HIV-1 Group M Subtype Fitness, Disease Progression, and Entry Efficiency

Colin M. Venner
The University of Western Ontario

Supervisor
Arts, Eric J.
The University of Western Ontario

Graduate Program in Microbiology and Immunology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
© Colin M. Venner 2019

Follow this and additional works at: https://ir.lib.uwo.ca/etd

Part of the Epidemiology Commons, Immune System Diseases Commons, Immunology of Infectious Disease Commons, Laboratory and Basic Science Research Commons, Virology Commons, and the Virus Diseases Commons

Recommended Citation
https://ir.lib.uwo.ca/etd/6179

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlswadmin@uwo.ca.
Abstract

Human immunodeficiency virus type 1 (HIV-1) emerged in the human population shortly after the turn of the 19th century. Distribution of HIV-1 across the globe over the past 30–35 years can be traced to founder events with primordial HIV strains from sub-Saharan Africa. Even considering the burden of HIV in Africa, our knowledge of HIV-1 disease is still largely limited to subtype B HIV-1, a strain responsible for 3 million infections in North America and Europe as compared to the 33 million that are infected with HIV-1 subtypes A, C, D, and circulating and unique recombinant forms.

This dissertation analyzes data and archived samples from a cohort of HIV+ women in Uganda and Zimbabwe to assess the role of HIV-1 Group M subtype in disease progression, viral replicative fitness and cellular entry efficiency. Generalized estimating equation models are employed to calculate average rates of CD4+ T-cell loss over the course of disease, investigate circulating viral load, and compare depletion of memory T-cell subsets in CD4+ and CD8+ T-cells. Fluorescent tissue culture assays of HIV-1 entry were used to phenotypically evaluate entry efficiency and its association with replicative fitness.

Ugandan and Zimbabwean women infected with HIV-1 subtype C had 2.5-fold slower rates of CD4 T-cell declines and higher frequencies of long-term non-progression than those infected with subtype A or D, a difference not associated with any other clinical parameters. Relative replicative fitness and entry efficiency of HIV-1 variants directly correlated with virulence in the patients, subtype D > A > C. These relationships were maintained in both assays evaluating HIV-induced cell-fusion rates, viral particle entry rates and CD4 receptor affinity.

The primary burden of HIV infection is placed on T-cell memory populations that are focal points for progeny virion production, the latent proviral reservoir, and immune activation. All subtypes depleted effector memory T-cells preferentially, however subtype D infections lost these cells at about twice the rate of subtype C infections. Finally, subtype D was found to uniquely deplete CD8+ memory T-cells.
Keywords

HIV, Pathogenesis, Viral Diversity, Group, Subtype, SIV, Viral Entry, Fitness, Viral Load, Uganda, Zimbabwe, Africa, Binding Kinetics, Memory T-cell Subset, Linear Modelling

Co-Authorship Statement

Chapter 2

Drs. Tsungai Chipato, Josaphat Byamugisha, Peter Mugyenyi, Robert A. Salata, Charles S. Morrison, Sandra Rwambuya, Eric J. Arts, and Cynthia Kwok designed the clinical study, directed the recruitment of patients, and maintained the human ethics approvals. Dr. Arts directed and supervised all the analyses presented herein especially those related to replicative fitness. Dr. Morrison was the overall PI of the GS study with Dr. Chipato as site PI in Zimbabwe; Drs. Byamugisha, Mugyenyi, and Salata as site PIs in Uganda, and Dr. Barbara Van der Pol as the laboratory consultant for both sites. Drs. Korey Demers, Immaculate Nankya, and Fred Kyeyune coordinated the clinical assays in Uganda and collaborated with Dr. Marshall Munjoma who did the same in Zimbabwe. Dr. Pai-Lien Chen validated all the statistical analyses. Dr. Kwok maintained the clinical databases. Drs. Nankya, Kyeyune, and Demers processed all the clinical samples on site and performed initial subtyping.

Colin Venner performed the cloning for HIV isolates, generated a working database for disease progression analyses and viral load studies. Colin also performed all the replicative fitness assays, entry/fusion assays, designed and conducted the CD4 binding experiments, and analyzed the clinical data. Colin and Dr. Arts wrote the manuscript with editing by all the other authors.

Chapter 3

Dr Immaculate Nankya and Eva Nabulime stained and ran the archived samples on the flow cytometer and extracted cell population frequencies.

Colin Venner calculated circulating cell numbers and analyzed the clinical data. Colin also designed and analyzed the Kaplan Meier data. Finally, Colin wrote the manuscript with editing by Dr. Eric Arts.
Appendix A

Dr. Sharon Koivu, Dr. Michael Silverman and Klajdi Puka generated the hypotheses presented. Dr. Silverman, Ryan Wong, Laura Ball, and Brian Hallam designed and conducted the interviews for the case-control study. Dr. Silverman, Dr. Mark Speechley, Laura Ball, and Klajdi Puka analyzed the case control data and calculated hazard ratios. Dr. Rommel Tirona and Laura Ball measured opioid persistence in intravenous drug preparation equipment.

Colin Venner designed and performed all HIV experiments, both for persistence in the presence of drug as well as interventions. Colin also made the figures. Colin, with Dr. Silverman, Dr. Eric Arts, Dr. Sharon Koivu and Laura Ball wrote the manuscript. All authors reviewed and approved the final manuscript.

Introduction and Discussion

Colin Venner wrote and edited all other parts of this dissertation. Additional editing was provided by Drs. Jimmy Dikeakos and Eric Arts.
Acknowledgments

Thank you to all the participants in the studies presented here. Thank you for seeing the value in this research even during a time of extreme personal difficulty. Thank you to members of the HIV research community who have provided comments, questions, and feedback at scientific conferences. Even while disagreeing, I have always been treated with respect and as a valued part of the field.

I would also like to thank my teachers, employers, mentors, and committee members: Drs. Andrew McCall, Nora Underwood, Brian Inouye, Sudha Iyengar, Kewal Asosingh, Serpil Erzurum, Jimmy Dikeakos and Greg Dekaban. Everything I know about research science I learned from you. The number of things I learned studying Ecology at Denison University and Florida State that I utilized during this research shocked me, especially given my lack of appreciation at the time. Not everything I learned was easy, or directly applicable, but taught me resilience, analytical thought, and the value of a positive attitude. As Dr. Asosingh once said, “Ice cream cake is full of protein, it’s healthy”.

In a category of his own, I want to thank Dr. Eric Arts. You trusted me enough to take me on as a student, gave me a direction and trusted me again to work largely independently. Your faith in me outlasted my own on more than a few occasions, your advice was insightful, and your knack for experimental design were the sources of many of my successes.

Finally, I want to thank my friends and my family. I haven’t always been easy or happy during the last 5 years, but you were always supportive. Katie Bain and Arad Moghadasi provided invaluable uplift in times of turmoil for which I’ll always be grateful. My parents, Heidi and the girls, thanks for keeping me grounded every Monday night, at 9pm EST. To my brother Eric, thanks for blazing a trail.
Table of Contents

Abstract .................................................................................................................................................. ii
Co-Authorship Statement ................................................................................................................ iii
Chapter 2 ........................................................................................................................................... iii
Chapter 3 ........................................................................................................................................... iii
Appendix A ........................................................................................................................................ iv
Introduction and Discussion ........................................................................................................... iv
Acknowledgments ............................................................................................................................ v
Table of Contents ............................................................................................................................. vi
List of Figures .................................................................................................................................... ix
Chapter 1 .......................................................................................................................................... xii
Chapter 2 .......................................................................................................................................... xii
Chapter 3 .......................................................................................................................................... xiii
Appendix A ....................................................................................................................................... xiii
List of Appendices ............................................................................................................................. xv
Abbreviations .................................................................................................................................... xvi

Chapter 1

Introduction .......................................................................................................................................... 1
1.1. The Cut Hunter Hypothesis .................................................................................................... 1
1.2. Alternative Theories ................................................................................................................ 3
1.3. SIV Diversity and Pathogenesis ............................................................................................ 4
1.4. HIV Diversity .......................................................................................................................... 7
Chapter 2

Infected HIV-1 Subtype Predicts Disease Progression in Women of Sub-Saharan Africa.
Chapter 3

Results

3.3.1 Analyses of CD4+ and CD8+ T-cell Subsets During HIV-1 Disease .......................................................................................... 99

3.3.2 HIV Subtype Impacts Depletion of CD4+ and CD8+ Populations ..................................................................................................... 100

3.3.3 Study Unenrollment Agrees With Other Pathogenicity Measurements ......................................................................................... 105

Discussion

3.4.1 Preferential Depletion of Effector Memory T-cells During HIV Infection ........................................................................................... 109

3.4.2 Implications............................................................................... 110

References .............................................................................................. 114

Chapter 4

Discussion .............................................................................................. 119

4.1 Viral Fitness, Disease Progression, and CD4 Binding Affinity........... 119

4.2 Subtype C Epidemic Spread ............................................................ 121

4.3 GS Study Cohort ............................................................................... 122

4.4 Future Directions ............................................................................... 123

4.5 Implications ...................................................................................... 126

4.5.1 Latency .................................................................................. 126

4.5.2 HIV Cure ............................................................................... 126

4.5.3 HIV Policy ............................................................................... 128

4.6 Concluding Remarks ........................................................................ 128
Appendix A

Sharing of Equipment Used for the Preparation of a Controlled-Release Oral Opiate for Injection is Associated with HIV Transmission

5.1 Preface

5.2 Abstract

5.3 Introduction

5.4 Methods

5.4.1 Setting

5.4.2 Association Between Sharing IDPE and HIV Infection

5.4.3 Power Calculation

5.4.4 Residual Hydromorphone in IDPE & Effects of Heating on the Amount of Hydromorphone Injected

5.4.5 HIV Persistence in IDPE & Effects of Heating on HIV

5.4.6 Community Interventions

5.5 Results

5.5.1 Association Between Sharing IDPE and HIV Infection

5.5.2 Residual Hydromorphone in IDPE & Effects of Heating on the Amount of Hydromorphone Injected

5.5.3 HIV Persistence in IDPE & Effects of Heating on Persistence

5.6 Discussion

5.6.1 Association Between Sharing IDPE and HIV Infection

5.6.2 Residual Hydromorphone in IDPE

5.6.3 HIV Persistence in IDPE

5.6.4 Harm Reduction Implications

5.6.5 HIV Transmission in Other Regions
5.6.6 Study Limitations ................................................................. 159

5.7 Conclusion ................................................................................. 160

5.8 Acknowledgements .................................................................. 160

5.9 References ................................................................................ 162

Curriculum Vitae ............................................................................ 165
List of Figures

Chapter 1

Figure 1.1 – SIV Origins of HIV Infections and Relative Pathogenicity ........................................5
Figure 1.2 – HIV-1 Genome, Viral Particle and Env Glycoprotein ......................................... 14
Figure 1.3 – Schematic of HIV Entry ..................................................................................... 17
Figure 1.4 – Schematic HIV Replication............................................................................... 23
Figure 1.5 – CD4+ T-Cell and Viral Load Dynamics During HIV Disease Progression ....... 31
Figure 1.6 – Relative Fitness of Group M Subtypes............................................................... 36
Figure 1.7 – HIV-1 Group M, Group O and HIV-2 Fitness .................................................... 37

Chapter 2

Figure 2.1 – Global HIV-1 Group M Subtype Distribution and Changes, 1990-2015......... 65
Figure 2.2 – Analysis of Disease Progression by Country and Infecting Subtype ............... 71
Figure 2.3 – CD4+ T-cell Counts and Viral Load Progressions from GS Study ................. 73
Figure 2.4 – Replicative Fitness of Samples from GS Study ................................................. 75
Figure 2.5 – Cell-to-cell Transmission and Viral Entry is Impacted by Subtype ............... 78
Figure 2.6 - CD4 Binding Strength via Competitive ELISA Between HIV-1 Subtypes ...... 79
Supplementary Figure 2.1 – Follow-up Time to cART Initiation by Country and Subtype .. 80
Supplementary Figure 2.2 – Total CD4 T-Cell Counts by Country ........................................ 81
Supplementary Figure 2.3 – Total Viral Loads by Subtype and Country ............................ 82
Supplementary Figure 2.4 – Subtype Classifications from Env Sequencing ......................... 83
Supplementary Figure 2.5 – Subtype Classifications from PR and RT Sequencing.............. 84
Supplementary Figure 2.6 – CD4 Competitive ELISA Reverse Transcriptase Blot............. 85

Chapter 3

Figure 3.1 – Flow Cytometry Gating Strategy for CD4+ and CD8+ T-Cell Subsets......... 95
Figure 3.2 – CD4+ T-Cell Subset Depletion by Group M Subtype ................................. 98
Figure 3.3 – CD8+ T-Cell Subset Depletion by Group M Subtype ............................... 102
Figure 3.4 – CD4+ and CD8+ T-Cell Counts from GS Study.................................... 103
Figure 3.5 - Correlations Between CD4+ and CD8+ Memory T-Cell Counts............. 106
Figure 3.6 – Kaplan Meier Analysis for Patient Enrollment in the GS Study............ 107
Supplementary Figure 3.1 – CD4+ Central Memory Dynamics ............................... 111
Supplementary Figure 3.2 – CD8+ T-Cell Activation by HIV Subtype .................... 112
Supplementary Figure 3.3 – Viral Load Dynamics by Subtype Over Infection......... 113

Appendix A

Figure 5.1 – HIV Infections in Middlesex-London and Ontario, 2005-2017............... 142
Figure 5.2 – Method for Preparing Hydromorphone Controlled-Release for Injection .... 144
Table 5.1 – Characteristics of HIV+/− Persons who Inject Drugs .......................... 147
Table 5.2 – Odds Ratio of HIV+ Status with Injecting Behavior and Risk Factor Variables ................................................................................................................................ 149
Figure 5.3 – Opioid Recovered from Syringe with and without Heating............... 151
Figure 5.4 – Infectious HIV Persistence in Injection Drug Preparation Equipment .......... 153

Figure 5.5 – Reduction in Infectious HIV-1 after 10 Second Heat Treatment .................. 154

Supplementary Figure 5.1 – Infectious HIV in Filters and Opiate Preparations ............... 161
List of Appendices

Appendix A: Sharing of Equipment Used for the Preparation of a Controlled-Release Oral Opiate for Injection is Associated with HIV Transmission………………………………...136
Abbreviations

[α-32P] dTTP  32-Phosphorus labeled deoxy-thymidine triphosphate
Δ32  32 bp deletion in CCR5
Ψ-site  HIV major packaging signal
5-FOA  5-Fluoroorotic Acid
AHI  Acute HIV Infection
AIDS  Acquired immunodeficiency syndrome
ANOVA  Analysis of Variance test
aOR  Adjusted odds ratio
APC  Allophycocyanin
ART  Anti-retroviral therapy
ATCC  American Type Culture Collection
B13  Monoclonal antibody with a linear CD4bs epitope
B-K44  Lab-adapted subtype B strain of HIV-1
BlaM  E. coli β-lactamase
bp  Base pair
B-Q0  Lab-adapted subtype B strain of HIV-1
BSL  Biosafety Level
C# (1-5)  Conserved regions of gp120
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA</td>
<td>Capsid or p24</td>
</tr>
<tr>
<td>cART</td>
<td>Combination antiretroviral therapy</td>
</tr>
<tr>
<td>CCR5</td>
<td>C-C chemokine receptor type 5</td>
</tr>
<tr>
<td>CCR7</td>
<td>C-C chemokine receptor type 7</td>
</tr>
<tr>
<td>CD38</td>
<td>Cyclic ADP ribose hydrolase</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of differentiation 4</td>
</tr>
<tr>
<td>CD45R0</td>
<td>Protein tyrosine phosphatase, receptor type, C, isotype R0</td>
</tr>
<tr>
<td>CD4bs</td>
<td>CD4 binding site of gp120</td>
</tr>
<tr>
<td>CD8</td>
<td>Cluster of differentiation 8</td>
</tr>
<tr>
<td>CDC</td>
<td>U.S. Centers for Disease Control</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>COC</td>
<td>Combined oral contraceptives</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effects</td>
</tr>
<tr>
<td>CRF</td>
<td>Circulating recombinant form</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T-lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-Lymphocyte Associated Protein 4</td>
</tr>
<tr>
<td>CXCR4</td>
<td>C-X-C chemokine receptor type 4</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DMPA</td>
<td>Depomedroxyprogesterone acetate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's phosphate-buffered saline</td>
</tr>
<tr>
<td>DRC</td>
<td>Democratic Republic of the Congo</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded DNA</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EFV</td>
<td>Enfuvirtide (or T-20)</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Env</td>
<td>Envelope glycoprotein</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ESCRT-I</td>
<td>Endosomal sorting complexes required for transport 1</td>
</tr>
<tr>
<td>FACS</td>
<td>Flow-assisted cell sorting</td>
</tr>
<tr>
<td>Fas</td>
<td>Apoptosis antigen 1</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>GEE</td>
<td>Generalized Estimating Equation</td>
</tr>
<tr>
<td>Gp120</td>
<td>Envelope glycoprotein with mass of 120 kDa</td>
</tr>
<tr>
<td>Gp160</td>
<td>Uncleaved precursor of gp120 and gp41</td>
</tr>
<tr>
<td>Gp41</td>
<td>Envelope glycoprotein with mass of 41kDa</td>
</tr>
<tr>
<td>GS</td>
<td>Genital Shedding and Disease Progression study</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HC-HIV</td>
<td>Hormonal Contraception and the Risk of HIV Acquisition study</td>
</tr>
<tr>
<td>HCR</td>
<td>Hydromorphone controlled-release</td>
</tr>
<tr>
<td>HEK293T</td>
<td>Human embryonic kidney derived cell line</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HR1 &amp; HR2</td>
<td>Heptad repeat regions of gp41</td>
</tr>
<tr>
<td>HSV-2</td>
<td>Herpes simplex virus 2</td>
</tr>
<tr>
<td>HTLV-III</td>
<td>Human T-lymphotropic virus type 3</td>
</tr>
<tr>
<td>HVTN-502</td>
<td>Merck vaccine Phase IIb trial (also called STEP study)</td>
</tr>
<tr>
<td>HXB2</td>
<td>Lab-adapted strain of HIV-1 used for nucleotide numbering</td>
</tr>
<tr>
<td>IDPE</td>
<td>Injection drug preparation equipment</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin 2</td>
</tr>
<tr>
<td>Int or IN</td>
<td>Integrase</td>
</tr>
<tr>
<td>IRB</td>
<td>Institutional review board</td>
</tr>
<tr>
<td>IVDU</td>
<td>Intravenous drug use</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography–tandem mass spectrometry</td>
</tr>
<tr>
<td>LGBTQ</td>
<td>Lesbian, Gay, Bisexual, Transgender and Queer</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeats</td>
</tr>
<tr>
<td>MA</td>
<td>Matrix or p17</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MHC I</td>
<td>Major histocompatibility complex class I</td>
</tr>
<tr>
<td>MHC II</td>
<td>Major histocompatibility complex class II</td>
</tr>
<tr>
<td>ML</td>
<td>Middlesex-London</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MSM</td>
<td>Men who have sex with men</td>
</tr>
<tr>
<td>MTC</td>
<td>Mother-to-Child</td>
</tr>
<tr>
<td>MUSCLE</td>
<td>Multiple Sequence Comparison by Log-Expectation</td>
</tr>
<tr>
<td>NC</td>
<td>Nucleocapsid or p7</td>
</tr>
<tr>
<td>Nef</td>
<td>Negative Regulatory Factor</td>
</tr>
<tr>
<td>NHP</td>
<td>Non-human primate</td>
</tr>
<tr>
<td>NIH</td>
<td>U.S. National Institutes of Health</td>
</tr>
<tr>
<td>NL4-3</td>
<td>Lab-adapted subtype B strain of HIV-1</td>
</tr>
<tr>
<td>NP-40</td>
<td>Tergitol type nonyl phenoxyethoxylethanol</td>
</tr>
<tr>
<td>ON</td>
<td>Ontario</td>
</tr>
<tr>
<td>OPV</td>
<td>Oral Polio Vaccine</td>
</tr>
<tr>
<td>p32</td>
<td>32-Phosphorus</td>
</tr>
<tr>
<td>p55</td>
<td>Gag precursor protein</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Primer binding site</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell death protein 1</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PEPFAR</td>
<td>U.S. President’s Emergency Plan for AIDS Relief</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin-Chlorophyll-protein</td>
</tr>
<tr>
<td>PHA</td>
<td>Polyhydroxyalkanoates</td>
</tr>
<tr>
<td>PIC</td>
<td>Pre-integration complex</td>
</tr>
<tr>
<td>Poly-dT</td>
<td>Poly-deoxy-thymidine</td>
</tr>
<tr>
<td>Poly-rA</td>
<td>Poly-ribo-adenylic acid</td>
</tr>
<tr>
<td>PPT</td>
<td>Poly-purine tract</td>
</tr>
<tr>
<td>P-TEFb</td>
<td>Positive transcription elongation factor</td>
</tr>
<tr>
<td>PWID</td>
<td>People who inject drugs</td>
</tr>
<tr>
<td>qVOA</td>
<td>Quantitative viral outgrowth assay</td>
</tr>
<tr>
<td>Rev</td>
<td>Regulator of Expression of Virion</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNaseH</td>
<td>Ribonuclease H</td>
</tr>
<tr>
<td>RRE</td>
<td>Rev-response element</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>RV144</td>
<td>Sanofi Pasteur vaccine Phase III trial (also called Thai trial)</td>
</tr>
<tr>
<td>SARS</td>
<td>Severe Acute Respiratory Syndrome</td>
</tr>
</tbody>
</table>
SD  Standard deviation
SIV  Simian immunodeficiency virus
SIVagm  SIV that infects *Chlorocebus spp.* (African green monkeys)
SIVcpzPts  SIV that infects *Pan troglodytes schweinfurthii* (Eastern Chimpanzee)
SIVcpzPtt  SIV that infects *Pan troglodytes troglodytes* (Central Chimpanzee)
SIVgor  SIV that infects *Gorilla gorilla gorilla* (Western Lowland Gorilla)
SIVmac239  SIVsmm strain that infects *Macaca mulatta* (Rhesus macaque)
SIVsmm  SIV that infects *Cercocebus atys* (Sooty mangabey)
SSC  Saline-sodium citrate
ssDNA  Single stranded DNA
ssRNA  Single stranded RNA
START  Strategic Timing of Antiretroviral Treatment study
T-20  Enfuvirtide (or EFV)
TAR  Transactivation response element
Tat  Trans-Activator of Transcription
TB  Tuberculosis
TCID  Tissue culture infective dose
TCR  T-cell receptor
TNF  Tumor necrosis factor
TRAIL  TNF-related apoptosis-inducing ligand
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>Trisaminomethane hydrochloride</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
</tr>
<tr>
<td>TZM-bl</td>
<td>HIV infection indicating HeLa cell line</td>
</tr>
<tr>
<td>U87</td>
<td>Astrocyte derived cell line</td>
</tr>
<tr>
<td>UNAIDS</td>
<td>Joint United Nations Program on HIV/AIDS</td>
</tr>
<tr>
<td>URF</td>
<td>Unique recombinant form</td>
</tr>
<tr>
<td>V# (1-5)</td>
<td>Variable loops of gp120</td>
</tr>
<tr>
<td>Vif</td>
<td>Viral Infectivity Factor</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus-like particle</td>
</tr>
<tr>
<td>Vpr</td>
<td>Viral Protein R</td>
</tr>
<tr>
<td>Vpu</td>
<td>Viral Protein U</td>
</tr>
<tr>
<td>VQA</td>
<td>Virology Quality Assurance</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
Chapter 1
Introduction

Approximately 35 million people have died from HIV/AIDS. Over 37 million more are currently infected [1]. The past, present and future of the entire African continent have been shaped by it. But for the first time, HIV appears beatable: new infections have slowed, global prevention initiatives funded and implemented, and science has made tremendous progress controlling and preventing infections. Whether the future holds a vaccine, sterilizing cure or additional international programs pushing to prevent transmissions, the tide has already turned. It’s only in the light of this potential that we can look back to learn from this disease, to inform future HIV policies, but also to prevent other zoonotic, or animal borne diseases in the future.

1.1. The Cut Hunter Hypothesis

A young man, starving and exhausted from overwork in the rubber tree plantations of the Belgian Congo, hunts in the jungle [2, 3]. It’s approximately 1931 (best estimates place it between 1915 and 1941) and food has become so scarce that he is forced to expand his typical dietary choices [4-7]. Bush meat, or the practice of hunting and eating jungle animals, has become a mainstay in his diet. Chimpanzees were a particularly common prey since they are large, relatively abundant, and accustomed to human interaction along the edges of the jungle [8-12]. In this account, the hunter is pursuing the local chimpanzee subspecies, the Central chimpanzee (*Pan troglodytes troglodytes*), armed with rudimentary equipment: dull knives or sharpened metal implements. Exhaustion provides enough of an impediment for the hunter to make a deadly mistake. While killing a chimpanzee the hunter injures himself, and the strain of the simian immunodeficiency virus (SIV) this chimpanzee harbors was able to reach his bloodstream. Unaware of the virus he is carrying, this man disregards the mild fever he gets over the next week. These symptoms resolve without long-term complications. Sometime within the next few years, this man leaves the rubber plantations and relocates by train to the local metropolitan center of Kinshasa, Zaire (now the Democratic Republic of the Congo or DRC) [10, 13]. The future will call him patient zero, the first human to begin spreading the human immunodeficiency virus (HIV).
Social, political and environmental changes in the Congo Basin during this period suggest that this account of the “Cut Hunter” hypothesis may oversimplify the drivers of zoonosis [10, 14]. Transmission of the virus very likely involved blood-blood contact, due to the far greater likelihood of successful infection, but butchering of primate meat is a much more likely cause of transmission, not an incident during hunting itself. Today bush meat hunting is still practiced and the butchering process is a source of many viral zoonoses [15-17]. The abrupt economic retraction stemming from the Great Depression in the 1920s, not colonial abuses, were the likely push to expand primate hunting, though not dramatically, which had been practiced to some extent for at least 3,000 years [18]. By the 1920s these hunts were likely safer than they had been in the past, as firearms were commonly used in protection, warfare, and hunting since the mid-19th century [19]. Belgian rule over Congo didn’t dramatically change firearm usage or ownership among rural African people, and most historians conclude that the slow expansion and accumulation of modern weaponry in Western and Central Africa remained steady through the late 19th and early 20th centuries [20]. Additionally, intentional food shortages and extreme cruelty to the native Congolese were curbed in 1908 after the transition from the Congo Free State, ostensibly privately managed by King Leopold II, to the Belgian Congo. The new leadership, while still oppressive, sought to improve the conditions of Congolese workers to curtail international criticism of slavery and abuse.

These historical data, while sparse, suggest there is no force that accounts for a dramatic increase in SIV exposure in the early 20th century that had not existed previously. The changes in the Congo during this period were primarily in the movement of people, both through forced relocations to work in the metal mines in the South and voluntary relocation to major population centers [20]. Colonialism poured money into the resource-rich Congo to rapidly develop huge plantations to produce rubber, palm oil and cotton while the South was torn open for extensive copper mining. Thus, it is logical that humans have likely been infrequently exposed to SIV for millennia, but widespread dissemination of these diseases was limited. Forest societies in the region had always been mobile, but the construction of extensive railway networks in the 1910s-20s allowed for greater transportation. Quickly growing population centers like Kinshasa, a railroad hub that doubled in population between 1920-1940, became focal points for viral transmission. While it would be easy to
blame colonial cruelty as the root of the global HIV-1 pandemic, it’s likely that many shifting environmental and social disturbances under colonialism, rather than the human rights’ abuses, were the underlying drivers.

1.2. Alternative Theories

Occasionally, researchers and conspiracy theorists have offered alternative theories about the origins of HIV. Most pervasive among them is the theory put forward by Tom Curtis in *Rolling Stone* magazine in 1992; contaminated plasma from an SIV infected chimpanzee was used in developing the oral polio vaccine (OPV) in the Stanleyville region of the Belgian Congo [21]. The vaccine was deployed to approximately a million Africans between 1957-1960, an appealing theory to those who believe that a single infected individual couldn’t be the origin of the pandemic. By exposing such a large population to SIV, the theory states, it becomes much more likely to encounter an immunocompromised individual who could support viral replication, regardless of how maladapted this virus was to infecting humans. Despite the appeals of the contaminated polio vaccine theory, there is no evidence of accidental or negligent infection of African populations with SIV/HIV due to medical treatment. Employees at the facility in Stanleyville recall that chimpanzee tissues were never cultured due to safety concerns, and that the vaccines administered in the region were produced outside of Africa [22, 23]. These facts did little to dissuade those who believed this theory and pushed researchers to identify the source of HIV-1 infections in Africa.

The final blow to the contaminated OPV theory came in 2010. Published in *Nature*, work from the groups of Drs. Beatrice Hahn and George Shaw conclusively demonstrated that, while the Eastern chimpanzees (*Pan troglodytes schweinfurthii*) of the Stanleyville region are endemically infected with SIV (SIVcpzPts), this strain does not resemble the SIVcpz strain that led to the Group M epidemic (SIVcpzPtt, Central Chimpanzee, Figure 1.1), or any transmissions establishing the HIV-1 or HIV-2 epidemics [24-28]. The geographic isolation of the chimpanzee subspecies that harbor these different SIVcpz strains, about 800km between their ranges, makes it impossible for these subspecies to mix and there are no known incidents of SIVcpzPts strains infecting either humans or any other primate species [27, 28]. In later work, Hahn and Shaw demonstrated that the SIVcpzPts strain in
the Eastern chimpanzees of the Stanleyville region cluster as an outgroup to SIVcpzPtt, HIV-1 Group M and SIVgor [3]. This data conclusively identified SIVcpzPtt from *Pan troglodytes troglodytes* chimpanzees from the Kinshasa region as the origins of both HIV-1 Group M and N, as well as infecting gorillas to establish SIVgor which in turn later transmitted to humans to found the Group O and P outbreaks. Finally, the timing of the deployment of the oral polio vaccine in the late 1950s is several decades too late to coincide with the origins of the Group M epidemic, situated around 1931 (95% confidence interval between 1915-1941) [3-7]. The story of the cut hunter, while flexible in details, provides the most realistic framework available to understand the origins of HIV group diversity.

1.3. SIV Diversity and Pathogenesis

Distinct SIV strains infect over 40 different primate species and subspecies ranging throughout Africa [29]. Most of these viruses are non-pathogenic in their hosts. For example, Gorillas and sooty mangabeys in Africa, infected with SIVgor and SIVsrm respectively, maintain high circulating viral loads but only rarely experience immunodeficiency symptoms, and in those cases these symptoms manifest only in the presence of exacerbating factors (e.g. advanced age or other infections) [30]. Loss of virulence due to evolutionary attenuation has been observed in many viruses, though this cannot be demonstrated experimentally in SIV [31-33]. Using genomic data from all SIV lineages and models accounting for both host specific and independent molecular clocks, the common ancestor of all SIVs is estimated to have existed between 2.5 and 5 million years ago [34-36]. This loosely coincides with the diversification of the primate species themselves, suggesting the virus and NHPs have coexisted through evolutionary time. It’s important to note that in cases of cross infection with SIVs from other primate species, the subsequent recombination has made estimates of SIV origin notoriously difficult and the findings controversial [37].
Figure 1.1) SIV origins of HIV infections with relative pathogenicity between groups. Arrows denote transmission events between species, both between non-human primates and, in the rightmost arrows, into humans in Central and Western Africa. The average pathogenicity of each HIV variant can be ranked by the reported rates of CD4+ T-cell depletion that they cause, illustrated on the far right [38-60]. Approximate numbers of HIV infection denoted in parenthesis. Dashed lines denote groups with such small numbers of infections that relative pathogenicity cannot be determined, only estimated.
A small number of these SIVs can cause immunodeficiency when infecting a non-host primate species, particularly Sooty mangabey SIVs infecting the three Asian macaque species. The most common example of this is in the Asian primate Rhesus macaques, which, when infected by SIVmac239 (a misnomer as this strain originated in Sooty mangabeys) are the prototypic immunodeficiency model used by researchers globally [61-63]. SIVmac239 infected macaques display many of the same markers of disease progression as human immunodeficiency including the loss of CD4+ T-cells, loss of CD8+ T-cell function, degenerative changes in lymph node architecture, and neurological symptoms. Much like HIV infections in humans, SIVmac infected macaques die from a range opportunistic diseases [61, 62].

In the mid-1990s, researchers focused on identifying new SIV strains in reclusive monkey species. By sequencing these new SIV strains, key discoveries were made regarding global SIV diversity. Early phylogenies showed that each primate species’ SIV strain was monophyletic, evolving with their primate host. However, more complete sequences of the new SIV genomes showed that a few SIV strains thought to be evolutionarily distinct were closely related and shared a recent common ancestor [63-66]. The assembled phylogenies from these studies showed 5 groups of SIVs that displayed evidence of recent transmission events between NHP species [29, 67]. The rest of the 40 known SIV strains are species specific and it has been hypothesized that these viruses are adapted to their host species and are non-transmissible [68, 69]. For example, SIV in the Sykes’ monkeys (Cercopithecus albogularis) is phylogenetically unique and has never successfully transmitted from its host species, despite regular interactions in the wild with other SIV infected primates [70]. Monkeys that are preyed upon but do not hunt other primates themselves, like the African green monkeys (Chlorocebus spp.), also harbor unique SIV strains (SIVagm) that do not show evidence of recombination with other SIVs [65, 67].

SIVcpz, the ancestral source of the most pathogenic HIV-1 groups, M and N, diverges significantly from the timeline of other SIVs [70]. Dating of SIVcpz divergence suggests that chimpanzees acquired the virus long after diversifying from gorillas and bonobos, their next closest primate relatives [4, 6]. This is especially likely due to the lack of SIV strains infecting the Western and Nigerian/Cameroonian Chimpanzee subspecies, which diverged
from the Eastern and Central Chimpanzee species during the last 500,000 years [35, 36]. Observations by the eminent primatologist/anthropologist Jane Goodall suggest that hunting of other primate species by chimpanzees is the most likely source of SIV zoonotic transmission [71, 72]. SIVcpz, when aligned to other closely related SIV strains, resembles a mosaic of SIVs from Greater spot-nosed monkeys, Mustached monkeys, Mona monkeys and Red-capped mangabeys, all prey species for Eastern and Central Chimpanzees [65, 67, 73] (Figure 1.1). Hunting as a mode of viral transmissions also agrees with the “missing” SIV in Nigerian/Cameroonian Chimpanzee subspecies since they exclusively prey on monkeys lacking SIV [74].

If SIV attenuated over time to become less pathogenic, then this more recent acquisition of SIV correlates with the intermediate level of pathogenicity observed in naturally infected chimpanzees relative to other SIVs. When infected, these apes display high viral loads and some symptoms of immunodeficiency, including reduced numbers of circulating CD4+ T-cells and structural changes in their lymph nodes [24]. One key difference, however, is the lack of AIDS-defining illnesses and opportunistic infection based-mortality in these monkeys. Lifespan is significantly reduced but typically due thrombocytopenia leading to heart failure, a comorbidity during SIV infection that is also occasionally observed in chronically infected HIV patients.

1.4. HIV Diversity

The most conservative estimates require at least twelve independent transmission events of SIV into human populations of Africa, and very likely many more. The earliest transmissions (1920-1930) led to the establishment of HIV-1 Groups M and N (from SIVcpzPtt) and HIV-1 Group O (from SIVgor) [75-77]. Other HIVs appear to have arisen from transmission events far more recently; HIV-2 (from SIVsmm in eight independent events) likely transmitted in the 1960s and HIV-1 Group P and the SIV strain infecting Western lowland gorillas (SIVgor) separating in approximately 1970 [6, 38, 78, 79] (Figure 1.1). The eight HIV-2 transmissions are geographically isolated from the rest of the SIV transmissions, located much further northwest in Guinea-Bissau. A war for independence in the region lasted from 1963-1974 against the Portuguese, displacing large numbers to neighboring countries. It has been hypothesized that this abrupt displacement to larger
population centers, much like in the Belgian Congo in the 1920s and 1930s, provided the ideal environment for establishing a new HIV epidemic [6, 38, 79].

All forms of HIV lead to immunodeficiency, however they vary significantly in the rate at which the disease they cause progresses (Figure 1.1). HIV-2 has been shown to cause a very slowly progressing disease and has been typically geographically restricted to West Africa due to poor transmission efficiency [39-41]. Those infected with HIV-2 generally have very low viral loads, near the limit of detection, likely a major factor in the limited transmissibility of these viruses [42]. This is especially evident in mother-to-child transmissions, where ~30% of infants born to HIV-1-infected women become infected in the absence of preventive interventions, while only about 1% of HIV-2-infected mothers pass the virus to their infants [43-46]. Other studies in Western Africa have shown a reduction in the time to AIDS when patients are infected with HIV-1 and 2 simultaneously compared to HIV-1 alone [48]. The average time to the onset of AIDS in the absence of treatment is about 8 years in a patient infected with a HIV-1 Group M subtype B virus [49]. In stark contrast, the same patient infected with an HIV-2 virus would reach clinical AIDS in approximately 14-23 years [47].

Globally, HIV-2 makes up fewer than 5% of all HIV infections and has been declining over the last decade, in contrast to HIV-1 that has increased dramatically in prevalence over that same period. HIV-1 infections make up the remaining ~95% of global infections and are approximately 55-75% divergent from HIV-2 at the nucleotide level [38, 39]. Very small numbers of geographically isolated human infections by HIV-1 Groups N and P viruses have been observed, each genetically differing from each other group by ~45% [50-54]. Group M remains the only HIV group that has established epidemics outside of Western Africa.

The diseases caused by non-Group M HIV-1 strains are more difficult to classify due to the small numbers of infections documented relative to Group M. The most prevalent of these groups is Group O, with almost a million documented infections since its identification in Belgium in 1990 [55]. Almost all Group O infections are restricted to West Africa, particularly Cameroon. The incidence of Group O infections has been steadily
declining since its identification, even as the numbers of Group M infections continued to rise in the same areas [56]. Group O disease largely mimics Group M infections though with reduced rates of tropism switching and mother-to-child transmissions. There are also increased numbers of elite controllers (patients who, without treatment, do not progress to AIDS) and patients who develop broadly neutralizing antibodies as a result of Group O infections relative to M [57, 58]. All of these factors likely contribute to a documented slower progression to AIDS in Group O infections [59].

Group N and P infections are very rare, fewer than 20 Group N and 5 Group P infections have been recorded. Perhaps due to this rarity these cases are closely documented, however no concrete conclusions can be drawn about their disease progression. Case reports of Group N virus infections resemble the disease caused by Group M. Mother-to-child Group N transmissions have been documented, suggesting a far higher likelihood than other non-Group M infections [51, 53]. Infections with Group N and M viruses typically require phylogenetic testing in order to differentiate due to the similarity in disease at early timepoints. Trajectories of CD4+ cell loss do not significantly differ between Group M and N infections and many of the same comorbidities and AIDS-defining-illnesses have been recorded [51-54, 75]. All of these measurements of disease progression are only available for the ~20 patients with Group N infections and could easily be skewed by population and selection biases. Group P, while capable of causing AIDS, appears to be the least pathogenic of the HIV-1 groups [50]. However, it is important to note that the very small numbers of infections make these conclusions impossible to validate [60].

1.5. Group M Diversity

The origins of HIV-1 Group M infections have been traced by Sharp and Hahn to a small region of southeastern Cameroon within the Congo Basin [77]. Hahn and Sharp compared SIV strains isolated from chimpanzee feces from across Central and Western Africa using next-generation sequencing technologies [3, 25, 27, 28]. From this study they identified the location of the SIV strain, that of the Central Chimpanzee, which most resembled HIV-1 Group M, likely the location the virus established within a human host. While the first decades of human transmission of HIV-1 Group M were sluggish, an epidemiological threshold appears to have been passed in the 1950s that led to rapid increases in
transmission [13, 80]. Hypotheses have been generated to explain this threshold, including adaptation of the HIV-1 accessory genes nef and vpu [81]. The same epidemiological threshold has not been reached by HIV-2 or other HIV-1 groups despite a similar early trajectory. The increased transmission and global spread led to massive diversification within Group M, yielding 9 viral subtypes: named A-D, F-H, J and K [82]. These subtypes can differ by up to 35% of the nucleotide sequence and are heterogeneously distributed globally [82-84]. Within each subtype, genetic variability is still quite significant, ranging from 8-17%. Subtypes A and F each contain distinct variants, termed sub-subtypes, though these variants differ enough to be considered unique subtypes by themselves (18-34% nucleotide divergence). Subtype A is broken into sub-subtypes A1, A2, A3, A4 and A6 while subtype F is differentiated into sub-subtypes F1 and F2. Circulating recombinant forms (CRFs) and unique recombinant forms (URFs) can also form when a single patient is infected with multiple subtypes simultaneously, allowing the viral genome to recombine. As of 2018 there are 96 identified CRFs in HIV-1 and one in HIV-2.

Conspicuously, HIV-1 Group M subtype nomenclature omits subtypes E and I. These subtypes, referred to as “problematic”, were identified as distinct through incomplete sequencing alignments [85]. When more complete sequencing was performed on the samples, these subtypes were found to be recombinants including either subtype A or numerous other Group M subtypes. For accuracy, subtype E was renamed circulating recombinant form AE (CRF01_AE), while subtype I was renamed circulating recombinant form complex (CRF04_cpx).

1.6. Group M Geographic Distribution

After crossing an epidemiological threshold in the 1950s, HIV-1 Group M diversified greatly, primarily due to the very high mutation and replication rates coupled with large numbers of newly infected individuals [76]. During this period the virus spread out of Central and Western Africa. Dating the epidemic in South Africa suggests a subtype C founder entered the population in approximately 1960 (+/- 8 years) [86]. Currently, South Africa routinely has one of the highest HIV infection rates in the world and is almost exclusively subtype C. Many published studies of the South African epidemic demonstrate that the virus has spread much more rapidly in the same time period relative to Central
Africa, where there is a greater diversity of circulating HIV subtypes circulating [86]. Recently, subtype C viruses have spread into China and India and appear poised to expand further in many countries in Southeast Asia [87, 88].

Establishment of a subtype B virus from Central Africa in Haiti in ~1964 led to the first epidemic in North America [89]. Sequencing of samples of known HIV+ individuals from the mid-1970s demonstrated a clear link between Haiti subtype B viruses and the first infection in the United States in New York City (dated to ~1970) [90-92]. In both Haiti and the United States, the epidemic doubling time is placed at about one year (0.86-1.12) suggesting extremely rapid expansion. The most likely theories about the arrival of HIV in the Caribbean involve missionaries returning to Haiti from Congo in the 1960s and future dissemination to Hispaniola, the Dominican Republic, and, eventually, the United States [89]. There also exist many legends about the introduction of subtype B into the United States and its early spread, typically involving a flight attendant patient zero, none of which are substantiated by facts [93]. Following its introduction, subtype B quickly became the dominant HIV subtype in not only North America and the Caribbean but also in South America, Europe and Australia. HIV phylogenies show subtype B is a recent divergence, likely pushed further by geographic evolutionary pressures and founder effects. This recency manifests in the short branch distance between subtypes B and D, making them the most closely related of the Group M subtypes, about 7% nucleotide distance in the pol gene [80].

The predominance of subtype B in North America has led to a disproportionate focus in research funding and efforts even though fewer than 11% of individuals living with HIV globally harbor a subtype B virus [84]. The disease caused by subtype B provides almost all estimates of “time to AIDS” used by clinicians worldwide, approximately 8 years in the absence of treatment. This disease has been noted to be significantly more aggressive than most other HIV groups and subtypes, but exactly to what degree remains controversial [94, 95]. Sub-Saharan Africa, the region with the greatest number of infected individuals, is the source of all ancestral HIV diversity. While this region still contains the greatest viral diversity, it is ironically generally devoid of subtype B infections, despite clear evidence
of its origin in Kinshasa, Zaire [89]. This region currently primarily contains infections of subtypes A, C and D [5, 96].

1.7. Modern Epidemic and Research

In 1981, Acquired Immune Deficiency Syndrome (AIDS) was documented for the first time. A series of patients presenting to hospitals across the United States displayed opportunistic lung infections, particularly Pneumocystis carinii pneumonia, and highly aggressive soft tissue cancer, Kaposi’s sarcoma, previously only seen in severely immunocompromised individuals [97, 98]. Now, clinical AIDS is defined by the World Health Organization (WHO) and the American Centers for Disease Control (CDC) as either a CD4+ T-Cell count below 200 cells/mL in the circulation, a T-cell percentage of total lymphocytes below 14%, or the presence of one or more of the 20 AIDS-defining-illnesses. In order to validate the AIDS diagnosis, a patient must test positively for anti-HIV antibodies, known as seroconversion [99].

In 1983 the etiological agent of AIDS was identified independently by Drs. Luc Montagnier and Robert Gallo, a retrovirus originally branded the lymphadenopathy associated virus (LAV by Montagnier) or human T-lymphotropic virus type 3 (HTLV-III by Gallo), later renamed the human immunodeficiency virus (HIV) [100-102]. Prior to the identification of HIV, no other lentivirus caused disease in humans. Identifying HIV as the causative agent of AIDS finally provided a research target. Since 1983, 22 single drugs and several multi-drug therapies have been approved by the U.S. Food and Drug Administration (FDA), targeting multiple steps of the HIV replication cycle. New methods of preventing infection have been developed including microbicides and pre-exposure prophylactic drugs. Unfortunately, resistance mutations have been identified, at least in the laboratory, to each of these drugs individually [103]. Two drug regimens targeting independent steps in the replication cycle historically have been overwhelmed by HIV mutation rates and develop resistance mutations to both drugs, though new combinations may overcome this block [104]. For now, however, HIV positive patients’ regimens must include three drugs to suppress viral replication and stem the development of drug resistance.
In the search of a cure, there have been four international vaccine efficacy trials which administered Env protein based vaccines to thousands of people around the world [105]. Each of the first three trials failed, perhaps most spectacularly in the HVTN-502 study (or “STEP study”) which increased the risk of infection in recipients. The final trial, RV144 or the “Thai trial”, combined lessons from each of the previously failed vaccines, combining different Env immunogens to develop non-neutralizing and cellular immune responses in its recipients. The results of the trial were promising, showing a 31% reduction in the incidence of HIV in those vaccinated relative to controls [106]. Despite being the most successful HIV vaccine trial, the efficacy remained too low for widespread deployment and the results have been dissected and hotly debated, even before they were fully released. While many researchers had concerns with funding allocation, some epidemiologists raised significant concerns about selective patient exclusion during the final analyses, which were necessary for statistical significance [107].

1.8. HIV Genome, Viral Structure and Replication

The HIV genome is relatively small, even for viruses, at 10kb, varying slightly depending on the viral strain (Figure 1.2A). Structural proteins are encoded by the \textit{gag} gene on the 5’ end of the genome, enzymatic proteins encoded in the \textit{pol} gene, while the \textit{env} gene encodes the structural Env protein, which is required for viral entry. Efficient viral protein production requires the activator proteins Rev and Tat which promote transcription and transport of mRNAs out of the nucleus. The remaining genes, \textit{vif}, \textit{vpr}, \textit{vpu}, and \textit{nef} are grouped together and called accessory genes. None of the accessory genes have enzymatic or structural functions, and most exist to antagonize cellular resistance factors.

The process of HIV infecting a susceptible and permissive cell are classified into distinct steps: 1) attachment and entry, 2) reverse transcription, 3) integration, 4) transcription and translation, 5) assembly and packaging, and 6) budding, release and maturation (Figure 1.3). Each of these steps are now targetable with different classes of antiretrovirals, though resistance mutations have been identified to all developed drugs [103].
Figure 1.2 A) HIV-1 Genome organization. Rows show reading frames with HXB2 nucleotide numbering shown below. Individual protein product abbreviations are denoted below, gene names denoted above. Inset shows the env gene coding sequence with HXB2 nucleotide numbering. Variable regions and major features are labeled.

B) Assembled HIV-1 Virion Structure. The assembled viral particle consists of a Gag matrix (MA) protein lattice with embedded env proteins (gp120 and gp41) protruding from a host-derived membrane. The ribonucleic acid genome is contained within a Gag capsid (CA) protein core. The interior of the viral core contains Protease, Tat, and Integrase with Reverse Transcriptase (RT) and Nucelocapsid (NC) proteins in association with the RNA.

C) HIV-1 Env is comprised of a heterotrimer consisting of external gp120 and transmembrane gp41 subunits. Gp120 contains a series of variable loops around its outer surface and is heavily glycosylated. These variable regions interact with host cell proteins, particularly the entry receptors CD4 and, in sexual transmissions, CCR5. Gp41 contains two heptad repeats, a fusion peptide that inserts into the host cell membrane and a viral membrane transmembrane region.
1.8.1. Attachment and Entry

The first stage of HIV infection is viral entry, which comprises attachment, receptor and coreceptor binding, membrane fusion and capsid/genome release into the cytoplasm. These steps are mediated by the HIV Env glycoprotein, the only viral protein exposed on the external surface of the virion. In order to evade the host immune system, Env is heavily glycosylated and must undergo a series of conformational changes during the viral entry process in order to expose the residues capable of binding and fusing with the host cell [108].

The mature Env protein is heterotrimeric, consisting of three outer surface gp120 subunits and three transmembrane gp41 subunits (Figure 1.2C). Gp120 is composed of a bridging sheet linking an inner and outer domain. Notably, the inner domain contains both the N and C-termini pointing inwards towards gp41 [109]. The inner domain consists of a 5 stranded β-sandwich and a two strand two helix bundle which are formed from the conserved regions of gp120 (C1 to C5) [109, 110]. Five highly variable regions (V1 to V5) extend outwards from the inner domain, making up the gp120 outer domain [111], forming loops that interact with extracellular host cell proteins, and mediating conformational changes and membrane fusion. The gp41 subunit is composed of a cytoplasmic tail, a transmembrane region, a membrane proximal external region (MPER) and two complementary heptad repeat regions (HR1 and HR2). These subunits, gp120 and gp41, are translated together as a gp160 precursor and are subsequently proteolytically cleaved at the cell surface. Following processing, noncovalent interactions are formed to hold these glycoproteins together as heterodimeric subunits. Trimers of these subunits, also called envelope spikes, are then reinternalized and trafficked to sites of viral assembly where they bind structural Gag matrices.

The first interaction between a CD4+ T-cell and a viral particle involves non-specific electrostatic interactions between the sugar residues of the HIV Env gp120 glycoprotein with a number of host cell receptors, including the transmembrane receptor α4β7 Integrin [112]. These nonspecific interactions concentrate the number of viral particles at the cell surface, facilitating the interaction between the CD4 attachment receptor and the CD4
binding site (CD4bs) of gp120 (Figure 1.3.1). This binding site is highly conserved and the target of numerous identified neutralizing antibodies. Conservation at this site has also rendered this an attractive candidate for drug design, however CD4 mimetics and other so-called attachment inhibitors have largely failed during clinical trials [113]. The only FDA approved antiretroviral that prevents viral attachment is Enfuvirtide (EFV or T20), marketed as Fuzeon. EFV has largely fallen out of regular use due to the rapid development of drug resistance mutations in the virus [114].

Isothermal titration microcalorimetry has demonstrated that binding of the CD4 receptor induces gross changes in both the exposed loops, including the V3 loop and the coreceptor binding site, as well as within the protein core. These studies also suggest that before binding to CD4, the viral envelope is capable of existing in multiple different conformations, between open and closed, with roughly equal free energy [115]. Following engagement with the CD4 receptor, the free energy of the system drops precipitously and locks the Env protein into an open state. This state exposes more of the V3 loop and allows the virus to bind its coreceptor (Figure 1.3.2). Although over fourteen different transmembrane receptors have been shown to support HIV infection in CD4+ cell lines, only CCR5 and CXCR4 have been shown to act as coreceptors in vivo [116-118]. Coreceptor binding initiates further conformational changes in the envelope, permitting the insertion of the gp41 fusion peptide into the cellular membrane (Figure 1.3.3). Through intermediate gp41 structural rearrangements, a fusion pore develops the viral and host cell membranes mix (Figure 1.3.4). The capsid core structure, containing the viral genome, is then able to pass through the fusion pore and into the cytoplasm of the cell.
The process of HIV entry into a target cell is divided into four main events: 1) attachment to a host cell via the CD4 receptor engaging with the CD4bs of the HIV Env, 2) binding to a seven transmembrane chemokine coreceptor, typically CCR5 during sexual transmission, 3) fusion peptide insertion into the host cell membrane, and finally, 4) fusion of the viral and cellular membranes.

**Figure 1.3** The process of HIV entry into a target cell is divided into four main events: 1) attachment to a host cell via the CD4 receptor engaging with the CD4bs of the HIV Env, 2) binding to a seven transmembrane chemokine coreceptor, typically CCR5 during sexual transmission, 3) fusion peptide insertion into the host cell membrane, and finally, 4) fusion of the viral and cellular membranes.
1.8.2. Reverse Transcription

The defining trait of retroviruses is the conversion of their single stranded RNA (ssRNA) genome into a double-stranded DNA (dsDNA) viral genome via the process of reverse transcription (Figure 1.4.3). The reverse transcriptase (RT) enzyme, encoded in the pol gene, is a DNA polymerase with both RNA-dependent and DNA-dependent activities. RT also has an associated ribonuclease H (RNaseH) with degradation activity of RNA in RNA-DNA duplexes. Reverse transcription occurs in the cytosol and requires an RNA primer to initiate polymerization from the ssRNA genome template. In the case of HIV-1, a specific host tRNA serves as this primer and binds to an 18 nucleotide region known as the primer binding site (PBS) near the 5’ end of the genome (Figure 1.2A). This tRNA is incorporated into budding virions and some primer extension occurs prior to viral entry [119].

Upon binding to the PBS, polymerization of minus (-) strand DNA proceeds towards the 5’ end of the genome. Digestion of the genomic RNA by RNaseH frees this short ssDNA, known as minus strand strong stop DNA, which then hybridizes with the 3’ end of the genome via a short repeat region of homology. DNA synthesis proceeds along the remaining length of RNA genome in a 3’ to 5’ direction. RNase H leaves a region of RNA in the env coding region known as the poly-purine tract (PPT). The PPT serves as a primer for positive-strand DNA synthesis which proceeds in a 5’ to 3’ manner using the minus-strand DNA as template. A second strand transfer event occurs where the positive-strand DNA binds to the minus-strand DNA at the PBS region which becomes present in both DNAs. Second strand DNA synthesis completes through bidirectional synthesis by the circular binding of the minus-strand to the positive-strand DNA. The double-stranded DNA product of reverse transcription contains identical regions at the 5’ and 3’ ends termed the long terminal repeats (LTR). These regions are essential for integration and mRNA transcription and contain the enhancer, promoter, transcription initiation, transcription termination and polyadenylation signals [120].

1.8.3. Integration

Following reverse transcription, the newly synthesized viral dsDNA must be imported into the cell nucleus for integration into the host chromosome. This is accomplished through
the formation of the pre-integration complex (PIC), a complex of host and viral proteins that actively transport the dsDNA into the cell nucleus. The PIC consists of the HIV viral dsDNA, the integrase (IN) enzyme, the viral Vpr protein, matrix protein, and host cellular factors. Due to the size of the PIC complex, it cannot passively transfer from the cell cytoplasm into the nucleus but must be actively carried into the nucleus through a nuclear import pathway fueled by a RanGTP gradient (Figure 1.4.4 & 1.4.5). Nuclear localization signals, found in matrix, vpr, and integrase, recruit nuclear importin proteins to the PIC. This active nuclear transport process permits HIV infection of non-dividing cells such as macrophages [121].

The process of integration initiates with a reaction known as 3’-end processing. The integrase enzyme cleaves off two nucleotides from the 3’ ends of both strands of linear viral DNA in a staggered manner, resulting in non-blunt ends. The 3’ recessed ends of viral DNA bind with the cleaved cellular DNA in a strand transfer event. Integration is completed when the gaps between the integrated viral DNA and cellular DNA are filled in by cellular repair enzymes. Once integrated, the viral DNA is referred to as “proviral” DNA [122].

1.8.4. Transcription and Translation

Following successful integration into the host chromosome, the proviral DNA is used as a template for the synthesis of viral RNAs which encode the necessary structural, enzymatic, and regulatory proteins for the production of progeny virus. The cellular machinery is utilized for the transcription of viral RNAs, RNA-splicing, and nuclear export.

Basal transcriptional activity from the HIV LTR promoter is very poor. However, the Tat protein is delivered to the entering virion and produced from a small number of RNA transcripts generated early in infection [123]. Tat potentiates a positive feedback mechanism promoting high level HIV mRNA transcription. Tat binds to a transactivation response (TAR) element, a cis-acting RNA element that forms a stable stem-loop structure located downstream of the transcription initiation site [124]. The Tat-TAR interaction promotes the recruitment of a multicomponent kinase complex termed P-TEFb or positive
transcriptional elongation factor b [125]. P-TEFb activates RNA polymerase II, promoting processive mRNA elongation (Figure 1.4.6).

Three major groups of RNA transcription products are produced from the proviral DNA: 1) unspliced RNAs, 2) partially spliced mRNAs, and 3) multiply spliced mRNAs. The unspliced RNAs serve as genomic RNA for packaging in progeny virions and encode the Gag and Pol proteins. Partially spliced mRNAs encode the Env, Vif, Vpu and Vpr proteins while short, multiply spliced mRNAs encode the Rev, Tat, and Nef proteins. Differential splicing patterns can result in as many as thirty or more unique mRNA transcripts being produced [126].

Most cellular mRNAs are fully spliced prior to leaving the nucleus. However, HIV replication requires that partially and unspliced RNAs are transported out of the nucleus to the cytoplasm. In order to overcome this dilemma, the Rev protein (regulator of expression of viral proteins) binds to unspliced viral RNAs and shuttles them out of the nucleus. Rev binds to a cis-acting RNA element known as the rev-response element (RRE) located in the env gene. The RRE is a series of highly structured stem loops found in all unspliced and partially spliced viral RNAs [127]. Multiple rev proteins bind to the RRE and form a complex capable of interacting with the nuclear export machinery. Rev shuttles the unspliced or partially spliced RNAs to the cytoplasm and return to the nucleus by way of its nuclear localization signal. Once in the cytoplasm, viral protein synthesis from mRNA transcripts ensues through normal cellular translational machinery.

1.8.5. Assembly and Packaging

Production of Gag and Pol proteins promotes assembly of new virus particles at the plasma membrane. The matrix (MA) domain of Gag, which is located at the N-terminus of the p55 precursor protein, is cotranslationally modified by the addition of myristic acid. Mutational studies suggest that a positively charged face of MA interacts with negatively charged phospholipids of the plasma membrane, stabilizing membrane binding [128].

In addition to associating with the plasma membrane, Gag must also participate in Gag-Gag and Gag-RNA interactions. The Gag-Gag interaction is thought to involve multiple
regions of the precursor to form a matrix along the cell membrane [129]. In tandem, interactions between NC and a cis-acting element located 5’ of the gag initiation site known as the major packaging signal, or Ψ-site, mediate the incorporation of genomic RNA into virus particles. The envelope precursor protein, gp160, is synthesized in the endoplasmic reticulum (ER) and translocated into the ER lumen by means of an N-terminal signal peptide comprised of approximately the first thirty amino acids of the polyprotein. In the lumen, gp160 folds, disulfide bonds are formed, the protein is glycosylated, and oligomerizes to form trimers. After gp160 is properly folded and trimerized, it is transported to the Golgi complex where the polyprotein is cleaved by cellular proteases into the gp120 and gp41 subunits. The gp41 subunit is anchored to membrane and noncovalently associates with gp120. The envelope complex is trafficked to the plasma membrane through the secretory pathway. Mutational studies indicate that the MA domain of gag promotes envelope incorporation into budding virions through an interaction with the cytosolic tail of gp41 [130].

1.8.6. Viral Budding, Release, and Maturation

Accumulation of Gag and associated RNA at the plasma membrane enhances the final stage of virion assembly known as budding. A proline rich domain at the C-terminus of Gag known as p6 encodes a small protein that promotes viral particle release from the plasma membrane [131]. Multiple studies have indicated a connection between the endosomal sorting pathway and the viral budding process. The observation that p6 interacts with a component of the ESCRT-I complex (endosomal sorting complex required for transport) further points to the importance of the endosomal sorting pathway in virion release [132].

A cellular defense mechanism to prevent release and maturation of budded virus particles involves a cellular factor, Tetherin, which physically tethers budding virus particles to the cell membrane. These nascent particles are subject to internalization through endocytic pathways. While it has been known for some time that the accessory protein Vpu promotes release of virus particle [133], it has only recently been demonstrated that Vpu does so through antagonization of Tetherin [134].
Shortly after virus release from the membrane, the protease enzyme cleaves the Gag and Pol precursors into the mature proteins. Once released, the structural proteins induce rearrangements in the virion morphology in a process known as maturation. Mature virions contain a conical shaped core encapsulating the RNA genome. Immature virions are not capable of establishing productive infections and thus maturation is a requirement for infectious particle production [135].

1.9. HIV Disease

While modern medicine can reduce HIV replication to undetectable levels, in the absence of treatment HIV+ people living with HIV undergo a predictable progression of disease. Key features of the disease, such as the amount of infectious virus in the circulation (known as viral load), transmission efficiency, time to the onset of AIDS, serious HIV-related complications, and viral fitness, can be compared to quantify relative pathogenicity between strains.

1.9.1. HIV Transmission

HIV transmissions between humans are classified into several possible routes: sexual intercourse (both anal and vaginal for both partners), intravenous drug use (IVDU), Mother-to-Child (MTC) during pregnancy or delivery, accidental inoculation with contaminated bodily fluids (typically in health care workers) or during organ transplantation [136]. The most prevalent pathway for transmission is sexual but other modes may play a larger role in individual epidemics, like that of IVDU transmission in the former Soviet republics [137, 138]. Regardless of route, transmitted virus undergoes an immense genetic bottleneck, typically with a single viral variant causing infection in the recipient [139-143]. In those rare instances in which more than one viral variant is transmitted, patients experience a more rapid disease progression and reduced time to the onset of AIDS. This bottleneck has significant consequences for viral replication, as the transmitted-founder virus, or the virus establishing the new infection [144]. The most apparent of these consequences is the near universal utilization of the CCR5 chemokine coreceptor and reduced replicative fitness in the new host [145].
Figure 1.4) The major steps of HIV replication: 1) Viral attachment to nonspecific extracellular receptors, 2) Specific viral interactions with CD4 (red) and a chemokine receptor (typically CCR5 in green) that leads to release of the viral capsid into the cytoplasm, 3) Reverse Transcription of the RNA viral genome into a DNA proviral genome, 4) Import of the pre-integration complex (PIC), 5) Integration of the DNA provirus into the host genome, 6) Viral transcription and translation generating viral proteins and full length RNA genomes, 7) Viral assembly at the cell surface, 8) Budding of the progeny virus and 9) Maturation into fully infectious new virions.
Many transmissions of HIV occur during the early phases before symptoms are displayed and without knowledge of the infected party’s status. Large scale testing efforts have been implemented globally, but have limits since diagnosing HIV can be prone to challenges and false-negative results [146, 147]. The long periods of asymptomatic infection during HIV disease amplify these challenges and are responsible for the relatively poor rates of HIV diagnosis. Of the 37 million people living with HIV, about 25% do not know that they are infected [1]. These numbers are thought to be even higher in Eastern and Southern Africa, the regions with the greatest HIV/AIDS burden.

1.9.2. Acute HIV Infection

The progression of HIV infection in the typical patient is classified into two phases, Acute and Chronic, each with their own associated symptoms, CD4+ T-cell responses and viral load dynamics. The Acute/Early phase, also called acute HIV infection (AHI), is classified as the period between viral transmission and stable viremia following the viral set point. Researchers frequently define the conclusion of this phase in terms of months post-infection, however the exact number of months can vary between 2 and 4 [148]. Immediately following transmission, within the first 2 weeks, the host innate immune system is largely overwhelmed by the rapid replication rate of the virus and mutations that evade host immune responses. Viral loads in the patient’s circulation can spike to $10^7$ copies per mL and flu-like symptoms are common, however the precise symptoms vary depending on the viral strain and host genetics [149, 150]. Typically, this fever is described as resembling mononucleosis, with mainly minor symptoms including fatigue, headache and swollen lymph nodes, though meningoencephalitis and other serious complications have been observed [151]. Acute viremia can last up to several weeks before declining due to the host adaptive immune system [152]. Seroconversion, or the detection of anti-HIV antibodies in the patients’ circulation, typically occurs within the first 2 weeks following infection [153]. Most antibodies produced during this disease phase have epitopes that target the structural protein Gag and are non-neutralizing. Despite the inability to neutralize virus, these antibodies are very important markers for disease progression and their titers are one of the most reliable markers of pathogenesis [154]. The latent HIV reservoir, long-lived memory T-cells containing integrated but not actively transcribed provirus, is also
established within AHI. Even when antiretroviral therapy is implemented within 3 days of virus exposure, the size and decay rate of the latent reservoir remains unaffected [155], though its T-cell subset makeup shifts away from central memory populations and towards resting memory T-cells [156, 157]. Both the size and makeup of the latent reservoir are impacted by viral subtype and have significant implications for treatment, disease progression, and long-term patient outcome [158-161].

Many clinical measurements during AHI have been associated with either slower or more rapid disease progression. Peak viremia immediately following infection and viral load at the set point have both been implicated as predictors of CD4+ T-cell loss [41, 162, 163]. Unfortunately, the reproducibility of these results is inconsistent and multiple other studies have found no association between disease progression and AHI measurements, particularly when compared across different viral and host populations [164, 165]. These conflicts illustrate the difficulties predicting and evaluating disease progression in natural cohorts due to patient and virus variability.

1.9.3. Chronic HIV Infection

Chronic HIV infection typically refers to HIV disease after AHI, or the period of disease after the viral load set point. The set point represents an equilibrium between viral replication and the host immune system. This post-set point disease is typically called the asymptomatic phase of infection, which is marked by clinical latency of the disease. Viral loads are reduced relative to the other phases of infection due to suppression by the host innate and adaptive immune system. The asymptomatic phase of HIV disease progression may not display any outward symptoms of infection, but many of the defining processes of immunodeficiency occur during this phase [99]. Despite viral loads remaining low during the initial months following AHI, viral fitness and diversity increase rapidly [29, 166, 167]. This is possible due to the sustained viral replication in lymphoid tissue even during the asymptomatic phase of infection [168]. Viral replication is also sustained even during ART [169-171], suggesting the viral replication and the symptoms of immunodeficiency are not inextricably linked. This continued replication may be one of the leading causes of drug resistance in patients who maintain high levels of drug adherence.
The asymptomatic phase of HIV infection is the longest phase of HIV disease. Since there are typically few HIV-related symptoms, relatively few patients are aware that they have been infected. Studies have shown that while both early and late stage HIV infections are more transmissible, due to high circulating viral loads, most transmissions occur from a patient in the asymptomatic phase due to the greatly expanded time scale [172]. The asymptomatic phase typically lasts multiple years, even in the most aggressive of cases. CD4+ T-cells are gradually depleted in a near linear rate over this period. Since these cells are necessary for survival and proliferation signals to other immune cells, their depletion leads to widespread immune system collapse, permitting opportunistic infections and rare cancers with an associated infectious agent etiology to be observed. Most patients experience one or several AIDS-related symptoms which include chronic wasting, diarrhea, swollen lymph nodes, skin lesions or rashes, oral sores, neurological complications, fatigue, fever and night sweats [173, 174]. The underlying driver of these symptoms is the broad inflammation and immune activation perennially experienced by HIV patients.

1.9.4. HIV Disease Cellular Biology

HIV disease has been characterized extensively over the decades following its emergence. The virus is able to compromise the host immune system due to its capacity to infect and deplete a number of immune cell types, primarily CD4+ or “helper” T-cells [175-178], though the virus can also infect macrophages and dendritic cells, albeit less productively [179]. The infection of CD4 T-cells eventually begins a multifaceted path to immunodeficiency related to cell death of directly infected cells [180-182], bystander cell apoptosis [183, 184], long-term immune activation [185-187] and impaired T-cell function [188-190]. As the disease progresses, the CD4+ T-cell population is gradually depleted. It has been documented that depletion occurs in particular T-cell subsets, namely the memory populations, and T-cells with HIV specific T-cell receptors (TCRs) [191-193]. These memory T-cells are a heterogeneous population of both effector memory and central memory T-cells. Effector memory T-cells (CD45R0+/CCR7-) are more differentiated, home to peripheral tissues, and can generate more effector memory cells in-vitro. Central memory T-cells (CD45R0+/CCR7+) are able to self-renew and reside in primary lymphoid
organs. Depletion of these populations is directly responsible for many of the characteristic symptoms associated with HIV disease and AIDS.

The principal drivers of CD4+ T-cell loss in HIV patients has been subject to much investigation, but many facets remain mysterious. Key discoveries in the field demonstrated that the loss of CD4+ cells is centered in the lymphoid organs of the gut with other local sites of replication including the lymph nodes [194]. Serving as a primary site of viral replication, lymphoid organs undergo a progressive degradation of their structures [168]. The lymphoid tissue degradation, as well as persistent inflammation, leads to the characteristic “leaky gut” in chronically infected HIV patients [194]. Disruption of the structure of the digestive tract leads to further inflammation and bacterial translocation into the patient circulation, a vicious cycle that drives immune cell loss, particularly the cells that are resident in the nearby lymphoid organs (Figure 1.5). Live animal imaging in primate models has further validated lymphoid tissues as hotspots of viral replication, but it is still unclear what molecular mechanisms push these infected cells towards death [195]. Recent discoveries have pointed towards infected cells undergoing pyroptosis, a caspase-1 mediated, highly inflammatory form of cell death [196]. Other studies responding to this discovery suggested apoptosis, T-cell mediated clearance and viral cytopathic effects as the most important drivers of CD4+ T-cell loss [197, 198].

Bystander effects, or the death of uninfected, neighboring cells, has also been noted as a primary cause of death in lymph nodes during HIV and SIV infections [199]. These uninfected T-cells die via apoptosis, a caspase-3 mediated death pathway, that limits the release of inflammatory signals. The rates of uninfected cells undergoing apoptosis remains unaffected by the number of CD8+ T-cells, or cytotoxic T-lymphocytes (CTLs), suggesting that the death is caused by signals released by nearby infected cells, namely inflammatory cytokines [183]. During these same experiments, researchers also noted that productively infected cells were not necessarily dying after producing virus. Instead, a balance between latent and activated states causes these cells to persist [199]. These results suggest that the depletion of CD4 T-cells during HIV disease has been largely misunderstood, and that CTL responses and inflammatory cytokine signals drive the death of uninfected cells while the cytopathic effects of HIV are minimal. CTLs induce apoptosis via the extrinsic pathway,
typically via engagement of Fas, TNF or TRAIL with their cognate receptors, to actively clear infected cells [200]. CTL response breadth, as well as the absence of CTL escape mutations, have been identified numerous times as predictors of HIV disease severity and the likelihood of viral control [201-205].

Finally, T-cells undergo a process of activation and expansion followed by contraction via apoptosis in response to T-cell receptor (TCR) activation with their cognate antigen in the context of MHC class II. HIV, being a highly inflammatory disease releasing huge amounts of antigen, causes broad inflammatory responses throughout the body, activating immune cells. The T-cells that activate in response will eventually undergo apoptosis or, in a minority, will become long-lived, memory T-cells. The extent and intensity of chronic immune activation strongly correlates with the timing of AIDS and CD4+ T-cell depletion [185, 186, 194, 206].

Early experiments with HIV identified that these viruses require an activated cell to replicate [207]. Access to chromatin is necessary for the integration process which is only freed upon T-cell activation and active transcription. The long-lived memory T-cell population has a lower threshold for activation, making these cells ideal targets for HIV infection [208]. The effector memory subset (CD4+/CD45R0+/CCR7-) migrate to the periphery of the infected patient, homing to sites of inflammation, and likely encountering virus. Upon infection these memory cells can return to a quiescent state, forming the latent reservoir of HIV [209, 210]. This reservoir is what renders HIV incurable, due to its rapid formation after infection and the extremely long half-life of memory T-cells [171, 211].

1.9.5. Clinical Measurements of Disease Progression

HIV disease severity is typically quantified through a few clinical markers and diagnostic tests. The most classic of these is viral load, or the number of viral particles in a patient’s circulation, measured as copies per milliliter of blood. The test uses quantitative PCR to measure the number of copies of the non-integrated HIV RNA genome that are present in a sample. The development of this test was groundbreaking for its sensitivity in the diagnosis of HIV infection and drug regimen effectiveness, detecting as few as 50 RNA copies per mL of blood. Immediately clinicians and researchers identified both cumulative
viral load, or viral load at the set point closely associates with the timing to the onset of AIDS [41, 49, 160, 163, 212]. Recent investigations have identified many additional factors which may skew viral load for patient prognosis in some rare instances, particularly when concerning diverse HIV-1 isolates [160, 164].

Many host behavioral and genetic factors also exacerbate HIV disease progression. Stress, mental illness, drug and alcohol use, sexual behaviors, vitamin deficiencies, co-infections, rates of T-cell exhaustion, susceptibility to chronic inflammation, circulating cytokine levels, and other viral and host genotypes have all been implicated in driving rapid disease progression in different cohorts [163, 166, 213-236]. It is important to note, however, that almost all of these factors have also been found not to have an impact on disease progression in other cohorts/studies (e.g. alcohol and drug use in [237]). Many of these associations are likely population specific and lack the human diversity in the study group to properly control for host genotype.

Some host human leukocyte antigen (HLA) alleles, encoding for major histocompatibility complex class I (MHC class 1) appear protective against HIV disease progression [238]. In particular HLA-B variants 57, 27 and 81 (HLA-B*57, HLA B*27 and HLA-B*81) are commonly associated with stable CD4+ cell counts, slow disease progression, and lower viral loads [239]. These alleles are non-homogenously spread across the globe. For example, in Africa, HLA-B*57 is found frequently in some regions of Africa (e.g. Zambia and South Africa) and at a low prevalence in other regions (e.g. Uganda and Zimbabwe) [240]. In populations in which HIV is less prevalent, these HLA variants are likely selected against due to their associations with many autoimmune disorders [241, 242].

Perhaps the most impactful host factor in determining HIV disease progression is the CCR5 gene itself. Discovered in 1996, a 32 base pair deletion creating a premature stop codon in CCR5 generates a nonfunctional receptor that HIV is unable to bind to [243]. Since engagement with a chemokine coreceptor is essential for HIV entry, individuals that are homozygous for the Δ32 mutation are resistant to infection by CCR5-tropic viruses. Since CCR5-tropic viruses make up nearly all transmitted viral strains, these individuals have been documented to resist HIV infection even after repeated, high-risk exposures.
However, rare instances have been observed in which CXCR4-tropic viruses can infect Δ32 homozygotes [244]. Individuals who are heterozygous for the mutation, possessing both full length and truncated versions of the CCR5 gene are semi-resistant to HIV infection, with reductions in infection rates near 50%. Researchers soon identified the mechanism of this partial inhibition; due to the required dimerization of CCR5 receptors while being transported to the cell surface. A truncated copy of the CCR5 receptor is unable to dimerize, thus greatly reducing cell surface CCR5, rendering the T-cells in these patients largely non-permissive [245]. Additionally, upon infection, heterozygotes progress to AIDS more slowly, delaying its onset by about 2-3 years relative to homozygous wildtype CCR5 patients [246, 247].

While the Δ32 mutation is highly advantageous in preventing or limiting HIV infection, it remains a relatively rare allele. Depending on ethnicity, heterozygotes make up about 10% of the population in Europe, the continent with the highest frequency (about 1% homozygotes). This rate varies depending on ethnic background within Europe, with the highest levels of the mutation identified in people of Finnish descent (16% heterozygotes) and the lowest in Sardinian people (4% heterozygotes) [248]. All other continents have significantly lower rates of the mutation. The origins of the Δ32 mutation are hotly debated but appear to be recent and linked to reductions in pathogenicity of other human diseases, namely the bubonic plague (Yersinia pestis) or smallpox [249]. Conversely, individuals with Δ32 mutations also appear to be at greater risk of infection or mortality from flaviviruses, demonstrating the positive and negative selection dynamics on this allele [250, 251].
Figure 1.5) Summary of HIV Pathogenesis. Red lines represent CD4 T-cell counts per mL of blood in the circulation (solid red) and CD4 T-cell density in the lymphoid tissues (dashed line) over the course of infection. HIV viral load (black solid line) is also shown in infectious units per mL of blood (limit of detection at about $10^2$ infectious units) with major disease features labelled.
1.10. Group M Subtype Pathogenesis

Infections by different HIV Group M subtypes have long been considered to differ in pathogenicity, however exactly why and to what degree remains poorly understood. A series of epidemiological studies report differing patterns in the rates of disease progression and disease severity across Group M subtypes [252-258]. Unfortunately, the patterns suggested by each study directly contradict others, and seem to be swamped by confounding variables, poor controls, and conclusions that are not adequately supported by the data.

The laboratory of Dr. Eric Arts has published direct comparisons of the relative replicative fitness via primary viral competition assays across all Group M subtypes (Figure 1.6) as well as between Groups M, N, O and HIV-2 (Figure 1.7) [255, 256]. Replicative fitness, in this sense, refers to a virus’s ability to replicate in a given environment. During these competitions, a single culture of primary activated CD4+ cells is infected with equal amounts of two viruses of differing groups or subtypes. These infections progress through multiple rounds of replication before viral nucleic acid is isolated and sequenced using high-throughput, next-generation sequencing (NGS) technology [255-257]. These data demonstrate the greatly reduced (up to 1,000 fold) replicative fitness of subtype C relative to subtypes A, B and D (Figure 1.6C).

Intragroup competitions also show that subtype C clones more closely cluster with HIV-2 isolates than with other Group M subtypes (Figure 1.7D). The well characterized reduction in pathogenic capacity of HIV-2 viruses strongly suggests similarly slow rates of disease progression during subtype C infections [39-41]. It has also been observed in subtype B infections that a greater proportion of untreated, infected individuals that do not progress to AIDS (Elite controllers) are infected with less fit viruses [259, 260]. These data highlight the direct effect of viral replicative fitness on patient disease progression and outcome. Elite controllers have also been found to harbor viruses with envelope proteins that interact with their cell surface receptors with low affinity and therefore infect cells with greatly reduced efficiency [261]. These results demonstrate a direct link between viral entry kinetics, fitness and viral suppression. However, these studies are unequipped to associate
reductions in viral fitness with pathogenicity and rates of disease progression in non-elite controlling patients.

While uncommon, a few experimental explorations of HIV-1 Group M subtype pathogenicity have been previously published. The earliest of these investigations suffered from errors of convenience recruiting all HIV+ patients available in a given study site [94]. These recruitment tactics were typically recognized as less than ideal but were necessary to generate the statistical power necessary to tease apart an interaction as complex as immune system failure and HIV disease progression. The flaw in this study design is based around calculations of the time of infection based on clinical measurements like viral load and CD4+ cell counts. The variability due to host and environmental factors makes these estimates heavily biased, far outweighing any subtler effects of viral genotype.

Other studies, while properly controlling for date of infection, failed to generate the necessary statistical power for robust analyses. In a study published in the Journal of Infectious Disease in 1999, Kanki et al. recruited all registered sex workers in Dakar, Senegal for biannual HIV monitoring (study ran from 1985-1997). Of the nearly 2000 women enrolled in the study, 81 seroconverted during the study period and 11 of those reached clinical AIDS, defined as CD4 T-Cell counts below 200/mL. The study concluded that subtype C displayed a rapid disease progression simply because 3 of the 4 enrolled patients infected with subtype C viruses reached clinical AIDS during follow-up, their only measure of disease progression. Subtype A, the most represented subtype among seroconverters in the study, showed an eight-fold decrease in the likelihood of reaching clinical AIDS relative to non-A subtypes. This finding was soon refuted by multiple studies in Europe and Thailand showing either no difference in disease progression between subtypes or increased rates of pathogenesis in subtypes A and CRF01_AE relative to subtype B. Most recently, analyses in the Rakai cohort in rural Uganda demonstrated rapid disease progression in patients infected with subtype D relative to all other subtypes in the region. The rapid disease progression by subtypes B and D are the most pronounced and frequently reported, while subtypes A and C are typically considered less aggressive. These findings, like most studies quantifying disease progression, are still actively debated [94].
1.11. Thesis Overview and Rationale

1.11.1. Chapter 2 – Infecting HIV-1 Subtype Predicts Disease Progression in Women of Sub-Saharan Africa

The broader HIV diversity, that is comparisons between HIV-1 and 2 and between the groups of HIV-1, has clear implications in disease progression. This differential pathogenicity has largely relegated non-HIV-1 Group M infections to small regions of Africa and limited the numbers of transmissions. What is unclear however, is whether the genetic diversity within Group M confers a similar range of disease progressions, what that pattern is, and how it impacts the cellular replicative cycle of HIV. Previous work has shown that this diversity manifests in a distinct pattern of replicative fitness in which subtype D and A outcompete subtype C in activated PBMCs. While low replicative fitness has been linked to viral control, there are no published direct measurements of the rate of CD4+ T-cell loss between multiple Group M subtypes in a single human population.

Herein we seek to explore the role of HIV-1 Group M diversity on the diseases they cause, whether there is a continued link to viral replicative fitness, and whether the association with viral attachment and cellular entry hold when modelling CD4+ T-cell declines. In response to other published work we will also investigate viral load dynamics over the study period, specifically how diversity may impact circulating viral levels in both acute and chronic phases of disease.

Cellular entry, the first stage of viral replication, is most closely linked with replicative fitness, but has not been directly implicated in severity of HIV disease. We will use three novel assays to interrogate viral rates of cell entry, affinity for the CD4 receptor, and induction of multi-cell fusion.

Despite decades of research, the diversity of HIV-1 Group M around the world is rarely considered. Viruses of subtype B from North America makes up nearly all published work. Subtype diversity clearly will impact cure strategies, testing efforts, antiretroviral prioritization and will direct future spread of the pandemic.
1.11.2. Chapter 3 - HIV-1 Group M subtypes deplete memory and naive T-cell populations at different rates

Previous investigations into CD4+ T-cell decline will be expanded to interrogate the dynamics of specific subsets with a focus on long-lived memory populations. Flow cytometry separations of archived cohort samples will be used to measure CD4+ and CD8+ T-cell dynamics, as well as CD8+ cell patterns of activation. These memory populations, when infected, allow for HIV to outlast even the best treatment regimens and rapidly rebound upon cessation. The role of HIV-1 Group M diversity on memory T-cell depletion has not been explored and will be a key determinant in the success of future cure efforts. These analyses seek to identify T-cell populations that are differentially depleted between subtypes, highlighting the differences in pathogenicity identified in Chapter 2.

Finally, in response to other published work, we will examine different measurements of pathogenicity, that is time to unenrollment from the cohort as a proxy for the time to clinical AIDS. This methodology has been utilized previously in reporting contradictory findings to our own. We will use a Kaplan Meier estimator to track unenrollment based on infecting HIV-1 Group M subtype and statistically compare between groups.

The future of HIV research will be disproportionately focused on memory T-cell subsets. These populations harbor integrated provirus for decades, evading combined antiretroviral therapy (cART). If they are to be eradicated, it is critical to understand the scope of the global challenge. Most measurements of memory subsets only focus on subtype B viruses while we will use a diverse group of viruses from Uganda and Zimbabwe to compare pathogenicity.
Figure 1.6) Ex vivo replicative fitness derived from pairwise competitions between HIV Group M subtypes (A) Each CCR5 tropic primary isolate was competed against all of the other CCR5 tropic isolates. (B) Each CXCR4 tropic isolate was competed against all of the other CXCR4 tropic isolates. Fitness differences for the pairwise competitions were determined based on relative quantities of viral nucleic acid isolated post competition. Green boxes represent competitions in which subtype C was outcompeted by a non-subtype C isolate while Red boxes represent subtype C outcompeting non-subtype C isolates. (C) Results from the above matrices plotted by a given isolates replicative fitness calculated from all competitions. The lower left quadrant represents the least fit viruses while the upper right quadrant represents the most fit. Figure adapted with permission from [255].
Figure 1.7) Mean relative fitness values (0=less fit, 2=more fit) for intratype/intragroup and intertype/intergroup competitions in PBMCs involving both CCR5 and CXCR4 tropic (A) HIV-1 group M, (B) group O and (C) HIV-2 primary isolates. (D) Intertype/intergroup versus intratype/intragroup mean relative fitness of all CCR5 isolates. Clustering represents viruses of similar fitness, with the most fit viruses in the top right quadrant and the least fit viruses in the lower left quadrant. Figure adapted with permission from [256].
1.12. References


Chapter 2
Infecting HIV-1 Subtype Predicts Disease Progression in Women of Sub-Saharan Africa

2.1 Background

Human immunodeficiency virus type 1 emerged in the human population from the Congo River basin shortly after the turn of the 19th century following an established zoonotic jump of simian immunodeficiency virus from *Pan troglodytes troglodytes* [1]. Sixty to seventy years of deforestation, exploitation, and urbanization likely led to evolution, adaptation, and prevalence of HIV in the human host as well as the eventual spread of HIV across Africa [2]. Distribution of HIV-1 across the globe over the past 30-35 years can still be traced to founder events with primordial HIV strains from sub-Saharan Africa [1-3].

Our knowledge of HIV-1 disease and treatment is still largely limited to the 3 million subtype B HIV-1 infections that dominate North America and Europe despite the mixture of HIV-1 subtype A, C, D, and circulating and unique recombinant forms (CRFs and URFs) responsible for 30 million historic and 28 million current infections in sub-Saharan Africa [1-3].

The extreme genetic diversity between HIV-1 subtypes has phenotypic consequences in vitro including differential HIV-1 mRNA transcriptional control [4, 5], protease activity [6], integration site selection [7], MHC class I downregulation [8], and entry efficiency [9]. Overall, subtype C HIV-1 isolates display a significant reduction in replicative fitness in primary human CD4+ T-cells relative to HIV-1 isolates of any other subtype [10-12]. Despite these studies, comprehensive analyses on the natural history of non-subtype B HIV infections are still not available to guide treatment and prevention strategies in the >28 million people currently living with HIV-1 in sub-Saharan Africa. A few longitudinal and cross-sectional studies on patients in sub-Saharan Africa have suggested that subtype A, C, D and URFs/CRFs HIV-1 infections could have different disease courses and treatment outcomes as compared to subtype B infected populations [13-19]. Faster progression to AIDS and higher treatment failures were observed in subtype D versus subtype A infections [15, 16, 20].
From 1999-2003, over 4,400 HIV-negative women in Uganda and Zimbabwe were enrolled in the Hormonal Contraception and the Risk of HIV Acquisition (HC-HIV) Study [21], during which 303 women were identified with incident HIV infection and participated in the Hormonal Contraception and HIV-1 Genital Shedding and Disease Progression among Women with Primary HIV Infection (GS) Study [22-24]. The 76 subtype A, 177 subtype C, 31 subtype D and two URF HIV infected women (286 of 303 women) were followed for an average of 5 years, currently the largest natural history cohort of non-subtype B infections. Consistent with WHO recommendations at the time of the study, treatment was provided when HIV-infected participants reached CD4 cell counts below 200/ml on two consecutive tests or were diagnosed with stage IV or advanced III disease (WHO Classification). Antiretroviral therapy (ART) was not yet routinely available in these countries during the study period, however, the study team felt that it was ethically imperative to provide treatment to women participating in the project and committed study resources to provide that treatment. This study was designed to monitor immunologic and virologic parameters and to compare these biomarkers of disease progression to country of origin, contraceptive use, infecting subtype, and phenotypic properties of the virus.

2.2 Methods

2.2.1 Participants and clinical tests

A complete description of the Ugandan and Zimbabwean participants is provided in the Supplementary methods and previously described [23, 24]. Women who became HIV infected while participating in the Hormonal Contraception and Risk of HIV Acquisition Study in Uganda and Zimbabwe [21] were enrolled upon primary HIV-1 infection into a subsequent study, the Hormonal Contraception and HIV-1 Genital Shedding and Disease Progression among Women with Primary HIV Infection (GS) Study [22-24]. Blood and cervical samples were collected every month for the first six months, then every three months for the first two years, and then every six months up to 9.5 years. Women who had CD4 lymphocyte counts of 200 cells/ml and/or who developed severe symptoms of HIV infection (WHO clinical stage IV or advanced stage III disease) were offered combination ART (cART) and trimethoprim-sulfamethoxazole (for prophylaxis against bacterial
infections and Pneumocystis jiroveci pneumonia). In addition, information was collected at every visit related to changes in sociodemographic information, sexual behavior, sexually transmitted and reproductive tract infections, opportunistic infections, and diet.

Following primary infection, 112 Uganda and 174 Zimbabwean women were followed for an average 1,826 and 1,899 days (respectively) (Supplementary Figure 2.1) for a maximum of 3,453 days (9 ½ years) (Supplementary Figure 2.1). Antiretroviral treatment was initiated in 23% of the women in both countries but 100 days sooner on average in the Ugandan versus Zimbabwean women (1,458 versus 1,558 days) (Supplementary Figure 2.1). As described [22], cervical and plasma viral loads were determined with the Roche Amplicor HIV-1 Monitor Test, version 1.5 using cryopreserved samples from visits from every three months. CD4 T-lymphocyte counts were determined by standard flow cytometry using FACSCalibur (Becton Dickinson, Sparks, MD, USA).

2.2.2 Models to determine viral load increases and CD4 cell declines.

A marginal model with the generalized estimating equation (GEE) approach including autoregressive error structures (accounting for repeated measurements on the same individual) was used to determine the plasma viral load changes and compare rates of CD4 cell decline [22]. Multivariable analyses included demographic, sexual, contraceptive and medical history data and underwent stepwise model simplification with a significance threshold of p≤0.05. All analyses used Stata Version 14 (StataCorp LP, College Station, TX).

2.2.3 DNA sequencing and subtyping.

DNA was extracted from patient PBMC samples obtained at the seroconversion visit and every 3 months post HIV infection. Primers specific to the protease (PR) and polymerase regions of reverse transcriptase (RT), and C2-V3 envelope (env) coding regions were used to PCR 300, 800, and 450 nt regions as described [12] in a Virology Quality Assurance (VQA) certified laboratory in Kampala, Uganda [22]. The HIV-1 sequences were edited using BioEdit v7.0.4 and PR-RT coding regions were uploaded into the Stanford
University HIV Drug resistance database (http://hivdb.stanford.edu) to obtain drug resistance profiles. Phylogenetic alignments were performed using maximum likelihood methods (MUSCLE [25]) and trees constructed using SEAVIEW 4 [26] and FigTree 1.4 (http://tree.bio.ed.ac.uk/software/figtree/). Each participant PR, RT, and Env sequence was aligned to curated set of subtype A, B, C, D, G, and CRFs HIV-1 reference sequences (hiv.lanl.gov).

### 2.2.4 Virus cloning and propagation

The entire gp120 coding region and the extracellular domain of gp41 (referred to as Env DNA) was PCR amplified with the ENV REC primer set from PBMC DNA derived from the patient samples at the seroconversion visit. Briefly, pREC_nflΔenv/URA3 was linearized with SacII (Fermentas) and transformed into Saccharomyces cerevisiae MYA-906 cells (ATCC) using lithium acetate along with Env PCR products [27]. Following homologous recombination, plasmids were extracted from the yeast cells growing on plates containing minimal media, 5-Fluoroorotic Acid (5-FOA) but lacking leucine. The yeast derived plasmid DNA was then transformed into electrocompetent Escherichia coli Stbl4 cells (Invitrogen) and amplified for high purity plasmid purification using the Qiagen midiprep kits. Detailed procedures of the yeast recombination/gap repair for HIV-1 cloning has been described [27]. The resulting pREC_nfl plasmid contains a subtype B NL4-3 backbone with the patient derived gp120/gp41 ecto coding region. This plasmid produces virus-like particles and can be used to access Env glycoprotein function and to measure the rate of host cell entry in virus [28] and cell-to-cell infections [29]. pREC_nfl (containing the patient derived Env gene) can also be co-transfected with pCMV_cplt complementing vector in 293T cells to produce replication-competent virus as describe and used for the studies on replicative fitness [27].

### 2.2.5 Reverse Transcriptase Assay

To quantify viral particles, we utilized a previously published radioactive nucleotide incorporation assay to measure viral reverse transcriptase [262, 263]. Briefly, 10µL viral concentrates were added to a 96 well plate. 25µL RT buffer was added to each well of the plate (50 mM Tris-HCl (pH 7.8), 75 mM KCl, 2 mM DTT, 5 mM MgCl₂, 5 µg of poly(rA)
per ml, 0.5% NP-40) and allowed to incubate for 10 minutes at room temperature. 1µL radiolabeled thymidine triphosphate is then added (10-mCi/ml [α-³²P] TTP). Plates were then incubated at 37°C for 2 hours. The synthesized radioactive DNA is then blotted onto DEAE filter mats (Wallac) and dried on a hotplate. The mats are then washed in SSC buffer (x5) and 85% Ethanol (x2) and dried again to remove unbound radiation. Finally, these radioactive mats are placed in plastic bags and exposed on a cassette which are read on a Phosphimager (GE Storm 840). Quantification of radioactive spot intensity was done using ImageJ. A more detailed protocol is available in previous publications [262, 263].

2.2.6 Replicative fitness

47 primary HIV-1 isolates of subtypes A (12), C (27), and D (8) derived from patients from cohorts from Europe, South America, Asia, and all regions of Africa were titered on PHA-activated, IL2-treated PBMCs of HIV-negative donors using the standard Reed-Munch TCID₅₀ determination [30] then competed against 25 subtype B HIV-1 in the same PBMCs. We also competed two reference subtype B HIV-1 isolates, B-QO and B-K44 against chimeric viruses derived from the acute/early infection samples of 5 subtype A, 3 subtype D, and 5 subtype C infections of this cohort. The relative production of two viruses in each competition was measured by a radiolabeled heteroduplex tracking assays or using next generation sequencing [10-12]. Assay sensitivity is 100-fold differences in replicative fitness expressed as percentages.

2.2.7 Cell-to-cell infections

Veritrop involved the transfection (Fugene 6, Promega) of the pRECnfl constructs containing patient Envs into 6.5x10⁴ HEK293T cells [29] then cultured for 8 hours. To synchronize Env engagement and cell fusion, the effector HEK293T cells were placed on ice before being spun onto 6.5x10⁴ of U87 CD4+ CCR5+ target cells (500 x g for 5 minutes at 4°) transfected with a HIV tat-responsive luciferase plasmid (pDM128fluc). Co-cultures were then brought to 37°C and allowed to form syncytia for 12 hours. Fully inhibitory concentrations of Enfuvirtide (3uM) were added at discrete time points (t=4, 4.75, 5.5, 6.25 and 7 hours post combination) to replicates of each construct throughout this incubation to
stop fusion events. Medium was removed after 12 hours and the Bright-Glo Luciferase Assay System (Promega) was used to quantify luciferase production on a plate reader (Biotek Synergy 2). Drug treated cells were compared to untreated controls to calculate percent maximal fusion. Relative rates of fusion by Env subtype were statistically compared based on time required to reach 50% maximal fusion (ANOVA, STATA Version 14, College Station, TX).

2.2.8 Virus entry into host cells

Cotransfection (Fugene 6, Promega) of the pMM310 plasmid (NIH AIDS Repository, cat #11444) with the pRECnfl patient constructs into 3.0x10^5 HEK293T producer cells generated viruses containing the HIV-1 accessory protein Vpr fused to E. coli β-lactamase (BlaM). The viruses were purified via ultracentrifugation and quantified using p24 ELISA (NCI Frederick Cancer Research and Development Center, AIDS Vaccine Program). 1.5x10^4 U87-CD4-CCR5 cells were plated on black, clear-bottom plates and loaded with CCF2-AM dye (Live-BLAzer, ThermoFisher) for 60 minutes. The pRECnfl/BlaM viruses were added to the culture at a multiplicity of infection of 2. Cells were placed on a plate reader (Synergy 2, Bio-Tek) and virus entry was quantified every 15 minutes by comparing emission at 460nm (cleaved dye) relative to emission at 530nm (uncleaved dye) following excitation with a 405nm laser. Dye cleavage curves were normalized relative to cell-free and virus-free controls, means calculated for each subtype and statistically compared based on time required to reach 75% maximal viral entry (ANOVA, STATA Version 14, College Station).

2.2.9 Competitive CD4 ELISA

96-well plates were coated with Goat Anti-Human IgG Fc Ab 1:10,000 and 100µL was added per well. These plates coated overnight at 4°C. Following the incubation, the plates were washed three times with 0.05% Tween-20 (Sigma) in DPBS. 100µL of 10% FBS in DPBS was added to the plates to block and they were incubated at room temperature for 1 hour. Washes were repeated. 100µL of IgG-anti-Fc antibodies at 0.2 μg/mL in DPBS were added to each well and incubated at room temperature for 1 hour. Washes were repeated. Blocking steps were repeated for 1 hour at room temperature. 100µL of CD4-Fc fusion
protein diluted in DPBS was added (20μg/mL) to all wells, followed by repeating wash steps. 50μl of purified virus was added in triplicate for 5 timepoints, 15 wells per sample. Plates were then sealed with plate seals and incubated overnight at 4°C. Following the incubation, wash steps were repeated. 100μL of soluble CD4 (sCD4) diluted in DPBS was added (20μg/mL) to all but the non-competed controls which received 100μL DPBS. Competitions incubated at 37°C for 4, 12, 24, and 48-hours post-addition of sCD4. Wells were washed with plain DPBS to remove unadhered virus and filled with 100μL DPBS to prevent desiccation. Once all competitions finished the DPBS was aspirated and each well received 25μL of lysis buffer and the quantitative reverse transcriptase assay was employed to measure remaining bound virus. Results from a subsample of these trials are shown in Supplementary Figure 2.6. To conserve reagents, future experiments would only use the 24-hour timepoint, where differences between subtypes was found to be most pronounced. Comparisons between subtypes were made with ANOVA using Tukey multiple comparison tests, significance p<0.05. Schematic of the assay is shown in Figure 2.6A.

2.3 Results

2.3.1 Monitoring disease progression in the absence of treatment

Newly infected Ugandan (N=112) and Zimbabwean (N=174) women, 71% (N=203) enrolled within 18 weeks of the infection date were followed for an average of 5.1 years up to 9.5 years in the absence of combination antiretroviral therapy (cART), initiated when CD4 cell counts dropped below 200/mm$^3$ or concurrent counts below 300/mm$^3$ (Supplementary Figure 2.1). Complete sociodemographic, clinical chemistries, and other clinical data has been reported [22-24, 31].

Lower CD4 T-cell levels were reported in uninfected women and in our early infection cohort of Zimbabwean women as compared to Ugandan women [31]. Despite this difference between countries, declines in CD4 T-cells was 2-fold slower in the Zimbabwean women population than in the Ugandan women in the absence of treatment (Figure 2.1a&b) (GEE model; P<0.001).
Figure 2.1) The graphs around the map display the number of people living with HIV-1 between 1980 and 2015 in the four UNAIDS Regions with the greatest number of infections as well as globally. The distribution of subtypes in 1990 and 2015 are shown in the inset pie graphs, scaled by the number of infections. Relative numbers of subtype C infections per UNAIDS Region are reported on the pie graphs and via red shading on the map. Data compiled from the World Health Organization, UNAIDS and published literature (References in Supplementary Files).
Virus levels at both the set point and all future time points in the plasma and endocervix did not significantly differ by country (Figure 2.2a) [22]. The cohort was also analyzed for rapid progressors (CD4 decline to below 200 cells/ml within 2 years of infection) and controllers/non-progressors (stable CD4 cell counts above 350 with viral loads <2000 copies/ml in plasma for more than 3 years post-infection) in the absence of treatment. Approximately 7.4% (n=13) and 8.1% (n=9) were classified as rapid progressors in Zimbabwe and Uganda but controllers were observed in less than 3% (n=3) of Ugandan women versus 10% (n=18) of Zimbabwean women (Figure 2.1d). When rapid progressors and controllers were removed from the analyses, the CD4 declines were still significantly slower in Zimbabwean versus Ugandan women (P<0.001). As reported by numerous studies, the rate of CD4 decline is directly associated with HIV disease progression [1, 3].

2.3.2 Possible factors contributing to country-specific difference in disease progression

To explain these differences in CD4 T-cell decline between countries, we analyzed numerous factors previously described as correlates of disease progression. Opportunistic infections and secondary infections (malaria, TB, various STIs) were not significantly different in the cohorts and each infection was treated accordingly upon diagnosis. Other factors such as diet, recurrent sexually transmitted diseases (chlamydia, trichomonas, HSV-2, bacterial vaginosis), smoking, sexual behavior, depomedroxyprogesterone acetate (DMPA) use, and combined oral contraceptives (COC) use did not show significant differences in HIV-1 disease progression between countries [22-24, 31, 32]. There may be a perceived difference in human genetics between the two countries that may explain this differential disease progression. Our informed consent and institutional review board (IRB) approvals did not permit genetic testing in these patients. These women self-reported being members of the Shona tribe of Zimbabwe and Buganda tribe of Uganda which are both of Bantu origin which has the highest HLA diversity among humans as well as lower frequencies of protective HLA B*57 (3-5%) and B*27 alleles (<1%) [27, 33, 34] than in Caucasian populations (7.0 and 9.2%, respectively) (www.allelefrequencies.net/). Thus, it is unlikely that any of these genetic polymorphisms could explain the striking differences in CD4 decline between HIV-infected Ugandan and Zimbabwean women.
2.3.3 Infecting HIV-1 subtype correlates to disease progression

The infecting HIV-1 isolates in these women were subtyped using the HIV-1 PR, RT and C2-V3 gp120 env coding regions (Supplementary Figure 2.2&3). As expected, the Ugandan women, all residing in Kampala, were predominantly infected with subtype A (68%, n=76) followed by subtype D (28%, n=31), 3 cases of subtype C, and 2 intersubtype A/D recombinants (Supplementary Figure 2.3a&b). All 174 Zimbabwean women were infected with HIV-1 subtype C. Based on the infecting subtype, women with subtype C HIV-1 infections had CD4 T-cell declines (-0.489 cells/week, GEE model) 2.5-fold and 1.6-fold slower than with subtype D and A infections, respectively (-1.231 cells/week and -0.781 cells/week, GEE model) (Figure 2.1c). The faster CD4 T-cell declines in subtype D infections also resulted in more patients (45%, n = 14) requiring cART than in women infected with subtype A and C (28% and 32%) (Supplementary Figure 2.1b). Slow or non-progression (defined as controllers) was not observed among the subtype D infected women as compared to the 4% (n=3) and 10% (n=18) in the subtype A and C infected women, respectively. In contrast, subtype A, D, and C infected women had similar frequencies of rapid progressors (8.5, 11, and 7.5%, respectively) (Figure 2.1d). Again, viral levels at set point (Figure 2.2c) in plasma or endocervical mucosa or in plasma during disease (Figure 2.2a,b,d,e&f) did not differ by subtype (statistical analyses in Figure 2.2g).

To illustrate subtype-specific differences, CD4 T-cell and viral RNA levels were plotted for 9 subtype C controllers, 9 subtype C typical progressors, and 9 subtype D typical progressors (Figure 2.3) (subtype A, C, and D rapid, slow, and typical progressors are shown in Supplementary Figure 2.3). The controller status in subtype C infected patients is quite evident with less than 0.03 CD4 cells lost per week (based on all 13 controllers) (Figure 2.3a) as compared to the 0.48 lost per week (linear regression model or 0.49 cells/week, GEE) in the 148 typical subtype C progressors (Figure 2.3b). This rate of CD4 T-cell decline in subtype C is obviously slower than that observed in the typical subtype D (Figure 2.3c) and subtype A (Supplementary Figure 2.3) progressors (-1.0 and -0.60 CD4 cell loss/week, respectively, linear regression). Viral load increases (following establishment of viral set point) were similar in all the subtype A, C, and D progressors (Figure 2.3d,e,f and Supplementary Figure 2.3) but viral loads remained the lowest in the
subtype C controllers with an actual decrease over time (-4 HIV-1 RNA copies/ml/week; Figure 2.3d).

### 2.3.4 Phenotypic differences between HIV-1 subtypes

For this study, we analyzed 143 published and 42 unpublished dual HIV-1 competitions involving 47 subtype A, D, and C HIV-1 isolates against 25 subtype B isolates (Figure 2.4a&b). In these dual infection/competitions, absolute virus production can vary 100-fold in PBMCs of different human donors but the relative production (i.e. fitness) of one virus versus another typically varies less than 10% [10-12]. Subtype C HIV-1 isolates are clearly less fit in PBMCs than subtype A and D HIV-1 isolates (P<0.002) when competing against subtype B HIV-1 (Figure 2.4a). Subtype C viruses were not detected (i.e. 100-fold less fit) in 60% (n=74/123) of the competitions despite the fact that these same subtype C viruses were readily detected in mono-infections or competitions against other subtype C isolates [10-12]. These competitions of subtype A, C, and D versus subtype B HIV-1 suggest a fitness order of HIV-1 subtype B = D > A > C in human PBMC or primary CD4 T-cells (Figure 2.4) [10-12]. We have previously reported that the replicative fitness of primary HIV-1 isolates is dominated by the env gene and the rate of cell entry, i.e. the rate limiting step in HIV-1 replication [9, 11]. Five subtype A, 5 C, and 3 D HIV-1 env genes (from 13 patients) derived from acute/early infection were used to produce chimeric virus (subtype B NL4-3 backbone) which were then competed against the B-Q0 and B-K44 subtype B reference viruses (Figure 2.4c&d, respectively). The subtype D Env chimeric viruses were able to compete with both the B-Q0 and B-K44 reference viruses whereas the subtype A Env chimeric viruses were less fit (Figure 2.4c&d). In contrast, subtype C Env chimeric viruses derived from acute/early infection had extremely low replicative fitness or were completely outcompeted by the B-K44 and B-Q0 reference viruses. Thus, both the HIV-1 subtype C found at acute/early infection and in chronic disease are less fit than subtype A and D HIV-1 co-circulating in these Bantu populations.

HIV-1 infection can be mediated by cell-to-cell virus transfer or by direct virus infection. We examined cell-to-cell infection using the more physiological Veritrop system where transfection of the pREC-nfl HIV-1 vector into 293T effector cells can produce HIV-1 particles (non-infectious) and can mediate fusion with the U87.CD4.CCR5 target cell [28].
The absolute level of cell-to-cell HIV transmission was similar with 34 acute/early env genes in pREC-nfl over a 12 h incubation (Supplementary Figure 2.5). Cell-to-cell fusion kinetics was then measured using a time-of-drug-addition experiment where Enfuvirtide, an inhibitor of HIV-1 entry, was added at different times post effector/target cell co-incubation (Figure 2.5a). Subtype C HIV-1 Env glycoproteins derived from acute/early infection mediated significantly slower rates of cell-to-cell HIV-1 infection than did subtype A (P<0.001) or subtype D (P<0.001) Env glycoproteins based on the T\(^{1/2}\) of Enfuvirtide inhibition.

Next, we measured the rate of free virus entry into host cells (U87.CD4.CCR5) using these same 34 acute/early env genes in pREC-nfl. In this case, pREC-nfl was co-transfected with pMM310 BlaM-Vpr to produce HIV-1 particles harboring a Vpr-BlaM fusion protein (Figure 2.5b). Upon de novo infection, the HIV_nfl is fully capable of host cell entry which releases the Vpr-Beta-lactamase to cleave the fluorescent CCF2 dye and change fluorescent spectra [31] (Figure 2.5b). The rate of virus entry into host cells was the slowest with the 16 HIV_nfl harboring the acute/early subtype C envelopes as compared to those carrying the subtype A (P<0.05) and subtype D envelopes (P<0.001) (Figure 2.5c&d).

This cohort demonstrated large fitness reductions in the infecting subtype C viruses which correlated with reductions in entry efficiency. Work developing assays to clarify Group M virus entry rates and CD4 affinities in different CD4+ T-cell memory cell populations is ongoing. Preliminary data comparing CD4 attachment receptor affinity is shown below. These experiments were pursued to explore previous finding that HIV-1 Group M subtypes differ in their entry rates and rates of HIV induced cell-cell fusion. To clarify the stage of viral entry that differs most between subtypes, a novel competitive CD4 binding ELISA was used to quantify the first step of viral entry between viruses from the cohort (Figure 2.6A). Initial experiments were used to establish a protocol and competition timepoints (Supplementary Figure 3.4). These explorations were used to set a 24-hour competition period to identify subtype differences. Significant differences after a 24-hour competition were found between subtypes C and D (p<0.05), and subtypes A and D (p<0.001, T-tests) (Figure 2.4B). Subtypes C and A did not appear to differ in their affinity for CD4. Subtype
D showed the greatest affinity for CD4, only losing about 8% of the bound virus when competed with sCD4. Subtypes A and C lost 30.5% and 48% of bound virus, respectively.

2.4 Discussion

By the 1980s, all the HIV-1 subtypes had emerged, were circulating and recombining in the Congo basin as well as spreading to neighboring countries/regions [1]. Various HIV-1 subtypes were introduced in multiple geographic regions (South Africa, Zimbabwe, Brazil, India, etc) [3] by the late 1990s and yet, subtype C HIV-1 rose in prevalence faster than any other subtype in the heterosexual population (<10% in early 1990s to >50% today) (Arien et al., 2007) (Figure 2.1). We have previously reported that HIV-1 subtype C isolates had lower replicative fitness in human T-cells and macrophages than other group M subtypes [10-12] but could only speculate that HIV-1 subtype C may cause slower disease progression [3]. Aside from regions in Brazil, Tanzania, and Kenya, HIV-1 subtype C typically dominates in regional pandemics and does not co-circulate at high frequencies with other HIV-1 subtypes in human populations [3]. As a consequence, comparing disease progression related to different HIV-1 subtype infections in a single country or region is very difficult. Thus, we screened for and recruited 300 AHIs in Ugandan and Zimbabwean women and then followed the natural history of these HIV infections for 5–9 years in the absence of treatment. With this cohort in Uganda and Zimbabwe, we have a population of only women, all of Bantu origin, all recruited within AHI (following heterosexual transmission), and finally, representing the subtype A, C and D HIV cohorts for sufficient statistical power to measure differences in disease progression. Independent statisticians at two different institutions (FHI 360 and Case
Figure 2.2) CD4+ cell declines in HIV-1 infected Ugandan (n =112) and Zimbabwean women (n=174) during the natural course of disease. (a) The mean loss of CD4 cells per mm³ per week of infection in Ugandan and Zimbabwean women and (c) in those women infected with subtypes A, C, and D. (b) The percentage of rapid progressors and slow progressors/controllers in Ugandan and Zimbabwean women and (d) in those women infected with subtypes A, C, and D. Rapid progressors are defined as HIV-1 infected women with a drop to 200 CD4 cell counts/mm³ within 2 years of infection and sustained viral RNA loads greater than 2000 copies/ml. Slow progressors/controllers had viral RNA loads stable CD4 cell counts above 350 and viral loads < 2000 copies/ml in plasma for >3 years.
Western Reserve University) analyzed the cohort data presented herein. The 2-fold difference in disease progression between these two countries was not attributable to diet, secondary/opportunistic infections, sexual habits, or age [24]. Of course, we could not screen for presence or absence of all pathogens, sociodemographic or tribal/population differences but the questionnaires administered by medical officers at each patient visit was extensive as was the tests for various clinical chemistries and other health indicators. Examples of the questionnaire are included in the attached Supplementary Files. We are currently interested in screening the microbiota in the vaginal tract of these women to assess possible difference which may be associated with disease progression but again, we do anticipate differences that segregate by country given the similar diet and diverse human genetics. Of all the parameters tested, only HIV-1 subtypes could clearly delineate differences in the rate of CD4 cell decline in blood. Infection with subtype C HIV-1 resulted in the slowest declines in CD4 T-cell counts (0.489 cells/week) as compared to subtype A (0.781 cells/week) and then subtype D HIV-1 infection with the most rapid declines in CD4 T-cell counts (1.231 cells/week).

Like other natural history cohorts, rapid disease progression was observed at a 7–9% frequency regardless of HIV-1 subtype but slow progressors/controllers appeared only in subtype A and C infections. The differential rates of CD4 T-cell declines (ΔNΔC) was still significant when rapid progressors and controllers were removed from the analyses. We have proposed that a combination of “good” host genetics (e.g. HLA B27 or B57) and infection with HIV of low replicative fitness may result in “elite” HIV control [35] i.e. a rare condition in Africa but closely approximated by the 13 subtype C infections with no declines in CD4 cell counts and b103 copies/ml of virus for N 5 years of infection (Figure 2.3a & f).

Based on previous human genetic studies across Africa [27, 33, 34, 36, 37] (www.allelefrequencies.net/), we now know that the Bantu population has the greatest genetic diversity of Homo sapiens. Low prevalence of “HIV protective” polymorphisms/alleles in the Shona and Buganda tribes (both Bantu) (e.g. <5% of CCR5Δ32, CCR2a 64I, HLA-B57 and B27) could not explain the dramatic differences in disease progression. Interestingly, there were no significant differences between countries
Figure 2.3) Comparing disease progression in a subset of 56 women in Uganda and Zimbabwe infected by different subtypes. (a)(b)(c)(d)(e) CD4 cell counts/ml were plotted over time in subtype C infected women defined as slow progressors/controllers (a), as typical progressors (b), in subtype A infected women defined as slow progressors/controllers (c), as typical progressors (d), and in subtype D infected women defined as typical progressors (e). (f)(g)(h)(i)(j) Viral RNA loads in plasma of subtype C slow progressors, C typical progressors, A slow progressors, A typical progressors and D typical progressors, respectively.
or HIV-1 subtypes in the viral RNA levels at set point or over the course of infection. In our previous report using 188 patients from this cohort and with minimal analyses of follow up (b3 years), we observed slightly higher viral loads at set point in subtype C and D versus A infections (p=0.04, ANOVA) that has not held significance when expanding to the 286 patients in this study [22]. Instead, expansion of the cohort size now showed a trend for lower viral load set points in subtype C infections versus A or D (Supplementary Figure 2.3c). Regardless, it is again important to stress that we did not observe any significant differences in viral load during disease based on infection by specific HIV-1 subtype.

Most studies examining disease progression in natural history cohorts are now impossible and unethical based on WHO guidelines for the initiation of cART in all HIV positive patients regardless of CD4 T-cells counts [38]. With the start of this study in 2000, we followed WHO/UNAIDS guidelines to treat with CD4 cell counts ≤200/ml which was controversial because few charities, governments, and international organizations (e.g. WHO, PEPFAR) had rolled out their treatment programs in Africa. During this ten-year cohort study, 33% of the participants received treatment at an average of 1,500 days post infection. Subtype D infected women were ~1.7-fold more likely to receive treatment than subtype A or C infected women. By modelling the rates of CD4 declines in this natural history cohort, the projected time to reach ≤200/ml or AIDS in these women was 1.3-fold longer with subtype C (estimated mean of 12.3 years) than A (9.5 years) and 2.0 fold longer with C than subtype D (6.2 years). Historical data of subtype B infections in North America and Europe suggest 6-8 years as an approximate time to reach AIDS (CD4 T-cells <200/ml) [39, 40] but there are no natural history cohorts from diagnosis (prior to treatment) to establish accurate estimates. Interestingly, despite different geographical regions, different human populations, and higher rates of parasitic and other infectious diseases, secondary and opportunistic infections in Uganda, infections with subtype B in North America and D in Uganda may have similar rates of CD4 T-cell declines and time to AIDS. Subtype B and D HIV-1 share the most sequence homology of all subtypes and multiple studies suggest that subtype B was a sub-branch and “member” of the subtype D super-cluster. Earlier reports have also described faster disease progression and reduced response to treatment in
East Africans infected with HIV-1 subtype D than subtype A [15, 16, 20].

Figure 2.4) Relative replicative fitness of HIV-1 subtype A, B, C, and D in human peripheral blood mononuclear cells. (a) 12 subtype A, 8 subtype D, and 27 subtype C HIV-1 primary isolates were competed against 25 subtype B HIV-1 isolates in PHA-activated, IL2-treated PBMCs of HIV-negative donors in series of published and unpublished studies. All competitions in PBMCs involved the use of 0.001 infectious units of virus to 1 cell. The relative production of two viruses in each competition was measured by a radiolabeled heteroduplex tracking assays or using next generation sequencing. Production of individual HIV-1 isolates in a dual infection \( f_0 \) divided by its initial proportion in the inoculum \( i_0 \) is referred to as the relative fitness \( w = f_0/i_0 \). In this study, \( w \) is expressed as percent replication of the subtype A, C, or D HIV-1 isolates relative to subtype B HIV-1. Assay sensitivity is 100-fold differences in replicative fitness. (b) The relative replicative fitness of the subtype B HIV-1 isolates in competition with the subtype A, C, and D HIV-1 (the reciprocal of a). Symbols with thick outlines represent replicative fitness of the classified virus at greater than 100-fold or less than 0.01-fold to competitor virus. The shaded symbols represent the replicative fitness values of primary HIV-1 isolates. The open/white symbols represent the replicative fitness values of replication-competent chimeric viruses containing the \( env \) gene of a subtype A, C, or D virus within the NL4-3 subtype B backbone. (c) and (d) Replicative fitness of chimeric viruses derived from \( env \) gene of 5 subtype A, 3 subtype D, and 5 subtype C AHI of this cohort. These 13 AHI
chimeric viruses were competed against the reference subtype B HIV-1 isolates, B-QO (c) and B-K44 (triplicate).

HIV-1 subtype C isolates are less fit than subtype A which are both less fit than subtype B and D HIV-1 isolates, a finding established by over 2000 direct head-to-head dual virus competitions in primary CD4+ T-cells and macrophages from HIV-negative donors of different races/ethnicities [10-12]. These findings suggest a direct association between the replicative fitness of HIV-1 and subsequent disease progression. In addition, the HIV-1 env genes derived from 47 women with acute/early infections also showed that subtype C as compared to subtype A and D were slower in host cell entry by free virus and slower in cell-to-cell transfer of virus.

In vitro assays of HIV Env engaging with cell surface receptors have also demonstrated a rate imbalance in the receptor/ligand interactions. Using the Affinofile system, a transformed cell type with manipulatable levels of surface CD4 and CCR5, the required receptor densities for infection have been quantified for diverse viral strains [264]. The results demonstrate that the affinity of the open confirmation of Env is much higher for CCR5 than the closed confirmation is for CD4. While future work should explore whether Group M subtype impacts CCR5 affinity, work in this manuscript began with the stage of viral entry that is the most rate-limiting.

The first stages of HIV infection, those of attachment and cellular entry, have also been most associated with replicative fitness [265]. These correlations warrant investigations into the specific step of the entry process that confers this competitive advantage. Our initial experiments show that the attachment to CD4 receptors correlate with overall entry rates, patient disease progression and viral replicative fitness in this cohort. While there are still downstream stages of viral replication to explore, there appears to be little published variability in the rates of these steps. Future work will explore how variable entry rates, fusion dynamics, and induced apoptosis differ across the T-cell subsets investigated here. This will be accomplished using a flow cytometry model and HIV entry-responsive dye system. These observations, as well as reduced fusion and viral entry efficiency, suggest that infecting HIV-1 subtype may set the course of subsequent disease progression.
In summary, this largest natural history cohort of non-subtype B infected women revealed a significant difference in HIV-1 subtype virulence with ramifications for HIV-1 spread and treatment in the global epidemic. Women infected with HIV-1 subtype C progressed to AIDS at least 1.5-fold slower than women infected with subtype A or D. Longer asymptomatic periods of a subtype C infection will lead to greater probability of transmission, especially among those discordant couples with unknown infection status (http://www.who.int/hiv/pub/progressreports/update2014/en/). Using human cervical and penile explant tissues, transmission “fitness” appears to be similar among most HIV-1 subtypes [12, 41]. Thus, the expansion of HIV-1 subtype C infections in the global epidemic may be related to the low virulence of subtype C, resulting in long periods of asymptomatic periods, and leading to increased opportunity for heterosexual transmission over other HIV-1 subtypes. Ultimately, HIV-1 subtype C may be more “fit” in the human population based on low subtype virulence and potential high transmission efficiency due to an protracted asymptomatic phase.
Figure 2.5) Function, cell-to-cell transmission efficiency and viral fusion of the Env glycoprotein derived from acute subtype A, C, and D HIV-1 infections. The HIV-1 env gene of acute/early infections in Ugandan and Zimbabwean women was cloned into the pREC-nfl HIV-1 genomic vector by yeast-based recombination/gap repair as described. The function and co-receptor usage of the Env glycoprotein from these 14 subtype A, 4 subtype D, and 16 subtype C acute/early infections was accessed using the Veritrop cell-to-cell fusion assay. None of the 34 Env produced in the context of virus-like particles (VLPs) could mediate cell fusion via the CXCR4 co-receptor within U87.CD4.CXCR4 cells (below negative control and 1000 RLUs) (data below range of graph in (a)). (a) Cell-to-cell fusion between the U87.CD4.CCCR5 target cells expressing Firefly Luciferase upon Tat/Rev-mediate expression from pDM1.1 and the 293T effector cells expressing the 34 Env glycoproteins in context with HIV-1 VLPs. The NL4-3 nfl VLPs are morphologically identical to wild type HIV but is incapable of reverse transcription and cannot induce luciferase expression in the target cells. (b) Schematic of the time-of-drug-addition experiment using the Veritrop assay. 2 uM of Enfuvirtide was added at 30 min to 10 hrs post incubation of the effector and target cells. (c) Box plot of the time to 50% inhibition by Enfuvirtide of cell-to-cell fusion mediated by 34 Env glycoproteins expressed in context with the HIV-1 VLPs. (c) Schematic of the viral fusion assay which can be monitored by HIV-1 carrying a BlaM-Vpr fusion protein that cleaves the CCF2 dye in target cells and changes the fluorescent spectrum. (d) Relative entry over time (0-600 min) into U87.CD4.CCCR5 cells by the chimeric HIV nfl carrying the 14 subtype A, 4 subtype D, and 16 subtype C Env glycoproteins from acute/early infections. (e) Spectral shift curves and box plot of the time required for maximal virus entry into U87.CD4.CCCR5 cells carrying the CCF2 dye.
Figure 2.6) CD4 binding strength via competitive ELISA between HIV-1 subtypes. A) Schematic of the assay 1) Plates were coated with anti-human-IgG-Fc antibody 2) CD4-Fc was then attached 3) HIV adheres to the CD4-Fc and 4) Is competed off the plate with free sCD4. Non-competed controls normalized input via Reverse Transcriptase Assay. Input virus was also normalized in the same way. B) Percentage of virus removed after 24 hours of competition with sCD4, smaller numbers suggest greater binding strength. Means compared via t-test, *p>0.05, ***p>0.001. n=23.
Supplementary Figures

**Supplementary Figure 2.1** The follow up time period and time of cART initiation in the Ugandan and Zimbabwean women enrolled following acute/early infection. (a) The length of follow-up time for each HIV-infected woman by country and by HIV-1 subtype is shown in this box plot. The number of days of infection preceding the initiation of cART is also shown. cART was only initiated in those women with CD4 cell counts below 200 cells/ml and/or who developed severe symptoms of HIV infection (WHO clinical stage IV or advanced stage III disease). (b) The percentage of HIV-infected woman by country and by HIV-1 subtype that required cART during the study period.
Supplementary Figure 2.2) CD4+ cell declines in HIV-1 infected Ugandan (n=112) and Zimbabwean women (n=174) during the natural course of disease. (a) and (b) CD4 cell counts per mm3 as measured in whole blood by FACS Caliber in Ugandan and Zimbabwean women from AHI to 9 years post infection.
Supplementary Figure 2.3) Viral RNA loads in blood and cervix during HIV-1 infections. 
(a) and (b) Bulk viral RNA viral loads in the plasma of HIV-1 infected Ugandan (n=112) 
and Zimbabwean women (n=174) during the course of disease. (c) Viral RNA levels at set 
points in the plasma and endocervical swab samples of women from the different countries 
and infected by different subtypes (as determined by Loess procedure)\textsuperscript{22}. (d)(e)(f) Bulk 
viral RNA loads in the plasma of patients infected with Subtype A, C and D HIV-1 
respectively. (g) Statistical significance of GEE models relating infecting HIV-1 subtype 
with differences in viral RNA loads over the study period.
Supplementary Figure 2.4) The HIV-1 subtype classification based on the C2-V3 env nucleotide sequence. A 450 nt C2-V3 envelop (env) coding regions were amplified from patient samples and DNA sequenced (see Materials and Methods). The HIV-1 sequences were edited using BioEdit v7.0.4, aligned using Neighbor joining (ClustalX\textsuperscript{43}) and maximum likelihood methods (MUSCLE\textsuperscript{38}). Neighbor joining and maximum likelihood trees were constructed with SEAVIEW 4\textsuperscript{39} and visualized with FigTree 1.4 shown in this figure. These env sequences have been previously published\textsuperscript{22}. 
Supplementary Figure 2.5) The HIV-1 subtype classification based on the PR and RT nucleotide sequences. (a) 300 nt PR and (b) 800 nt RT sequence in the pol gene was PCR amplified from patient samples, aligned using Neighbor joining (ClustalX\textsuperscript{43}) and maximum likelihood methods (MUSCLE\textsuperscript{38}), and presented as trees in the figure. Two HIV-1 RT sequences from Ugandan women did not cluster with subtypes A, C, or D and were analyzed for possible intersubtype recombination using RIP and SimPlot (www.hiv.lanl.gov/content/sequence/HIV/HIVTools.html). (c) Patient 1-515-10526-4 was infected with a A1/D recombinant. (d) Patient 1-516-10208-5 was infected with a D/A1 recombinant.
Supplementary Figure 2.6) Example Reverse Transcriptase Assay outputs for CD4 competitive ELISA. This proof-of-concept experiment used timepoints of 12, 24 and 48 hours of sCD4 competition to find the greatest separation in subtype binding strengths. Assays quantified in Figure 3.4 used a 24 hour endpoint measurement.
2.5 References


10. Ariën, K.K., A.A. Abraha, M.E. Quiñones-Mateu, L. Kestens, G. Vanham, and E.J. Arts. The replicative fitness of primary human immunodeficiency virus type 1 (HIV-


Chapter 3

HIV-1 Group M subtypes selectively deplete memory and naive T-cell populations at different rates

3.1 Introduction

Rapidly upon infection, the Human Immunodeficiency Virus (HIV) establishes a transcriptionally silent, latent reservoir of integrated provirus [1]. This pool of infected cells is what renders HIV infections incurable and is so rapidly generated that even the best therapies, administered just days after seroconversion, do not prevent its formation [2, 3]. While most circulating cells are transient and have half-lives of a few days to weeks, the most concerning cells of the HIV reservoir are the CD4+ T-Cell memory subsets which confer immunological memory for decades [4]. The half-life of these cells is approximately 44 months; estimates suggest remaining on antiretroviral therapy for 70+ years would be insufficient to clear this latent pool [5]. Therefore, the dynamics of infection and cellular death in these populations have profound implications for both the individual patient as well as global HIV eradication strategies.

Integration of the viral genome into the host allows for the virus, even after years of suppression, to reactivate upon cessation of antiretroviral therapy [1, 6]. Modern combination antiretroviral therapy (cART) is capable of fully inhibiting viral replication to the limits of detection, but require lifelong adherence to a regimen of three drugs that may have pronounced side-effects and can shorten the patient’s lifespan [7-9]. Even with the success of antiretroviral treatment, only half of the HIV infected population receive cART and still another of the quarter of the 37 million infected do not know their status [10]. HIV related deaths have decreased to less than 1 million/year while the number of new infections remains relatively constant at nearly 2 million a year [10]. Thus, renewed efforts have focused in an HIV vaccine, due also in part to the successes of the RV144 vaccine trial. Vaccination strategies that educate the recipients immune system against HIV would be able to eradicate any reactivated infected cells. Unfortunately, in another cruel twist, the immense diversity of HIV-1 Group M, which makes up >95% of HIV infections
worldwide, is so vast that no monovalent vaccine would be capable of targeting every strain.

This same HIV diversity in the epidemic confers differential drug resistance rates, transmission abilities, CD4+ T-cell depletion rates, and virulence [11-17]. Perhaps due to these differences, or other unexplored driver(s), HIV-1 Group M subtypes have spread at different rates around the world since the virus was identified in 1983 [18, 19]. Most apparently, subtype C has risen to be the most prevalent strain in the world, representing ~55% of all HIV-1 infections. While other HIV-1 subtypes from Central Africa may have been introduced earlier, they appeared to have spread slower in many geographical regions. Subtypes A and D circulate in many of the same regions of Africa but only make up 10 and 4% of all infections worldwide, respectively [18]. As described in the previous chapter, subtype C HIV-1 appears to replicate slower than subtype A and D in CD4+ T cells which is directly related to slower disease progression. Of course, the administration of cART will halt disease progression regardless of the HIV-1 subtype responsible for infection. However, there may be a differential rate of memory CD4+ T cell infection by these HIV-1 subtypes prior to treatment, which has implications for the size of the HIV reservoir during cART. In this chapter we initiated studies to determine the relative infection and depletion of memory T-cell subsets in women infected with subtype A, C, and D prior to treatment.

Previous work from our laboratory has demonstrated that infections with HIV-1 Group M subtype C viruses, relative to other Group M subtypes, have a prolonged time to the onset of clinical AIDS without differing in viral loads at set point or during chronic infection. These analyses extensively controlled for host and environmental factors that can skew measurements of disease progression, including coinfections (particularly Malaria and Tuberculosis), sexual habits, diet, and hormonal birth control use. However, despite these controls, some researchers raised questions about how these results align with other published work. Previously published investigations have suggested that subtype C infected patients quickly reach clinically defined AIDS (CD4+ T-cell counts below 200 cells/mL in the circulation). With these past analyses in mind, we sought to more deeply
explore the differential disease progression between subtypes A, C, and D, focusing on the memory T-cell subsets.

As HIV disease progresses, the canonical measure of pathogenesis is the rate of CD4+ T-cell loss [20]. Differences in these rates have been associated with numerous viral and host traits, with broad implications for treatment and research. Confoundingly, researchers have noted that different T-cell populations have varying degrees of infectibility and rates of apoptosis. Central and effector memory T-cell populations are both more infectible by HIV than naive CD4+ T cells and have increased densities of CD4 and CCR5 entry receptors on their surface [21, 22]. Although HIV can gain entry, virus replication only occurs upon activation of any of these T cell subsets. In general, the memory T-cell subsets are more commonly activated by common recall antigens (e.g. malaria and TB as well as the continual HIV replication) than naive T cells responding to novel antigens [23]. Thus, memory T cells are thought carry the greater burden of HIV replication. Over the course of HIV disease, the CD4+ T-cell memory subsets may be depleted more quickly than other subsets due to direct infection and bystander apoptosis. An important question remains whether HIV Group M subtypes deplete these populations at different rates. Most studies have measured differential T-cell subset infection in anatomical sites of viral replication, but this effect is also notable in the circulation.

This also raises the question whether the differences in disease progression extend to the loss of T-cell memory subsets or if the memory subset decline is independent from disease progression. Most researchers associate differences in rates of cell death between T-cell subsets with receptor density on the surface, known to differ between memory T-cell populations [21, 24]. This may also suggest that the differences observed between HIV Group M subtypes may be due to differing affinities for these receptors. Downstream viral cytopathic effects (CPE), or even signaling through Env receptor binding, may compound over time to drive T-cell depletion.

While the molecular causes of HIV induced cell-death have been explored at-length, it remains unclear how the vast diversity within Group M infections impacts which cells are depleted from infected patients, and what drives these differential rates of subset depletion.
In this manuscript we will investigate how Group M diversity, in particular subtypes A, C and D from Uganda and Zimbabwe, differentially deplete T-cell memory subsets.

3.2 Methods

3.2.1 Cohort

A cohort of 303 number of HIV positive women from Uganda and Zimbabwe were monitored throughout their disease progression in the Genital Shedding Study (GS Study), as described previously (Section 2.2.1, [25-28]). Blood draws were taken at each visit for viral load titers and white blood cell counts. Over the course of the study, many of these aliquots were frozen at -80°C and stored for future analysis and a database was created of CD4, CD8 and viral load test results associated with each sample. A complete history of this cohort is available in Chapter 2, section 2.2.1 or published previously [25-29].

3.2.2 Archived Samples and Flow Cytometry

Flow cytometry panels were run to quantify CD4+ and CD8+ memory T-cell populations on a subset of patient white blood cell samples. 69 patients who were enrolled in the study for a minimum of 1,000 days and had CD4 and CD8 T-cell counts documented from each visit had a total of 388 samples analyzed, an average of 5.6 timepoints per patient. These timepoints were selected to fall over the linear range of disease progression; at least 100 days post-infection to avoid the set point. To quantify memory populations in these archived samples, isolated buffy coats were thawed, spun down and stained with a cocktail containing CD4-PerCP, CD45RO-FITC, and CCR7-PE or CD8-PerCP, CD45RO-FITC, CD38-PE, & HLA-DR-APC antibodies per manufacturers protocol. Cells were incubated, washed and run on an LSR-II (BD Biosciences). Relative numbers of each memory population were calculated by multiplying the proportion of cells in each gate (Figure 3.1) by the archived CD4 and CD8 cell counts in the study databases. All lymphocytes were gated by lymphocyte size using forward and side scatter. Gates described are Naïve CD4+ T-cells (CD4+/CD45RO-/CCR7+), Central Memory CD4+ T-cells (CD4+/CD45RO+/CCR7+), Effector Memory CD4+ T-cells (CD4+/CD45RO+/CCR7-), Naïve CD8+ T-cells (CD8+/CD45RO-), and Memory CD8+ T-cells (CD8+/CD45RO+).
Figure 3.1) Flow cytometry gating strategy to differentiate memory subsets of CD4+ and CD8+ T-cells. From the left, lymphocyte gating using Forward and Side Scatter, CD4 and CD8 positivity using PerCP conjugated antibodies and memory subset quantification using either CCR7-PE and CD45RO-FITC for CD4+ cells or CD45RO-FITC and CD8-PerCP for CD8+ cells.
Markers of activation (HLA-DR and CD38) were also added to CD8+ T-cell Naive and Memory tubes (Figure 3.1 for quantification and gating).

### 3.2.3 Models

Marginal generalized estimating equation models were employed to associate viral diversity with changes in CD4+ T-cell populations, similarly to previous (See Section 2.2.2). Patients codes were used as the subject level to control for varying numbers of observations per patient. Models were constructed by running univariate correlations between subtype groups and progressively adding variables. While data normality was checked, it is not strictly necessary in GEE models. Goodness of fit scores were compared when adding variables to the model to minimize residuals. Variables were excluded if they were both insignificant between subtype groups and failed to improve model fit [30]. Error structures were also compared. Autoregressive error structure (AR(1)) both improved model fit and most accurately represented the theory of CD4+ T-cell decline in the literature [31]. In the final model, T-cell counts over time, circulating viral load (RNA copies/mL), and country of origin were included. All analyses were done using STATA Version 14 (StataCorp LP, College Station, TX).

### 3.2.4 Virus Cloning, Propagation and Purification

Viral stocks from patients in the GS cohort were isolated from blood draws at each visit. Patient viruses from timepoints within 3 months of seroconversion were used in all in-vitro experiments. Patient Env DNAs in pREC_nfl vectors, lacking the 5’LTR, were generated (as described in Section 2.2.4). In these vectors the 5’LTR promoter is replaced with a CMV promoter to generate viral particles that can only infect for a single round but can be transfected with a vector containing the 5’LTR to reform infectious particles. Patient HIV isolated env genes were cloned in this way for 30 patients in the cohort that also had memory T-cell data collected. Virus particles were formed by transfecting into HEK-293T cells with Fugene 6 (Promega).

Infectious particles from the GS study (full length patient virus) as well as reformed infectious particles (including only patient Env in a NL4-3 backbone) were propagated for
these studies. Viruses were propagated in U87*CD4*CCR5 cells (NIH AIDS Cat. No. 4035) in DMEM with 15% fetal bovine serum, 1 µg/ml puromycin, 300 µg/ml G418, glutamine, and pen/strep. Infections were continued for 7 days and supernatant was purified and concentrated. Briefly, the supernatant was cleared of cellular debris by centrifugation at 3,000 RPM. Supernatant was then loaded into a syringe and filtered with a 0.45µm syringe filter into an Amicon Ultra 15 Filter Unit (100kDa filter, Millipore). Amicon tubes were spun in a bucket rotor centrifuge at 3,700 RPM for 12 minutes to pass supernatant through the filter. Using a pipette, the concentrated supernatant from the upper Amicon filter unit was transferred into a 1.5mL snap-cap tube and filled with DPBS (Sigma). Tubes were spun at 32,000g in a fixed rotor centrifuge for 1 hour at 4°C. Supernatant was discarded and viral pellets were resuspended in a small volume of DPBS.

3.2.5 Kaplan Meier Enrollment Analysis

Rates of unenrollment from the GS study were compared using Kaplan Meier curves generated with Graphpad Prism. Enrollment and unenrollment data came from GS Study archives. In the GS Study, unenrollment was required upon reaching T-cell counts of 200, or concurrent counts >300 cells/mL in the circulation or at the discretion of the patient or clinicians. Patients who left the study with high CD4 counts were excluded from the study, and only those with CD4 counts below 300 cells/mL and remained in the study for at least 1,000 days were analyzed. Upon unenrollment patients were provided with treatment. Comparisons were made between infecting subtypes. A total of 282 (114 Ugandan and 168 Zimbabwean) patients were included in this analysis. Curves were statistically compared using Chi-Square tests with significance cutoffs of p<0.05.
Figure 3.2) CD4+ T-cell GEE calculated decline rates separated into A) the cohort subset compared to the previously published entire cohort analysis, B) total CD4+, memory (CD45R0+), and naive populations (CD45R0-) or C) separating the memory population into its constitutive central (CCR7+) and effector (CCR7-) memory parts. Infecting subtypes, A, C, or D, are listed below. P values calculated by GEE models are shown above. n=69.
3.3 Results

3.3.1 Analyses of CD4+ and CD8+ T-cell subsets during HIV-1 disease

As described in Chapter 2, we recruited 303 women following acute/early infection into the GS Study. Subsequent follow-up visits were scheduled monthly for the first six months, every three months for the first two years, and then every six months up to 9.5 years between 2001 and 2010. At the time, and based on the availability of antiretroviral drugs, all women were eligible for cART when CD4+ T cell counts fell below 200 per mL of blood or concurrent counts below 300 per mL. In contrast, the Joint Clinical Research Centre in Kampala, Uganda has a test-and-treat policy where cART is provided to an HIV infected individual regardless of the CD4+ T cell count. Thus, this study provides one of the most comprehensive analyses of the natural history of HIV disease in those infected with non-subtype B HIV-1. Sample collections during the cohort study were also extensive with blood, vaginal, and cervical samples collected every 3 months. PBMCs were purified from blood every 3 months.

In this study we compared rates of T-cell loss between Group M subsets to identify the role of memory T-cell depletion in HIV disease progression. Using cryopreserved, purified PBMCs, we stained the cells with either anti-CD4, CD45R0, and CCR7 antibodies (for CD4+ subsets) or anti-CD8, CD45R0, CD38 and HLA-DR antibodies (for CD8+ subsets). Flow assisted cell sorting (FACS) identified the percentages of T-cell subsets. CD4+ populations identified were CD4+/CCR7+/CD45R0- T-cells (naive), CD4+/CCR7+/CD45R0+ T-cells (central memory), and CD4+/CD45R0+/CCR7- (effector memory) as described in Figure 3.1A. CD8+ populations identified were CD8+/CD45R0- (naive) and CD8+/CD45R0+ (memory) as described in Figure 3.1B.
3.3.2 HIV Subtype impacts depletion of CD4+ and CD8+ populations

We first compared the CD4+ T-cell declines between the subgroup samples (69 patients) relative to the total patient population (303 patients) over the course of infection (Figure 3.2A). Generalized estimating equation models are considered the gold standard for longitudinal data analysis in order to account for covariance due to multiple measurements within a single patient over time. This model also corrects for varying numbers of observations per patient and allows for flexible error structures. Autoregressive error structures (AR-1) suggest that the correlation between two cell count measurements in a given patient is a function of the time between the visits, with reducing correlations over greater time. These relationships most accurately resemble the data and dynamics of the human immune system generally and were employed in all the analyses herein.

The results reconfirm previous conclusions about differential disease progression between subtypes even with a limited number of patient samples available (Figure 3.2A). Subtype C infections showed a highly statistically significant reduction in the rate of all CD4+ T-Cell loss, about 0.63 cells per week, relative to that of subtype A (p<0.0001, GEE), losing about 1.03 cells per week, both of which differ from subtype D, which loses about 1.22 cells per week (all pairwise comparisons p<0.0001, GEE) (Figure 3.2B). Our analyses also found that while viral load correlates with the loss of CD4+ cells, these relationships do not differ between subtypes (Supplementary Figure 3.3).

Pooled memory subsets, CD4+/CD45R0+ T-cells, also showed reductions in pathogenesis by subtype C relative to A and D (C vs. A, p<0.0001, GEE; C vs. D, p=0.019, GEE, Figure 3.2B). In other words, the decline in the memory CD45R0+ T-cells was slower when a patient was infected with subtype C. Analyses of this cohort subset showed that the more rapid declines in memory CD45R0+ T-cells with HIV-1 A and D infections did not significantly differ from each other and does not suggest, based on the memory CD45R0+ T-cell decline data alone, that subtype D HIV is more pathogenic. This pooled memory
population also displays a wide range of memory CD45R0+ T-cell losses during subtype C infection (+/- 0.75 cells per week) with some subtype C infected individuals having no decline of memory CD4+ T-cells during ~6.5 years of infection. In contrast, all 118 subtype A and D infections had a progressive loss in the memory CD4+ T-cell populations. The CD4+ naive population did not change in frequency over the course of infection regardless of infecting subtype (Figure 3.2A).

To further interrogate the individual memory subsets, the central and effector memory populations were separated and compared directly by staining for CCR7 and CD45R0 (Figure 3.2C). Statistically significant differences based on infecting subtype can be observed in the effector memory CD4+ T cells over the ~6.5 years of infection (C vs. A, p<0.0001, GEE; C vs. D, p=0.004, GEE, Figure 3.2C). Separating memory T-cells populations showed that central memory T-cell counts did not significantly decline during HIV infection (p=0.1271, Linear reg., Supplementary Figure 3.1) and there were also no significant differences related to the infecting HIV-1 subtype (Figure 3.2C).

CD8+ T-cells, while not directly infected by HIV, have been shown to lose function and decline due to bystander effects during untreated HIV disease [34]. Our measurements of CD8+ T-cell populations are unable to show these losses of cells in subtype A and C infections, but the most pathogenic subtype, subtype D, shows consistent and significant CD8+ cell depletion of about 0.5 cells/week (Figure 3.3). These declines in subtype D CD8+ T-cell populations are visible in total cell measurements but are largely driven by the loss of memory cells, which only declined in subtype D infections. Numbers of naive CD8+ T-cells did not change in prevalence in these patients.
Figure 3.3) CD8+ T-cell GEE calculated decline rates separated into memory (CD45R0+), and naive (CD45R0-) populations. Infecting subtype is listed below. P values calculated by GEE models are shown above. n=69.
Figure 3.4) CD4+ and CD8+ memory T-cell dynamics (CD45R0+) for 27 patients with typical disease progression for infections by each subtype in women who participated in the GS Study in Uganda and Zimbabwe.
during 6.5 years of infection and regardless of the infecting subtype.

Typical HIV disease depletion of CD4+ and CD8+ memory T-cells for patients infected with each Group M subtype are shown in Figure 3.4. These example patient measurements provide a better visualization of the steady linear depletion of these subsets, as well as the subtype D specific depletion of CD8+ memory T-cells. Patient subtype A infections largely resembled subtype D depletion of CD8+ memory T-cells. Subtype C infections remain largely steady over the course of infection, with clear proliferative events between visits that are not seen in the other Group M subtypes. All HIV infected patients experienced CD4+ memory T-cell depletion over the 6.5 years of monitoring. These cell losses appear consistent and steady in both subtype A and D infections, while subtype C, mirroring CD8+ memory T-cells, show greater stochasticity.

The simultaneous expression of CD38 and HLA-DR was used a marker for the activated CD8+ T-cell population but did not identify subtype specific activation in naive or memory populations during HIV infection (Supplemental Figure 3.2). There was however a trend for higher CD8+ T-cell populations in the subtype C infected patients as compared to those infected with subtype A and D. This trend was most apparent in the memory and activated CD8+ T-cell populations.

As described above, subtype C infections led to apparent slower declines in the effector memory CD4+ T-cell population than did infections with subtypes A or D. Indeed, the slower declines in the entire CD4+ T lymphocyte population suggest slower disease progression by subtype C, which may be attributable to both a reduced activation and transition of memory CD4+ T-cells to effector memory T-cells. The effector memory T-cells could die due to TCR initiated-programmed apoptosis, direct HIV cytopathogenicity, or HIV-related direct or bystander apoptosis. Importantly, viral load analyses on both the cohort subsets analyzed here, as well as the broader cohort, have not identified lower circulating virus concentrations during subtype C infections (p=0.219, ANOVA, Supplementary Figure 3.3).
Given the wide range in the CD4+ T-cells memory and CD8+ memory T-cell counts (CD45R0+) over the course of subtype C infections, we performed a correlative analysis between these two cell types to investigate differing relationships between these cell types during Group M subtype infections (Figure 3.4). Linear regressions by infecting subtype showed statistically significant correlations between the dynamics of the CD4 and CD8 memory subsets (Linear regression, p=0.002). These correlations highlight the CD4+ T-cell independent depletion of CD8+ T-cells during subtype D infections. Infections with subtype D are the only subtype to show a negative correlation between CD4+ and CD8+ memory T-cells (Figure 3.5). The depletion of CD8+ T-cells during subtype D disease has major implications for viral control but, without increased activation markers in CD8+ T-cell populations, relies on a yet undiscovered mechanism.

3.3.3 Rates of study unenrollment agree with other pathogenicity measurements

Other studies on pathogenicity of HIV-1 Group M subtypes used the time to clinical AIDS as a measure of pathogenicity. We used unenrollment from the study as a proxy measurement for clinical AIDS since patients were unenrolled to receive treatment after concurrent CD4+ T-cell counts below 300 cells/mL in their circulation. Subtypes C and A were statistically indistinguishable (p=0.9451), while subtype D infected patients unenrolled earlier than subtype C (p=0.0444) and nearly differed from subtype A (p=0.0541) (Figure 3.6).
Figure 3.5) Correlations between CD4+ memory T-cells and CD8+ memory T-cells (CD45R0+ populations) between Group M subtype infections. Observations made between 100 days post infection and 2,100 days post infection. Comparisons between the slopes of best fit lines used linear regression, \( p=0.002 \), \( n=338 \).
Figure 3.6) Kaplan Meier analysis for patient enrollment by infecting subtype. Patients included in this analysis must have remained in the study for >1000 days and reached a CD4 count below 300 cells/mL. Subtype A median enrollment was 2,239 days, subtype C was 2,169 days, and subtype D was 1,367 days. n=282.
3.4 Discussion

In both HIV and SIV infections, many of the immunodeficiency symptoms and opportunistic infections are directly related to the loss of effector memory cells and the ability of these cells to rapidly respond to non-HIV recall antigens [35, 36]. Primate models and other investigations have shown that these cells are the first to become exhausted and undergo apoptosis. Here we show that effector memory cells are the primary CD4+ population to be depleted during untreated infection while naive and central memory CD4+ T-cells are nearly unimpacted, regardless of infecting subtype.

The same pattern of differential pathogenicity of the most prevalent Group M subtypes in Africa is visible in T-cell memory (CD45R0+) population declines. Patients infected with subtype A and D viruses lose their CD4+ memory T-cells at about double the rate of subtype C. While subtype C viruses appear highly variable in their effects on memory T-cells, the more pathogenic subtypes A and D specifically deplete CD4+ effector memory T-cells without depleting central memory or naive populations. These populations that remain unchanged over HIV-1 infection have been documented to be poorly infected, largely transcriptionally inactive, and non-dividing [24, 37]. While some depletion of these subsets may be expected due to bystander apoptosis, the lack of viral replication limits cytopathic effects, and the intrinsic apoptosis pathway.

Infections with subtype C viruses induced varying CD8+ T-cell responses, over a much broader range than subtype A or D disease. This heterogeneous response to subtype C infection warrants future work to identify correlates of CD8+ T-cell proliferation. Published literature has detailed the prevalence of CTL epitopes in conserved regions of the subtype C accessory protein Nef, and the overwhelming preference of the CTL response to Nef epitopes in subtype C infections (97.5% of epitopes) [38, 39]. Recent subtype C reference strains from Brazil have also been described that harbor mutations in Nef residue A84 that greatly reduce MHC class I downregulation [40]. These types of
mutations may leave subtype C open to CTL clearance of infected cells and contribute to the differential pathogenesis observed here.

Investigations into pathogenicity and fitness have routinely shown that subtype D is the most fit and pathogenic strain of HIV-1 Group M [41-43]. Using unenrollment from the GS study as an endpoint mimicking clinical AIDS, we compared enrollment by infecting subtype. While subtypes A and C did not differ in their rates of unenrollment, subtype D patients unenrolled at much greater rate. Results presented here also suggest that subtype D is unique in its depletion of CD8+ memory populations. In the absence of viral escape mutations, CD8+ T-cells are the primary mode of infected cell clearance, the breadth of which is linked to disease control [44]. However, it remains possible that the skew towards a memory phenotype (CD45R0+) CD8+ T-cell is a product of the disruption of natural lineage maturation in subtype C HIV infected patients [45].

3.4.1 Preferential depletion of effector memory T-cells during HIV infection

Simple models of T-cell homeostasis and activation show an accelerating depletion of the T-cell populations [31, 46]. As the immune system loses cells to activation-induced apoptosis, bystander apoptosis, and HIV cytopathic effects (CPEs), the bone marrow releases new uninfected naive target cells which, according to the second hypothesis, should migrate to inflammatory sites and become infected themselves. This “fuel to the fire” model would grow exponentially and deplete the immune system in months rather than years. However, patients rarely display this cell-loss dynamic and almost always have a slower, near linear progressive effector and memory effector T-cell depletion over the asymptomatic period of infection.

One explanation draws from the finding that mature, peripheral T-lymphocytes are the primary source of new CD4+ T-cells in the periphery [47]. Specifically, mature T-cells transferred between HIV discordant twins were found to persist for months and expand significantly. HIV patients with unsuppressed viremia undergo chronic immune activation
and inflammation due to huge amounts of antigen produced from productively infected cells. The antigen naive HIV-specific T-cell population, in response to cognate antigen presentation, proliferate into transcriptionally active effector cells. These cells are well documented to be preferentially infected by HIV, fueling viral replication, cytopathic effects and releasing more HIV antigens [22]. This cycle of activation seeds HIV-specific effector memory populations, long-lived cells capable of producing new virions for decades, further fueling post-activation apoptosis. It also results in the persistence of HIV infected cells in the periphery while gradually depleting the activated effector T-cell populations. This theory of T-cell depletion particularly highlights the role of inflammation and T-cell activation in their eventual apoptosis, while downplaying the impact of viral cytopathic effects.

3.4.2 Implications

Replicative fitness, CD4+ effector memory T-cell depletion, and cellular entry efficiency strongly correlate in a cohort of HIV-1 infected women. Subtype C displayed the least efficient attachment affinities, entry rates, relative fitness scores, and caused a disease that progressed at about half the rate of subtype D infections. The consistent patterns across widely differing analyses lends credence to these conclusions. Maintenance of CTLs during subtype C disease, and their gradual depletion during subtype D disease, may be major drivers of pathogenesis differences.

However, regardless of subtype, HIV infections remain incurable. To fully consider strategies to eradicate HIV in lieu of a cure, the field must better understand the diversity of diseases caused by this virus, whether current estimates of undiagnosed infections are accurate and how treatment must be prioritized to minimize future transmission. To target the latent reservoir of integrated HIV-1 provirus, it’s critical to understand the dynamics of the cell types that harbor it. Most importantly, these considerations must be made using the full diversity of HIV-1 Group M, not just subtype B.
Supplementary Figure 3.1) Cohort subset CD4+ T-cell Central Memory (CD4+/CD45R0+/CCR7+) cell counts over the course of HIV-1 infection. Observations within AHI, <100 days post infection, were excluded. Linear regression comparison with zero slope, p=0.1271. n=338.
Supplementary Figure 3.2) CD8+ T-cells trends in activation markers CD38 and HLA-DR over the course of infection by HIV Group M subtypes. Gating strategy shown on the left and GEE calculated rates of change shown in the box plot on the right. n=57.
Supplementary Figure 3.3) Cohort subset viral loads for all visits post AHI, <100 days post infection. Lines and dots denote Group M subtype, green = D, red = C, blue = A. Nonlinear regression between curves for each subtype, p=0.219. n=388.
3.5 References


Chapter 4

Discussion

4.1 Viral Fitness, Disease Progression, and CD4 Binding Affinity

This dissertation conclusively supports the link between replicative fitness and disease progression in HIV-1 Group M subtypes A, C, and D from a cohort of women in Uganda and Zimbabwe. Patterns of T-cell depletion strongly correlate with patterns of replicative fitness in activated PBMCs, such that subtype D is fitter and more pathogenic than subtype A, both of which are greater than subtype C (Figures 2.2 & 2.4). The patterns of cell depletion extend to memory subsets of CD4+ T-cells, but not naive or central memory T-cells (Figure 3.2). These data substantiate the notion that effector cells, and particularly effector memory cells, are the most depleted populations during untreated HIV disease. Chronic inflammation and immune activation drive the expansion and subsequent apoptosis of effector T-cells in the periphery, generate more infectible targets, release HIV antigens, and signal bystander apoptosis [1-4].

The size of the cytotoxic T-lymphocyte (CTL) population that target HIV-1 are associated with disease control and limiting viremia [5]. Analyses of CD8+ memory T-cell population dynamics in the GS cohort demonstrate that only subtype D causes a statistically significant reduction in the number of circulating cells, while naive and activated subsets remained unchanged (Figure 3.3). These results demonstrate a possible mechanism of the rapid disease progression associated with subtype D infections, due to a depleted CTL response. This CD8+ cell depletion in subtype D infections appears to begin early in infection and does not correlate with losses of CD4+ T-cells or CD4+ memory T-cell subsets. Subtype C infections displayed a particularly wide range of effect on CD8+ T-cell counts, suggesting in some participants that CTLs proliferate even during active infection. This data may clarify why subtype C disease progresses so slowly relative to other Group M subtypes and warrants additional exploration. Studies attempting to predict CTL epitopes in subtype C viruses highlight the flaws in studying
only subtype B infections, thus the unique immune pressure on HIV subtype C by CTLs remains to be explored [6].

Links between fitness and pathogenesis have been previously suggested, however few studies have adequately addressed such a question [7-9]. In the analyses presented herein, we addressed many of the failings of previous studies through extensive medical surveying, PCR and seroconversion tests to closely estimate time of infection and accounted for host diversity more effectively than prior investigations. Finally, despite extensive analyses we did not find an impact of viral load, diet, environment, host genotype, sexual habits, country of origin, or hormonal birth control use that better explains the differences associated with HIV-1 Group M subtype. HIV disease, as with all human disease, is a complex system with many factors determining immune cell apoptosis and depletion. These analyses required very large numbers of observations in a large cohort to reach statistical significance while controlling for the other measurable factors known to impact pathogenesis.

Previous work from our laboratory utilized diverse chimeric viruses to map the primary drivers of viral suppression in lieu of treatment. That study identified the Env glycoprotein, the viral protein responsible for virion attachment and cellular entry, to be the largest single contributor to elite viral suppression between HIV-1 clones [10]. Data presented here, using viruses from the GS Study cohort, also strongly implicated a role of viral attachment, entry, and fusion in CD4+ T-cells to account for fitness differences and, in turn, differences in virulence. The rate of cell-cell fusion, as a corollary to syncytia formation in the lymph nodes, showed statistically significant rate increases in subtypes A and D relative to subtype C (Figure 2.5b&c). These syncytia are a major marker of advanced HIV disease, disrupting lymph node architecture and interrupting immune system function. We also found that these rate changes extend to a viral particle entry model, to cover all modes of cell infection (Figure 2.5d&e). These rates of fusion and viral particle entry are likely driven by CD4 binding affinity differences (Figure 3.4).

The differences between HIV-1 subtypes presented in this work are consistent with the patterns reported by our group and others [8, 11-17]. Investigations into Gag-Protease,
replicative fitness, disease progression, susceptibility to fitness-reducing mutations, viral entry rates, CD4 affinity, and proviral decay all identified subtype C as the least and subtype D as the most effective or fit subtype of those compared [8, 11-13, 15-25], each of which may be contributors to fitness differences between subtypes. It must be hypothesized that the combined reductions in fitness in subtype C might be adaptive.

4.2 Subtype C Epidemic spread

Over the past four decades HIV-1 has spread around the world. However, this spread has not been equally distributed between the Group M subtypes. Subtype C expanded the most and is currently the most prevalent in the world (55% of HIV-1 infections) [21, 24], with little research focusing on identifying why. The work presented here has identified significant reductions in pathogenicity in subtype C that may have driven this expansion [11, 12, 26, 27]. Mathematical models that estimate the effects of prolonged asymptomatic disease have shown strong positive correlations with disease spread [28, 29]. It is worth noting that these models typically use data from more rapid disease outbreaks like Influenza A or SARS and may not accurately predict future HIV transmissions.

South Africa has the highest incidence of HIV infections in Africa, 18.8% of adults 15-49, while containing the highest prevalence of subtype C HIV in the world, 95%. The prevalence of subtype C infections in Brazil has also increased in the last decade while other subtypes plateaued [21, 24, 30]. But these findings remain limited due to the small number of appropriate populations, limited funding, and limitations of ethical human research.

Past work from our laboratory suggest that these fitness differences between subtypes are consistent between regions [21, 22], but further analyses are required to demonstrate consistent patterns from other continents. Work from others has demonstrated the rapid disease progression of subtype D in Kenya, Rwanda, South Africa, Uganda, and Zambia [17, 31, 32]. The patterns of fitness in other regions appear to be strong indicators of pathogenesis as well. However, future work should continue to examine HIV-1 subtypes A, C, and D fitness and disease progression outside of Eastern and Southern Africa.
Ideally, future metanalyses of archived monitoring data from untreated HIV+ patients from around the world could assemble a much broader database than any single study. The results from the GS cohort suggest that human genotype has a significant impact on basal CD4+ T-cell counts but when accounted for, no other patient background measurements were necessary to identify subtype differences in pathogenesis. Understandably, the managers of trial data will be reluctant to share their patient records, and these analyses are unlikely to come to fruition, but are likely one of the few remaining avenues to investigate untreated HIV disease progression.

It is also possible that the predominance of subtype C has been fueled by founder effects alone, since most HIV epidemics are established by a relatively small number of infected people with limited viral diversity [33]. Subtype B is documented to be one of the most pathogenic forms of HIV-1, and expanded into North America, Europe, the Caribbean, South America and Australia. These epidemics provide contradictory evidence to the hypothesis that fitness reductions spread HIV. Unfortunately, it is difficult to compare HIV transmission patterns between eras and populations. The difference made by HAART cannot be discounted and the overall decline of HIV transmissions may provide different selection pressures on the virus than was present in the 1960s and 70s.

### 4.3 GS Study Cohort

Research of human pathogens requires innovative research strategies and extreme care to maintain ethical research practices. In the design of the GS Study, from which the disease progression data was mined, it was extremely important to the study directors to provide as many resources to the participants as possible. While not required for ethics approval, counselling, frequent medical examinations and treatment upon unenrollment were provided to all participants. This was the first study of its kind to provide treatment to participants after enrollment, thus the study coordinators could state that the research and use of its data were navigated in the most ethical manner available. Despite this, later studies, like the 2015 Strategic Timing of Antiretroviral Treatment (START) study, found that delaying HIV treatment in the infected had severe consequences on long-term survival and disease management, even when these participants maintained CD4+ T-cell counts above 500 cells/mL [34]. Following these results, the WHO established new rules
about the ethical research of HIV+ populations that require treatment to be provided upon HIV diagnosis. These rules would make repeating the GS study, or any study for that matter, with similar approaches, impossible. Since these data are irreplaceable and unrepeateable, it is critical to maximize what we can learn from it.

The GS study is also unique in its location and participants. Few human populations are as well suited to this study design as the enrolled Ugandan and Zimbabwean women. The human genetic diversity in the region is so vast as to mimic a much broader study area. This allowed for an economically efficient study design while also controlling for human genotype, a criterion not assessed in previous investigations. Genetic diversity and other covariates between the Ugandan and Zimbabwean populations did impact our analyses, primarily in the baseline CD4+ T-cell counts, prior to infection. Zimbabwean women naturally had lower CD4+ T-cell counts, which were suggested to play a significant role in how their diseases progressed. During the analysis we worked to address these concerns, both by including baseline T-cell counts in our model and by measuring CD4+ cell loss as a percentage of the participants starting cell count. Each of these strategies did little to change the results of the analysis, and only served to add variability and reduce overall model fit scores.

Finally, the GS study data identified the date of HIV infection in a more analytically appropriate manner. Rather than enrolling HIV+ populations, the GS study enrolled participants from the HC-HIV study upon early PCR or seroconversion test. These diagnoses were then confirmed via PCR and a best estimate at the date of infection was calculated. This differs dramatically from other studies which estimate HIV infection date by viral load retrograde extrapolation [35, 36]. These errors in enrollment strategy typically overestimate the time since infection in participants with higher viral loads and more rapid disease progression, effectively dampening differences between participants.

4.4 Future Directions

Work is ongoing to explore the results presented herein. Varying rates of entry, cell-cell fusion and affinities for CD4 can be explained either through variation of viral surface Env expression or mutations that confer greater binding affinity and kinetic rate. HIV-1
subtypes that have low Env density on the viral surface would slow the receptor-ligand interaction rate and could account for the whole-virus affinity for CD4. The competitive ELISA presented in Chapter 2 measured CD4 affinity by an entire viral particle and not purified Env protein (Figure 2.6). Future work could also explore the per-Env trimer affinity differences for CD4 and would be worth pursuing regardless of the results of Env quantification experiments. Preliminary analyses to quantify the number of Env trimers per viral particle have failed to find differences in Env density. However, the fluorescent western blot protocol used has a relatively high limit of detection for the picogram quantities of Env we are trying to quantify. Transition to a chemiluminescent assay using antibodies conjugated to horseradish peroxidase (HRP) to amplify the band signal [37].

Overall, these studies are especially difficult due to the variability between the HIV subtypes being measured. DNA sequence homology between subtypes A, C and D is about 85% with the greatest differences found in the env gene sequence. The impact on the binding strength of many monoclonal antibodies with epitopes in HIV Env has been documented and varies depending on subtype [38]. The monoclonal antibody B13 binds to the CD4 binding site (CD4bs) and is the most consistent antibody when binding diverse HIV strains [10, 39, 40]. Unfortunately, this antibody requires high titers for efficient binding and the linear epitope requires significant denaturing steps before blotting. This relatively poor interaction highlights the necessity of signal amplification through chemiluminescence.

Expression differences of HIV-1 Env between subtypes would raise future questions about the subtype specific mechanisms at work. Both degradation in an endocytic compartment following reinternalization and weakened Gag-Env interactions during viral assembly and budding have been suggested [41]. Protocols developed in our laboratory for surface plasmon resonance (SPR) experiments would be able to measure both Env-CD4 and Env-Gag interactions per trimer to clarify these binding results. Sample preparation would need to be highly purified to detect signal differences, likely requiring purified protein of different subtypes to be ordered from the NIH AIDS Reagent repository. These experiments could explore CD4 affinity for all available viruses from
around the world to see if the differences found in Uganda and Zimbabwe are maintained.

A flow cytometry panel utilizing HIV entry sensitive dye and fluorescent antibodies differentiating lymphocyte populations is also being developed. This assay, when fully developed, could measure viral entry by cell type. Viruses used in these experiments contain dsRed2 sequences between the env and nef genes to quantify productive infection as well [42]. Long-term infection assays could use markers of pyroptosis (activated caspase 1) and apoptosis (activated caspase 9 or caspase 3) to clarify the mode of cell death, measured between HIV-1 subtypes and across all CD4 and CD8 T-cell subsets.

Finally, the in vitro assays presented in this dissertation were developed to move into activated PBMC cultures from uninfected participants to explore differential virus entry, fusion and CD4 binding in the T-cell memory populations. Implementation and troubleshooting of these assays were beyond the scope of this work but is progressing to clarify the mode of cell death in these memory lymphocytes. The typical forms of T-cell death during HIV infection are bystander apoptosis, HIV cytopathic effects and post-TCR stimulation apoptosis. The first two of these modes have cannot be addressed by data presented in this thesis and are hotly debated in the field. The final, the role of TCR stimulation and immune activation, has been a focus of this research, however conclusive evidence of immune stimulation-based T-cell contraction is elusive.

The engagement of the viral particle with the CD4 receptor, which we demonstrate here is heavily influenced by HIV subtype, may serve to “prime” these cells for future activation and expansion. Assays allowing HIV attachment but preventing cell entry show a significant increase in future activation levels in diverse T-cell populations. Future work will also explore whether the CD4 binding strength of the viruses themselves functions to modulate this chronic activation observed in HIV disease. While evidence in this dissertation is simply correlative, that subtype C has both a reduced CD4 binding strength as well as a slower pathogenesis, future studies will investigate subtype induced levels of immune activation that could exacerbate disease.
4.5 Implications

4.5.1 Latency

The transcriptionally latent viral reservoir remains the final challenge in curing HIV [43, 44]. “Shock and kill” strategies seeking to reactivate and destroy this pool of integrated provirus are in development. New data from a Rakai, Uganda cohort of primarily infections by Group M subtypes A and D, revealed that North Americans, infected with subtype B viruses, typically have larger pools of latent provirus than participants in the African cohort [45]. The relative size of the subtype C latent proviral pool remains to be quantified. This result strongly suggests a role for viral diversity in the size of the latent reservoir, with many future questions to be addressed.

The impact of HIV diversity on latency may provide targets for cure strategies that push the integrated HIV provirus so deep into a latent state that it is unable to reactivate. These strategies, catchily termed “Block and Lock” therapies, are attempting to identify host factors to promote histone translocation, methylation and deacetylation near the integrated viral promoter to prevent future reactivation [46]. If Group M subtypes have naturally evolved ways for the virus to promote greater integration and latency, then this may become a viable therapeutic to limit viral rebound after ART cessation and lower the bar for “Shock and Kill” effectiveness.

4.5.2 HIV Cure

Cure strategies, elevated by the results of the RV144 Thai Trial vaccine, have begun to focus on non-neutralizing IgG antibodies due to their negative correlations with infection [47]. These antibodies are unable to neutralize the virus directly, but instead capture the virus in aggregates. Viruses bound by these antibodies cannot infect new cells and are instead phagocytosed [48]. Critically, these antibodies do not bind infected cells and promote antibody-dependent cellular cytotoxicity, instead relying on other forms of infected cell clearance. The data presented herein suggest that a vaccine that successfully stimulates this subclass of IgG antibodies may struggle to control advanced subtype D disease due to the unique depletion of CD8+ T-cells (Figure 3.3). CD8+ T-cells, or cytotoxic T lymphocytes (CTLs) play a key role in destroying infected cells [3]. Potential
pitfalls like these highlight the importance of considering the broader HIV-1 Group M diversity while moving forward. Neutralizing antibody research has already reached this conclusion and future HIV vaccine trials based in South Africa will utilize subtype C Env proteins in their formulation [49, 50]. These upcoming trials are likely going to be the most impactful in the history of the field, due to the better understanding of HIV control from the RV144 trial and incorporation of the most prevalent subtype in the pandemic, subtype C, in the vaccine itself.

While optimism abounds following RV144, it is still important to find methods to limit HIV transmission in case a vaccine doesn’t materialize. The WHO goal for 2020 is for all countries to achieve 90/90/90 targets: 90% of HIV+ individuals aware of their status, 90% of those patients to be on treatment and 90% of patients on treatment to be fully virally suppressed [51]. These goals are made especially difficult when considering the data presented on subtype C disease progression [52]. Expanded time to reach clinical AIDS following infection with a subtype C HIV-1 virus primarily expands the asymptomatic phase of infection. Estimates of prevalence of HIV can be badly skewed given a disease progression that doubles the length of time patients remain asymptomatic [53]. Misjudging the prevalence of HIV may be an especially dangerous mistake as subtype C moves into regions with huge populations, like India, China and southeast Asia. The delay in apparent symptoms of disease, when combined with local governments that are resistant to international interference, can allow epidemics to immerse quickly and overwhelm the limited resources to reach 90/90/90 targets. While these warnings may seem dire, the rapid expansion of the HIV epidemic in Russia and other former soviet states among intravenous drug using populations underscores this reality [54, 55]. Refusal to allow international organizations into Russia to accurately test HIV prevalence and incidence rates leaves this region and the whole world at risk.

Research has historically, and continues to, focus on subtype B as a model of all HIV infections. Despite research highlighting major differences between subtypes, nearly every model strain of HIV-1 used in laboratories around the world is subtype B in origin [56]. Subtype specific traits impact how drug resistance develops, and in countries with limited available ART regimens, controlling HIV drug resistance will be the key driver in
reducing infection rates [9]. For example, China, a country with a burgeoning HIV epidemic, limits the import of foreign made HIV drugs [57]. These limits seek to bolster the Chinese pharmaceutical industry but prevent new therapeutics from reaching Chinese patients. The net effect is the prevailing use of older drug regimens that are prone to resistance. Viral resistance to newly developed ART drugs, like dolutegravir, an integrase inhibitor, are much less common relative to previous generations. Unfortunately, these drugs are currently blocked from import by the Chinese government. The broad genetic diversity in the infecting HIV strains in the country allows for recombination and possesses even more drug resistance capacity than a single HIV type would. Estimating future drug resistance on studies of subtype B alone woefully misjudge the problem at hand.

### 4.5.3 HIV Policy

HIV is not curable, regardless of subtype. However, that doesn’t imply that differences in pathogenicity between viral strains are not useful to inform policy and political decision about the epidemic. Renewed focus must be placed on diagnosing HIV+ patients, particularly in areas with circulating subtype C viruses, as HIV-related symptoms are likely not to appear until years after infection, perhaps reducing rates of diagnosis. Efforts must be made to place every diagnosed patient onto treatment as soon as possible, per the recommendations of the START study and WHO research guidelines. However, given the limited funding available, the aggressiveness of subtype D infections may aid in triaging patients of Sub-Saharan Africa. Economic retractions have pushed the President’s Emergency Plan for AIDS Relief (PEPFAR), the largest single contributor to HAART in Africa, to decline in recent years, limiting the availability of life-saving drugs [58]. In the face of impossible choices, informed policy is the last, best option.

### 4.6 Concluding Remarks

Work by generations of HIV researchers have pushed back against the pandemic, developing effective therapies and multinational eradication strategies. The number of new infections drops each year as those with the disease can live longer and healthier lives. While HIV is no longer the global menace it once was, it remains fundamentally
important to understand how the different forms of the disease manifest. In order to understand the details of HIV disease, explorations of the immune cells depleted may provide clarity in a search for a vaccine or future treatment. Finally, illustrating the differences between diseases caused by the North American forms of the virus and those found in Africa may prompt future research efforts to identify mechanisms and impacts of the work presented.
4.7 References


Appendix A

Sharing of equipment used for the preparation of a controlled-release oral opiate for injection is associated with HIV transmission

5.1 Preface

During my graduate research I was lucky to collaborate with multiple institutions and groups of researchers on many HIV-focused projects. Fortunately, I had the greatest involvement in a collaboration with the London Health Science Center, University Hospital, and St. Joseph’s Hospital. The city of London, Ontario was experiencing an HIV outbreak linked to injection drug use and clinicians were looking for basic science collaborators to experimentally examine the feasibility of their hypotheses. I’ve included the subsequent manuscript from this collaboration because of its importance in my graduate work despite its incongruity with the HIV diversity project presented above.

5.2 Abstract

In London, Canada, an outbreak of HIV among people who inject drugs (PWID) occurred despite widespread distribution of sterile needles, syringes and IDPE. Hydromorphone controlled-release capsules (HCR) are the local opioid of choice (followed by immediate-release hydromorphone) and is associated with frequent reuse and sharing of IDPE. Sharing of needles/syringes is a known and well documented mechanism for transmission of HIV amongst persons who inject drugs (PWID). Injection drug preparation equipment (IDPE; i.e., cookers, filters) are more often shared than needles/syringes, but the risk of HIV via this mode of transmission is unknown. The objective of this study was to determine whether the practice of sharing injection drug preparation equipment (IDPE) to inject controlled-release opiates is associated with HIV transmission; and to examine the properties of controlled-release opiates that may lead to both IDPE sharing and HIV transmission.
A case control study of HIV infected and HIV uninfected PWID cases was conducted using a questionnaire to examine behaviors associated with injection drug use. The primary exposure of interest was IDPE sharing over the last three months. To assess motivation to share/reuse IDPE, residual opioid left in the IDPE after aspiration with a needle/syringe was measured in conditions replicating PWID practices using liquid chromatography–tandem mass spectrometry. To assess conditions favouring HIV transmission, HIV was added to IDPE in the presence or absence of opioid. HIV viral persistence was then measured in IDPE by HIV reverse transcriptase activity and infectivity of indicator Tzm-bl cells. All experiments were done with HCR versus immediate-release hydromorphone and a control.

Sharing IDPE (without sharing needles/syringes) was associated with HIV infection \[\text{aOR}=22.1; \ p<0.001\]. IDPE reuse was almost universal among HCR users \[31/31 (100\%) \text{ HIV+} \text{ and } 62/66 (94\%) \text{ HIV–}\]. Patient report of barriers and negative beliefs regarding heating HCR in the IDPE was also associated with HIV infection \(p<0.05\). Approximately 45\% of HCR remained in the IDPE following initial injection, with no change after heating, whereas only 16\% of immediate-release hydromorphone was retained. HIV reverse transcriptase activity and infectivity were preserved in IDPE in the presence of HCR, but not in the presence of immediate-release hydromorphone. Heating the IDPE rapidly inactivated HIV despite the presence of residual HCR.

IDPE sharing is associated with HIV infection. Hydromorphone controlled-release preparation leaves a significant drug residue in IDPE which encourages IDPE reuse and sharing among PWID. HCR excipients preserve HIV viability which could potentiate HIV transmission in this population. Heating IDPE prior to drug aspiration may be an effective harm reduction strategy.

5.3 Introduction

Injection of prescription opioids has reached epidemic levels, resulting in an almost fourfold rise in hospital admissions in the United States between 2004–2014 \[1\]. This epidemic has also been associated with several large outbreaks of HIV \[2,3\] infection among people who inject drugs (PWID), including one in London, Ontario that resulted in
the declaration of a Public Health emergency (June 14, 2016) (Figure 1) [4]. The control of these outbreaks generally depends on a multi-pronged approach of needle distribution, opiate substitution therapy, and provision of antiretroviral therapy. Our outbreak was unusual as it occurred despite London already having at baseline the highest distribution of sterile needles and syringes per capita in Canada, and an opiate substitution therapy prescription rate in the top quartile for Ontario [5]. As well, London has a government-funded, multidisciplinary HIV care program with access to funded HIV antiretroviral therapy. The reason for this outbreak despite the excellent pre-existing control programs was the subject of this investigation.

A study of injection drug users in London identified hydromorphone controlled-release (HCR) capsules (Hydromorph Contín® [marketed as Palladone SR® in Europe], Purdue Pharma, Stamford CT, and marketed as Jurnista® in Australia, Janssen-Cilag Pty Ltd, High Wycombe, UK.) as the preferred opioid for illicit use. The majority of PWID (79%) reported HCR use in the past six months [6]. In addition, prescription rates of HCR capsules are unusually high in London compared to other regions in Ontario, which may indicate widespread availability of the drug for illicit use [5].

HCR capsules have a low solubility in water, and thus preparation of the drug for injection requires numerous steps (Figure 2) [7]. First, the time released beads must be removed from the capsule and crushed, then mixed with water in a cooker to create a slurry, and then drawn into a syringe through a cotton filter to remove visible particulate matter (Figure 2) [7]. The filters, cookers and water are referred to as the injection drug preparation equipment (IDPE). The large dose of hydromorphone in the controlled-release formulation and the need to dissolve the capsule in a large volume of water increases the likelihood that the residual drug will be shared between users [7]. In addition, patients report large amounts of non-solubilized drug are retained in the filter and cooker after the first use, thereby encouraging the reuse and/or sharing of used IDPE [7,8]. Reuse of IDPE is done by adding water to the previously used cooker with residual drug still inside, placing a needle into the previously used filter and aspirating the residual drug (“doing another wash”) (Figure 2).
Sharing of needles/syringes amongst persons who inject drugs has been well established as a means of HIV transmission [9]. Thus, distribution of sterile needles/syringes has been a major focus of HIV prevention programs. However, sharing of IDPE is more common than sharing of needles/syringes [10–14]. In some centers, sharing of IDPE has been identified as the primary risk factor for Hepatitis C (HCV) transmission [10,15–17]. The incentives for sharing IDPE despite the existence of free IDPE distribution programs, and the impact of IDPE sharing on HIV transmission, have not been extensively studied. We propose that IDPE may initially become contaminated with HIV via a used needle from a PWID who reuses their own needle [18,19] and then HIV is transmitted when the IDPE is shared with a second user placing their own needle into the used filter/cooker to aspirate another “wash”. We further hypothesized that heating the contaminated IDPE prior to each use could inactivate HIV and help prevent transmission.

The objectives of this study were to clarify the following: (1) whether sharing IDPE is associated with HIV infection, (2) the amount of residual hydromorphone in IDPE after the first injection [and thus the incentive for reuse or sharing of IDPE] and the effects of heating of the IDPE on the amount of hydromorphone available for injection, (3) the persistence of HIV in IDPE and the efficacy of heating to inactivate HIV to prevent transmission.

5.4 Methods

5.4.1 Setting

The HIV outbreak in London, Ontario, was confirmed by comparing new diagnoses provided by the local Public Health Unit to regional and provincial figures. Cases were referred to as incident infections if no previous positive result for HIV serology or HIV viral load testing was available in the provincial database. Sociodemographic characteristics and risk factors for incident HIV cases were determined by reporting on the province wide, standardized serological HIV testing form.


5.4.2 Association Between Sharing IDPE and HIV Infection

A case-control study of HIV-infected (HIV+) and HIV-uninfected (HIV-) injection drug users was conducted to examine whether sharing IDPE is associated with HIV infection. The primary exposure of interest was IDPE sharing within the last 3 months. Inclusion criteria were age >17 years, residence in London-Middlesex county, Canada, and reported injection drug use within the last three months. A total of 127 participants were recruited from two sites: A) consecutive patients admitted to one of two local hospitals with a diagnosis of injection drug use associated endocarditis (as these were patients with high risk injection practices) and B) from a local needle exchange (where sterile needles/syringes/cookers/filters and sterile water were distributed). Recruitment occurred between August 11, 2016 and June 7, 2017. Routine HIV testing was performed using rapid diagnostic tests at the safe injection site and fourth generation testing at the hospital sites. Participants completed a detailed questionnaire regarding injection behaviors. The study was approved by the Research Ethics Board, Western University. Written informed consent was obtained from all participants.

5.4.3 Power Calculation

Based on a previous study that found a 40% prevalence of sharing IDPE amongst those injecting hydromorphone controlled-release [8], we estimated an exposure prevalence of 20% in controls and 50% in cases, corresponding to an odds ratio of 4.0. A minimum of 33 cases and 66 controls were needed to detect this association between sharing IDPE and HIV status, with 80% power, a 2-tailed alpha of 5%, and the continuity correction [20]. Final recruitment was 35 cases (HIV+) and 84 controls (HIV-).

Analyses were conducted using SAS 9.4 (SAS Institute Inc. Cary, NC, USA). Sociodemographic characteristics and reported sharing behaviors between cases and controls were compared using logistic regressions. Chi-square tests were used to compare other categorical variables such as multiple washes of IDPE, and attitudes and beliefs about heating. Multivariable logistic regressions were conducted for the primary exposure of interest: IDPE sharing. If the addition of any sociodemographic variable demonstrated a confounding influence (>10% change), or interaction with, the IDPE sharing parameter
estimate, or if it was a significant independent predictor itself, it was retained in the multivariable models. Variables that met these criteria included: employment, housing, LGBTQ, and MSM; only housing and MSM were retained in the multivariable model as a result of multicollinearity with employment and LGBTQ variables, respectively. Two-tailed tests were employed, and the level of significance was set to 0.05.

5.4.4 Residual Hydromorphone in IDPE & Effects of Heating on the Amount of Hydromorphone Injected

All experiments were performed with unused needles/syringes and IDPE equipment (sterile water, cookers, filters) (Sterifilt™ Apothicom, Paris, Fr) that were distributed as part of the provincial harm reduction program, and thus commonly used by local PWIDs. To quantify the amount of residual drug present in the cooker and filter after an initial injection, we replicated the technique of crushing the drug, placing it in a cooker, dissolving it in water, and aspirating the solution through a filter (Figure 2). We developed this technique in consultation with local PWIDs. We performed this experiment with hydromorphone controlled-release capsule (24 mg) (Purdue Pharma, Stamford, CT) and immediate-release hydromorphone tablet (8 mg) (Pharmascience Inc., Montreal, PQ) as they are the doses and preparations most commonly injected locally. Hydromorphone concentrations were measured using liquid chromatography–tandem mass spectrometry (LC-MS/MS). The effect of heating on drug stability was examined by heating the preparation with a cigarette lighter until bubbles formed.
Figure 5.1) Incidence and count of HIV infection in Middlesex-London and Ontario, 2005-2017. The Ontario (ON) and Middlesex-London (ML) HIV rates (per 100,000) are shown in the red and green lines respectively. Middlesex-London HIV incident cases per year are shown in the green bars. * denotes significantly higher (p<0.05) incidence of HIV in Middlesex-London relative to Ontario.
5.4.5 HIV Persistence in IDPE & Effects of Heating on HIV

All experiments with replication competent HIV-1 were performed in a BSL2+ facility with appropriate safety and waste protocols. Hydromorphone controlled-release capsules and immediate-release hydromorphone tablets were prepared as above (schematic in Figure 1). Two controls were used for each experiment 1) 250 mg of the excipient microcrystalline cellulose (Sigma) was added to the cookers, or 2) the cookers remained empty. Microcrystalline cellulose is a common filler/stabilizer in controlled-release drug formulations including hydromorphone controlled-release. HIV-1 (CXCR4 tropic genotype #1389) at titer of 1*10^8 infectious particles per mL was diluted 1:5 in sterile water (Sigma) and 1.5 mL was added to each cooker.

Following a five-minute, room temperature incubation, the water was aspirated through the filter with a syringe (sans needle per containment lab requirements). This injectate preparation protocol was repeated with serial washes at 1 hour, 4 hours, 24 hours, 48 hours, and 72 hours for a total of six preparations for each filter. Filters were collected after the final aspiration. Insoluble residual material from the hydromorphone controlled-release and microcrystalline cellulose cookers was collected after the 24, 48, and 72-hour time points (50 mg per time point). To measure HIV viral persistence, injectates were split into aliquots for HIV reverse transcriptase and infectivity assays using Tzm-bl cells. Protocols for these assays have been previously published [21].

To assess the effects of heating on HIV persistence, cookers were heated with an ethanol flame until bubbles formed around the edge of the cooker. All laboratory experiments were performed in triplicate with standard error bars displayed. Heating experiments were performed according to BSL2+ safety protocols in a biosafety cabinet with an ethanol flame. Cookers were heated until bubbles formed around the edge of the cooker.

Prior to 2012, the filters distributed by harm reduction sites in Ontario were originally intended for the dental industry. These filters are made of rolled, less dense cotton with no chemical binding agents, additives, or whiteners (Maxill 53190). The filters distributed since 2012 are made of pressed cotton and coated with methylethyl-cellulose (Apothicom, France). Filters were cut to normalize by weight between generations.
Figure 5.2) Method for preparing Hydromorphone controlled-release for injection. 1) Open hydromorphone controlled-release capsule and empty time release beads into a pill crusher to crush the beads. Crush beads into a fine dust. Many individuals opt to use the bottom of a disposable lighter or the tops of syringe plungers to crush the beads as opposed to a pill crusher. Empty the crushed beads into a cooker. 2) Place a cotton injection filter into the solution. 3) Add approximately 1.5mL of water to the cooker. Allow cooker to sit for 5 minutes at room temperature to dissolve the opioid. 4) Draw up the solution into a syringe and inject. 5) Individuals usually opt to perform multiple “washes” whereby further water is added to the residual drug in the cooker/filter and the used needle is reintroduced into the same filter. The individual will then draw the additional drug solution and re-inject. Multiple users may participate with “serial washes” using the same filter/cooker/crushed-drug complex and patients reusing their own needle/syringe into a common filter.
5.4.6 Community Interventions

Based upon the results of the above investigations a community education project was carried out starting on June 15, 2017. This involved peer mentors, injection equipment distribution staff and public health officials spreading a message to PWIDs to “cook your wash” ie heat the IDPE containing the drug and filter and water, with a cigarette lighter until bubbling prior to any injection of hydromorphone. Local mass media was also used to spread this message (see “London researchers may have pinpointed why rates of HIV, other diseases rising among drug users” in the London Free Press, October 27th, 2017). In October 2017, a program of health care navigators to enable HIV infected patients to attend their medical appointments was initiated. These staff were also involved in the education campaign.

5.5 Results

HIV incidence in London rose above that in other regions of Ontario in 2015 and 2016 (Figure 1) (p<0.05). The incidence remained high in early 2017 but fell in the latter half of 2017 and was no longer significantly different from that of Ontario for 2017 as a whole.

Data from 2016 suggested that the most frequently reported exposure category among new HIV cases in London was “injection drug use” (n=45/60; 75%), whereas <10% of new HIV cases occurred amongst persons who inject drugs in the same time period in the rest of Ontario. Mean age of new HIV cases was 37.6 years (SD=9.7). Sixty four percent were male.

5.5.1 Association Between Sharing IDPE and HIV Infection

One hundred and twenty-seven patients were recruited into the case control study. Eight participants were excluded due to unknown HIV status. The remainder were divided into cases (HIV infected; n=35) and controls (HIV uninfected; n=84).

Cases (HIV+) and controls (HIV–) for this study were all persons who inject drugs, living in London, and were similar with regards to age, sex, housing status, race, employment, and recent endocarditis (Table 1). There was no difference in the proportion of inpatients
(recruited from the hospital site) and participants recruited at the safe-injection site, in the case and control groups.

Unadjusted (Table 1) and adjusted (Table 2) models showed that HIV infection was strongly and consistently associated with sharing IDPE among persons who inject drugs. HIV infection was associated with men-who-have-sex-with-men status, although the association was weaker than sharing of IDPE. Sharing needles/syringes in the absence of IDPE sharing was not associated with HIV status. Removing either MSM status or housing status from the regression model did not change the results.
Table 5.1) Characteristics of HIV infected and HIV uninfected Persons Who Inject Drugs

<table>
<thead>
<tr>
<th>Injection Equipment Sharing</th>
<th>Case (HIV +) (n = 35)</th>
<th>Control (HIV –) (n = 84)</th>
<th>Odds Ratio (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>14 (42%)</td>
<td>61 (73%)</td>
<td>Reference</td>
</tr>
<tr>
<td>Only needles/syringes</td>
<td>4 (12%)</td>
<td>16 (19%)</td>
<td>1.09 (0.32, 3.76)</td>
</tr>
<tr>
<td>Only IDPE¹</td>
<td>10 (30%)</td>
<td>3 (4%)</td>
<td>14.52 (3.53, 59.77)*</td>
</tr>
<tr>
<td>Both IDPE² and needles/syringes</td>
<td>5 (15%)</td>
<td>3 (4%)</td>
<td>7.26 (1.55, 34.03) *</td>
</tr>
</tbody>
</table>

Sociodemographic factors

<p>| Age                        | 38.4 (10.2)          | 41.2 (10.5)              | 1.03 (0.99, 1.07)                    |
| Female                     | 6 (17%)              | 21 (25%)                 | 0.62 (0.23, 1.70)                    |
| MSM²                       | 5 (18%)              | 2 (3%)                   | 6.63 (1.20, 36.60) *                 |
| LGBTQ³                     | 3 (12%)              | 7 (9%)                   | 1.29 (0.61, 5.39)                    |
| Stable housing⁴            | 19 (54%)             | 34 (41%)                 | 1.71 (0.77, 3.79)                    |
| Aboriginal (REF=Caucasian)⁵| 9 (26%)              | 17 (21%)                 | 1.33 (0.53, 3.39)                    |
| Stable employment⁶         | 2 (6%)               | 5 (6%)                   | 1.02 (0.19, 5.53)                    |
| Endocarditis               | 9 (25%)              | 14 (17%)                 | 1.73 (0.67, 4.48)                    |</p>
<table>
<thead>
<tr>
<th>Injection drug use</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydromorphone controlled-release</td>
<td>31 (89%)</td>
<td>66 (79%)</td>
<td>2.11 (0.66, 6.77)</td>
</tr>
<tr>
<td>Immediate-release hydromorphone</td>
<td>20 (57%)</td>
<td>64 (76%)</td>
<td>0.42 (0.18, 0.96) *</td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>27 (77%)</td>
<td>63 (75%)</td>
<td>1.13 (0.44, 2.85)</td>
</tr>
</tbody>
</table>

* Highlights significant values
1 IDPE = Injection Drug Preparation Equipment
2 Standard Deviation
3 Sharing data available for 33 cases and 83 controls
4 MSM (men who have sex with men) data available for 28 cases and 63 controls
5 LGBTQ (lesbian, gay, bisexual, transgender and queer) data available for 26 cases and 76 controls
6 Housing data available for 35 cases and 83 controls
7 Race data available for 34 cases and 80 controls
8 Employment data available for 33 cases and 84 controls
Note: MSM defines a behaviour while LGBTQ is an umbrella term for many sexual and gender identities
Table 5.2) Associations of HIV status with injecting behavior and Risk Factor variables. Logistic Regression.

<table>
<thead>
<tr>
<th>Sharing (Ref=neither)</th>
<th>Odds Ratio (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Both IDPE and needle/syringes</td>
<td>23.88 (2.36, 241.82)</td>
<td>.007</td>
</tr>
<tr>
<td>Only IDPE</td>
<td>22.12 (4.51, 108.59)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Only needle/syringes</td>
<td>0.91 (0.16, 5.37)</td>
<td>0.92</td>
</tr>
<tr>
<td>MSM</td>
<td>11.34 (1.79, 71.69)</td>
<td>0.01</td>
</tr>
<tr>
<td>Stable housing</td>
<td>1.83 (0.55, 6.10)</td>
<td>0.32</td>
</tr>
</tbody>
</table>

Adjusted odds ratio (OR) and associated 95% confidence interval (CI) are presented. IDPE: injection drug preparation equipment; MSM: men who have sex with men.
5.5.2 Residual Hydromorphone in IDPE & Effects of Heating on the Amount of Hydromorphone Injected

Forty-five percent of the total hydromorphone in the hydromorphone controlled-release capsule remained in the IDPE after the first aspiration (and thus was available for use in a subsequent “wash”), whereas only 16% remained in the IDPE when using immediate-release hydromorphone tablets (Figure 3). Heating during the injection preparation did not significantly change the amount of hydromorphone controlled-release in the injectate or the amount recovered from the IDPE (Figure 3). The preparation boiled (reached bubbling) in less than 10 seconds when using a cigarette lighter.

Patient behavior reflected the findings of high drug residual in hydromorphone controlled-release preparations. The case control subjects reported performing multiple washes of used IDPE more frequently when using hydromorphone controlled-release than with other drugs. Multiple washes were reported when using hydromorphone controlled-release [93/97 (96%) (of whom 31/31 HIV+, and 62/66 controls)], in comparison to immediate-release hydromorphone [41/84 (49%) (12/20 HIV+, and 29/64 controls); p<0.001] and crystal methamphetamine [19/92 (21%) (9/29 HIV+, and 10/63 controls); p<0.001] (denominators reflect number of patients using the respective drug in each group).
Figure 5.3) Relative amount of hydromorphone recovered in syringe (injectate sample) and injection drug preparation equipment (filter and residue samples) with and without heating with A) 24mg of hydromorphone controlled-release and B) Immediate-release hydromorphone 8mg. Error bars represent standard error.
Simulating drug preparation habits with a known inoculum of HIV-1 (Figure 2) allowed us to quantify relative viral persistence in different IDPE environments (Figure 4). Tzm-bl cells are highly susceptible to HIV infection and were used to quantify viral infectivity in the injectate samples under conditions employed by PWIDs. When preparing HCR for injection, infectious HIV was recovered from the zero-, one-, and four-hour time points, representing washes one, two, and three of HIV-inoculated IDPE. Significantly less infectious HIV was recovered from HIV-inoculated IDPE used when preparing immediate-release hydromorphone compared to hydromorphone controlled-release. Infectious HIV recovery was intermediate from HIV-inoculated IDPE that contained microcrystalline cellulose as a control (Figure 4A).

The limiting sensitivity of the Tzm-bl assay prevented us from measuring infectivity beyond four hours (third wash). Previous work has indicated that reverse transcriptase activity in HIV-1 is a strong predictor of virus infectivity and has greater sensitivity/specificity than other surrogate assays such as p24 ELISAs or viral RNA load [22]. In the presence of HCR, and to a lesser extent microcrystalline cellulose, HIV reverse transcriptase activity persisted in the filters for 72 hours after inoculation and six serial washes, whereas HIV was not detected in the same conditions when originally mixed with immediate-release hydromorphone or the water control (Figure 4B).
Figure 5.4) Infectious HIV persistence after addition to Injection Drug Preparation Equipment.

A) Infectivity of HIV-1 in injectates as determined by Tzm-bl cell luciferase production. HIV-1 was spiked into cookers containing opioids and expedients to measure viral persistence. Serial washes with sterile water were collected 5 minutes, 1 hour and 4 hours post addition of virus. Maximal preservation of infectivity over time seen with Hydromorphone controlled-release across all measured timepoints. Error bars represent standard error. * significant at p<0.05 via two-way ANOVA with Tukey’s correction for multiple comparisons. B) HIV-1 reverse transcriptase activity after 72 hours in injectates (after 6 washes). Only hydromorphone controlled-release and Microcrystalline Cellulose continued to show above-background reverse transcriptase activity at this last timepoint. Dashed lines represent assay detection limits. Error bars represent standard error. * significant at p<0.05 via two-tailed t-tests.
Figure 5.5) Reduction in infectious HIV-1 (measured via Tzm-bl cell luciferase production) after 10 second heat treatment. HIV-1 was added to cookers containing hydromorphone controlled-release 24mg and an Apothicom filter and washed with sterile water (Injectate Washes 1 & 2 were collected 5 minutes and 1 hour after HIV-1 addition respectively). Cookers were then heated above an ethanol flame for 10 seconds or left at room temperature. Persisting infectious HIV-1 was quantified via luciferase production from Tzm-bl cells. Dashed line represents assay detection limit. Error bars represent standard error. ** significant at p<0.001 via two-tailed t-test.
To identify the location of the HIV-1 reservoir in the IDPE, we measured reverse transcriptase activity in the insoluble drug material and filters independently, both “housed” in the cooker, after 72 hours. Higher levels of reverse transcriptase activity (i.e. HIV) were observed in the filters in the presence of hydromorphone controlled-release compared to the other compounds (Supplemental Figure 1). Heating the cooker liquid with HCR or hydromorphone immediate-release to a boil reduced the residual HIV-1 titers (infectious or reverse transcriptase activity) by two logs, to the limits of detection (Figure 5).

5.6 Discussion

5.6.1 Association Between Sharing IDPE and HIV Infection

We demonstrate that sharing of IDPE among injection drug users was a strong risk factor for HIV infection. We cannot differentiate risk associated with specific drug preparation equipment (i.e., filters versus cookers, rinse water, or residual drug), as these items are almost always used and shared concurrently [17]. However, the significant amount of viable virus found within the filters at 72 hours suggests that inserting an unused needle into a shared filter could lead to infection (Supplemental Figure 1). We also found that sharing both IDPE and needles/syringes was associated with an increased risk of HIV transmission. However, this result was likely driven by sharing IDPE, as sharing needles/syringes alone was not a significant factor in HIV infection in our study, and the adjusted odds ratio (aOR) of sharing both IDPE and needles/syringes was similar to that of sharing IDPE alone. Sharing of needles and syringes has been shown to be a risk for HIV infection in other settings, however our study occurred in an area with an exceptionally large needle/syringe distribution program enabling assessment of the impact of IDPE sharing when needle/syringe sharing is relatively infrequent. In contrast to our study, a recent HIV outbreak in Indiana demonstrated a 90% rate of needle/syringe sharing amongst HIV+ patients and 66% amongst HIV negative controls during the outbreak. Our much lower rates of needle/syringe sharing allowed the impact of IDPE sharing to be detected. Therefore, as needle/syringe distribution programs are rolled out, interventions to prevent HIV transmission associated with IDPE sharing will become increasingly important to eliminate further HIV transmission.
As in previous studies [8,10,12–14] we found that sharing filters and other IDPE occurs often. This is likely because the risk of HIV transmission with IDPE is not recognized. Participants who avoided needle/syringe sharing frequently reported IDPE sharing several times per day. Those who share needle/syringes likely do so very rarely, due to the known significant associated risk. Therefore, even if the risk of HIV infection associated with a single episode of needle/syringe sharing may be higher than that of a single episode of IDPE sharing, the high frequency of IDPE sharing contributes to a greater cumulative risk. We also suspect that due to awareness of the risks associated with needle/syringe sharing, individuals may only do so with partners whom they believe are sero-concordant. Due to a lack of awareness of the risk associated with IDPE sharing, we believe that individuals do not avoid this behavior, nor do they attempt to sero-sort with IDPE sharing. Further studies are necessary to confirm this hypothesis.

5.6.2 Residual Hydromorphone in IDPE

These results provide the first evidence that 45% of the contents of a hydromorphone controlled-release capsule remain in the IDPE after an initial use, thereby incentivizing the behavior of frequent reuse of the IDPE to access retained HCR. The local street value of a capsule of hydromorphone controlled-release is approximately $60 CAN ($45.60 US) and therefore, disposal of the remaining drug in the IDPE is very uncommon. This leads to IDPE being commonly shared and sold among persons who inject drugs, despite nearly exclusive use of fresh needles/syringes [7]. In contrast, the low quantity of residual immediate-release hydromorphone on the IDPE, due to its lower dose and the high solubility of immediate release opioids, provides less incentive to reuse the IDPE. Our study confirmed that reuse was indeed most common with hydromorphone controlled-release and less common with hydromorphone immediate-release or crystal methamphetamine.

5.6.3 HIV Persistence in IDPE

It has previously been demonstrated that HIV RNA can be found in high titer in IDPE used by persons who inject drugs, although the specific drugs used and their relative abilities to preserve HIV were not described in these studies [18,19]. HIV survival has been found to
be reduced in the presence of heroin, but it is unclear whether this was related to the heating process required for home production or the many caustic chemicals added to produce the solution [23]. To our knowledge, this study is the first to assess HIV persistence in the presence of prescribed opioids that do not require heating or the addition of extra chemicals for solubility. We demonstrated prolonged HIV survival in IDPE used in the preparation of controlled-release opioid (up to 3 days) compared to that used for an immediate-release opioid preparation. The dual effect of a controlled-release opioid which not only increases HIV survival in IDPE but is also retained, thereby providing an incentive for sharing IDPE, appears to be synergistic in facilitating HIV transmission.

HIV persistence may be related to excipients in the long-acting preparation, as persistence was not seen with hydromorphone immediate-release tablets. Specifically, excipients in hydromorphone controlled-release that are not in immediate-release hydromorphone include ethyl cellulose, hydroxypropyl methylcellulose, and microcrystalline cellulose. Addition of microcrystalline cellulose alone to the IDPE retained more viable HIV and for longer than IDPE used with water or IDPE used with immediate-release hydromorphone. We were unable to obtain pure hydromorphone in the absence of the other constituents in the formulation, which affected our ability to determine if the drug alone impacts HIV viability and retention on the IDPE. However, we believe that it is more likely that excipients in the long-acting drug preparations may contribute to HIV persistence. Cellulose-containing drugs may maintain hydration, resulting in virus preservation. In fact, methyl cellulose derivatives are a major constituent in media to maintain hydration and limit diffusion for viral plaque assays used for clinical diagnostics [24].

5.6.4 Harm Reduction Implications

While HIV transmission from sharing needles/syringes has been well established, transmission associated with sharing IDPE in the absence of needle/syringe sharing has only been suggested [25,26]. As a result, harm reduction education for persons who inject drugs often emphasizes the risk of sharing needles/syringes, but not IDPE. Future harm reduction education should emphasize the importance of not sharing IDPE, as this behavior could reduce the incidence of HIV among persons who inject drugs. However, it is unlikely that a campaign focusing on the prevention of IDPE sharing alone would be successful,
given the economic pressures to maximize utilization of the very expensive residual hydromorphone.

We found that HIV+ cases were more likely to believe that heating hydromorphone during preparation was not necessary or was harmful to drug availability (believing it “boiled off the dope”). A previous study showed that HIV is inactivated with heating IDPE, although this was not studied in the presence of prescribed opioids [27]. We demonstrated that heating IDPE until boiling is an effective means of inactivating HIV, even in the presence of a controlled-release opioid. Heating maintained the same dose of active hydromorphone in the injectate, and therefore would not contribute to increased rates of overdosing or discourage drug users who are afraid of reducing drug availability. Thus, HIV transmission may be attenuated by promoting the message to “cook the wash” (i.e. heat the wash with a lighter until bubbling) if reusing IDPE.

Based upon the results of the above investigations a community education project was carried out starting on June 15, 2017. This involved peer mentors, injection equipment distribution staff, HIV healthcare navigators, and public health officials spreading a message to PWIDs to “cook your wash” i.e., heat the IDPE containing the drug and filter and water with a cigarette lighter until bubbling prior to any injection of hydromorphone. Local mass media was also used to spread this message. Results of this campaign will be presented at a later date.

Using a sterile needle for every aspirate of a drug is also useful in preventing viral contamination of the IDPE, but this alone will not reduce the risk of bacterial contamination of the injectate from repeated handling; boiling is necessary to reduce this potential bacterial load (data not shown). Cleaning the IDPE with bleach prior to reuse has been occasionally recommended, although it would not be acceptable in the context of retained drug and injectate being exposed to the caustic effects of bleach. Recognizing that the formulation of hydromorphone controlled-release is a specific risk factor for reuse, the provision of injectable hydromorphone should also be considered, as per the SALOME trials [28].
5.6.5 HIV Transmission in Other Regions

We suspect that HIV transmission in association with sharing IDPE may be occurring in other regions as well. There have been several recent large injection drug use-associated HIV outbreaks including an ongoing outbreak in Saskatchewan, where hydromorphone controlled-release is the opioid of choice [30]. Similarly, the opioid of choice in a recent outbreak in Indiana was a controlled-release preparation containing microcrystalline cellulose [oxymorphone hydrochloride extended-release (Opana ER®, Endo International PLC, Malvern Pennsylvania)]; [31] a drug that has also been associated with IDPE sharing and HCV transmission [2,10,31]. The risk of HIV and HCV infection associated with illicit use of oxymorphone extended-release resulted in its removal from the US market [29], although it is still available in Canada and Europe.

5.6.6 Study Limitations

The major exposure of interest was sharing of IDPE. As this was identified retrospectively in a case control design, the possibility of recall bias cannot be ruled out. However, it is notable that HIV infected patients were no more likely to recall sharing of needles/syringes than HIV uninfected patients and so we do not believe that recall bias was leading case patients to non-specifically report more risky behaviors.

As the focus of this study was on HIV transmission associated with Injection Drug Use, some sexual risk factors for HIV were not fully investigated (e.g. unprotected sex, multiple partners). Report of beliefs and attitudes regarding heating IDPE prior to injection was not available for several participants, and so this could not be placed into the multiple regression analysis and only an unadjusted analysis of these data could be presented.

Hydromorphone immediate-release use was associated with a protective effect in unadjusted analysis, as it tends to be used by local persons who inject drugs in substitution for Hydromorphone controlled-release when the latter is unavailable or unaffordable. Therefore, increased hydromorphone immediate-release use would suggest decreased controlled-release availability, and we believe that the controlled release formulation was most important in transmission.
5.7 Conclusion

The recent outbreaks of HIV in PWID emphasize the need for understanding all mechanisms of transmission and the development of new tools in the fight against HIV transmission in this population. This community suffered an outbreak of HIV despite having excellent pre-existing traditional programs for harm reduction and HIV prevention. Our data demonstrated that the widespread local abuse of a controlled release opioid which encouraged reuse and sharing of IDPE when injecting, and the increased HIV survival in the IDPE, contributed to the significant infectiousness of the practice. Preventing IDPE sharing will be difficult due to the retention of valued drug in the IDPE after use. Heating IDPE prior to each use may provide an effective harm reduction approach to reducing HIV infection. As the popularity of injecting prescription opioids continues to increase [5,30], implementation of harm reduction strategies that target sharing IDPE as a mechanism of transmission will become increasingly important.

5.8 Acknowledgements

We would like to thank the Middlesex-London Health Unit for sharing data on the epidemic curve and characteristics, and Dr G Hovhannisyan for her input; B Lester, S Burke, B Henry, K Burton at the Regional HIV AIDS Connection for assistance in recruiting participants for interviews; Dr S. Pearl for editing and the many individuals who volunteered for participation and shared their experiences.
A) HIV-1 was spiked into IDPEs with varying opioids and syringe filters to measure viral persistence. HIV-1 isolated from the syringe filters after 72 hours was quantified via Reverse Transcriptase assay. * significant at p<0.05, ** significant at p<0.01 via two-way ANOVA with Tukey’s correction for multiple comparisons. B) HIV-1 isolated from the injectates pulled through each filter type after 72 hours was quantified via Reverse Transcriptase assay. Apothicom® filters are made of tightly woven cotton coated in methylethyl-cellulose while dental filters are loosely woven, undyed cotton. Virus Reverse Transcriptase activity was best preserved using Hydromorphone controlled-release and Apothicom® filters. Dashed line represents assay detection limit. Error bars represent standard error. * significant at p<0.05, ** at p<0.001 via via two-way ANOVA with Tukey’s correction for multiple comparisons.
5.9 References


EDUCATION

Ph.D (Microbiology and Immunology) August 2014 - April 2019
University of Western Ontario
Schulich School of Medicine
Supervisor: Dr. Eric Arts

Bachelor of Science (Biology Major) August 2005 - May 2009
Denison University
Supervisor: Dr. Andrew McCall

PUBLICATIONS


McCall AC, Murphy SJ, Venner C, Brown M. Florivores Prefer White Versus

SCHOLARSHIPS AND AWARDS

International Ontario Graduate Scholarship (Provincial Award) 2017-2018
University of Western Ontario

R.G.E. Murray Fellowship (Institutional Award) 2016-2017
University of Western Ontario

R.G.E. Murray Travel Awards (Departmental Awards) 2014-2018
University of Western Ontario

Anderson Grant (Institutional Award) 2008-2009
Denison University

Battelle Memorial Institute Award (Institutional Award) 2007-2008
Denison University

PRESENTATIONS


Honorable Mention for Poster Presentation


Venner, C.M., Nankya, I., & Arts, E.J. 2017. HIV Subtypes Display Differential Receptor Binding and Entry Rates: Contributions to Pathogenesis. Oral Presentation, Canadian Association for HIV Research Conference, Montreal, QC.

Venner, C.M., Nankya, I., & Arts, E.J. 2016. Slow disease progression of women infected with HIV-1 subtype C versus subtype A and D: Implications for the epidemic. Poster, Canadian Association for HIV Research Conference, Winnipeg, MB.

**Second Prize for Oral Presentation**


**Venner, C.M.,** Nankya, I., & Arts, E.J. 2015. HIV-1 Group M subtypes display differential rates of CD4 T-Cell decline. Poster, Canadian Association for HIV Research Conference, Toronto, ON.


**Venner, C.,** & McCall A.C. 2009. Plant traits that affect pollination and seed set in *Echinacea angustifolia*. Poster, Denison University Senior Research Symposium, Granville, OH.


**TEACHING ASSISTANTSHIPS**

**Teaching Assistant** - University of Western Ontario
Biological, Social, and Economic Determinants of Infectious Diseases
GHS3500 - Spring 2018

Bacterial Pathogenesis
MICRO4100 - Fall 2015, Fall 2016

**Teaching Assistant** - Denison University
Plant Evolution and Reproduction
BIOL326 – Spring 2009

Introduction to Biology
BIOL150 – Fall 2008