Correlation of serpin A1 and A3 levels in cervical lavage samples:

Serpin A1 and A3 plasma levels increase during infection or inflammation and can increase three to four fold during acute phase events (200). It is unknown if they have any correlation with levels in the mucosal compartment of the female genital tract. To answer this question, serpin A1 and A3 levels normalized to volume (figure 6a) and to the total protein concentration (figure 6b) of CVL samples from all the four groups (n=232) were used and the nonparametric Spearman correlation test (at 95% confidence intervals) was performed, where p values <0.05 were deemed significant. Figure 6a and 6b showed that serpin A1 and serpin A3 levels normalized to volume or to the total protein concentration were tightly correlated (p=0.0001***). When the correlation of serpin A1 and A3 levels was performed in different groups (HIV-R, HIV-P and HIV-N), the results showed that serpin A1 and A3 levels were positively correlated.

a) **b)**

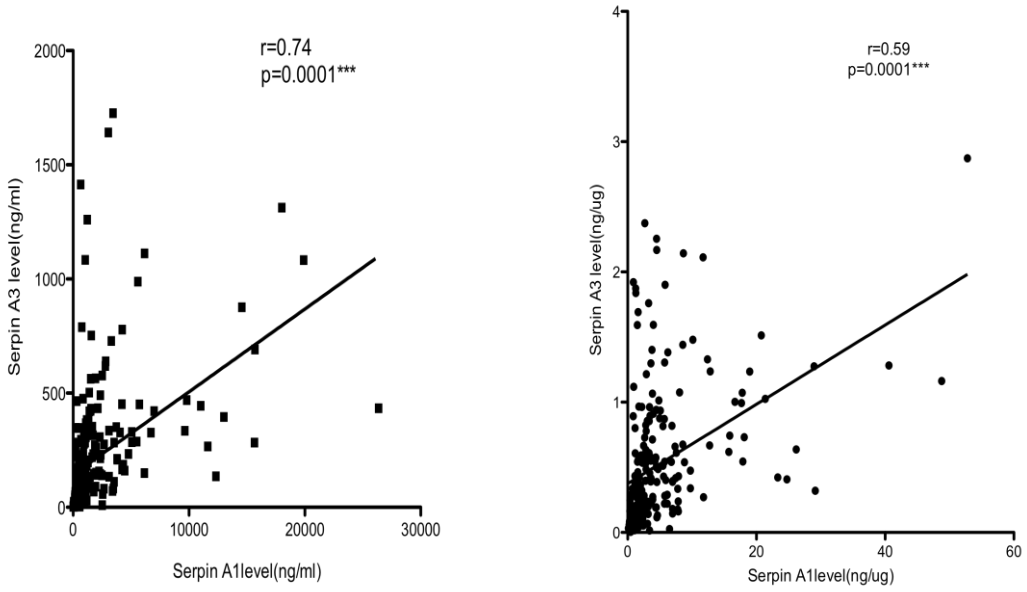


Figure 6: Correlation of serpin A1 and serpin A3 levels in CVL. Both normalized to volume (ng/ml) (a) or to total protein concentration (ng/μg) (b), serpin A1 and A3 levels were compared in CVL fluid of commercial sex workers and showed a strong correlation. Nonparametric Spearman correlation tests (at 95% confidence intervals) were performed, where p values <0.05 were deemed significant.

Section III

Comparison of serpin A1 and serpin A3 levels in mucosal and systemic compartments

14.3.1. Comparison of plasma and mucosal levels of serpins

To determine if mucosal serpin levels correlated to systemic levels, plasma samples from selected individuals (total=27, HIV-R=9, HIV-P=7, and HIV-N=11) were chosen and plasma serpin A1 and A3 levels normalized to volume were determined by ELISA and compared to mucosal serpin levels. To see the correlation, it was necessary to select the plasma samples that were collected simultaneously with CVL samples. Only 27 plasma samples selected for this study were obtained at the same time as the CVL samples. Nonparametric Spearman correlation tests (at 95% confidence intervals) were performed, where p values <0.05 were deemed significant. Figure 7a and 7b showed no correlation of CVL serpin A1 levels ($p=0.30$) and serpin A3 levels ($p=0.76$) with plasma serpin A1 or A3 levels.

a) **b)**

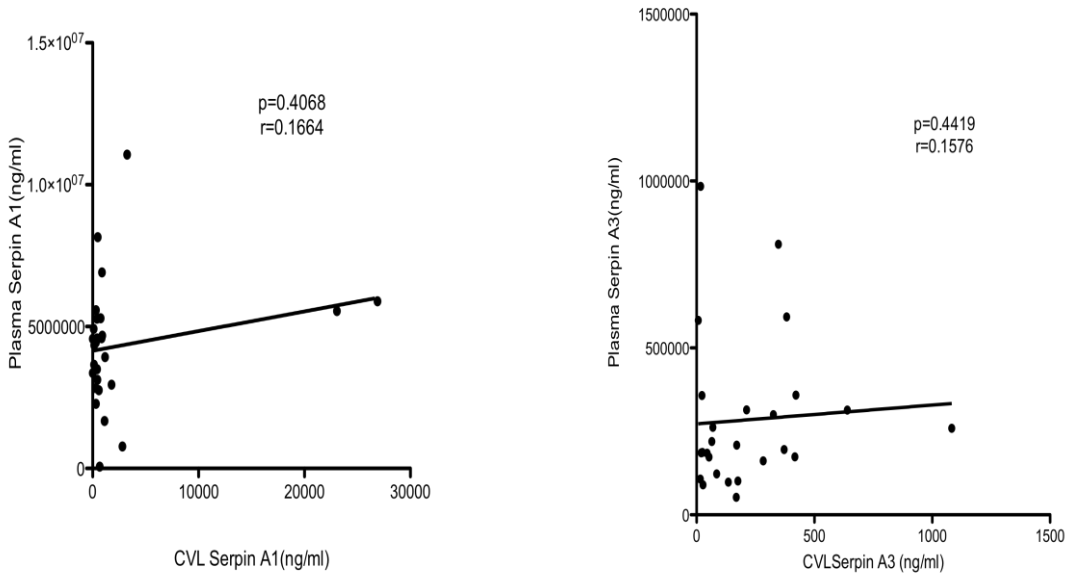


Figure 7: Correlation of serpin A1 and A3 levels in CVL and plasma. Serpin A1 and A3 levels were determined in 27 plasma samples (HIV-R=9, HIV-P=7, and HIV-N=11) by ELISA and compared with CVL serpin A1 and A3 levels. Correlation of plasma and CVL serpin A1 levels (a) or A3 levels (b) normalized to volume was measured by Nonparametric Spearman correlation test (at 95% confidence intervals), where p values <0.05 were deemed significant.

14.3.2. Comparison of plasma serpin A1 and A3 levels

Serpin A1 and A3 expression correlated mucosally, but it is unclear if this trend is evident in the plasma compartment. As these are acute phase proteins our hypothesis was serpin A1 and A3 levels would be similar in this compartment. SerpinA1 levels from the same 27 plasma samples were compared against serpin A3 levels and nonparametric Spearman correlation test (at 95% confidence intervals) was performed, where p values <0.05 were deemed significant. Figure 8 revealed no association between the two serpins in plasma ($p=0.7607$).

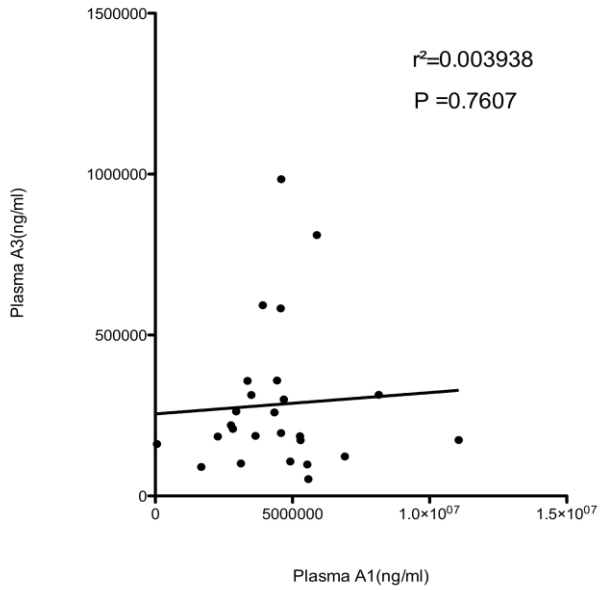


Figure 8: Correlation of serpin A1 and A3 in plasma. Serpin A1 and A3 levels (per volume) were measured in 27 plasma samples (HIV-R=9, HIV-P=7 and HIV-N=11) by using ELISA kits and compared by Nonparametric Spearman correlation test (at 95% confidence intervals), where p values <0.05 were deemed significant.

Section IV

Association of serpin A1 and serpin A3 levels with epidemiological variables

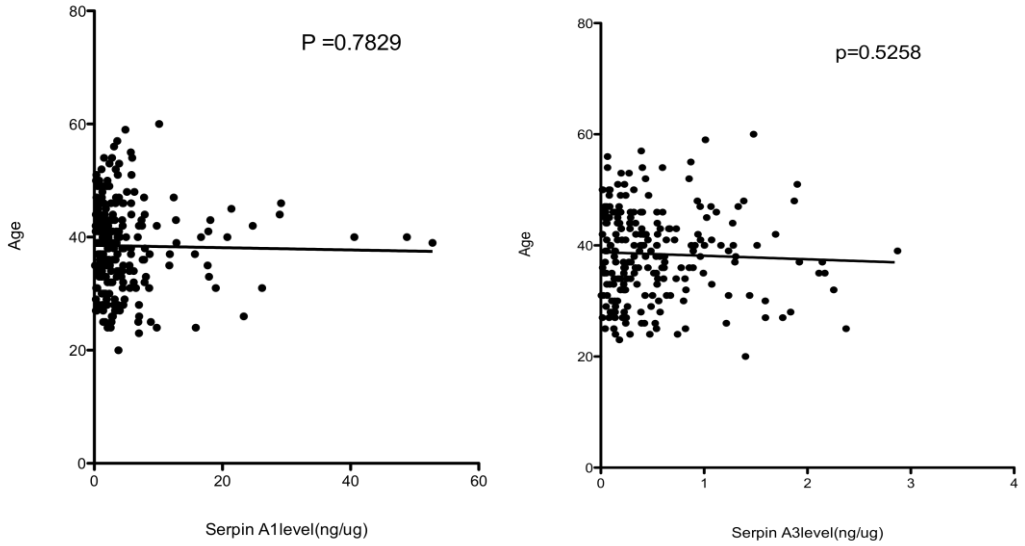
14.4. Correlation of serpin A1 and A3 levels with biological confounders

Many behavioral and biological factors contribute to changes in protein expression like pregnancy, nutrition status, and contraceptive use (200). Therefore, it was necessary to determine if epidemiological variables like age, duration of sex work, client numbers, condom use, contraceptive use, menstrual cycle, concurrent infection, and menopausal status play any role in serpin expression in CVL samples from HIV-resistant and susceptible women. All correlations were performed on 232 women pooled together and then with 84 HIV-resistant women alone to investigate if these confounders were affecting resistant women only.

14.4.1. Relationship of serpin expression levels with age

The mean age of the HIV-resistant group was significantly higher than other groups ($p<0.05$), since by definition the resistant group was enrolled in the sex worker cohort for at least 7 years of follow-up and engaged in sex work for > 7 years. To determine whether age had any role behind the higher level of serpins, serpin A1 and serpin A3 expression levels were compared against age and a nonparametric Spearman correlation test (at 95% confidence intervals) was used, where p values <0.05 were deemed significant. In figure 9a, serpin A1 ($p=0.7829$) and serpin A3 ($p=0.5258$) expression levels were found to be not associated with age. Resistant women also showed lack of association of serpin expression levels with their age (Serpin A1 $p=0.71$, A3 $p=0.39$) (Figure 9b).

a)



b)

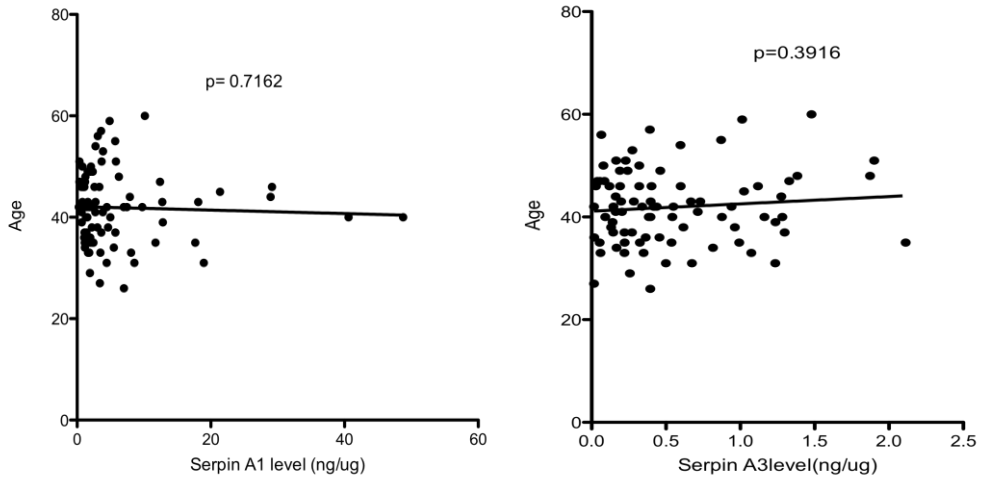


Figure 9: **Correlation of serpin expression levels with age in a) HIV-R, HIV-P and HIV-N groups and b) HIV- resistant group.** Association of serpin A1 and A3 levels (normalized to total protein concentration) in **a)** 232 CVL samples (HIV-R=84, HIV-P=66 and HIV-N=82) and **b)** 84 CVL samples from HIV-1 resistant individuals with age was calculated using a nonparametric Spearman correlation test (at 95% confidence intervals), where p values <0.05 were deemed significant.

14.4.2. Relationship of serpin expression level and repeated immune stimuli in the FGT

Longer duration of sex work may account for changes in the immune system and protein expression of the FGT as evidence suggests that sexual intercourse has an impact on FGT immunology (162). To determine whether exposure to HIV for different time periods had any effect on serpin expression levels, duration of their sex work was compared against serpin levels in all HIV groups (HIV-R=84, HIV-P=66 and HIV-N=82) and only in the 84 HIV-R group by using a non-parametric Spearman correlation test. Figure 10a revealed that there is no association of serpin expression levels with duration of sex works in 232 women (serpin A1: p=0.5746 and A3: p=0.1409). In the HIV-resistant group serpin A1 (p=0.6153) and A3 (p=0.2490) expression levels showed lack of association with duration of sex work (Figure 10b).

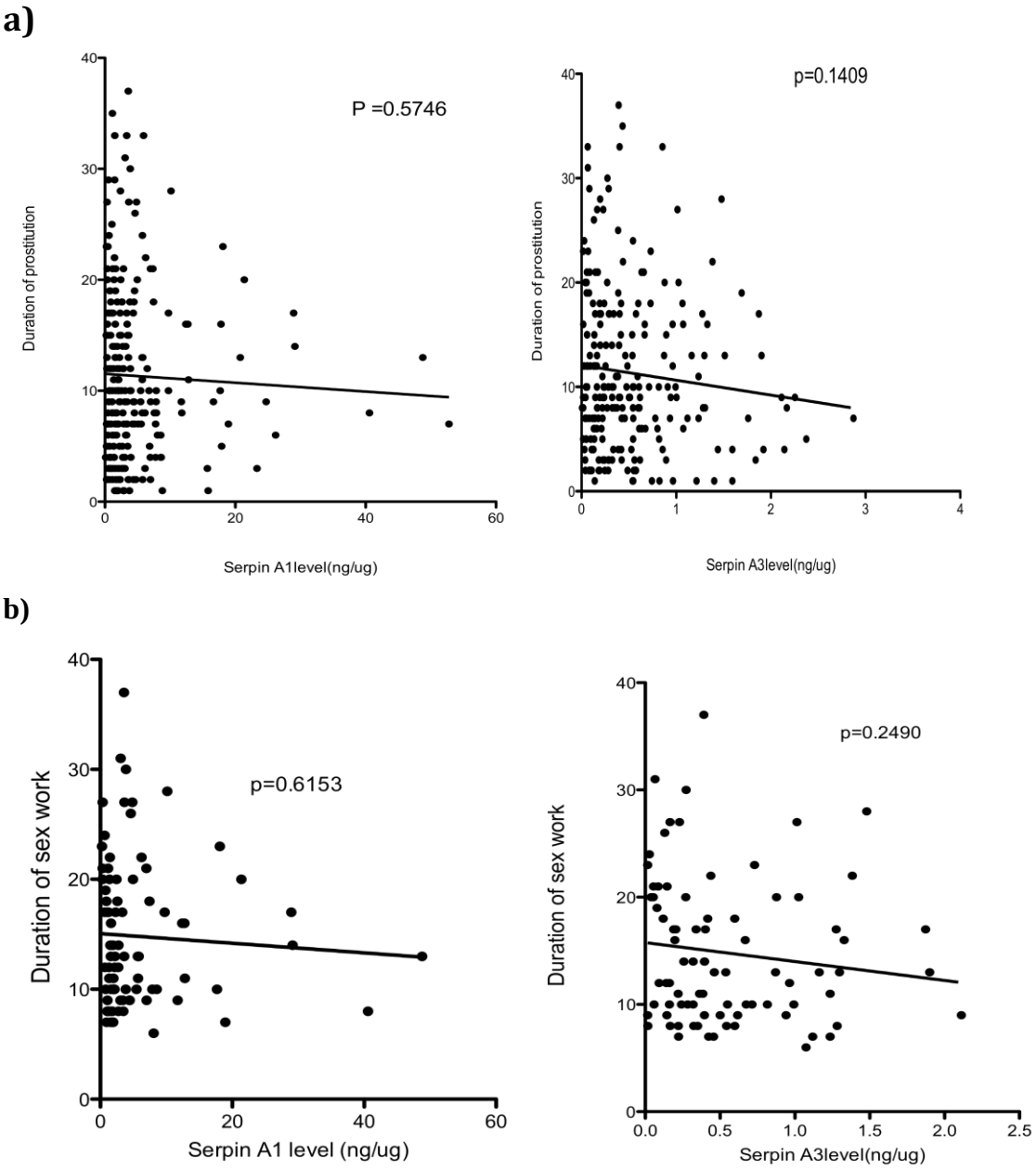


Figure 10: Correlation of serpin expression levels with duration of sex works in a) all HIV groups and b) HIV-resistant group. A comparison of serpin A1 and A3 expression levels in CVLs from **a)** 232 individuals (84 HIV-R, 66 HIV-P and 82 HIV-N) and **b)** 84 HIV-R commercial sex workers with duration of sex work was conducted using a nonparametric Spearman correlation test (at 95% confidence intervals), where p values <0.05 were deemed significant.

14.4.3. Relationship of serpin expression with number of clients

To find out whether women who had a higher number of clients/day and clients/week also had higher serpin A1 and A3 levels in CVL, serpin A1 and A3 expression levels were compared with the number of clients/day and clients /week and a non-parametric Spearman correlation test was applied. Figure 11a revealed that serpin A1 ($p=0.9251$) and A3 ($p=0.3713$) expression levels did not have any association with client number per day in 232 individuals. When they were measured in the HIV-resistant women only, they revealed no association in between the variables (Figure 11b) (A1 $p=0.9674$, A3 $p=0.8131$). When serpins were compared with client numbers per week, figure 12a showed no association of serpin A1 ($p=0.6033$) and A3 ($p=0.5915$) expression levels with clients/week in all groups. In HIV-resistant women, serpin A1 and A3 expression levels also did not show any association (A1 $p=0.6292$, A3 $p=0.8131$) (figure 12b).

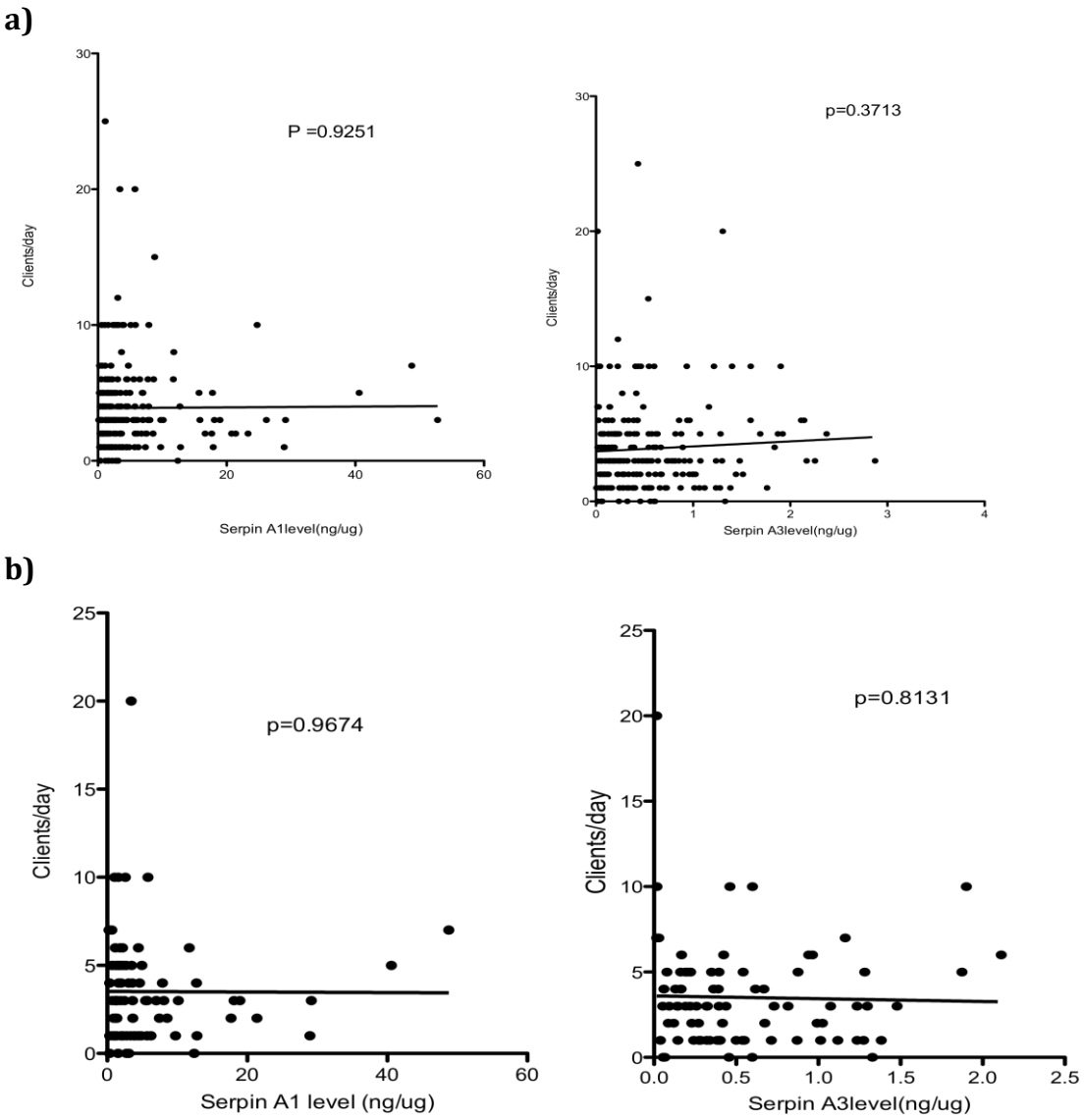
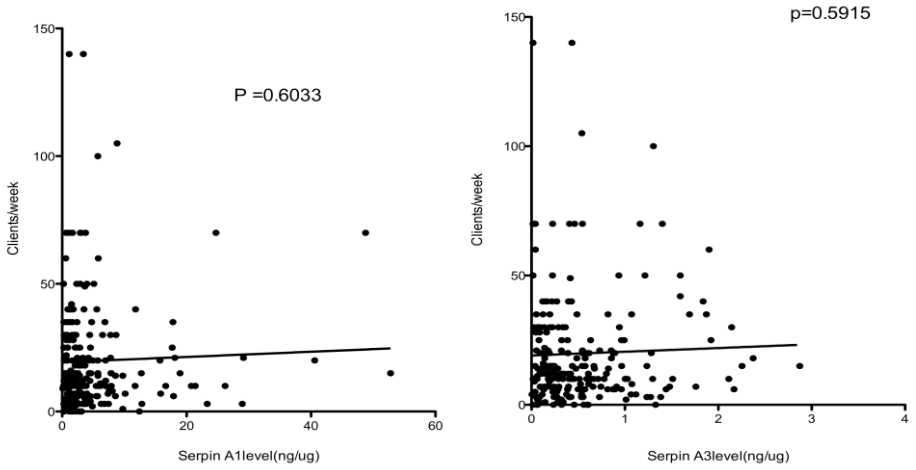


Figure 11: Correlation of serpin expression with clients/day in a) all HIV groups and b) HIV-R group. An association of serpin A1 and A3 expression levels in CVL from **a)** 232 commercial sex workers and **b)** 84 HIV-resistant women with the number of clients/day was calculated by a nonparametric Spearman correlation test (at 95% confidence intervals), where p values <0.05 were deemed significant.

a)



b)

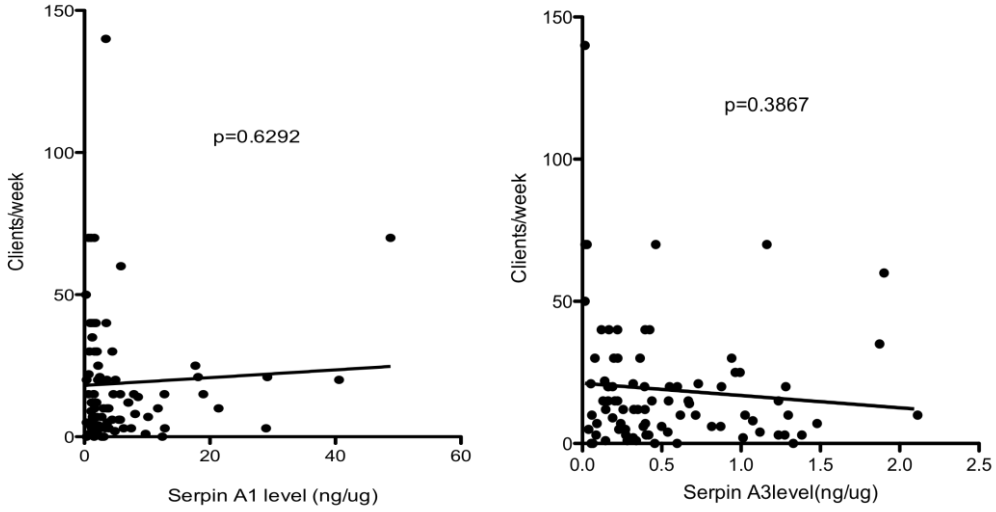
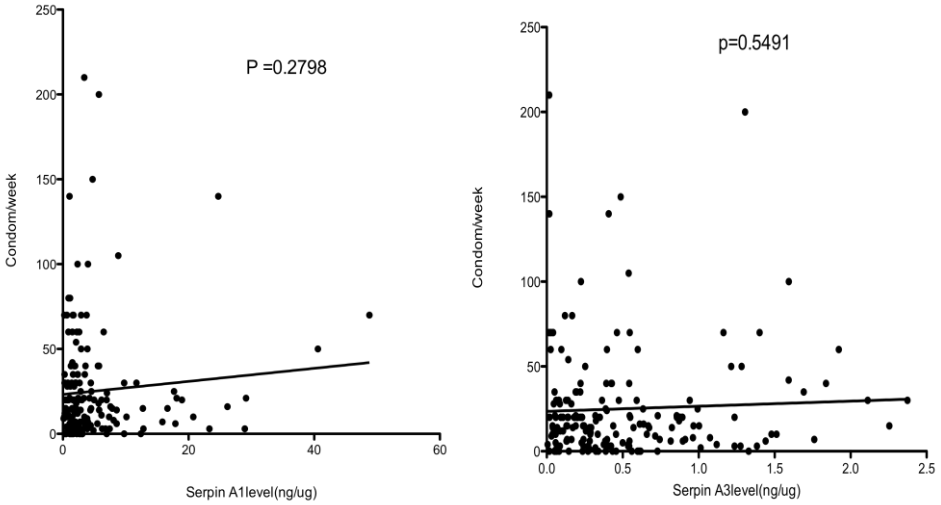


Figure 12: Correlation of serpin expression levels and client/week in a) all HIV groups and b) HIV-resistant group. A comparison of serpin A1 levels and A3 levels in CVL of a) 232 women (HIV-R=84, HIV-P=66 and HIV-N=82) and b) 84 HIV-R group with the number of client per week was conducted using a nonparametric Spearman correlation test (at 95% confidence intervals), where p values <0.05 were deemed significant.

14.4.4. Relationship of serpin expression levels and condom use

Proper use of condoms can prevent sexually transmitted diseases (HIV, Syphilis, gonorrhea) and unwanted pregnancy. Seminal fluid contains many protein factors and can impact the mucosal environment of the female genital tract (201). For these reasons it is possible that seminal exposure may impact mucosal serpin expression levels. Therefore the reported frequency of condom usage was used as a measure of cumulative seminal exposure and compared to serpin levels. Serpin A1 and A3 expression levels were compared with number of condom use per week first in all groups (irrespective of HIV status) and then in HIV-resistant group only. Figure 13a showed that serpin A1 and A3 expression levels in the total group did not associate with condom usage ($p=0.2798$, 0.5491 respectively). Serpin A1 and A3 expression levels versus condom usage in HIV-resistant women also revealed a lack of association (A1 $p=0.2496$ and A3 $p=0.1744$) (Figure13b).

a)



b)

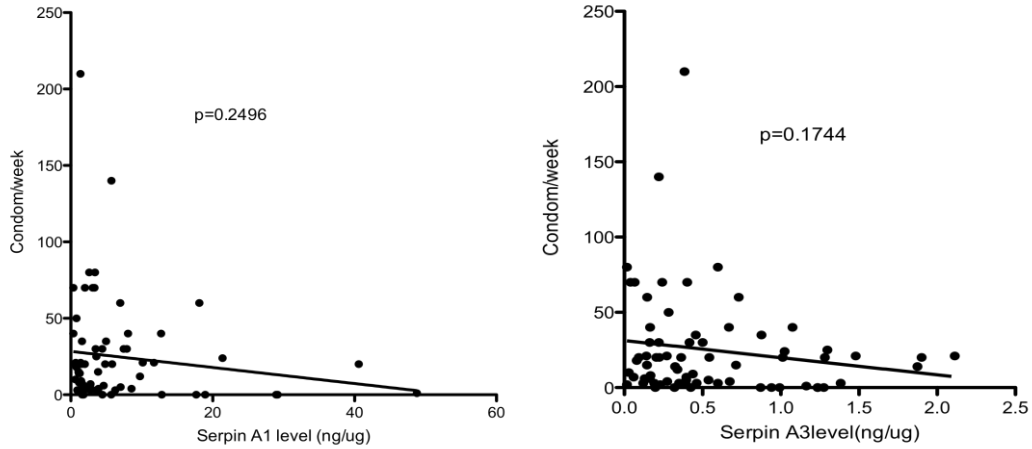
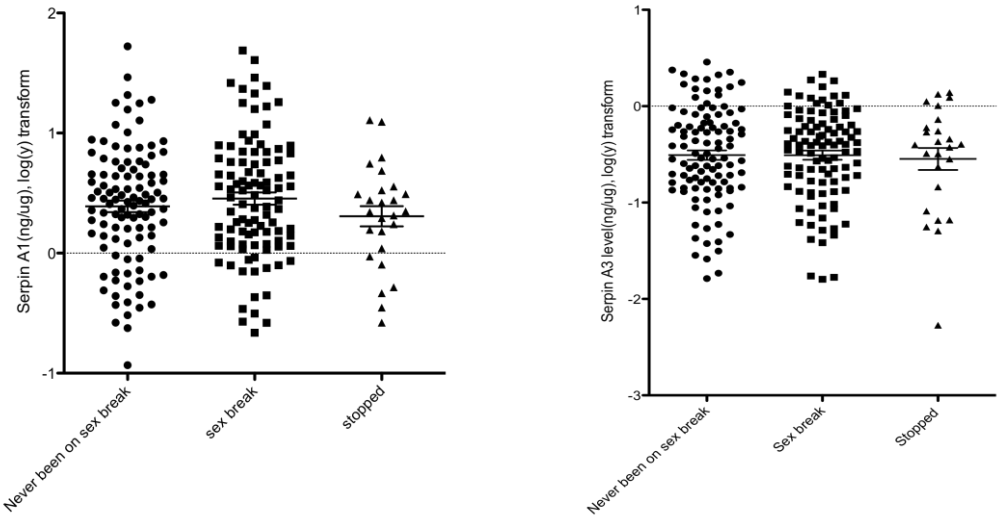


Figure13: Correlation of serpin expression levels and condoms/week in a) all HIV groups and b) HIV-resistant group. Association of serpin A1 and A3 expression levels in CVL from a) 232 commercial sex workers and b) 84 HIV-resistant women with self reported condom use per week was calculated by a nonparametric Spearman correlation test (at 95% confidence intervals), where p values <0.05 were deemed significant.

14.4.5. Relationship of serpin expression levels and breaks from sex work

Sex workers from the Pumwani cohort sometimes take temporary breaks like 2 weeks to 6 months due to various reasons (illness, moving from one place to another, starting a business). Some stopped sex work due to age or to get married. We also found some women who did not take any breaks in the year preceding this study. For this part of the study we focused only on previous year's data from the date of sample taken. To investigate whether serpin levels vary with a change in sexual activity, women from this cohort were divided into three groups: i) 106 women who did not take any break in a year, ii) 100 women who took a short break (2 weeks to 6 months) and iii) 26 women who stopped sex work. Figure 14a showed no significant difference between all HIV groups (A1; p=0.67, A3; p=0.99). In the HIV-resistant group the breakdown was: i) 30 women did not take any break in a year, ii) 38 women took a short break and iii) 16 women who stopped sex work. Figure 14b also showed a lack of difference of serpin levels in HIV-resistant individuals (serpin A1, p= 0.67; A3 p=0.54) and therefore, sex breaks did not have any effects on serpin levels. Horizontal bars represent mean levels +/- the standard error of the mean.

a)



b)

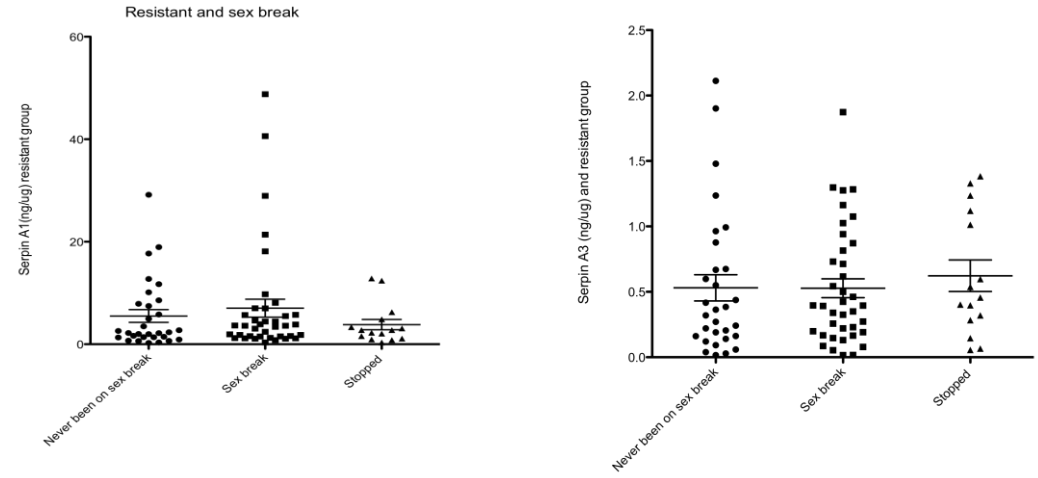


Figure14: Correlation of serpins expression levels and sex break in a) all groups and b) HIV-Resistant group. a) Serpin A1 and A3 levels in 232 CVLs were categorized into three groups to determine the impact of sex break on serpin expression levels: Never been on a sex break (n=106), had a sex break (n=100) and stopped sex work (n=26). **b)** In figure **b**, serpins from 84 HIV-R women were also divided into: Never been on a sex break (n=30), had a sex break (n=38) and stopped sex work (n=16). Both Kruskal-Wallis

(1-way Anova) and unpaired two-tailed Mann-Whitney statistical tests were performed to assess expression levels between groups (95% confidence level, or $p < 0.05$).

14.4.6. Relationship of serpin expression levels and concurrent infections in CVLs

In view of the fact that the women from this cohort are sex workers and exposed to countless STIs and non-STIs that may alter their inflammatory response, it was necessary to examine concurrent infection and inflammation of the FGT had any influence on serpinA1 and A3 levels. STIs that were selected for this study included: any symptomatic HSV-2 infections and non-STIs included: pelvic inflammatory disease (PID), vulvovaginitis, bacterial vaginosis, cervicitis, or candidiasis. The women with urinary tract infection (UTI) were also added to this study to find out if serpin had any role with UTI. Comparison of serpins levels in CVL from 232 women divided into i) 184 women with no history of infections at the time of sample collection, ii) 25 women with STIs and non-STIs and iii) 23 women with UTI in figure 15a showed no difference in between groups and indicated that the concurrent infections in the female genital tract were not likely to have affects on serpin levels (A1 p=0.4031, A3 p=0.1244). When compared in the HIV-resistant group (i) 68 women with no history of infections at the time of sample collection, ii) 8 women with STIs and non-STIs and iii) 8 women with UTI), further lack of association was noticed in figure 15b (Serpin A1 p=0.2720, A3 p=0.2275). Both Kruskal-Wallis (1-way Anova) and unpaired two-tailed Mann-Whitney statistical tests were performed to assess expression differences between groups (95% confidence level, or $p < 0.05$). Horizontal bars represent mean levels +/- the standard error of the mean.

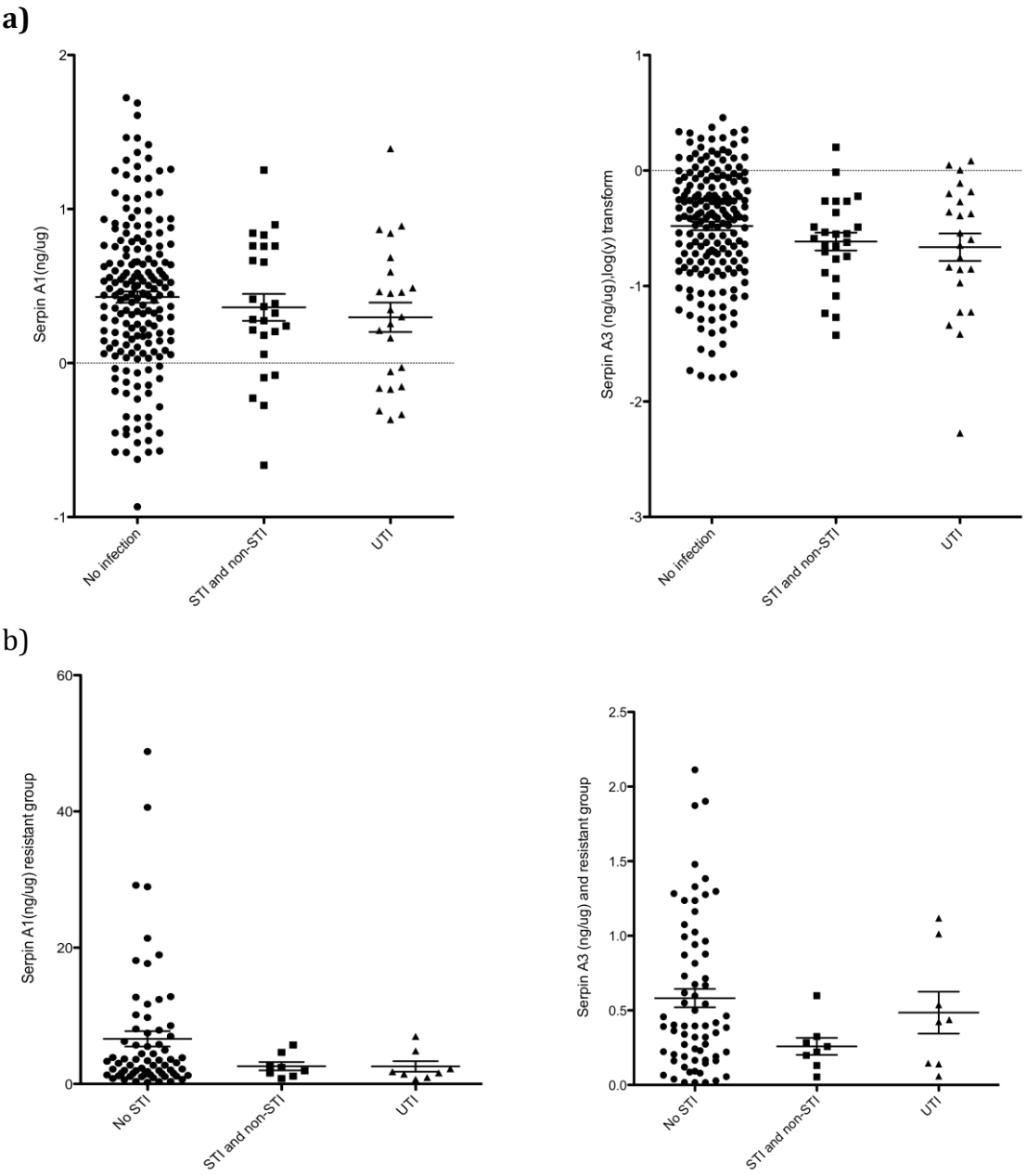


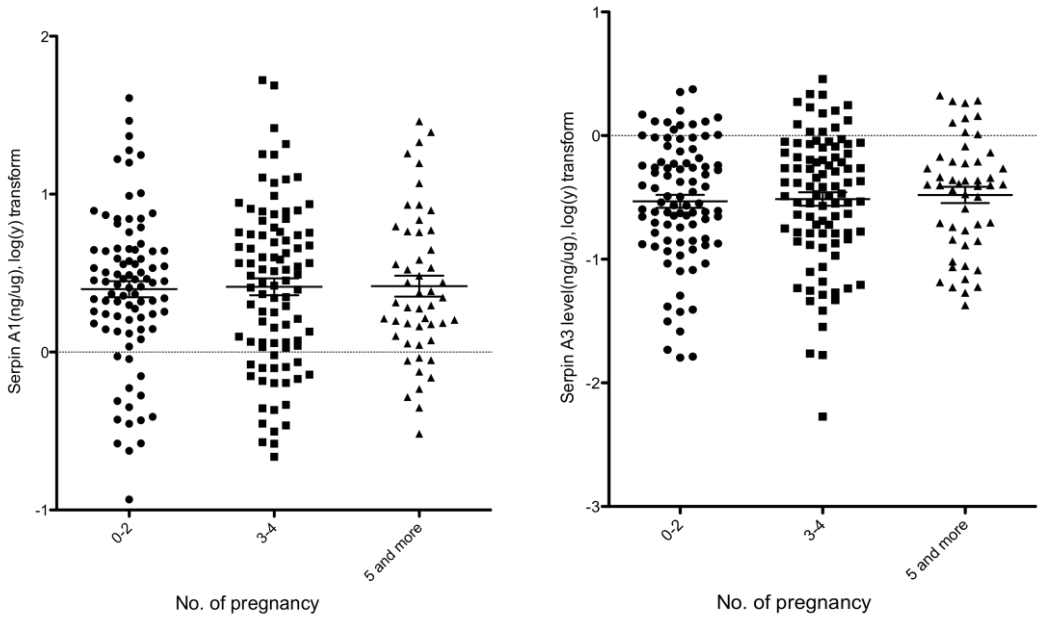
Figure 15: Correlation of serpins and concurrent infections in a) all HIV groups and b) HIV-resistant group a) Serpins in CVL from 232 women were grouped into: 184 women with no history of infections at the time of sample collection, 25 women with STIs and non-STIs and 23 women with UTI and compared to determine the effects of

infections on serpin expression levels. **b)** In HIV-R group, serpins expression levels from 68 women with no history of infections at the time of sample collection, 8 women with STIs and non-STIs and 8 women with UTI serpin levels were compared by Kruskal-Wallis (1-way Anova) and unpaired two-tailed Mann-Whitney statistical tests (95% confidence level, or $p < 0.05$). Horizontal bars represent mean levels +/- the standard error of the mean.

14.4.7. Relationship of serpin expression levels and number of pregnancies

During pregnancy, numerous changes occur to protect the uterus and prevent infections and it seems that pregnancy results in a pre-inflammatory or anti-inflammatory state depending on the period of gestation (7). Therefore, it is possible for an alteration to the FGT with an increased number of pregnancies. To investigate whether the number of pregnancies had any effect on serpin A1 and A3 levels, 232 women were grouped into three groups: i) women with no history of pregnancy or with 1 or 2 pregnancies (n=89), ii) women who were pregnant for 3-4 times (n=93), and iii) women with 5 or more pregnancies (n=50). Here the number of pregnancies applied only to how many times they got pregnant but not on the outcome of the pregnancy (some turned into full term pregnancy, some into still birth, abortion). Figure 16a showed no difference in serpin A1 ($p=0.9722$) and A3 levels ($p=0.8912$) in these groups and excludes the possibility of elevated level of serpins due to number of pregnancy. We also looked at HIV-resistant group only, where i) 26 women with no history of pregnancy or with 1 or 2 pregnancies, ii) 34 women who got pregnant 3-4 times and iii) 24 women with 5 or more pregnancies were included for this study. Serpin A1 ($p=0.6896$) and A3 ($p=0.3228$) revealed a lack of association (Figure 16b). Horizontal bars represent mean levels +/- the standard error of the mean.

a)



b)

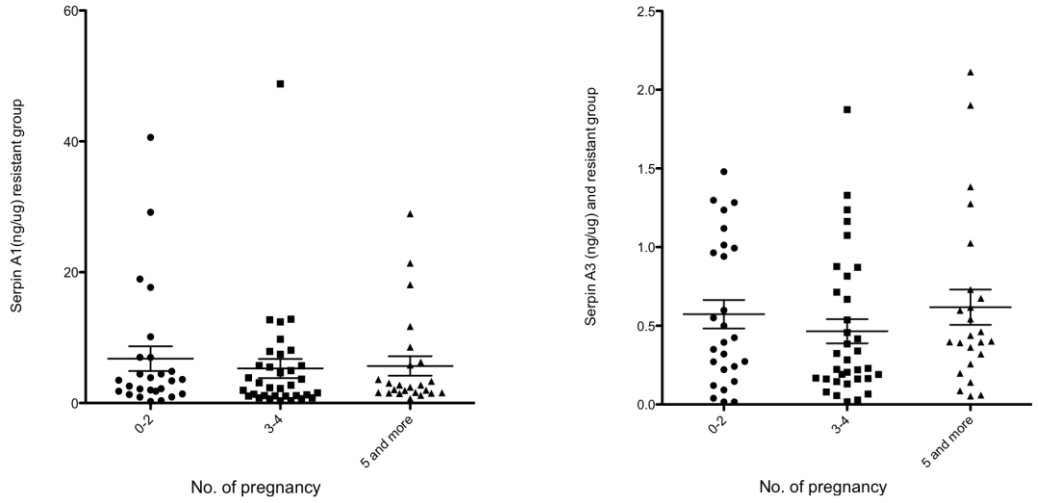


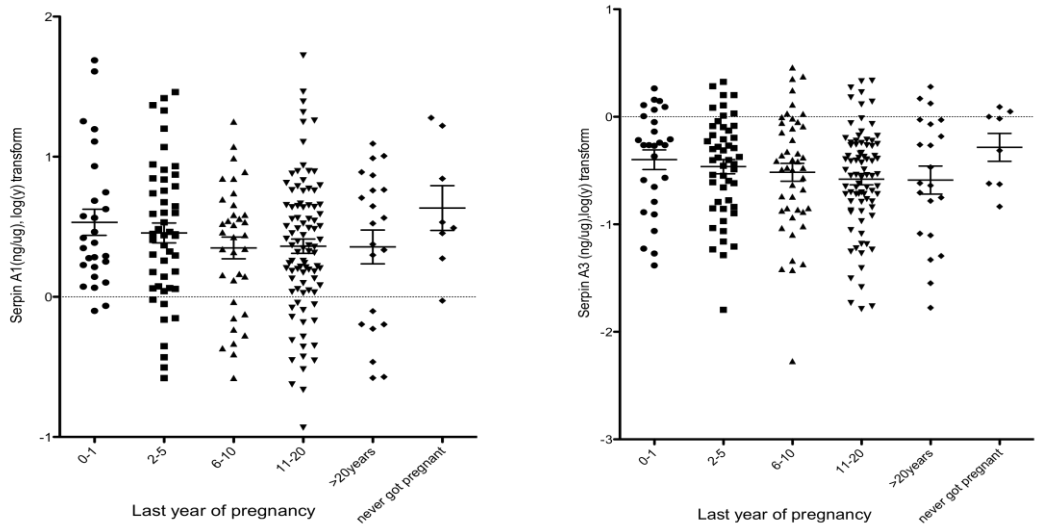
Figure 16: Correlation of serpin expression levels and number of pregnancies in a) all groups and b) HIV-resistant group. Comparison of serpin A1 and A3 levels a) in 232 CVL samples divided into: women with no history of pregnancy or with 1 or 2 pregnancies (n=89), women who were pregnant for 3-4 times (n=93), and women with 5 or more pregnancies (n=50) and b) in 84 HIV-resistant women divided into: 26 women

with no history of pregnancy or with 1 or 2 pregnancies, 34 women who got pregnant 3-4 times and 24 women with 5 or more pregnancies was conducted to find out the effects of number of pregnancies on serpin levels. Both the Kruskal-Wallis (1-way Anova) and unpaired two-tailed Mann-Whitney statistical tests were performed to assess expression differences between the groups (95% confidence level, or $p < 0.05$).

14.4.8. Relationship of serpins levels and last year of pregnancy

To investigate further whether the changes of uterus, cervix and vagina after the pregnancy were related to elevated serpin A1 and A3 levels, 232 women from the cohort were divided into six groups according to the number of their last year of pregnancy. The groups included: i) women who were currently pregnant or had a baby 1 year earlier (n=27), ii) women who were pregnant 2-5 years earlier (n=49), iii) women who had their pregnancy 6-10 years earlier (n=35), iv) women who were pregnant 10-20 years earlier (n=92), v) women who got pregnant 20 years earlier (n=21) and vi) those who never got pregnant (n=8). Figure 17a showed no significant difference in the serpin A1 (p= 0.6264) and A3 levels (p=0.3103) in these groups. In HIV-resistant group, everybody had a history of getting pregnant. So we divided them into five groups instead of six. We found i) 8 women from the resistant group who were pregnant at the time of sample collection or had a baby 1 year earlier, ii) 20 women who were pregnant 2-5 years earlier, iii) 12 women who had their pregnancy 6-10 years earlier, iv) 28 women who had their pregnancy 10-20 years earlier and v) 11 women who got pregnant 20 years earlier. The serpin A1 (p=0.6602) and A3 levels (p=0.5515) in the HIV-resistant group did not show any significant difference between groups. Horizontal bars represent mean levels +/- the standard error of the mean.

a)



b)

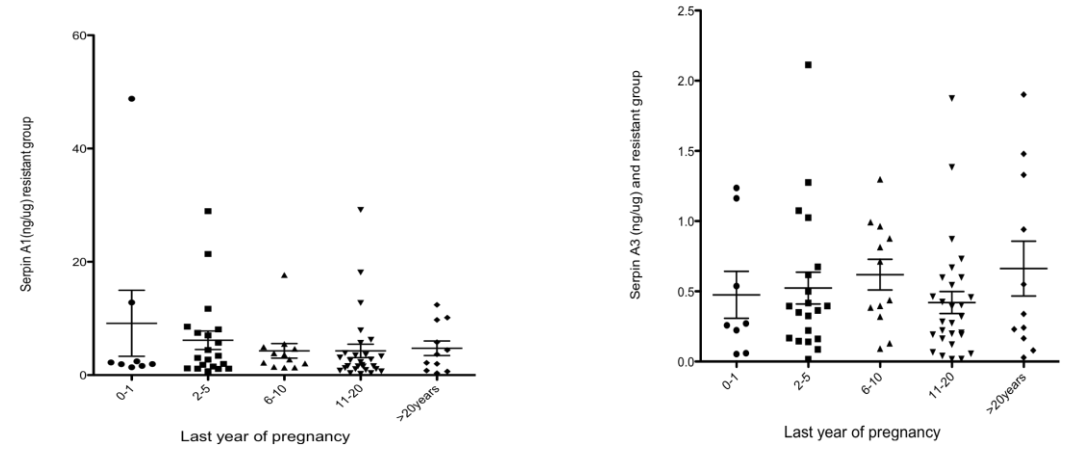


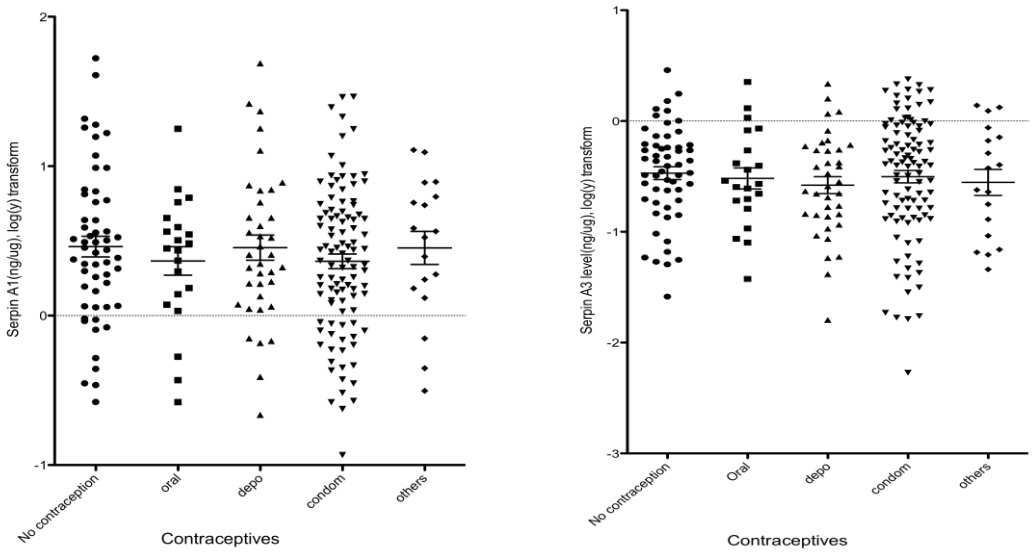
Figure 17: Correlation of serpins expression levels and last year of pregnancy in a) all HIV- groups and b) HIV- resistant group. a) Serpin A1 and A3 levels from 84 HIV-R, 66 HIV-P and 82 HIV-N individuals were divided into six groups based on the last year of pregnancy to assess the effects on serpin expression levels by using the Kruskal-Wallis (1-way Anova) and unpaired two-tailed Mann-Whitney statistical tests (95% confidence level, or $p < 0.05$). **b)** 84 women from HIV-R group were divided into

five groups and serpin A1 and A3 levels were compared to see the effects on serpin expression levels by the changes of FGT after last pregnancy.

14.4.9. Relationship of serpins levels and contraception

Women use many forms of contraception that modify hormonal cycles and alter many constituents of the genital immune system (202). Therefore, the relationship between serpin levels in CVL and different forms of contraceptives used by the women of the cohort was evaluated. The types of contraception that were considered for this study included: i) 21 women were on oral contraceptive pill (OCP) that comes in a combined form containing estrogen and progesterone or a progesterone only pill. Their main function is to suppress ovulation by keeping these hormones above normal baseline level and inhibit luteinizing hormone (LH) and follicle stimulating hormone (FSH), ii) 37 women were on depovera which is an injection form of progesterone hormone that inhibits follicular maturation, and thinning of cervical mucosa and uterine lining, iii) 101 women were found to use condoms which is the barrier method that influences genital flora and immune system, iv) other methods like tubal ligation, intrauterine devices (IUD), and herbal methods were placed under the "other" group (n=18). v) 55 women were found who did not use any type of contraception. Figure 18a revealed no difference in serpins levels (A1 p=0.8514, A3 p=0.7426) because of different forms of contraceptives. In HIV-resistant women, there was no significant difference of serpin levels in five groups (A1 p=0.4443, A3 p=0.0969) (Figure 18b).

a)



b)

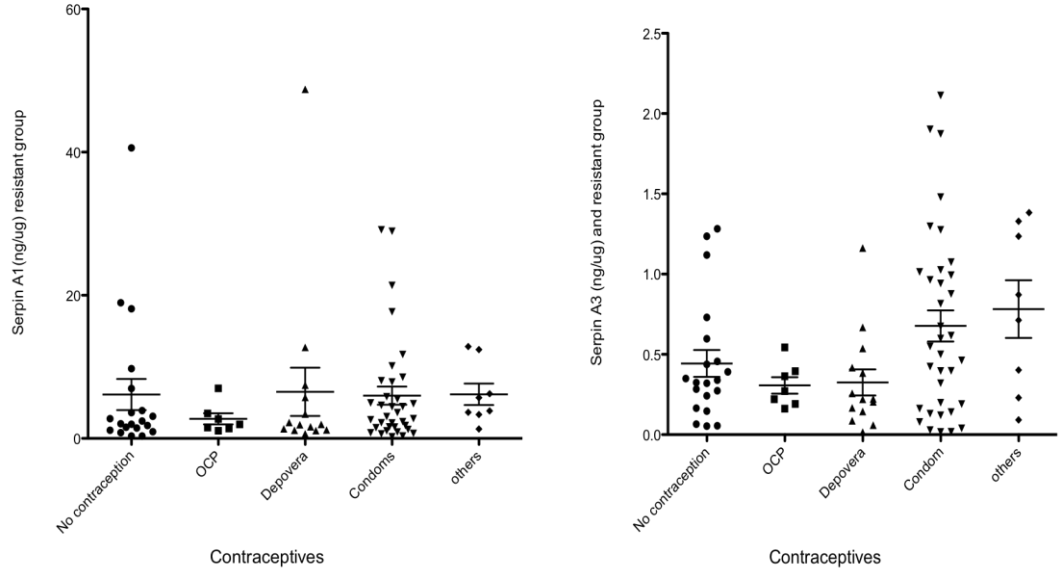


Figure 18: Correlation of serpins expression levels and contraception in a) all HIV-groups and b) in HIV-resistant group. a) Comparison of serpin A1 and A3 levels from 232 women divided into five groups based on contraceptive use (no contraception user (n=55); OCP user (n= 21); depovera user (n=37); condom user (n=101), and other contraceptive user (n=18)) was conducted by using the Kruskal-Wallis (1-way Anova)

and unpaired two-tailed Mann-Whitney statistical tests (95% confidence level), where p values <0.05 were deemed significant. **b)** Serpin levels from 84 HIV-resistant women were also grouped into: 20 women who did not use any contraception, 7 women were on oral contraceptive pill, 14 women used depovera, 35 women used condoms and 8 women used other methods like tubal ligation, intrauterine devices (IUD), and herbal methods and the Kruskal-Wallis (1-way Anova) and unpaired two-tailed Mann-Whitney statistical tests were performed to see if contraception use had any effects on serpin expression levels. Horizontal bars represent mean levels +/- the standard error of the mean.

14.4.10. Relationship of serpin expression levels and menopause

There are systemic and local hormonal changes that take place during menopause (7). Owing to the fact that estrogen levels decrease and that there are changes in the genital immune functions due to these hormonal changes, it was necessary to investigate whether serpin A1 and A3 levels in the CVL differed due to menopausal status. Unpaired two-tailed Mann-Whitney statistical test was performed to assess expression differences between the groups (95% confidence level, or $p < 0.05$). A comparison of serpin A1 ($p=0.1124$) and A3 levels ($p=0.0879$) in premenopausal ($n=202$) and menopausal women ($n=30$) from 232 women in the cohort reveals no significant difference (figure 19a). When we investigated the serpin levels in resistant women only we found 66 premenopausal and 18 menopausal women. Serpin A1 ($p=0.1177$) and A3 levels ($p=0.1311$) showed no significant differences between the two groups (figure 19b).

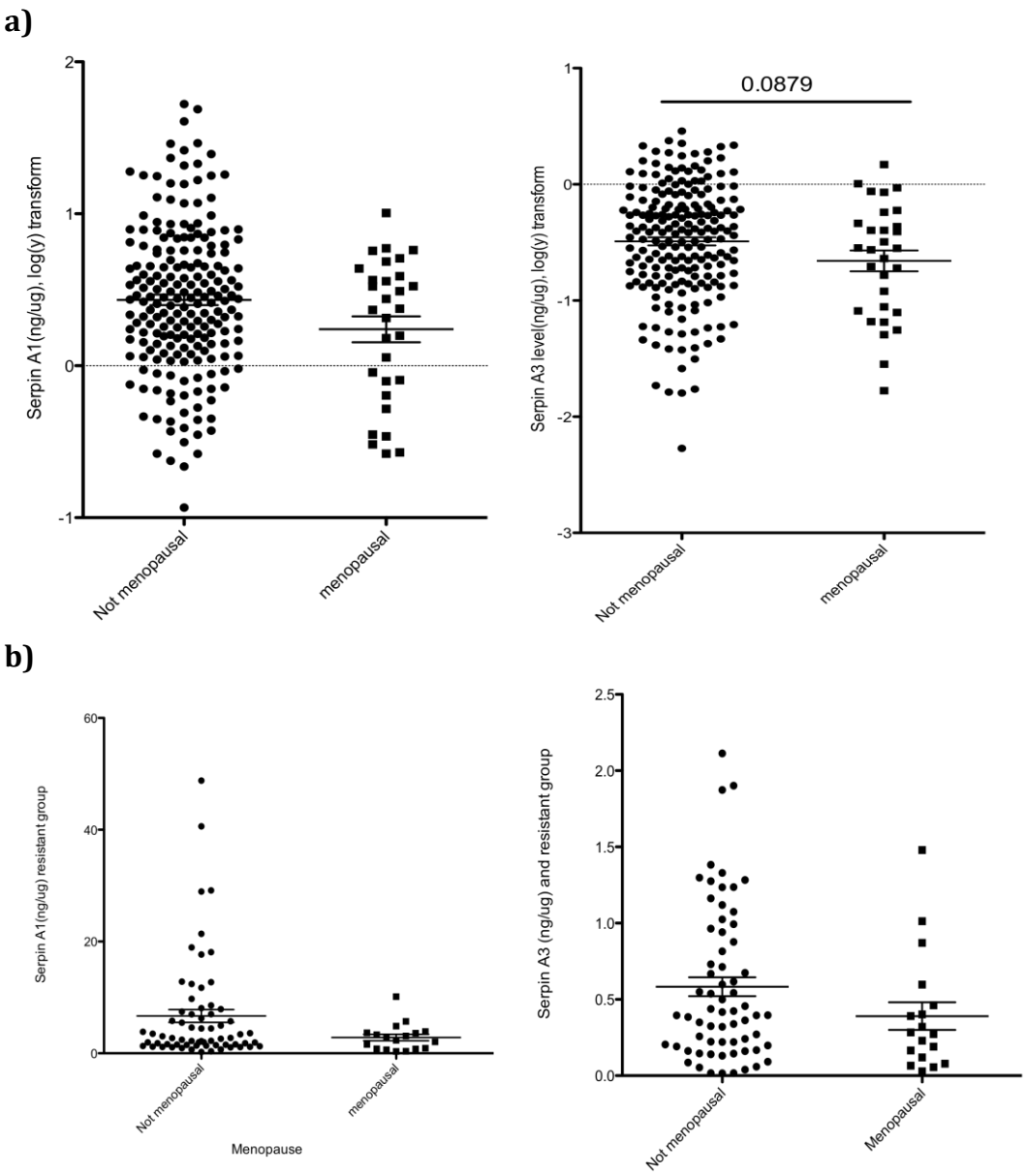


Figure 19: Correlation of serpin expression levels and menopause a) in all HIV groups and b) in HIV-resistant group. Unpaired two-tailed Mann-Whitney statistical test was performed to assess expression differences of serpin A and A3 between **a)** 202 pre-menopausal and 30 post-menopausal women from HIV-R, HIV-P and HIV-N groups and **b)** 66 premenopausal and 18 postmenopausal women from the HIV-R group.

14.4.11. Relationship of serpin expression levels and the menstrual cycle:

Different phases of the menstrual cycle are regulated by different sex hormones and many immunologic changes occur in the genital mucosa due to these hormonal changes. Cervico- vaginal secretion analysis reveals that antimicrobials including SLPI, human β-defensin-2, lactoferrin, chemokine and cytokine (IL-6 and IL-8) drop significantly at the day 13 (beginning of secretory phase) and remain low for 7-10 days and then rise at the proliferative phase starting from menstruation (7, 203). To investigate whether these hormonal changes during the menstrual cycle had any effect on serpin levels, 214 women were divided into four groups: i) 27 women were on oral contraceptive pills (OCP) and were not having any menstruation, ii) 74 women were on proliferative phase, iii) 85 women were on secretory phase and iv) 28 women were menopausal. Both Kruskal-Wallis (1-way Anova) and unpaired two-tailed Mann-Whitney statistical tests were performed to assess expression differences between groups (95% confidence level, or $p < 0.05$). Figure 20(a) showed that serpin A1 levels were significantly high in the proliferative phase when compared with women who were on OCP and not having any menstruation ($p=0.0229^*$), women in secretory phase ($p=0.0275^*$) and women who were menopausal ($p=0.0135^*$). There was no significant difference between the other groups. On the other hand, serpin A3 level in proliferative phase was higher than the menopausal women ($p= 0.0490^*$). Serpin A3 level was also tended to be higher in proliferative phase ($p=0.0546$) and in secretory phase ($p=0.0878$) than women who were on OCP and not having any menstruation. The levels were also tended to be high in secretory phase when compared with the menopausal women ($p=$

0.0809). There was no significant difference in the serpin A3 level between proliferative and secretory phases of menstrual cycle.

In the HIV-resistant group, 11 women were on oral contraceptive pills (OCP) and were not having any menstruation, 25 women were on proliferative phase, 30 women were on secretory phase and 17 women were menopausal. In figure 20b, serpin A1 levels tended to be higher in the proliferative phase when compared with the secretory phase (p= 0.0832), but reached the significance level when compared with menopausal group (0.0211*). Serpin A3 levels were increased significantly in the proliferative phase when compared t with those who were on OCP and not having any regular menses (p=0.0214*) and the menopausal group (p=0.0258*).

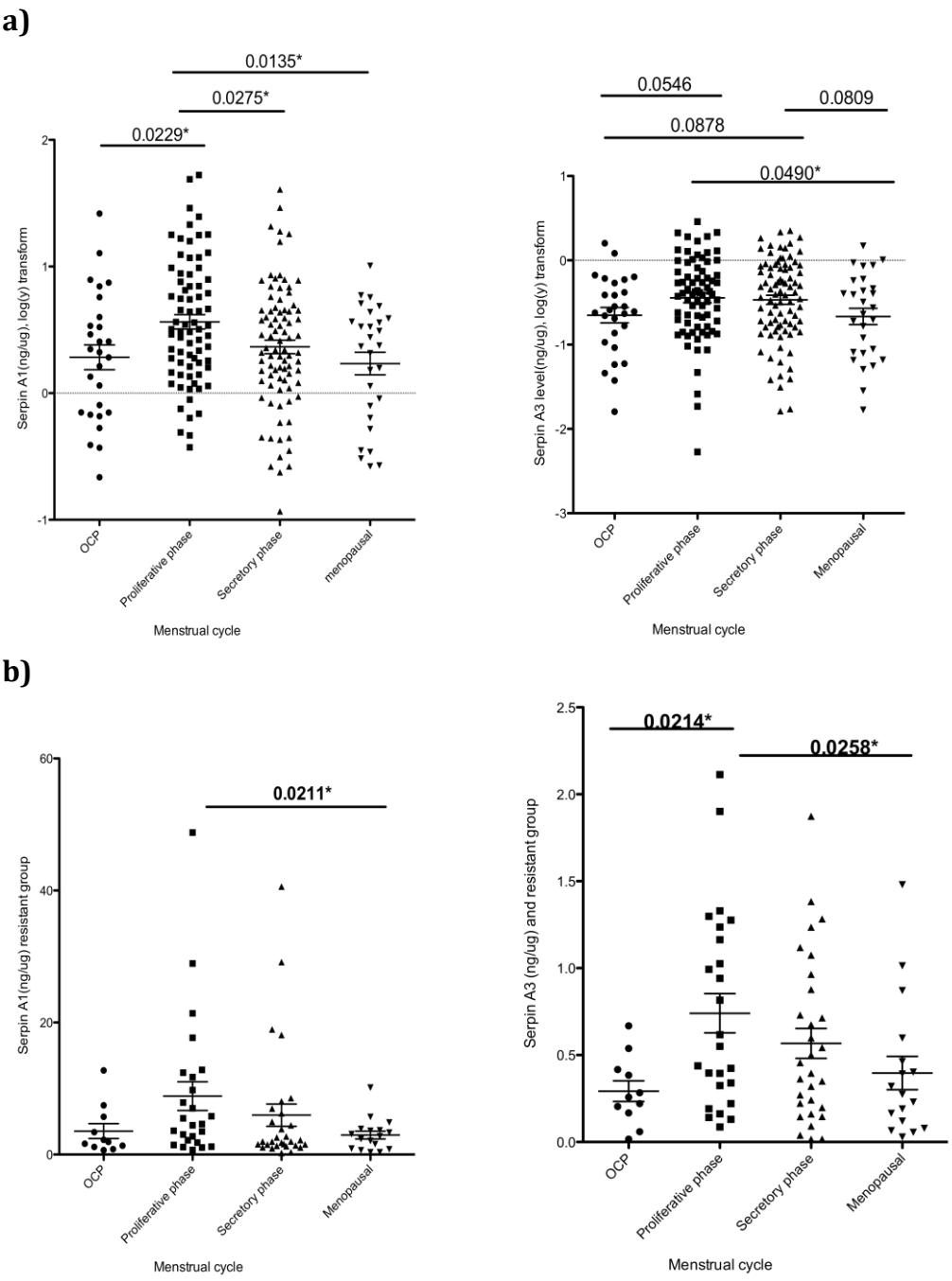


Figure 20: Correlation of serpin expression levels in CVL and menstrual cycle in a) all HIV groups and b) HIV-resistant group: To assess the impact of menstrual cycles on serpin expression levels, serpin A1 and A3 levels from all HIV groups were divided into four classes based on menstrual cycle: women were on oral contraceptive pills (OCP)

and were not having any menstruation (n=27), women were on proliferative phase (n=74), women were on secretory phase (n=85) and menopausal women (n=28) (figure 20a). In figure 20b, 84 women from HIV-resistant group were divided into: 11 women were on oral contraceptive pills (OCP) and were not having any menstruation, 25 women were on proliferative phase, 30 women were on secretory phase and 17 women were menopausal and compared to evaluate whether the variation of serpin expressions was due to the menstrual cycle. Both the Kruskal-Wallis (1-way Anova) and unpaired two-tailed Mann-Whitney statistical tests were performed to assess expression differences between groups (95% confidence level, or $p < 0.05$).

15. Discussion

The global HIV/AIDS epidemic has to date accounted for approximately 25 million deaths (7). Therefore, it is an urgent need to develop new strategies to prevent HIV-1 infection. Owing to the fact that only 1 in 1000 per coital act results in male to female HIV transmission, there is hope for HIV prevention by identifying factors that protect against HIV-1 infection via mucosal surfaces.

The global hypothesis for this thesis "the levels of clade A serpins are elevated in the cervical mucosa of highly HIV-1 exposed seronegative (HESN) women and that these levels correlate to HIV-1 resistance" was validated after examination of serpin expression levels in the cervical secretions of HIV-resistant sex workers. Serpin A1 expression levels were significantly higher in the HIV-resistant group than HIV-N<7 years HIV exposure group ($p=0.0470^*$) but did not differ with HIV-positive and HIV-N>7 years HIV exposure group. However, our hypothesis was not validated with respect to serpin A3. Serpin A3 levels were at the ranges from 2-4 μg/ml and were not significantly different between the resistant and control groups. However, as serpin A3 has known inhibitory functions against HIV-1 it does not eliminate the possibility that it might be synergistically involved with other antivirals found in CVL. We have determined the serpin A1 level in HIV-resistant women to be in the 20-200 μg/ml range which falls within the range that is physiologically relevant against HIV-1 infection. For serpin A1 10% inhibition was observed at 50 μg/ml and 50% at 500 μg/ml in both primary T-cell and dendritic cells (195). The levels of serpin A1 we found is in the concentration range capable of affecting HIV-1 replication partially based on *in vitro* studies. This supports a role for Serpin A1 in host defense against HIV infection.

Serpins or serine protease inhibitors are one of the key mediators of immune activation. They are relatively large molecule found in many cells types including epithelial/endothelial cells and a wide variety of immune cells. Their main function is to regulate the function of serine (elastage, cathepsin G, granzymes) and cysteine (caspase and papain) proteases (171). The serine proteases are released from neutrophils and cytotoxic T lymphocytes following bacterial invasion and protect the body by activating immune functions including complement activation, enhancement of endothelial-leukocyte interactions, and the secretion of inflammatory mediators, to propagate this process (173, 204). But the function of these proteases must be balanced because an imbalance can lead to severe consequences like severe inflammation, tissue damage and disease like emphysema and cystic fibrosis (171, 197). Serpin A1 inhibits elastase and chymotrypsin like proteases and prevents elastase-induced lung tissue damage. Knock out mice studies have shown that serpins are important immune regulators during bacterial infection by abrogating protease mediated tissue damage (174). Serpin A1 (α_2-antitrypsin) is also essential for regulation of hemostasis and fibrinolysis which are the biological processes necessary for proper blood flow (180).

In vitro studies of serpin A1 revealed that it can inhibit HIV transmission by inhibiting the proteases that are necessary for receptor binding (192), and the HIV LTR driven transcription process (193). The C terminal 26-residue of serpin A1 were found to increase STAT1 phosphorylation and reduce viral expression in an HIV -1 infectected monocytic cell line (193). NFκB is a major activator of HIV transcription and serpin A1 can inhibit NFκB activation through altered ubiquitination (195). Serpin A1 inhibits pro-inflammatory cytokines and increases anti-inflammatory cytokines and acts as an

important inflammatory modifier (174). A recent cross-sectional study confirmed the significantly lower serum serpin A1 levels in HIV infected patients compared to healthy individuals (196). Serpin A1 also has immunoregulatory properties that are independent of protease inhibition. It can inhibit LPS/endotoxin induced TNF-α expression and enhances IL-10 expression in monocytes, and neutralizes LPS mediated over-activation of monocytes via regulation of CD14 expression (188).

Inhibition of proteases may be one mechanism of protective effects against HIV-infection in the genital tract. With respect to HIV-1, proteases such as cathepsins and elastase can enhance infectivity by acting as chemo attractants for neutrophils and macrophages that are HIV-target cells (198). They can also cleave/inactivate anti-viral factors (RANTES, MIP-3β, and SLPI) (199), enhance infection of target cells (CD4+ T cells and macrophages) (205), promote pro-inflammatory cytokine expression (such as TNF-α and IL-1β), and impair wound healing (179). Many of the serpins are inhibitors of these proteases, and can abrogate these processes, such as serpin A1, A3, B1, B4 and B13 (174, 190, 192, 206) and they were found overabundant in HIV-resistant women (14, 102). Overall, these serpins may work synergistically by reducing effects mediated by excess proteases, such as inhibiting antiviral responses, degrading of the epithelial barrier, or stimulating inflammatory pathways that could contribute to reduced HIV transmission. Reduced immune activation in the female genital tract has been found to protect from HIV-infection in animal models and higher level of these serpins may be performing this role by preventing inflammation events (207).

In tissues and biological fluids, serpins adopt variant conformations: native inhibitory, non-inhibitor forms such as cleaved, polymerised and oxidised and inactivated

or latent forms due to the physical properties, chemical reactivity, and molecular structure of serpins (208). In our Western blot (WB) analysis of serpins, we found several bands with different intensities that may be representing the cleaved/isoform variation of serpins. As we performed this WB to validate the expression of serpin concentration measured by ELISA, it was expected that ELISA assay might quantify all of these native and isoforms/cleaved forms of serpins. However, the volumetric analysis of band intensities did not match the serpin levels measured by ELISA. One of the possibilities was that WB antibodies might bind with more/different cleaved/isoforms of serpins than the two ELISA antibodies. Additionally, another caveat was the sample size that might not give the actual result. More works need to be done to be able to generate reagents, or assays to more precisely detect serpin A1/A3 and all their potential isoforms found in CVL samples.

We determined that serpin A1 and A3 levels were strongly correlated in CVL suggesting a common regulator of expression. However, we found serpin A1/A3 expression was compartment-specific, and we did not find any correlation between mucosal and plasma levels. This indicated that the mechanism of regulation of serpins in cervical mucosa might be different from the systemic compartment. This was further evidenced by a cytokine/chemokines study by Lajoie et al where cytokine/chemokine expression in the systemic compartment did not correlate with the mucosal compartment (209).

It is known that the immune system and protein expression weaken with age (210-211). In our study, women in HIV-resistant group were statistically older than the other groups ($p=0.0001^{***}$). The negative groups had the youngest women as they were

new enrolees into the Pumwani cohort. However, we did not find any significant association of serpin levels with age. This result is consistent with the study by Iqbal et al, which showed that age does not have any effect on the levels of elafin/trappin2 (12). Therefore expression of these innate factors may be independent of age and consistently expressed over time.

While each normal coital act can cause micro ulceration and breaking of vaginal mucosa (212), it was predicted that higher levels of inflammation-associated proteins would be observed in the CVL as they encounter unprotected sex. As serpins are acute phase proteins and increase during inflammation in plasma, one would expect to find higher serpin levels in women who are involve in sex work for a long period of time. Statistical analysis did not reveal any correlations between serpin levels and duration of prostitution, clients/day, clients/week, and condom usage per week. Also, sex workers who reported to be on a 'sex break' (a 2 weeks-6 months break from sex work) did not have statistically altered levels of serpin to those that were not on a sex break and those that completely stopped sex work, suggesting that these factors may be at constitutively higher levels prior to engaging in sex work, and therefore prior to HIV-1 exposure. However, some women from the group who stopped sex work were found to be having sex with their husband or boyfriend and therefore, could not discount the possibility that the women on sex break were engaged in sexual activity with other partners. Further follow-up with sex workers on prolonged sex-breaks may shed light on this issue.

The sex workers from this cohort are not only exposed to HIV virus but also Herpes simplex virus (HSV-2), Human papilloma virus (HPV), Neisseria gonorrhea, Chlamydia trachomatis, and many more through sexual intercourse. The presence of

these STIs can enhance the epithelial-secreted mediators that have antimicrobial and anti-inflammatory effects (7). We asked whether elevated level of serpins in CVL was due to the presence of concurrent infections. For this study, we also looked at other non-STIs like bacterial vaginosis, candidiasis as some studies showed that these infections contribute significantly to heterosexual HIV-transmission (213). Women who had any complaint of vulval itching, vaginal discharge, pelvic inflammatory disease (PID), vulvovaginitis, vaginosis or cervicitis, or candidiasis or UTI's revealed no difference in serpin levels. This eliminated the prospect that higher level of serpins observed in HIV resistant women were due to presence of STIs, non-STIs or UTI. This is contradictory to what we expected as serpins are acute phase proteins. One of the reasons why we may not have observed an increase in serpin levels is that the samples might have been collected when women were already on antibiotics and in recovery. Another reason could be that most of the infections reported here was vulvovaginitis with vulval itching or vaginal discharge. Vulvovaginis is a state of inflammation of vulva and vagina and presents with redness and itchiness of these areas as well as, in the form of vaginal discharge. The causative agents for this inflammation are Candida infection, substance like laundry soap, vaginal contraceptives, and poor hygiene (214). We are not sure whether the vulvovaginitis reported here was due to poor hygiene or douching practice (in these cases there would be no increase level of serpins) or was due to the presence of Candida infection in which case one would expect to see higher levels of serpins. Further investigation of CVL samples of women with known causative agent of STI (bacteria, virus or fungus) and who are not on antibiotics may help to determine the relationship of serpins and concurrent infections.

As mentioned earlier, antimicrobials in the FGT are regulated by sex hormones: estrogen and progesterone (203). The ovary produces estrogen and progesterone in a cyclical manner over the course of the menstrual cycle. Besides antimicrobials, the migration of immune cells (T lymphocytes, B lymphocytes, dendritic cells (DC) and macrophages/monocytes), cytokines and chemokines, and CTL activity are controlled by these sex hormones (203). Overall these hormones prepare the reproductive tract for fertilization and implantation by regulating the immune system in the uterus, fallopian tubes, cervix and vagina (215). Although pregnancy is considered a state of immune suppression and increases the risk of HIV acquisition (216), data from recent studies have proven otherwise. The protection of fetus and prevention of infection are the main priorities during the pregnancy and the trophoblast (the cellular component of the placenta) acts as an immune regulator by producing cytokines, chemokines and antimicrobials. Both α and β defensins are found in amniotic fluid, chorion, placenta and also in the mucous plug. SLPI and elafin levels are also elevated during pregnancy but SLPI is detected in deciduas, amniotic epithelium and in the cervical mucosal plugs whereas elafin is found in fetal membrane and placenta. Both SLPI and elafin are increased in pregnant CVLs but decrease in the presence of bacterial vaginosis and SLPI is found to be down-regulated in HIV infected pregnant women (7). In this cohort, CVL samples from pregnant women were not collected as only three women were pregnant at the time of collection. So it was not possible to measure serpin levels during pregnancy. However, we examined the effect of the number of past pregnancies upon serpin levels. Since other antimicrobials change during pregnancy, we hypothesized that multiple pregnancies may cause more physiological changes in the genital tract. We found no

significant difference in serpin levels with number of past pregnancies, or duration since last pregnancy, and excluded the possibility that these influence levels of serpins. However, we could not discount the possibility that they vary during active pregnancy.

The women in the Pumwani cohort take different types of contraceptives. The most common contraceptives are condoms, oral combined pill, depovera, whereas intrauterine device (IUD), tubal ligation and herbal medicine are uncommonly used. Some women do not use any kind of contraceptives due to menopause or cessation of sex work. Various contraceptives have different effects on the immune system and protein expression within the FGT depending on their preparation. Oral combined pills (OCP) contain both estrogen and progesterone whereas some oral pills only consist of progesterone. Oral hormonal pills mainly cease the menstrual cycle by suppressing the hypothalamus pituitary axis and ovulation. Progesterone only pills and depovera, which is an injection form of progesterone hormone, inhibit follicular maturation, and cause thinning of cervical mucosa and uterine lining. Condoms, which are a barrier method, can protect the vaginal and cervical environment from the immunogenic effect of semen and STIs (217). Condom usage is also linked to reduced immune activation (characterized by lower CD38 on CD4 T cells) when compared with healthy control subjects (218). Many of these contraceptives not only function to prevent pregnancy but also regulate body's immune system. Several in vitro and in vivo studies have shown that OCPs containing estrogen and progesterone have both pro- and anti-inflammatory effects. An increased level of estrogen can suppress the synthesis of TNF-α and IL-1β, up-regulate regulatory T-cell function and increases the levels of the anti-inflammatory cytokines IL-10 and IL-1ra and also IL-10-dependent total immunoglobulin production. On the other hand, in

low and high doses, progesterone decreases both pro- and anti-inflammatory cytokine productions (202). Progesterone based pills also increase cervico-vaginal HIV viral shedding and the number of inflammatory cells in the CVL (219). After reviewing all these facts, the effect of contraceptives on serpin levels was measured. No alteration of serpin levels were found in those who used different kinds of contraceptives and those who used none. We expected to see higher level of serpins in women who were taking hormonal contraceptives in the form of depovera or in oral form as these contraceptives can heighten STI risks and alter the vaginal microenvironment by decreasing the H_2O_2 producing lactobacillus (220). However, these hormonal contraceptives mainly contain progesterone to prevent pregnancies. Progesterone inhibits both pro and anti-inflammatory cytokines, which may decrease serpin levels as TNF-α, IL-1β, are the potent inducers of serpin in monocytes. Furthermore, monocytes are not only source and epithelial cells are also large producers of serpins, so inflammation may not be the only factor in regulating serpin levels. Our data corresponds with the elafin/trappin-2 study which also showed no relationship for this protein with contraceptives (12).

We also looked at pre-menopausal and post-menopausal women that reflect a hormonal effect on the female genital tract to assess the relationship to serpin expression. Menopause means permanent cessation of menstruation so there is no sex hormone production by ovaries and as a result there may be vaginal atrophy, vaginal itchiness or discharge and increased risk of candidiasis or urinary tract infection. The loss of estrogen due to menopause results in decreased TLR mediated function, low vaginal pH due to decreased lactobacilli, and loss of secretory antimicrobials (7). As a high estrogen level is immune stimulatory, we hypothesized that those women who were post-menopausal may

have lower level of serpin than women who were pre-menopausal. However, there was no statistical difference in the serpin A1 and A3 expression in menopausal women. Perhaps we did not observe differences between pre-menopausal and menopausal women because the estrogen levels were not significantly different. However, when we divided the groups according to menstrual cycle and examined those in the proliferative phase (high estrogen level) we saw differences of this phase with menopause. This may be due to selecting women who had high estrogen levels from the groups who were at secretory phase or menopausal or on oral pill and had low estrogen levels.

Every woman goes through the process of the menstrual cycle for reproduction that starts from age 8-16 and the cycle is under the control of endocrine system. The cycle is a 28 days cycle that is measured from the first day of menstruation and divided into three phases: menstruation (1-5 days), proliferative phase (5-14 days) and secretory phase (14-28 days). The proliferative phase is mainly regulated by estrogen whereas progesterone regulates the secretory phase. Many immune cells, cytokines, chemokines and antimicrobials are controlled by different phases of the menstrual cycle depending on the location of the genital tract. In culture media, estrogen has been shown to have stimulatory effects on antimicrobial (SLPI, elafin, human β-defensin) secretion by uterine epithelial cells but inhibits their secretion in vaginal squamous epithelium (203). On the other hand estrogen suppresses the cytokine and chemokine responses to TLR agonist and IL-1β in both uterine and vaginal epithelium (203). Cervico- vaginal secretion analysis revealed that antimicrobials including SLPI, human β-defensin-2, lactoferrin, chemokine and cytokine (IL-6 and IL-8) dropped significantly at the day 13 (beginning of secretory phase) and remain low for 7-10 days and then rise at proliferative phase starting

from menstruation (7, 203). In our study it was essential to determine how the menstrual cycle regulated serpin levels in the FGT. Serpin A1 level was found to be significantly higher in the proliferative phase than secretory phase whereas serpin A3 did not vary between menstrual phases. Both serpin levels were higher in the proliferative phase in non-menopausal compared to menopausal women implying that serpin levels might be regulated by estrogen. OCP can change the immune response in the genital tract and also causes cessation or irregular menstruation and comparison of serpin levels of this group with those who had normal menstrual cycle revealed serpin A1 levels to be higher for women in the proliferative phase in comparison to those who were OCP users. We also investigated the relationship of serpins with the menstrual cycle in resistant women. Here we found serpin A1 levels tended to be higher (but not significantly) in the proliferative phase over the secretory phase and reached the significant level when compared them with menopausal group. Serpin A3 levels were increased significantly in the proliferative phase when compared them with those who were on OCP and not having any regular menses and the menopausal group. These results indicate that serpin A1 levels but not A3 levels are increased during the proliferative phase possibly due to estrogen stimulation.

These results suggested that protection against HIV-1 infection at times during the menstrual cycle might vary as serpin A1 level increases during the proliferative phase but not during the secretory phase. Also postmenopausal women are more susceptible to HIV-1 infection as they have decreased levels of serpins. Not only serpins, but also, SLPI, Human β defensin 2, lactoferrin, chemokine and cytokine levels (IL-6 and IL-8) are also found to decrease significantly at mid-cycle and secretory phase and become high at proliferative phase (203). On the other hand, increased level of elafin is detected

in the human endometrium at the late secretory and menstrual phases of the menstrual cycle (221). This result also validated our hypothesis that serpin A1 is an important player in FGT immunity.

16. Conclusions and future directions

We believe that resistance to HIV is multi-factorial. It has been correlated with polymorphism of the IRF -1 gene (166), HIV specific IgA (110) and HIV specific cellular CD4 and CD8 immune responses (9). Anti-HIV factors like RANTES (10), CC-chemokines SLPI (11), Elafin/trappin-2 (12) and MIP-α/β (13) are also found to be elevated in HIV resistant women. By exploring all the correlates of protection and the exact mechanism how this relates to immune activation at the mucosal compartment responsible for the resistance; it might be possible to develop preventive strategies or vaccines against HIV.

Overall, the works completed in this thesis support the hypothesis that serpin A1 was correlated with HIV-resistance but that serpin A3 was not. Mucosal serpins were independent of clinical factors such as infections or number of pregnancy. Serpin levels were also not affected by sexual practices such as duration of sex work, client number, condom usage, or sex break in commercial sex workers. However, serpin expression was associated with the menstrual cycle and was elevated during the proliferative phase of menstrual cycle, suggesting that mucosal serpins may be regulated by hormones such as estrogen.

Elevated serpin A1 levels in HIV-resistant women may have a critical role at the front lines of defence during HIV-1 exposure by controlling inflammatory events and at the same time providing a more potent antiviral environment at mucosal surfaces. The implication of this is that it may reduce the risk of spreading infected founder populations in the sub-mucosa to the systemic compartment and allow time for adaptive responses to take control.

There remains a large gap in our understanding of how serpins would impact inflammation in genital tract tissue. Examining their impact in biologically relevant tissue models would help to evaluate serpin function upon inflammation in the genital tract. Both serpins A1 and A3 demonstrated anti-HIV activity in cell types that are present in female genital tract; therefore the logical next step would be to evaluate how these serpins affect HIV-1 replication in cervical tissue model. Future studies may also include measuring other serpins like B1, B4, B13, C and their association with HIV resistance. It may be useful to determine isoform expression differences of these serpins as cleavage products of serpin A1 (193) and C1 (222) are shown to have the most potent activity in HIV infection.

In this study, the multi- factorial nature of HIV-resistance highlights the important resistant factors that have been identified by multivariate analysis. Examining the complex interplay of each of these factors and how this balance contributes to HIV-resistance will increase our understanding of mucosal immunity. The hope is that this information will be useful to help design a female controlled prevention technology against HIV-infection.

17. References

1. UNAIDS. 2010. UNAIDS REPORT ON THE GLOBAL AIDS EPIDEMIC 2010.
2. Buve A, Bishikwabo-Nsarhaza K, Mutangadura G. 2002. The spread and effect of HIV-1 infection in sub-Saharan Africa. *Lancet* 359: 2011-7
3. Oguntibeju OO VSFVDHW. 2003. The HIV Epidemic: factors responsible for the epidemic and the impact of HIV/AIDS. *Rawal Medical journal* Volume 28, Number 2
4. Buve A. 2002. HIV epidemics in Africa: what explains the variations in HIV prevalence? *IUBMB Life* 53: 193-5
5. Oguntibeju OO, Esterhuyse AJ, Truter EJ. 2009. Microbicides: a possible prevention approach to HIV transmission among African women. *West Indian Med J* 58: 277-82
6. Gray RH, Wawer MJ, Brookmeyer R, Sewankambo NK, Serwadda D, Wabwire-Mangen F, Lutalo T, Li X, vanCott T, Quinn TC. 2001. Probability of HIV-1 transmission per coital act in monogamous, heterosexual, HIV-1-discordant couples in Rakai, Uganda. *Lancet* 357: 1149-53
7. Wira CR, Patel MV, Ghosh M, Mukura L, Fahey JV. 2011. Innate immunity in the human female reproductive tract: endocrine regulation of endogenous antimicrobial protection against HIV and other sexually transmitted infections. *Am J Reprod Immunol* 65: 196-211
8. Fowke KR, Nagelkerke NJ, Kimani J, Simonsen JN, Anzala AO, Bwayo JJ, MacDonald KS, Ngugi EN, Plummer FA. 1996. Resistance to HIV-1 infection among persistently seronegative prostitutes in Nairobi, Kenya. *Lancet* 348: 1347-51
9. Kaul R, Plummer FA, Kimani J, Dong T, Kiama P, Rostron T, Njagi E, MacDonald KS, Bwayo JJ, McMichael AJ, Rowland-Jones SL. 2000. HIV-1-specific mucosal CD8+ lymphocyte responses in the cervix of HIV-1-resistant prostitutes in Nairobi. *J Immunol* 164: 1602-11
10. Iqbal SM, Ball TB, Kimani J, Kiama P, Thottingal P, Embree JE, Fowke KR, Plummer FA. 2005. Elevated T cell counts and RANTES expression in the genital mucosa of HIV-1-resistant Kenyan commercial sex workers. *J Infect Dis* 192: 728-38
11. Borrow P, Shattock RJ, Vyakarnam A. 2010. Innate immunity against HIV: a priority target for HIV prevention research. *Retrovirology* 7: 84
12. Iqbal SM, Ball TB, Levinson P, Maranan L, Jaoko W, Wachihi C, Pak BJ, Podust VN, Broliden K, Hirbod T, Kaul R, Plummer FA. 2009. Elevated elafin/trappin-2 in the female genital tract is associated with protection against HIV acquisition. *Aids* 23: 1669-77
13. Fowke KR, Dong T, Rowland-Jones SL, Oyugi J, Rutherford WJ, Kimani J, Krausa P, Bwayo J, Simonsen JN, Shearer GM, Plummer FA. 1998. HIV type 1 resistance in Kenyan sex workers is not associated with altered cellular susceptibility to HIV type 1 infection or enhanced beta-chemokine production. *AIDS Res Hum Retroviruses* 14: 1521-30

14. Burgener A, Boutilier J, Wachihi C, Kimani J, Carpenter M, Westmacott G, Cheng K, Ball TB, Plummer F. 2008. Identification of differentially expressed proteins in the cervical mucosa of HIV-1-resistant sex workers. *J Proteome Res* 7: 4446-54
15. Smith JA, Daniel R. 2006. Following the path of the virus: the exploitation of host DNA repair mechanisms by retroviruses. *ACS Chem Biol* 1: 217-26
16. Brass AL, Dykxhoorn DM, Benita Y, Yan N, Engelman A, Xavier RJ, Lieberman J, Elledge SJ. 2008. Identification of host proteins required for HIV infection through a functional genomic screen. *Science* 319: 921-6
17. Guberski TD GD. 2006. *Clinical Challenges in HIV/AIDS: A Practice Handbook for Nurse Practitioners and Physician Assistants Cranbury*
18. Gilbert PB, McKeague IW, Eisen G, Mullins C, Gueye NA, Mboup S, Kanki PJ. 2003. Comparison of HIV-1 and HIV-2 infectivity from a prospective cohort study in Senegal. *Stat Med* 22: 573-93
19. Kosakovsky Pond SL, Smith DM. 2009. Are all subtypes created equal? The effectiveness of antiretroviral therapy against non-subtype B HIV-1. *Clin Infect Dis* 48: 1306-9
20. Poss M, Gosink J, Thomas E, Kreiss JK, Ndinya-Achola J, Mandaliya K, Bwayo J, Overbaugh J. 1997. Phylogenetic evaluation of Kenyan HIV type 1 isolates. *AIDS Res Hum Retroviruses* 13: 493-9
21. Watkins JD, Siddappa NB, Lakhashe SK, Humbert M, Sholukh A, Hemashettar G, Wong YL, Yoon JK, Wang W, Novembre FJ, Villinger F, Ibegbu C, Patel K, Corti D, Agatic G, Vanzetta F, Bianchi S, Heeney JL, Sallusto F, Lanzavecchia A, Ruprecht RM. 2011. An anti-HIV-1 V3 loop antibody fully protects cross-clade and elicits T-cell immunity in macaques mucosally challenged with an R5 clade C SHIV. *PLoS One* 6: e18207
22. Geretti AM, Harrison L, Green H, Sabin C, Hill T, Fearnhill E, Pillay D, Dunn D. 2009. Effect of HIV-1 subtype on virologic and immunologic response to starting highly active antiretroviral therapy. *Clin Infect Dis* 48: 1296-305
23. Gallo R, Wong-Staal F, Montagnier L, Haseltine WA, Yoshida M. 1988. HIV/HTLV gene nomenclature. *Nature* 333: 504
24. Freed EO. 2001. HIV-1 replication. *Somat Cell Mol Genet* 26: 13-33
25. Farrow MA, Sheehy AM. 2008. Vif and Apobec3G in the innate immune response to HIV: a tale of two proteins. *Future Microbiol* 3: 145-54
26. Majumder B, Venkatachari NJ, Schafer EA, Janket ML, Ayyavoo V. 2007. Dendritic cells infected with vpr-positive human immunodeficiency virus type 1 induce CD8+ T-cell apoptosis via upregulation of tumor necrosis factor alpha. *J Virol* 81: 7388-99
27. Miyagi E, Andrew AJ, Kao S, Strebel K. 2009. Vpu enhances HIV-1 virus release in the absence of Bst-2 cell surface down-modulation and intracellular depletion. *Proc Natl Acad Sci U S A* 106: 2868-73
28. Foster JL, Garcia JV. 2008. HIV-1 Nef: at the crossroads. *Retrovirology* 5: 84
29. Patterson S, English NR, Longhurst H, Balfe P, Helbert M, Pinching AJ, Knight SC. 1998. Analysis of human immunodeficiency virus type 1 (HIV-1) variants and levels of infection in dendritic and T cells from symptomatic HIV-1-infected patients. *J Gen Virol* 79 (Pt 2): 247-57

30. Goodenow MM, Collman RG. 2006. HIV-1 coreceptor preference is distinct from target cell tropism: a dual-parameter nomenclature to define viral phenotypes. *J Leukoc Biol* 80: 965-72
31. Coakley E, Petropoulos CJ, Whitcomb JM. 2005. Assessing chemokine co-receptor usage in HIV. *Curr Opin Infect Dis* 18: 9-15
32. Cho MW, Lee MK, Carney MC, Berson JF, Doms RW, Martin MA. 1998. Identification of determinants on a dualtropic human immunodeficiency virus type 1 envelope glycoprotein that confer usage of CXCR4. *J Virol* 72: 2509-15
33. Schuitemaker H, van 't Wout AB, Lusso P. 2011. Clinical significance of HIV-1 coreceptor usage. *J Transl Med* 9 Suppl 1: S5
34. Garrido C, Roulet V, Chueca N, Poveda E, Aguilera A, Skrabal K, Zahonero N, Carlos S, Garcia F, Faudon JL, Soriano V, de Mendoza C. 2008. Evaluation of eight different bioinformatics tools to predict viral tropism in different human immunodeficiency virus type 1 subtypes. *J Clin Microbiol* 46: 887-91
35. Melby T, Despirito M, Demasi R, Heilek-Snyder G, Greenberg ML, Graham N. 2006. HIV-1 coreceptor use in triple-class treatment-experienced patients: baseline prevalence, correlates, and relationship to enfuvirtide response. *J Infect Dis* 194: 238-46
36. Svicher V, Balestra E, Cento V, Sarmati L, Dori L, Vandenbroucke I, D'Arrigo R, Buonomini AR, Van Marck H, Surdo M, Saccomandi P, Mostmans W, Aerssens J, Aquaro S, Stuyver LJ, Andreoni M, Ceccherini-Silberstein F, Perno CF. 2011. HIV-1 dual/mixed tropic isolates show different genetic and phenotypic characteristics and response to maraviroc in vitro. *Antiviral Res* 90: 42-53
37. Bjorndal A, Deng H, Jansson M, Fiore JR, Colognesi C, Karlsson A, Albert J, Scarlatti G, Littman DR, Fenyo EM. 1997. Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. *J Virol* 71: 7478-87
38. van Rij RP, Blaak H, Visser JA, Brouwer M, Rientsma R, Broersen S, de Roda Husman AM, Schuitemaker H. 2000. Differential coreceptor expression allows for independent evolution of non-syncytium-inducing and syncytium-inducing HIV-1. *J Clin Invest* 106: 1569
39. Gorry PR, Ancuta P. 2011. Coreceptors and HIV-1 pathogenesis. *Curr HIV/AIDS Rep* 8: 45-53
40. Karlsson I, Grivel JC, Chen SS, Karlsson A, Albert J, Fenyo EM, Margolis LB. 2005. Differential pathogenesis of primary CCR5-using human immunodeficiency virus type 1 isolates in ex vivo human lymphoid tissue. *J Virol* 79: 11151-60
41. Samson M, Libert F, Doranz BJ, Rucker J, Liesnard C, Farber CM, Saragosti S, Lapoumeroulie C, Cognaux J, Forceille C, Muyldermans G, Verhofstede C, Burtonboy G, Georges M, Imai T, Rana S, Yi Y, Smyth RJ, Collman RG, Doms RW, Vassart G, Parmentier M. 1996. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* 382: 722-5
42. Keele BF, Giorgi EE, Salazar-Gonzalez JF, Decker JM, Pham KT, Salazar MG, Sun C, Grayson T, Wang S, Li H, Wei X, Jiang C, Kirchherr JL, Gao F, Anderson

JA, Ping LH, Swanstrom R, Tomaras GD, Blattner WA, Goepfert PA, Kilby JM, Saag MS, Delwart EL, Busch MP, Cohen MS, Montefiori DC, Haynes BF, Gaschen B, Athreya GS, Lee HY, Wood N, Seoighe C, Perelson AS, Bhattacharya T, Korber BT, Hahn BH, Shaw GM. 2008. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proc Natl Acad Sci U S A* 105: 7552-7

43. Margolis L, Shattock R. 2006. Selective transmission of CCR5-utilizing HIV-1: the 'gatekeeper' problem resolved? *Nat Rev Microbiol* 4: 312-7
44. Zheng YH, Lovsin N, Peterlin BM. 2005. Newly identified host factors modulate HIV replication. *Immunol Lett* 97: 225-34
45. Hiscott J, Kwon H, Genin P. 2001. Hostile takeovers: viral appropriation of the NF-kappaB pathway. *J Clin Invest* 107: 143-51
46. Dayton AI, Sodroski JG, Rosen CA, Goh WC, Haseltine WA. 1986. The trans-activator gene of the human T cell lymphotropic virus type III is required for replication. *Cell* 44: 941-7
47. Purcell DF, Martin MA. 1993. Alternative splicing of human immunodeficiency virus type 1 mRNA modulates viral protein expression, replication, and infectivity. *J Virol* 67: 6365-78
48. Hladik F, McElrath MJ. 2008. Setting the stage: host invasion by HIV. *Nat Rev Immunol* 8: 447-57
49. Sugaya M, Lore K, Koup RA, Douek DC, Blauvelt A. 2004. HIV-infected Langerhans cells preferentially transmit virus to proliferating autologous CD4+ memory T cells located within Langerhans cell-T cell clusters. *J Immunol* 172: 2219-24
50. Tsegaye TS, Pohlmann S. 2010. The multiple facets of HIV attachment to dendritic cell lectins. *Cell Microbiol* 12: 1553-61
51. Facts CHAM. 2008. . HIV and AIDS in the United States: A picture of today's Epidemic.
52. Munch J, Rucker E, Standker L, Adermann K, Goffinet C, Schindler M, Wildum S, Chinnadurai R, Rajan D, Specht A, Gimenez-Gallego G, Sanchez PC, Fowler DM, Koulov A, Kelly JW, Mothes W, Grivel JC, Margolis L, Keppler OT, Forssmann WG, Kirchhoff F. 2007. Semen-derived amyloid fibrils drastically enhance HIV infection. *Cell* 131: 1059-71
53. Nazli A, Chan O, Dobson-Belaire WN, Ouellet M, Tremblay MJ, Gray-Owen SD, Arsenault AL, Kaushic C. Exposure to HIV-1 directly impairs mucosal epithelial barrier integrity allowing microbial translocation. *PLoS Pathog* 6: e1000852
54. Anderson JA, Ping LH, Dibben O, Jabara CB, Arney L, Kincer L, Tang Y, Hobbs M, Hoffman I, Kazembe P, Jones CD, Borrow P, Fiscus S, Cohen MS, Swanstrom R. 2010. HIV-1 Populations in Semen Arise through Multiple Mechanisms. *PLoS Pathog* 6: e1001053
55. Auvert B, Taljaard D, Lagarde E, Sobngwi-Tambekou J, Sitta R, Puren A. 2005. Randomized, controlled intervention trial of male circumcision for reduction of HIV infection risk: the ANRS 1265 Trial. *PLoS Med* 2: e298

56. Service CfDCaPAUDoHaH, http://www.cdc.gov.proxy1.lib.umanitoba.ca/hiv/topics/surveillance/resources/reports/. *HIV/AIDS Surveill Rep 2007,19.*
57. McGowan I. 2011. Rectal microbicides: can we make them and will people use them? *AIDS Behav* 15 Suppl 1: S66-71
58. Bomsel M. 1997. Transcytosis of infectious human immunodeficiency virus across a tight human epithelial cell line barrier. *Nat Med* 3: 42-7
59. Butler LM, Osmond DH, Jones AG, Martin JN. 2009. Use of saliva as a lubricant in anal sexual practices among homosexual men. *J Acquir Immune Defic Syndr* 50: 162-7
60. Campo J, Perea MA, del Romero J, Cano J, Hernando V, Bascones A. 2006. Oral transmission of HIV, reality or fiction? An update. *Oral Dis* 12: 219-28
61. Lu FX, Jacobson RS. 2007. Oral mucosal immunity and HIV/SIV infection. *J Dent Res* 86: 216-26
62. Scully C, Porter S. 2000. HIV topic update: oro-genital transmission of HIV. *Oral Dis* 6: 92-8
63. Chen H, Zha J, Gowans RE, Camargo P, Nishitani J, McQuirter JL, Cole SW, Zack JA, Liu X. 2004. Alcohol enhances HIV type 1 infection in normal human oral keratinocytes by up-regulating cell-surface CXCR4 coreceptor. *AIDS Res Hum Retroviruses* 20: 513-9
64. Pisani E SB, Cherney S et al. Report on the global HIV/AIDS epidemic.
65. Santmyire BR. 2001. Vertical transmission of HIV from mother to child in sub-Saharan Africa: modes of transmission and methods for prevention. *Obstet Gynecol Surv* 56: 306-12
66. Parry S, Zhang J, Koi H, Arechavaleta-Velasco F, Elovitz MA. 2006. Transcytosis of Human immunodeficiency virus 1 across the placenta is enhanced by treatment with tumour necrosis factor alpha. *J Gen Virol* 87: 2269-78
67. Santmyire BR. 2001. Vertical Transmission of HIV from Mother to Child in Sub-Saharan Africa: Modes of Transmission and Methods for Prevention. *Obstetrical & gynecological survey.* 56(5):306-12
68. Teasdale CA, Marais BJ, Abrams EJ. 2011. HIV: prevention of mother-to-child transmission. *Clin Evid (Online)* 2011
69. John-Stewart G, Mbori-Ngacha D, Ekpini R, Janoff EN, Nkengasong J, Read JS, Van de Perre P, Newell ML. 2004. Breast-feeding and Transmission of HIV-1. *J Acquir Immune Defic Syndr* 35: 196-202
70. Wamai RG, Morris BJ, Bailis SA, Sokal D, Klausner JD, Appleton R, Sewankambo N, Cooper DA, Bongaarts J, de Bruyn G, Wodak AD, Banerjee J. 2011. Male circumcision for HIV prevention: current evidence and implementation in sub-Saharan Africa. *J Int AIDS Soc* 14: 49
71. Lehner T, Wang Y, Whittall T, Seidl T. 2011. Innate immunity and HIV-1 infection. *Adv Dent Res* 23: 19-22
72. Borrow P, Bhardwaj N. 2008. Innate immune responses in primary HIV-1 infection. *Curr Opin HIV AIDS* 3: 36-44
73. Mitchell JA, Fitzgerald KA, Coyle A, Silverman N, Cartwright N. 2006. TOLLing away in Brazil. *Nat Immunol* 7: 675-9

74. Firoz Mian M, Ashkar AA. 2011. Induction of innate immune responses in the female genital tract: friend or foe of HIV-1 infection? *Am J Reprod Immunol* 65: 344-51
75. Cameron PU, Handley AJ, Baylis DC, Solomon AE, Bernard N, Purcell DF, Lewin SR. 2007. Preferential infection of dendritic cells during human immunodeficiency virus type 1 infection of blood leukocytes. *J Virol* 81: 2297-306
76. de Witte L, Nabatov A, Pion M, Fluitsma D, de Jong MA, de Gruijl T, Piguet V, van Kooyk Y, Geijtenbeek TB. 2007. Langerin is a natural barrier to HIV-1 transmission by Langerhans cells. *Nat Med* 13: 367-71
77. Huang J, Yang Y, Al-Mozaini M, Burke PS, Beamon J, Carrington MF, Seiss K, Rychert J, Rosenberg ES, Lichterfeld M, Yu XG. 2011. Dendritic Cell Dysfunction During Primary HIV-1 Infection. *J Infect Dis* 204: 1557-62
78. Gurney KB, Elliott J, Nassanian H, Song C, Soilleux E, McGowan I, Anton PA, Lee B. 2005. Binding and transfer of human immunodeficiency virus by DC-SIGN+ cells in human rectal mucosa. *J Virol* 79: 5762-73
79. Wallace M, Bartz SR, Chang WL, Mackenzie DA, Pauza CD, Malkovsky M. 1996. Gamma delta T lymphocyte responses to HIV. *Clin Exp Immunol* 103: 177-84
80. Scott-Algara D, Truong LX, Versmisse P, David A, Luong TT, Nguyen NV, Theodorou I, Barre-Sinoussi F, Pancino G. 2003. Cutting edge: increased NK cell activity in HIV-1-exposed but uninfected Vietnamese intravascular drug users. *J Immunol* 171: 5663-7
81. Alter G, Teigen N, Ahern R, Streeck H, Meier A, Rosenberg ES, Altfeld M. 2007. Evolution of innate and adaptive effector cell functions during acute HIV-1 infection. *J Infect Dis* 195: 1452-60
82. Alter G, Heckerman D, Schneidewind A, Fadda L, Kadie CM, Carlson JM, Oniangue-Ndza C, Martin M, Li B, Khakoo SI, Carrington M, Allen TM, Altfeld M. 2011. HIV-1 adaptation to NK-cell-mediated immune pressure. *Nature* 476: 96-100
83. Sips M, Sciaranghella G, Diefenbach T, Dugast AS, Berger CT, Liu Q, Kwon D, Ghebremichael M, Estes JD, Carrington M, Martin JN, Deeks SG, Hunt PW, Alter G. 2011. Altered distribution of mucosal NK cells during HIV infection. *Mucosal Immunol*
84. Bonaparte MI, Barker E. 2004. Killing of human immunodeficiency virus-infected primary T-cell blasts by autologous natural killer cells is dependent on the ability of the virus to alter the expression of major histocompatibility complex class I molecules. *Blood* 104: 2087-94
85. Fan Z, Huang XL, Kalinski P, Young S, Rinaldo CR, Jr. 2007. Dendritic cell function during chronic hepatitis C virus and human immunodeficiency virus type 1 infection. *Clin Vaccine Immunol* 14: 1127-37
86. Gendelman HE, Baca LM, Turpin J, Kalter DC, Hansen B, Orenstein JM, Dieffenbach CW, Friedman RM, Meltzer MS. 1990. Regulation of HIV replication in infected monocytes by IFN-alpha. Mechanisms for viral restriction. *J Immunol* 145: 2669-76
87. Peters AM, Jager FS, Warneke A, Muller K, Brunkhorst U, Schedel I, Gahr M. 1991. Cytokine secretion by peripheral blood monocytes from human

immunodeficiency virus-infected patients is normal. *Clin Immunol Immunopathol* 61: 343-52
88. Choe W, Volsky DJ, Potash MJ. 2002. Activation of NF-kappaB by R5 and X4 human immunodeficiency virus type 1 induces macrophage inflammatory protein 1alpha and tumor necrosis factor alpha in macrophages. *J Virol* 76: 5274-7
89. Roulston A, Lin R, Beauparlant P, Wainberg MA, Hiscott J. 1995. Regulation of human immunodeficiency virus type 1 and cytokine gene expression in myeloid cells by NF-kappa B/Rel transcription factors. *Microbiol Rev* 59: 481-505
90. Mahajan SD, Agosto-Mojica A, Aalinkeel R, Reynolds JL, Nair BB, Sykes DE, Martinez J, Adams J, Singh N, Bernstein Z, Hsiao CB, Schwartz SA. 2010. Role of chemokine and cytokine polymorphisms in the progression of HIV-1 disease. *Biochem Biophys Res Commun* 396: 348-52
91. Chiu YL, Soros VB, Kreisberg JF, Stopak K, Yonemoto W, Greene WC. 2005. Cellular APOBEC3G restricts HIV-1 infection in resting CD4+ T cells. *Nature* 435: 108-14
92. Chiu YL, Greene WC. 2008. The APOBEC3 cytidine deaminases: an innate defensive network opposing exogenous retroviruses and endogenous retroelements. *Annu Rev Immunol* 26: 317-53
93. Neil SJ, Zang T, Bieniasz PD. 2008. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* 451: 425-30
94. Stremlau M, Owens CM, Perron MJ, Kiessling M, Autissier P, Sodroski J. 2004. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* 427: 848-53
95. Perez-Caballero D, Zang T, Ebrahimi A, McNatt MW, Gregory DA, Johnson MC, Bieniasz PD. 2009. Tetherin inhibits HIV-1 release by directly tethering virions to cells. *Cell* 139: 499-511
96. Zhang X, Kondo M, Chen J, Miyoshi H, Suzuki H, Ohashi T, Shida H. 2010. Inhibitory effect of human TRIM5alpha on HIV-1 production. *Microbes Infect* 12: 768-77
97. Javanbakht H, Yuan W, Yeung DF, Song B, Diaz-Griffero F, Li Y, Li X, Stremlau M, Sodroski J. 2006. Characterization of TRIM5alpha trimerization and its contribution to human immunodeficiency virus capsid binding. *Virology* 353: 234-46
98. Zuccotti GV, Vigano A, Borelli M, Saresella M, Giacomet V, Clerici M. 2007. Modulation of innate and adaptive immunity by lactoferrin in human immunodeficiency virus (HIV)-infected, antiretroviral therapy-naive children. *Int J Antimicrob Agents* 29: 353-5
99. Groot F, Geijtenbeek TB, Sanders RW, Baldwin CE, Sanchez-Hernandez M, Floris R, van Kooyk Y, de Jong EC, Berkhout B. 2005. Lactoferrin prevents dendritic cell-mediated human immunodeficiency virus type 1 transmission by blocking the DC-SIGN--gp120 interaction. *J Virol* 79: 3009-15
100. Chang TL, Vargas J, Jr., DelPortillo A, Klotman ME. 2005. Dual role of alpha-defensin-1 in anti-HIV-1 innate immunity. *J Clin Invest* 115: 765-73

101. Quinones-Mateu ME, Lederman MM, Feng Z, Chakraborty B, Weber J, Rangel HR, Marotta ML, Mirza M, Jiang B, Kiser P, Medvik K, Sieg SF, Weinberg A. 2003. Human epithelial beta-defensins 2 and 3 inhibit HIV-1 replication. *AIDS* 17: F39-48
102. Burgener A, Rahman S, Ahmad R, Lajoie J, Ramdahin S, Mesa C, Brunet S, Wachihi C, Kimani J, Fowke K, Carr S, Plummer F, Ball TB. 2011. Comprehensive Proteomic Study Identifies Serpin and Cystatin Antiproteases as Novel Correlates of HIV-1 Resistance in the Cervicovaginal Mucosa of Female Sex Workers. *J Proteome Res* 10: 5139-49
103. Ahlers JD, Belyakov IM, Berzofsky JA. 2003. Cytokine, chemokine, and costimulatory molecule modulation to enhance efficacy of HIV vaccines. *Curr Mol Med* 3: 285-301
104. Geginat J, Sallusto F, Lanzavecchia A. 2001. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells. *J Exp Med* 194: 1711-9
105. Janeway CA, Travers, P., Walport, M., and Shlomchik, M.J. . (2001). *Immunobiology (5th ed.)*: New York and London: Garland Science.
106. Munier CM, Andersen CR, Kelleher AD. 2011. HIV vaccines: progress to date. *Drugs* 71: 387-414
107. Tomaras GD, Yates NL, Liu P, Qin L, Fouda GG, Chavez LL, Decamp AC, Parks RJ, Ashley VC, Lucas JT, Cohen M, Eron J, Hicks CB, Liao HX, Self SG, Landucci G, Forthal DN, Weinhold KJ, Keele BF, Hahn BH, Greenberg ML, Morris L, Karim SS, Blattner WA, Montefiori DC, Shaw GM, Perelson AS, Haynes BF. 2008. Initial B-cell responses to transmitted human immunodeficiency virus type 1: virion-binding immunoglobulin M (IgM) and IgG antibodies followed by plasma anti-gp41 antibodies with ineffective control of initial viremia. *J Virol* 82: 12449-63
108. Veazey RS, Shattock RJ, Pope M, Kirijan JC, Jones J, Hu Q, Ketas T, Marx PA, Klasse PJ, Burton DR, Moore JP. 2003. Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. *Nat Med* 9: 343-6
109. Matoba N, Magerus A, Geyer BC, Zhang Y, Muralidharan M, Alfsen A, Arntzen CJ, Bomsel M, Mor TS. 2004. A mucosally targeted subunit vaccine candidate eliciting HIV-1 transcytosis-blocking Abs. *Proc Natl Acad Sci U S A* 101: 13584-9
110. Devito C, Hinkula J, Kaul R, Lopalco L, Bwayo JJ, Plummer F, Clerici M, Broliden K. 2000. Mucosal and plasma IgA from HIV-exposed seronegative individuals neutralize a primary HIV-1 isolate. *Aids* 14: 1917-20
111. Devito C, Broliden K, Kaul R, Svensson L, Johansen K, Kiama P, Kimani J, Lopalco L, Piconi S, Bwayo JJ, Plummer F, Clerici M, Hinkula J. 2000. Mucosal and plasma IgA from HIV-1-exposed uninfected individuals inhibit HIV-1 transcytosis across human epithelial cells. *J Immunol* 165: 5170-6
112. Mestecky J, Wright PF, Lopalco L, Staats HF, Kozlowski PA, Moldoveanu Z, Alexander RC, Kulhavy R, Pastori C, Maboko L, Riedner G, Zhu Y, Wrinn T, Hoelscher M. 2011. Scarcity or absence of humoral immune responses in the

plasma and cervicovaginal lavage fluids of heavily HIV-1-exposed but persistently seronegative women. *AIDS Res Hum Retroviruses* 27: 469-86
113. Fast PE, Kaleebu P. 2010. HIV vaccines: current status worldwide and in Africa. *AIDS* 24 Suppl 4: S50-60
114. Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, Salazar-Gonzalez JF, Salazar MG, Kilby JM, Saag MS, Komarova NL, Nowak MA, Hahn BH, Kwong PD, Shaw GM. 2003. Antibody neutralization and escape by HIV-1. *Nature* 422: 307-12
115. Walker LM, Phogat SK, Chan-Hui PY, Wagner D, Phung P, Goss JL, Wrin T, Simek MD, Fling S, Mitcham JL, Lehrman JK, Priddy FH, Olsen OA, Frey SM, Hammond PW, Kaminsky S, Zamb T, Moyle M, Koff WC, Poignard P, Burton DR. 2009. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. *Science* 326: 285-9
116. Hessell AJ, Rakasz EG, Tehrani DM, Huber M, Weisgrau KL, Landucci G, Forthal DN, Koff WC, Poignard P, Watkins DI, Burton DR. 2010. Broadly neutralizing monoclonal antibodies 2F5 and 4E10 directed against the human immunodeficiency virus type 1 gp41 membrane-proximal external region protect against mucosal challenge by simian-human immunodeficiency virus SHIVBa-L. *J Virol* 84: 1302-13
117. Baum LL. 2010. Role of humoral immunity in host defense against HIV. *Curr HIV/AIDS Rep* 7: 11-8
118. Copeland KF, Heeney JL. 1996. T helper cell activation and human retroviral pathogenesis. *Microbiol Rev* 60: 722-42
119. Martin-Fontecha A, Thomsen LL, Brett S, Gerard C, Lipp M, Lanzavecchia A, Sallusto F. 2004. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. *Nat Immunol* 5: 1260-5
120. Reynolds MR, Rakasz E, Skinner PJ, White C, Abel K, Ma ZM, Compton L, Napoe G, Wilson N, Miller CJ, Haase A, Watkins DI. 2005. CD8+ T-lymphocyte response to major immunodominant epitopes after vaginal exposure to simian immunodeficiency virus: too late and too little. *J Virol* 79: 9228-35
121. Belyakov IM, Ahlers JD. 2008. Functional CD8+ CTLs in mucosal sites and HIV infection: moving forward toward a mucosal AIDS vaccine. *Trends Immunol* 29: 574-85
122. Vigano A, Principi N, Crupi L, Onorato J, Vincenzo ZG, Salvaggio A. 1995. Elevation of IgE in HIV-infected children and its correlation with the progression of disease. *J Allergy Clin Immunol* 95: 627-32
123. Becker Y. 2004. The changes in the T helper 1 (Th1) and T helper 2 (Th2) cytokine balance during HIV-1 infection are indicative of an allergic response to viral proteins that may be reversed by Th2 cytokine inhibitors and immune response modifiers--a review and hypothesis. *Virus Genes* 28: 5-18
124. Min B, Paul WE. 2008. Basophils and type 2 immunity. *Curr Opin Hematol* 15: 59-63
125. El Hed A, Khaitan A, Kozhaya L, Manel N, Daskalakis D, Borkowsky W, Valentine F, Littman DR, Unutmaz D. 2010. Susceptibility of human Th17 cells to human immunodeficiency virus and their perturbation during infection. *J Infect Dis* 201: 843-54

126. Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, Fouser LA. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* 203: 2271-9
127. Workman CJ, Szymczak-Workman AL, Collison LW, Pillai MR, Vignali DA. 2009. The development and function of regulatory T cells. *Cell Mol Life Sci* 66: 2603-22
128. Moreno-Fernandez ME, Zapata W, Blackard JT, Franchini G, Chougnet CA. 2009. Human regulatory T cells are targets for human immunodeficiency Virus (HIV) infection, and their susceptibility differs depending on the HIV type 1 strain. *J Virol* 83: 12925-33
129. Walker CM, Moody DJ, Stites DP, Levy JA. 1986. CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication. *Science* 234: 1563-6
130. Bagasra O, Pomerantz RJ. 1993. The role of CD8-positive lymphocytes in the control of HIV-1 infection of peripheral blood mononuclear cells. *Immunol Lett* 35: 83-92
131. Saksena NK, Wu JQ, Potter SJ, Wilkinson J, Wang B. 2008. Human immunodeficiency virus interactions with CD8+ T lymphocytes. *Curr HIV Res* 6: 1-9
132. Chang TL, Mosoian A, Pine R, Klotman ME, Moore JP. 2002. A soluble factor(s) secreted from CD8(+) T lymphocytes inhibits human immunodeficiency virus type 1 replication through STAT1 activation. *J Virol* 76: 569-81
133. Baeten JM, Hassan WM, Chohan V, Richardson BA, Mandaliya K, Ndinya-Achola JO, Jaoko W, McClelland RS. 2009. Prospective study of correlates of vaginal Lactobacillus colonisation among high-risk HIV-1 seronegative women. *Sex Transm Infect* 85: 348-53
134. Shen R, Richter HE, Smith PD. 2011. Early HIV-1 target cells in human vaginal and ectocervical mucosa. *Am J Reprod Immunol* 65: 261-7
135. Kaushic C, Ferreira VH, Kafka JK, Nazli A. HIV Infection in the Female Genital Tract: Discrete Influence of the Local Mucosal Microenvironment. *Am J Reprod Immunol*
136. Cu-Uvin S, Hogan JW, Caliendo AM, Harwell J, Mayer KH, Carpenter CC. 2001. Association between bacterial vaginosis and expression of human immunodeficiency virus type 1 RNA in the female genital tract. *Clin Infect Dis* 33: 894-6
137. Ding J, Rapista A, Teleshova N, Mosoyan G, Jarvis GA, Klotman ME, Chang TL. 2010. Neisseria gonorrhoeae enhances HIV-1 infection of primary resting CD4+ T cells through TLR2 activation. *J Immunol* 184: 2814-24
138. Tobian AA, Quinn TC. 2009. Herpes simplex virus type 2 and syphilis infections with HIV: an evolving synergy in transmission and prevention. *Curr Opin HIV AIDS* 4: 294-9
139. Zhu J, Hladik F, Woodward A, Klock A, Peng T, Johnston C, Remington M, Magaret A, Koelle DM, Wald A, Corey L. 2009. Persistence of HIV-1 receptor-positive cells after HSV-2 reactivation is a potential mechanism for increased HIV-1 acquisition. *Nat Med* 15: 886-92

140. Chakraborty H, Sen PK, Helms RW, Vernazza PL, Fiscus SA, Eron JJ, Patterson BK, Coombs RW, Krieger JN, Cohen MS. 2001. Viral burden in genital secretions determines male-to-female sexual transmission of HIV-1: a probabilistic empiric model. *AIDS* 15: 621-7
141. O'Hanlon DE, Lanier BR, Moench TR, Cone RA. 2010. Cervicovaginal fluid and semen block the microbicidal activity of hydrogen peroxide produced by vaginal lactobacilli. *BMC Infect Dis* 10: 120
142. Roan NR, Munch J, Arhel N, Mothes W, Neidleman J, Kobayashi A, Smith-McCune K, Kirchhoff F, Greene WC. 2009. The cationic properties of SEVI underlie its ability to enhance human immunodeficiency virus infection. *J Virol* 83: 73-80
143. Haase AT. 2005. Perils at mucosal front lines for HIV and SIV and their hosts. *Nat Rev Immunol* 5: 783-92
144. Haase AT. 2011. Early events in sexual transmission of HIV and SIV and opportunities for interventions. *Annu Rev Med* 62: 127-39
145. Shacklett BL. 2009. Cell-mediated immunity to HIV in the female reproductive tract. *J Reprod Immunol* 83: 190-5
146. Girard MP, Osmanov S, Assossou OM, Kieny MP. 2011. Human immunodeficiency virus (HIV) immunopathogenesis and vaccine development: a review. *Vaccine* 29: 6191-218
147. Hladik F, Doncel GF. 2010. Preventing mucosal HIV transmission with topical microbicides: challenges and opportunities. *Antiviral Res* 88 Suppl 1: S3-9
148. Cone RA, Hoen T, Wong X, Abusuwwa R, Anderson DJ, Moench TR. 2006. Vaginal microbicides: detecting toxicities in vivo that paradoxically increase pathogen transmission. *BMC Infect Dis* 6: 90
149. Feldblum PJ, Adeiga A, Bakare R, Wevill S, Lendvay A, Obadaki F, Olayemi MO, Wang L, Nanda K, Rountree W. 2008. SAVVY vaginal gel (C31G) for prevention of HIV infection: a randomized controlled trial in Nigeria. *PLoS One* 3: e1474
150. Schwartz JL, Poindexter A, Schmitz SW, Mauck C, Callahan MM. 2005. Male tolerance of ACIDFORM gel. *Contraception* 71: 443-6
151. Mitsuya H, Looney DJ, Kuno S, Ueno R, Wong-Staal F, Broder S. 1988. Dextran sulfate suppression of viruses in the HIV family: inhibition of virion binding to CD4+ cells. *Science* 240: 646-9
152. McCormack S, Ramjee G, Kamali A, Rees H, Crook AM, Gafos M, Jentsch U, Pool R, Chisembele M, Kapiga S, Mutemwa R, Vallely A, Palanee T, Sookrajh Y, Lacey CJ, Darbyshire J, Grosskurth H, Profy A, Nunn A, Hayes R, Weber J. 2010. PRO2000 vaginal gel for prevention of HIV-1 infection (Microbicides Development Programme 301): a phase 3, randomised, double-blind, parallel-group trial. *Lancet* 376: 1329-37
153. McGowan I, Gomez K, Bruder K, Febo I, Chen BA, Richardson BA, Husnik M, Livant E, Price C, Jacobson C. 2011. Phase 1 randomized trial of the vaginal safety and acceptability of SPL7013 gel (VivaGel) in sexually active young women (MTN-004). *AIDS* 25: 1057-64
154. Veazey RS, Ling B, Green LC, Ribka EP, Lifson JD, Piatak M, Jr., Lederman MM, Mosier D, Offord R, Hartley O. 2009. Topically applied recombinant

chemokine analogues fully protect macaques from vaginal simian-human immunodeficiency virus challenge. *J Infect Dis* 199: 1525-7
155. Forssmann WG, The YH, Stoll M, Adermann K, Albrecht U, Barlos K, Busmann A, Canales-Mayordomo A, Gimenez-Gallego G, Hirsch J, Jimenez-Barbero J, Meyer-Olson D, Munch J, Perez-Castells J, Standker L, Kirchhoff F, Schmidt RE. 2010. Short-term monotherapy in HIV-infected patients with a virus entry inhibitor against the gp41 fusion peptide. *Sci Transl Med* 2: 63re3
156. Abdool Karim Q, Abdool Karim SS, Frohlich JA, Grobler AC, Baxter C, Mansoor LE, Kharsany AB, Sibeko S, Mlisana KP, Omar Z, Gengiah TN, Maarschalk S, Arulappan N, Mlotshwa M, Morris L, Taylor D. 2010. Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science* 329: 1168-74
157. Arien KK, Jespers V, Vanham G. 2011. HIV sexual transmission and microbicides. *Rev Med Virol*
158. Padian NS, van der Straten A, Ramjee G, Chipato T, de Bruyn G, Blanchard K, Shiboski S, Montgomery ET, Fancher H, Cheng H, Rosenblum M, van der Laan M, Jewell N, McIntyre J. 2007. Diaphragm and lubricant gel for prevention of HIV acquisition in southern African women: a randomised controlled trial. *Lancet* 370: 251-61
159. Koning FA, Jansen CA, Dekker J, Kaslow RA, Dukers N, van Baarle D, Prins M, Schuitemaker H. 2004. Correlates of resistance to HIV-1 infection in homosexual men with high-risk sexual behaviour. *Aids* 18: 1117-26
160. de la Torre MS, Torres C, Nieto G, Vergara S, Carrero AJ, Macias J, Pineda JA, Caruz A, Fibla J. 2008. Vitamin D receptor gene haplotypes and susceptibility to HIV-1 infection in injection drug users. *J Infect Dis* 197: 405-10
161. Furci L, Lopalco L, Loverro P, Sinnone M, Tambussi G, Lazzarin A, Lusso P. 2002. Non-cytotoxic inhibition of HIV-1 infection by unstimulated CD8+ T lymphocytes from HIV-exposed-uninfected individuals. *AIDS* 16: 1003-8
162. Horton RE, Ball TB, Wachichi C, Jaoko W, Rutherford WJ, McKinnon L, Kaul R, Rebbapragada A, Kimani J, Plummer FA. 2009. Cervical HIV-specific IgA in a population of commercial sex workers correlates with repeated exposure but not resistance to HIV. *AIDS Res Hum Retroviruses* 25: 83-92
163. Baker BM, Block BL, Rothchild AC, Walker BD. 2009. Elite control of HIV infection: implications for vaccine design. *Expert Opin Biol Ther* 9: 55-69
164. Suresh P, Wanchu A. 2006. Chemokines and chemokine receptors in HIV infection: role in pathogenesis and therapeutics. *J Postgrad Med* 52: 210-7
165. MacDonald KS, Fowke KR, Kimani J, Dunand VA, Nagelkerke NJ, Ball TB, Oyugi J, Njagi E, Gaur LK, Brunham RC, Wade J, Luscher MA, Krausa P, Rowland-Jones S, Ngugi E, Bwayo JJ, Plummer FA. 2000. Influence of HLA supertypes on susceptibility and resistance to human immunodeficiency virus type 1 infection. *J Infect Dis* 181: 1581-9
166. Ball TB, Ji H, Kimani J, McLaren P, Marlin C, Hill AV, Plummer FA. 2007. Polymorphisms in IRF-1 associated with resistance to HIV-1 infection in highly exposed uninfected Kenyan sex workers. *Aids* 21: 1091-101
167. Fowke KR, Kaul R, Rosenthal KL, Oyugi J, Kimani J, Rutherford WJ, Nagelkerke NJ, Ball TB, Bwayo JJ, Simonsen JN, Shearer GM, Plummer FA. 2000. HIV-1-

specific cellular immune responses among HIV-1-resistant sex workers. *Immunol Cell Biol* 78: 586-95
168. McLaren PJ, Ball TB, Wachihi C, Jaoko W, Kelvin DJ, Danesh A, Kimani J, Plummer FA, Fowke KR. 2010. HIV-exposed seronegative commercial sex workers show a quiescent phenotype in the CD4+ T cell compartment and reduced expression of HIV-dependent host factors. *J Infect Dis* 202 Suppl 3: S339-44
169. Card CM, McLaren PJ, Wachihi C, Kimani J, Plummer FA, Fowke KR. 2009. Decreased immune activation in resistance to HIV-1 infection is associated with an elevated frequency of CD4(+)CD25(+)FOXP3(+) regulatory T cells. *J Infect Dis* 199: 1318-22
170. Whisstock JC, Silverman GA, Bird PI, Bottomley SP, Kaiserman D, Luke CJ, Pak SC, Reichhart JM, Huntington JA. 2010. Serpins flex their muscle: II. Structural insights into target peptidase recognition, polymerization, and transport functions. *J Biol Chem* 285: 24307-12
171. Law RH, Zhang Q, McGowan S, Buckle AM, Silverman GA, Wong W, Rosado CJ, Langendorf CG, Pike RN, Bird PI, Whisstock JC. 2006. An overview of the serpin superfamily. *Genome Biol* 7: 216
172. Irving JA, Pike RN, Lesk AM, Whisstock JC. 2000. Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. *Genome Res* 10: 1845-64
173. Wiedow O, Meyer-Hoffert U. 2005. Neutrophil serine proteases: potential key regulators of cell signalling during inflammation. *J Intern Med* 257: 319-28
174. Mangan MS, Kaiserman D, Bird PI. 2008. The role of serpins in vertebrate immunity. *Tissue Antigens* 72: 1-10
175. van Gent D, Sharp P, Morgan K, Kalsheker N. 2003. Serpins: structure, function and molecular evolution. *Int J Biochem Cell Biol* 35: 1536-47
176. Heutinck KM, ten Berge IJ, Hack CE, Hamann J, Rowshani AT. 2010. Serine proteases of the human immune system in health and disease. *Mol Immunol* 47: 1943-55
177. Benarafa C, Priebe GP, Remold-O'Donnell E. 2007. The neutrophil serine protease inhibitor serpinb1 preserves lung defense functions in Pseudomonas aeruginosa infection. *J Exp Med* 204: 1901-9
178. Janciauskiene SM, Bals R, Koczulla R, Vogelmeier C, Kohnlein T, Welte T. 2011. The discovery of alpha1-antitrypsin and its role in health and disease. *Respir Med* 105: 1129-39
179. Herrick S, Ashcroft G, Ireland G, Horan M, McCollum C, Ferguson M. 1997. Up-regulation of elastase in acute wounds of healthy aged humans and chronic venous leg ulcers are associated with matrix degradation. *Lab Invest* 77: 281-8
180. Rau JC, Beaulieu LM, Huntington JA, Church FC. 2007. Serpins in thrombosis, hemostasis and fibrinolysis. *J Thromb Haemost* 5 Suppl 1: 102-15
181. Oelschlager C, Romisch J, Staubitz A, Stauss H, Leithauser B, Tillmanns H, Holschermann H. 2002. Antithrombin III inhibits nuclear factor kappaB activation in human monocytes and vascular endothelial cells. *Blood* 99: 4015-20

182. Whitney JB, Asmal M, Geiben-Lynn R. 2011. Serpin induced antiviral activity of prostaglandin synthetase-2 against HIV-1 replication. *PLoS ONE* 6: e18589
183. Hattori T, Koito A, Takatsuki K, Kido H, Katunuma N. 1989. Involvement of tryptase-related cellular protease(s) in human immunodeficiency virus type 1 infection. *FEBS Lett* 248: 48-52
184. Congote LF. 2007. Serpin A1 and CD91 as host instruments against HIV-1 infection: are extracellular antiviral peptides acting as intracellular messengers? *Virus Res* 125: 119-34
185. Bristow CL. 2001. Slow human immunodeficiency virus (HIV) infectivity correlated with low HIV coreceptor levels. *Clin Diagn Lab Immunol* 8: 932-6
186. Noorbakhsh F, Vergnolle N, Hollenberg MD, Power C. 2003. Proteinase-activated receptors in the nervous system. *Nat Rev Neurosci* 4: 981-90
187. Churg A, Dai J, Zay K, Karsan A, Hendricks R, Yee C, Martin R, MacKenzie R, Xie C, Zhang L, Shapiro S, Wright JL. 2001. Alpha-1-antitrypsin and a broad spectrum metalloprotease inhibitor, RS113456, have similar acute anti-inflammatory effects. *Lab Invest* 81: 1119-31
188. Nita IM, Serapinas D, Janciauskiene SM. 2007. alpha1-Antitrypsin regulates CD14 expression and soluble CD14 levels in human monocytes in vitro. *Int J Biochem Cell Biol* 39: 1165-76
189. Pott GB, Chan ED, Dinarello CA, Shapiro L. 2009. Alpha-1-antitrypsin is an endogenous inhibitor of proinflammatory cytokine production in whole blood. *J Leukoc Biol* 85: 886-95
190. Bergin DA, Reeves EP, Meleady P, Henry M, McElvaney OJ, Carroll TP, Condron C, Chotirmall SH, Clynes M, O'Neill SJ, McElvaney NG. 2010. alpha-1 Antitrypsin regulates human neutrophil chemotaxis induced by soluble immune complexes and IL-8. *J Clin Invest* 120: 4236-50
191. Munch J, Standker L, Adermann K, Schulz A, Schindler M, Chinnadurai R, Pohlmann S, Chaipan C, Biet T, Peters T, Meyer B, Wilhelm D, Lu H, Jing W, Jiang S, Forssmann WG, Kirchhoff F. 2007. Discovery and optimization of a natural HIV-1 entry inhibitor targeting the gp41 fusion peptide. *Cell* 129: 263-75
192. Cordelier P, Strayer DS. 2003. Mechanisms of alpha1-antitrypsin inhibition of cellular serine proteases and HIV-1 protease that are essential for HIV-1 morphogenesis. *Biochim Biophys Acta* 1638: 197-207
193. Congote LF. 2006. The C-terminal 26-residue peptide of serpin A1 is an inhibitor of HIV-1. *Biochem Biophys Res Commun* 343: 617-22
194. Shapiro L, Pott GB, Ralston AH. 2001. Alpha-1-antitrypsin inhibits human immunodeficiency virus type 1. *Faseb J* 15: 115-22
195. Zhou X, Shapiro L, Fellingham G, Willardson BM, Burton GF. 2011. HIV Replication in CD4+ T Lymphocytes in the Presence and Absence of Follicular Dendritic Cells: Inhibition of Replication Mediated by {alpha}-1-Antitrypsin through Altered I{kappa}B{alpha} Ubiquitination. *J Immunol* 186: 3148-55
196. Bryan CL, Beard KS, Pott GB, Rahkola J, Gardner EM, Janoff EN, Shapiro L. 2010. HIV infection is associated with reduced serum alpha-1-antitrypsin concentrations. *Clin Invest Med* 33: E384-9

197. Baker C, Belbin O, Kalsheker N, Morgan K. 2007. SERPINA3 (aka alpha-1-antichymotrypsin). *Front Biosci* 12: 2821-35
198. Chertov O, Ueda H, Xu LL, Tani K, Murphy WJ, Wang JM, Howard OM, Sayers TJ, Oppenheim JJ. 1997. Identification of human neutrophil-derived cathepsin G and azurocidin/CAP37 as chemoattractants for mononuclear cells and neutrophils. *J Exp Med* 186: 739-47
199. Lim JK, Lu W, Hartley O, DeVico AL. 2006. N-terminal proteolytic processing by cathepsin G converts RANTES/CCL5 and related analogs into a truncated 4-68 variant. *J Leukoc Biol* 80: 1395-404
200. Fleck A. 1989. Clinical and nutritional aspects of changes in acute-phase proteins during inflammation. *Proc Nutr Soc* 48: 347-54
201. Sabatte J, Lenicov FR, Cabrini M, Rodriguez CR, Ostrowski M, Ceballos A, Amigorena S, Geffner J. 2011. The role of semen in sexual transmission of HIV: beyond a carrier for virus particles. *Microbes Infect* 13: 977-82
202. Marks MA, Gravitt PE, Burk RD, Studentsov Y, Farzadegan H, Klein SL. 2010. Progesterone and 17beta-estradiol enhance regulatory responses to human papillomavirus type 16 virus-like particles in peripheral blood mononuclear cells from healthy women. *Clin Vaccine Immunol* 17: 609-17
203. Wira CR, Fahey JV, Ghosh M, Patel MV, Hickey DK, Ochiel DO. Sex Hormone Regulation of Innate Immunity in the Female Reproductive Tract: The Role of Epithelial Cells in Balancing Reproductive Potential with Protection against Sexually Transmitted Pathogens. *Am J Reprod Immunol*
204. Ashton-Rickardt PG. 2010. Serine protease inhibitors and cytotoxic T lymphocytes. *Immunol Rev* 235: 147-58
205. Moriuchi H, Moriuchi M, Fauci AS. 2000. Cathepsin G, a neutrophil-derived serine protease, increases susceptibility of macrophages to acute human immunodeficiency virus type 1 infection. *J Virol* 74: 6849-55
206. Janciauskiene SM, Nita IM, Stevens T. 2007. Alpha1-antitrypsin, old dog, new tricks. Alpha1-antitrypsin exerts in vitro anti-inflammatory activity in human monocytes by elevating cAMP. *J Biol Chem* 282: 8573-82
207. Li Q, Estes JD, Schlievert PM, Duan L, Brosnahan AJ, Southern PJ, Reilly CS, Peterson ML, Schultz-Darken N, Brunner KG, Nephew KR, Pambuccian S, Lifson JD, Carlis JV, Haase AT. 2009. Glycerol monolaurate prevents mucosal SIV transmission. *Nature* 458: 1034-8
208. Janciauskiene S. 2001. Conformational properties of serine proteinase inhibitors (serpins) confer multiple pathophysiological roles. *Biochim Biophys Acta* 1535: 221-35
209. J Lajoie JJ, A Burgener , S Rahman, K. Mogk, M Kimani , J, Mwanjewe J, FA Plummer , Kimani , TB Ball, KR Fowke. 2011. A distinct cytokine and chemokine profile at the genital mucosa is associated with HIV-1 protection among HIV exposed seronegative commercial sex workers.
210. Tessari P. 2000. Changes in protein, carbohydrate, and fat metabolism with aging: possible role of insulin. *Nutr Rev* 58: 11-9
211. Wong CP, Ho E. 2011. Zinc and its role in age-related inflammation and immune dysfunction. *Mol Nutr Food Res*

212. Hirbod T, Broliden K. 2007. Mucosal immune responses in the genital tract of HIV-1-exposed uninfected women. *J Intern Med* 262: 44-58
213. Johnson LF, Dorrington RE, Bradshaw D, Coetzee DJ. 2011. The role of sexually transmitted infections in the evolution of the South African HIV epidemic. *Trop Med Int Health*
214. Karlsson I GJ, Chen SS, et al. Vulvovaginitis. MedlinePlus. A service of the U.S. National Library of Medicine, National Institutes of Health
215. Agerberth B, Gudmundsson GH. 2006. Host antimicrobial defence peptides in human disease. *Curr Top Microbiol Immunol* 306: 67-90
216. Gray RH, Li X, Kigozi G, Serwadda D, Brahmbhatt H, Wabwire-Mangen F, Nalugoda F, Kiddugavu M, Sewankambo N, Quinn TC, Reynolds SJ, Wawer MJ. 2005. Increased risk of incident HIV during pregnancy in Rakai, Uganda: a prospective study. *Lancet* 366: 1182-8
217. Anderson BL, Cu-Uvin S. 2011. Clinical parameters essential to methodology and interpretation of mucosal responses. *Am J Reprod Immunol* 65: 352-60
218. Camara M, Dieye TN, Seydi M, Diallo AA, Fall M, Diaw PA, Sow PS, Mboup S, Kestens L, Jennes W. 2010. Low-level CD4+ T cell activation in HIV-exposed seronegative subjects: influence of gender and condom use. *J Infect Dis* 201: 835-42
219. Hel Z, Stringer E, Mestecky J. 2010. Sex steroid hormones, hormonal contraception, and the immunobiology of human immunodeficiency virus-1 infection. *Endocr Rev* 31: 79-97
220. Lavreys L, Chohan V, Overbaugh J, Hassan W, McClelland RS, Kreiss J, Mandaliya K, Ndinya-Achola J, Baeten JM. 2004. Hormonal contraception and risk of cervical infections among HIV-1-seropositive Kenyan women. *AIDS* 18: 2179-84
221. King AE, Critchley HO, Sallenave JM, Kelly RW. 2003. Elafin in human endometrium: an antiprotease and antimicrobial molecule expressed during menstruation. *J Clin Endocrinol Metab* 88: 4426-31
222. Geiben-Lynn R, Brown N, Walker BD, Luster AD. 2002. Purification of a modified form of bovine antithrombin III as an HIV-1 CD8+ T-cell antiviral factor. *J Biol Chem* 277: 42352-7