EVALUATING THE CLINICAL AND IMMUNOLOGICAL CORRELATES OF SEMEN HIV-1 RNA SHEDDING

By

Brendan James William Osborne

A thesis submitted in conformity with the requirements

for the degree of Doctor of Philosophy

Institute of Medical Science

University of Toronto

© Copyright Brendan Osborne 2014
Evaluating the clinical and immunological correlates of semen HIV-1 RNA shedding

Brendan James William Osborne

Doctor of Philosophy

Institute of Medical Science

University of Toronto

2014

Abstract

Globally it is estimated that 35 million people were living with HIV at the end of 2012. Unprotected sexual transmission of HIV accounts for the majority of all new infections, with semen acting as a vector. HIV levels in blood of an HIV-infected individual is the best-defined predictor of transmission risk, however, as blood is not often a factor during sex, this association likely reflects the level of HIV RNA in semen. It has been shown that while blood viral load reaches a set point during chronic untreated infection, semen can remain highly variable. Factors responsible for this heterogeneity have not fully well characterized. Furthermore, while highly active antiretroviral therapy has been associated with suppression of blood and genital viral loads, some men continue to shed HIV in their semen, despite undetectable viremia, a phenomenon called isolated semen HIV RNA shedding (IHS).

In my doctoral thesis I evaluated the impact of semen collection method on viral load measurements, correlations between antiretroviral therapy intensification and its effect on
immune activation, viral load and IHS as well as factors contributing to semen viral load variability in drug naive men. Overall, I found that semen collected directly into transport medium had a large impact on the measurement of viral load, evidencing a $0.42\log_{10}$ increase in viral load over neat collection. Initiation of intensified antiretroviral therapy was shown to decrease viral load in blood and semen faster than a standard regimen. Intensification, however, did not resolve the occurrence of ISH, nor did it reduce systemic immune activation faster than a conventional therapy. It was observed that men on longer durations of ART had no evidence of IHS, suggesting the phenomenon is transient and may resolve with longer duration of therapy. Finally I explored factors that may be linked to the variability seen in semen viral load in drug naive men. Of all parameters examined, the macrophage-associated cytokine IL-8 was the only factor independently predictive of semen viral load (aside from blood viral load) in longitudinal studies. This suggests IL-8 as a common pathway linking clinical causes of high semen HIV levels in therapy-naive men.
Acknowledgments

I want to start by first of all thank my supervisor Dr Rupert Kaul. I am grateful to him for not only taking a chance on an atypical PhD student but also for his understanding when things may have not gone as planned. He has been one of the most supportive, intelligent and helpful men I have come across in my career and I wish him and his lab all the best into the future.

I would like to thank the members of my program advisory committee Drs Irving Salit and Mario Ostrowski. Their experience and knowledge have been invaluable throughout my PhD from my shaky beginnings to where I ended.

A huge Thank You to all the members of the HIV Research group and Kaul lab who have helped me over the years with their advice, insight, helpful suggestions and support. In particular I would like to thank Connie Kim for her critical insight into my work, Jessica Prodger for not only her delicious baked goods and amazing birthday cards but also for sitting down with me, and explaining what a flow cytometer was and what a FITC was. Tae Joon Yi, you have been a great source of support and encouragement over the years. Brett Shannon you have been a wonderful addition to the Kaul lab and a source of entertainment since day 1. Shariq Mujib you have been a great friend and someone I have been able to rely on for bouncing ideas with or just getting advice from. Sanja Huibner you have been a great lab manager and friend over the years, you were always there to listen to my problems and frustrations and provide support when I needed it most. On top of all that, you brought me wine when I needed it most, my birthday. Finally I would like to thank Angie Marsh for keeping me sane the past 4 years. I’m pretty sure you are responsible for that shreds of sanity I had left on my last day.
This wouldn’t be a proper acknowledgement without thanking my parents, Mrs. Laurie Vivian Osborne and Mr. Dennis James Osborne. While I don’t believe to this day you understand anything I have done in my PhD, or for that matter what it is in, your support throughout the process has definitely been appreciated. I hope you can now find relief and pride in the fact I have reached the highest level of academic education and there is literally no where else I can or will go but work. Yes mom, a real job. To my sister Lindsay, while we may see things differently on almost every topic you have provided support when I most needed it. Finally to my nephew Bryden, may you grow up knowing the power and influence knowledge and research has.

“If we knew what it was we were doing, it would not be called research, would it?”

-Albert Einstein
LIST OF ABBREVIATIONS

3TC - Lamivudine

ABC – Abacavir

ACD – Acid Citrate Dextran

AIDS – Acquired Immunodeficiency Syndrome

ARS – Antiretroviral syndrome

ART – Antiretroviral Therapy

ARV – Antiretroviral

ATZ – Atazanavir

AZT – Zidovudine

bDNA – Branched Deoxyribose Nucleic Acid Assay

BTB – Blood testes barrier

CCL – Chemokine (C-C motif) ligand

CCR – CC Chemokine Receptor

cDNA – Complementary Deoxyribonucleic Acid

CD – Cluster of Differentiation

CDR – Cerebellar degeneration-related protein

CMV – Cytomegalovirus

COPD – Chronic obstructive pulmonary disorder
CT/NG – *C. trachomatis* and/or *N. gonorrhoeae*

CVD – Cardiovascular disease

CXCR – CXC Chemokine Receptor

DHHS – Department of Health and Human Services

DHS – Disproportionate HIV-1 RNA Semen Shedding

DMSO – Dimethyl Sulfoxide

dNTP – Deoxyribonucleotide triphosphate

DNA – Deoxyribose Nucleic Acid

EBV – Epstein-Barr Virus

ENV – HIV envelope glycoprotein

EFV – Efavirenz

ELISA – Enzyme Linked Immunosorbent Assay

FACS - Fluorescence-Activated Cell Sorting

FBS – Fetal Bovine Serum

FCS – Fetal Calf Serum

FDA – Food and Drug Administration

FITC – Fluorescein isothiocyanate

FTC – Emtricitabine

GALT – Gut Associated Lymphoid Tissue

GP160 – Envelope Glycoprotein 160
GP120 – Envelope Glycoprotein 120

GP41 – Envelope Glycoprotein 41

GUD – Genital Ulcer Disease

HAART – Highly Active Antiretroviral Therapy

HIV – Human Immunodeficiency Virus-1

HHV6 – Roseolovirus / Herpes lymphotropic Virus

HHV7 – Pityriasis Rosea

HHV8 – Kaposi’s Sarcoma-Associated Herpesvirus

HLA-DR – Human Leukocyte Antigen

HPTN – HIV Prevention Trials Network

HSV1 – Human Simplex Virus Type 1

HSV2 – Human Simplex Virus Type 2

iART – Intensified Antiretroviral Therapy

IHS – Isolated HIV-1 RNA Shedding

IL – Interleukin

INSTI – Integrase Strand Transfer Inhibitors

IP – Interferon gamma-induced protein

LC/MS – Liquid chromatography / Mass spectrometry

LPS – Lipopolysaccharide

LPV – Lopinavir
LTNP – Long Term Non-Progressor

M cells – Microfold cells

MCP – Macrophage Chemotactic Protein

MDC – Macrophage-Derived Chemokine

MGT – Male Genital Tract

MHC – Major Histocompatibility Complex

MIG – Monokine Induced by Gamma Interferon

MIP-1β – Macrophage Inflammatory Protein 1-beta

MIP-3α – Macrophage inflammatory protein 3-alpha

MLMC – Maple Leaf Medical Clinic

MSM – Men who have Sex with Men

mRNA – Messenger Ribonucleic Acid

MSM – Men who have sex with men

MVC – Maraviroc

NAAT - Nucleic acid amplification-based *N. gonorrhoeae* tests

*N. gonorrhea* – *Neisseria gonorrhea*

NHP – Non-Human Primates

NRTI – Nucleoside Reverse Transcriptase Inhibitor

NNRTI – Non-Nucleoside Reverse Transcriptase Inhibitor

NVP – Nevirapine
OR – Odds Ratio

p24 – Gag Capsid Protein

p17 – Gag Matrix Protein

p7 – Gag Nucleocapsid Protein

PBMC – Peripheral Blood Mononuclear Cells

PCR – Polymerase Chain Reaction

PE - Phycoerythrin

PerCP – Peridinin Chrolophyll Protein

PHA - Phytohemagglutinin

PI – Protease Inhibitor

PIC – Pre-integration Complex

qPCR – Quantitative Real Time Polymerase Chain Reaction

RAL – raltegravir

RANTES – Regulated on Activation, Normal T cell Expressed and Secreted

RNA – Ribonucleic Acid

RPMI 1640 – Roswell Park Memorial Institute Media

RPR – Rapid Plasma Reagin

rRNA – Ribosomal Ribonucleic Acid

RT – Reverse Transcriptase

RTV – Ritonavir
sART – Standard Antiretroviral Therapy
sCD14 – Soluble Cluster of Differentiation 14
sTNF-RII – Soluble tumor necrosis factor II
SIV – Simian Immunodeficiency Virus
SMC – Semen Mononuclear Cells
SQV – Saquinavir
STI – Sexually Transmitted Infection
SVL – Semen Viral Load
Tat – Trans-Activator of Transcription
TDF – Tenofovir
Th – T helper Cell
TNFα – Tumor Necrosis Factor-alpha
T.palladum – Treponema palladium
T Reg – T regulatory Cells
UNAIDS – Joint United Nations Program on HIV/AIDS
VDRL – Venereal Disease Research Laboratory Test
VIF – Viral Infectivity Factor
VL – Viral Load
VPR – Viral Protein R
VPU – Viral Protein U
VZV – Varicella Zoster Virus

WBC – White Blood Cells

WHO – World Health Organization
Table of Contents

ACKNOWLEDGMENTS............................................................................................................................... IV

LIST OF ABBREVIATIONS .......................................................................................................................... VI

TABLE OF CONTENTS.................................................................................................................................. XIII

LIST OF TABLES ........................................................................................................................................ XVIII

LIST OF FIGURES ......................................................................................................................................... XVIII

MANUSCRIPTS ARISING FROM THIS THESIS......................................................................................... XX

CHAPTER 1: INTRODUCTION TO HIV ...................................................................................................... 1
  1.1  THE EPIDEMIOLOGY OF HUMAN IMMUNODEFICIENCY VIRUS......................................................... 2
  1.2  THE HIV VIRION AND LIFECYCLE...................................................................................................... 3
    1.2.1 Structure and genes.............................................................................................................................. 3
    1.2.2 Viral Attachment and Cellular Entry .................................................................................................. 5
    1.2.3 Nuclear Import and Integration ......................................................................................................... 5
    1.2.4 Transcription and Release ................................................................................................................ 6
  1.3  CLINICAL STAGES OF HIV-1 INFECTION ....................................................................................... 7
    1.3.1 Acute HIV Infection .......................................................................................................................... 7
    1.3.2 Chronic HIV Infection and AIDS ...................................................................................................... 7
    1.3.3 Innate Immune Responses to HIV ................................................................................................... 8
    1.3.4 Acute CD8 T-cell response to HIV .................................................................................................. 10
    1.3.5 CD8 T-cell in the genital mucosa ..................................................................................................... 11
    1.3.6 The Effect of Chronic Immune Activation ....................................................................................... 12
  1.4  UNDERSTANDING HIV TRANSMISSION ............................................................................................ 15
  1.5  HIV SUSCEPTIBILITY .......................................................................................................................... 17
  1.6  SEMEN AS A VECTOR FOR TRANSMISSION ..................................................................................... 17
    1.6.1 Origins of HIV in the MGT .............................................................................................................. 23
    1.6.2 HIV in the Male Genital Tract ......................................................................................................... 24
    1.6.3 The effect of HIV on the semen immune milieu .............................................................................. 26
  1.7  HIV ACQUISITION IN THE GENITAL TRACT ..................................................................................... 27
    1.7.1 The Male Genital Tract .................................................................................................................... 27
    1.7.2 The Rectal Mucosa .......................................................................................................................... 28
1.7.3 The Female Genital Tract.................................................................29
1.8 HIV-1 RNA Shedding in Genital Secretions......................................................31
  1.8.1 HIV Shedding in the female genital tract....................................................31
  1.8.2 Semen HIV-1 RNA Shedding.................................................................33
  1.8.3 Impact of cytokines on semen HIV..........................................................35
  1.8.4 The Role of Co-Infection.............................................................................36
1.9 Highly Active Antiretroviral Therapy .............................................................43
  1.9.1 Introduction to Antiretroviral Therapy and Regimen Choice........................43
  1.9.2 Major ARV Drug Classes for Treatment of HIV.......................................44
  1.9.3 Differential Penetration of ART in the MGT..............................................49
  1.9.4 The Effect of HAART on HIV Transmission and Genital VL.......................52
  1.9.5 Development of Resistance and Viral Evolution.........................................54

CHAPTER 2: RESEARCH AIMS AND HYPOTHESES ...........................................55
  2.1 Thesis Summary and Rationale.......................................................................56

CHAPTER 3: IMPACT OF COLLECTION METHOD ON ASSESSMENT OF SEMEN HIV RNA VIRAL LOAD .................................................................59
  3.1 Abstract........................................................................................................60
  3.2 Introduction....................................................................................................61
  3.3 Methods..........................................................................................................62
    3.3.1 Human Subjects.......................................................................................62
    3.3.2 Sample Acquisition, processing and viral load measurement.......................62
    3.3.3 Statistical Analysis..................................................................................63
  3.4 Results............................................................................................................63
  3.5 Discussion......................................................................................................64

CHAPTER 4: IMPACT OF ANTIRETROVIRAL THERAPY DURATION AND INTENSIFICATION ON ISOLATED SHEDDING OF HIV-1 RNA IN SEMEN.......................................................67
  4.1 Abstract........................................................................................................68
  4.2 Introduction....................................................................................................69
  4.3 Methods.........................................................................................................70
    4.3.1 Human Subjects.......................................................................................70
    4.3.2 Study Design..........................................................................................71
    4.3.3 Sample Acquisition, Sample Processing, and HIV Load Measurement........71
    4.3.4 Antiretroviral Levels..............................................................................72
CHAPTER 6: CLINICAL AND MUCOSAL CORRELATES OF HIV-1 RNA SEMEN SHEDDING IN ANTIRETROVIRAL NAIVE MEN

6.1 ABSTRACT.................................................................................................................................99
6.2 INTRODUCTION..........................................................................................................................100
6.3 METHODS....................................................................................................................................101
6.3.1 Study participants and design .................................................................................................101
6.3.2 Sample collection and diagnostic testing ................................................................................101
6.3.3 Real-time quantification of herpesvirus Viral load .................................................................102
6.3.4 Cytokine and trappin-2/elafin levels in semen plasma ...........................................................103
6.3.5 Bacterial load quantification .................................................................103
6.3.6 T-cell populations in blood and semen ..................................................104
6.3.7 Statistical analysis..................................................................................104
6.4 RESULTS ..................................................................................................105
6.4.1 Cross-sectional cohort demographics and viral loads..............................105
6.4.2 Semen cytokines and the semen viral load ..............................................106
6.4.3 sVL and local herpesvirus reactivation ..................................................106
6.4.4 Semen T-cell immune parameters and the sVL ......................................107
6.4.5 Bacterial load in semen and the sVL .......................................................108
6.4.6 Longitudinal cohort demographics and viral Loads ................................108
6.4.7 Patterns and correlates of HIV shedding in semen ..................................108
6.4.8 Longitudinal correlates of HIV shedding in semen .................................109
6.5 DISCUSSION.............................................................................................110

CHAPTER 7: DISCUSSION & FUTURE DIRECTIONS ........................................121
7.1 The impact of transport media on viral RNA ............................................131
7.2 Isolated HIV semen shedding and the microbiome ....................................133
7.3 Assessment of longitudinal shifts in the semen microbiome .......................134
7.4 Investigation into mucosal macrophages ..................................................134
7.5 Sexual behaviour and the impact on the semen microbiome ......................136
7.6 Investigation into the semen proteome and the effects on low-level semen shedding 138
7.7 Research Applications...............................................................................139
List of Tables

**TABLE 1-1** - Contribution of HIV invasion sites to global HIV infection ........................................... 20

**TABLE 1-2**: Differences observed in common cell types found in the semen of HIV negative and positive men.......................................................................................................................... 27

**TABLE 5-1**: Baseline (drug naive) clinical demographics of study participants in either the standard (sART) or intensified (iART) group ........................................................................................................ 97

**TABLE 6-1**: Cross-sectional Cohort Demographics and Semen Parameters ........................................... 114

**TABLE 6-2**: Semen Levels of Cytokines and Chemokines ...................................................................... 115
List of Figures

**Figure 1-1**: The HIV Virion Structure......................................................................................... 4

**Figure 1-2**: Clinical progression of HIV .......................................................................................... 14

**Figure 1-3**: The effect of quality and quantity of mucosal targets on HIV transmission............ 19

**Figure 1-4**: The male genital tract anatomy .................................................................................. 22

**Figure 1-5**: Female to male HIV-1 transmission incidence by plasma and endocervical HIV-1 RNA ......................................................................................................................... 32

**Figure 1-6**: HIV sexual transmission rate and blood viral load.................................................... 34

**Figure 1-7**: Male to Female HIV-1 transmission incidence by plasma and semen HIV-1 RNA. 37

**Figure 1-8**: Life cycle of HIV and ART site of action..................................................................... 45

**Figure 1-9**: Chart summarizing the penetration of ARVs into the male genital tract.................. 51

**Figure 3-1**: Blood and semen viral load by visit ............................................................................. 66

**Figure 4-1**: Early impact of standard vs intensified ART on the semen HIV viral load............. 81

**Figure 4-2**: Semen and blood HIV RNA viral load in men initiating iART ................................. 82

**Figure 4-3**: Semen vs blood levels of raltegravir and maraviroc after 6 months ................. 83

**Figure 4-4**: Isolated HIV semen shedding (IHS) and semen levels of raltegravir and maraviroc 84

**Figure 4-5**: Impact of therapy duration on the occurrence of isolated HIV semen shedding in men taking standard ART........................................................................................................ 85

**Figure 4-6**: Representative envelope V3 nucleotide and amino acid alignments of bulk HIV RNA plasma and semen sequences from participant 002 (high-level IHS) ........................................... 86
FIGURE 5-1: Associations between ART regimen (sART or iART) and markers of immune activation ................................................................. 95

FIGURE 5-2: Associations between ART regimen (sART or iART) at baseline (drug naive) and month 6 in soluble markers of immune activation ........................................................................... 96

FIGURE 6-1: Cross sectional linear regression of semen viral load and IL-8.............................. 116

FIGURE 6-2: Cross sectional association between CMV and semen viral load ......................... 117

FIGURE 6-3: 16s rRNA bacterial load positively correlates with semen viral load ....................... 118

FIGURE 6-4: Representative prospective HIV-1 RNA viral loads in blood and semen .............. 119

FIGURE 6-5: Longitudinal association between semen IL-8 and sVL ........................................ 120
Manuscripts Arising From This Thesis


Chapter 1: Introduction to HIV
1.1 The Epidemiology of Human Immunodeficiency Virus

Globally it is estimated that 35 million people were living with HIV at the end of 2012. Since 2001, worldwide incidence of HIV in adults has fallen by more than 25% in 39 counties, with 23 of those countries being in sub-Saharan Africa, an area that sees the highest incidence of HIV infections worldwide. North America alone accounted for 1.4 million adults and children living with HIV with a yearly incidence of 51,000 new infections, an increase since 2001. While infection routes may vary, heterosexual sex remains the dominant form of transmission of the virus worldwide, accounting for 85% of all HIV-1 infections; women make up more than 50% of those infected worldwide, the majority of whom live in sub-Saharan Africa. HIV infection among men who have sex with men (MSM) in major capital cities remain consistently higher than that of the general population, with a prevalence of infection 13 times higher than that in the general population. While prevention strategies have been in place to encourage education and condom use in the MSM and heterosexual communities, condom use over 75% of the time has only been reported in 13 of the 96 countries surveyed.

Since the introduction of highly active antiretroviral therapy (HAART), mortality rates and AIDS-related hospital admissions have dropped substantially in developed and developing countries. While HAART is not able to eradicate the virus, worldwide access to HAART has the potential to control the advance of the worldwide pandemic as treatment reduced the risk of sexual transmission twenty-five fold in clinical trials. As of 2012, 9.7 million people in low- and middle-income countries and approximately 875,000 people in high-income countries were receiving antiretroviral therapy. While these numbers are encouraging, only 30% of those infected are being treated, leaving over two-thirds of the infected population posing potential HIV transmission risk.
Despite the cost of antiretroviral therapy having decreased remarkably and the availability increased in resource poor settings, the cost of delivering these drugs to all those living with HIV is still too high for most public health systems to absorb\textsuperscript{11}. It has been estimated that to achieve universal access to HIV therapy and treat at least 14 million people would require upwards of 58 billion dollars\textsuperscript{12}.

### 1.2 The HIV Virion and Lifecycle

#### 1.2.1 Structure and genes

HIV-1 is a member of the genus \textit{Lentivirus}, family Retroviridae. The HIV genome is roughly spherical with a diameter of 120nm, 60 times smaller than that of a red blood cell (\textbf{Figure 1.1}). The virus is comprised of two copies of positive single-stranded RNA coding for nine genes (gag, pol, env, tat, rev, nef, vif, vpr, vpu) which code for 19 proteins enclosed in a conical capsid composed of 2,000 copies of viral protein p24\textsuperscript{13}. Three genes; gag, pol and env code for structural components of the virion with gag encoding the matrix, capsid and nucleocapsid; pol encoding HIV protease, reverse transcriptase and integrase and finally env encoding the HIV surface antigen gp160 (broken down by viral enzymes into gp120 and gp41). The remaining genes; tat, rev, nef, vif, vpr and vpu encode various HIV regulatory proteins.
FIGURE 1-1: The HIV Virion Structure. The HIV-1 virion is composed of two single-stranded RNA enclosed by a capsid comprising viral protein p24. Structural and other HIV virion proteins are identified.
1.2.2 Viral Attachment and Cellular Entry

To establish a productive infection, HIV must first come in close proximity to its primary cellular receptor CD4. For successful binding, the V1 region (CDR2 domain) of the CD4 molecule (expressed predominantly by T-helper cells and macrophages)\(^{14,15}\) must interact with the a CD4 binding region near the C-terminal end of the HIV envelope gp120 glycoprotein\(^{16,17}\). Binding of HIV to CD4 causes a conformational change in gp120 allowing the virus to bind to the chemokine receptor CCR5 or CXCR4, which function as co-receptors for the virus\(^{18,19}\). Of the two co-receptors, CCR5 has been shown to be responsible for almost all sexual transmission events\(^{20,21}\). It has been documented that individuals who present with a homozygous CCR5 mutation that abrogates CCR5 function are resistant to HIV infection. Furthermore, CCR5 antagonists such as maraviroc (discussed in 1.7.2) have shown great efficacy as an antiretroviral therapy agent in both nonhuman primates\(^ {22}\) and humans\(^ {23,24}\). Following binding of gp120 to one of its co-receptors causes an irreversible conformational change, exposing the N-terminal fusion peptide gp41 that is needed for virus to cell fusion\(^ {25,26}\). Following these events, the extracellular portion of gp41 collapses into a 6 helical coil-coil structure often referred to as a ‘hairpin like’ structure. The virus and host cell fuse and the viral core is then released into the cytoplasm\(^ {27-30}\). The exact mechanism by which this fusion occurs is not yet fully understood.

1.2.3 Nuclear Import and Integration

Following the release of the viral core into the cytoplasm, the core begins to un-coat through the loss of matrix and capsid proteins and is converted to a reverse transcription complex and pre-integration complex\(^ {29}\). Catalyzed by reverse transcription, viral RNA is transcribed into double-stranded DNA. The process of reverse transcription is extremely error prone as reverse
transcriptase does not correct errors by exonucleolytic proofreading. It is estimated that HIV reverse transcriptase generates $3 \times 10^5$ errors per base pair per replication cycle. The process of reverse transcription results in a highly heterogeneous HIV population or “quasispecies”\textsuperscript{31,32}. Throughout this process of reverse transcription, the viral genome remains associated with the reverse transcription complex. Integration of reverse transcribed viral cDNA occurs in a two catalytic step process; 3’ processing and strand transfer. During 3’-processing, the enzyme integrase removes a dinucleotide at the 3’ end of each viral long terminal repeat within the pre-integration complex in the cytoplasm\textsuperscript{33}. While the actual process of integration remains unclear, it has been suggested that Vpr plays an important role in nuclear import\textsuperscript{34}. Following integration of the complex, integrase catalyzes the insertion of the double stranded viral DNA into the host cell chromosome. The newly integrated viral DNA, now called provirus, will now serve as a template for the transcription of new viral particles.

### 1.2.4 Transcription and Release

For the production of a functional virus, the HIV provirus must utilize the host cell transcriptional machinery to replicate upon activation. In the host genome, transcription is initiated by the 5’ long terminal repeat\textsuperscript{35}. These promoters function to position RNA polymerase II at the site where transcription is to begin. It is here that HIV-1 utilizes the viral trans-activator protein (TAT) to stabilize the elongation of RNA polymerase II and increase the transcription rate of the viral genome\textsuperscript{35,36}. Following completion of transcription over a dozen HIV-specific transcripts are generated. Multiply spliced mRNA sequences encode Nef, Tat and Rev and are transported rapidly into the cytoplasm\textsuperscript{37}. Singly spliced or unspliced transcripts initially remain in the nucleus encoding structural enzymatic accessory proteins and the viral RNA genome. Upon production of sufficient levels, a shuttling protein known as Rev containing nuclear localization
sequence and nuclear-export sequence\textsuperscript{37,38}, shuttles transcripts and releases them into the cytoplasm. Following the transportation to the cytoplasm, translated viral proteins, viral enzymes and RNA move to the plasma membrane where they accumulate and assemble into an immature virus. This immature virus will bud off the cell and remain immature until HIV polyproteins are cleaved into a matrix, capsid and nucleocapsid proteins.

1.3 Clinical Stages of HIV-1 Infection

1.3.1 Acute HIV Infection

Within 48 hours following exposure and successful infection, HIV begins to spread to the lymph nodes and gastrointestinal lymphoid tract (GALT) where rapid replication or Acute HIV infection begins. Acute HIV infection is defined as the earliest stages of HIV infection, from the establishment of a productive infection to the point at which anti-HIV antibodies develop, after which a period of clinical latency occurs (Figure 1.2)\textsuperscript{39}. During acute infection a massive and preferential depletion of CD4\textsuperscript{+} T-cells occurs, observed in blood the lamina propria of the gastrointestinal mucosa. Concurrently, high viral loads (10\textsuperscript{6} – 10\textsuperscript{7} HIV-1 RNA copies/mL) are observed in blood plasma rising until a peak in viremia is observed around 21 days post-infection\textsuperscript{40}. In many individuals this rise in viremia is accompanied by acute retroviral syndrome (ARS), which is characterized by non-specific symptoms such as fever, rash, swollen lymph nodes, muscle aches and joint pain\textsuperscript{39,41–43}.

1.3.2 Chronic HIV Infection and AIDS

Following acute infection, a period of chronic or clinical latency begins and can last between 6 months to >10 years depending on rate of HIV disease progression. This rate of
progression depends on viral characteristics, host genetics and yet unknown factors. During this chronic phase of infection, viral loads drop to a steady state. This drop in viral load is seen during the emergence of HIV-1 specific cytotoxic T lymphocytes. During clinical latency, circulating CD4$^+$ T-cells may rebound slightly following ARS, however, without treatment, CD4 counts will then continue to drop by roughly 25-60 cells/μL per year. Drops of CD4$^+$ T-cell count below an absolute level of 200 cells/mm$^3$ leave patients at high risk for AIDS-related opportunistic infections and malignancies. During AIDS, a rapid increase in viremia with a decrease in HIV-specific immune response is observed.

1.3.3 Innate Immune Responses to HIV

The innate immune system represents the first host line of defense against viral infection. Differentiating itself from an adaptive immune response, the innate response is rapid and can act without major histocompatibility complex (MHC) restriction. Unlike the adaptive arm, the innate system uses an array of pattern recognition receptors to detect pathogens instead of antigen-specific receptors. Upon infection, HIV triggers these innate receptors including toll-like receptors (TLR) 7 and 8, leading to activation of dendritic cells and release of type 1 interferon (IFN) and tumor necrosis factor alpha (TNF-α). This initial response serves to shut down viral replication and promote immune activation in an attempt to clear the virus. It has been observed that women generally produce more IFN-α than men, possibly providing enhanced control and one possible reason behind lower viral set points observed in female populations. IFN-α has particular usefulness as it has been shown to directly interfere with virus replication (mediated by IFN-stimulated genes), increase antiviral activity of natural killer (NK) cells and induce adaptive antiviral T- and B-cell response to HIV.
This early activation of dendritic cells and other toll-like receptor expressing immune cells is associated with the rapid induction of large amount of cytokines IFN-α, IL-15, IP-10, followed by an increase in proinflammatory cytokines. This wave of cytokines causes cells of the innate immune system to begin to respond, including; phagocytes (monocytes, macrophages, DCs) for antigen clearance, cytolytic cells (natural killer cells and neutrophils) for destruction of infected cells, and professional antigen-presenting dendritic cells\(^47\).

1.3.3.1 Natural Killer cells

Natural killer cells, in particular, have been implicated in early HIV antiviral control expanding rapidly following acute infection\(^54\). HIV is able to evade NK cells with the HIV-1 protein Nef through down regulation of the T-cell receptor ligands HLA-A and –B molecules and forcing retention of MHC class I molecules in the Golgi\(^55\). Mounting data strongly implicates a role for NK cells in early HIV control, since the virus specifically evolves mutations at sites of NK-mediated immune pressure in vivo\(^47\).

1.3.3.2 Gamma Delta (γδ) T-cells

Another cell type of particular interest in the innate response is the γδ T-cell. γδ T cells differ from standard αβ T-cells in that their T-cell receptor (TCR) is invariant, encoded by different gene segments, and may recognize lipid-based epitopes. Much like natural killer cells, these cells have effector functions including cytotoxic activity and cytokine production\(^46\). γδ T cells represent a small percentage among peripheral blood lymphocytes and account for <5% of total blood lymphocytes. However, larger proportions of γδ T-cells have been observed in mucosal surfaces\(^56\).

γδ T-cells secrete Th1 cytokines (TNF-α and IFN-γ) and directly induce monocyte-derived dendritic cell maturation and activation\(^57\). It was found that there exists a significant
correlation between γδ T-cell number depletion and dysfunction in HIV-infected individuals and disease progression, both immunologically (rates of CD4 decline) and virologically (HIV plasma RNA viral load). Mucosally, γδ T-cells are found in high numbers, especially in the lamina propria where they represent a major lymphocyte sub-population. Lower levels of mucosal γδ T-cells have been found associated with short survival expectancy in advanced AIDS patients.

The direct impact of γδ T-cells on HIV acquisition and/or the control of viral replication after acquisition is largely unknown, with very limited research in vivo and studies limited to in vitro model systems.

1.3.4 Acute CD8 T-cell response to HIV

Acute HIV infection is associated with uncontrolled viral replication, reflected in very high plasma viral loads (often exceeding $1 \times 10^6$ RNA copies/ml) until adaptive host immune responses are triggered and a viral set point is achieved (discussed previously), at which point an individual is defined as having entered the chronic phase of infection. Current evidence suggests an important role for HIV-specific CD8+ cytotoxic T-cells (CTLs) in control of viral replication, with the emergence of HIV-specific CTLs directly correlating with a decline in plasma viremia and much of the early sequence variation in HIV plasma quasispecies being driven by CD8+ T-cell-mediated immune pressure. Additional strong evidence for the role of CD8+ T cells in viral control comes from SIV models of infection. Non-human primates (NHP) depleted of CD8+ T-cells with monoclonal antibodies are unable to clear infection, demonstrate an increased plasma VL, and rapidly progress to AIDS due to a complete absence of a CTL response.

Immunologically, CTLs work through recognition of viral proteins in the form of short 8-11 amino acid segments presented in association with MHC-class I molecules on the surface of infected cells. Direct cell lysis by CD8+ T-cells is achieved through the release of perforin,
glycoprotein that inserts itself into the plasma membrane of a target cell, forming pores and inducing cell death through osmotic lysis.\textsuperscript{66} Granzyme, a protease released with perforin, enters target cells and induces apoptosis and plays an important role in perforin-mediated cytotoxicity.\textsuperscript{67} The role of the CTL response during chronic infection is less clear. Despite CTL presence, control of viral replication is lost. Several studies have reported an inverse correlation between HIV-specific CTL response and viral load, despite some patients presenting with high levels of viral load with strong CTL responses. When viral infections such as HIV persist, CD8 T-cell response can be substantially altered. Excessive antigenic stimulation and chronic immune activation induce a state of anergy, causing CTLs to be become exhausted and to lose key effector functions, such as the production of effector cytokines (IFN-γ, TNF-α) and granzyme/perforin-mediated cytotoxicity.\textsuperscript{68}

1.3.5 CD8 T-cell in the genital mucosa

HIV-specific CD8\textsuperscript{+} T-cells have been characterized in the vaginal mucosa of SIV-infected NHPs\textsuperscript{69}, HIV-infected women\textsuperscript{70} and seminal plasma from an HIV-infected man.\textsuperscript{71} While the exact function of HIV-specific CD8\textsuperscript{+} T-cells in the genital mucosa has not been defined, it is hypothesized they serve a similar role in viral control as seen in blood. To date few studies have examined seminal plasma HIV-specific CD8 T-cells and failed to find an association with shedding of the HIV virus in semen.\textsuperscript{72} Neither the presence or frequency correlated with reduced semen HIV RNA, in fact, higher absolute numbers of HIV-specific CD8\textsuperscript{+} T-cells was associated with increased HIV RNA and pro-inflammatory cytokines.\textsuperscript{72} Based on these observations, it has been hypothesized that inflammatory cytokines in semen may induce local HIV mucosal replication and viral shedding, with a subsequent increase in mucosal CD8\textsuperscript{+} T-cell numbers as a host response.
1.3.6 The Effect of Chronic Immune Activation

One of the defining features of progressive and non-progressive HIV-1 infection is a state of chronic systemic immune activation; defined as the active state of molecular and cellular processes that include T-cell activation, proliferation, death, and secretion of soluble inflammatory molecules. In HIV-infected patients and SIV-infected macaques, increased expression of T-cell activation markers CD38 and HLA-DR is observed, correlating with disease progression and CD4 T cell depletion\(^73-76\). Immune activation measured by the co-expression of CD38 and HLA-DR on CD8 T cells is the best predictor of HIV disease progression. CD38, a transmembrane glycoprotein has been found to be upregulated during the earliest stages of T-cell activation in HIV infection and one of the strongest surrogate markers of immune activation and disease progression\(^75,76\). It has been observed in non-pathogenic SIV hosts (sooty mangabeys for SIV) the profile of activation seen with pathogenic infection does not occur nor does an increase in viral load\(^77,78\), stressing the importance of activation state to disease progression.

Many theories as to the cause of the observed chronic immune activation state in HIV infection have been postulated. Gp120 from the envelope protein of HIV has shown the potential to activate T-cells and enhance their responsiveness to activation even without productive infection\(^79-81\). Nef, an accessory protein of HIV, has further been shown to lead to activation states in lymphocytes both directly\(^82,83\) and through infection of resident macrophages\(^80\). It has been further suggested that translocation of microbial products, such as lipopolysaccharide (LPS), from breaks in the gut mucosa, are the root cause of systemic immune activation, with microbial products inducing a pro-inflammatory environment in systemic circulation\(^84,85\). In HIV negative individuals phagocytes within the lamina propria, remove and breakdown microbes that translocate in. Coupled with an intact epithelial barrier, these natural host defenses prevent
translocation of microbial products into systemic circulation\textsuperscript{86}. Damage of the epithelial barrier integrity and decreased local immune defense as a result of HIV infection may allow for translocation of microbial products and subsequent increases in systemic immune activation.
**Figure 1-2: Clinical progression of HIV.** A representative relationship depicted between HIV-1 viral load and CD4 count over the average course of untreated HIV infection

Adapted from Fauci et al. 1996

In order to control heightened levels of immune activation, viral replication and reduce transmission and mortality rates, antiretroviral therapy is utilized. Antiretroviral therapy (ART) consists of a combination of antiretroviral (ARV) drugs to suppress HIV replication and disease progression. Initiation of antiretroviral therapy has been shown to reduce the levels of immune activation significantly, but does not necessarily normalize inflammation levels to that of an HIV uninfected individual\textsuperscript{88}. Persistent chronic immune activation, even at low levels can leave patients at increased risk for cardiovascular/cerebrovascular disease, cancer, diabetes, liver disease, osteoporosis and neurocognitive disorders\textsuperscript{89,90}. Non-AIDS related illness now make up over half of the deaths in HIV-infected patients with liver disease and cardiovascular disease (CVD) leading the trend. Effective (drug) therapy to reduce levels of immune activation is key to control of secondary and non-AIDS related complications.

1.4 Understanding HIV Transmission

Understanding how viruses are transmitted at a population level is important in the study of HIV transmission. Mathematical modeling has produced an equation to help predict how viruses will spread in a population without pre-existing host immunity, called the \textit{Reproductive Ratio} ($R_0$). In short, if the $R_0$ is larger than 1, the virus can invade, persist, maintain itself in a population, and emerge\textsuperscript{91} as an epidemic.
\[ R_0 = \beta Dc \]

\( R_0 = \) Secondary spread of an agent

\( \beta = \) efficiency of transmission \((i.e. \ the \ likelihood \ that \ an \ HIV^{+} \ individual \ will \ spread \ the \ virus \ to \ his/her \ sexual \ partner \ during \ a \ single \ contact)\)

\( D = \) Duration of infectiousness \((i.e. \ How \ long \ an \ HIV^{+} \ individual \ remains \ infectious)\)

\( c = \) number of individuals exposed to infection \((i.e. \ The \ number \ of \ sexual \ events/exposures \ or \ partners)\)

While some of the immune parameters associated with altered host HIV susceptibility have been defined, the spread of an infectious agent like HIV can be influenced by a plethora of complex social and cultural factors. Some of these may include number and frequency of sexual partners, frequency of unprotected intercourse, regular condom use or based on perceived HIV status of sexual partner (serosorting), sexual role assignments (insertive vs. receptive or based on HIV status “seropositioning”), varying HAART treatment strategies, social and sexual networks and the influence of the internet\(^{92}\).

It has been estimated in one study that during primary infection (during peak viremia) HIV is 26 times more likely to be transmitted than during the chronic, asymptomatic phase, with a probability of 10.6 transmission events per 100 persons-years\(^{93}\) (e.g. 10.6% per year). It has further been estimated that the \( R_0 \) for populations where serial monogamy occurs is 1.09, however, in random mixing populations with contacts occurring all the time the \( R_0 \) has been
estimated at 2.15, a value much more reflective of the general populations. This number must be taken with caution as many local factors drive this number much higher or in some groups lower.

1.5 HIV Susceptibility

The probability of HIV transmission during a single unprotected sexual encounter is surprisingly low, considering the size of worldwide pandemic, with estimates for penile-vaginal sex or insertive anal sex ranging from 1/200 to 1/2000 (receptive anal sex is about ten-fold riskier, with a per-act transmission rate ~1/100). These numbers, however, do not take into account the multitude of factors that can change one’s susceptibility to HIV infection. The number and frequency of sexual partners, hormonal contraceptive, vaginal and anal douching, bacterial vaginosis, STIs, and specific sexual practices all contribute and effect how susceptible an individual may be. Our lab group has demonstrated that mucosal factors in particular have a large role in transmission risk. It has been hypothesized that mucosal immunology – specifically, the presence and/or number of HIV-susceptible target cells in an exposed mucosal tissue – can increase the probability of HIV transmission in two different ways. The first is that the influence of various clinical infections can lead to an overall increase in all mucosal CD4+ T-cells above a “transmission threshold”; alternatively, the overall number of cells may stay the same, with enrichment of a highly susceptible CD4+ T-cell subset (e.g. Th17 cells), despite no change in overall cell numbers (Figure 1.3).

1.6 Semen as a Vector for Transmission

Sexual transmission of HIV-1 accounts for between 60-90% of new infections in the world, with semen acting as the vector for the majority of transmission events at a global level (Table 1.1). Despite the high level of sexually transmitted HIV infections, the probably of transmitting
HIV through sex is highly variable between 0.1-0.001%\textsuperscript{96}. In a study of serodiscordant MSM couples, it has been estimated that the per contact risk for unprotected anal sex with ejaculation to be 1.43% and 0.65% if withdrawal occurred without ejaculation. The per-contact risk for an insertive partner has been estimated at 0.11% in men who were circumcised and 0.62% in uncircumcised men\textsuperscript{97}.

Many factors affect the efficiency of transmission, such as; acute vs chronic HIV disease stage, semen viral load, active STIs and mucosal epithelial damage. In recent years, a large focus has been put on determining the effects semen and its constituents have on HIV-1 transmission. Semen comprises a diverse mixture of cytokines, chemokines, growth factors, immunomodulatory compounds, mononuclear cells, spermatozoa and other biologically active molecules and secretions from the seminal vesicles, prostate, testis, epididymis and bulbourethreal glands (Figure 1.4). Semen is generated in a multi-step process throughout the male genital tract with volume of ejaculate ranging from 100µL to 11mL\textsuperscript{98}. Protecting the developing sperm are Sertoli cells that line the seminiferous tubules. These cells sequester sperm from immune cells in the interstitial space and blood vessels forming the blood-testes barrier (BTB).
**Figure 1-3** - The effect of quality and quantity of mucosal targets on HIV transmission.
### Table 1-1 - Contribution of HIV invasion sites to global HIV infection

<table>
<thead>
<tr>
<th>HIV invasion site</th>
<th>Anatomical sub-location</th>
<th>Type of epithelium</th>
<th>Transmission medium</th>
<th>Transmission probability per exposure event</th>
<th>Estimated contribution to HIV cases worldwide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female genital tract</td>
<td>Vagina</td>
<td>Squamous, non-keratinized</td>
<td>Semen</td>
<td>1 in 200–1 in 2,000</td>
<td>12.6 million</td>
</tr>
<tr>
<td></td>
<td>Ectocervix</td>
<td>Squamous, non-keratinized</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Endocervix</td>
<td>Columnar, single layer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>Various</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male genital tract</td>
<td>Inner foreskin</td>
<td>Squamous, poorly keratinized</td>
<td>Vaginal and rectal secretions and desquamation</td>
<td>1 in 700–1 in 3,000</td>
<td>10.2 million*</td>
</tr>
<tr>
<td></td>
<td>Penile urethra</td>
<td>Columnar, stratified</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>Various</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestinal tract</td>
<td>Rectum</td>
<td>Columnar, single layer</td>
<td>Semen</td>
<td>1 in 20–1 in 300</td>
<td>3.9 million*</td>
</tr>
<tr>
<td></td>
<td>Upper GI tract</td>
<td>Various</td>
<td></td>
<td>1 in 2,500</td>
<td>1.5 million</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Maternal blood; genital secretions (intrapartum)</td>
<td>1 in 5–1 in 10</td>
<td>960,000*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Breast milk</td>
<td>1 in 5–1 in 10</td>
<td>960,000*</td>
</tr>
<tr>
<td>Placenta</td>
<td>Chorionic villi</td>
<td>Two layer epithelium (cyte-</td>
<td>Maternal blood (intrauterine)</td>
<td>1 in 10–1 in 20</td>
<td>480,000*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and syncytiotrophoblast)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood stream</td>
<td></td>
<td>Blood products, sharps</td>
<td>95 in 100–1 in 150</td>
<td>2.6 million*</td>
<td></td>
</tr>
</tbody>
</table>

*Table adapted from the UNAIDS/WHO AIDS epidemic update and REFS 121–128. *Includes men having sex with men (MSM), bisexual men and heterosexual men. **Includes MSM, bisexual men and women infected via anal receptive intercourse. ***Mother-to-child transmission. ****Mostly intravenous drug use, but includes infections by transfusions and health-care-related accidents. GI, gastrointestinal.

© Hladik, F., et al. 2008
This barrier creates a compartmentalized site within the male genital tract (MGT)\(^9\). Despite this barrier, HIV has been detected as cell-free RNA in semen and in semen macrophages and lymphocytes. Many factors have been hypothesized as to what can compromise this barrier, allowing HIV to gain access. The exact method by which HIV-1 crosses the blood-testes barrier and enters the compartmentalized MGT is largely unknown. One hypothesis suggests that during acute infection, widespread inflammation compromises the tight junctions between Sertoli cells that line the blood testis barrier. Breaks in this barrier due to acute inflammation allow virus in to the systemic circulation to cross and infect local HIV-susceptible target cells expressing the CD4\(^+\) receptor. The resolution of inflammation at the end of the acute phase traps the virus, creating a compartment for replication distinct from peripheral circulation\(^10\). Another hypothesis by Muciaccia et al. (2007) suggests that chronic orchitis (inflammation of the testes) that arises during HIV-1 infection compromises the BTB allowing viral exchange between the peripheral blood and MGT\(^10\). While there is some debate over the exact mechanism by which HIV-1 establishes a site of replication in the MGT, several studies have documented distinct viral sequences in semen separate from blood evidencing compartmentalization of the virus\(^10\).\(^2\)-\(^10\)^5.
**Figure 1-4: The male genital tract anatomy**

Adapted from Coombs, R.W. et al. *Acquir Immune Defic Syndr* 2006¹⁰⁶

Sagittal view of the male lower genitourinary tract. (a) Prostate gland, (b) seminal vesicle, (c) testicle, (d) epididymis, (e) vas deferens, (f) bulbourethral (cowper) gland, (g) urethral gland and (h) ejaculatory duct
1.6.1 Origins of HIV in the MGT

Semen represents the main vector of HIV-1 infection worldwide, yet the origin of the virus that is present in semen remains poorly understood. It has been well documented in several studies that semen represents a distinct and separate compartment from blood (discussed in section 1.4); suggesting local sources of replication of HIV-1 in the MGT are contributing to the production of infectious virus\textsuperscript{100,102-105,107}.

In 2008, a study by Tortorec et al. (2008)\textsuperscript{108} examined SIV infection in the reproductive tracts of male non-human primates (NHP) during the acute and chronic stages of SIV infection. In this prospective study SIV was found in the testes, epididymides, prostate and seminal vesicles (Figure 1.4) of both acutely and chronically infected macaques. The prostate and seminal vesicles were the most highly infected sites, followed by the epididymis with the testis displaying the lowest level of infection. Predominantly, infected cell types varied depending on organ samples. Throughout the genital tract, T-lymphocytes were the main infected population with macrophages predominating in the epididymis and accessory glands\textsuperscript{100}. The prostate and seminal vesicles are known to contribute 60\% and 30\% of seminal fluid respectively\textsuperscript{109} and are likely the source of the majority of infectious cell-associated viral particles in NHP. Low-level infection of the testis has been further supported by studies of NHP with AIDS\textsuperscript{110}. It has been suggested that low levels of infection observed in the testis may be due to a small pool of HIV target cells combined with local immunosuppressive factors that inhibit inflammation and suppress T-cell activity\textsuperscript{111-113}. Whether these NHP findings translate into human HIV-1 infection has been of some debate and yet to be fully documented in humans due to inherent difficulties in collection of human biopsy samples.

Studies determining the origin of HIV in the human MGT are scant, with ethical and practical caveats limiting sampling. Early studies assessing the origins of HIV in the MGT
examined men receiving a vasectomy, a safe and efficacious method of contraception. Vasectomy disrupts the vas deferens at a distal site in the MGT causing the elimination of sperm and reducing local leukocyte counts by more than 20-fold\textsuperscript{114,115}. This procedure allows for an indirect assessment of proximal sites MGT (Germinal cells, secretions from testes, rete testes, epididymides and proximal vasa deferentia) as potential major contributors to the pool of HIV RNA observed in semen. It has been observed that following vasectomy, semen HIV RNA before and after the procedure has no effect on recovery of cell-free HIV RNA in seminal plasma\textsuperscript{116}. This suggests that proximal sites contribute insignificant levels of HIV to the HIV RNA quantified in seminal plasma. Distal sites such as seminal vesicles, prostate, urethra and periurethral and bulbourethral glands are therefore likely to be more important sources of HIV. More recent studies examining these sites have further determined that it is the organs that lie distal to the prostate to be the larger contributors to the HIV RNA pool in the MGT then the prostate\textsuperscript{106}.

1.6.2 HIV in the Male Genital Tract

Semen represents the main transmission vector for HIV infection, evidenced by transmission occurring more efficiently from men to women and men than from women to men\textsuperscript{117}. Once HIV is established in the MGT, it takes the form of either; (1) cell-free virion within the seminal plasma; (2) as proviral HIV DNA within infected resident cells or, (3) as spermatozoa associated HIV. The origin of the free virus and infected cells that contaminate semen still remains unclear. Semen is a mix of cells and secretions originating from various sites including the testes, epididymis, prostate, seminal vesicles and bulbo-urethral glands. With both practical and ethical limitations preventing sampling, studies determining the source and HIV reservoirs in the MGT remain limited. Numerous studies have examined HIV present in blood and semen and
have shown data that semen virus evolves separately from strains seen in blood or other anatomical compartments, suggesting the MGT constitutes a distinct viral compartment from blood\textsuperscript{107}. The testes represent one potential reservoir for HIV. The presence of the blood testis barrier and drug efflux pumps, expressed by a number of testicular cell types, limit drug access\textsuperscript{118,119}. It is, however, currently unknown what concentration HIV ARVs may be present in the testis due to difficulty in sampling. HIV infection examined ex vivo by organotypic culture has shown that human testis can be infected by HIV ex vivo and produce low levels of infectious virus with the main virus-producing cell being that of resident testicular macrophages\textsuperscript{120}. In vivo studies of SIV-infected NHP has further confirmed the productive infection of the testis during both asymptomatic and chronic stage of infection with establishment of the reservoir occurring during acute primary infection\textsuperscript{108}. The accessory glands of the MGT pose another potential site for HIV to reside. SIV NHP infection models have shown infection and subsequent viral production from the epididymis, prostate and seminal vesicles in both primary and chronically infected NHPs\textsuperscript{110}.

While it is yet unclear as to the exact cellular or anatomical sanctuary for HIV in the MGT, various studies continue to provide evidence that the MGT constitutes a viral reservoir. The persistence of HIV in semen under HAART has potentially important consequence for transmission of the virus. One particular question is to whether natural conception is an acceptable or negligible risk for HIV-serodiscordant couples, whom the HIV+ partner is treated with HAART and virologically suppressed\textsuperscript{121}. The Swiss national AIDS Commission released a statement in 2010 indicating that serodiscordant couples in whom the infected partner had undetectable viremia under prolonged HAART could safely have unprotected intercourse in the absence of other STIs\textsuperscript{122}. This statement has come under heavy debate among both the scientific and general public. A model proposed by Wilson et al in 2008 calculated that the incidence of
HIV would quadruple if this advice was followed by 10,000 copies over 10 years\textsuperscript{123}. Numerous clinical studies have shown the ability of HAART to drastically reduce the possibility of transmitting HIV, however, as best illustrated by a case study by Sturmer in 2008 where a homosexual man under effective HAART infected his partner, shows that HAART may not completely prevent the ability to transmit the virus\textsuperscript{124}.

A fundamental question is whether cell free or cell-associated HIV is responsible for the observed HIV transmission. Several hypotheses exist as to ways each form of virus found in semen can contribute to productive infection during unprotected sexual intercourse. Examination of serodiscordant MSM couples has helped shed light on the transmitting form of HIV (cell-free vs. cell-associated). Phylogenic analysis of samples taken from serodiscordant couples following transmission have shown cell-free virus from the transmitting partner consistently bearing the strongest sequence resemblance of the HIV found in the plasma of the newly infected partner.\textsuperscript{125} Despite these findings the literature remains open to debate as to whether it is the cell-free or cell-associated virus in semen that is causing infection.

1.6.3 The effect of HIV on the semen immune milieu

The HIV negative MGT is typically populated with white blood cells (WBC), immature germ cells and spermatozoa. Studies have shown HIV negative men to have roughly $10^5$ WBC/mL, comprising polymorphonuclear leukocytes, macrophages and CD4$^+$ T-cells (Table 1.2)\textsuperscript{126-129}. Upon infection with HIV, seminal CD4$^+$ and CD8$^+$ T-cell populations are depleted with macrophage to CD4$^+$ lymphocytes reaching up to a 22:1 ratio\textsuperscript{130}.
Table 1-2: Differences observed in common cell types found in the semen of HIV negative and positive men

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>HIV-Negative(^a) (n=17) Median (Range) per mL</th>
<th>HIV- Positive(^b) (n=98) Median (Range) per mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total WBCs</td>
<td>170,000 (9,000 – 20,520,000)</td>
<td>104,000 (0 – 55,380,000)</td>
</tr>
<tr>
<td>Monocytes/Macrophages</td>
<td>52,000 (300-998,000)</td>
<td>22,000 (0 – 24,349,000)</td>
</tr>
<tr>
<td>CD4+ T Cells</td>
<td>4,000 (0-52,000)</td>
<td>0 (0 – 6,187,000)</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>2,000 (0-57,000)</td>
<td>0 (0 – 1,547,000)</td>
</tr>
</tbody>
</table>

\(^a\)Wolff and Anderson\(^b\)Politch et al.

1.7 HIV Acquisition in the Genital Tract

1.7.1 The Male Genital Tract

Globally, it has been estimated 70% of HIV-infected men have acquired HIV through heterosexual intercourse with the penis being the most likely site of entry\(^1\). Randomized trials from South Africa\(^2\), Kisumo (Kenya)\(^3\) and Rakai (Uganda)\(^4\) have shown male circumcision to reduce the risk of HIV acquisition by 60%. This protective effect of circumcision may be due in part to keratinization of the glans when not protected by the foreskin, reduction of total surface area of skin, reduction of target cells (numerous in the foreskin\(^5\)) or indirect factors such as decreased acquisition of STI’s that can increase HIV susceptibility\(^6\). It’s further been documented that circumcision decreases HSV-2 acquisition by 28-34%, HPV prevalence by 32-
35% and *Neisseria gonorrhoea* incidence by as much as 61% all contributing factors to the protective effect circumcision imparts. Despite these findings, mixed results have been reported in the United States. In one study by the HIV Network for Prevention Trials, the HIV-vaccine preparedness cohort showed uncircumcised men to be twice as likely as circumcised men to acquire HIV infection\textsuperscript{137} these findings have also been observed in a cross-sectional study of heterosexual men attending an STD clinic among patients with known HIV exposure. However, in a prospective cohort study of MSM, no association was found between circumcision status and the incidence of HIV infection\textsuperscript{137}. Studies examining the effect of circumcision in MSM populations are often limited by the assumption that MSM assume either an exclusive insertive or receptive sexual role, with respect to anal sex. Often is the case in studies of MSM that the majority of men have engaged in receptive anal sex during their life, thus introducing bias into associations between circumcision and HIV, favouring non-significant observations. Studies investigating sites of acquisition other then foreskin are limited by the availability of penile tissue samples. It has been shown that HIV target cells, CD4\textsuperscript{+} T-cells and macrophages, are present on the columnar urethral mucosa\textsuperscript{138-140}, with HIV co-receptors CCR5 and CXCR4 being expressed on cells present at the urethral opening\textsuperscript{141}. In a study by Fischetti *et al* 2009 fresh penile explant models showed that tissue from the foreskin, glans, meatus and urethra were all susceptible to R5 HIV-1 infection in vitro\textsuperscript{138}.

### 1.7.2 The Rectal Mucosa

Among MSM populations, unprotected receptive anal sex with an HIV+ partner has been shown to have a per contact risk of 0.82% versus 0.06% for unprotected insertive anal sex\textsuperscript{142}. While more difficult to study due to privacy, religion and various social factors, anal sex in
women has been previously investigated in sex workers in South Africa and associated with a 1.4 – 5.1-fold higher risk of HIV infection compared to vaginal sex\textsuperscript{143}.

Unlike the vaginal epithelium, but similar to the cervix, the rectal mucosa is lined with a single layer of columnar epithelial cells, providing little physical protection against possible trauma/breaches caused during sex, giving HIV access to underlying target cells that express high levels of CCR5 and CXCR4 present in the sub-mucosa\textsuperscript{144}. Several factors beyond physical trauma allow for transmission of HIV through the mucosa. For instance, the gastrointestinal mucosa is rich in membranous or microfold cells (M-cells), that function to deliver luminal antigens through the epithelium to underlying immune cell by encasing antigens in vesicles on the apical side and transcytose them to the basolateral side\textsuperscript{145}. The ability of these cells to transcytose particles across the epithelium make them a favourable entry point for HIV as they can shuttle the virus across the barrier and allow HIV access to target cells.

1.7.3 The Female Genital Tract

Vaginal intercourse is the predominant means of HIV transmission in developing countries\textsuperscript{146}. A number of events that occur during and post-coitus contribute to transmission of HIV. Epithelial micro-abrasions can be observed in 60% of women following coitus\textsuperscript{147}, allowing HIV better access to target cells initially protected by multiple layers of stratified squamous epithelium. Following ejaculation, a pH rise is observed in the female genital tract shifting the natural pH from an acidic to a more basic environment. This shift in pH creates an environment within the female genital tract that is now more favourable to male-to-female HIV transmission by preventing acidity-associated loss of infectivity of HIV\textsuperscript{148}. In response to the presence of semen, the vaginal epithelium has further been shown to secrete chemokine (C-C motif) ligand 20 (CCL20), which enhances recruitment of Langerhans cells to the vaginal mucosa, which may
assist in the transportation of virions across the mucosa or be internalized into Birbeck granules and degraded\textsuperscript{149,150}. Other factors such as trauma, genital ulcers, inflammation, sexually transmitted infections, hormonal contraceptives can also increase susceptibility to infection\textsuperscript{147,151}.

A number of studies have examined the initial steps of HIV acquisition in the female genital tract (FGT). Despite vaginal intercourse carrying a lower HIV transmission probability per exposure event than anal sex, it contributes more cases to the global pandemic than anal intercourse or parenteral inoculation\textsuperscript{96}. The relative contribution of the vaginal, ectocervical and endocervical mucosa to successful transmission is largely speculative, however, transmission events have been documented at all sites\textsuperscript{96}. The thick squamous epithelium covering the vaginal mucosa and ectocervix offers much better mechanical protection than the single-layered epithelium that covers the endocervix, although virus can diffuse directly through an intact epithelium. In vivo observations in female macaques have shown HIV infection can be achieved by both free and cell-associated virus\textsuperscript{152}, this observation has been confirmed through cervical explant models\textsuperscript{153}. Transcytosis is one means by which HIV can cross the columnar epithelium of the endocervical epithelium\textsuperscript{154}. Certain cell-surface molecules such as glycosphingolipids have been implicated in some studies to facilitate the binding of HIV to epithelial cells\textsuperscript{155}. Whether through an intact epithelium or via penetration through microabrasions, another set of events occurs once HIV has crossed the cervicovaginal epithelium. Once across the epithelium, HIV encounters immature dendritic cells and CD4\textsuperscript{+} target cells\textsuperscript{156}. Langerhans cells (LCs) are a particular cell of interest due to their susceptibility to HIV entry and infection\textsuperscript{157}. More specifically, LCs can internalize HIV into their cytoplasmic organelles\textsuperscript{158} and transport the virus to the basal side of the epithelium, where a productive infection can be established. These dendritic cells are able to bind HIV though C-type lectin receptors langerin (CD207) and DC-specific ICAM-grabbing non-integrin (DC-SIGN), which functions as adhesion receptors for HIV.
and mediating both DC migration and T-cell activation\textsuperscript{159}. Another primary target in the FGT are the intraepithelial CD4\textsuperscript{+} T-cells. Studies have shown that HIV infection of these epithelial cells and LCs is in part due to their high levels of expression of HIV co-receptors CD4 and CCR5\textsuperscript{158}. Once infected these cells amplify local infection and subsequently disseminate infection systemically\textsuperscript{158}

1.8 HIV-1 RNA Shedding in Genital Secretions

1.8.1 HIV Shedding in the female genital tract

Within HIV-infected women, the probability of sexual and mother-to-child transmission is highly correlated with plasma virus levels, likely because blood virus levels are linked to the level of HIV RNA in female genital secretions\textsuperscript{160,161}. Many factors can increase genital shedding of HIV RNA in women including; antiretroviral medication pharmacology\textsuperscript{162}, genital infections\textsuperscript{163}, oral contraceptive use\textsuperscript{164}, and pregnancy\textsuperscript{165}. As seen in men\textsuperscript{166-168}, HIV RNA can be sometimes be detected in the genital tract of women receiving HAART, even with undetectable plasma viremia and no concurrent STIs\textsuperscript{169}. Women with undetectable viremia have presented with genital viral loads ranging in one study from 480,000 to 748,000 copies/mL. In the only study to date examining genital HIV shedding in women while on therapy, it was found that 54\% of women had detectable HIV-1 RNA at least once in the genital tract during the study with 37\% having detectable RNA when blood vireamia was undetectable\textsuperscript{169}. While it has been suggested that certain subcompartments of the female genital tract may be more conducive to viral shedding, such as the ectocervix, (versus the endometrium)\textsuperscript{170}, recent data suggests this is not the case with no one subcompartment being more likely to shed the virus than the other. As seen with semen, a strong step-wise effect is observed when comparing female genital tract HIV RNA levels and
incidence of transmission. Each $1.0 \log_{10}$ increase in endocervical HIV RNA is associated with a 1.67-fold increased risk of female-to-male transmission$^{171}$ (Figure 1.5).

**Figure 1-5 - Female to male HIV-1 transmission incidence by plasma and endocervical HIV-1 RNA.** HIV-1 incidence for each HIV-1 RNA quantity category (undetectable, $<3$, 3-4, 4-5, and $>5 \log_{10}$) presented for blood and semen HIV RNA. A step-wise relationship between HIV quantity and transmission was observed for both blood and semen.
1.8.2 Semen HIV-1 RNA Shedding

The HIV RNA level in blood of an HIV-infected individual is the best-defined predictor of transmission risk in HIV sero-discordant couples. The annual transmission risk increases from 0 to 25% as the blood viral load in an ART naïve partner increases from undetectable levels to >50,000 copies/mL (Figure 1.6)\(^{160}\). As there is minimal blood contact during most sexual HIV transmission, the association of transmission risk with blood viral load likely reflects the level of HIV-1 RNA in seminal plasma\(^{172-175}\).

Work from the Partners and Prevention HSV/HIV Transmission study team has demonstrated that the semen viral load of a male partner was a stronger independent predictor of HIV transmission than blood viral load, with each \(\log_{10}\) increase in semen viral load being associated with a 1.8 fold increased risk of HIV transmission (Figure 1.7)\(^{171}\). In vitro studies have shown a threshold of 3.8\(\log_{10}\) HIV RNA copies in semen required for infection of CD4\(^+\) T-cells in co-culture\(^{176}\).

The correlation between blood and semen is moderate at best, with few studies showing strong correlations. It has been documented that the mean HIV RNA level in semen is approximately 1.3-1.5 \(\log_{10}\) copies/mL lower than blood\(^{177-179}\). Despite these observations some notable caveats do exist. In 2006 our lab found that 35% (9/26) of participants shed semen HIV at levels exceeding blood viral load\(^{166}\). In a separate study, analysis of sexual risk behaviours showed that men with greater concentrations of HIV in their semen relative to blood reported significantly greater rates of unprotected vaginal intercourse and total number of unprotected sexual encounters as the insertive partner than men with equal to or greater concentrations of virus in their plasma than semen\(^{180}\). These findings suggest unprotected intercourse may result in
© Quinn et al, NJEM 2000

**Figure 1-6** - HIV sexual transmission rate and blood viral load.
acquisition of a yet unknown factor such non-pathogenic bacterium that is directly influencing HIV replication in the MGT.

1.8.3 Impact of cytokines on semen HIV

As discussed in 1.6.3, HIV has a large impact on the dynamics of the semen immune milieu. Many factors, both infectious and non-infectious can serve to enhance viral replication and increase viral load. One of these factors includes the highly regulated cytokine network found in the MGT. Semen has been shown in several studies\textsuperscript{181-183} to have a distinct cytokine profile from that of blood with elevated levels of several proinflammatory cytokines including; MCP-1, IL-8, IL-6, Fractalkine, MIP-1β and GM-CSF; adaptive cytokines IL-7 and IL-15 and decreased levels of RANTES, Exotaxin, and G-CSF relative to blood\textsuperscript{182}. Our lab, as well as others, have found associations between semen cytokine concentrations and semen viral load including; TNF-α, IFN-γ, G-CSF and IL-10\textsuperscript{168,182}. While the exact mechanism by which these cytokines may be influencing viral load both directly or indirectly have yet to be fully established, some theories have been postulated as to their role. G-CSF, a proinflammatory cytokine produced by macrophages and endothelial cells at sites of infection, attract and promote the survival of neutrophils\textsuperscript{184}. High concentrations of G-CSF in seminal plasma have been positively associated with β-chemokines MIP-1α and MIP-1β as well as with high frequencies of CD8\textsuperscript{+}CCR5\textsuperscript{+} T-cells. β-chemokines, which have a large role in chemotaxis, have been thought to be recruiting these activated CD8\textsuperscript{+}CCR5\textsuperscript{+} T-cells into the MGT which potentially suggests that HIV target cells are not activated locally in the MGT, but rather recruited in an activated state\textsuperscript{182}. The effects of semen cytokines are not limited to influencing viral replication and cell recruitment. A few studies have observed that cytokines present in high concentration in an HIV\textsuperscript{+} man can actually increase the
susceptibility to infection in ex vivo models. Both IL-7 and IL-8 have shown the ability to enhance infection in cervio-vaginal explant models \(^{185,186}\) evidencing the important role cytokines may have in the mucosa during the initial stages of infection. A more comprehensive understanding of the exact roles of these cytokines as it relates to HIV infection and transmission is needed.

1.8.4 The Role of Co-Infection

The varying effects of concurrent infections on HIV viral load, CD4 count and susceptibility to acquisition in HIV have been well documented. With respect to semen HIV, both symptomatic infections (Syphilis, urethritis, etc.) and asymptomatic (CMV/EBV) infections play a large role in HIV replication in the genital tract.

1.8.4.1 Sexually Transmitted Infections and HIV

The per coital act rate of HIV transmission in heterosexual HIV-discordant couples is surprisingly low, estimated to be one productive infection for every 1,000 coital acts\(^ {187}\). Sexually transmitted infections (STIs) that cause inflammation or clinical ulcers change this frequency; increasing the efficiency of transmission both in respect to susceptibility and infectiousness.

Ulcerative STIs have a dramatic effect on increasing semen HIV viral load while increasing local inflammation. Cohort studies have shown men who present with genital ulcers and non-gonococcal urethritis to shed significantly higher levels of HIV in semen compared to men with urethritis alone\(^ {188}\). HIV seropositive men with urethritis have been shown to shed as much as eight times higher levels of semen HIV-1 RNA than those without. Similar findings have been found in men testing positive for both gonococcal and chlamydial urethritis with semen viral loads.
increasing by as much as 5-fold compared to men without urethritis. Treatment of STIs with antimicrobial therapy has been shown to be efficacious in resolving infection and decreasing


**Figure 1-7: Male to Female HIV-1 transmission incidence by plasma and semen HIV-1 RNA.** HIV-1 incidence for each HIV-1 RNA quantity category (undetectable, <3, 3-4, 4-5, and >5 log10) presented for blood and semen HIV RNA. A step-wise relationship between HIV quantity and transmission was observed for both blood and semen
semen viral load significantly by as much as 3-fold within the first few weeks of treatment\textsuperscript{188}. These increases in semen viral load as a result of STIs pose considerable public health concerns, as increases in viral load are associated with an increased risk of transmission.

1.8.4.2 STIs Effects on HIV Acquisition

Many factors affect the host innate and adaptive immune response and can increase susceptibility to HIV. Breaks in the anal and vaginal mucosal lining can alter the bodies’ main defense against sexually transmitted infections. Genital trauma and irritations by means of aggressive or dry sex\textsuperscript{190} or through the use of spermicides\textsuperscript{191} compromise the natural mucosal lining. With respect to HIV acquisition, the presence of an STI increases the susceptibility of an HIV-negative individual by 2-11 fold\textsuperscript{192}. Individuals presenting with recurrent genital ulcer disease (GUD) have been shown to have a seven-fold increased risk of acquiring HIV, and the presence of cervicitis/urethritis was associated with a threefold increase in the risk\textsuperscript{193}.

Herpes simplex virus type-2 (HSV-2) is a chronic infection, being asymptomatic in the majority of those infected\textsuperscript{194}. Prevalence of HSV-2 has been estimated to be greater than 60% in African adults and approximately 15-20% in North American, with higher rates around 40-50% observed in MSM communities\textsuperscript{192}. In a study of sex-specific effect of herpes simplex virus 2 (HSV-2) on acquisition of HIV infection, it was found that HSV-2 was associated with a three-fold increased risk of HIV acquisition after sexual exposure in both men and women. This data suggests that in areas with high prevalence of HSV-2, a large proportion of HIV infections may be attributed to this infection\textsuperscript{194}.

Disruptions in mucosal integrity caused by STIs underpin many HIV transmission events. STIs that cause ulcers, for instance, usually do so through necrosis of epithelial cells exposing subepithelial cells and CD4\textsuperscript{+} T-cells to HIV. Irrespective of the presence of ulcers, STIs cause T
cell infiltration, increasing the local quantity of HIV-susceptible target cells. Some STIs such as syphilis increase expression of CCR5, providing additional co-receptors for HIV to attach and enhance potential infection.

1.8.4.3 Herpesvirus Co-Infection and HIV Synergy

Despite the exhaustive literature on the impact of STIs on HIV, little attention until recently has been given to the role of asymptomatic non-HIV viral infections. Several studies have examined the effect of life-long viral co-infections and the effect that their reactivation may have on HIV viral load and pathogenesis. Of particular interest are the herpes viruses, due to their ability to establish life-long infections, the fact they are found in a number of bodily fluids (saliva, semen, vaginal fluids and urine) as well as their ability to periodically reactivate. Many of these viruses have been associated with secondary complications in immunocompromised individuals (reviewed in 1.5.2.2.1-2). Herpesviridae is a family of eight double-stranded DNA viruses also known as herpesviruses, more specifically; Herpes simplex viruses 1 and 2 (HSV1/2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (CMV), human herpesvirus 6, human herpesvirus 7 and kaposi’s sarcoma-associated herpes virus (human herpesvirus 8).

Several studies in recent years have begun exploring what influence herpes viruses may be having on semen HIV-1 RNA viral load.

1.8.4.3.1 Epstein-Barr Virus

Epstein-Barr Virus (EBV), also referred to as human herpesvirus 4 (HHV-4), is a member of the gamma herpes virus family, best known for causing infectious mononucleosis. It infects more than 90% of the world’s population and is transmitted orally via saliva. EBV is a double-
stranded DNA virus 172 kb in length encoding 85 genes\textsuperscript{198,199}. Much like CMV, EBV exhibits a biphasic life cycle. During primary infection brief replication occurs at the site of infection but is confined in an immunocompetent host. The second phase of infection establishes latency (non-productive infection) in another cell type in which the viral genome is maintained throughout the life of the host with periodic reactivation. Primary infection usually occurs with EBV targeting B lymphocytes and some epithelial cells within the lymphoid organs in the oropharynx\textsuperscript{199}. The frequency of EBV-infected B cells is 1 per $10^5$ to $10^6$ B-cells in healthy EBV-seropositive adults\textsuperscript{200}. In HIV-infected individuals, cellular EBV copy number from blood mononuclear cells is increased but not associated with blood viremia or CD4 T-cell count with EBV DNA not commonly detected in serum or blood plasma in immunocompromised patients\textsuperscript{201}. Several studies in recent years have documented reactivation of EBV in seminal plasma. Lisco, A. et al (2011) found that among the herpes family of viruses only EBV and CMV reactivation (Reviewed below) was detectable at high levels in seminal plasma, suggesting the MGT as a potential site of reactivation compared other herpes viruses; HSV-2, HHV-6, HHV-7, HHV-8, that were either not detected or at relatively low levels\textsuperscript{181}. These findings have been further substantiated by Gianella,A. et al. (2013) who found CMV and EBV reactivation to be the most prevalent of all herpes viruses found in semen. Reactivation was associated with increased HIV seminal shedding and transmission in sero-discordant partners\textsuperscript{202}.

In the context of HIV infection, limited data exists in terms of the direct effects EBV infection and reactivation may have on HIV. EBV has been associated with several proliferative disorders in AIDS patients including oral hairy leukoplakia (OHL), a benign lesion of the tongue epithelium, as well as AIDS-related non-Hodgkin’s lymphoma (ARNHL)\textsuperscript{203,204}. ARNHL develops largely at extra-nodal sites and associated with EBV gene expression in tumor cells\textsuperscript{205}.
1.8.4.3.2 Cytomegalovirus

Like all human herpes viruses, CMV (human herpesvirus-5) a member of the beta herpesvirus family, establishes lifelong latent infection. Following primary CMV infection, reactivation of the CMV virus is typically asymptomatic in children and adults\textsuperscript{206,207}. Reactivation of CMV has been documented in saliva, urine, semen and several other secretions. In the United States, seroprevalence of CMV has been estimated at 58.9\% in children $\geq$ 6 years old and 90.8\% in those aged $\geq$ 80 years\textsuperscript{208}. Seroprevalence amongst those co-infected with HIV is much higher than the general population with $>$ 90\% of HIV-infected individuals testing positive for CMV\textsuperscript{209}. CMV transmission occurs through person-to-person contact with urine, saliva, breast milk, vaginal fluids, semen and other bodily fluids. The virus itself is a double stranded DNA virus, 235 Kbp in length making it the largest human herpesvirus\textsuperscript{210}.

1.8.4.3.2.1.1 CMV Cellular Entry and Latency

Upon interaction of the CMV envelope glycoproteins with fibroblast cellular receptors, the virion and cell membranes fuse\textsuperscript{211}, releasing the capsid and viral proteins directly into the cellular cytoplasm. Cell entry occurs through low-pH-dependent endocytosis mediated by viral glycoproteins in epithelial and endothelial cells\textsuperscript{212}. Upon entry, the tegument protein (TP) pp71 has been implicated as one of the most important determinants of whether infection will become lytic or latent, although the exact mechanism still remains unclear\textsuperscript{213}. Translocation of pp71 into the cell nucleus is thought to induce down-regulation of cellular repressive proteins and promote synthesis of immediate early proteins (IE). These IE proteins mediate the expression of viral genes that induce replication of viral DNA\textsuperscript{213}. While the method by which entry into latently infected cells occurs remains unclear, in vitro studies have suggested IL-8 and IL-6 can induce reactivation of CMV from these cells\textsuperscript{214}. 41
The impact of the opportunistic pathogens CMV on progression of HIV has been controversial, and difficult to study in vivo since >95% of HIV-infected people are CMV co-infected. Considerable research has explored the interaction between HIV and CMV in vitro, showing both positive and negative effects on HIV replication and coinfection of the same cells. In general terms, it is thought that HIV facilitates CMV replication, and in turn, CMV enhances transcription of HIV virions. Laboratory cell lines infected with CMV have been observed to produce 20-times more HIV p24 capsid protein than those uninfected lines. This increased HIV replication is possibly due to the stimulation of cytokines that induce cellular activation, creating a more favourable environment of cell targets for HIV replication. It has further been observed that the CMV viral load in AIDS patients may in fact be a better predictor of survival than circulating HIV RNA viral load. Mortality of patients who have progressed to AIDS has been shown to be 2.5-fold greater when CMV reactivation is detected in plasma and that each log_{10} increase in CMV DNA is associated with a 2.2-fold increase in the risk of death. While reactivation is often asymptomatic during early HIV infection, as immunodeficiency progresses CMV can cause significant disease, including CMV retinitis and colitis. Prior to HAART, CMV retinitis occurred in over one-third of AIDS patients and was responsible for 90% of all reported HIV-related blindness. While now not a significant issue in western countries, due to the availability of HAART, CMV retinitis still poses considerable concern in resource-poor countries due to lack of routine examination, diagnosis and treatment. Once a person has had CMV disease, virus-specific treatment with ganciclovir, which reduces plasma CMV DNA to undetectable levels, is further associated with improved survival in these patients.

In 2009 our lab found that the shedding of higher levels of HIV-1 RNA in seminal plasma compared to blood plasma was strongly associated with mucosal CMV reactivation (p<0.01) with
HIV and CMV viral loads closely correlated, independent of blood viral load and CD4 count (p<0.01)\(^1\). Shedding of both CMV and HIV in this study was correlated with levels of IFN-gamma in blood and semen of study participants. Studies in the female genital tract have suggested that HIV replication in the context of CMV reactivation may be induced via the proinflammatory cytokine IL-8\(^1\), a cytokine previously found associated with semen HIV shedding\(^7\). It was further noted that compared to CMV monoinfected patients, CMV reactivation was much more common in HIV/CMV coinfected patients\(^1\).

1.9 Highly Active Antiretroviral Therapy

Antiretroviral medications for the treatment of HIV are organized into six major drug classes by the Food and Drug Administration for the treatment of HIV\(^2\). These classes are described below along with a brief overview of their mechanisms of action.

1.9.1 Introduction to Antiretroviral Therapy and Regimen Choice

Since the introduction of reverse transcription inhibitors in the early 1990’s, drug development for the treatment of HIV-1 infection has been substantial. As of June 2013, thirty-six antiretroviral drugs had been approved for treatment of HIV by the Federal Drug Administration (FDA) in the United States. According the Department of Health and Human Services, treatment of an HIV-1 infected drug naïve adult generally consists of two NRTIs in combination with an NNRTI, a PI or a INSTI. Regimen choice will depend on several factors including; virologic efficacy, toxicity, pill burden, dosing frequency, drug-drug interaction potential, resistance testing results and comorbidities\(^2\).
1.9.2 Major ARV Drug Classes for Treatment of HIV

1.9.2.1 Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTI/NtRTI)

Nucleoside Reverse Transcriptase inhibitors are the first class of antiretroviral drugs to be approved by the FDA in 1987 and often considered to be the “backbone” of most HIV regimens\(^{226}\). NRTI’s are analogs of endogenous 2’-deoxy-nucleosides and -nucleotides, functioning as defective derivatives of nucleic acids able to inhibit the action of reverse transcriptase\(^{227}\). In their initial form they are inactive and require phosphorylation by host cell kinases and phosphotransferases to form deoxynucleoside triphosphate (dNTP) analogues. Cellular entry of NRTIs is dependent on passive diffusion or carrier-mediated transport\(^{228}\).

Following cellular entry (either passive or carrier-mediated) and phosphorylation, newly formed dNTP analogues compete with endogenous dNTP for incorporation by HIV reverse transcriptase. Once incorporated, the lack of a 3’ hydroxyl group results in obligate chain-termination, inhibiting proviral DNA synthesis preventing integration into the host cell genome\(^{229}\). Initial NRTI treatment side effects often include fatigue, headache, gastrointestinal upset with long-term effects including myelotoxicity (bone marrow suppression), lactic acidosis, polyneuropathy and pancreatitis\(^{227}\).
Adapted from Furtado, MR., et al. 230,231

**Figure 1-8 - Life cycle of HIV and ART site of action.** ARV drug class indicated in red indicated at which step in the life cycle of HIV it targets.
1.9.2.2 Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI)

First approved by the U.S. FDA in 1996, NNRTIs unlike NRTIs are not incorporated in the growing HIV DNA strand and do not require intracellular metabolism, but rather directly inhibit the action of HIV reverse transcriptase by inducing allosteric changes. NNRTIs function by non-competitively binding to the reverse transcriptase enzyme downstream of its active catalytic site in a hydrophobic pocket in the p66 subunit of the reverse transcriptase. Binding of NNRTI to HIV RT causes a reposition of the three stranded β-sheet in the p66 subunit, locking the active catalytic site in an inactive conformation\(^{232}\). This conformational change causes inhibition of transcription of viral RNA and reduces the replication speed of the virus\(^{233}\). Some NNRTIs such as efavirenz and etravirine have been shown to further act in the later stages of HIV-1 replication by interfering with HIV-1 Gag-Pol polyprotein processing\(^{234}\).

1.9.2.3 Protease Inhibitors (PI)

Protease inhibitors are substrate analogues of the HIV aspartyl enzyme. Protease inhibitors function by blocking HIV aspartyl protease, a viral enzyme responsible for the cleavage of HIV gag and gag-pol poly-proteins that produces shorter, functional proteins. During normal viral replication polyproteins are assembled, packaged at the cell surface and immature virions are produced and released into the plasma. At this stage, HIV-1 protease cleaves the polyproteins into smaller functional proteins that allow the virions to mature. PIs mimic the phenylalanine-proline peptide bond present in the polyproteins, preventing cleavage and arresting maturation of virions resulting in HIV particles that lack infectious capabilities\(^{235}\). The introduction of protease inhibitors as a treatment for HIV has dramatically decreased the odds of mortality for individual with HIV both before and after AIDS\(^{236}\).
Long term PI-based HAART has been associated with several side effects including hyperbilirubinaemia\textsuperscript{237}, insulin resistance, hyper – or hypo-lipidaemia, fat body redistribution, osteopenia and osteoporosis\textsuperscript{238}.

1.9.2.4 Fusion Inhibitors

Currently the only FDA approved fusion inhibitor is enfuvirtide (formerly T-20 or DP178). Fusion inhibitors act extracellularly, blocking HIV from binding to the target cells. Enfuvirtide is a 36 amino acid peptide analogous to a portion of the natural sequence of gp41 HIV\textsuperscript{239}. Fusion inhibitors exploit the conformational change that occurs following gp120-CD4 binding and co-receptor binding (preceding pore formation). Before fusion of HIV and the cell can occur two peptides (HR1/HR2) must interact to form a six-helix bundle that will promote membrane fusion. During this conformational change, hydrophobic grooves of the HR1 area are exposed where fusion inhibitors bind, locking gp41 in this transitional form and preventing the formation of the six-helix hairpin needed for fusion\textsuperscript{240}. Despite being efficacious, the use of enfuvirtide is limited, as it must be administered twice daily through subcutaneous injection.

1.9.2.5 HIV Integrase Strand Transfer Inhibitors (INSTI)

The HIV integrase enzyme is responsible for the integration of viral DNA into the host chromosome (reviewed in 1.2.3). Currently there are two FDA approved HIV integrase strand transfer inhibitors (INSTI); raltegravir and dolutegravir with a third, elvitegravir, currently approved by the FDA as part of a multi-class combination drug Stribild. Integrase inhibitors work to prevent integration through prevention of the formation of covalent bonds to host DNA that allow integration\textsuperscript{241}. Briefly, INSTIs binds to a highly conserved region of the HIV integrase called the catalytic core. The binding to the core prevents covalent bonds from forming between
integrase and host DNA. It is hypothesized that divalent cations in the core enable integrase to form covalent bonds with the phosphodiester backbone of DNA. This process prevents HIV integrase from incorporating viral DNA into the host cell chromosome\textsuperscript{242,243}. Further details into the mechanism of action of integrase inhibitors have been hampered by the lack of structural information on the interactions between integrase, metal cofactors and DNA substrates (viral and host DNA)\textsuperscript{243}.

1.9.2.6 Entry Inhibitors – CCR5 Co-Receptor Antagonist

Entry inhibitors represent a relatively new class of ARV targeting HIV binding to target cells. Maraviroc, the first approved entry inhibitor, functions by binding chemokine ligand 3, CCL4 and CCL5 to the cell-membrane preparations of CCR5-expressing cells. This binding blocks CCR5-signaling events after chemokine binding and intracellular calcium redistribution\textsuperscript{244}. It had been hypothesized that maraviroc, representing a new drug class, would not only be able to better control HIV viral load but have a dramatic effect on deceasing T-cell immune activation. In a placebo-controlled trial of 45 HIV-infected subjects it was noted that maraviroc-treated subjects experiences greater median increases in CD8+CD38+HLADR+ T-cells. Furthermore it was noted that during maraviroc intensification sCD14 levels and T-cell activation increased in both blood and rectal tissue\textsuperscript{245}. The clinical implications of this increased activation remain unclear but could pose a particular risk in individuals who are receiving pre-exposure prophylaxis treatment that includes maraviroc. While CCR5 inhibition should prevent HIV entry through CCR5, increased activation of target cells, especially in the rectal tissue, may actually increase the risk of HIV acquisition through unprotected receptive anal sex. Despite these observations maraviroc has been successfully used in both first and second-line treatment programs to control viral replication.
1.9.3 Differential Penetration of ART in the MGT

HAART therapy has been clearly shown to reduce HIV-1 RNA in blood plasma. However, studies have shown that not all ARV’s penetrate into the male genital tract to the same degree, leaving the potential for ongoing viral replication, transmissibility and development of drug resistance; an important treatment and public health consideration. Studies have shown that in men on combination therapy, proviral DNA could still be detected in seminal cells with HIV recovered in culture\textsuperscript{246}. Of particular concern is the emergence of ARV resistance mutations. Plasma viral rebound after suppression by protease inhibitors have been shown to be possibly linked to repopulation of HIV from a viral sanctuary (such as the MGT) where protease inhibitors penetrate to a low degree and viral suppression may be inadequate\textsuperscript{247}. A reduction in sexual transmission of HIV depends on antiretroviral penetration into the MGT and supressing HIV replication\textsuperscript{248}. For an ARV to pass into the MGT it must diffuse either by simple (non-mediated) or carrier mediated mechanism. Shedding of HIV in the genital tract is significant in patients taking ARV monotherapy\textsuperscript{249}, dual therapy\textsuperscript{180} and with only agents known to have poor penetration into the genital tract (e.g. protease inhibitors)\textsuperscript{128}. The differences observed with ARV penetration vary based on individual ARVs and ARV class determined by drug pharmacokinetics and physiochemical properties\textsuperscript{248}. In general, maximal penetration would be achieved by an ARV that is weakly basic, lipophilic and minimally protein-bound\textsuperscript{250,251}. Protein binding in particular is thought to be one of the major determinates of an ARV penetrating the MGT. After dosing, an ARV enters the blood and distributes into tissues. Only unbound drug can penetrate into cell membranes and exert their anti-HIV effect. Protein bound drugs are too large to enter into the cell and pass through the blood-testis barrier\textsuperscript{250,251}. Pharmacokinetic studies have documented differential penetration of ARV drugs into the male genital tract from drugs that are almost
undetectable (such as protease inhibitors\textsuperscript{252}) to others that concentrate in the male genital tract (such as nucleoside analogues)\textsuperscript{248,253,254} (\textbf{Figure 1.6}). While penetration of ARV’s may vary, no study to date has shown conclusively that a regimen with higher penetration is more effective at reducing genital tract shedding of HIV. These findings are confounded by the fact that one or more drugs used are found reaching therapeutic concentrations in the genital tract (Reviewed in 1.7.4)\textsuperscript{248,250,255}
**FIGURE 1-9: Chart summarizing the penetration of ARVs into the male genital tract**

Adapted from Taylor and Davies 2010. E/F, Entry and Fusion inhibitors; PI, Protease Inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; INSTI, Integrase strand inhibitors; MVC, maraviroc; ENF, enfurvitide; IDV, indinavir; APV, amprenavir; ATV, atazanavir; DRV, darunavir; LPV, lopinavir; NFV, nelfinavir; RTV, ritonavir; SQV, saquinavir; NVP, nevirapine; EFV, efavirenz; 3TC, lamivudine; TDF, tenofovir; ZDV, zidovudine; ABC, abacavir; d4T, stavudine; RAL, raltegravir.
1.9.4 The Effect of HAART on HIV Transmission and Genital VL

HAART has revolutionized the treatment of HIV, both increasing quality of life and reducing mortality rates\(^\text{256}\). Initiation of HAART results in rapid reduction of blood viremia and reduction of systemic immune activation and inflammation. This initial decline reduces plasma virus by over 90% in the first two weeks of therapy in the majority of those treated with HAART\(^\text{257-259}\). Following this decline a second phase of decay occurs until the virus becomes undetectable by current assays. This slow decay is thought to be due to chronically infected cell compartments with a half life of 1-4 weeks, compared to free virions that have a decay rate of 6 hours or less\(^\text{260}\). Finally a third phase of decay occurs that results in the slow elimination of HIV from these areas. It has been estimated that these compartments have a half-life of 2.3-3.1 years\(^\text{257}\).

Numerous studies have documented that the initiation of HAART reduces the HIV viral load in blood and semen to undetectable levels in the majority, dramatically reducing the risk of sexual transmission of HIV at a population level. The partners in prevention HSV/HIV Transmission Study studied transmission rates in 3400 HIV-1 serodiscordant heterosexual couples from seven African countries, and found that ART use by the infected partner was accompanied by a 92% reduction in risk of transmission to their partner\(^9\). The strongest data available on the effect of ARTs on transmission comes from the HIV Prevention Trials Network (HPTN) multi-continent, randomized, controlled trial; HPTN052. This study found a 96% reduction in the number of transmission events when ART was initiated early (CD4 count between 350-500) compared to delayed ART\(^\text{10}\). Despite a substantial decrease in transmission events as a result of ART (only 1 genetically linked in HPTN052), the potential for transmission events still exist, evidenced by incomplete reduction of all transmission events. In addition to these transmission
events observed in the context of these trials, some data suggest that ART for preventing transmission via anal intercourse may have limited efficacy compared to vaginal intercourse. Without the use of ART, HIV transmission is estimated to occur in a range of 1/20 – 1/300 acts of condomless anal intercourse, compared to 1/200 – 1/2000 for penile-vaginal exposure\(^{94,97,117,261}\). The pharmacology of ART in the MGT (as described in 1.9.3) and the gastrointestinal tract (anal intercourse) differs from blood. It has been further documented that while ART can reduce HIV levels, HIV DNA and RNA can still be recovered from the MGT\(^{262,263}\) and GI tract\(^{264-266}\). Furthermore it has been documented for some ARV such as tenofovir and maravirocer have greater penetration into rectal tissue compared to blood or semen plasma\(^{267,268}\). These transmission events could further be due in part to continued shedding of HIV in the genital tract despite undetectable viremia and lack of concurrent STI’s. Several studies over the past few years have documented that while ART does reduce blood to undetectable levels, a significant subset of men continue to shed HIV RNA in semen\(^{167,269-272}\) with similar observations seen in the female genital tract\(^{169}\). In 2009 our lab\(^{167}\) found 48% of men enrolled in a prospective observational study shed HIV-1 RNA in semen despite undetectable blood viral load at more than one visit and at levels greater than 5,000 HIV-1 RNA copies/mL in 16% of men with no concurrent STI. Interestingly this phenomenon termed “Isolated HIV Semen Shedding” or IHS was observed in men with semen virus that was drug-sensitive and not associated with ARV drug level, regimen choice or altered blood and semen cytokine levels\(^{168}\). This study, however, only examined the effect of HAART over a 6-month course, questioning whether longer duration of therapy would resolve the occurrence of IHS. While studies such as HPTN052 have demonstrated the ability for ART to significantly reduce transmission in serodiscordant heterosexual couples, future studies exploring efficacy in MSM populations where anal intercourse is the primary route of infection are needed.
1.9.5 Development of Resistance and Viral Evolution

Diversity of the HIV virus throughout different cells, organs and tissues plays a significant role in HIV pathogenesis. Within an infected individual HIV can differ from one tissue or cell type to another, creating viral compartments. Compartmentalization of HIV within the genital tract has been demonstrated in several studies comparing blood and semen. This compartmentalization of viral sequences often leads to the development of somewhat distinct resistance development patterns when compared to blood. Phylogenic analysis of reverse transcriptase and protease sequences has evidenced that the majority HIV variant in seminal plasma is almost always distinct from that of the blood\textsuperscript{273}. Substantial genetic differences between blood and semen have been observed in several cross-sectional studies\textsuperscript{102,103}. Selective compartmental differences are likely to influence viral evolution and resistance development. Difference in ARV penetration concentration is likely a large factor. Zidovudine (AZT) an NRTI, for instance, penetrates to above therapeutic concentrations in MGT with ZDV resistance mutations often coinciding with (or following) their appearance in blood\textsuperscript{273}. As protease inhibitors typically do not penetrate into the MGT, resistance mutations that develop in blood are often not observed in semen in the same way they are seen in blood\textsuperscript{102,273}. Together this data stress the need for a better understanding of the differences that exist between blood and semen and factors that control local viral replication.
Chapter 2: Research Aims and Hypotheses
2.1 Thesis Summary and Rationale

The genital mucosa lies at the front line for host-virus interactions, yet mucosal correlates of susceptibility and infection remain poorly understood. The level of HIV RNA in semen is thought to be substantially lower than blood in the majority, and often undetectable in men receiving effective antiretroviral therapy. Important caveats to this exist, with previous work by our lab showing; (1) a subset of antiretroviral therapy-naive men shed semen HIV RNA at varying levels and at times disproportionately higher than blood viral load; (2) a smaller subset of men on effective ART have high HIV levels in semen despite undetectable blood viral (isolated HIV semen shedding, “IHS”). The goal of this research is to investigate the clinical and immunological correlates that may underpin the heterogeneity observed in semen viral load in both antiretroviral naive and treated men.

To examine our hypothesis, the following questions will be addressed:

1) **Does collection of semen neat or directly into a viral transport medium influence quantitative measurements of semen HIV RNA?** Within the literature, various techniques have been used to collect semen samples for measurement of HIV viral load. To determine whether collection method significantly impacted viral load measurements we compared collection techniques to ensure methodology was optimized before proceeding with subsequent studies.

2) **Can ART intensification through the addition of semen active antiretrovirals reduce or eradicate isolated HIV semen shedding (IHS)?** Previous studies by our lab documented IHS in men on standard ART. While some drugs in the study penetrated quite well into semen, others such as protease inhibitors were at low levels, if detected at all. Given these observations, we investigated whether IHS was a result of inadequate
penetration of antiretrovirals into the semen compartment by examining men who were receiving an intensified ART regimen that included two semen active ARVs, maraviroc and raltegravir.

3) **Does drug intensification (greater than standard 3-drug regimen) provide any short-term benefit in reducing systemic immune activation?** Initiation of effective antiretroviral therapy rapidly reduces the HIV RNA to undetectable levels, but systemic immune activation takes much longer to resolve. Delayed resolution of immune activation may underlie increased rates of functional impairment, cardiovascular and neuropsychiatric diseases observed despite effective ART. To determine whether drug intensification (>3 ARV) could resolve immune activation to a greater degree than standard ART in the short-term, we examined markers of cellular and soluble immune activation on men followed prospectively receiving either standard or intensified ART regimens.

4) **How variable is semen viral load when examined prospectively and are there any mucosal correlates (clinical or immunological) in the semen of ART-naive men that are predictive of semen viral load?** Semen HIV RNA in an ART-naive man is an independent predictor of HIV transmission risk, however, sVL only moderately correlates with blood viral load and its variability is only partly explained by compartmentalized factors. While effective antiretroviral therapy dramatically reduces both the sVL and probability of HIV transmission, globally most HIV-infected individuals do not have access to ART. Understanding of factors that cause variability in sVL of ART-naive men is an important area of research that may potentially lead to novel avenues for prevention.
The identification of factors that influence semen HIV RNA viral load has the potential to provide a powerful tool for further investigation into mucosal viral replication and potentially the design of new treatment strategies that would optimize ARV choice to help reduce HIV transmission. The following studies highlight the importance of understanding mucosal viral load, antiretroviral choice and the important differences between the blood and semen compartments.
Chapter 3: Impact of Collection Method on Assessment of Semen HIV RNA Viral Load

3.1 Abstract

Background

The blood HIV RNA viral load is the best-defined predictor of HIV transmission, in part due to ease of measurement and the correlation of blood and genital tract (semen or cervico-vaginal) viral load, although recent studies found semen HIV RNA concentration to be a stronger predictor of HIV transmission. There is currently no standardized method for semen collection when measuring HIV RNA concentration. Therefore, we compared two collection techniques in order to study of the impact of antiretroviral therapy on the semen viral load.

Methodology/Principal Findings

Semen was collected by masturbation from HIV-infected, therapy-naïve men who have sex with men (MSM) either undiluted (Visit 1) or directly into transport medium (Visit 2). Seminal plasma was then isolated, and the HIV RNA concentration obtained with each collection technique was measured and corrected for dilution if necessary. Collection of semen directly into transport medium resulted in a median HIV RNA viral load that was 0.4 log10 higher than undiluted samples.

Conclusions/Significance

The method of semen collection is an important consideration when quantifying the HIV RNA viral load in this compartment.
3.2 Introduction

Globally there were an estimated 2.6 million new HIV-1 (HIV) infections in 2009\textsuperscript{146}, most acquired through sex. The blood HIV RNA viral load is the best-defined predictor of HIV transmission\textsuperscript{160}, probably because it is easily measured and tends to correlate with the genital tract (semen or cervico-vaginal) viral load\textsuperscript{172}. However, recent studies have found that the semen HIV RNA viral load is a stronger independent predictor of HIV transmission than the blood viral load\textsuperscript{171}.

Following the initiation of antiretroviral therapy (ART) blood HIV RNA concentrations generally decrease to undetectable levels, in association with a 92% reduction in HIV transmission risk in a recent observational study\textsuperscript{9}. However, a significant minority of individuals continue to have detectable levels of viral RNA in semen despite an undetectable HIV RNA blood VL, sometimes at very high levels\textsuperscript{167}. Whether this phenomenon underpins the inability of ART to completely prevent HIV transmission is not clear. Research studies to clarify these issues will require well-validated assays to measure semen HIV RNA viral load, something which is more technically challenging than measurement of the blood VL due to the presence in semen of PCR inhibitors, endonucleases and other factors\textsuperscript{274}. While commercially available molecular assays may be more reliable and reproducible than in-house assays\textsuperscript{275}, in this study we evaluated the impact of different semen collection methods on the HIV RNA level in ART-naïve men. Our goal was to determine whether semen collected neat or diluted would impact viral load measurements and to what extent. We hypothesize that semen collected directly into transport medium would provide a more accurate representation of viral load than neat due to a more favorable and stable transport environment than neat collection.
3.3 Methods

3.3.1 Human Subjects

HIV-infected, antiretroviral therapy-naïve men who have sex with men (MSM) were recruited through the Canadian Immunodeficiency Research Collaborative at the Maple Leaf Medical Clinic in Toronto, Canada. Participants were excluded if at either visit they had clinical urethritis, genital ulcer disease, laboratory evidence of infection by C. trachomatis, or N. gonorrhoeae by urine nucleic acid amplification testing (NAAT: Amplicor CT/NG assay, Roche Diagnostic Systems), or active T. pallidum infection by serology (RPR; rapid plasma reagin). A first-void urine dipstick for leukocytes was also performed to screen for asymptomatic urethritis. All participants provided informed, written consent; ethical approval for this study was obtained through the research ethics board of the University of Toronto.

3.3.2 Sample Acquisition, processing and viral load measurement

Paired blood and semen specimens were collected within an hour of each other at two separate study visits. Semen samples were collected by masturbation into a dry sterile container (undiluted) at visit 1, and directly into 10mL of sterile RPMI 1640 (Gibco) containing 100 U/mL penicillin and 100mg/mL streptomycin (Gibco) (transport medium) at visit 2. All study participants agreed to abstain from sexual intercourse or masturbation for 48 hours prior to sample donation. All samples were processed within 2 hours of collection. Seminal plasma was cryopreserved at -80°C after sample centrifugation at 850g for 10 minutes. Blood plasma was collected and cryopreserved after ficoll density gradient centrifugation at 500g for 25 minutes. Blood and semen plasma HIV-1 RNA concentrations were measured in the Mount Sinai Hospital Department of Microbiology (accredited by the Ontario Public Health Lab for clinical HIV-1 viral
load measurement) using the Versant HIV-1 RNA 3.0 assay (bDNA; Bayer Diagnostics; lower limit of detection, 50 RNA copies/mL). Correction for semen dilution at visit 2 was calculated based on the total sample volume provided; since transport medium was occasionally spilled during semen collection, where the returned total volume (semen and transport medium) was lower than the original volume of transport medium, we assumed a semen volume of 2 ml (the mean volume of undiluted samples collected during visit 1).

3.3.3 Statistical Analysis

All analyses were formed with SPSS software (version 18; SPSS). Data were statistically analyzed using the non-parametric paired Wilcoxon signed rank test for median measurements and changes in mean VL measurements. Statistical significance was defined as p < 0.05.

3.4 Results

Twenty-seven participants were recruited; the median CD4⁺ T cell count was 550/mm³ (range, 320-1210 mm³) at visit 1 and 470/mm³ (range, 160-780 mm³) at visit 2. There was no statistically significant difference in the CD4 counts between visits (Wilcoxon paired p=0.383), although one individual at visit 2 had progressed to AIDS based on a CD4 count <200 mm³ (160 mm³). No participant had a prior history of an AIDS-defining illness (at either study visit), and no participant had syphilis, N. gonorrhea or C. trachomatis infection by NAAT, clinical urethritis, genital ulcer disease or leukocytes detected on dipstick of first void urine. Study visits were a median of 6 months apart, and there was no difference in the blood HIV RNA VL (4.26 vs. 4.35 log₁₀ RNA copies/mL, Visit 1 vs. Visit 2; p = 0.274) between visits. Five participants (18.5%) had an undetectable semen VL by at least one of the two collection methods (i.e.: at ≥1 study visit), and in 4/5 the semen VL was undetectable at both study visits: for practical reasons these 4
participants were not included in the comparison of sampling techniques.

In those participants with a detectable semen VL during at least one study visit, the median HIV load as measured in undiluted semen (Visit 1) was 0.42 log_{10} copies/mL (2,236 copies/mL) lower than that measured in semen that had been collected directly into transport medium. Median semen HIV RNA collected undiluted was 3.14 log_{10} copies/mL (1,396 RNA copies/mL, range, 50-210,350 RNA copies/mL) vs. 3.56 log_{10} copies/mL (3,631 RNA copies/mL, range, <300 - 1,002,030 RNA copies/mL) when collected into transport media (Figure 2.1; p= 0.012).

The proportion of participants with any detectable HIV RNA in semen did not vary by collection technique (21/27 undiluted vs. 20/27 diluted; p=NS). As expected, having an undetectable semen VL at one visit increased the probability of remaining undetectable at the next (LR=6.0; p=0.014). An estimated semen volume of 2mL was used at visit 2 for 14/27 participants who had spilled transport medium during sample collection (see Methods section, above). When these participants were excluded, our overall results were unchanged with a median semen HIV concentration of 2.88 log_{10} at visit 1 and 3.56 at visit 2 (median difference 0.68 log_{10}, p=0.033).

3.5 Discussion

This study demonstrates that the method of semen collection can have a substantial impact on semen HIV RNA VL measurements, and this should be an important consideration when performing and assessing studies of semen HIV RNA load. The cause of the reduced level when measuring the HIV RNA VL in semen that had been collected undiluted is not clear, but might relate to PCR inhibitors present in undiluted semen\(^{276}\) or to the presence in semen of various enzymes and other immune factors\(^{72,183}\); certainly, seminal plasma is well described to have substantial cytotoxic effects\(^{277}\). Interestingly, while the semen viral load was significantly higher
when collected into transport medium, there was no difference between collection methods in the proportion of participants who had any detectable semen HIV RNA. We hypothesize that this may be because although collection into RPMI medium was associated with increased semen HIV RNA level, this also diluted the sample approximately six-fold, decreasing our assay limit of detection from "50 to "300 HIV RNA copies/mL.

The second study visit, when semen VL was assayed in a diluted sample, was 6 months after the semen VL was measured undiluted. This raises the possibility that a higher semen VL might represent HIV disease progression. However, the fact that both the blood VL and CD4+ T cell count were unchanged between visits strongly suggests that this was not the case. The semen HIV concentration may be more variable than that in blood plasma, and in addition there is considerable variability of all HIV RNA assays currently in clinical use. However, neither of these sources of variability would explain our observation that the semen HIV viral load was significantly and consistently higher when samples were collected into transport medium rather than undiluted; indeed, such random variability would tend to have blunted our ability to find such a difference. In addition, in those participants with a spilled sample at visit two, if we assumed a volume equal to their first sample, the difference in semen viral loads across the two collection methods was still statistically significant.

Overall, our findings suggest that semen collection technique is an important consideration if the semen viral load is to be assessed quantitatively, since immediate collection of semen into transport medium was associated with higher semen HIV RNA concentration that measured into semen collected undiluted. However, this would less critical if the goal were to deem semen HIV RNA as being “detectable” or “undetectable”, since these proportions were not altered by collection technique.
Blood was collected and the HIV RNA viral load assayed the same way at both study visits (BVL1 and BVL2, respectively); semen was collected undiluted at visit 1 (SVL1) and directly into transport medium and visit 2 (SVL2). Participants with an undetectable semen viral load at both study visits were excluded from statistical analysis.
Chapter 4: Impact of antiretroviral therapy duration and intensification on isolated shedding of HIV-1 RNA in semen

©Journal of Infectious Diseases 2013
4.1 Abstract

**Background:** Effective antiretroviral therapy (ART) dramatically reduces HIV transmission. However, isolated semen HIV-1 shedding (IHS) can occur in the absence of detectable viremia or genital infections. We hypothesized that ART intensification with semen-active medications might prevent IHS.

**Methods:** Paired blood and semen samples were collected monthly for 6 months from HIV-infected men starting ART that was intensified (iART) with maraviroc and raltegravir, in an open-label fashion. Semen parameters were compared to historical controls starting standard ART (sART).

**Results:** Compared to sART (n=25), semen VL was more rapidly suppressed with iART (n=13; p=0.043). IHS was detected at >1 visit in 2/13 participants (15%) taking iART and 12/25 (48%) sART (p=0.040). With iART, IHS was associated with lower raltegravir concentrations in blood and semen (p=0.03). Prolonged, high-level IHS was observed in one iART participant (1/13; 8%) despite rapid viremia suppression and therapeutic drug levels; this virus remained R5-tropic, drug sensitive and similar in sequence to blood virus over ten months. IHS was not seen after >3 years of effective ART in a parallel, prospective cohort study.

**Conclusions:** iART transiently reduced the occurrence of IHS early after ART initiation, but did not prevent high-level IHS. IHS was not seen after more prolonged sART.
4.2 Introduction

The risk of HIV-1 (HIV) sexual transmission is strongly correlated with the genital (semen or cervico-vaginal) HIV RNA level, and in heterosexual African couples each $\log_{10}$ increase in genital HIV RNA levels increased the probability of both male-to-female and female-to-male transmission by approximately 1.7-fold\textsuperscript{171}. As semen is the most common vector for HIV sexual transmission in the global pandemic, reducing HIV levels in semen is an important public health priority.

Antiretroviral therapy (ART) reduces the HIV transmission probability by more than twenty-fold within heterosexual couples\textsuperscript{10}. However, a subset of individuals on effective ART continue to have detectable levels of HIV RNA in genital secretions, despite an undetectable blood VL and a lack of detectable sexually transmitted infections (STIs). This phenomenon is known as isolated HIV RNA shedding (IHS), and has been described in both men\textsuperscript{167,270,272,278-282} and women\textsuperscript{169}. Levels of virus during IHS are often low and of uncertain clinical importance, however, “high level” shedding (exceeding 5,000 RNA copies/mL) has been described in a substantial subset\textsuperscript{167}, and this level was associated with a increased risk of HIV transmission \textit{in vitro}\textsuperscript{283}. Interestingly, the 92-96% reduction in actual HIV transmission events that was seen in clinical studies of ART for prevention\textsuperscript{9,284} was very similar to the 92% reduction in high-level HIV semen shedding that was seen in men starting ART\textsuperscript{285}, and the rare transmission events that have occurred in recent studies all took place soon after starting therapy.

The cause of IHS has not been defined. Studies comparing antiretroviral drug levels in semen and blood\textsuperscript{253,269} have documented differences in the penetration of several commonly used medications into these compartments. For example, protease inhibitors are present in semen at
levels <5% of those in blood\textsuperscript{252}, while semen levels of nucleoside/nucleotide analogues (NRTIs, NtRTIs) such as lamivudine (3TC) may exceed those in blood by 600% or more\textsuperscript{286-289}. While this suggests that drug regimen might play a role in the phenomenon of IHS, no clear associations with drug regimen have been apparent in the small studies performed to date\textsuperscript{167,169}, although the poor semen penetration of efavirenz has been implicated as a possible cause of a slow decline in the semen viral load\textsuperscript{290}. To further study the effect of antiretroviral drug regimen on IHS, we added two compounds with documented high semen penetration, raltegravir and maraviroc\textsuperscript{291,292}, to standard ART in men starting therapy and prospectively assessed the impact of this intensified drug regimen on IHS in an open label fashion; these men were compared to a previously-described group of men who had initiated standard 3-drug ART\textsuperscript{167}.

4.3 Methods

4.3.1 Human Subjects

HIV-infected, antiretroviral therapy-naïve men who have sex with men (MSM) were recruited through the Canadian Immunodeficiency Research Collaborative at the Maple Leaf Medical Clinic in Toronto, Canada as previously described\textsuperscript{167}. Participants were excluded if at any visit they had clinical urethritis, genital ulcer disease, laboratory evidence of infection by \textit{C. trachomatis}, or \textit{N. gonorrhoeae} by urine nucleic acid amplification testing (NAAT: Amplicor CT/NG assay, Roche Diagnostic Systems), or active \textit{T. pallidum} infection by serology (RPR; rapid plasma reagin) at any study visit. A first-void urine dipstick for leukocytes was also performed to screen for asymptomatic urethritis. All participants provided informed, written consent; ethical approval for this study was obtained through the research ethics board of the University of Toronto.
4.3.2 Study Design

This observational, open label study of iART followed an identical format to a previously published study \(^{167}\). All study participants provided informed, written consent. Participants consisted of men starting an intensified ART regimen that added both maraviroc (MVC) and raltegravir (RAL) to a standard ART regimen. The use of an intensified regimen was at the discretion of the participant, in discussion with their physician. Participants provided semen and blood samples at baseline (drug naïve) and then at weeks 0, 2, 4, 8, 12, 16, 20 and 24 after therapy initiation; after this point a subset of consenting participants were followed every three months up to a maximum of two years. Parameters in this intensified ART (iART) group were then compared to a group of historical controls who had been treated with standard ART (sART) in 2008-9 \(^{167}\). All study conditions; sample processing and virologic/immune assays were identical between studies.

In addition to this new iART (n=13) group and sART (n=25) historical controls, a separate cohort was enrolled (n=26), consisting of HIV-infected men who had been taking sART for varying lengths of time (1-3 years, 3-5 years and >5 years). These participants provided semen and blood samples monthly for six consecutive months, in order to assess the possible association of HIV treatment duration with IHS occurrence.

4.3.3 Sample Acquisition, Sample Processing, and HIV Load Measurement

Paired blood and semen specimens were collected within an hour of each other at each study visit. Semen samples were collected by masturbation into 10mL of sterile RPMI 1640 (Gibco) containing 100 U/mL penicillin and 100mg/mL streptomycin (Gibco) (transport medium). All study participants agreed to abstain from sexual intercourse or masturbation for 48
hours prior to sample donation. All samples were processed within 2 hours of collection. Seminal plasma was cryopreserved at -80°C after sample centrifugation at 850g for 10 minutes. Blood plasma was collected and cryopreserved after ficoll density gradient centrifugation at 500g for 25 minutes. Blood and semen plasma HIV RNA concentrations were measured in the Mount Sinai Hospital Department of Microbiology (accredited by the Ontario Public Health Lab for clinical HIV viral load measurement) using the Versant HIV RNA 3.0 assay (bDNA; Bayer Diagnostics; lower limit of detection, 50 RNA copies/mL). Correction for semen dilution was calculated based on a mean semen volume of 2mL, as in previous studies.  

### 4.3.4  Antiretroviral Levels

Blood and semen plasma concentrations of maraviroc, raltegravir, etravirine, lopinavir, efavirenz and ritonavir were determined using validated high performance liquid chromatography with tandem mass spectrometry (LC/MS) after protein precipitation extraction. This method was adapted from previous studies by adding MVC, RAL and ETV in the original calibration standards. All detected drug concentrations both in blood and semen were within the calibration curve ranges and all the recoveries, accuracies, and precisions were well within the predefined limits. All the semen drug levels were corrected for dilution as described above.

### 4.3.5  HIV RNA Amplification, Sequencing, and Analysis

Total nucleic acids were extracted from baseline plasma and longitudinal semen specimens from a patient exhibiting high-level IHS using a standard commercial kit (Invitrogen). HIV RNA sequences spanning protease and the first 400 codons of RT, RT codon 401 through the end of integrase, and envelope V3 loop were amplified in independent nested RT-PCR reactions using sequence specific primers optimized for HIV subtype B. Reactions were performed in
triplicate (minimum). Successful amplicons were “bulk” (directly) sequenced bi-directionally using an ABI 3130xl genetic analyzer (Applied Biosystems). Chromatograms were analyzed using Sequencher 4.10.1 Software (Genecodes). Bases were called as mixtures if the height of the secondary peak was ≥25% of the primary peak. Sequences were aligned using MUSCLE\textsuperscript{294}. HIV co-receptor usage was inferred using geno2phenocoreceptor (http://coreceptor.bioinf.mpi-inf.mpg.de/) using optimized FPR cutoffs. Drug resistance genotype interpretation was performed using the Stanford HIV database (http://hivdb.stanford.edu/). Twenty-seven sequences have been deposited in GenBank (Accession numbers JX519532 – JX519558).

4.3.6 Statistical Analysis

All analyses were performed using IBM SPSS Statistics software for Mac (version 18; SPSS). Data were statistically analyzed using Wilcoxon matched-pairs signed rank, Mann-Whitney and Chi-Square tests. Statistical significance was defined as p < 0.05. We hypothesized that starting iART with two semen-active antiretroviral agents would completely prevent the phenomenon of IHS. Were this to be the case, we would be powered to see a significant difference between groups with an iART sample size of just 5 participants, given the IHS frequency of 48% in our previous study\textsuperscript{167}. However, in case occasional IHS occurred, we expanded enrolment to include 13 participants.
4.4 Results

4.4.1 Study Participants

This prospective, observational study enrolled 13 men starting an iART regimen (Table 3.1), and compared these participants with 25 previously reported men starting sART \(^{167}\). The nadir (baseline) CD4\(^+\) T cell count was lower in men starting sART (median 213 cells/mm\(^3\) vs. 340 cells/mm\(^3\) in men starting iART; \(p=0.001\)) and the baseline blood HIV RNA VL (bVL) was also higher (50,000 vs. 7,122; \(p=0.05\)). HSV-2 and CMV seroprevalence were 36% (9/25) and 100% in the sART group, respectively, and were 69% (9/13) and 84.6% (11/13) in the iART group. The 2-drug nucleoside reverse transcriptase inhibitor (NRTI) “backbone” was similar for both sART and iART groups utilizing combinations of tenofovir, abacavir, zidovudine and lamivudine; however, all participants in the iART group were taking a boosted protease inhibitor, while the sART group was more evenly split between an NNRTI (efavirenz or nevirapine; 12/25) and a boosted protease inhibitor (lopinavir, atazanavir, or saquinavir; 13/25) \(^{[8]}\).

4.4.2 Overall impact of ART on Blood and Semen HIV RNA Level

The median baseline levels of HIV RNA in semen were similar between groups, at 2,979 copies/mL (range, 300-70,710) in the iART group and 5,136 copies/ml (range, 300 – 86,856; \(p=0.36\)) in the sART group (Figure 4.1). The semen viral load dropped more rapidly in the iART group, and was more likely to be undetectable by 2 weeks into treatment when compared to sART at the same time point (\(p=0.043\)). However, the majority of participants had achieved an undetectable bVL and sVL by week 16 in both the standard (20/25) and intensified (12/13) ART groups.
4.4.3 Isolated HIV RNA Semen Shedding

As previously reported \(^{167}\), IHS was detected at ≥1 visit for 12/25 (48%) participants in the sART group, with high-level shedding in 4/25 (16%); IHS was present during 19/116 (16.4%) study visits with an undetectable bVL, and at a high level in 5/16. In the iART group IHS was seen in fewer participants (2/13, 15%; p=0.048), but there was no reduction in the occurrence of high-level shedding (1/13, 8%; p=0.472; Figure 4.2). IHS was present during 5/73 (6.8%) iART study visits with an undetectable bVL (OR 2.6 vs. sART group; p=0.072), and at a high level in 4/73 (OR 0.94 vs. sART group; p=1.0).

4.4.4 Maraviroc and Raltegravir Levels

Median MVC and RAL drug levels after 6 months of therapy were 109 ng/mL (range, 44.4-377 ng/mL) and 246 ng/mL (range, 40.3-1380 ng/mL) in blood and 804 ng/mL (range, 153-4920 ng/mL) and 723 ng/mL (range, 282.6-1890 ng/mL) in semen, respectively (Figure 4.3). Levels of MVC and RAL in both blood and semen were above the minimal therapeutic concentration required to suppress HIV in all samples tested (Maraviroc IC\(_{90}=2.03\)nM, Raltegravir IC\(_{95}=31\)nM)\(^{295,296}\). Median semen-to-plasma ratios for MVC and RAL were 9.7 (range, 0.49-46.9) and 4.9 (range, 0.49-8.9), respectively (p<0.05).

We compared individuals with IHS (n=2) to time-matched samples from participants who had never had an IHS episode (n=11). In keeping with our prior study \([15]\), no differences were observed in blood or semen plasma drug levels of maraviroc (blood p=0.637, semen p=0.346), etravirine (blood p=0.670, semen p=0.530), lopinavir (blood p=0.478, semen p=0.166) or ritonavir (blood p=0.157, semen p=0.530) for iART. However, lower levels of raltegravir were observed in both blood and semen plasma (p=0.03, Figure 4.4). Within these two individuals,
raltegravir levels during the IHS episode were then compared to flanking time-points with no detectable semen virus, and no significant differences were observed. This suggests that raltegravir levels were consistently lower in participants with IHS, although the IHS episode itself was not associated with further reductions.

4.4.5 Sequence Analysis of Virus Detected in Semen From a Patient With Prolonged High-Level IHS

One individual had prolonged, high level HIV RNA detected in semen despite undetectable viremia in blood plasma (Figure 3.2C). Follow up for this individual was extended to two years, with persistent IHS detected until month 14. Viruses from pre-ART semen and blood, as well as semen virus during IHS episodes at month 2 and month 10, were assessed for the presence of drug resistance mutations. Bulk sequencing of blood plasma HIV V3 sequences revealed at least two baseline circulating variants, while all semen samples were identical and matched one of these blood plasma variants (Figure 4.6). Blood and semen viruses were identified as CCR5-tropic by V3 genotyping. Sequencing of RT-integrase revealed greater HIV diversity, as measured by the presence of nucleotide mixtures297, in plasma compared to semen, but no evidence of any major or minor drug resistance mutations in any sample at any time-point (not shown). Overall, there was no evidence for the development of drug resistance or virus evolution in the semen of this individual despite prolonged, high level IHS for over ten months.

4.4.6 Duration of standard ART and the incidence of IHS

Despite prolonged IHS in this one individual, semen virus levels fell below the level of detection by 16 months, suggesting that IHS may resolve after sustained ART. Therefore, to explore whether a longer duration of standard antiretroviral therapy would be associated with
reduced IHS, we recruited a new cohort of ART-experienced men for this purpose. Monthly semen and blood samples were collected for 6 months from twenty-six HIV-infected, sART-experienced men within the following three groups: 1-3 years of prior sART (Group 2; N=10), 3-5 years of prior sART (Group 3; N=9) and >5 years of prior sART (Group 4; N=7). All men enrolled in each of groups 2-4 had had an undetectable blood viral load and no sexually transmitted infection for six months prior to enrollment. These groups were compared to the previously described participants initiating sART (Group 1; N=25). Overall, the occurrence of any IHS during the six months of follow up was associated with a shorter prior duration of effective sART. At least one episode of IHS was observed in 12/25 (48%) participants on prior effective ART for <6 months, 2/10 (20%) men on prior ART for 1-3 years, and no participants on effective ART for >3 years (Mann-Whitney, p<0.001; Figure 4.5). At clinical visits with an undetectable blood viral load, the proportion of visits where IHS was detected also declined with the duration of prior treatment (19/116, 2/60, 0/54 and 0/42 in Groups 1-4 respectively, p<0.001). IHS was much more likely to occur during the first year on therapy (OR=15.1, p<0.001).

4.5 Discussion

Semen is the most common vector for HIV transmission, and the semen viral load is an independent predictor of HIV transmission risk. While standard antiretroviral therapy generally reduces the semen viral load to undetectable levels, isolated HIV semen shedding (IHS) may be seen despite an undetectable blood viral load, and occasionally at high enough levels to represent a significant transmission risk. The present study evaluated the ability of an intensified antiretroviral therapy (iART) regimen, defined as the addition of two antiretroviral drugs with enhanced semen penetration to standard ART, to reduce the incidence of IHS during the first six months of therapy. We confirmed that both maraviroc and raltegravir were present in the semen at
high levels, as has been shown in previous studies\textsuperscript{291,292}. In addition, we found that the incidence of IHS was significantly reduced in participants taking iART, generally due to the prevention of low-level (<1,000 HIV RNA copies/ml) semen HIV RNA. However, one participant demonstrated high-level (often >10,000 HIV RNA copies/ml) IHS that persisted for 14 months after starting therapy. This indicates that occasional HIV sexual transmission might still be possible despite effective ART with an undetectable blood VL, although semen infectivity was not directly assessed\textsuperscript{[6]}.

The cause of IHS is not known. In this one individual we found no drug resistance mutations or evidence of viral evolution despite more than a year of sustained, high level semen viral RNA in the presence of therapeutic semen and blood drug levels. We hypothesize that the virus may have originated from a latent mucosal reservoir without active replication cycles, perhaps due to the activation of immune cells in the genito-urinary mucosal compartment that contains integrated HIV DNA. This would be in keeping with previous studies showing that IHS in men taking standard ART was associated with transient, compartmentalized T cell activation\textsuperscript{285}, and may be plausible in the semen compartment, since drugs that act post-integration (protease inhibitors) were present at very low or undetectable levels\textsuperscript{167}, while levels of several drugs that act pre-integration (3TC, maraviroc and raltegravir) exceeded those seen in blood\textsuperscript{167}.

Which cell subset constitutes the mucosal HIV reservoir, if such a reservoir exists, is not known. Since IHS became progressively less common with an increasing duration of effective ART, with no IHS seen beyond 3 years of ART. We hypothesize that the size of the genital reservoir may decrease over this period in the absence of productive virus replication, as has been seen in both the blood and gut reservoirs of individuals taking ART\textsuperscript{298}. However, this decay in other reservoirs is too slow to permit eradication, whether this is also the case for the putative
genital reservoir is a topic that will require future study. The high virus levels seen during some IHS episodes certainly suggest that the mechanism is distinct to that of low-level viral RNA “blips” in blood, which are thought to reflect random biological and statistical variation around a mean HIV level that falls just below the sensitivity of current clinical assays\textsuperscript{299}.

Individuals initiating an intensified ART regimen were more likely to achieve virologic suppression in semen (HIV RNA <300 copies/ml) by two weeks, in keeping with prior studies showing delayed semen virus suppression in men taking an efavirenz-based ART regimen\textsuperscript{290}. However, the majority of participants in both the sART and iART arms had an undetectable semen VL within 2 months of starting treatment, and so it is not clear that the marginal benefit of more rapid semen virus suppression in iART-treated participants would have any significant public health benefit in terms of reducing HIV secondary transmission compared to sART. The fact that participants with IHS had significantly lower semen (and blood) levels of raltegravir suggests that low semen levels of this medication may play a role, although within the two iART individuals with IHS there was no difference in raltegravir levels between visits with and without IHS, and clinical trials have shown no significant association between blood viral load suppression and raltegravir trough levels\textsuperscript{300,301}.

The sample size of groups treated with iART and sART was relatively small, and data regarding IHS frequency in sART-treated individuals comes from historical controls in a previously published study\textsuperscript{167}. This introduces potential bias, and increases uncertainty into the analysis of IHS frequency and differences between iART and sART-treated groups. Furthermore, semen viral loads were calculated based on an average volume of 2mL rather than exact volumes as previously described [26]. However, it remains clear that IHS, potentially at very high levels,
can occur despite effective antiretroviral therapy, even with the addition of agents with enhanced semen penetration.

In summary, the persistence of HIV RNA in semen despite effective ART has been documented by many groups\textsuperscript{107,167,246,263,270,302}, with at least one case of transmission documented in this context\textsuperscript{124}. We previously described the clinical and mucosal associations of isolated HIV shedding (IHS) in men on effective ART\textsuperscript{167,285}, and now provide observational evidence that an intensified ART regimen reduces, but cannot wholly prevent, high-level IHS. Whether the phenomenon of IHS relates to actual transmission is not known. Although the HIV RNA levels seen in one individual on effective iART were well above a previously-defined \textit{in vitro} threshold for HIV infection\textsuperscript{283}, it is important to note that the frequency of high-level semen HIV virus was reduced by 89\% in participants taking iART, and by 92\% in participants taking sART, compared to previously-described ART-naive men\textsuperscript{167}. This implies that ART, whether provided as a standard or an intensified regimen, will have a substantial effect in reducing HIV sexual transmission early after ART initiation, as has been shown in both observational and randomized clinical studies\textsuperscript{9,10}. It is also encouraging that IHS was not observed after prolonged ART, regardless of initial regimen intensification. Nonetheless, the biological basis of IHS, including the nature of a possible HIV reservoir within the semen compartment, merits further investigation.
FIGURE 4-1: Early impact of standard vs. intensified ART on the semen HIV viral load

Semen HIV RNA viral load (Log10) at enrolment (when drug naïve), and at week 2 and 1 month after ART initiation in standard (sART) and intensified (iART) therapy groups.
Median levels of HIV RNA in the blood and semen of all 13 participants starting an intensified antiretroviral regimen (iART), demonstrating rapid viral load suppression in semen and blood (A). One participant demonstrated a single episode of low level IHS (B), while another demonstrated sustained high-level semen shedding even beyond the six month duration of the study (C). ■, blood plasma; ■, semen plasma.
Concentrations of raltegravir and maraviroc measured six months after starting an intensified antiretroviral regimen (iART). Significantly higher levels of both RAL and MVC were present in semen compared to blood.

**Figure 4-3: Semen vs. blood levels of raltegravir and maraviroc after 6 months**
**Figure 4-4**: Isolated HIV semen shedding (IHS) and semen levels of raltegravir and maraviroc

Concentrations of raltegravir and maraviroc in a time matched sample from (a) blood plasma and (b) semen plasma from individuals with an IHS episode (n=2) and those who never had an IHS episode (suppressed) (n=11). Significantly lower concentrations of raltegravir were observed in both blood and semen of participants with IHS.
**Figure 4-5:** Impact of therapy duration on the occurrence of isolated HIV semen shedding in men taking standard ART

Participants (N=49) were divided into four groups based on the prior duration of effective ART (<6 months, 1-3 years, 3-5 years, >5 years); each participant was asked to provide monthly semen and blood samples for 6 months. Only visits with an undetectable blood viral load were included in the analysis. The Y axis shows the semen viral load, and the X axis shows the number of participants in each group, with the total number of study visits (semen samples) in parentheses.
**FIGURE 4-6**: Representative envelope V3 nucleotide and amino acid alignments of bulk HIV RNA plasma and semen sequences from participant 002 (high-level IHS)

Nested RT-PCR reactions were performed in triplicate (minimum). All replicates yielded identical sequences, therefore only one representative sequence is shown for each compartment at each studied time-point. Base “M” in the nucleotide alignment represents the IUPAC code for a mixture of bases A
Chapter 5: The effect of an intensified antiretroviral regimen on early changes in soluble and cellular markers of systemic immune activation after HIV treatment initiation

Submitted to the Journal of Infectious Diseases
5.1 Abstract

CD4\(^+\) T-cell depletion and increased immune activation are defining features of HIV pathogenesis. The initiation of effective antiretroviral therapy rapidly reduces the HIV RNA viral load to undetectable levels, but systemic immune activation takes much longer to resolve. Cellular and soluble markers of immune activation were followed prospectively for six months in twenty-nine men initiating either a standard 3-drug (n=11) or intensified >3-drug (n=18) antiretroviral regimen. An intensified regimen did not accelerate the resolution of T cell immune activation (CD38, HLADR), the fall in soluble immune parameters (sCD14, sIL-2R, \(\beta_2\)-microglobulin, sTNF-RII), or the restoration of CD4\(^+\) or \(\alpha_4\beta_7\) T cells.

5.2 Introduction

Systemic immune activation is a defining feature of HIV pathogenesis, and is an important driver of CD4\(^+\) T-cell depletion and progression to AIDS\(^{303}\). Effective antiretroviral therapy (ART) results in a rapid fall in the HIV plasma viral load to undetectable levels, which is associated with a more gradual, and generally incomplete, resolution of systemic immune activation\(^{304,305}\). This delayed resolution of immune activation may underlie the increased rates of functional impairment\(^{306}\) and of cardiovascular\(^{89}\) and neuropsychiatric\(^{90}\) disease that are seen despite effective ART.

Several laboratory parameters have been shown to predict adverse HIV outcomes, including the HIV RNA plasma viral load (VL), the peripheral blood CD4 T-cell count, plasma levels of inflammatory biomarkers and T-cell immune activation. Various studies have documented strong soluble markers of immune activation, increased during HIV infection that are reduced following initiation of antiretroviral therapy including; tumor necrosis factor (TNF)-\(\alpha\)
receptor II (sTNF-RII)\textsuperscript{307,308}, soluble interleukin (IL)-2 receptor (sIL-2R)\textsuperscript{307} and β\textsubscript{2}-microglobulin\textsuperscript{309,310}. In this study we quantified both soluble and cellular surface markers of immune activation to determine the effect of an intensified antiretroviral therapy regimen compared to the conventional 3-drug ART regimen.

In a previous study\textsuperscript{311} the effect of intensifying a standard ART regimen with the addition of antiretroviral drugs (maraviroc and raltegravir) known to have good semen penetration was examined, to determine the effect on the rate of blood and genital viral load suppression. The intensified ART regimen was associated with more rapid reduction in the semen viral load, and a reduced incidence of isolated HIV semen shedding. To determine whether an intensified ART regimen would allow for greater and more profound decreases in systemic immune activation we compared reductions of both soluble and cellular markers of immune activation in groups receiving either a standard 3-drug or an intensified ART regimen.

5.3 Methods

HIV-infected MSM and HIV-negative controls were recruited into an observational, open label study through the Maple Leaf Medical Clinic in Toronto, Canada as previously described\textsuperscript{167}. All study participants provided informed, written consent. Blood samples were collected into Acid Citrate Dextran Solution A (BD Bioscience, La Jolla, CA) prior to ART initiation (drug-naïve time point) and after one and six months of ART. The use of an intensified regimen (iART), defined as any antiretroviral regimen including more than the standard 3-drug regimen (sART) was at the discretion of the participant, in discussion with their physician. Blood plasma HIV-1 RNA concentrations were assayed using the Versant HIV-1 RNA 3.0 assay (bDNA; Bayer Diagnostics; lower limit of detection, 50 RNA copies/mL). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation of whole blood at 500g for 25 minutes,
counted, and washed twice in RPMI 1640 with 10% heat-inactivated fetal bovine serum (FBS; Sigma), 100U/mL penicillin, 100 mg/mL streptomycin, and 1X GlutaMAX-1 (Gibco). PBMC’s were cryopreserved in FBS with 10% dimethyl sulfoxide (DMSO) and stored at -150°C until use. Upon use, PBMCs were washed and re-suspended in a sterile 25cm² tissue culture flask at 37°C in an atmosphere of 5% carbon dioxide. Cells were then washed with 1% FBS-1X phosphate buffered saline and stained for 30 minutes with fluorochrome-labeled monoclonal antibodies and a viability dye (Invitrogen) followed by fixation in 1% paraformaldehyde. Antibodies included CD3, CD4, CD8, CD38, HLA-DR and β7-integrin. Cells were acquired on the BD™ LSR-II (BD systems). Data analysis was performed using FlowJo analytical software v.9.4.11 (Treestar). Commercially available ELISA kits were used to measure blood plasma levels of sCD14 (R&D Systems), sIL-2R (eBioscience), β2-microglobulin (R&D Systems), sTNF-RII (R&D Systems). All assays were performed according to manufacturer’s instructions in duplicates.

Statistical comparisons between patient groups were performed using the nonparametric Mann-Whitney U test or the Kruskal-Wallis test, as appropriate. Correlations between variables were evaluated using Pearson correlation test. All statistical analyses were performed using IBM SPSS statistics software for Mac (version 20; SPSS).

5.4 Results

5.4.1 Participant demographics and clinical parameters

Twenty-nine chronically HIV-infected, antiretroviral therapy-naïve men were enrolled into this prospective observational six-month pilot study. Eleven (38%) initiated a standard three-drug ART regimen. The receipt of four or more antiretroviral drugs was classified as iART; 18/29 (62%) received iART. Of the intensified patients, 77.8% (14/18) were intensified with a combination of maraviroc and raltegravir, 16.7% (3/18) with raltegravir alone and 5.6% (1/18)
with an NNRTI (etravirine). The majority of individuals (83%, 15/18) in the iART group received a protease inhibitor (PI) backbone vs. 18% (2/11) of individuals in the sART group. The baseline CD4+ T cell count of the sART and iART groups was similar (Median Absolute Counts; 270 Vs. 330, Respectively, p=0.1); only two individuals had a pre-ART CD4 count below 200/mm³, both from the sART group. The median baseline blood VL was similar between groups at 4.48 log_{10} RNA copies/mL (range, 3.75-5.59) and 4.14 (range, 2.64-5.7) in the sART and iART group respectively, p=0.243. Overall, no significant differences were found between groups at baseline (drug naïve) or at six months of ART therapy for absolute CD4/CD8 T-cell count, blood viremia or HSV/CMV seroprevalence (Table 5.1).

5.4.2 Impact of iART on clinical parameters

Irrespective of treatment regimen, initiation of ART was strongly correlated with increases in CD4 Count (p<0.0001), CD4:CD8 T-Cell Ratio (p<0.0001) and decreased blood viral load (p<0.0001) by month 6. In those participants receiving iART, a higher month 1 absolute CD4 counts was observed (p=0.033); however, by month 6 neither group showed a selective advantage in regards to increased CD4 counts (p=0.33). No significant differences were observed in viral loads between groups at baseline (p=0.243) or month 6 (p=0.177). However, a lower blood viral load was observed in the first month of treatment in the iART group (p=0.04). The decrease in blood viral load from baseline (drug naïve) to month 1 was strongly correlated with the number of ARVs in an individual’s regimen (p=0.025).

5.4.3 Impact of iART on T cell activation in blood

Systemic immune activation was assessed by flow cytometry based on the co-expression of the cell surface markers CD38 and HLA-DR on CD4+ and CD8+ T-cells. Prior to ART initiation, CD4+ T cells in sART participants demonstrated significantly higher levels of immune
activation than those in the iART group (1.51% vs. 1.04%, p=0.027). Despite this difference, by month 6 there was no longer any significant difference between groups (0.51% vs. 0.47%, p=0.461). Similarly, there were no observable differences on CD8+ T-cells at baseline (2.42% vs. 2.45%, p=0.924) or at month 6 (0.83% vs. 0.98%, p=0.920) (Figure 5.1A,B). When the percent change in expression of these markers was compared, no significant differences were observed with respect to CD4+ (p=0.079) or CD8+ (p=0.269) T-cells.

5.4.4 Reconstitution of α4β7+ T-cells in the peripheral blood

In addition to immune activation status, we examined the gut homing receptor, α4β7 on CD4+T-cells depleted during primary infection. Compared to HIV negative controls, there was a significantly lower proportion of CD4+ T-cells expressing β7hi in HIV+ ART naive participants, 1.82% (0.82-5.74%) vs. 3.11% (range, 1.86-6.42%), p=0.006.

No difference in β7hi expression between treatment groups at baseline (p=0.549) was observed. Initiation of ART showed significant increases in CD4+β7hi+ expression levels regardless of therapy regimen (p=0.001), with significant increases present in both the sART (2.83%, p=0.039) and iART (2.73%, p=0.0158) group after six months. Furthermore, no significant differences in absolute CD4+β7hi+ levels between groups (p=0.327) or in percentage increase from baseline (p=0.403) were observed (Figure 5.1C).

5.4.5 Soluble markers of immune activation and microbial translocation

Plasma markers of immune activation assessed included; soluble β2-microglobulin, IL-2R and TNF-RII. Compared to HIV negative controls, HIV positive participants had increased β2-microglobulin (p=0.003), sIL2 (p=0.003) and TNF-RII (p=0.017).
Plasma markers of immune activation strongly correlated with each other at baseline, with both sIL-2R and sTNF-RII correlating with HIV viral load (p=0.0004 and 0.011, respectively) and with expression of CD3+CD4+CD38+HLADR+ (p=0.008 and 0.032, respectively). Both groups showed significant decreases in all markers on ART. iART was not associated with a more profound reduction by month 6 of antiretroviral therapy for any of β2-microglobulin, IL-2R or TNF-RII (Figure 5.2A,B,C).

Soluble CD14 (sCD14), a soluble protein, found increased with chronic HIV infection and attributed to increased immune activation was also examined between treatment groups. Examination of sCD14 within our cohort revealed comparable levels between groups at baseline (p=0.268), however, the proportional change from baseline to month 6 showed higher levels in the iART group (p=0.023). Within the iART group 48.3% (14/29) of individuals were receiving maraviroc, an ARV previously shown to be associated with increased sCD14 levels. We observed by month 6 individuals treated with maraviroc had increases in several soluble markers of immune activation including; sCD14 (p=0.002), sTNF-RII (p=0.024), β2-Microglobulin (p=0.027) compared to those not receiving maraviroc in the iART group.

5.5 Discussion

Initiation of antiretroviral therapy has many effects, including reducing HIV viral load in both blood and semen, as well as decreasing systemic cellular and soluble immune activation markers. The decrease in blood viremia reflects the clearance of not only infected cells but also plasma virions. However, small reservoirs of latently infected memory lymphocyte and replication competent HIV persist despite ART, serving to replenish the pool of infected cells and preventing therapy from eradicating the virus and returning immune homeostasis. In theory, drug intensification might provide a better means of reducing viremia and eradicating viral reservoirs,
however, trials have shown this not to be the case. Nonetheless, intensified therapy might provide a means to reduce immune activation as elevated levels of immune activation are still observed in individuals receiving standard antiretroviral therapy regimens. We hypothesized that an intensified ART regimen would result in a more substantial reduction in systemic immune activation. This pilot study examined whether drug intensification can reduce systemic immune activation to a greater degree than standard antiretroviral therapy in the short-term. Within our small cohort, we have been able to determine that drug intensification does not provide any observable clinical advantage over standard therapy. While some parameters such as CD4⁺CD38⁺HLADR⁺ T-cells differed between groups at baseline, these effects were muted by month 6 suggesting no clinical benefit. In addition, we observed increased levels of sCD14, sTNF-RII and β2-microglobulin in participants that had maraviroc included in their therapy regimen. Further supporting this finding, Hunt et al. 2013 recently released findings from a randomized trial of maraviroc intensification showing its addition to therapy regimens caused increases in sCD14 in both blood and rectal tissues\textsuperscript{312}. The mechanism by which this increased immune activation occurs still remains unclear but has important clinical implications, especially in studies looking at drug combinations for pre-exposure prophylaxis.

Our lab has previously shown that intensification with agents with high mucosal penetration (maraviroc and raltegravir) provided a greater reduction in semen viral load in the short term but provided no selective advantage after 6 months of treatment in regards to blood and semen viral load, CD4 and CD8 T-cell counts and occurrence of high-level isolated HIV-1 semen shedding\textsuperscript{311}. Our current study shows that in addition, intensification of therapy did not provide a clinical benefit in regards to a more rapid reduction of systemic immune activation (soluble or cellular) or CD4 reconstitution. In conclusion, this observational study suggests that drug intensification may not reduce systemic immune activation to a greater degree than standard ART in the short term.
FIGURE 5-1: Associations between ART regimen (sART or iART) and markers of immune activation
**Figure 5-2:** Associations between ART regimen (sART or iART) at baseline (drug naive) and month 6 in soluble markers of immune activation
**Table 5-1:** Baseline (drug naive) clinical demographics of study participants in either the standard (sART) or intensified (iART) group

<table>
<thead>
<tr>
<th></th>
<th>Standard ART (n=11)</th>
<th>Intensified ART (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), median ± S.D.</td>
<td>37 ± 10.36</td>
<td>34 ± 9.17</td>
</tr>
<tr>
<td>HSV-1</td>
<td>7 (63.6%)</td>
<td>14 (82.3%)</td>
</tr>
<tr>
<td>HSV-2</td>
<td>4 (36.4%)</td>
<td>11 (64.7%)</td>
</tr>
<tr>
<td>CMV</td>
<td>11 (100%)</td>
<td>16 (88.9%)</td>
</tr>
<tr>
<td>PI-based ART</td>
<td>2 (18.18%)</td>
<td>15 (83.33%)</td>
</tr>
<tr>
<td>NNRTI-based ART</td>
<td>9 (81.81%)</td>
<td>3 (16.67%)</td>
</tr>
<tr>
<td># ARV drugs, median ± S.D.</td>
<td>3 ± 0</td>
<td>5 ± 0.56</td>
</tr>
<tr>
<td>Blood HIV-1 RNA copies/mL (log_{10} copies/mL)</td>
<td>4.48 (3.75-5.59)</td>
<td>4.14 (2.64-5.7)</td>
</tr>
<tr>
<td>Absolute CD4 Count (cells/mm³)</td>
<td>270 (170-620)</td>
<td>330 (210-610)</td>
</tr>
<tr>
<td>Absolute CD8 Count (cells/mm³)</td>
<td>910 (230-1550)</td>
<td>850 (340-1980)</td>
</tr>
<tr>
<td>CD4:CD8 T-Cell Ratio</td>
<td>0.28 (0.17-0.91)</td>
<td>0.41 (0.18-0.96)</td>
</tr>
</tbody>
</table>
Chapter 6: Clinical and Mucosal Correlates of HIV-1 RNA semen Shedding in Antiretroviral Naive Men

Submitted to Mucosal Immunology
6.1 Abstract

The semen HIV RNA viral load (sVL) of an antiretroviral therapy-naïve man is an independent predictor of HIV transmission risk. The sVL correlates moderately with blood viral load, and its variability only partly explained by compartmentalized factors such as bacterial STIs and reactivation of mucosal herpesviruses. Thirty HIV+ therapy-naive naive men were recruited for cross-sectional analyses, with thirteen men followed longitudinally for up to a year. Paired blood and semen samples were collected for quantification of HIV RNA, bacterial load by 16S RNA and herpesviruses VL. Semen cytokines/chemokines were assayed by multiplex ELISA and semen T-cells measured by flow cytometry. Semen HIV RNA was detected at 93% of visits, with >50% of men shedding high levels of virus (>5,000 copies/mL). In the baseline cross-sectional analysis, T-cells in semen were significantly more activated than blood, with an increased frequency of Th17 cells and γδ-T-cells. CMV reactivation, bacterial load and semen inflammatory cytokines correlated with the sVL. The prospective sub-study found striking inter-individual variability in HIV and CMV shedding; only semen IL8 levels and the blood VL were independently associated with sVL. While variability in the sVL of therapy-naïve men had several associations, IL-8 was the only stable predictor longitudinally of sVL.
6.2 Introduction

Semen containing HIV-1 (HIV) is the major vector worldwide for transmission from an infected man to his sexual partner(s). The HIV RNA level in the blood of an infected individual is the best-defined predictor of transmission risk in HIV sero-discordant couples, with the annual transmission risk increasing from 0 to 25% as the blood viral load (bVL) in an antiretroviral therapy (ART) naïve partner increases from undetectable levels to >50,000 HIV-1 RNA copies/mL\textsuperscript{160}. However, as there is minimal blood contact during most sexual HIV transmission, the association of transmission risk with the blood viral load likely reflects the correlation of the latter with semen viral load (sVL)\textsuperscript{172-175}. In keeping with this, work from the Partners in Prevention HSV/HIV Transmission Study Team demonstrated that the sVL of a male partner was a stronger independent predictor of HIV transmission than the bVL, and that each log\textsubscript{10} increase in the semen viral load at participant enrolment was associated with a 1.8 fold increased risk of HIV transmission\textsuperscript{171}.

While sVL has been shown as a critical determinant of transmission risk\textsuperscript{172}, substantial sVL variability may exist over time, independent of bVL, and at any given time there is at best a moderate correlation between the two\textsuperscript{168}. Genital infections are an important source of this variability, whether they are chronic and recurrent (e.g. semen cytomegalovirus reactivation) or transient (e.g. gonorrhoea). Treatment of gonococcal urethritis reduces the sVL almost ten-fold without any effect on bVL\textsuperscript{188}. Despite this, approximately one third of antiretroviral therapy-naïve men shed levels of HIV RNA in semen that are disproportionately higher than those in blood\textsuperscript{166}, even in the absence of symptomatic urethritis or asymptomatic “classical” STIs\textsuperscript{313}. Studies examining the effect of herpesviruses on semen viral load have documented associations of the sVL with both cytomegalovirus (CMV) and Epstein-Barr virus (EBV) reactivation\textsuperscript{166,181}. In
addition, men with a disproportionately high sVL but no classical STIs reported a higher rate of unprotected insertive (but not receptive) sexual acts, both anal and vaginal\textsuperscript{180}.

Although effective antiretroviral therapy dramatically reduces both the sVL and the probability of HIV transmission, globally most HIV-infected individuals do not have access to ART, and the goal of increased access faces important logistic and economic barriers. Therefore, understanding the factors that cause variability in the sVL of ART-naïve men remains important and may lead to novel avenues for prevention. The current study examined clinical and mucosal correlates of semen HIV RNA shedding, both cross-sectionally and longitudinally, with the goal of elucidating factors that influence sVL variability in HIV-infected, ART-naïve men who have sex with men (MSM).

6.3 Methods

6.3.1 Study participants and design

Chronically HIV-infected men who have sex with men were recruited through the Maple Leaf Medical Clinic, an HIV primary care clinic in Toronto, Canada. Participants were antiretroviral drug naïve, and were screened for urethritis and sexually transmitted infections (STIs) at each study visit. All participants provided paired blood and semen samples at a single time point, with a subgroup of men electing to be followed monthly for up to one year. High-level semen shedding was defined as a semen HIV viral load of ≥5,000 HIV-1 RNA copies/mL.

6.3.2 Sample collection and diagnostic testing

Blood was collected into acid citrate dextran vacutainers by venipuncture, and semen was collected by masturbation into a sterile container containing 10mL of RPMI with
penicillin/streptomycin following at least 48 hours of abstinence, as previously described \textsuperscript{293}. Samples were processed within 2 hours of collection. Semen plasma was isolated by centrifugation at 850 x g for 10 minutes and blood plasma following Ficoll density gradient centrifugation at 500 x g for 25 minutes. Blood and semen plasma HIV-1 RNA viral load was measured in the Mount Sinai Hospital Department of Microbiology (accredited by the Ontario Public Health Lab for clinical HIV viral load measurement) with the Abbott RealTime HIV-1 assay on an automated m2000 system (Abbott Molecular Diagnostics). Correction for semen dilution was calculated based on a mean semen volume of 2mL, as previously described \textsuperscript{293}. The lower limit of detection for blood and semen viral load were 50 and 300 HIV RNA copies/mL, respectively. High-level semen shedding was defined as a viral load >5,000 HIV RNA copes/mL as semen VL tends to be ~10-fold lower than blood and individuals with a blood VL >50,000 HIV RNA copies/ML are more likely to transmit\textsuperscript{160}.

6.3.3 Real-time quantification of herpesvirus Viral load

Viral load quantification of Human Herpesvirus (HHV) 1 through 8 was performed using RT-PCR analysis with commercially available genesig qPCR detection kits (HSV-1, HSV-2, HHV-3, EBV, CMV, HHV-7, and HHV-8), which were designed and validated by PrimerDesign Ltd. Similarly, HHV-6 (A and B) was quantitated using the RealStar PCR kit (Altona Diagnostics), as per manufacturer's protocol\textsuperscript{314}. Briefly, DNA was extracted from 400 µL of seminal plasma using the Qiagen DNA mini extraction kit (Qiagen), as per the manufacture’s protocol. For the genesig detection kits, extracted seminal plasma DNA (5 µl) containing the provided internal control was combined with a PCR mix (PrimerDesign) containing 10µL 2xPrecision\textsuperscript{TM} MasterMix, 1µL Primer/Probe mix, and 4µL RNAse/DNAs free water. For the RealStar PCR kit, 10 µL of extracted seminal plasma DNA was added to the mastermix containing 5 µL of master A, 15 µL of master B, and 1 µL of the internal control. All viruses were
quantified using the Applied Biosystems 7900 HT Real-Time PCR system with the following reactions conditions: 95°C for 10 minutes followed by 50 cycles of 95°C for 10 seconds (genesig) or 15 minutes (RealStar) and 60°C for 1 min, and results were analyzed using the Sequence Detection System v2.4 (Applied Biosystems). Lower limit of detection for herpesvirus viral loads was 200 copies/mL plasma.

6.3.4 Cytokine and trappin-2/elafin levels in semen plasma

Cytokine levels in semen plasma were measured using the Meso Scale Discovery multiplex System (Gaithersburg, MD). Fourteen cytokines/chemokines were measured in semen plasma using two human ultra-sensitive custom 7-spot kits, lower limit of quantification listed in brackets, including; IL-1α (3.6pg/mL), IL-6 (3.6pg/mL), IL-1β (3.6pg/mL), IL-8 (1.8pg/mL), MCP-1 (1.8pg/mL), MDC (1.8pg/mL), MIG (1.8pg/mL), MIP-1β (29.28pg/mL), MIP-3α (14.64pg/mL), RANTES (3.6pg/mL), IL-10 (1.8pg/mL), IL-17 (3.6pg/mL), IP-10 (14.64) and TNF-α (1.8pg/mL). All samples were run in duplicate according to the manufacturer’s protocol. For cytokine levels that were below the lower limit of detection of the assay, values were reported as the lower limit of quantification based on the standard curve generated for the particular cytokine. Cytokine concentrations were log transformed for analysis. Commercially available Trappin-2/Elafin ELISA kits (Hycult Biotech) were used to measure seminal plasma levels of Elafin according to manufacturer’s instructions in duplicates.

6.3.5 Bacterial load quantification

For bacterial load quantification, 500µL of thawed seminal plasma was lysed using a combination of chemical and mechanical methods and purified using Qiagen AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA, USA). DNA was eluted in 100µL of buffer EB, while RNA was eluted in 50µL. Reverse transcription was performed using qScript cDNA SuperMix according to
the manufacturer’s instructions (Quanta Biosciences, Geithersburg, MD, USA). Using the DNA fraction bacterial load was quantified, measured as bacterial 16S rRNA gene copy per mL of seminal plasma using a broad-coverage qPCR assay as described previously\textsuperscript{315}.

6.3.6 **T-cell populations in blood and semen**

Mononuclear cells were isolated from blood and semen as previously described\textsuperscript{72}. Briefly, peripheral blood mononuclear cells were collected into Acid Citrate Dextran solution A (ACD; BD Bioscience, La Jolla, CA), and semen samples collected in 10mL of RPMI solution with 100U/mL penicillin, 100mg/mL streptomycin, and 1x GlutaMAX (Giboco, Gran Island, NY) and transported to the laboratory within 2 hours of collection\textsuperscript{293}. Mononuclear cells from blood and semen were isolated by layering samples over Ficoll-plaque Plus (Amersham Bioscience, oster City, CA) and centrifuged at 500g for 25 minutes without breaks, counted and washed once in RPMI 1640 with 10% heat-inactivated fetal bovine serum (Sigma, St Louis, MO), 100 U/mL penicillin, 100mg/ml streptomycin, and 1x GlutaMax (Gibco) and a second time in BD Nanocrystal Buffer + 5% heat-inactivated fetal bovine serum (Sigma). Mononuclear cells were then stained for 30 minutes with fluorochrome-labeled monoclonal antibodies (CD3G-FITC, CD8-PETR, CD195-PerCPCy5.5, CD69-PECy7, $\beta$7integrin-APC, HLADR-APCH7, CD196-Pacific Blue, CD3-605NC, CD4-650NC) and a viability dye (Invitrogen) followed by fixation for 30 minutes in 1% paraformaldehyde and analyzed using BD\textsuperscript{TM} LSR-II (BD systems).

6.3.7 **Statistical analysis**

Flow cytometry data analysis was performed using FlowJo analytical software v.9.4.11 (Treestar). Non-parametric Mann-Whitney U and Wilcoxon Signed Ranks statistical analysis were performed using IBM SPSS Statistics v.20 (IBM Corp.). Backward stepwise linear regression analyses were performed for multiple independent co-variants using SPSS, with
p<0.05 used as the threshold for statistical significance. Count and/or concentration variables were logarithmically transformed. For the longitudinal multivariate analysis, a mixed model analysis was used to take into account that individuals produced multiple observations/measurements. Variables associated with the outcome variable on univariate analysis (p<0.1) were selected for the multivariable analysis.

6.4 RESULTS

6.4.1 Cross-sectional cohort demographics and viral loads

Thirty HIV-infected, antiretroviral-naïve men provided paired blood and semen samples at the baseline time point. All participants had been infected with HIV for at least 1 year (median 3 years; range 1-23 years). Study participants had median absolute CD4 and CD8 T-cell counts of 440 cells/mm³ (range, 190-860) and 905 cells/mm³ (range 230-2,690), respectively. The median blood HIV RNA viral load was 30,544 HIV-1 RNA copies/mL (Range, 824 - >500,000 copies/mL), and median semen HIV RNA viral was 5,496 HIV-1 RNA copies/mL (range, 300-208,152 copies/mL). All study participants were CMV seropositive, 80% (24/30) HSV-1 seropositive and 33.3% (10/30) HSV-2 seropositive. Clinical parameters are summarized in Table 6-1.

Semen HIV-1 RNA was detected in 93% (28/30) of participants, and 50% (15/30) met criteria for high-level semen shedding with an sVL ≥5,000 copies of HIV-1 RNA/mL. Two individuals tested positive for infection with Chlamydia trachomatis, both of whom had high-level semen shedding, and these participants were eliminated from subsequent data analysis. In 10.7% (3/28) of men the sVL exceeded the paired bVL, meeting pre-defined criteria for disproportionate HIV-1 semen shedding. Viral load data are summarized in Table 6-1.
6.4.2 Semen cytokines and the semen viral load

To begin to investigate the predictive correlates of the semen HIV RNA viral load, levels of 14 cytokine/chemokines (see Methods) and of Elafin/Trappin-2 were first assayed in seminal plasma (Table 2). Bivariate analysis demonstrated that the sVL was positively associated with semen levels of the pro-inflammatory cytokines/chemokines IL-1α (p=0.009), IL-8 (p=0.033), IL-1β (p=0.005), IL-6 (p=0.037) and MIP-1β (p=0.031). Next, to determine the strongest predictive correlates of the semen viral load, a partial correlation analysis controlling for blood viral load was performed. After controlling for the bVL, semen levels of the cytokines IL-8 (p=0.002), IL-1α (p=0.024), IL-1β (p=0.011), IL-6 (p=0.013) and MIP-1β (p=0.006) remained significantly correlated with the semen viral load. The independent association(s) of these cytokines with the semen VL was then assessed in a stepwise multivariate linear regression model, with IL-8 (p<0.0003) as the strongest independent predictive sVL correlate (Figure 6-1) followed by IL-1β (p=0.03). Trappin-2/Elafin was readily detectable in seminal plasma, but levels did not correlate with either the sVL or semen cytokine/chemokine levels.

6.4.3 sVL and local herpesvirus reactivation

CMV and EBV qPCR was performed on all seminal plasma samples; 63% (19/30) successfully passed internal control standards for CMV and 67% (20/30) for EBV. Internal control or run failure for the remaining samples was thought to be due to the presence of PCR inhibitors, a common issue with seminal plasma qPCR assays. The median CMV and EBV viral load was 486 copies CMV DNA/mL (range, 200 – 266,761) and 200 copies EBV DNA/mL (range, 200-409,157), respectively; CMV was detected in the semen plasma of 11/19 (58%) and EBV in 6/20 (30%) of participants. The CMV but not EBV semen viral load correlated with the HIV sVL (p=0.014 and p=0.238, respectively (Figure 6-2). CMV levels were correlated with semen cytokines IL-10 (0.048), IL-1β (<0.0001), IL-6 (0.009), MIP-1β (0.004) and TNF-α
The independent association of these cytokines with semen CMV viral load was assessed in a stepwise multivariate linear regression model. Semen IL-1β level (p<0.0001) was the strongest immune predictor of CMV viral load of the cytokines assessed.

6.4.4 Semen T-cell immune parameters and the sVL

Semen mononuclear cells (SMC) were analyzed by flow cytometry in the subset of men (13/30; 43.3%) who were subsequently followed longitudinally, to evaluate the potential associations of semen T-cell phenotype and activation status with the sVL. Semen T-cells were immunologically much more activated than those in the blood of the same participant. Specifically, co-expression of CD38 and HLADR was much higher on both semen CD4+ T-cells (median 16.25 vs. 0.89%; p<0.0001) and CD8+ T-cells (median, 13.75 vs. 3.91%; p=0.039). This was also the case for expression of the early activation marker CD69 on both CD4+ (32.80 vs. 3.14%; p<0.0001) and CD8+ (57 vs. 5.17; p<0.0001) T-cells, and semen CD4+ T cells demonstrated higher expression of the HIV co-receptor CCR5 (24.15 vs. 4.3%; p<0.0001). The proportion of CD3+ T-cells expressing the gamma-delta receptor (CD3G) tended to be higher in PBMCs than semen (2.84 vs. 0.94; p=0.18). CCR6, a surface marker for IL-17 secreting cells, was expressed at significantly increased levels on both semen CD4+ T-cells (28.8 vs. 4.3; p=0.003) and CD3G+ T-cells (83.7 vs. 0.43; p<0.0001) when compared to blood. Expression of β7, a proxy for the mucosal homing integrin α4β7+, was actually lower on semen than blood CD4+ T cells (0.9 vs. 2.1%; p=0.013).

Upon examination of cell surface marker expression in blood and semen mononuclear cells, a correlation was observed between blood CD8+HLADR+ (p=0.048) and semen CD3G+CCR6+ (p=0.011) with semen viral load. No other parameter examined was found to correlate with the level of semen HIV. In general, the HIV sVL was positively correlated with an
increased total number of semen T cells, including semen CD3^-CD8^+ T-cells (p=0.026), CD3^-CD4^+ T-cells (p=0.028). However, the proportion of γδ T-cells expressing CCR6^+ were negatively correlated with the sVL (p=0.011)

6.4.5  Bacterial load in semen and the sVL

The semen compartment has an endogenous microbiome. To assess whether the total semen bacterial load impacts semen immunology and the sVL, this bacterial load was quantified by 16S RNA in all semen samples as a global measure of the microbiome. The total bacterial load was positively correlated with the sVL (p=0.013; Figure 6-3). Furthermore, cross sectional analysis showed that the semen bacterial load was significantly correlated with semen levels of the cytokines IL-8 (p=0.004), MCP-1 (p=0.02), MDC (p=0.017), MIP-3α (p=0.008), IL-6 (p=0.012), IP-10 (p=0.007) and TNF-α (0.046). Semen bacterial load did not correlate with baseline clinical parameters, semen T-cell immunology or the reactivation of herpesviruses.

6.4.6  Longitudinal cohort demographics and viral Loads

A subgroup of participants (n=13) agreed to provide monthly, paired semen and blood samples for up to 12 months, or until initiation of antiretroviral therapy, to permit assessment of sVL and semen immunology changes within an individual. HSV-1 and HSV-2 seroprevalences in this subgroup were 92% (12/13) and 46% (6/13) respectively, and the median duration of follow-up was seven months (range, 2-12 months) with a total of 95 sample visits.

6.4.7  Patterns and correlates of HIV shedding in semen

High-level semen shedding (>5,000 copies/mL) had been detected at enrolment in 45.45% (5/11) of the prospective participants: during follow up high level shedding was detected at one study visit or more in 69% (9/13), and overall at 51% (48/95) of study visits. Two individuals in the longitudinal sub-study presented at enrolment with STI infections and had their first visit
eliminated from all data analyses. These participants had enrolment semen viral loads of 4.4 and 4.66 log_{10}, respectively and had resolved their infections by their second visit. While the median sVL was 0.95 log_{10} (0.79-fold) lower than the bVL (median bVL 4.45 log_{10} copies/mL, range, 2.92-5.18 log_{10} copies/mL; vs. median sVL 3.5 log_{10} copies/mL, range, 2.48-5.00 log_{10} copies/mL), patterns of semen shedding showed substantial inter-individual variability. In almost half of the participants, the sVL was always substantially lower than the blood (6/13 participants; representative example; **Figure 6-4a**). Although only 23% (3/13) of study men had an undetectable semen viral load at any time point, one individual maintained an undetectable sVL at each of 9 study visits despite a median blood viral load of 3.69 log_{10} copies/mL (range, 3.06-3.87 log_{10} copies/mL; **Figure 6-4b**). Only 1/13 of men consistently demonstrated a disproportionately high sVL (**Figure 6-4c**), while in the remaining 6/13 participants the sVL was sometimes lower than blood and sometimes disproportionately high (**Figure 6-4d**).

### 6.4.8 Longitudinal correlates of HIV shedding in semen

Semen parameters that had been associated with the sVL in the baseline, cross-sectional analysis also varied over time. CMV reactivation was more common than EBV in semen, with detection in 9/13 vs. 4/13 participants at any time, and at 28.7% (27/94) vs. 7.4% (7/94) of total study visits, respectively. The median semen CMV VL was 241 copies/mL (range 200-266,761 copies/mL), and exceeded 5,000 copies/mL during 29.6% (8/27) reactivations; the median semen EBV VL was also 200 copies/mL (range 200-353,125 copies/mL), and exceeded 5,000 copies/mL during 28.6% (2/7) reactivations. In general, the semen bacterial load, semen cytokine levels and T-cell parameters were relatively stable over time (data not shown).

The sVL and several clinical and immune parameters that had been associated with the HIV sVL at baseline demonstrated substantial variability over time. Therefore, a multivariate mixed
model that incorporated the longitudinal viral, immune and bacterial load data was used to assess independent associations of the HIV sVL (see Methods). In this prospective analysis, using a random intercept linear mixed model, the only independent associations of HIV sVL were the blood viral load ($p=0.0004$) and semen levels of the pro-inflammatory chemokine IL-8 ($p<0.0001$). For every log$_{10}$ unit increase in blood viral load the semen viral load increases by 0.40 log$_{10}$, and for every log$_{10}$ increase in the IL-8, the sVL increases by 0.75 log$_{10}$ (Figure 6-5).

6.5 DISCUSSION

The semen of an HIV-infected, ART-naive man is responsible for most global HIV transmission, with the HIV-1 RNA viral load in both blood and semen being independent predictors of HIV transmission risk$^{171}$. While the sVL is generally considerably lower than that in blood, virus levels in this compartment are more variable$^{176,318,319}$. Cross-sectional studies have shown that there is considerable inter-individual variability in the sVL, and that a subset of men manifest disproportionately high levels of HIV in semen$^{166,320}$ with increased levels of semen virus linked to transient STIs, herpesvirus reactivation and local immune activation$^{188,189,321,322}$. However, cross-sectional studies have often not been comprehensive in their approach, and it is not known whether these patterns are stable in an individual over time. In the current study we demonstrated multiple correlates of the semen HIV RNA viral load in ART-naïve men, some of them novel and some confirmatory: these included the blood VL, semen CMV reactivation, levels of pro-inflammatory cytokines and chemokines, the total bacterial load (in the absence of classical STIs), and semen T-cell numbers. There was considerable inter-individual variability in the patterns of HIV semen shedding over time, and the only independent associations of the sVL were the blood HIV RNA viral load and semen levels of the pro-inflammatory chemokine IL-8, suggesting that the latter may constitute a “common pathway” linking heterogeneous local causes
of increased semen shedding.

IL-8 is a proinflammatory cytokine produced by a variety of cells, but with macrophages/monocytes as the dominant source\textsuperscript{323}. Macrophages are common in semen, constituting 20-30\% of white blood cells in this compartment, and are more numerous in semen than T-lymphocytes (2-5\%)\textsuperscript{109}. Increased semen levels of IL-8 have been not only been observed in the context of HIV infection, but are also elevated in the seminal plasma of infertile patients with leukospermia\textsuperscript{324}. Furthermore, in cervical explant tissue models IL-8 has been shown to increase HIV replication, and competitive inhibition of the IL-8 receptor CXCR2 decreasing explant susceptibility to HIV infection by 45-70\%\textsuperscript{185}. Interestingly, the majority of semen chemokines/cytokines present in high levels in this study are secreted by macrophages. While these findings suggest that macrophage-derived IL-8 may play a compartmentalized role in influencing the semen viral load, future studies will need to confirm the cellular origin of IL-8 and assess whether therapeutic strategies targeting IL-8 could reduce semen HIV levels.

Several of the individual upstream mucosal parameters that were associated with the sVL had been previously described, while others were novel. The local reactivation of CMV has been previously associated with the sVL\textsuperscript{9,20}, and increased semen cytokines have been associated with semen levels of both CMV and HIV\textsuperscript{166,202}. The absolute number of HIV-specific T-cells in semen has been associated with an increased sVL\textsuperscript{166}, although this was not seen when a substantial proportion of men were on ART\textsuperscript{182}. The finding of an association between the total semen bacterial load and the sVL in the absence of classical STIs strongly suggests that the semen microbiome plays a role in HIV transmission, an area that will be very interesting for future research. The fact that local CMV reactivation, the semen bacterial load and semen T-cell numbers were all associated with semen proinflammatory cytokines, and that semen IL-8 levels
were the only semen parameter associated with the sVL on multivariate analysis, again suggests that IL-8 serves as a final common pathway for these associations.

There was considerable inter-individual heterogeneity in patterns of semen HIV shedding. The sVL has generally been observed to be significantly lower than the bVL\textsuperscript{177,178}. However, in our study we found that less than half of all participants consistently had an sVL lower than the bVL, while the majority (>53%) either consistently or intermittently demonstrated a disproportionately high sVL. This visit-to-visit variability has implications for predicting HIV transmission at an individual level, and stresses the importance of prospective studies. However, it is important to note that the early initiation of antiretroviral therapy is now the norm in developed countries\textsuperscript{225}, both for the health benefit of the HIV-infected person and to reduce HIV transmission. While there is no question that effective ART trumps the parameters studied and rapidly reduces the sVL to undetectable levels\textsuperscript{104,246,270,271,318}, the unfortunate truth is that most people infected by HIV globally are unable to access therapy, and that ART rollout may not be able to keep pace with the expanding burden of new HIV infections.

The sample size of our study was relatively limited, particularly for the prospective analysis of semen shedding. Despite this, this comprehensive, prospective analysis of multiple semen parameters related to the sVL provides new insights of substantial public health importance. Indeed, the prospective cohort component clearly confirmed the blood HIV RNA VL as a strong predictor of semen HIV levels, while adding semen IL-8 levels as a strong, independent mucosal (compartmentalized) predictor.

In summary, our study confirms that there is considerable variability in the semen viral load among HIV-infected, ART-naïve men, although patterns and stability of shedding vary considerably between men in prospective analysis. Fluctuation in the sVL may relate to numerous
factors within the semen compartment, including T-cell influx, herpesvirus reactivation, the semen microbiome, and effects of these parameters may be mediated through increased levels of the cytokine IL-8. This suggests that IL-8 may constitute a common pathway for increased sVL in therapy-naïve men, and may constitute a potential therapeutic target.
### TABLE 6-1    Cross-sectional Cohort Demographics and Semen Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cross-Sectional Visit 1 (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>34 (range, 24-56)</td>
</tr>
<tr>
<td>Years Infected*</td>
<td>3 (IQR, 1-4)</td>
</tr>
<tr>
<td>HSV-1</td>
<td>80% (24/30)</td>
</tr>
<tr>
<td>HSV-2</td>
<td>33.3% (10/30)</td>
</tr>
<tr>
<td>HSV1+/HSV2+</td>
<td>26.7% (8/30)</td>
</tr>
<tr>
<td>HSV1-/HSV2-</td>
<td>13.3% (4/30)</td>
</tr>
<tr>
<td>CMV</td>
<td>100% (30/30)</td>
</tr>
<tr>
<td>Absolute CD4*</td>
<td>440 (190-860)</td>
</tr>
<tr>
<td>Absolute CD8*</td>
<td>905 (230-2,690)</td>
</tr>
<tr>
<td>CD4:CD8 Ratio*</td>
<td>0.56 (0.17-1.97)</td>
</tr>
<tr>
<td>Blood Viral Load (copies/mL)</td>
<td>30,544 (824 – 500,000)</td>
</tr>
<tr>
<td>Semen Viral Load (copies/mL)</td>
<td>5,496 (300-208,152)</td>
</tr>
<tr>
<td>Semen Bacterial Load (16s copies/mL)</td>
<td>379,252 (19,672 – 12,736,702)</td>
</tr>
<tr>
<td>Semen CMV Viral Load (copies/mL)</td>
<td>282 (200 – 266,761)</td>
</tr>
<tr>
<td>Semen EBV Viral Load (Copies/mL)</td>
<td>200 (200-409,156)</td>
</tr>
</tbody>
</table>

*Median values reported
### Table 6-2  
Semen Levels of Cytokines and Chemokines

<table>
<thead>
<tr>
<th>Cytokine/Protein</th>
<th>Median (pg/mL)</th>
<th>Percent Detectable (%)</th>
<th>Range (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1α</td>
<td>139</td>
<td>100%</td>
<td>12-1,135</td>
</tr>
<tr>
<td>IL8</td>
<td>1,451</td>
<td>100%</td>
<td>176-180,408</td>
</tr>
<tr>
<td>MCP-1</td>
<td>3,378</td>
<td>100%</td>
<td>289-79,593</td>
</tr>
<tr>
<td>MDC</td>
<td>26,034</td>
<td>100%</td>
<td>2,298-127,178</td>
</tr>
<tr>
<td>MIG</td>
<td>33,511</td>
<td>100%</td>
<td>2,939-114,179</td>
</tr>
<tr>
<td>MIP-3α</td>
<td>1,167</td>
<td>93%</td>
<td>30-3,769</td>
</tr>
<tr>
<td>RANTES</td>
<td>373</td>
<td>100%</td>
<td>25-1,935</td>
</tr>
<tr>
<td>IL-10</td>
<td>49</td>
<td>82%</td>
<td>15-188</td>
</tr>
<tr>
<td>IL-17</td>
<td>37</td>
<td>82%</td>
<td>15-190</td>
</tr>
<tr>
<td>IL-1β</td>
<td>47</td>
<td>64%</td>
<td>24-592</td>
</tr>
<tr>
<td>IL-6</td>
<td>119</td>
<td>100%</td>
<td>14-3,866</td>
</tr>
<tr>
<td>IP-10</td>
<td>35,768</td>
<td>100%</td>
<td>4,019-222,383</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>251</td>
<td>68%</td>
<td>90-7,216</td>
</tr>
<tr>
<td>TNF-α</td>
<td>8.9</td>
<td>82%</td>
<td>1.8-96</td>
</tr>
<tr>
<td>Trappin-2</td>
<td>1,871</td>
<td>100%</td>
<td>839-7,823</td>
</tr>
</tbody>
</table>
**Figure 6-1: Association between semen viral load and IL-8**

In study patients, seminal plasma IL-8 remained the strongest independent predictor of semen viral load cross-sectionally and remained stable when examined over time.
In study participants a positive correlation was observed between CMV reactivation and sVL. This effect, however, was not maintained when examined longitudinally. EBV reactivation did not correlate with sVL cross sectionally or longitudinally in study participants.
**Figure 6-3:** 16s rRNA bacterial load positively correlates with semen viral load

As a direct measure of the semen microbiome, 16s rRNA bacterial load was measured and found to correlate cross-sectionally with sVL. This correlation was not maintained when examined longitudinally.
FIGURE 6-4: Representative prospective HIV-1 RNA viral loads in blood and semen
(a) Expected semen viral load approximately 1.3-1.5 log10 lower than blood viral load; (b) undetectable semen viral load maintained despite detectable blood viral load; (c) high-level semen viral load despite low level blood viral load; (d) highly variable semen viral load. — , blood plasma; — , semen plasma
**Figure 6-5:** Longitudinal association between semen IL-8 and sVL. Using a random intercept linear mixed model, levels of the macrophage-associated cytokine, IL-8, strongly correlated with sVL prospectively.
Chapter 7: Discussion & Future Directions
Sexual transmission of HIV with semen as a transmission medium accounts for more than half of the worldwide infections. In 2008, the Swiss Federal Commission for HIV/AIDS released a consensus statement that “HIV-positive individuals on effective antiretroviral therapy and without sexually transmitted infections (STIs) are sexually non-infectious”. This statement was based on the assumption that HIV is eliminated from genital secretions with effective antiretroviral therapy. While effective ART does reduce semen viral load in the majority, this is not always the case. In fact, the conclusions drawn by the Swiss Commission did not account for several confounding possibilities such as; acquisition of a new STI, the differences in transmission likelihood between anal and vaginal sex as well as the actual viral load response in semen and cervicovaginal secretions to antiretroviral therapy. With the likelihood of transmission increasing stepwise with increases in viral load, understanding mucosal correlates that influence semen viral load is an important public health endeavor to guide future treatment strategies. The overarching aim of my doctoral thesis has been to identify factors influencing the variability observed in semen viral load both on and off antiretroviral therapy and possible predictive correlates. Understanding the relationship between blood and semen viral load as well as the impact varying antiretroviral therapy regimens have is of key importance for effective use of ART to reduce future transmission events worldwide.

Semen collection methods have differed in the literature bringing into question whether collection methods may impact proper assessment of viral RNA. In Chapter 3 I investigated whether collection of semen neat (undiluted) or directly into a viral transport medium would impact semen viral load measurements. Upon examination of semen samples collected neat (undiluted), HIV RNA viral load was reduced when compared to that of semen collected directly into viral transport medium. The exact reasons for this discrepancy are unclear, but may relate to the presence of PCR inhibitors present in undiluted semen\textsuperscript{179}, or due to certain immune factors,
enzymes or other cytotoxic effects previously described in the literature\textsuperscript{180-182}. With respect to detectable versus undetectable semen viral load, there was no difference between collection methods whether samples were collected neat or directly into transport medium. It was our hypothesis that this observation may be because collection directly into RPMI medium, while associated with increased semen HIV RNA viral load, is also diluting samples collected by approximately six-fold, thus decreasing our assay limit of detection from 50 to 300 HIV RNA copies/mL. At the second study visit, semen was collected and assayed from semen donated directly into RPMI transport medium 6 months following the original undiluted viral load measurements. While this raises the possibility that higher semen viral load might represent HIV disease progression, the fact that blood viral load and CD4\textsuperscript{+} T-cell count were unchanged suggests this was not the case. While semen HIV concentration may be more variable than blood plasma, as well as inherent variability in all HIV RNA assays in clinical use, neither of these sources would explain our observation that semen HIV VL was consistently higher when collected in transport medium rather than undiluted. When accounting for those patients who spilled their sample at visit two, if we were to assume a volume equal to their first sample, the difference in sVL across the two collection methods remained statistically significant. This initial study suggests the importance of choice of collection method on proper semen viral load measurement, as immediate collection into transport medium was statistically significantly associated with higher semen HIV RNA.

With proper collection technique optimized we were able to explore various aspects of semen shedding including the effects of intensified HAART regimens on both semen shedding and systemic immune activation as well as looking at local immune and clinical correlates that may influence semen viral load in drug naive men. In \textbf{Chapter 4}, I explored whether drug intensification with semen active ARV drugs would reduce semen shedding while on therapy.
(IHS) and impact common clinical parameters. In looking at the effects of drug intensification we examined if drug choice or regimen may underpin isolated semen HIV shedding (IHS). While standard therapy on its own does reduce semen viral loads in the majority of men treated, almost 50% of men continue to shed the virus while on therapy, some intermittently and some persistent. To test whether drug intensification may impact the frequency of IHS, we prospectively followed men receiving both raltegravir and maraviroc in addition to their standard 3-drug regimen prescribed by their physician. Both maraviroc and raltegravir have good penetration into the MGT and this was further confirmed in our study with drug levels far exceeding the levels we observed in blood. It was observed that in men on the intensified regimen, compared to historical standard ART controls, low-level IHS was significantly reduced (<1,000 copies/mL). However, despite this reduction, one participant shed high-levels of semen HIV RNA (>10,000 copies/mL) for 14 months following initiation of iART. While speculative, shedding to this level may indicate that occasional sexual transmission of HIV is still possible despite effective ART, undetectable blood VL and no concurrent-STIs. Upon examination of this individual’s semen HIV, we found no drug resistance mutations or viral evolution during his 14 months of persistent shedding with therapeutic drug levels present in semen. It is possible that the observed virus may have originated from latent mucosal reservoirs without active replication cycles, possibly due to activation of genitourinary immune cells containing integrated HIV DNA. What cell subset may constitute this mucosal reservoir, if one should exist, is not known. This hypothesis would be consistent with previous studies from our lab evidencing compartmentalized T-cell activation in men with IHS\textsuperscript{168}. This theory is further supported by the fact that drugs that act post-integration such as protease inhibitors were found to be at low or undetectable in all individuals examined, with only drugs acting pre-integration exceeding those in blood plasma.
Interestingly, upon examination of men receiving standard ART for varying lengths of time, no IHS was observed beyond three years of effective ART. It is possible that the genital HIV reservoir decreases over time in the absence of ongoing viral replication, an observation consistent with previous work. The high levels of virus found in the high-level isolated shedder may suggest that the mechanism is distinct from low-level HIV RNA “blips” observed in blood, an observation thought to reflect biological and statistical variation around a mean HIV level that falls below the sensitivity of assays currently used in clinical practice. Of note, in our study individuals who were receiving intensified regimens were more likely to achieve and undetectable semen viral load by two weeks, an observation consistent with studies showing delayed semen HIV suppression in efavirenz-based regimen. However, while this observation is interesting, the majority of participants in both treatment groups had achieved an undetectable semen VL within two months, suggesting that any public health benefit in reducing HIV transmission compared to sART is likely to be marginal at best.

Overall our study demonstrated that an intensified drug regimen can reduce but not prevent the occurrence of high-level IHS. Whether standard or intensified, ART substantially reduces the risk of HIV transmission. It is unknown whether semen HIV RNA shedding at the level that was observed in this study may lead to transmission, particularly given the protective effect of ART observed in heterosexual serodiscordant couples. Interestingly, the few transmission events observed in clinical studies of treatment as prevention have occurred soon after therapy initiation, and the 92-96% reduction of HIV transmission events seen in these studies was similar to the 92% reduction in high-level HIV semen shedding we observed during the six month period after starting ART. Therefore, ART in my view does not negate the use of condoms as a preventative measure. In both the HPTN052 and Donnell study, the few transmission events that occurred did so during the early period when our research has shown IHS
to be most likely to occur, implying that the phenomenon may have real world implications.

While my studies found that the frequency of IHS dropped to zero after a longer period of effective ART, other studies have demonstrated persistent IHS despite ART. Therefore, it remains possible that I observed no IHS due to a limited sample size, because participants in our studies had very high levels of ART compliance, or for other reasons.

At the public health level, a decision to forego condom use during anal sex in the context of long-term effective ART should be based on adequately powered observational clinical studies with HIV transmission as an endpoint. Relevant data from MSM HIV-discordant couples was presented at the recent CROI meeting by Mark Mascolini. This study, entitled “Nearly Nil HIV Transmission Risk Without Condoms and With HIV+ Partner on Suppressive ART” included both heterosexual and gay couples showing strong evidence to support that ART use can prevent transmission. In this study of 1110 HIV-discordant European heterosexual and gay couples in which the ART-treated partner had a viral load lower than 200, found no genetically linked HIV transmission events even with partners not using condoms during the observation time analyzed. However, several caveats apply, most importantly the limited data regarding MSM practicing receptive anal intercourse with ejaculation, a group considered to have the most significant risk for HIV transmission. While overall risk of HIV transmission through condomless sex from HIV-positive people on ART with plasma viral load below 200 copies/mL was low as evidenced from the Mascolini study finding within-couple HIV transmission risk of 0 during periods of condom-free sex for any sex act, uncertainty over the upper limit of risk remains, particularly over receptive anal sex with ejaculation. The level of uncertainty decreases as the sample size and duration of follow-up increase, and until the entire confidence interval around transmission risk moves much closer to zero; I believe that condom use is still warranted.
While no clear clinical benefit was observed in the use of an intensified drug regimen mucosally, with respect to IHS, we decided to explore whether any notable reductions in systemic immune activation were observed in men on more than 3 antivirals. In Chapter 5 we examined immune activation, both cellular and soluble, in men on standard 3-drug ART and those men receiving in excess of 3-drugs. We hypothesized that these intensified regimens would provide a greater reduction in systemic immune activation. The ability to reduce systemic immune activation quickly would prove extremely useful in the reduction of several secondary complications associated with delayed resolution of immune activation, such as functional impairment and cardiovascular and neuropsychiatric disease. Immune activation was assessed in study participants with markers of cellular immune activation quantified by flow cytometry and soluble markers assessed through commercially validated ELISAs. We observed no difference between those men receiving standard or intensified therapies by month 6 post-initiation of ART for any of the parameters examined. Upon sub-analysis we noticed increased levels of soluble immune activation parameters in individuals who had maraviroc as part of their treatment. This finding has been previously reported in groups looking at levels of sCD14 in blood and rectal tissue\(^{245}\). While the mechanism by which this occurs is largely speculative, it is an important observation for design of future treatment strategies. We concluded based on these two studies that from a clinical and public health prospective, drug intensification does not provide any advantage over standard therapy in the short-term, following therapy initiation.

While not reducing systemic immune activation in the short-term, it has been suggested in the literature that one potential major benefit to treatment intensification could be a reduction in the size of the latently infected CD4 T-cell reservoir. A study in 2013 showed that intensification with maraviroc or raltegravir (similar to our own study) for 48 weeks in chronically infected patients was able to significantly decrease the HIV reservoir size from 1.1 to 0.0 infectious units.
per million, and even after 24 weeks after discontinuation of the intensification drug the median reservoir size was still significantly lower than at baseline. Furthermore, T-cell activation decreased at 48 weeks and remained low even after discontinuation [327]. From studies like these it is reasonable to hypothesize that the benefits of intensification – while limited in the short-term – may become significant after an increased duration of therapy. We observed a decreased IHS incidence after increasing lengths of therapy, and I hypothesize that this may represent a decrease in the size of genito-urinary HIV reservoirs.

In terms of transmission, blood HIV RNA from an infected individual is the best-defined predictor of transmission in serodiscordant couples [160]. However, as there is minimal contact with blood during sex, this observation is likely reflective of mucosal viral load. A recent study has shown that in fact semen viral load is a stronger independent predictor of transmission than blood with each log_{10} increase in viral load associated with a 1.8-fold increased transmission risk [171]. Many factors have been documented that influence semen viral load, including genital infections both chronic (e.g. CMV) and transient (e.g. gonorrhoea). Even in the absence of concurrent infection, semen viral load varies considerably with some men even shedding more semen HIV RNA than found in their blood plasma [166]. In **Chapter 6**, I investigated various factors that may play a role in semen HIV viral load variability. In this study it was our hope to determine how variable semen viral load was when examined longitudinally and whether there may be any clinical or immunological correlate(s) that would help predict semen viral load. Understanding factors influencing viral load variability has important implications in terms of transmission, treatment and usefulness of blood viral load as a surrogate marker. In this study we observed several important findings. It was noted that there was considerable inter-individual heterogeneity in patterns of semen HIV shedding. Our study showed that only 50% of participants met the “expected” level of semen virus based on their blood viremia. Furthermore, the majority of men
shed HIV RNA at levels higher than their blood viral load or fluctuated between disproportionately high levels and sometimes much lower. These month-to-month differences in sVL stress the importance in prospective studies and predicting HIV infectiousness. Upon examination of mucosal chemokines/cytokines and proteins in the semen of men in the study, it was found that while many parameters correlate cross-sectionally with sVL, only IL-8 remained a stable predictor of sVL prospectively.

Macrophages constitute 20-30% of WBCs in semen, and so high semen IL-8 levels are not surprising as these cells are a major producer of this cytokine. The strong association between IL-8 and sVL in drug naive men both confirmed and extended pre-existing data in the literature. Previous work by our lab72 and others181-183 has shown high levels of IL-8 in semen of HIV+ drug naive men, and a correlation between IL-8 and the sVL72,166,175. In addition, the semen VL has been associated with an increased level of numerous other cytokines, including; G-CSF, TNF-α, IFN-γ, IL-6 and IL-1072,182,328,329. Our work and the work of others have shown that levels of these cytokines themselves are correlated, making it hard to determine independent causation in the context of a cross-sectional study. Specifically, it has not been clear whether the individual cytokine associations with the sVL represent causation, something that perhaps seems unlikely given that several different cytokines have been linked to the sVL72,182,328,329, with different studies measuring different panels of cytokines. Alternatively, the association of different cytokines with the sVL may have reflected a generalized increase in semen cytokines, all of which tend to be inter-related and synergistically exacerbating local immune dynamics. My own work has been able to shed some light on this question. I showed that, while that several cytokines (IL-8, IL-1α, IL-1β, IL-6 and MIP-1β) were associated with the sVL in cross-sectional analysis, the level of IL-8 was the only independent immune association of the sVL in our prospective analysis.
The independent association between semen IL-8 levels and the sVL increases the probability that the relationship is causative, although my observational study cannot prove this, and how the effect is mediated is not clear. Interestingly a similar observation of high levels of IL-8 in lymphoid tissue has even been observed in limited numbers of AIDS patients with a mean viral load of 1,000,000 copies/mL. Examination of lymphoid tissues found increased cellular IL-8 production in these patients with high viral loads advanced disease progression. IL-8 has been further shown to stimulate HIV replication in both T-lymphocytes and macrophages, and compounds that inhibit its action via receptor blockade inhibit HIV replication in both cell types. One important role of IL-8 could be its ability to influence CMV reactivation. Infection with CMV has been shown to induce both IL-8 and CXCR1 (IL-8 receptor) expression. In turn, IL-8 enhances CMV replication in a dose- and time-dependent manner and can attenuate antiviral activity of produced interferon-α. Since many studies, including our own, show a higher HIV sVL during local semen CMV reactivation, this is an intriguing way in which IL-8 might exert an effect on semen HIV levels. Whether the association with increased HIV replication is direct or via CMV reactivation, I believe that the independent association that I found between semen IL-8 levels and the sVL is causative, and reflects IL-8-induced increases in HIV replication.

Further research is needed to more robustly examine the role of IL-8 in HIV replication and to determine whether it can be exploited as a therapeutic target to reduce HIV transmission. It has been shown in various studies that IL-8 has the ability to increase HIV replication in vitro and that inhibition of one of its receptors, CXCR2, decreases susceptibility to HIV infection by as much as 70%. It is reasonable to hypothesize that administration of anti-IL8ab or synthetic inhibitor may provide an efficacious therapeutic supplement to ART worth exploring as a method of reducing mucosal viral load faster in the short term and as a proof-of-concept study showing the impact of IL-8 on HIV viral load. Overall this component of my thesis stressed that a number
of factors may drive sVL variability in ART-naïve men, including T-cell influx, herpesvirus reactivation and local semen microbiome shifts, and suggested that increased levels of IL-8 may act as a “common pathway” to drive these associations.

In conclusion, my doctoral research found several important findings; (1) for accurate semen HIV-1 RNA viral load measurements, semen samples need to be collected directly into transport medium, (2) antiretroviral intensification neither eliminates the occurrence of IHS nor resolves systemic immune activation to a greater degree than conventional regimens, (3) IL-8 as well as blood viral load are the only correlates identified to be predictive of semen viral load and stable when examined longitudinally.

In the following section, several projects have been described in brief as an extension of the findings from the presented work. The goal of these projects is to further the conclusions drawn and determine the precise factors controlling and/or influencing HIV-1 RNA variability in semen.

7.1 The impact of transport media on viral RNA

In Chapter 3 we were able to show that collection into a transport medium has an effect on the measurement of viral RNA, perhaps through preservation of RNA by diluting out endonucleases and PCR inhibitors at the time of collection. One limitation of this study was a robust look at various available media for collection and storage conditions prior to processing and measurement of viral RNA. One potential future study could assess the effects of different transport media on viral RNA and perhaps downstream infectivity experiments. A panel of media would be chosen based on media currently being used in the literature, these would include the previously assessed RPMI 1640, along with other transport media currently including; Hank’s balanced salt solution (HBSS), phosphate buffered saline (PBS) and Copan’s viral transport
medium (VTM) to cite a few examples, many others are commercially available for testing. All buffers would be supplemented with L-glutamine and penicillin/streptomycin (as described in Chapter 3) and inoculated separately with HIV RNA in either high or low known dilutions, to represent the range of viral titers likely to be seen in semen samples from HIV+ ART naive men. HIV RNA would be assayed by RT-PCR to identify which media is superior in maintaining the integrity of viral samples for a positive diagnostic outcome. In addition to transport media and important extension of this work would be to assess the impact various storage temperatures have on RNA measurements. This is an important extension of the work as samples may experience changes in temperature from collection in clinic to arrival at the lab for processing. Following inoculation with virus (in triplicates), samples would be distributed across three separate temperature conditions, one at room temperature, one packaged with an ice pack and another refrigerated at 4°C. Samples would be left at the various conditions for 2 hours, processed and frozen at -80°C in equal volumes to mimic actual conditions of collection, processing and storage prior to viral RNA measurements. Samples would then be sent for viral load measurement as per our clinical studies to determine if in fact media choice and storage conditions prior to processing significantly impact viral RNA. As a supplement to this, frozen aliquots of the various conditions could be used to determine whether media and condition have any impact on the ability to use the sample for downstream infectivity assays. It is possible that media choice may directly impact viral integrity and influence results gleaned from downstream infectivity assays. While there is a chance we would see similar results with samples inoculated with high titrated virus, differences between conditions on RNA measurement and infectivity may be seen largely in samples with low viral titers and have important implications for future studies assessing the infectivity of low semen HIV RNA in clinical sample. The two best media and conditions may further benefit from a comparative collection of real clinical samples in a group of 30 men or more providing two
samples, 1 week apart, each visit into a different media with viral RNA compared in a similar fashion to Chapter 3.

7.2 Isolated HIV Semen Shedding and the Microbiome

Results generated from the examination of drug intensification with semen active ARVs showed that while sVL declines more rapidly, the phenomenon of IHS is not resolved. While low-level IHS is reduced, high-level IHS can persist in some individuals to potentially infectious levels (>5,000 copies/mL) for up to a year post-effective ART. Previous work by our lab evidenced compartmentalized immune activation highly reminiscent of the immune changes in the female genital tract observed during bacterial, parasitic or viral co-infections\textsuperscript{172,333-335}. Standard co-infection diagnostics has found no evidence of urethral infection by gonorrhoea or chlamydia nor any associations with CMV and HSV-2 reactivation. This evidence suggests that the cause of IHS may be microbial, albeit not a classical STI. Furthermore it has been noted in the literature that men who engage in unprotected insertive sex have significantly higher semen viral loads when drug naive\textsuperscript{180} and have more frequently detected semen virus when on ART\textsuperscript{130}. These observations could suggest acquisition of non-pathogenic bacteria possibly exerting an impact on HIV replication. Recent collaborations with the TGen Centre for Human Health and Microbiomics has allowed us to begin to explore the semen microbiome. A paper characterizing the HIV negative and positive semen microbiome has already been submitted for peer review. This study has assessed the impact HIV has on the microbiome, changes to the microbiome in the short term folling initiation of antiretroviral therapy and associations between the microbiome and semen viral load. Comparative analysis between the microbiome of someone with persistant IHS versus someone with complete suppression may help delinate wether a microbial cause is underpinning the observations and frequency of IHS. Such findings would allow for the
development of targeted therapies to supplement conventional ART to help reduce the possibility of semen shedding while on therapy during the first year of ART.

7.3 Assessment of longitudinal shifts in the semen microbiome

Data gathered from my work investigating correlates of sVL shedding in ART-naive men revealed that bacterial load, a direct measurement of the microbiome was positively correlated with sVL. While this association was lost when examined prospectively, many possible explanations could account for this, including, subtle shifts in dominant microbial species, changes in overall diversity and other yet to be determined factors. Seminal plasma collected during this study has been sent to the TGen Centre for Microbiomics and Human Health with seminal plasma RNA/DNA extracted and sequencing of the microbiome already underway. Prospective analysis of the microbiome will allow us for the first time to determine how stable the microbiome is over time and whether different families or species of bacteria shift in dominance. It may be that during periods of species overgrowth a direct impact on viral load is observed. Findings such of this would not be captured in an overall bacterial load assessment and require species-specific data and associations with their respective viral loads. This data will allow us to determine whether sVL and to a further extent, other semen parameters examined in the previous study, have any relationship with the state of microbiome. This knowledge would help not only in our understanding of semen HIV-1 variability but also as to whether targeted therapies and/or microbicides could be developed in an effort to decrease semen viral load and the risk of transmission events.

7.4 Investigation into mucosal macrophages

Results from the examination of chronic HIV infected drug naive men found that IL-8 and several macrophage-associated factors were the strongest correlate of semen viral load. semen
levels of IL-8, bacterial load, CMV viral load, IL-1α, IL-1β, IL-6, and MIP-1β were all found correlated cross-sectionally with sVL IL-8, a macrophage associated cytokine, was found to be the strongest independent predictor of semen viral load aside from bVL suggestive a common pathway linked to semen HIV levels in therapy naive men. Despite these and other similar observations in the literature, semen macrophages and other sources of IL-8 in the MGT have not been well characterized. Macrophages are terminally differentiated, non-dividing cells, derived from monocytes and capable of being infected by or harbouring HIV\textsuperscript{336}. In semen, macrophages make up 20-30% of the total cell population with T-lymphocytes constituting only 5% of resident cell populations\textsuperscript{109,337,338}. Analysis of the cellular fraction of semen and immunocytochemical and in situ molecular investigations of human and nonhuman primate tissues suggest that cells of the macrophage lineage may be an important source of virus in the male genital tract\textsuperscript{339,340}. Recent studies confirm that macrophages and dendritic cells are able to support high-level HIV-1 replication under certain conditions, especially in the presence of immune activation\textsuperscript{341-343}. If HIV-1 in the genital tract were to originate from relatively long-lived macrophages, the dynamics of local viral production and clearance might differ significantly from those in systemic lymphoid tissues. This may influence both short- and long-term effects of antiviral regimens on HIV levels in the semen, as well as transmission events. Analysis of semen macrophages through multi-parameter flow cytometry will allow not only the phenotyping of these resident macrophages but also determine how and to what extent they differ from blood macrophages in both HIV- and HIV+ individuals. Furthermore, I propose an evaluation of IL-8, a macrophage associated cytokine, to determine its effect on HIV replication and to quantify the relative contribution from semen lymphocytes, macrophages, neutrophils and epithelial cells. In vitro exploration into the impact IL-8 has on HIV replication in the MGT may help provide evidence to determine whether an anti-IL8 antibody if administered would decrease HIV replication, providing evidence of a
common pathway between HIV levels and IL-8. Such a therapy may help reduce the occurrence of IHS and achieve undetectable viremia sooner in the short-term, thereby reducing the risk of transmission following initiation of ART. To determine the effect IL-8 has on HIV replication, an *in vitro* model must be set up using semen mononuclear cells. Following isolation of lymphocytes from HIV-seronegative donors by ficoll-gradient centrifugation, lymphocytes should be plated in 96-well V-bottom plates in the presence of IL-2, CD3/CD28 with RPMI culture medium, until sufficient cell numbers have been cultured. Following culture, CD4 T-cells should be isolated using selective depletion methods and infected with a subtype-B laboratory derived HIV clone available from the National Institutes of Health (NIH) as described previously. This method of infection should routinely yield 15-35% HIV-antigen positive cells (detected by immunofluorescence) at peak replication in 5-7 days. Following infection, cells should be cultured in the presence or absence of IL-8, along with IL-2 and RPMI culture medium for an additional 15 days. Following cytokine culture, supernatant should be harvested and reverse transcriptase activity measured using commercially available diagnostic kits. A ratio of RT activity between cultures with and without IL-8 will help determine whether presence of the cytokine may inhibit or increase HIV replication in semen CD4+ T-cells. Increased levels of reverse transcriptase activity will determine whether further investigation into the effect an anti-IL8 antibody or other small-molecule inhibitors have on viral load in vivo is warranted.

### 7.5 Sexual behaviour and the impact on the semen microbiome

The microbiome is defined as the collective genomes of the microbes (bacteria, bacteriophage, fungi, protozoa) that live within our body. Studies investigating the impact of fertility on the microbiome have documented a plethora of bacterial species in semen samples
with gram-positive anaerobic cocci and *Corynebacterium spp*\textsuperscript{317} dominating. My research has shown that bacterial load, a direct measure of the semen microbiome, is significantly correlated with semen viral load in a cross-sectional study format (Reviewed in Chapter 5). In a study by Kalichman et al. (2001) it was noted that men who had higher semen viral loads relative to their plasma viral load were distinguished by having engaged in significantly higher rates of unprotected sex, either anally or vaginally\textsuperscript{180,320}. I hypothesize that unprotected sex is leading to the colonization of the MGT by various non-pathogenic bacteria that naturally colonize the vaginal tract/sigmoid colon, and its presence in the male genital tract is driving immune activation and HIV replication of the insertive partner, similar to the findings of STIs such as gonococcal urethritis increasing sVL\textsuperscript{188}. To better define the impact of perturbations of the semen microbiome as it relates to semen viral load, I propose the recruitment of HIV negative MSM controls and HIV+ drug naive MSM. All study participants should have paired blood and semen samples collected along with penile and perianal swabs collected for microbiome analysis to help determine possible anatomical microbiome differences. Sexual risk questionnaires should be administered to gather data related to sexual preference, frequency of intercourse, insertive vs. receptive sex, circumcision status and frequency of unprotected sex. This data taken together will provide a powerful dataset that will allow one to relate the occurrence and abundance of bacterial species with the kinds of sexual practices participants have engaged in, while clearly defining the semen microbiome and the impact of HIV. Identification of a particular family of bacteria would allow for us to identify possible targeted treatment options that could be administered either prior to- or with the initiation of antiretroviral therapy to help reduce semen viral and lower the risk of transmission.
7.6 Investigation into the semen proteome and the effects on low-level semen shedding

By definition, semen is a complex mixture of spermatozoa and fluid secretions from the testes and accessory sex glands. Proteomics provides a useful and powerful approach to elucidate novel mucosal factors associated with semen HIV-1 RNA shedding. Human semen has an approximate average protein concentration of 35-55 g/L, representing 923 proteins. Proteomics work in the field of reproductive biology has already made huge leaps in defining the proteome. For instance, in HIV negative individuals, lactoferrin (seminal vesicle protein) is found in high abundance and is known for its antimicrobial properties and ability to inhibit HIV replication in vitro. Proteomics have been further used in several studies to identify novel anti-HIV inhibitory proteins such as Trappin-2/Elafin. In a study of 500 HIV resistant Nairobi sex workers and low risk women, elevated levels of Trappin-2 were found up-regulated in resistant women and shown to have anti-HIV properties in vitro. For this study I propose a small pilot study to investigate the impact of HIV infection on the semen proteome and possible proteomic differences between high and low semen HIV-1 RNA shedding and factors responsible for low-level replication.

Three groups of men including HIV Negative MSM controls, HIV+ drug naive low shedders (<5,000 copies/mL) and HIV+ drug naive high shedders (>5,000 copies/mL) controls would serve as a pilot group to isolate proteins which may be differently regulated between high and low shedding patterns. Once proteomic differences are determined between groups in vitro characterization of the proteins can be done if not currently documented in the literature. In vitro infectivity assays (As previously described) would be important steps in determining what affects the identified proteins have on HIV replication and T-cell susceptibility. Identification of novel proteins in the MGT correlating with reduced viral loads and anti-HIV properties may provide a
unique target for the development therapeutics or microbicide against HIV-1 mucosal transmission.

### 7.7 Research Applications

The research conducted in my doctoral thesis has many implications to our understanding of semen HIV, treatment strategies and public health. Isolated HIV shedding identified in both men and women while on effective antiretroviral therapy may help partially explain transmission events that have been observed in clinical trials occurring during the first year of therapy and one potential explanation as to why effective antiretroviral therapy does not completely prevent HIV transmission in the short term. Our previous work had shown that certain antiretroviral compounds penetrate into the male genital tract to varying degrees with classes such as protease inhibitors being detected at low frequency if even at all. Lack of drug penetration was thought initially by us to underpin the phenomenon of IHS. We hypothesized that not only could poor drug penetration potentially influence IHS but could also could select for HIV drug-resistant strains to actively replicate in the genital tract, potentially leading to virologic failure, something that would not be picked up by routine clinical blood work. Standard care for individuals living with HIV measures blood plasma viral load regularly (i.e., every three months), but does not measure genital viral load. As a result of this, it is not clear if being on HAART will reduce HIV transmission among all serodiscordant sexual partners. It is because of these reasons that it is important to understand the immunological factors that influence semen viral load and what ARV compounds penetrate to therapeutic levels in mucosal secretions. We observed a decreased frequency of low-level IHS in our study of men receiving intensified ART regimens, however, high-level IHS still persisted for up to 14 months in one individual. Data collected from men on varying lengths of HAART suggested that with longer duration of standard therapy, the
phenomenon of IHS dissipates, suggestive of a transient phenomenon. This knowledge is important for any newly treated HIV-positive individual as they may potentially carry a transmission risk despite undetectable blood viral load. It has been reported in one study that the rectal mucosa contains higher HIV viral loads than both blood and semen of infected men\textsuperscript{266}. Due to only a thin epithelium providing protection during anal sex, it has been suggested that the infectious titer required for a productive infection in MSM is lower than vaginal intercourse\textsuperscript{266}. As the rectal mucosal also typically carries higher viral loads, an uninfected insertive partner is at particular risk with the urethra potentially serving as a primary HIV infection site, even with low but detectable viral loads\textsuperscript{266,282}. These findings stress the importance of counseling and safe sex practices such as condom use as a means of protecting their serodiscordant sexual partner(s) in the case they are unknowingly shedding HIV in their semen or rectal secretions. One question that has been raised by many groups is whether “more drugs is better” for treatment of HIV. Our study investigating the impact of iART on immune activation and clinical parameters revealed that intensification showed no clinical benefit in terms of reducing systemic immune activation, increasing CD4 T-cell count or preventing IHS. The efficacy of a standard ART regimen was re-established as effective means of viral control and suppression of systemic activation. This finding is of particular importance as there has been much work done over the last few years determining whether intensification would lead to better long term prognosis and decreased HIV viral reservoir sizes. With access to HIV ARV compounds and cost being a large factor in worldwide treatment strategies, knowing the most efficacious treatment is important in establishing public health programs to reduce HIV, especially in low-income developing countries where access to a full arsenal of ARV would be limited. While developed countries have access to a wide range of ARV compounds at their disposal, it is important for treating physicians to factor into their equation whether or not the ARV prescribed will in fact penetrate into the mucosal
surfaces of the HIV positive individual. In the absence of routine clinical assays capable of predicting mucosal viral load, choice of appropriate ARV may be important for the individual’s in reducing the transmission likelihood in an infected individual and suppressing compartmentalized virus.

One observation made in the literature routinely is that of the heterogeneity of semen viral loads observed and the impact various factors have on compartmentalized virus relative to blood. We were particularly interested in understanding factors that may in fact be directly influencing semen viral load that could potentially be a future target for therapeutic intervention. In our study of antiretroviral therapy naive men, two observations were found of particular importance. The first was a correlation between semen bacterial load and HIV viral load. We observed that with increasing levels of bacteria (a direct measure of the microbiome), semen HIV virus levels increased. It is our hope that future work will be able to accurately define the semen microbiome and delineate whether certain species or genera of bacterium correlate directly with semen HIV viral load (Refer to section 7.2 and 7.4). If such a finding were to be documented, novel avenues for control of mucosal viral load would open. Clinical trials would be able to investigate whether targeted antibiotic compounds to reduce levels of various families of bacterium could lower the semen viral load, a potential supplementary treatment to current ART practices. Studies looking at tooth decay (dental caries) have explored similar ideas by investigating whether specifically-targeted antimicrobial peptides (STAMPs) could eliminate *Streptococcus mutans*, a bacterium found to be responsible for tooth decay in the oral cavity. STAMPs consist of two functionally independent moieties conjoined in a linear peptide sequence: a non-specific antimicrobial peptide serves as the killing moiety, while a species-specific binding peptide comprises the targeting moiety that provides specific binding to the selected bacterium and facilitates delivery of the antimicrobial peptide. Should our microbiomics assessment of semen from drug-naive men
reveal particular families driving viral load, we may theoretically be able to develop a STAMP for use in newly diagnosed HIV positive individuals as a prophylactic means of eliminating bacterium that increase viral load. This form of treatment could potentially aid in reducing the occurrence of transmission events during the first year of therapy when the risk of shedding HIV in the semen is greatest, as seen our studies of IHS. Another important finding from our study was the strong correlation of IL-8, a macrophage associated cytokine with semen viral load both cross-sectionally and prospectively and potentially involved in the mucosal replication pathway. IL-8 has been implicated as a target in many disease areas with clinical trials already examining the safety and tolerability of a fully humanized monoclonal antibody recognizing IL-8 and small molecule antagonists of the IL-8 receptor CXCR2 and CXCR1. Knowing that these molecules are being utilized and found to be well tolerated and safe in humans opens up the opportunity for studies looking at the impact inhibition of IL-8 in HIV+ individuals and the effect it has on mucosal HIV viral load. In 7.3 I have outlined in-vitro studies to characterize the source of IL-8 and its impact on HIV replication. This first study would provide data as to what extent IL-8 increases (or does not effect) HIV viral load and to whether inhibition would impact viral kinetics. With safety studies completed in humans, should inhibition of IL-8 be found to reduce HIV in vitro, small pilot studies could be initiated; looking at the impact an anti-IL8 monoclonal antibody or small molecule antagonist would have on reducing semen viral load. If proven efficacious this could potentially provide another form of supplementary treatment for individual’s initiating an antiretroviral therapy regimen as of means of suppressing mucosal viral load, especially in treatment circumstances where ARV with high mucosal penetration is limited due to access. It is my hope that the results gleaned from my research will provide future treatment options, aside from ART, as a supplementary means of reducing mucosal viral load, and as a direct impact, reducing the risk of transmission events.
The field of HIV prevention is one that is rapidly evolving with an ever-changing ART formulary. For instance, once a month long-acting injectable antiretroviral combinations are already in phase II clinical trials\textsuperscript{352}. Such an advance in ART would help not only improve adherence to therapy but also potentially extend opportunities for therapeutic or prophylactic intervention to underserved patient populations. As new prophylactic treatments and ART enter the market we hopefully will begin to see decreases in HIV incidence in Canada. Long-lasting effective ART and safe sex practices have huge potential to reduce transmission. It is important that even in the wake of advances in HIV treatment, that safe sex through condom use is always being encouraged. It is our hope the research presented will aid in the development of novel treatment options for people living with HIV and help reduce future transmission events both locally and abroad.
Chapter 8: References


75. Liu, Z., et al. Elevated CD38 antigen expression on CD8+ T cells is a stronger marker for the risk of chronic HIV disease progression to AIDS and death in the Multicenter AIDS Cohort Study than CD4+ cell count, soluble immune activation markers, or combinations of HLA-DR and CD38 expression. *Journal of acquired immune deficiency syndromes and human retrovirology: official publication of the International Retrovirology Association* 16, 83-92 (1997).


