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Erin M. Kahle

Understanding risk factors for HIV-1 infectiousness and transmission: prospective studies in
HIV-1 serodiscordant couples

Erin M. Kahle

A dissertation

submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington

2013

Reading Committee:

Jared M. Baeten, Chair

Connie Celum

Grace John-Stewart

Program authorized to Offer Degree:

Public Health – Epidemiology

University of Washington

Abstract

Understanding risk factors for HIV-1 infectiousness and transmission: prospective studies in HIV-1 serodiscordant couples

Erin Kahle

Chair of the Supervisory Committee:
Jared M. Baeten, Associate Professor
Departments of Global Health, Medicine and Epidemiology

The studies described in this dissertation focus on clinical and biologic factors associated with increased HIV-1 infectiousness among heterosexual HIV-1 serodiscordant couples. The specific aims include 1) evaluating whether specific characteristics of HIV-1 serodiscordant couples could be used to defined a higher-risk subgroup for targeted prevention research, 2) assessing the prevalence of unreported antiretroviral therapy (ART) in HIV-1 infected participants enrolling in an HIV-1 prevention trial, 3) determining whether HIV-1 subtype C is associated with increased HIV-1 transmission, and 4) assessing whether immune activation is associated with increased HIV-1 transmission.

The identification of a composite set of predictors for HIV-1 transmission is applicable to the design of efficient prevention programs targeting high-risk subpopulations to maximize limited prevention resources. We developed a risk score for identifying a high-risk subpopulation of HIV-1 serodiscordant couples which will provide greater predictive ability in identifying HIV-1 transmission risk than individual risk predictors (i.e. viral load, unprotected sex). A well-developed and validated risk scoring tool, such as ours, is a valuable addition to HIV-1 prevention intervention research in order to reduce sample size, decrease cost of study and provide more efficient recruitment.

Biologic factors, including both viral and host characteristics, may be associated with increased HIV-1 infectiousness. HIV-1 subtype, specifically subtype C, has been suggested as a factor in

differential HIV-1 transmission between populations, although no epidemiologic evidence supports this conclusion. We compared HIV-1 subtype C and non-C subtypes and found no significant difference in risk of HIV-1 transmission in a multinational population in sub-Saharan Africa. In an analysis of cytokines as markers for immune activation, we found elevated IL-10 and IP-10 concentrations to be associated with increased HIV-1 transmission and acquisition, suggesting a potential biologic mechanism in both HIV-1 infected and susceptible partners.

HIV-1 serodiscordant couples cohorts offer unique opportunities to assess correlates of HIV-1 infectiousness, as transmissions can be directly measured within partnerships. The application of our study findings will provide more efficient methods for identifying target populations and a better understanding of the virologic and immunologic mechanisms of HIV-1 infectiousness.

TABLE OF CONTENTS

List of tables.....	ii
List of figures.....	iii
Acknowledgements.....	iv
Dedication.....	v
Chapter 1: Introduction.....	1
Chapter 2: An empiric risk scoring tool for identifying high-risk heterosexual HIV-1 serodiscordant couples for targeted HIV-1 prevention.....	16
Chapter 3: Unreported antiretroviral use by HIV-1 infected participants enrolling in a prospective research study.....	38
Chapter 4: HIV-1 subtype C is not associated with higher risk of heterosexual HIV-1 transmission: a multinational study among African HIV-1 serodiscordnat couples.....	48
Chapter 5: Immune activation and risk of HIV-1 transmission: a nested case-control study among HIV-1 serodiscordant couples.....	71
Chapter 6: Conclusion.....	97
References.....	109

LIST OF TABLES

Table 1. Features of the HIV-1 pandemic, according to subtype of circulating recombinant form (CRF).....	9
Table 2. Selected cytokines modulating HIV replication <i>in vitro</i>	13
Table 3. Enrollment characteristics of derivative and validation study populations for risk score development.....	32
Table 4. Analysis of predictors and calculation of risk score.....	33
Table 5. Enrollment characteristics and type of antiretroviral (ART) detected among HIV-1 infected partners.....	47
Table 6. Characteristics of the HIV-1 subtype nested case-control cohort.....	65
Table 7. Adjusted multivariate models for the primary nested case-control and sensitivity case-cohort analyses comparing HIV-1 transmission for subtype C versus non-C subtypes.....	66
Table 8. Characteristics of the immune activation nested case-control cohort.....	87
Table 9. Mean (range) log ₁₀ concentration of cytokine analytes.....	88
Table 10. Logistic regression models associating cytokine concentrations with HIV-1 risk.....	89
Table 11. Select statistical methods.....	106

LIST OF FIGURES

Figure 1. Incidence of HIV-1 infection by risk score for a) full risk score, b) sensitivity analysis: excluding plasma HIV-1 viral load, c) external validation: Couples Observational Study, and d) external validation: Partners PrEP Study, placebo arm.....	34
Figure 2. ROC curves comparing risk score to individual continuous predictors.....	36
Figure 3. HIV-1 acquisition risk score worksheet.....	37
Figure 4. Distribution of <i>env</i> and <i>gag</i> subtype among cases and controls.....	68
Figure 5. Median plasma (2a) and median genital (2b) HIV-1 RNA by <i>env</i> subtype C and non-C subtypes.....	69
Figure 6. Mean log ₁₀ concentration of cases and controls for a) HIV-1 susceptible cases and controls, and b) HIV-1 infected cases and controls.....	91
Figure 7. Correlation of IL-10 and IP-10 log ₁₀ concentrations, a) IL-10 and IP-10 log ₁₀ concentrations in HIV-1 susceptible partners, b) IL-10 and IP-10 log ₁₀ concentrations in HIV-1 infected partners, c) IL-10 log ₁₀ concentrations within serodiscordant couples, and d) IP-10 log ₁₀ concentrations within serodiscordant couples.....	93
Figure 8. HIV-1 incidence and PrEP efficacy overall and among higher-risk subgroups.....	100
Figure 9. HIV-1 prevalence and subtype C distribution over time.....	102

ACKNOWLEDGEMENTS

I wish to thank my dissertation committee for their guidance and support: Jared Baeten, Connie Celum, Grace John-Stewart, Jim Hughes and Julie McElrath. I am particularly indebted to Jared for his dedication to mentorship, enthusiasm for teaching, and for helping me navigate complex data and challenging research questions.

I have been fortunate to have many incredible mentors along the way. Thank you to Deborah Donnell, Jairam Lingappa and Kathy Thomas for statistical and methodological guidance and support throughout my research..

The staff at the University of Washington International Clinical Research Center (ICRC) are some of the most dedicated people I have had the privileged to work with. Without their hard work, I would not have had the high quality data available from my research. Specifically, I would like to thank the ICRC Data Team for their support and compassion, both professionally and personally: Kathy Thomas, Lara Kidoguchi, Justin Brantley and Ting Hong.

I am thankful for my PhD student support group, Renee Heffron, Katie Curran and Pam Murnane, whose friendship and support have motivated and inspired me.

I appreciate to the many collaborators and co-authors for guidance, technical assistance, laboratory testing and constructive feedback. Thank you Mary Campbell, Michael Bolton and Angela Kashuba for patiently helping me understand laboratory methods and terminology.

My interest in Epidemiology started during my years working in public health, and I am grateful to those that I have guided me along the way. Thank you to Ron Griffin for giving me my start in public health and encouraging me to pursue graduate school and Susan Buskin for unwavering support and kindness.

My dissertation would not have been possible without the love and support of my family and friends.

DEDICATION

To my family, for unwavering support, patience and inspiration:

Chris and Rylan,

Mom and Dad,

Jennifer and Susan

CHAPTER 1: Introduction

The goal of this dissertation is to determine factors associated with increased HIV-1 infectiousness among African HIV-1 serodiscordant couples. Specifically, we address questions in two key areas: 1) optimizing recruitment of serodiscordant couples to find highest risk couples in order to maximize efficiency in study conduct and design, and 2) identification of novel biologic correlates of HIV-1 infectiousness. New HIV-1 prevention strategies, incorporating a broad-spectrum understanding of transmission risk factors, remain urgently needed, including both strategies that decrease HIV-1 susceptibility of uninfected persons and strategies that decrease the infectiousness, and thus risk of onward transmission to susceptible partners. Identifying novel study designs targeting high-risk participants using conservative estimates of HIV-1 incidence are essential to reduce sample size of efficacy trials without jeopardizing data quality. Greater understanding the role host-virus interaction plays in transmission is critical in developing new prevention strategies, including new treatments and effective vaccines. Our results will be used to evaluate risk factors for increased HIV-1 infectiousness and identify specific populations for targeted HIV-1 prevention research.

Few studies of risk factors influencing HIV-1 infectiousness have been conducted due to logistical challenges in recruitment and prospective follow-up of HIV-1 infected persons and their sexual partners. Thus, prospective studies of HIV-1 serodiscordant couples offer an important opportunity to directly study HIV-1 infectiousness and transmission risk, and longitudinal studies of HIV-1 serodiscordant couples with biologic specimen collection offer unique opportunities for assessing virologic and immunologic factors that increase infectiousness and contribute to transmission. Further, in sub-Saharan Africa, among the nearly 2 million new HIV-1 infections each year [1], a substantial proportion are estimated to occur within married or cohabiting heterosexual couples [2-4], making this population a high priority for targeted prevention research [5, 6].

Optimizing recruitment of serodiscordant couples to maximize study efficiency

Accurate HIV-1 incidence estimates in clinical trials. Recruitment and operational management for HIV-1 clinical prevention trials is costly, challenging and involves enrolling a large number of participants to achieve sufficient study power. Only a handful of funding sources make up the bulk of funding available, and current level funding is not adequate for addressing the costs of continuing to implement new large-scale HIV-1 clinical prevention trials [7]. The Global HIV Prevention Working Group has recommended identifying novel trial designs for accurately calculating HIV-1 incidence, while reducing the number of participants and follow-up time required to identify effective interventions [8]. Future HIV-1 prevention studies should be designed with optimized sample sizes without compromised study power. To optimize the number of participants, accurate estimations of HIV-1 incidence in proposed study populations are required. Overestimation of HIV-1 incidence in control groups during the study design phase may result in a clinical trial with limited statistical power to reliably detect an effect of an intervention [9]. Several studies have ended with no discernible effect of an intervention, likely in part due to low incidence rates. In the SAVVY vaginal gel trial, 2153 HIV-1 uninfected women were enrolled with an anticipated incidence of 5 per 100 person-years (n=66 infections). The trial was stopped due to operational futility when only 33 infections occurred, at an incidence of 1.87 per 100 person-years, below the number to achieve adequate power to detect the effect of the microbicide [10]. An HIV-1 clinical prevention trial assessing the efficacy of a peer education network intervention among 1027 injection drug users was stopped at interim study review due to a seroincidence of less than 1% at all sites [11]. Padian, et al. concluded that most HIV-related randomized clinical trials with flat results reported lower than expected HIV-1 incidence, arguing that effective interventions may have been “missed” by trials that were too small to demonstrate an effect [12].

An empiric risk scoring tool for identifying high-risk heterosexual HIV-1 serodiscordant couples for targeted HIV-1 prevention: Chapter 2

A discrete combination of clinical and behavioral characteristics can be selected to define higher-risk HIV-1 serodiscordant couples.

Achieving sufficient HIV-1 incidence in a study trial depends on identifying participants at highest risk of transmission. Risk factors for HIV-1 infection have been well documented, including sexual behavior, clinical characteristics and biologic factors. Some combinations of these risk factors have been used in the design and eligibility assessment for clinical trials (e.g., requiring potential participants to be sexually active), but few rigorous assessments of combinations of risk factors for selection of highest risk participants have been done. Selecting subgroups with highest risk within high prevalent populations increases the likelihood of achieving sufficient incidence. Clinical trials with preparatory or interim monitoring in cohort studies of behavior and risk have been more successful at enrolling high-risk participants than studies relying solely on population statistics of HIV-1 incidence and prevalence [13-15]. For studies of HIV-1 serodiscordant couples in particular, the additional recruitment and retention complexity of enrolling two individuals requires careful attention in assessing study sites for high risk within couples and calculating accurate estimates of HIV-1 incidence [16]. However, measures of HIV-1 exposure – a known HIV-1 infected partner, quantification of plasma HIV-1 levels, etc. – may allow for discernment of highest risk subpopulations for studies of serodiscordant couples.

Risk scores for enrollment in HIV-1 clinical trials and for implementation of preventive interventions. Enrollment criteria for clinical trials require clear definitions of risk factors for study eligibility. Simple tools that score individual risk have been used in clinical trials for a variety of diseases to target high-risk individuals for clinical trials or therapeutic intervention [17-19]. However, there is no published data on HIV-1 serodiscordant couples clinical trials or

prevention programs documenting the use of a risk score assessment to determine enrollment in such studies. A population-specific risk score tool to use in screening for participation in HIV-1 clinical trials is important for optimizing high HIV-1 incidence to reach sufficient study power. In addition, programs aimed at implementing successful prevention strategies may want to define highest risk populations of couples for targeting interventions with limited resources; the same risk scoring that may define optimal populations for clinical trials could be used for programmatic roll-out as well.

We created and validated a risk score using key predictors of HIV-1 transmission risk assessed at enrollment in an HIV-1 prevention trial. Chapter 2 describes the methods for developing the risk score and the benefit of utilizing a composite risk compared to individual risk factors in identifying the highest risk serodiscordant couples for prevention studies and intervention programs.

<p style="text-align: center;">Unreported antiretroviral use by HIV-1 infected participants enrolling in a prospective research study: Chapter 3</p>

What is the prevalence of unreported antiretroviral therapy use in HIV-1 infected participants enrolling in a clinical trial?

Unreported antiretroviral therapy as a potential cause for lower incidence in trials of HIV-1 serodiscordant couples. The quantity of HIV-1 in plasma is the primary determinant of HIV-1 transmission risk. Antiretroviral therapy (ART) reduces HIV-1 replication and has been associated with markedly reduced heterosexual HIV-1 transmission risk in serodiscordant couples [20]; thus, unreported ART use in a clinical trial population of HIV-1 serodiscordant couples hinders the ability to determine actual treatment effects and may confound any transmission outcomes. Prevention clinical trials aimed at studying the effect size of an intervention on HIV-1 transmission may require HIV-1 infected participants to be antiretroviral naïve at study enrollment. However, no studies of HIV-1 clinical trials have reported verification of ART-naïve status of participants through laboratory confirmation. Enrollment in clinical trials

often include frequent counseling and testing for HIV-1 infection, additional health care services, mental health care resources and financial reimbursement and incentives. In resource-limited communities, the personal incentives for participating in clinical trials may influence participation and encourage misreporting of eligibility criteria [21]. The potential for surreptitious ART use is an important consideration for the development of HIV-1 prevention clinical trials among serodiscordant couples specifically designed for the study of transmission. Plasma HIV-1 RNA levels are an important consideration in assessing transmission risk, and unreported ART use, by lowering plasma HIV-1 RNA levels, may explain low-risk participants in a clinical trial.

In Chapter 3, we assess the prevalence of unreported ART use among HIV-1 infected participants enrolling in an HIV-1 prevention clinical trial, specifically among the nearly quarter of HIV-1 infected participants with an enrollment plasma HIV-1 RNA <2000 copies/mL. The implications of our findings include study design of future clinical trials and better assessment of HIV-1 transmission risk.

Identification of novel biologic factors associated with HIV-1 infectiousness

Biologic correlates of HIV-1 infectiousness. Studies have shown the probability of transmission per exposure to be heterogeneous and dependent on a number of behavioral, clinical and biologic characteristics, even within populations of known similar risk (e.g. commercial sex workers) [22, 23]. Biologic and clinical factors, including viral load, co-infections, viral composition, circumcision and hormonal contraceptive use, have been shown to be associated with differential transmission, but none have completely explained individual variation in transmission risk. The development of new effective biomedical interventions and vaccines requires an understanding of the underlying factors that contribute to increased HIV-1 transmission risk, either through increased infectiousness or increased susceptibility.

HIV-1 infectiousness is associated with higher viral load, advanced disease progression and genital infection [24]. However, the biologic mechanism, whether due to host or viral factors, for influencing transmission has not been fully described. Viral load is the primary factor associated with HIV-1 infectiousness and heterosexual transmission [25]. Individuals with undetectable viral load, either through naturally controlled virus or the use of ART, are substantially less likely to transmit virus compared to those with detectable levels of virus [20, 26]. Biologic correlates of HIV-1 infectiousness may directly increase HIV-1 transmission or indirectly by increasing viral replication. These factors may be mechanisms occurring in the virus, such as subtype, or in the host, through immune dysregulation.

HIV-1 subtype may be associated with increased HIV-1 infectiousness, directly through enhanced transmissibility or indirectly by increasing ability to replicate in the host. Genetic differences in HIV-1 strain may impact the viral fitness (ability to reproduce and adapt to changing environments) that could contribute to increased transmission. During acute infection, the replicative fitness of HIV-1 is higher compared to chronic infection [27]. However, specific viral strains have shown persistent ability to evade the host response and replicate, contributing to disease progression and higher viral loads [28].

Additionally, other host factors, such as systemic and mucosal co-infections and hormonal contraceptive use, are associated with increased risk of HIV-1 transmission suggesting HIV-1 infectiousness is mediated by an increase immune response in the host [29-31]. Changes in immune function and inflammatory response to internal and external provocations may increase infectiousness through increased viral replication and shedding.

HIV-1 subtype C is not associated with higher risk of heterosexual HIV-1 transmission: a multinational study among African HIV-1 serodiscordant couples: Chapter 4

Is HIV-1 subtype C associated with an increased risk of HIV-1 transmission compared to other subtypes found in eastern and southern Africa?

HIV-1 subtype may influence pathogenesis. HIV-1 is characterized by wide genetic diversity, with 9 subtypes (A-K) and numerous circulating recombinant forms (CRFs) [32]. Of these, subtypes A, B, C and D account for nearly three-quarters of all HIV-1 infections worldwide [33]. Subtypes A, C and D are the predominant subtypes in sub-Saharan Africa, where heterosexual transmission is responsible for most HIV-1 infections [34, 35]. HIV-1 subtype diversity has been hypothesized as one potential explanation for geographic and regional differences in disease progression, response to antiretroviral therapies and disease transmission (Table 1). Additionally, response to antiretroviral therapy and viral rebound may be different among subtypes, although results of such studies have been inconsistent [36-39].

Biologic plausibility for the association of HIV-1 subtype and transmission. Regional variation in the distribution of HIV-1 subtypes suggests that some subtypes may be more transmissible than others. HIV-1 infection with subtype C has become the most common subtype worldwide, and the dominant subtype in southern Africa, and is responsible for nearly half of all global infections [39]. Throughout Africa and parts of Asia, significant increases in the incidence of subtype C HIV-1 infection suggests that subtype C may be more heterosexually transmissible than other subtypes [33]. Several factors could be attributed to changes in HIV-1 subtype distribution and differences in subtype transmissibility. First, distinct features of subtypes may allow for more efficient transmission [40, 41]. For example, studies have found that strains of HIV-1 that are better able to utilize CCR5 and CXCR4 coreceptors are more frequently transmitted [40, 42, 43]. Second, viral shedding of HIV-1 may vary among subtypes. Higher HIV-1 RNA and DNA viral loads have been shown to be associated with increased heterosexual HIV-1 transmission [25].

Subtype differences may account for higher levels of genital and plasma viral shedding [44, 45].

Table 1. Features of the HIV-1 pandemic, according to subtype or circulating recombinant form (CRF)

Subtype or CRF Subtype	Location	Global Prevalence	Tropism and Replication	Disease Progression	Response to Therapy
A	East and Central Africa, Central Asia, Eastern Europe	12.3%	Mostly uses CCR5, even in late infection	NA	No significant differences as compared with C and D
B	Americas, Western Europe, East Asia, Oceania	10.2%	Uses CCR5 early, with increasing use of CXCR4 in late infection	HLA-B7 associated with poor CTL response and increased viremia; HLA-B57 associated with slow progression; B strain in Brazil associated with slow progression	NA
C	India, Eastern and Southern Africa	49.9%	Mostly uses CCR5, even in late infection; increased vaginal shedding and mother-to-child transmission	HLA-B57 associated with slow progression	No significant difference compared with A and Ad; differential pathways to resistance
D	East Africa	2.5%	Uses CXCR4 in early infection	Progression more rapid than A in Uganda, Kenya and Tanzania	NA
G	West Africa	6.3%	NA	NA	NA
F,H,J and K	Various	Each <1.0%	NA	NA	NA
CRF					
CRF01_AE	Southeast Asia	4.7%	May have higher initial viral load than B but subtype may be a confounder	Possibly accelerated progression as compared with B	NA
CRF02_AG	West Africa	4.8%	Higher rate of replication in vitro than B	NA	NA
Other	Various	Each <0.1%	NA	NA	NA

Modified from: Taylor BS, et al. NEJM 2008 Apr 10;358(15):1590-1602 [29]

Third, subtypes associated with slower disease progression may allow for greater opportunities for transmission before the host succumbs to advanced illness. For example, studies have suggested that subtype C, responsible for most HIV-1 infections, is associated with slower disease progression [46, 47]. Finally, differences in rates of HIV-1 infection by subtype could be more related to founder effects or behavioral and other risk factors than the subtype itself, or due to multiple factors associated with sociobehavioral characteristics of the population, host and viral interactions and other factors.

Studies of HIV-1 subtype and transmission. Direct studies of HIV-1 subtype and transmission are limited by the requirement for data on both the transmitter and the seroconverter, and thus research has been confined to serodiscordant couple and mother-to-child transmissions. Multiple studies of mother-to-child transmission (MTCT) have found subtype to be associated with differences in probability of transmission, with the subtypes studied dependent on the local circulating strains [45, 48, 49]. A small number of studies of HIV-1 subtype and transmission have been conducted among serodiscordant couples within single African countries. Among 149 serodiscordant couples with transmitted HIV-1 infection within a Zambian serodiscordant couples cohort, 129 (87%) were confirmed to be genetically linked transmissions (the infected partner confirmed to have transmitted HIV-1 to the initially negative partner) [50]. Among these transmissions, 95% were subtype C (also 3 subtype G, 3 subtype A, 1 subtype D and 1 subtype J), the most common subtype in Zambia; however, phylogenetic sequencing for subtype was not conducted on the HIV-1 infected partner in the non-transmitting couples. In Rakai, Uganda, 92 (34%) of 268 serodiscordant couples transmitted HIV-1. This is the only published study where HIV-1 infected members of all serodiscordant couples were sequenced for subtype and viral load, regardless of transmission. Within the cohort, 74% were subtype D, 12% subtype A and 15% recombinant virus. Subtype A was found to be significantly associated with HIV-1

transmission compared to subtype D (aIRR=1.95, 95%CI 1.16-3.29) [51]. However, genetic linkage of HIV-1 transmission was not established for couples with a seroconversion event.

Only limited studies have directly assessed the relationship between HIV-1 subtype and heterosexual transmission. Studies of transmission are most effective when both the infected subject and the transmitting partner are available for participation, such as serodiscordant couples or MTCT. The only studies of subtype and transmission in African serodiscordant couples had limitations and were not able to sufficiently assess any associations between subtype and transmission. Most study populations within individual African countries lack the geographic diversity to allow for subtype differences, and therefore decrease the ability to conduct subtype analyses. We conducted a nested case-control analysis of HIV-1 subtype and transmission risk using among HIV-1 serodiscordant couples in a diverse geographic region of sub-Saharan Africa, including an analysis of subtype and viral load. The findings presented in Chapter 4 illustrate the importance of studies using HIV-1 serodiscordant couples and that the explosion of subtype C may be the result of factors other than subtype.

Immune activation and risk of HIV-1 transmission: a nested case-control study among HIV-1 serodiscordant couples: Chapter 5

Does immune activation, as measured by a panel of cytokine markers, increase the risk of HIV-1 transmission?

Immune activation, viral shedding and HIV-1 infectiousness. Chronic immune activation is partially caused, directly and indirectly, by the replication process of HIV-1 in the immune system [52-54]. Several studies have shown that immune stimulation by HIV-1 infection accelerates HIV-1 disease progression and contributes to depletion of CD4+ cells rather than purging the virus [55-58]. The relationship between host and virus that increases immune response drives HIV-1 replication and subsequent disease progression. While the role of

immune activation in HIV-1 disease progression has been well documented, few studies have looked at the role of increased immune response in the HIV-1 infected individual on HIV-1 transmission. Increased immune activation resulting in increased viral replication and disease progression could increase HIV-1 infectiousness [25, 59]. HIV-1 viral load, both in plasma and genital secretions, has been the primary marker of infectiousness [25]. Chronic immune activation may be associated with increased HIV-1 replication, although recent studies suggest that immune activation only indirectly increases viral load and may increase infectivity independent of viral load [57, 60]. The presence of other pathogens, either as chronic infections or multiple episodes of infection, is believed to increase immune activation and enhance the ability of HIV-1 virus to replicate [61-63]. Sexually transmitted infections (STIs) are thought to be particularly relevant in HIV-1 replication and spread through sexual transmission because of increase immune activation and inflammatory response in the genital tract [64-66]. In HIV-1 infected persons, increased immune activation resulting in heightened viral replication and faster disease progression may increase transmission risk in serodiscordant couples by enhancing the infectiousness of HIV-1 infected persons.

Cytokines, immune activation and HIV-1 infection. Cytokines encompass a wide range of small regulatory proteins released by cells of the immune system as intercellular messengers in the production of an immune response, and include colony stimulating factors, growth and differentiation factors, and immunoregulatory and proinflammatory cytokines. Cytokines are well-documented markers of immune activation in HIV-1 infection and contribute to the HIV-1 virus life cycle (Table 2). Specific cytokines have been identified as having a potential role in increased immune activation. Several studies have found cytokines, such as IL-6, IL-8, TNF- α (enhancers of HIV-1 replication) to be significantly elevated in HIV-1 infected persons compared to HIV-1 uninfected persons, while decreased levels in other cytokines, such as IL-10 (suppressor of HIV-1 replication) have been associated with HIV-1 infection [67-70]. Elevated

cytokine levels associated with HIV-1 infection have been noted in both blood plasma and mucosal cells of the genital tract [68, 69]. Dysregulation of cytokine levels has been shown to be associated with HIV-1 disease progression [55, 56, 58, 70-74].

Biologic plausibility for the association between cytokines and infectiousness. The mechanism by which immune activation, as measured by cytokine markers, enhances HIV-1 disease progression may also be associated with increased infectiousness and greater likelihood of transmission. Dysregulation of cytokine levels is associated with increases in viral replication and shedding levels [71, 73, 75].

Table 2. Selected cytokines modulating HIV Replication *In Vitro*

Cytokine	Effect on HIV Replication	Primary Mechanism of Action
IL-1 α/β	Enhancement	HIV transcription
IL-2		Induction of both TNF- α , IL-1 β and IFN- γ and of CD8-non-lytic suppression
IL-4	Multiple	Inhibition of TNF- α and IL-1 β , but post-transcriptional enhancement of HIV expression
IL-6	Enhancement	Post-transcriptional
IL-7	Enhancement	HIV transcription (with TNF- α)
IL-9	Enhancement	CD8-non-lytic suppression
IL-10	Multiple	Suppression of pro-inflammatory cytokines synthesis, synergy with TNF- α and IL-6
IL-13	Suppression	Post-transcriptional
IL-16	Suppression	HIV transcription
IL-18	Enhancement	HIV transcription
TNF- α/β	Multiple	Enhance HIV transcription but inhibit virus entry
GM-CSF	Multiple	Enhances HIV transcription, but inhibits virus entry
IFN- α/β	Suppression	Inhibition of multiple steps of the virus life cycle
IFN- γ	Multiple	Enhances HIV transcription, but inhibits virus entry
TGF- β	Multiple	Enhances or suppresses HIV transcription
MDC	Suppression	Post-entry inhibition
FasL	Suppression	Killing of infected cells

Modified from: Alfano and Poli, Curr Pharm Des. 2001 Jul;7(11):993-1013⁷⁵

Additionally, viral shedding in the genital tract, where heterosexual HIV-1 transmission occurs, is associated with cytokine levels. Multiple studies have found a significant positive association between HIV-1 shedding in the cervix and elevated levels of HIV- cytokines in cervical mucosa, specifically proinflammatory cytokines (IL-1 β , IL-6 and IL-8) and immune cell regulatory cytokines (TNF- α) [75-77]. Infection and cervical dysfunction have been shown to increase cervical cytokine concentrations in HIV-1 infected women, which is correlated with increase in HIV-1 viral load in genital secretions [77]. Other studies have shown higher concentrations in the genital compartment to be more closely associated with HIV-1 genital shedding than plasma HIV-1 viral loads, suggesting a local immune response may have a direct impact on viral shedding [78, 79]. Direct studies of cytokine concentrations and HIV-1 transmission have been limited to mother-to-child transmission (MTCT). Two studies of vertical transmission found elevated concentrations of two cytokines (IL-7 and IL-15) in non-transmitting mothers to be associated with protection against HIV-1 transmission through breastfeeding [80, 81]. Studies have also found elevated concentrations of cytokines, including RANTES and IL-8, to be associated with postnatal vertical transmission [80, 82]. There is a paucity of data looking at cytokines in HIV-1 infected persons and risk of transmission and no published research has looked at immune activation and infectiousness in HIV-1 infected persons in HIV-1 serodiscordant partnerships. Cellular immune activation, measured by cytokine levels, in the HIV-infected partner of serodiscordant couples is important for a better understanding of HIV-1 transmission and could provide information critical for treatment and vaccine development. In Chapter 5, we assess the role of immune activation in both HIV-1 transmission and acquisition in HIV-1 serodiscordant couples in a novel approach that provides additional mechanistic information on biologic factors related to HIV-1 risk.

The focus of this dissertation is to use prospective cohorts of African serodiscordant couples to assess transmission risk, determine high-risk cohorts for enrollment in clinical trials, and

evaluate the role of immunologic and virologic factors on infectiousness and transmission. Due to the complexities of conducting studies of serodiscordant couples, few studies have directly assessed transmission within partnerships, instead relying on correlates of transmission, such as HIV-1 viral shedding [24]. HIV-1 serodiscordant couples are unique cohorts that allow for simultaneous evaluation of transmission in both the HIV-infected partner and the HIV-uninfected partner. Serodiscordant couples studies provide opportunities to evaluate the biologic mechanisms of HIV-1 infection and how those processes impact transmission.

Chapter 2: An empiric risk scoring tool for identifying high-risk heterosexual HIV-1 serodiscordant couples for targeted HIV-1 prevention

Published citation:

Kahle EM, Hughes JP, Lingappa JR, John-Stewart G, Nakku-Joloba E, Njuguna S, Bukusi E, Manongi E, Celum C, Baeten JM. An empiric risk scoring tool for identifying high-risk heterosexual HIV-1 serodiscordant couples for targeted HIV-1 prevention. *J Acquir Immune Defic Syndr.* 2013;62(3):339-347.

An empiric risk scoring tool for identifying high-risk heterosexual HIV-1 serodiscordant couples for targeted HIV-1 prevention

Erin M. KAHLE¹, James P. HUGHES², Jairam R. LINGAPPA^{3,4,5}, Grace JOHN-STEWART^{1,3,4,5},
, Connie CELUM^{1,3,4}, Edith NAKKU-JOLOBA⁸, Stella NJUGUNA⁹, Nelly MUGO^{3,10}, Elizabeth
BUKUSI⁹, Rachel MANONGI¹¹, Jared M. BAETEN^{1,3,4} for the Partners in Prevention HSV/HIV

Transmission Study and the Partners PrEP Study Teams

Departments of ¹Epidemiology, ²Biostatistics, ³Global Health, ⁴Medicine and ⁵Pediatrics,
University of Washington, Seattle, USA; ⁸Department of Epidemiology and Biostatistics,
Makerere University, Kampala, Uganda; ⁹Research Care Training Program, Center for
Microbiology Research, Kenya Medical Research Institute, Nairobi, Kenya; ¹⁰Department of
Obstetrics & Gynecology, University of Nairobi & Kenyatta National Hospital, Nairobi, Kenya;
¹¹Kilimanjaro Christian Medical Centre, Moshi, Tanzania

Corresponding author:

Erin Kahle

University of Washington

908 Jefferson Street, 12th Floor, Box 359927

Seattle, Washington 98104

Tel – 206-520-3816, Fax – 206-520-3831, email – ekahle@u.washington.edu

Previous Presentation: This work was presented in part at the 19th Conference on
Retroviruses and Opportunistic Infections, Seattle, WA, USA, March 5-8, 2012.

Funding: The United States National Institutes of Health (grant R01-MH095507) and the Bill
and Melinda Gates Foundation (grants #26469 and 41185).

Conflict of Interest: None of the authors have commercial or other conflicts of interest related
to the content of this manuscript.

Manuscript length: (not including tables/acknowledgements): 3628 words

Abstract length: 236 words

Abstract

Background and objectives: Heterosexual HIV-1 serodiscordant couples are increasingly recognized as an important source of new HIV-1 infections in sub-Saharan Africa. A simple risk assessment tool could be useful for identifying couples at highest risk for HIV-1 transmission.

Methods: Using data from three prospective studies of HIV-1 serodiscordant couples from seven African countries and standard methods for development of clinical prediction rules, we derived and validated a risk scoring tool developed from multivariate modeling and composed of key predictors for HIV-1 risk that could be measured in standard research and clinical settings.

Results: The final risk score included age of the HIV-1 uninfected partner, married and/or cohabiting partnership, number of children, unprotected sex, uncircumcised male HIV-1 uninfected partner, and plasma HIV-1 RNA in the HIV-1 infected partner. The maximum risk score was 12; overall, 28% of serodiscordant couples had an elevated risk score (≥ 6), and this group accounted for 67% of HIV-1 transmissions. The area under the curve for predictive ability of the score was 0.74 (95% CI 0.70-0.78). Internal and external validation showed good predictive ability of the risk score, even when plasma viral load was excluded from the risk score.

Conclusions: A discrete combination of clinical and behavioral characteristics defines highest-risk HIV-1 serodiscordant couples. Discriminating highest-risk couples for HIV-1 prevention programs and clinical trials using a validated risk score could improve research efficiency and maximize the impact of prevention strategies for reducing HIV-1 transmission.

Keywords: HIV-1 serodiscordant couples, HIV-1 acquisition, clinical prediction rule

Introduction

Of the nearly 2 million new HIV-1 infections in sub-Saharan Africa each year, a substantial proportion occur within stable, cohabiting heterosexual couples, making this population a priority for targeted HIV-1 prevention research and implementation of effective HIV-1 prevention strategies [1, 2, 83]. As African countries are adopting couples HIV-1 counseling and testing as an HIV-1 prevention strategy, more couples of previously unknown serostatus are becoming aware of being HIV-1 serodiscordant [84, 85]. Couples aware of their serodiscordant status continue to face HIV-1 risk [86-90], and there is an urgent need to design optimal strategies for evaluation and delivery of HIV-1 prevention strategies for couples, particularly how to target prevention strategies to realize maximum population HIV-1 prevention benefits.

Risk factors for HIV-1 transmission in serodiscordant partnerships include high HIV-1 plasma concentrations in the HIV-1 infected partner, unprotected sexual activity, multiple partners and uncircumcised status for HIV-1 susceptible male partners [16, 60, 91]. A recent study showed that a risk algorithm, assessing the contribution of multiple risk factors, could be mathematically derived from the literature to identify partnerships at higher risk for transmission [92], but simple risk algorithms, for use in real-world settings and based on empiric data, have not been developed. While all serodiscordant couples are potentially at risk for HIV-1 transmission, defining those couples at the highest risk might permit more efficient recruitment of couples into clinical studies of novel prevention strategies and more cost-efficient, targeted delivery of expensive HIV-1 prevention interventions, such as earlier initiation of antiretroviral therapy (ART) for HIV-1 infected partners or antiretroviral pre-exposure prophylaxis (PrEP) for HIV-1 prevention in uninfected partners [20, 26, 93, 94].

Clinical prediction rules, also known as clinical decision rules, are evidence-based assessment tools that use patient medical history, physical examination, and diagnostic test results to assist

in medical decision-making [95, 96]. Clinical prediction rules are typically simple, efficient, and easy to implement and use in a clinical setting, but the methods for developing them can also be applied to assessing risk for prevention intervention [97-99]. Standardized, rigorous processes have been described for developing clinical prediction rules, including deriving and validating the prediction rule [99, 100]. We used standard methods for development of clinical prediction rules to create and validate a risk-scoring tool to identify highest-risk HIV-1 serodiscordant couples.

Methods

We used data from three prospective studies in Africa of stable heterosexual HIV-1 serodiscordant couples to assess the relationship between clinical and behavioral variables and the risk of HIV-1 acquisition, focusing on variables that could be measured in a standard clinical or research setting.

Study population

Partners in Prevention HSV/HIV Transmission Study (derivation cohort). Between November 2004 and April 2007, we enrolled 3408 heterosexual HIV serodiscordant couples from 7 African countries (Botswana, Kenya, Rwanda, South Africa, Tanzania, Uganda, and Zambia) into the Partners in Prevention HSV/HIV Transmission Study, a randomized, double-blind, placebo-controlled clinical trial of herpes simplex virus type 2 (HSV-2) suppressive therapy to reduce HIV-1 transmission, as previously described [101]. Eligible couples were at least 18 years of age, reported ≥ 3 vaginal sex acts in the three months prior to enrollment, and intended to remain a couple. At enrollment, all HIV-1 infected partners were HSV-2 seropositive, had CD4 counts ≥ 250 cells/ μL (making them ineligible for ART under national guidelines of the study countries at that time), and were not currently taking ART. HSV-2 suppressive therapy failed to reduce HIV-1 transmission within partnerships [102].

Couples Observational Study (validation cohort). In a parallel study at two of the Partners in Prevention HSV/HIV Transmission Study sites (Kampala, Uganda and Soweto, South Africa), an additional 485 HIV-1 serodiscordant couples who were not participants in the Partners in Prevention HSV/HIV Transmission Study were enrolled into an observational study of immune correlates of HIV-1 protection [31]. Similar to the clinical trial cohort, participants were ≥ 18 years of age and sexually active and HIV-1 seropositive partners were not using ART.

Partners PrEP Study (validation cohort). The Partners PrEP Study is a phase III, randomized, double-blind, placebo-controlled, three-arm clinical trial that assessed the safety and efficacy of oral PrEP for the prevention of HIV-1 acquisition using the antiretroviral medication tenofovir (TDF), either alone or in combination with emtricitabine (FTC/TDF). Between July 2008 and November 2010, 4758 HIV-1 serodiscordant couples from nine sites in Kenya and Uganda were enrolled. Eligible couples were at least 18 years of age and sexually active, with the intention to remain a couple [103]. Eligible HIV-1 uninfected partners were healthy and not pregnant or breastfeeding. HIV-1 infected partners were not using ART and did not otherwise meet Kenyan or Ugandan guidelines for initiation of ART; all had CD4 counts ≥ 250 cells/mm³ at the time of enrollment. The placebo arm of the Partners PrEP Study was discontinued in July 2011 due to significant reduction in HIV-1 acquisition risk for both TDF and FTC/TDF [93]. For this analysis, we included only couples in the placebo arm.

In the Partners in Prevention HSV/HIV Study and the Couples Observational Study cohorts, HIV-1 uninfected partners were seen quarterly for HIV-1 serologic testing. In the Partners PrEP Study, HIV-1 uninfected partners received monthly HIV-1 serologic testing.

Protection of Human Subjects

All participants received HIV-1 and risk-reduction counseling (both individual and as a couple), free condoms, and treatment for sexually transmitted infections (STIs), according to World Health Organization (WHO) guidelines. Written, informed consent was obtained from all participants. The study protocols were approved by the University of Washington Human Subjects Review Committee and ethical review committees at each of the study sites.

Laboratory methods

HIV-1 seroconversion of initially uninfected partners was determined by serologic testing using dual rapid HIV-1 antibody test with confirmatory HIV-1 EIA, Western blot, and RNA PCR. For HIV-1 infected partners, CD4 counts were quantified using standard flow cytometry and plasma HIV-1 RNA levels were quantified by PCR performed at the University of Washington using the COBAS Ampliprep/COBAS TaqMan real-time HIV-1 RNA assay, version 1.0 (Roche Diagnostics, Indianapolis, IN), with a lower limit of quantification of 240 copies/mL.

Risk score variables and data analysis.

Our goal was to develop a risk score that could be calculated as a simple scorecard, aiming for three to ten categorical predictors, validated on external data sources. We used methods described by McGinn, et al. for developing clinical prediction rules, including deriving the rule using clearly defined known and suspected predictors present in a significant proportion of the cohort and validating the rule both internally and externally [99].

The primary study outcome was HIV-1 seroconversion in previously HIV-1 seronegative participants. We did not utilize HIV-1 transmission linkage data available from viral sequencing [104] (i.e., to distinguish transmissions that occurred from the study HIV-1 infected partner versus an external partner); we considered that any HIV-1 acquisition event within serodiscordant couples would be important for HIV-1 prevention programs.

From the Partners in Prevention HSV/HIV Transmission Study cohort, we identified potential predictors of HIV-1 acquisition based on characteristics known to be associated with HIV-1 risk that could also be feasibly collected from couples in general clinical and research settings. The list of variables considered included demographic (age, gender of the HIV-1 infected partner, number of children, marital status, cohabitation and duration of partnership), behavioral (frequency of sex, unprotected sex reported in the prior 30 days), clinical factors (male circumcision status for HIV-1 uninfected men, hormonal contraceptive use), and laboratory measures of HIV-1 disease stage in HIV-1 infected partners (plasma HIV-1 level and CD4 count) collected at study enrollment. We restricted our consideration to enrollment variables in order to mimic the type of cross-sectional information that would be available for performing a risk assessment in a standard clinical setting. We converted continuous predictors to categorical variables using optimal cut-points identified through signal detection ROC analysis, weighting false positives over false negatives [105]. We determined signal detection to be the appropriate method for selecting cut-points for categorizing, because it allows for higher-level interactions among all variables and uses recursive partitioning identifying subgroups at highest risk, thus reducing potential misclassification of more arbitrary categories.

We assessed the relationship between our defined predictor variables and HIV-1 infection risk. We censored couples' follow-up at 12 months, anticipating that prevention programs would reevaluate couples' risk at approximately annual intervals. Additionally, couples in which the HIV-1 infected partner started ART were censored at initiation since clinical studies and HIV-1 prevention programs would likely consider couples in which the infected member initiated ART to be receiving a highly-effective prevention intervention [20]. Potential predictors that were significantly associated with HIV-1 transmission risk in univariate comparisons or those predictors we selected *a priori* for evaluation (gender, unprotected sex, circumcision status and

plasma HIV-1 RNA) were evaluated in a multivariate model. To determine the combination of variables that best predicted HIV-1 risk, potential predictors from the multivariate model were assessed in a fully stepwise sequence Cox proportional hazards model, where all predictors were evaluated at each step for inclusion or exclusion. We use the lowest Akaike Information Criterion (AIC) on all possible models from the final stepwise model to determine the predictors for the risk score. The score values for individual risk factors were obtained by dividing the coefficients from the hazard model for each predictor from the final proportional hazards model by the lowest coefficient among all predictors and rounding to the nearest integer. The sum of individual parameter score values for each predictor determined the final risk score for each couple. HIV-1 transmission incidence was calculated by risk score group. Due to the costs and limited availability of viral load assays in some settings, we also calculated HIV-1 incidence for risk score groups excluding scores for laboratory data (e.g. plasma viral load).

We used internal and external validation methods for assessing the robustness of our final risk score model. For internal validation, we used a 10-fold cross-validation of the final risk score and compared the area under the ROC curve (AUC) of our final model with the average AUC of the 10 different models for predictive ability and robustness. For external validation, we applied the risk scores separately to the Couples Observational Study cohort and the placebo arm of the Partners PrEP Study cohort, censoring at 12 months of follow-up as we had done for the derivation cohort.

All analyses were conducted using SAS (v.9.2, Cary, NC) and public domain ROC5 (Department of Veteran's Affairs and the National Institute of Aging of the United States).

Results

Population.

Of 3408 couples enrolled in the Partners in Prevention HSV/HIV Transmission Study, 61 were excluded because no follow-up visits were completed and 49 were excluded for missing predictor data. Of the remaining 3297 couples, most were married and cohabitating (Table 1). They reported a median of 4 (IQR 3-10) sex acts in the 30 days prior to enrollment with 35% reporting at least one unprotected sex act. The median number of children within the partnership was 1 (IQR 0-2), with 31% having no children together. Among HIV-1 infected partners, the median CD4 count was 462 cells/mm³ (IQR 347-631) and median plasma HIV-1 concentration was 11,746 copies/mL (IQR 2285-48,070) with 24% having a plasma HIV-1 concentration \geq 50,000 copies/mL. Among HIV-1 uninfected men, 63% were uncircumcised. Retention of initially HIV-1 uninfected partners at 12 months was 92%. During 3126 person-years of follow-up, a total of 107 HIV-1 seroconversions occurred (incidence 3.4 per 100 person-years).

Risk score model

Development of the final risk score model is detailed in Table 2. In univariate analyses, younger age (of either partner), fewer children in the partnership, recent unprotected sex, uncircumcised status of HIV-1 uninfected male partners and higher plasma viral load in the HIV-1 infected partner were each associated with HIV-1 risk. In the stepwise Cox proportional hazards analysis, age of the HIV-1 uninfected partner, married and/or cohabiting partners, number of children, unprotected sex, uncircumcised status of male HIV-1 uninfected partners, and HIV-1 plasma viral load were retained in the final prediction model. Notably, gender was not determined to be a key predictor of HIV-1 risk and was not included in the final model, as we determined that gender was accounted for in the score by other predictors, such as viral load.

We calculated the total risk score for each couple by summing the individual parameter scores determined in the final risk model. The HIV-1 transmission incidence for each risk score was generated to provide information for defining a cutoff for high risk couples (Figure 1A). For example, a score of ≥ 6 results had a statistically higher HIV-1 transmission incidence compared to a risk score of ≤ 6 (HIV-1 incidence 8.3 vs. 1.5 per 100 person-years, $p < 0.001$) and identified 67% of the observed HIV-1 seroconversion events among only 28% of the total study population. In the risk score in which HIV-1 plasma viral load was excluded, the overall incidences were lower but followed a similar pattern to the full risk score (Figure 1B).

Compared to our risk algorithm, we found that individual risk factors had more limited discriminatory potential in predicting HIV-1 seroconversion. Figure 2 shows ROC curves for the continuous predictors included in our model (plasma viral load, HIV-1 uninfected partner and number of children) along with the ROC curve for the composite risk score and the full multivariate model. The composite risk score based on the stepwise selection did not lose much information compared to the full multivariate model. Among binomial risk factors we found that unprotected sex alone predicted 55% of HIV-1 seroconversions, from 35% of the cohort (HIV-1 incidence 5.4 per 100 person-years), and uncircumcised status of male HIV-1 uninfected partners alone predicted 63% of male HIV-1 seroconversions, from 45% of the male cohort (HIV-1 incidence 4.3 per 100 person-years). Married and/or cohabiting partners made up 94% of HIV-1 seroconversions, but almost all couples in the study (92%) were married and/or cohabiting.

Model validation.

The area under the curve (AUC) for the probability of the risk score to correctly predict HIV-1 acquisition was 0.74 (95% CI 0.70-0.78). Internal cross-validation showed the average AUC for

10 subsets analyzed was 0.73, similar to the AUC of the full dataset and indicating robust generalizability of the risk algorithm within the dataset.

For external validation, we applied our risk score to the Couples Observational Study cohort and the placebo arm of the Partner PrEP Study (characteristics defined in Table 1). The observational cohort included 476 couples, of which 15 had an HIV-1 seroconversion event (incidence 3.2 per 100 person-years). Using the cutoff risk score of ≥ 6 , we predicted 80% of seroconversions from 37% of the population (Figure 1C). No HIV-1 seroconversion events occurred among couples having a risk score ≤ 2 . The AUC for the risk score applied to the Couples Observational Study cohort was 0.76 (95%CI 0.70-0.83). The Partners PrEP Study cohort included 1499 couples in the placebo arm, among whom 45 seroconverted in the first year of follow-up (incidence 2.6 per 100 person-years). A risk score cutoff of ≥ 6 , which was observed in 15% of the cohort, predicted 42% of HIV-1 seroconversions (Figure 1D), with an AUC of 0.70 (95%CI 0.64-0.76).

Discussion

The results of this analysis demonstrate that a discrete set of factors, considered in combination and quantified to develop a risk score, can efficiently identify a subpopulation of stable HIV-1 serodiscordant couples at higher risk for HIV-1 transmission. The predictors selected for our final risk score model are well-established risk factors for HIV-1 and included factors measurable in clinical settings: plasma HIV-1 RNA concentrations, unprotected sex, young age, marital status, no or few children in the partnership, and uncircumcised status of HIV-1 uninfected men [60, 106-112]. Importantly, the combination of risk factors in a single algorithm allowed for more precise predictive capability than individual predictors. The score had good predictive ability in internal and external validation, which lends strength to our findings. To our

knowledge, the model defined here is the first empirically-based risk assessment tool for identifying high-risk HIV-1 serodiscordant heterosexual couples, and it offers a simple, quantitative approach for defining couples at higher HIV-1 risk. Our findings are relevant to both clinical research studies (to improve efficiency of recruitment) and programmatic roll-out of new HIV-1 prevention strategies (to maximize cost-effectiveness by targeting those at greatest risk) [96-98].

New HIV-1 prevention strategies, such as early ART initiation and PrEP, offer the potential to markedly decrease HIV-1 transmission, particularly in regions hardest hit by the epidemic. To have the greatest impact on preventing HIV-1 transmissions among HIV-1 serodiscordant couples, while containing costs, targeting couples at highest risk may be important [113]. Recent WHO guidance on HIV-1 prevention for HIV-1 serodiscordant couples recommended consideration of ART initiation for couples regardless of CD4 count, and rapid advice guidance from WHO on use of PrEP in couples is under preparation [114]. While ART for HIV-1 infected partners with immediate clinical need is required, many countries have not yet implemented earlier ART, or PrEP for couples, due to cost constraints. A risk score for couples allows for rapid risk assessment making the identification of target populations feasible in clinical settings. An example of the simplicity of the risk scoring tool is demonstrated by an example risk scoring card in Figure 3.

In addition to programmatic roll-out, our results have relevance for recruitment for studies, such as large clinical trials of candidate prevention strategies for couples. Clinical research studies are costly, challenging and involve enrolling a large number of participants to have sufficient endpoints to assess efficacy. HIV-1 incidence is the main determinant of the size of HIV-1 prevention efficacy trials, and novel methods for accurately estimating HIV-1 incidence for planning of trials are needed, to reduce the number of participants and follow-up time required

to identify effective interventions [8]. Several HIV-1 prevention trials have ended with no discernible effect of an intervention, in part due to low HIV-1 incidence [9, 10, 12], and thus effective interventions may have been “missed” by trials that did not accurately anticipate HIV-1 incidence [12].

Although our risk score was derived from a study that was conducted in seven African countries, a limitation of our analysis is the lack of broad validation to different populations of couples; our research cohorts recruited couples in stable relationships with relatively low overall HIV incidence (~2-3% per year). However, our populations reflect the motivated subpopulation of HIV-1 serodiscordant couples who would present for research studies and to clinics to access novel HIV-1 prevention interventions – precisely the group for whom this scoring tool could be implemented -- and we demonstrated that an identifiable subset of our population had an annual HIV-1 incidence in excess of 8%. Our results do not derive from couples who are unaware of their serodiscordancy, who may face very high HIV-1 incidence [25], but such couples would also be unlikely to access prevention services – efforts to promote testing as a couple thus remain critically important. All HIV-1 infected partners in our derivation cohort were co-infected with HSV-2; however, HSV-2 seroprevalence is >80% among HIV-1 infected persons in sub-Saharan Africa [115] and thus this is unlikely to limit the generality of our findings. Although HSV-2 seropositivity is a risk factor for HIV-1 acquisition [116], we did not include HSV-2 serostatus of HIV-1 uninfected partners as a potential predictor in our models because HSV-2 serologic testing is not broadly available in most clinical settings in Africa. We retained HIV-1 plasma viral load in our final model, given the importance of this factor in predicting HIV-1 transmission; however, we were able to identify a risk score that would sufficiently identify highest-risk couples even without the inclusion of plasma viral load for setting where viral load assays are not available. Notably, we previously reported that 30% of HIV-1 transmissions in our derivation cohort occurred from outside the study partnership [104], emphasizing that

characteristics of the infected partner (like plasma HIV-1 levels) alone are likely not fully sufficient to predict transmission risk. Importantly, our risk score related predictor variables to all HIV-1 acquisitions, not just those determined to have occurred within the partnership, as ultimately HIV-1 prevention programs would want to prevent all new infections.

Operations research is needed to determine the feasibility of implementing this risk score in diverse research, clinical and HIV-1 testing settings and the impact on behaviors, costs, and programmatic and study efficiency. Currently, the risk score is being implemented for screening in PrEP demonstration projects in eastern African and will be evaluated for feasibility of use and validity in identifying higher risk couples. Further validation of our risk score in additional cohorts should be considered before widespread implementation. Importantly, this risk score was developed to identify couples at highest risk of HIV-1 transmission, but it is not necessarily a method for individual risk counseling. In our analyses, a low score did not indicate zero HIV-1 risk, and all serodiscordant couples should be counseled about risk-reduction strategies, including behavior change, condoms, and treatment of sexually transmitted infections that might facilitate HIV-1 transmission. Additionally, ongoing assessment of risk should be conducted among couples for changes in behavior and clinical progression that could impact HIV-1 transmission risk. Nonetheless, novel prevention strategies, such as PrEP, may have their greatest impact, as well as an appropriate balance of benefits versus potential toxicity, if targeted to those at greatest risk. Clinical research protocols frequently include behavioral risk characteristics in eligibility assessment, and evaluation of risk has been recommended in initial guidance documents related to PrEP for HIV-1 prevention [117]. Our risk score could be used to assess populations for targeted HIV-1 prevention programs and clinical research, specifically where prevention resources and funding are limited.

To maximize use of resources, there is a crucial need to identify those subpopulations at highest risk for targeted prevention. Implementation of new prevention strategies and programmatic roll-out of interventions must consider efficient risk assessment that will target high-risk populations to achieve the greatest impact on reducing new HIV-1 infections. A simple, quantitative risk score could offer a robust, usable method for identifying HIV-1 serodiscordant couples at highest risk for HIV-1 acquisition.

Acknowledgments: We gratefully acknowledge the invaluable contributions of the HIV-1 serodiscordant couples that participated in this study. We thank the teams at the study sites and at the University of Washington for work on data and sample collection and management.

Partners in Prevention HSV/HIV Transmission Study Team:

University of Washington Coordinating Center and Central Laboratories, Seattle, USA:

Connie Celum (principal investigator), Anna Wald (protocol co-chair), Jairam Lingappa (medical director), Jared M. Baeten, Mary Campbell, Lawrence Corey, Robert W. Coombs, James P. Hughes, Amalia Magaret, M. Juliana McElrath, Rhoda Morrow, James I. Mullins

Study sites and site principal investigators: Cape Town, South Africa (University of Cape Town): David Coetzee; Eldoret, Kenya (Moi University, Indiana University): Kenneth Fife, Edwin Were; Gaborone, Botswana (Botswana Harvard Partnership): Max Essex, Joseph Makhema; Kampala, Uganda (Infectious Disease Institute, Makerere University): Elly Katabira, Allan Ronald; Kigali, Rwanda (Rwanda Zambia HIV Research Group, and Emory University): Susan Allen, Kayitesi Kayitenkore, Etienne Karita; Kisumu, Kenya (Kenya Medical Research Institute, University of California San Francisco): Elizabeth Bukusi, Craig Cohen; Kitwe, Zambia (Rwanda Zambia HIV Research Group, and Emory University): Susan Allen, William Kanweka; Lusaka, Zambia (Rwanda Zambia HIV Research Group, and Emory University): Susan Allen, Bellington Vwalika; Moshi, Tanzania (Kilimanjaro Christian Medical College, Harvard University): Saidi Kapiga, Rachel Manongi; Nairobi, Kenya (University of Nairobi, University of Washington): Carey Farquhar, Grace John-Stewart, James Kiarie; Ndola, Zambia (Rwanda Zambia HIV Research Group, and Emory University): Susan Allen, Mubiana Inambao; Orange Farm, South Africa (Reproductive Health Research Unit, University of the Witwatersrand): Sinead Delany-Moretlwe, Helen Rees; Soweto, South Africa (Perinatal HIV Research Unit, University of the Witwatersrand): Guy de Bruyn, Glenda Gray, James McIntyre; Thika, Kenya (University of Nairobi, University of Washington): Nelly Rwamba Mugo

Partners PrEP Study Team:

University of Washington Coordinating Center and Central Laboratories, Seattle, USA:

Connie Celum (principal investigator, protocol co-chair), Jared M. Baeten (medical director,

protocol co-chair), Deborah Donnell (protocol statistician), Robert W. Coombs, Jairam R. Lingappa, M. Juliana McElrath.

Study sites and site principal investigators: Eldoret, Kenya (Moi University, Indiana University): Kenneth H. Fife, Edwin Were; Kabwohe, Uganda (Kabwohe Clinical Research Center): Elioda Tumwesigye; Jinja, Uganda (Makerere University, University of Washington): Patrick Ndase, Elly Katabira; Kampala, Uganda (Makerere University): Elly Katabira, Allan Ronald; Kisumu, Kenya (Kenya Medical Research Institute, University of California San Francisco): Elizabeth Bukusi, Craig R. Cohen; Mbale, Uganda (The AIDS Support Organization, CDC-Uganda): Jonathan Wangisi, James D. Campbell, Jordan W. Tappero; Nairobi, Kenya (University of Nairobi, University of Washington): James Kiarie, Carey Farquhar, Grace Johnston; Thika, Kenya (University of Nairobi, University of Washington): Nelly R. Mugo; Tororo, Uganda (CDC-Uganda, The AIDS Support Organization): James D. Campbell, Jordan W. Tappero, Jonathan Wangisi.

Data management was provided by DF/Net Research, Inc. (Seattle, USA) and site laboratory oversight was provided by Contract Lab Services (University of the Witwatersrand, Johannesburg, South Africa).

Table 3. Enrollment characteristics of derivative and validation study populations for risk score development

	Number (%) or median (IQR)					
	Partners in Prevention HSV/HIV Transmission Study		Couples Observational Cohort Study		Partners PrEP Study, placebo arm	
	Couples with HIV-1 acquisition, N=107	Couples without HIV-1 acquisition, N=3190	Couples with HIV-1 acquisition, N=15	Couples without HIV-1 acquisition, N=461	Couples with HIV-1 acquisition, N=57	Couples without HIV-1 acquisition, N=1442
Couple characteristics						
Female HIV-1 infected partner	65 (60.8%)	2153 (67.5%)	3 (20.0%)	246 (53.4%)	27 (47.4%)	880 (61.0%)
Married and/or cohabiting	101 (94.4%)	2921 (91.6%)	14 (93.3%)	434 (94.1%)	57 (100.0%)	1421 (98.5%)
Duration of partnership, years	3 (1.3-6.1)	5 (2.4-10.0)	1.9 (1.2-4.4)	4.4 (1.5-9.2)	4.2 (1.0-9.2)	7.4 (3.0-14.0)
Number of children within partnership	1 (0-2)	1 (0-3)	1 (0-1)	1 (0-3)	1 (0-3)	2 (1-4)
Number of sex acts in prior month	6 (3-12)	4 (3-9)	3 (2-4)	3 (1-4)	4 (3-10)	4 (3-8)
Any unprotected sex in prior month	59 (55.1%)	1101 (34.5%)	4 (26.7%)	69 (15.0%)	23 (40.4%)	378 (26.2%)
Characteristics of HIV-1 infected partner						
Age, years	30 (25-37)	32 (27-38)	29 (26-36)	32 (26-38)	32 (26-39)	33 (27-39)
Effective contraceptive use (women)*	14 (21.5%)	391 (18.2%)	0 (0.0%)	110 (24.7%)	8 (28.6%)	260 (29.6%)
Plasma viral load, copies/mL	44540 (7700-119025)	11388 (2184-45130)	60615 (14670-123320)	21210 (3545-96655)	30278 (11760-123420)	7669 (1534-31434)
CD4 count, cells/mm ³	419 (330-557)	463 (348-636)	472 (228-655)	391 (246-576)	496 (356-626)	500 (375-663)
Characteristics of HIV-1 uninfected partner						
Age, years	30 (26-38)	34 (28-41)	23 (19-28)	31 (26-38)	30 (25-37)	34 (28-40)
Uncircumcised (men)	41 (63.1%)	965 (44.8%)	0 (0.0%)	110 (44.7%)	14 (51.9%)	414 (47.1%)
Effective contraceptive use (women)*	7 (16.7%)	156 (15.0%)	3 (25.0%)	25 (11.6%)	16 (48.5%)	216 (38.5%)

*Includes oral, implantable or injectable hormonal contraceptives, intrauterine device (IUD) and/or condoms

Table 4. Analysis of predictors and calculation of risk score

	Univariate analysis		Multivariate analysis*		Stepwise multivariate analysis**			Risk score***
	Hazard ratio	95% CI	Hazard ratio	95% CI	Hazard ratio	95% CI	Regression coefficient	
Female HIV-1 infected partner	0.7	0.5-1.1	0.6	0.3-1.1				
Age of HIV-1 infected partner								
20 years or younger	2.4	1.0-5.7	1.7	0.6-4.6				
21-30 years	1.4	0.9-2.1	1.2	0.8-2.0				
More than 35 years	ref		ref					
Age of HIV-1 uninfected partner								
20 years or younger	5.5	2.8-10.8	3.3	1.5-7.2	4.5	2.2-9.1	1.51	4
21-35 years	1.6	1.1-2.4	1.3	0.8-2.0	1.5	1.0-2.2	0.40	1
More than 30 years	ref		ref		ref			
Married and/or cohabiting	1.5	0.7-3.4	1.8	0.8-4.2	1.8	0.8-4.1	0.58	1
Duration of partnership, years	0.9	0.90-0.98						
Number of children								
0	2.4	1.4-4.3	2.2	1.2-4.0	2.1	1.1-3.7	0.72	2
1-2	1.7	0.9-3.0	1.6	0.9-2.8	1.5	0.8-2.7	0.41	1
3 or more	ref		ref		ref			
Unprotected sex within partnership, prior 30 days	2.3	1.6-3.4	2.2	1.5-3.2	2.2	1.5-3.2	0.78	2
Number of sex acts within partnership, prior 30 days	1.02	1.00-1.04						
Uncircumcised male HIV-1 uninfected partner	1.4	1.0-2.1	1.9	1.1-3.1	1.5	1.0-2.3	0.44	1
Effective contraceptive use								
HIV-infected women	1.1	0.6-1.9						
HIV-uninfected women	1.3	0.6-2.8	1.1	0.5-2.6				
HIV-1 infected partner CD4 count (cells/mm ³)	1.0	0.99-1.0						
HIV-1 infected plasma viral load								
50,000 copies or higher	3.8	2.4-6.0	3.7	2.4-5.9	3.9	2.5-6.1	1.36	3
10,000 -49,999 copies	1.6	0.9-2.7	1.5	0.9-2.5	1.5	0.9-2.6	0.43	1
Less than 10,000 copies/mL	ref		ref		ref			

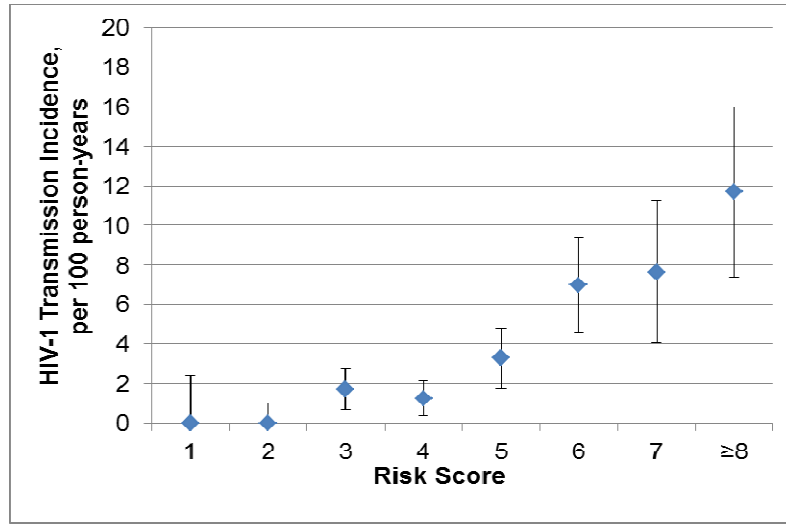
*Covariates selected for multivariate analysis were based on those factors that were selected a priori for evaluation (gender, unprotected sex, circumcision status and plasma HIV-1 RNA) and other factors that were statistically significant in univariate analysis

**Covariates selected for multivariate model based on lowest Akaike Information Criterion (AIC) score from stepwise procedure and not statistical significance of individual predictor

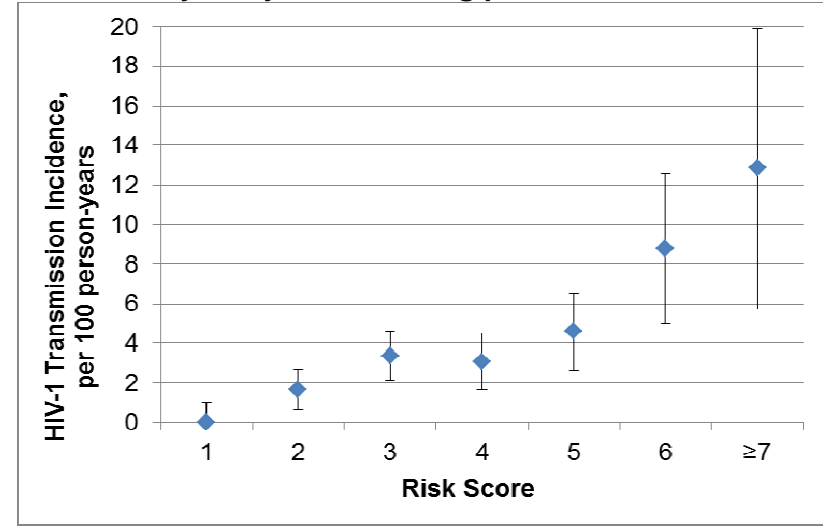
***Points were assigned to each risk factor by dividing each coefficient from the stepwise proportional hazard model by 0.29 (the lowest coefficient value, corresponding to HIV-1 uninfected age 21-35 years) and rounding to the nearest integer

Figure 1. Incidence of HIV-1 infection by risk score

A. Full risk score



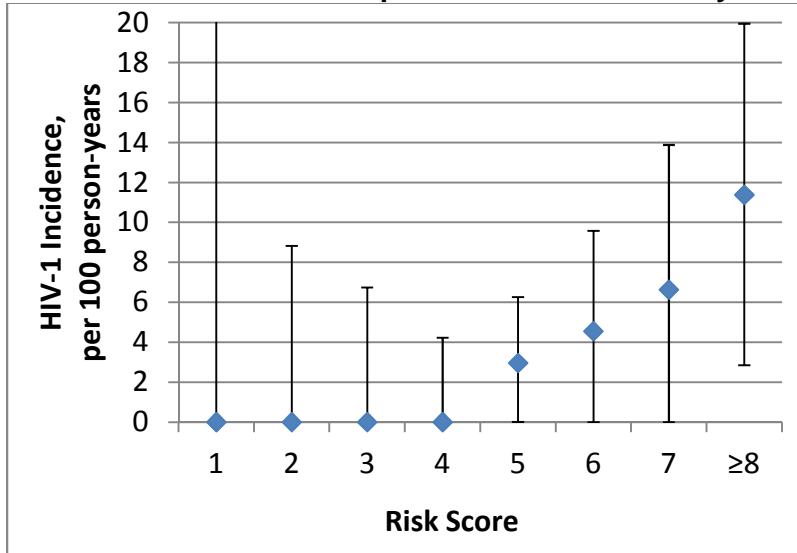
B. Sensitivity analysis: excluding plasma HIV-1 viral load



Risk score	Sero-conversions	Person-years	Incidence (95% CI)
1	0	152.0	0.00 (0.00-2.43)
2	0	370.3	0.00 (0.00-1.00)
3	10	584.1	1.71 (0.66-2.76)
4	8	633.3	1.26 (0.39-2.13)
5	17	518.6	3.28 (1.74-4.81)
6	31	444.6	6.97 (4.61-9.34)
7	16	208.8	7.66 (4.05-11.27)
≥ 8	25	213.9	11.69 (7.38-15.99)

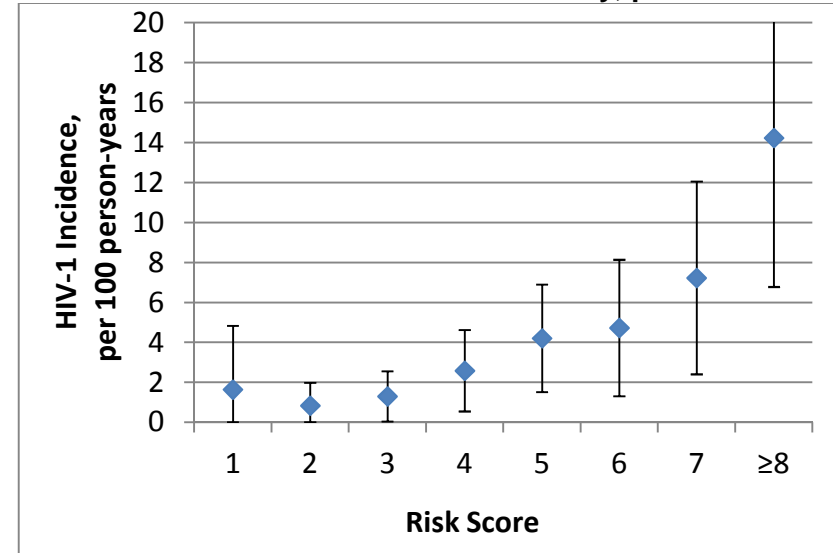
Risk score	Sero-conversions	Person-years	Incidence (95% CI)
1	0	355.0	0.00 (0.00-1.04)
2	10	601.3	1.66 (0.64-2.69)
3	28	826.9	3.39 (2.15-4.62)
4	18	582.5	3.09 (1.68-4.50)
5	21	457.5	4.59 (2.67-6.51)
6	19	216.6	8.77 (5.01-12.54)
≥ 7	11	85.8	12.82 (5.75-19.89)

C. External validation: Couples Observational Study



Risk score	Sero-conversions	Person-years	Incidence (95% CI)
1	0	7.5	0.00 (0.00-4.92)
2	0	41.8	0.00 (0.00-8.82)
3	0	54.8	0.00 (0.00-6.74)
4	0	87.4	0.00 (0.00-4.22)
5	3	101.5	2.96 (0.00-6.25)
6	3	65.9	4.55 (0.00-9.58)
7	3	45.3	6.63 (0.00-13.88)
≥8	6	52.7	11.38 (2.81-19.95)

D. External validation: Partners PrEP Study, placebo arm



Risk score	Sero-conversions	Person-years	Incidence (95% CI)
1	1	61.4	1.64 (0.00-4.82)
2	2	240.9	0.83 (0.00-1.98)
3	4	309.0	1.29 (0.03-2.56)
4	6	232.7	2.58 (0.54-4.62)
5	9	214.1	4.20 (1.52-6.89)
6	7	148.3	7.22 (2.40-12.04)
7	8	110.8	7.22 (2.40-12.04)
≥8	12	84.4	14.22 (6.77-21.68)

Figure 2. ROC curves comparing risk score to individual continuous predictors.

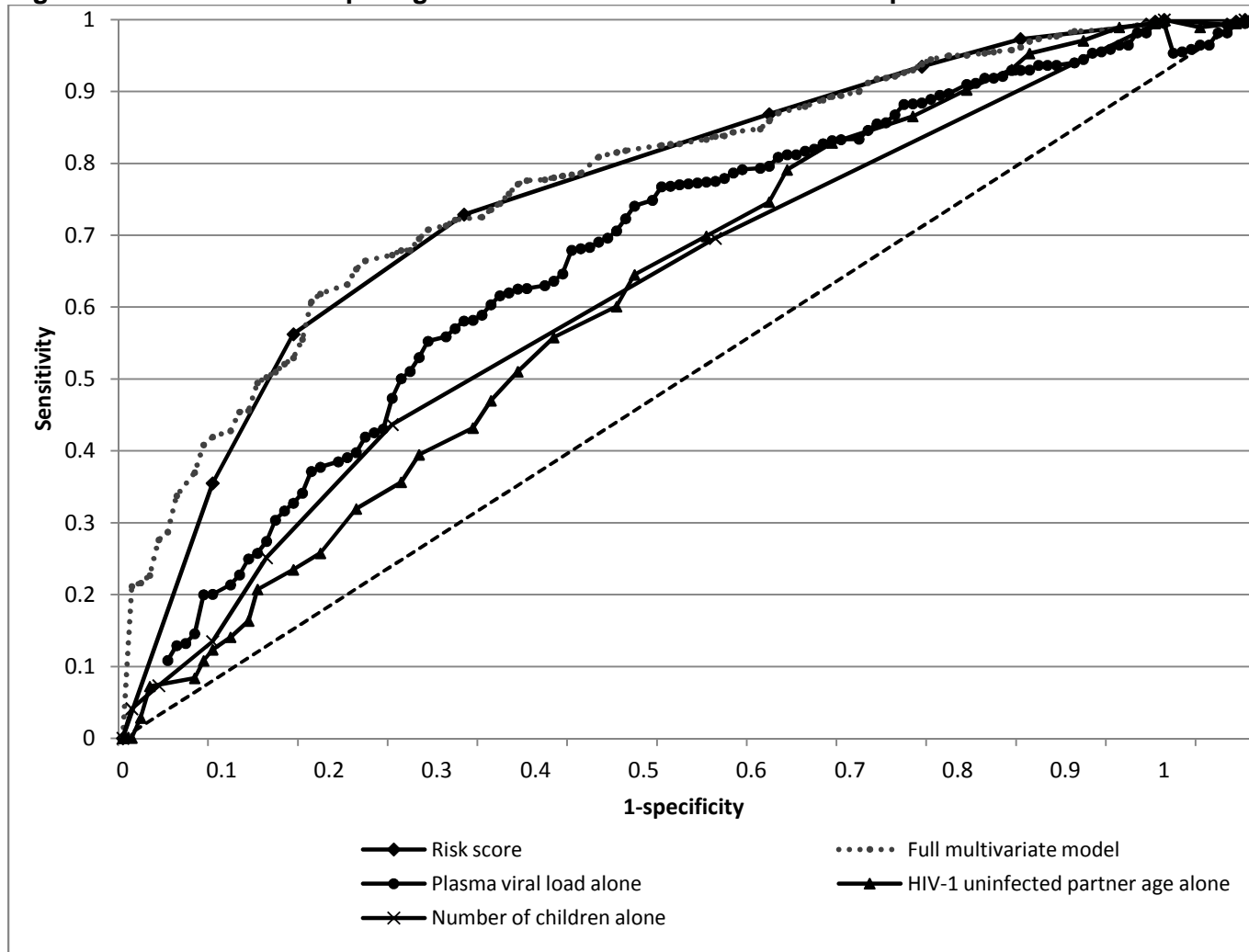


Figure 3. HIV-1 acquisition risk score worksheet.

Age of HIV-1 uninfected partner		
20 years or less	4	<input type="checkbox"/>
21-30 years	1	<input type="checkbox"/>
More than 30 years	0	<input type="checkbox"/>
Number of children		
0	2	<input type="checkbox"/>
1-2	1	<input type="checkbox"/>
3 or more	0	<input type="checkbox"/>
Male HIV-1 uninfected partner uncircumcised		
Yes	1	<input type="checkbox"/>
No	0	<input type="checkbox"/>
Married and/or cohabiting		
Yes	1	<input type="checkbox"/>
No	0	<input type="checkbox"/>
Unprotected sex within partnership, prior 30 days		
Yes	2	<input type="checkbox"/>
No	0	<input type="checkbox"/>
HIV-1 plasma viral load, HIV-1 infected partner		
50,000 copies or higher	3	<input type="checkbox"/>
10,000-49,999 copies	1	<input type="checkbox"/>
Less than 10,000 copies	0	<input type="checkbox"/>
Total score (≥ 6 = higher risk, ≥ 4 if viral load not done)		

Chapter 3: Unreported antiretroviral use by HIV-1 infected participants enrolling in a
prospective research study

Presented at the 2012 International Microbicides Conference, Sydney, Australia, 15-18 April 2012: Kahle E, Kashuba A, Baeten J, Delany S, Donnell D, John-Stewart G, Mujugira A, Mugo N, Celum C, Lingappa J. Unreported antiretroviral use by HIV-1 infected members of HIV-1 serodiscordant couples enrolling in an HIV-1 prevention clinical trial. Abstract 347, Session 35.

Citation:

Kahle EM, Kashuba A, Baeten JM, Fife K, Celum C, Mujugira A, Essex M, De Bruyn G, Wald A, Donnell D, John-Stewart G, Deleany-Moretlwe S, Mugo NR, Farquhar C, Lingappa JR. Unreported antiretroviral use by HIV-1 infected participants enrolling in a clinical trial of serodiscordant couples in sub-Saharan Africa. . *J Acquir Immune Defic Syndr (in press)*

Unreported antiretroviral use by HIV-1 infected participants enrolling in a prospective research study

Erin M. Kahle¹, Angela Kashuba⁵, Jared M. Baeten^{1,2,3}, Kenneth H. Fife⁶, Connie Celum^{1,2,3}, Andrew Mujugira², Max Essex⁷, Guy De Bruyn⁸, Anna Wald^{1,3,10}, Deborah Donnell¹⁰, Grace John-Stewart^{1,2,3,4}, Sinead Delany-Moretlwe⁹, Nelly R. Mugo^{2,11}, Carey Farquhar^{1,3}, Jairam R. Lingappa^{2,3,4} for the Partners in Prevention HSV/HIV Transmission Study Team

Departments of ¹Epidemiology, ²Global Health, ³Medicine and ⁴Pediatrics, University of Washington, Seattle, WA, USA; ⁵University of North Carolina at Chapel Hill, Chapel Hill, NC, USA; ⁶Department of Medicine, Indiana University, Indianapolis, IN, USA; ⁷Department of Immunology & Infectious Disease, Harvard School of Public Health, Harvard University, Cambridge, MA, USA; ⁸Perinatal HIV Research Unit and the ⁹Wits Reproductive Health & HIV Institute, University of Witwatersrand, Johannesburg, Republic of South Africa; ¹⁰Vaccine and Infectious Disease Institute, Fred Hutchinson Cancer Research Center, Seattle, WA, USA; ¹¹Department of Obstetrics & Gynecology, University of Nairobi & Kenyatta National Hospital, Nairobi, Kenya;

Manuscript length (not including table/acknowledgements): 1,489 words

Keywords: antiretroviral therapy, HIV-1, viral load, HIV-1 prevention clinical trials

Footnote Page

Conflict of Interest Statement: None of the authors have commercial or other conflicts of interest related to the contents of this manuscript

Funding statement: This study was supported by research grants from the Bill and Melinda Gates Foundation (grant ID #26469), the US National Institutes of Health (R01 MH095507), University of Washington Center for AIDS Research Clinical Retrovirology Core (UW CFAR, NIH AI-27757) and the University of North Carolina Center for AIDS Research (UNC CFAR P30-A150410)

Previous Presentation: This work was presented in part at the 2012 International Microbicides Conferences, Sydney, Australia, April 15-18 2012. Abstract 347.

Corresponding author:

Erin Kahle

University of Washington

325 Ninth Avenue, Box 359927

Seattle, Washington 98104

Tel – 206.520.3816, Fax – 206.520.3831

Email – ekahle@u.washington.edu

The concentration of viral RNA in plasma is the primary risk factor for sexual transmission of HIV-1 [16, 25, 60], and reductions in plasma HIV-1 RNA levels due to antiretroviral therapy (ART) result in marked decreases in HIV-1 transmission risk [20, 26]. Results from studies of HIV-1 transmission and disease progression may be more difficult to interpret if a substantial proportion of HIV-1 infected partners have low or undetectable viral loads on ART, and thus, ART use at study enrollment is often an exclusion factor.

Recent reports from clinical trial cohorts of HIV-1 transmission in HIV-1 serodiscordant couples have found that nearly a quarter of HIV-1 infected partners had low enrollment plasma HIV-1 RNA levels (<2000 copies/ml) [101, 103]. Low levels of plasma HIV-1 RNA in the HIV-1 infected partners, selected for not having transmitted HIV-1 to their partner for studies of candidate interventions to reduce HIV-1 transmission, may reflect natural host control of viral replication. However, an alternative explanation could be unreported ART use. Distinguishing between these potential sources of low viral load is important for studies seeking to understand the biology of HIV-1 transmission. We tested stored samples from a recent HIV-1 prevention clinical trial to determine the frequency of unreported ART use among HIV-1 infected individuals with low plasma HIV-1 RNA levels.

Between November 2004 and April 2007, we enrolled 3408 heterosexual HIV-1 serodiscordant couples from seven African countries in a randomized, double-blind, placebo-controlled clinical trial of herpes simplex virus type 2 (HSV-2) suppressive therapy to reduce HIV-1 transmission (Partners in Prevention HSV/HIV Transmission Study), as previously described [101]. Eligible couples were at least 18 years of age, sexually active, and intending to remain as a couple. All HIV-1 infected partners were HSV-2 seropositive, had CD4 counts ≥ 250 cells/ μL (making them ineligible for antiretroviral therapy under national guidelines of the study countries at that time), not pregnant, and self-reported not currently taking ART. Quarterly plasma and serum samples

were collected for up to 24 months and archived at -80°C for subsequent laboratory testing. HSV-2 suppressive therapy did not reduce HIV-1 transmission within the study partnerships [102]. At study screening and all follow-up visits, HIV-1 infected participants were asked if they were currently taking ART.

All participants received HIV-1 primary care, referral for ART according to national guidelines and risk reduction counseling and treatment for sexually transmitted infections during up to 24 months of study follow-up. Written, informed consent was obtained from all participants. The study protocol was approved by the University of Washington Human Subjects Review Committee and ethical review committees at each of the study sites.

All laboratory testing occurred at the end of study follow-up. Plasma HIV-1 RNA levels were quantified using the COBAS Ampliprep/COBAS TaqMan real-time HIV-1 RNA assay, version 1.0 (Roche Diagnostics, Indianapolis, IN), with a lower limit of quantification of 240 copies/mL. For those with plasma HIV-1 RNA <2000 copies/mL at enrollment, high-performance liquid chromatography with ultraviolet detection was used to measure antiretroviral (ARV) levels in an archived serum collected at enrollment [118]. The assay used to measure ART levels was validated for five nucleoside reverse transcriptase inhibitors (abacavir, didanosine, lamivudine, stavudine and zidovudine) and one non-nucleoside reverse transcriptase inhibitor (nevirapine). We considered any quantifiable concentration as indicative of ART use.

Low viral load was stratified into two groups defined as: 1) low detectable (240-2000 copies/mL), or 2) undetectable (<240 copies/mL). We calculated the overall prevalence of unreported ART use at enrollment among HIV-1 infected partners with low plasma HIV-1 RNA (<2000 copies/ml) overall and in each group. All analyses were conducted using SAS (v.9.2, Cary, NC).

Among 3371 HIV-1 infected partners who had results for enrollment plasma HIV-1 RNA tested, 798 (23.7%) had plasma HIV-1 RNA <2000 copies/mL, including 443/798 (13.1%) with low plasma HIV-1 RNA levels (240-2000 copies/mL) and 355/798 (10.5%) with undetectable RNA (<240 copies/mL). Those with enrollment plasma HIV-1 RNA <2000 copies/mL were more likely to be female compared to those with plasma HIV-1 levels >2000 copies/mL (78.1% vs. 64.1%, $p<0.05$); all other characteristics were similar for those with higher versus lower plasma HIV-1 RNA (Table 5).

ARV testing was performed on 771 (96.6%) of the persons with plasma HIV-1 RNA <2000 copies/mL where specimens were available. Antiretrovirals were detected in 171/771 (22.2%): 157/341 (46.0%) in those with undetectable plasma HIV-1 RNA (<240 copies/mL) and 14/430 (3.3%) in those with low detectable plasma HIV-1 RNA (240-2000 copies/mL). The most common ARVs detected were lamivudine (20.8%) and nevirapine (17.8%). Most, (83.6%), of the 171 participants with detectable ARVs had evidence of multiple drugs, specifically the combinations nevirapine/lamivudine (52.0%), nevirapine/lamivudine/stavudine (21.6%), stavudine/lamivudine (5.3%), and zidovudine/lamivudine with or without nevirapine (4.1%). Differences in ARV detection were found among the study sites, but there were no difference in ARV detection between men and women.

We have previously reported that nearly a quarter (23.7%) of HIV-1 infected partners in HIV-1 serodiscordant partnerships for the Partners in Prevention HSV/HIV Transmission Study had plasma HIV-1 levels <2000 copies/mL at baseline, in the absence of reported use of ART [6]. Our analysis here demonstrates that 22% of those, and nearly half of the subset with undetectable plasma HIV-1, had evidence of unreported ART use. Thus, undetectable plasma HIV-1 RNA is a potential marker of unreported ART use in HIV-1 infected partners. This finding could be significant for studies focused on describing host factors associated with natural viral control, since a large proportion of individuals with low plasma HIV-1 levels had pharmacologic

and not immunologically induced viral suppression. For example, in genetic studies of elite controllers, the inclusion of subjects with ART-induced viral suppression would undermine the ability of the study to identify any potentially valuable genetic markers.

It is important to note that while unreported ARV detection was strongly associated with plasma HIV-1 level (48% for undetectable versus only 3% for detectable but <2000 copies/mL), the proportion of individuals in the overall study cohort with unreported ART use and detectable ARVs was very small (171/3408, 5%) and equally distributed between the randomization arms in the clinical trial. For these reasons, it is unlikely that unreported ART use would have an important impact on the overall outcomes of this clinical trial, or other randomized clinical trials of this kind.

Since ART use was an exclusion criterion in this study and thus by definition was not reported, inferences about reasons or circumstances underlying this finding are largely speculative. Women had a significantly higher proportion of plasma HIV-1 RNA <2000, suggesting women may have been more likely to have received ART, possibly clinically indicated for prevention of mother-to-child transmission (PMTCT) for which nevirapine and lamivudine are included in recommended regimens and also prominently represented among the ARVs detected in this analysis [119, 120]. However, in our analysis, we did not find significant difference in ART by gender and do not have evidence that women were more likely to have unreported ART detected. Without knowing specific timing of recent doses, we cannot make any determination on whether or not persons with detected ARV discontinued drug use prior to study screening or what drug dose was taken. Nevirapine can be detected in women more than two weeks after receiving single-dose nevirapine for PMTCT [121, 122], while most nucleoside reverse transcriptase inhibitors such as lamivudine and stavudine can be detected, at most, for a few days after discontinuation [123].

In resource-limited communities, services and other benefits offered through clinical trial participation could provide an incentive to not disclose ART use, which would have made them ineligible for the trial [21]. Couples enrolled in the Partners in Prevention HSV/HIV Transmission Study did not receive a monetary incentive for participation but did receive benefits including free counseling, screening and treatment for sexually transmitted infections, condoms and travel reimbursement. One prior study of participation in a large-scale HIV-1 prevention trial in South Africa concluded that the level of reimbursement could be a motivating factor for some participants to misreport information during enrollment screening [124]. In multiple studies of willingness to participate in HIV-1 clinical trials in different settings, the majority of participants reported primarily altruistic motivations for participants, although a minority or respondents stated monetary incentives and access to health care as the primary motivator for participation [125-127]. We recommend further research to understand nondisclosure of ART by participants enrolling in an HIV-1 prevention study.

In summary, we found unreported use of ART to be prevalent among HIV-1 infected individuals with undetectable plasma virus. For randomized control trials of HIV-1 prevention interventions in HIV-1 serodiscordant couples or of novel HIV-1 treatments, assessing viral load may improve the efficiency of the study, by excluding those with low viral loads who would be unlikely to transmit or have a substantial virologic response to treatment. More importantly, for observational studies of pathogenesis and transmission, it may be critical to understand the etiology of undetectable viral loads, and thus particularly important to identify unreported ART use. Studies recruiting HIV-1 infected participants with low levels of plasma HIV-1 RNA, should consider laboratory testing for ART.

Acknowledgments: We gratefully acknowledge the contributions of couples that participated in this study and teams at the study sites and at the University of Washington.

Partners in Prevention HSV/HIV Transmission Study Team:

University of Washington Coordinating Center and Central Laboratories, Seattle, USA:

Connie Celum (principal investigator), Anna Wald (protocol co-chair), Jairam Lingappa (medical director), Jared M. Baeten, Mary Campbell, Lawrence Corey, Robert W. Coombs, James P. Hughes, Amalia Magaret, M. Juliana McElrath, Rhoda Morrow, James I. Mullins

Study sites and site principal investigators: Cape Town, South Africa (University of Cape Town): David Coetzee; Eldoret, Kenya (Moi University, Indiana University): Kenneth Fife, Edwin Were; Gaborone, Botswana (Botswana Harvard Partnership): Max Essex, Joseph Makhema; Kampala, Uganda (Infectious Disease Institute, Makerere University): Elly Katabira, Allan Ronald; Kigali, Rwanda (Rwanda Zambia HIV Research Group, and Emory University): Susan Allen, Kayitesi Kayitenkore, Etienne Karita; Kisumu, Kenya (Kenya Medical Research Institute, University of California San Francisco): Elizabeth Bukusi, Craig Cohen; Kitwe, Zambia (Rwanda Zambia HIV Research Group, and Emory University): Susan Allen, William Kanweka; Lusaka, Zambia (Rwanda Zambia HIV Research Group, and Emory University): Susan Allen, Bellington Vwalika; Moshi, Tanzania (Kilimanjaro Christian Medical College, Harvard University): Saidi Kapiga, Rachel Manongi; Nairobi, Kenya (University of Nairobi, University of Washington): Carey Farquhar, Grace John-Stewart, James Kiarie; Ndola, Zambia (Rwanda Zambia HIV Research Group, and Emory University): Susan Allen, Mubiana Inambao; Orange Farm, South Africa (Wits Reproductive Health & HIV Institute, University of the Witwatersrand): Sinead Delany-Moretlwe, Helen Rees; Soweto, South Africa (Perinatal HIV Research Unit, University of the Witwatersrand): Guy de Bruyn, Glenda Gray, James McIntyre; Thika, Kenya (University of Nairobi, University of Washington): Nelly Rwamba Mugo

Table 5. Enrollment characteristics and type of antiretroviral (ART) detected among HIV-1 infected partners

	≥ 2000 copies/mL, N=2573	<2000 copies/mL, N=443	Undetectable, N=355
Demographic characteristics			
Female gender	1650 (64.1%)	335 (75.6%)	288 (81.1%)
Age, years	32 (27-39)	31 (26-37)	32 (28-37)
Education, years	8 (6-11)	8 (7-12)	8 (6-11)
Has monthly income	940 (36.3%)	159 (35.9%)	120 (33.8%)
Couple characteristics			
Married and/or cohabiting	2340 (90.9%)	399 (90.1%)	324 (91.3%)
Duration of partnership, years	5.3 (2.3-10.4)	4.8 (2.2-9.6)	6.2 (2.6-10.6)
Number of children	1 (0-2)	1 (0-2)	1 (0-3)
Sex with outside partner, prior 30 days	92 (3.6%)	16 (3.6%)	7 (2.0%)
Any unprotected sex, prior 30 days	725 (28.2%)	120 (27.1%)	117 (33.0%)
Clinical characteristics			
CD4 count, cells/mm ³	458 (345-626)	471 (352-640)	467 (348-650)
Any sexually transmitted infection*	380 (14.8%)	56 (12.6%)	51 (14.4%)
Antiretroviral (ART) tested		N=430	N=341
NRTIs			
Abacavir (ABC)		0 (0%)	1 (0.3%)
Zidovudine (AZT)		0 (0%)	9 (2.6%)
Lamivudine (3TC)		12 (2.8%)	148 (43.4%)
Stavudine (D4T)		3 (0.7%)	43 (12.6%)
Didanosine (DDI)		0 (0%)	1 (0.3%)
NNRTIs			
Nevirapine (NVP)		11 (2.6%)	126 (37.0%)
Any ART detected		14 (3.3%)	157 (46.0%)

*Including *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*

Chapter 4: HIV-1 subtype C is not associated with higher risk of heterosexual HIV-1 transmission: a multinational study among African HIV-1 serodiscordant couples

Presented at the XIX International AIDS Conference, Washington, DC, USA, 22-27 July 2012: Kahle EM, Campbell M, Lingappa JR, Donnell D, Celum C, Kapiga S, Ondondo R, Mugo N, Mujugira A, Fife K, Mullins J, Baeten JM. HIV-1 subtype C is not associated with higher risk of heterosexual transmission: a multinational study among African HIV-1 serodiscordant couples. TUAC0101.

Citation:

Kahle, EM, Campbell M, Lingappa J, Donnell D, Celum C, Ondondo R, Mujugira A, Fife K, Mugo N, Kapiga S, Mullins J, Baeten JM. HIV-1 subtype C is not associated with higher risk of heterosexual HIV-1 transmission: a multinational study among African HIV-1 serodiscordant couples. *AIDS (in press)*

**HIV-1 subtype C is not associated with higher risk of heterosexual HIV-1 transmission:
a multinational study among African HIV-1 serodiscordant couples**

Erin Kahle^{1,2}, Mary Campbell³, Jairam Lingappa^{2,3,4}, Deborah Donnell⁷, Connie Celum^{1,2,3},
Raphael Ondondo⁸, Andrew Mujugira^{1,2}, Kenneth Fife⁹, Nelly Mugo^{2,10}, Saidi Kapiga¹¹,
James I. Mullins^{3,5,6}, Jared M. Baeten^{1,2,3}

for the Partners in Prevention HSV/HIV Transmission Study Team

Departments of ¹Epidemiology, ²Global Health ³Medicine, ⁴Pediatrics, ⁵Microbiology and
⁶Laboratory Medicine, University of Washington, Seattle, WA, USA; ⁷Vaccine and Infectious
Disease Institute, Fred Hutchinson Cancer Research Center, Seattle, WA, USA; ⁸Kenya Medical
Research Institute, Kisumu, Kenya; ⁹Department of Medicine, Indiana University, Indianapolis,
IN, USA; ¹⁰Department of Obstetrics & Gynecology, University of Nairobi & Kenyatta National
Hospital, Nairobi, Kenya; ¹¹London School of Hygiene and Tropical Medicine, London, United
Kingdom

Manuscript length: (not including tables/acknowledgements): 2,842 words

Abstract length: 241 words

Footnote page

Conflict of Interest: None of the authors have commercial or other conflicts of interest related to the content of this manuscript.

Funding: US National Institutes of Health (P30 A127757 to the University of Washington Center for AIDS Research, P01 057005, and K08 AI074424) and the Bill and Melinda Gates Foundation (grant 26469).

Previous Presentation: A portion of this work was presented at the XIX International AIDS Conference, 22-27 July 2012, Washington, DC, USA. Abstract TUAC0101.

Corresponding author:

Erin Kahle

University of Washington

325 Ninth Avenue, Box 359927

Seattle, Washington 98104

Tel – 206-520-3816, Fax – 206-520-3831, email – ekahle@u.washington.edu

Abstract

Background: HIV-1 subtype C has emerged as the most prevalent strain of HIV-1 worldwide, leading to speculation that subtype C may be more transmissible than other subtypes. We compared the risk of HIV-1 transmission for subtype C versus non-C subtypes (A, D, G and recombinant forms) among heterosexual African HIV-1 serodiscordant couples.

Methods: We conducted a nested case-control analysis using data from two prospective cohort studies of heterosexual HIV-1 serodiscordant couples from 6 countries in eastern and southern Africa. Cases (N=121) included incident HIV-1 transmissions that were established as linked within the serodiscordant partnership by viral sequencing; controls (N=501) were non-transmitting HIV-1 infected partners. Subtype was determined for partial *env* and *gag* genes. Multiple logistic regression controlled for age and gender of the HIV-1 infected partner and self-reported unprotected sex. Plasma and genital HIV-1 RNA concentrations were compared between subtype C and non-C subtypes using generalized estimating equations.

Results: HIV-1 subtype C was not associated with increased risk of HIV-1 transmission compared to non-C subtypes: *env* adjusted odds ratio (adjOR) 1.14 (95% confidence interval [CI] 0.74-1.75, p=0.6) and *gag* adjOR 0.98 (95% CI 0.63-1.52, p=0.9). Plasma and genital HIV-1 RNA levels did not differ significantly for subtype C versus non-C.

Conclusion: In a geographically diverse population of heterosexual African HIV-1 serodiscordant couples, subtype C was not associated with greater risk of HIV-1 transmission compared to non-C subtypes, arguing against the hypothesis that subtype C is more transmissible compared to other common subtypes.

Keywords: HIV-1 subtype, transmission, serodiscordant couples, Africa

Introduction

HIV-1 subtype C accounts for nearly half of all HIV-1 infections worldwide, primarily due to its predominance in sub-Saharan Africa, where the majority of HIV-1 infections occur [128, 129]. The explosion of heterosexually transmitted HIV-1 throughout southern Africa in the 1990s was almost exclusively due to HIV-1 subtype C, leading some to hypothesize that subtype C might be more transmissible compared to other subtypes [130-133]. Laboratory studies have suggested molecular and genetic characteristics of subtype C that could promote more efficient transmission [40, 134, 135]. However, clear evidence for differential transmissibility of HIV-1 subtypes in population-level epidemiological studies has not been shown [47, 136, 137]. HIV-1 genetic diversity, including subtype diversity, poses a challenge to the development of a globally-effective HIV-1 vaccine [39], and subtype-related differences in HIV-1 transmission, if present, would be a critical consideration in the selection of vaccine antigens [129, 138].

Epidemiologic studies directly measuring the relationship between HIV-1 subtype and heterosexual transmission risk have been challenging for two main reasons. First, prospective studies of HIV-1 transmission require following large numbers of HIV-1 infected persons and their uninfected sexual partners in order to identify rates of HIV-1 transmission occurring within the partnerships. Second, HIV-1 subtypes tend to be geographically specific, and thus studies must include populations from multiple regions in order to have sufficient subtype variation for comparison of transmission risk. Several studies of mother-to-child transmission have had mixed results when comparing vertical HIV-1 transmission by subtype [45, 48, 49, 139]. Even fewer studies of subtype and transmission exist for heterosexual transmission. One HIV-1 serodiscordant couples study in Uganda found higher transmission risk for subtype A compared to D [51], but subtype C was not present in the study population. Another study of serodiscordant couples in Zambia found subtype C in 95% of genetically-linked transmissions [50], but the Zambian epidemic is predominantly subtype C and thus comparing transmission

rates to other subtypes was not possible in that study. In the present study, among a multinational population of heterosexual HIV-1 serodiscordant couples from eastern and southern Africa, our aim was to assess whether subtype C, compared with non-C subtypes, was associated with greater HIV-1 transmission risk.

Methods

Study Population

We conducted a nested case-control study using data from two prospective cohort studies of African HIV-1 serodiscordant couples. Between November 2004 and April 2007, 3408 heterosexual HIV-1 serodiscordant couples from 6 African countries (Botswana, Kenya, South Africa, Tanzania, Uganda, and Zambia) were enrolled into the Partners in Prevention HSV/HIV Transmission Study, a randomized, double-blind, placebo-controlled clinical trial of herpes simplex virus type 2 (HSV-2) suppressive therapy to reduce HIV-1 transmission, as previously described [101]. Eligible couples were at least 18 years of age, reported at least three vaginal sex acts in the three months prior to enrollment, and intended to remain as a couple. At enrollment, all HIV-1 infected partners were HSV-2 seropositive, had CD4 counts ≥ 250 cells/ μL (making them ineligible for antiretroviral therapy (ART) under the national guidelines of the study countries at that time), and were not currently taking ART. HSV-2 suppressive therapy was found not to reduce HIV-1 transmission within the partnerships [102]. In a parallel study at two sites (Kampala, Uganda and Soweto, South Africa), an additional 485 HIV-1 serodiscordant couples were enrolled into an observational study of immune correlates of HIV-1 protection (Couples Observational Study)[140]. Similar to the clinical trial cohort, participants were ≥ 18 years of age and sexually active and HIV-1 seropositive partners were not using ART. In both cohorts, initially HIV-1 uninfected participants were followed quarterly, with HIV-1 serologic testing.

Protection of Human Subjects.

All participants received HIV-1 and risk-reduction counseling (both individually and as a couple), free condoms, and treatment for sexually transmitted infections (STIs), according to WHO guidelines. Written, informed consent was obtained from all participants. The study protocols were approved by the University of Washington Human Subjects Review Committee and ethical review committees at each of the study sites.

Selection of cases and controls

Cases were defined as all HIV-1 infected partners of HIV-1 seroconverters, limited to those couples in which it was determined, through viral genetic linkage, that HIV-1 transmission occurred within the partnership (as opposed to from an outside partner) [104]. A total of 121 cases were included: 106 from the Partners in Prevention HSV/HIV Transmission Study and 15 from the Couples Observational Study. Controls were selected randomly, in proportion to research site and gender distribution of each study, from non-transmitting HIV-1 infected partners to achieve a 1:4 case to control ratio. Since HIV-1 subtype was expected to be correlated with site, given the geographic association of HIV-1 subtypes in Africa, the proportional sampling of controls was used to select controls representative of the cohort. In total, 501 controls were selected.

Laboratory Testing

HIV-1 seroconversion of initially HIV-1 uninfected partners was determined by quarterly serologic testing using dual rapid HIV-1 antibody tests with confirmatory HIV-1 enzyme immunoassay (EIA), Western blot, and plasma HIV-1 RNA detection. Plasma HIV-1 RNA levels for HIV-1 infected partners were quantified using the COBAS Ampliprep/COBAS TaqMan real-time HIV-1 RNA assay version 1.0 (Roche Diagnostics, Indianapolis, IN). Plasma HIV-1 RNA viral loads were assessed at enrollment and visit months 3, 6, 9, 12 and study exit for the

Partners in Prevention HSV/HIV Transmission Study and at enrollment only for the Couples Observational Study. Genital HIV-1 RNA was quantified using the TaqMan assay from samples collected at a single study visit in the Partners in Prevention HSV/HIV Transmission Study: seminal plasma for HIV-1 infected men, collected at any visit ≥ 3 months after enrollment and endocervical swabs for HIV-1 infected women, collected at a visit 6 months after enrollment [141]. All viral loads were \log_{10} transformed, and results below the limit of quantification (< 240 copies/mL) were assigned a value of half the limit.

Viral sequencing using blood plasma was performed on partial HIV-1 *env* (C2-V3-C3) and *gag* (p17-p24) genes using samples collected at the first post-seroconversion study visit for cases and at the last follow-up visit for controls. Genetic linkage of HIV-1 transmission events was based on phylogenetic analysis and posterior probability of linkage using pair-wise nucleotide distances between sequences [104]. Subtypes were determined by the REGA subtype tool version 2.0 (<http://dbpartners.stanford.edu/RegaSubtyping/>). Sequence data were provided to GenBank and accession numbers are pending.

Data analysis

We compared HIV-1 transmission risk in cases versus controls between subtype C and all non-C subtypes (including A, D, G, and recombinants) separately for both *env* and *gag*. All cases had subtype information available in *gag*, *env* or both gene regions, but among controls, 43/501 (8.6%) were missing all subtype data, including 34/332 (10.2%) from eastern African and 9/169 (5.3%) from southern Africa, due to low HIV-1 plasma viral loads preventing adequate viral amplification. To avoid bias because of control exclusion due to missing subtype data, we performed multiple imputation with 20 datasets imputed using Markov chain Monte Carlo methods [142].

To assess differences in HIV-1 transmission between subtype C to non-C subtypes, we performed a standard nested case-control analysis using logistic regression, analyzing the 20 imputed datasets and combining the results to produce standard estimates and 95% confidence intervals. All models were adjusted for gender and age of the HIV-1 infected partner and self-reported unprotected sex in the month prior to study enrollment. We assessed other variables for potential confounding, including circumcision status of male HIV-1 uninfected partners, duration of partnership, number of children, presence of sexually transmitted infections, any ART initiation during follow-up by HIV-1 infected partners, and CD4 count of HIV-1 infected partners; however, none of these factors substantially changed the effect estimates and thus were not included in the final models. In additional analyses, we further adjusted for baseline plasma HIV-1 RNA concentrations to assess the association of subtype C and HIV-1 transmission independent of plasma viral load. With the available sample size, we estimated we would have 80% power to detect a 1.85-fold increased odds of HIV-1 transmission for subtype C versus non-C at the alpha 0.05 level.

In addition to the nested case-control analysis, in order to incorporate changes in longitudinal covariates, including time-dependent covariates such as plasma HIV-1 RNA and unprotected sex, we also employed a case-cohort analysis, as a secondary analysis. We used Cox proportional hazards analyses, adjusted for gender, age of the HIV-1 infected partner, and longitudinal report of unprotected sex and plasma HIV-1 RNA, to compare transmission by HIV-1 subtype. Case-cohort analysis methods were used [143].

Finally, we compared differences in plasma and genital HIV-1 RNA concentrations between subtype C and non-C subtypes for participants from the Partners in Prevention HSV/HIV Transmission Study. We assessed subtype differences related to longitudinal plasma HIV-1 RNA during study follow-up using repeated measures generalized estimating equations (GEE)

models with unstructured correlation matrix, adjusting for gender, age of the HIV-1 infected partner, and unprotected sex. Participants were censored at ART initiation. Genital HIV-1 RNA levels were available at a single time point for 416/624 (66.7%) of the HIV-1 infected partners, and we assessed differences among subtypes using a multiple linear regression for endocervical and semen HIV-1 RNA levels, controlling for age of the HIV-1 uninfected partner, unprotected sex reported at enrollment, and plasma HIV-1 viral load.

All analyses were performed using SAS v.9.2 (SAS Institute, Inc., Cary, N.C.).

Results

Of the 622 HIV-1 infected study participants in the nested case-control cohort, subtype information was available for 579 (93.1%), including all 121 (100.0%) cases and 458/501 (91.4%) controls. The majority of participants were from eastern Africa: 80 (66.1%) cases and 332 (66.3%) controls (Table 1). Most couples (92.0%) were married. Age was similar between cases and controls: median age of cases was 30 years (IQR 26-35) and the median age of controls was 32 years (IQR 26-38). Cases were more likely to report unprotected sex in the month prior to enrollment (52.8% versus 36.2%, $p=0.001$) and less likely to be female (49.6% versus 65.5%, $p<0.001$). The median baseline HIV-1 plasma RNA was significantly higher in cases (4.8 \log_{10} copies/mL, IQR 4.3-5.1) compared to controls (4.2 \log_{10} copies/mL, IQR 3.6-4.8, $p<0.001$).

The most common subtypes were A (*env* 44.0%, *gag* 38.3%) and C (*env* 39.2%, *gag* 39.7%), followed by D (*env* 13.9%, *gag* 11.1%), and G or recombinant subtypes (*env* 2.9%, *gag* 10.9%). Subtype was missing in *env* for 25 (4.3%) and in *gag* for 57 (9.8%). For participants with both *env* and *gag* subtypes available, concordance between genes was 82.5%, with concordance of 95.5% for subtype C *env* and *gag*. Nearly all participants from southern Africa were infected with

subtype C (*env* 98.5%, *gag* 99.5%). In eastern Africa, the predominant subtypes were subtype A (*env* 67.7%, *gag* 59.6%) and subtype D (*env* 21.5%, *gag* 17.4%). The distribution of subtype among cases and controls is shown in Figure 1.

Subtype C and HIV-1 Transmission Risk

In the nested case-control multivariate logistic regression analysis, subtype C was not associated with an increased risk of HIV-1 transmission compared to non-C subtypes, both when considering subtype based on *env* sequencing (adjusted odds ratio [adjOR] 1.14, 95% confidence interval [CI] 0.74-1.75, $p=0.6$) and *gag* sequencing (adjOR 0.98, 95% CI 0.63-1.52, $p=0.9$) (Table 2). Additionally, separate comparisons of subtype C to individual subtypes showed no statistically significant differences in the odds of HIV-1 transmission risk with subtype A (*env* adjOR 1.17, $p=0.5$ and *gag* adjOR 1.09, $p=0.7$) or subtype D (*env* adjOR 1.39, $p=0.3$ and *gag* adjOR 1.79, $p=0.08$). Due to the small proportion of participants with subtype G or recombinant variants, separate comparisons with subtype C were not possible. Further adjusting these same regression models for plasma HIV-1 RNA did not substantially change these results. Additionally, when we compared HIV-1 transmission for subtype A compared to subtype D, we did not find significant differences in *env* (adjOR 1.25, 95%CI 0.66-2.36, $p=0.5$) or *gag* (adjOR 0.89, 95%CI 0.48-1.67, $p=0.7$).

In the case-cohort analysis, which permitted adjustment for unprotected sex as a time-varying covariate, results were similar to those in the nested case-control approach: subtype C was not significantly associated with increased HIV-1 transmission compared to non-C subtypes, in *env* (adjHR 1.56, 95% CI 0.89-2.76, $p=0.1$) or *gag* (adjHR 0.92, 95% CI 0.51-1.67, $p=0.8$). In separate comparisons of HIV-1 transmission risk between subtype C and subtypes A and D, there were also no statistically significant differences for *env* or *gag*. These results were similar with the addition of time-dependent plasma HIV-1 RNA to the models.

Subtype C and HIV-1 Concentrations in Plasma and Genital Secretions

The median plasma HIV-1 RNA during the study was 4.3 log₁₀ copies/mL (IQR 3.7-4.8) among those with *env* subtype C and 4.2 log₁₀ copies/mL (IQR 3.4-4.9) among those with a non-C *env* subtype (Figure 2a; p=0.2). The median endocervical HIV-1 RNA for *env* subtype C was 3.3 log₁₀ copies/mL (IQR 2.5-4.0) and for non-C *env* subtypes was 3.4 log₁₀ copies/mL (IQR 2.5-4.0, p=0.9) (Figure 2b). The median semen HIV-1 RNA was 2.8 log₁₀ copies/mL (IQR 2.1-3.5) for *env* subtype C and 2.6 log₁₀ copies/mL (IQR 2.1-3.7) for non-C *env* subtypes. Individuals with *env* subtype C did not differ significantly from non-C subtypes by genital viral load in either endocervical fluid (p=0.9) or semen plasma (p=0.6). Results for *gag* subtype were similar to *env* (data not shown).

Discussion

In this analysis comparing transmitting and non-transmitting HIV-1 serodiscordant couples from eastern and southern Africa, we did not find evidence that subtype C was associated with increased HIV-1 transmission risk, compared with non-C subtypes. Our study population included a wide geographic region with sufficient subtype variation (primarily A, C and D) in order to perform the analyses, and genetic linkage information improved the precision of the results. Previous studies of subtype and HIV-1 transmission have either lacked the subtype diversity to compare subtype C to non-C subtypes or been based on ecological data of prevalent trends in subtype. To our knowledge, this is the first study to provide direct evidence for the question of whether subtype C is associated with increased heterosexual transmission risk compared to other non-C subtypes common in sub-Saharan Africa. Our results do not support the hypothesis that HIV-1 subtype C has greater transmissibility compared with other subtypes.

We conducted both a nested case-control analysis and a longitudinal analysis using a case-cohort study design to assess whether subtype C was associated with an increased risk for HIV-1 transmission. We adjusted for age, gender and reported unprotected sex, and we determined that other factors (e.g., male circumcision status) were not confounding. We did not initially include plasma HIV-1 RNA in our initial models because we hypothesized that if HIV-1 transmission differed by subtype, it could be mediated by subtype-related differences in viral load. However, after finding no association between subtype C and HIV-1 transmission, we further adjusted our models to control for plasma HIV-1 RNA and continued to see no significant relationship between subtype C and HIV-1 transmission risk, compared to non-C subtypes. In both the nested case-control and case-cohort analyses, we also compared subtype C and subtypes A and D separately and found no statistically significant difference in HIV-1 transmission risk.

A limited number of studies have found individuals with subtype C to have higher HIV-1 DNA or RNA concentrations in plasma and genital secretions, which could indicate higher transmission risk [25, 45, 144]; however, not all studies have found increased HIV-1 concentrations associated with subtype C infection [145]. In the present study, we assessed whether subtype C was associated with higher plasma and genital HIV-1 RNA concentrations, as a proxy for infectiousness and potential onward transmission. We found no statistically significant differences in plasma and genital HIV-1 RNA levels in participants with subtype C compared to non-C subtypes, further supporting the results of our nested case-control and case-cohort transmission analyses.

The rapid expansion of HIV-1 subtype C throughout sub-Saharan Africa has led some to hypothesize a causal relationship between subtype C and increased HIV-1 transmissibility. However, a combination of other factors may be as likely to contribute to the swift growth of HIV-1 subtype C. A founder effect, which has been hypothesized to explain the dominance of

specific subtypes throughout Africa, could be relevant [146, 147]. Additionally, Tatem et al. recently provided evidence to suggest that regions with greater accessibility allowing for increased mobility, such as in southern Africa, are associated with clusters of similar subtypes throughout the transportation infrastructure [148]. Another potential explanation is that subtype C has shown lower viral fitness, and therefore may result in slower disease progression compared to other subtypes [46, 47, 149, 150]; individuals with a slower progressing disease not only add person-years to prevalence estimates, but also have more opportunity to transmit their infection over a longer period of time. Finally, subtype C may be more prevalent in sexual networks with behavioral and demographic characteristics leading to higher risk for HIV-1 transmission [146, 151, 152].

Our analyses have limitations. First, it is likely that most HIV-1 infected partners in our study had chronic, as opposed to acute, HIV-1 infection. Some have speculated that subtype C is associated with higher viremia during acute infection that may contribute to increased transmission [153, 154]. However, in a separate analysis of seroconverters from our studies, we found no significant association between subtype C and plasma HIV-1 RNA levels during early HIV-1 infection [145]. Second, as subtypes are geographically distributed, there may be unmeasured differences across study sites that could potentially confound the results, in spite of our assessment of a number of behavioral, demographic, and clinical factors for potential confounding. In the primary cohorts from which our case-control sample derived, there was higher incidence of HIV-1 transmissions within couples in southern Africa (3.7 per 100 person-years, 95% CI 2.6-4.8) compared to couples in eastern African (2.2 per 100 person-years, 95% CI 1.7-2.7), a difference that was statistically significant in a proportional hazards model adjusted for age, gender, circumcision status and unprotected sex (adjusted HR 1.65, 95%CI 1.14-2.38, $p=0.007$); however, our results suggest that this difference is not explained by subtype. The selection of controls from our analysis was based on gender and geographic

distribution of the primary cohort to ensure a representative population of controls from the entire cohort.

In summary, we found no statistically significant differences in risk of heterosexual HIV-1 transmission associated with HIV-1 subtype C infection, nor was subtype C significantly associated with higher HIV-1 plasma and genital concentrations. A better understanding the impact of viral diversity on HIV-1 transmission and pathogenicity is important to HIV-1 prevention efforts, including treatment and vaccine development. The role of subtype on HIV-1 disease progression and pathogenicity should continue to be evaluated, particularly to inform the development of a globally applicable cross-protective vaccine.

Acknowledgments

We gratefully acknowledge the invaluable contributions of the HIV-1 serodiscordant couples who participated in this study. We also thank Airin L. Lam for her work sequencing the non-transmitting couples.

Partners in Prevention HSV/HIV Transmission Study Team:

University of Washington Coordinating Center, Seattle, USA: Connie Celum (principal investigator), Anna Wald (protocol co-chair), Jairam Lingappa (medical director), Amalia Magaret, James P. Hughes (protocol statisticians), Lawrence Corey, Jared M. Baeten, M. Juliana McElrath (co-investigators)

Study sites and site principal investigators:

Cape Town, South Africa (University of Cape Town): David Coetzee; **Eldoret**, Kenya (Moi University, Indiana University): Kenneth Fife, Edwin Were; **Gaborone**, Botswana (Botswana Harvard Partnership): Max Essex, Joseph Makhema; **Kampala**, Uganda (Infectious Disease Institute, Makerere University): Elly Katabira, Allan Ronald; **Kisumu**, Kenya (Kenya Medical Research Institute, University of California San Francisco): Elizabeth Bukusi, Craig Cohen; **Kitwe**, Zambia (Rwanda Zambia HIV Research Group, and Emory University): Susan Allen, William Kanweka; **Moshi**, Tanzania (Kilimanjaro Christian Medical College, Harvard University): Saidi Kapiga, Rachel Manongi; **Nairobi**, Kenya (University of Nairobi, University of Washington): Carey Farquhar, Grace John-Stewart, James Kiarie; **Ndola**, Zambia (Rwanda Zambia HIV Research Group, and Emory University): Susan Allen, Mubiana Inambao; **Orange Farm**, South Africa (Reproductive Health Research Unit, University of the Witwatersrand): Sinead Delany-Moretlwe, Helen Rees; **Soweto**, South Africa (Perinatal HIV Research Unit, University of the Witwatersrand): Guy de Bruyn, Glenda Gray, James McIntyre; **Thika**, Kenya (University of Nairobi, University of Washington): Nelly Rwamba Mugo

Data management was provided by DF/Net Research, Inc. (Seattle, USA) and site laboratory oversight was provided by Contract Lab Services (University of the Witwatersrand, Johannesburg, South Africa).

Table 6. Characteristics of the HIV-1 subtype nested case-control cohort

	Transmitting couples (cases), N=121		Non-transmitting couples (controls), N=501	
	N/median	%/IQR	N/median	%/IQR
<i>Demographic characteristics</i>				
HIV-1 infected female	60	49.6%	328	65.5%
Age in years, HIV-1 infected partner	30	26-35	32	26-38
East African (vs. southern African)	80	66.1%	332	66.3%
Married/living together	113	93.4%	459	91.6%
Duration of partnership, years	3.8	1.5-7.0	5	2.2-9.7
Number of children within partnership	1	0-2	1	0-2
Unprotected sex				
One month prior to enrollment	56	52.8%	160	36.2%
Across follow-up visits **	220/1057	20.8%	643/9280	6.9%
Antiretroviral therapy initiated during follow-up	1	0.8%	67	13.4%
<i>Baseline clinical characteristics</i>				
CD4 count, cells/mm ³	417	302-580	434	341-601
HIV-1 plasma viral load, log ₁₀ copies/mL	4.8	4.3-5.1	4.2	3.6-4.8
Any genital tract infection (either partner)*	23	20.4%	74	15.9%
Circumcision (male HIV-1 uninfected partners)	39	65.0%	239	72.9%

*Includes *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*

**Numerator=all follow-up visits with unprotected sex reported, denominator=total follow-up visits

IQR=interquartile range

Bold indicates statistical significant at the p<0.05 level, comparing transmitting to non-transmitting couples

Table 7. Adjusted multivariate models for the primary nested case-control and sensitivity case-cohort analyses comparing HIV-1 transmission for subtype C versus non-C subtypes

	Nested case-control				Case-Cohort			
	Odds ratio (95%CI) adjusted for gender, age and unprotected sex		Odds ratio (95%CI) adjusted gender age, unprotected sex <i>plus</i> plasma HIV-1 RNA		Hazard ratio (95%CI) adjusted for gender, age and unprotected sex		Hazard ratio (95%CI) adjusted gender age, unprotected sex <i>plus</i> plasma HIV-1 RNA	
	<i>env</i>	<i>gag</i>	<i>env</i>	<i>gag</i>	<i>env</i>	<i>gag</i>	<i>env</i>	<i>gag</i>
C vs. non-C	1.14 (0.74-1.75) p=0.6	0.98 (0.63-1.52) p=0.9	1.18 (0.76-1.83) p=0.5	1.03 (0.66-1.60) p=0.9	1.56 (0.89-2.76) p=0.1	0.92 (0.51-1.67) p=0.8	1.58 (0.86-2.90) p=0.1	0.97 (0.52-1.80) p=0.9
C vs. A	1.17 (0.74-1.84) p=0.5	1.09 (0.68-1.75) p=0.7	1.25 (0.78-1.99) p=0.3	1.13 (0.70-1.84) p=0.6	1.22 (0.65-2.29) p=0.5	0.88 (0.45-1.72) p=0.7	1.26 (0.65-2.44) p=0.5	0.91 (0.45-1.83) p=0.8
C vs. D	1.39 (0.76-2.56) p=0.3	1.79 (0.93-3.47) p=0.08	1.27 (0.68-2.38) p=0.5	1.60 (0.82-3.13) p=0.2	2.19 (0.95-5.06) p=0.07	1.29 (0.49-3.37) p=0.6	2.29 (0.95-5.51) p=0.07	1.28 (0.45-3.62) p=0.6

Adjusted for gender and age of HIV-1 infected partner

Unprotected sex and plasma HIV-1 RNA assessed at baseline in nested case-control model and longitudinally for case-cohort model

Figure legends**Figure 4. Distribution of *env* and *gag* subtype among cases and controls**

The percentage distribution of HIV-1 subtypes by cases (HIV-1 infected partner in transmitting couples, determined to be linked by viral sequencing) and controls (HIV-1 non-transmitting controls) for both the *env* and *gag* gene regions. Letters refer to the subtype for that gene region (RF=recombinant forms)

Figure 5. Median plasma and genital HIV-1 RNA by *env* subtype C and non-C subtypes

- a) Box plot distribution of log₁₀ plasma HIV-1 RNA for *env* subtypes C and non-C subtypes by study month. Mean values denoted by diamonds and median values denoted by bars.
- b) Median and interquartile range (IQR) for endocervical and semen HIV-1 RNA concentrations for those with *env* subtype C and non-C subtypes. Individual HIV-1 RNA concentrations plotted with median HIV-1 RNA concentration denoted and IQR denoted by black bars.

Figure 4. Distribution of *env* and *gag* subtype among cases and controls

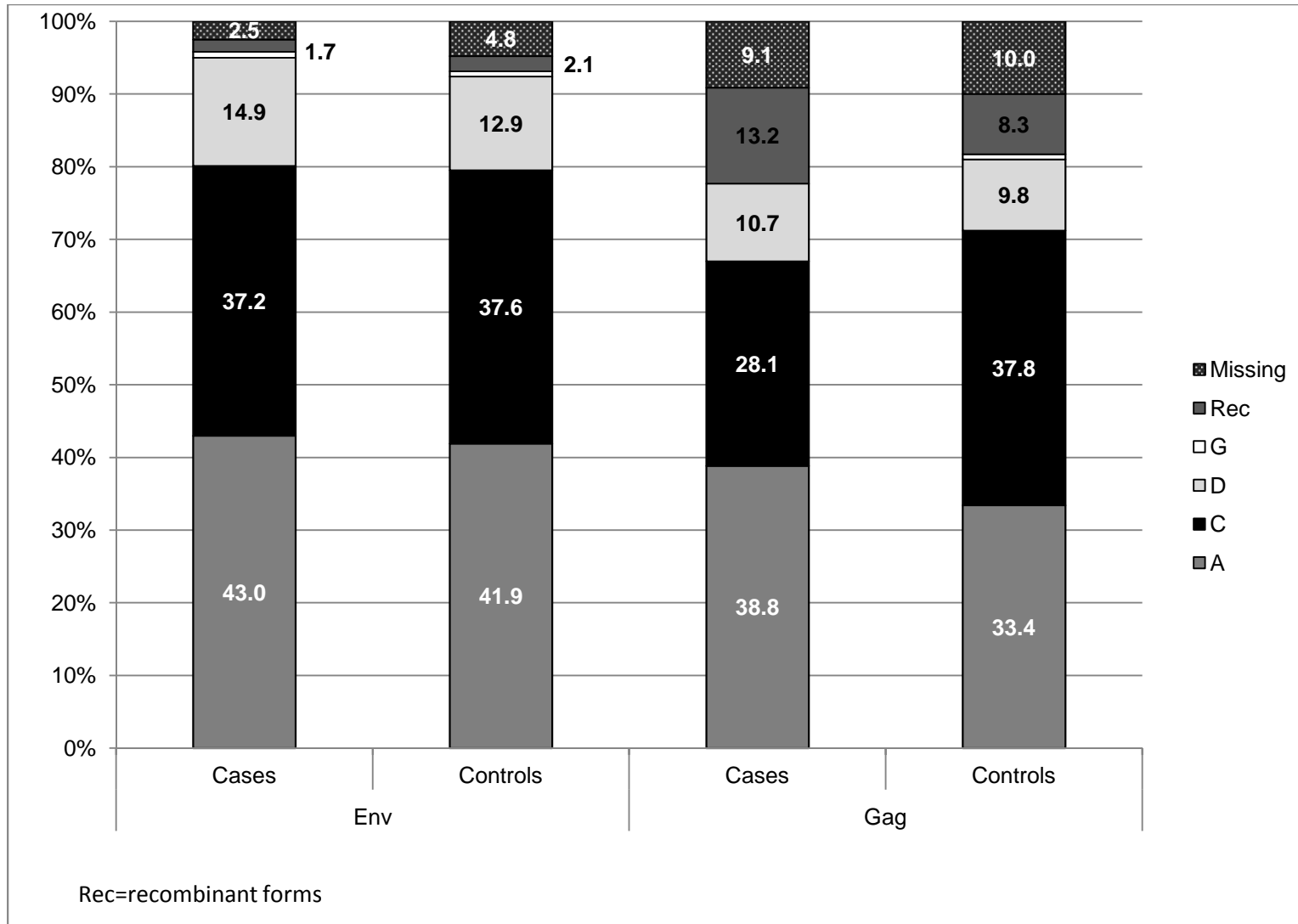
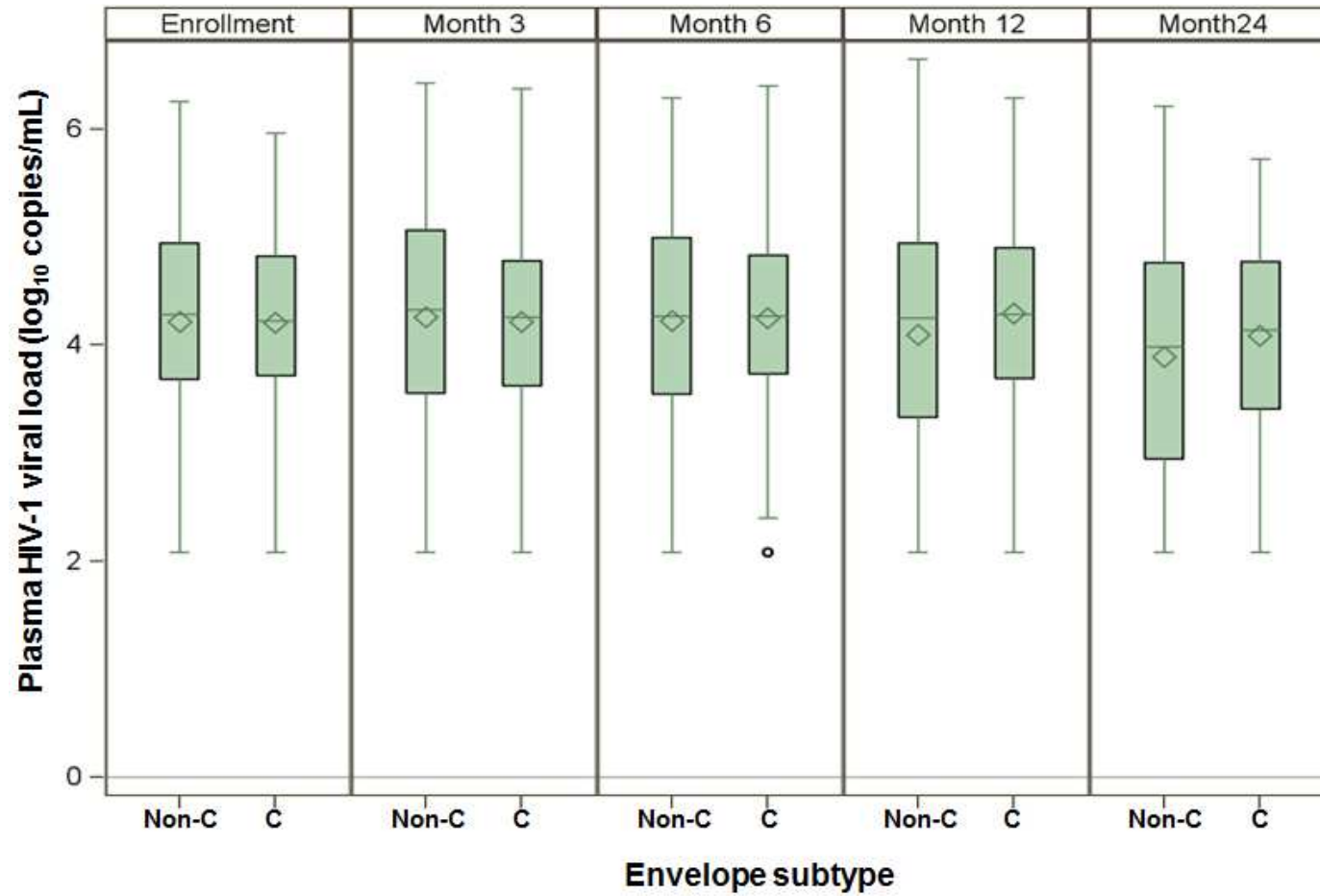
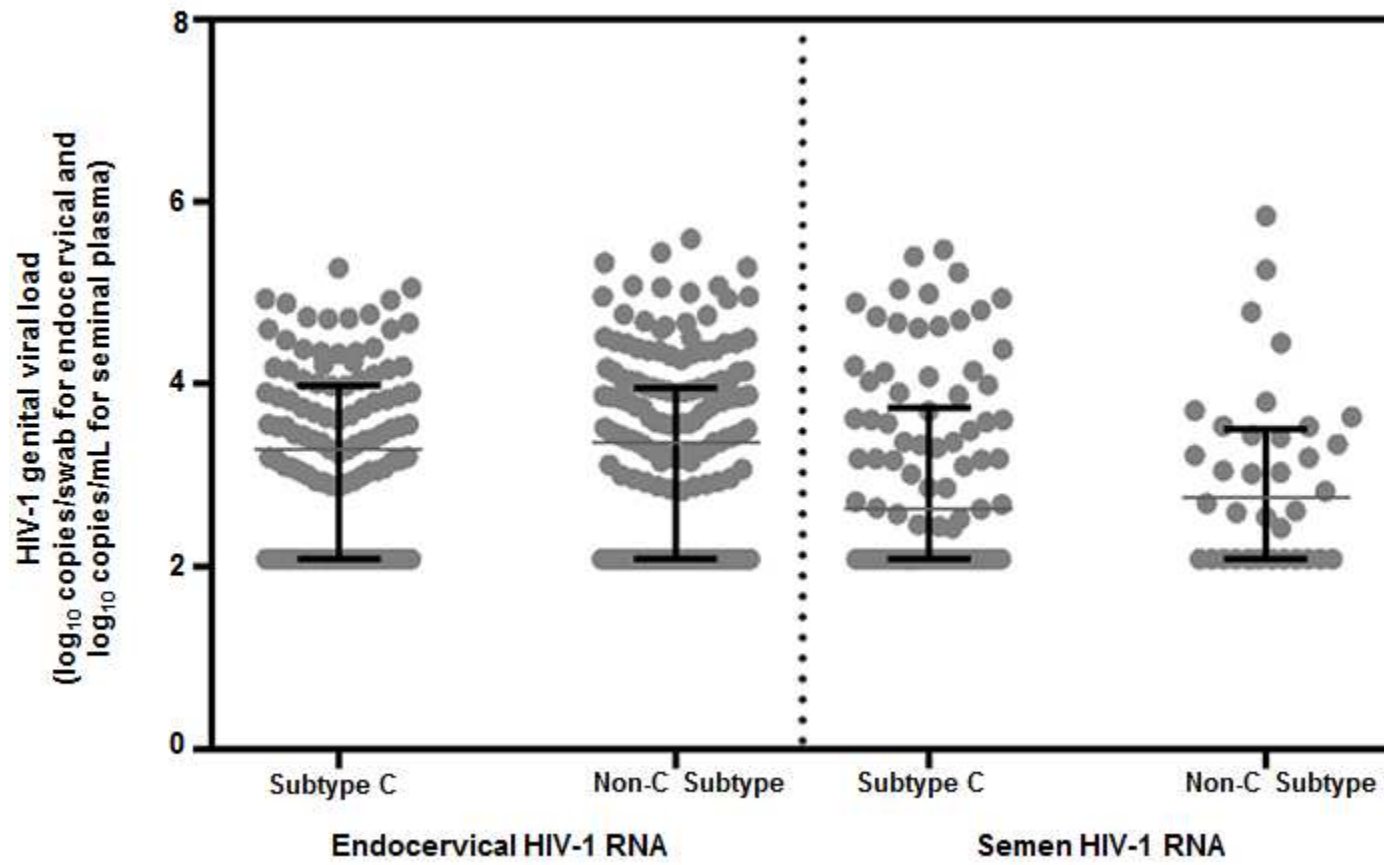


Figure 5. Mean plasma (2a) and median genital (2b) HIV-1 RNA by *env* subtype C and non-C subtypes

a.



b.



Chapter 5: Immune activation and risk of HIV-1 transmission: a nested case-control study
among HIV-1 serodiscordant couples

**Immune activation and risk of HIV-1 transmission: a nested case-control study among
HIV-1 serodiscordant couples**

TBD

for the Partners in Prevention HSV/HIV Transmission Study Team

Manuscript length: (not including tables/acknowledgements): 2,536 words

Abstract length: 230 words

Footnote page

Conflict of Interest: None of the authors have commercial or other conflicts of interest related to the content of this manuscript.

Funding: US National Institutes of Health (grant R01 AI096968 and JULIE TO FILL IN) and the Bill & Melinda Gates Foundation (grants 26469 and 41185)

Corresponding author:

Erin Kahle

University of Washington

325 Ninth Avenue, Box 359927

Seattle, Washington 98104

Tel – 206-520-3816, Fax – 206-520-3831, email – ekahle@u.washington.edu

Abstract

Background: Immune activation is a hallmark of chronic HIV-1 infection. A heightened pro-inflammatory state has been hypothesized to enhance HIV-1 transmission – both susceptibility of HIV-1-exposed persons and infectiousness of HIV-1-infected persons.

Methods: Using data collected prospectively from heterosexual HIV-1 serodiscordant couples from 6 countries in eastern and southern Africa, we conducted a nested case-control analysis to assess the relationship between immune activation and risk of HIV-1 acquisition and transmission. Cases (N=120) included incident HIV-1 transmissions; controls (N=321) were couples in which HIV-1 transmission did not occur. Immune activation was measured in both HIV-1 susceptible and infected partners by a panel of 30 cytokines.

Results: For both HIV-1 infected and susceptible partners, cases and controls had significantly different mean responses in cytokine panels (Hotelling T^2 $p < 0.001$), suggesting a broadly different pattern of immune activation for couples with HIV-1 transmission events compared to those who did not transmit HIV-1. When considering elevations in specific cytokines, \log_{10} mean concentrations were found to be significantly higher for HIV-1 susceptible cases when compared to controls for IL-10 ($p=0.001$) and IP-10 ($p=0.002$). Similarly, for HIV-1 infected partner cases and controls, \log_{10} mean concentrations were significantly higher for IL-10 ($p < 0.001$) and IP-10 ($p < 0.002$). In multivariate analysis, HIV-1 transmission was significantly associated with elevated IP-10 concentrations in HIV-1 susceptible partners ($p=0.001$) and elevated IL-10 concentrations in HIV-1 infected partners ($p=0.02$).

Conclusion: Immune activation – particularly elevated levels of IL-10 and IP-10 – are associated with both increased HIV-1 susceptibility and infectiousness.

Keywords: HIV-1 acquisition, immune activation, Africa

Introduction

Immune activation, characterized by polyclonal B cell activation, accelerated T cell turnover, dendritic cell depletion, and pro-inflammatory cytokine elevation, is a hallmark of HIV-1 infection [155]. Immune stimulation by HIV-1 contributes to depletion of CD4+ cells rather than purging the virus and has been associated with accelerated HIV-1 disease progression [55-58]. While the relationship between HIV-1 pathogenesis and persistent immune activation has been well described [55, 155], the role of immune activation in HIV-1 transmission is less understood. Increased immune activation in persons infected with HIV-1 could result in increased viral replication that could facilitate HIV-1 infectiousness and onward transmission [25, 59], but no data have directly explored this hypothesis. Furthermore, for HIV-1 uninfected persons, immune activation pre-infection has also been hypothesized to potentially heighten susceptibility and facilitate HIV-1 acquisition; however, few studies have directly compared immune activation between those who acquire HIV-1 and those that are exposed but remain HIV-1 uninfected.

Insight into the role of innate and adaptive immune function and HIV-1 susceptibility and infectiousness is an important factor in the development of effective prophylactic and therapeutic HIV-1 vaccines. Findings from the Step HIV-1 vaccine trial showed that in a prime-boost HIV-1 vaccine strategy based on an adenovirus type 5 (Ad5) vector, there was a trend towards increased HIV-1 acquisition among Ad5 seropositive individuals indicating that the amnestic immune response elicited was harmful [156], suggesting a challenge for vaccine development and a critical need to better understand how immune activation affects HIV-1 transmission. In African populations, where both HIV-1 and other infectious diseases are frequently prevalent, chronic immune activation may be heightened by the presence of other infections, both systemic and mucosal, that could contribute to increased susceptibility and infectiousness [157]

The aim of the present study was to assess whether differences in immune activation, as measured by a panel of cytokines, were associated with increased risk of HIV-1 transmission among heterosexual HIV-1 serodiscordant couples. Assessing these factors among virologically linked HIV-1 transmission in a prospective HIV-1 serodiscordant couples cohort permitted simultaneous evaluation of the relationship between systemic immune activation and heightened susceptibility of HIV-1 uninfected partners and infectiousness of HIV-1 infected partners.

Methods

Study population

We conducted a nested case-control study using data from two prospective studies of African HIV-1 serodiscordant couples. Between November 2004 and April 2007, heterosexual HIV-1 serodiscordant couples from 6 African countries (Botswana, Kenya, South Africa, Tanzania, Uganda, and Zambia) were enrolled into the Partners in Prevention HSV/HIV Transmission Study, a randomized, double-blind, placebo-controlled clinical trial of herpes simplex virus type 2 (HSV-2) suppressive therapy to reduce HIV-1 transmission, as previously described [101]. HSV-2 suppressive therapy was found not to reduce HIV-1 transmission within the partnerships [102]. In a parallel study at two sites (Kampala, Uganda and Soweto, South Africa), HIV-1 serodiscordant couples were enrolled into an observational study of immune correlates of HIV-1 protection (Couples Observational Study) [140]. In both studies, participants were ≥ 18 years of age and sexually active and HIV-1 seropositive partners were not using antiretroviral therapy at the time of study entry. HIV-1 uninfected participants were followed quarterly, with HIV-1 serologic testing.

Protection of human subjects.

All participants received HIV-1 and risk-reduction counseling (both individually and as a couple), free condoms and treatment for sexually transmitted infections (STIs), according to WHO guidelines. Written informed consent was obtained from all participants. The study protocols were approved by the University of Washington Human Subjects Review Committee and ethical review committees at each of the study sites.

Selection of cases and controls

Cases were defined as couples in which HIV-1 transmission occurred within the study partnership, as confirmed by viral sequencing [104]. Cases included 120 couples: 105 from the Partners in Prevention HSV/HIV Transmission Study and 15 from the Couples Observational Study. Controls were selected randomly, in proportion to research site and gender distribution of each study to be representative of the entire cohort of non-seroconverting couples. In total, 321 control couples were sampled. For both case and control couples, both the HIV-1 infected and initially HIV-1 uninfected members of the couple were included.

Laboratory Testing

HIV-1 seroconversion of initially HIV-1 uninfected partners was confirmed by dual rapid HIV-1 antibody tests, enzyme immunoassay, Western blot, and plasma HIV-1 RNA detection. Plasma HIV-1 RNA levels for HIV-1 infected partners were quantified using the COBAS Ampliprep/COBAS TaqMan real-time HIV-1 RNA assay version 1.0 (Roche Diagnostics, Indianapolis, IN).

Serum was collected at quarterly study visits and archived at -80°C for subsequent laboratory testing. For the present analysis, archived serum samples from cases were selected from the visits just prior to HIV transmission (i.e. initially HIV-1 uninfected partner serologically negative with undetectable plasma HIV-1 RNA), in order to evaluate immune activation *prior* to HIV-1

transmission. For controls, a single serum sample per control was selected so the proportion of study months across controls was similar to visit months for the cases. Samples were tested from both HIV-1 infected and uninfected partners. Immune activation was assessed from a panel of 30 cytokine analytes (EGF, eotaxin, fractalkine, G-CSF, GM-CSF, IFN- γ , IL-10, IL12(p40), IL-12(p70), IL-13, IL-15, IL-17, IL-1 α , IL-1 β , IL1R α , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IP-10, MCP-1, MIP-1 α , MIP-1 β , RANTES, scd40l, TGF- α , TNF- α and VEGF) that were measured using Luminex multiplex technology (MILLIPLEX™ Human Cytokine/Chemokine panel, Millipore, Billerica, MA), and standard curves were analyzed using the nCal package (<http://research.fhcr.org/youyifong/en/resources/ncal.html>) in the R statistical programming system [158]. Cytokine assays were performed blinded to case and control status.

Statistical analysis

Cytokine concentrations were \log_{10} transformed, and results below the limit of quantification were assigned a value of half the limit of detection. The relationship between immune activation and HIV-1 susceptibility (using data from the initially HIV-1 uninfected partners) and HIV-1 infectiousness (using data from the HIV-1 infected partners) were initially analyzed separately. Hotelling T^2 tests were performed to test for global equality of mean cytokine concentrations between cases and controls. To assess differences in specific cytokine concentrations, two-sided Student's t-tests were used to compare mean concentrations of each of the 30 cytokine analytes between cases and controls. P-values were adjusted for multivariate comparisons using a permutation method, as standard methods for controlling for multiple comparisons are overly conservative when using highly correlated data (i.e. inter-related cytokine concentrations) [159]. Cytokine analytes individually found to be significantly associated with seroconversion after controlling for multiple comparisons were then assessed as covariates in a multiple logistic regression (with case-control status as the outcome), adjusted for important

demographic, clinical, and behavioral predictors of HIV-1 risk in this population: gender of the HIV-1 uninfected partner, plasma HIV-1 RNA concentrations in the HIV-1 infected partner at the study visit selected for cytokine assessment, report of unprotected sex in the partnership at the study visit selected for cytokine assessment, and syndromic diagnosis of a sexually transmitted infection (STI) at the study visit selected for cytokine assessment, including urethritis, cervicitis, vaginitis, genital ulcer disease, pelvic inflammatory disease, genital herpes, or lymphogranuloma venereum) in either partner. Finally, we performed additional multivariate logistic regressions including the cytokine concentrations in both partners, to assess the potential contribution of each cytokine to HIV-1 risk within the partnership. Analyses were performed using SAS v.9.3 (SAS Institute, Inc., Cary, N.C.) and Prism graphing software (GraphPad Software Inc., La Jolla, CA).

Results

Cytokine results were available for 481 HIV-1 couples (120 cases, 321 controls). One HIV-1 uninfected control was excluded due to sample failure. Most couples (93.2%) were married or living with their HIV-1 infected partner and two-thirds were from eastern Africa (Table 1). A minority – 33 (10.3%) HIV-1 susceptible partners and 49 (15.3%) HIV-1 infected partners – had an STI diagnosis at the visit selected for cytokine testing. Compared to control couples, case couples were more likely to report unprotected sex (41.7% versus 19.0%, $p < 0.001$). For susceptible partners, those who acquired HIV-1 were more likely to have a syndromic STI diagnosis at study visit (15.0% versus 4.7%, $p = 0.002$) than controls who remained HIV-1 uninfected. Compared to HIV-1 infected controls, HIV-1 infected cases had a higher median plasma HIV-1 RNA concentrations (4.9 versus 4.0 \log_{10} copies/mL, $p < 0.001$).

Out of 30 cytokine analytes processed, 29 were assessed for differences between cases and controls (Table 2). IL-15 was not analyzed due missing results for 70 of 482 samples. When

considering the entire cytokine panel, for both HIV-1 susceptible and HIV-1 infected subjects, cases were statistically significantly different compared to controls (Hotelling T^2 $p < 0.001$ for both HIV-1 infected and susceptible partners). When specific cytokines were assessed for HIV-1 susceptible partners, cases had higher mean concentrations of G-CSF, IFN γ , IL-10, IL-12(p40), IL-12(p70), IP-10 and TNF- α compared to controls. After controlling for multiple comparisons, IL-10 and IP-10 remained significantly higher in susceptible cases compared to controls (Figure 1a). Among HIV-1 infected partners, cases had higher mean concentrations of G-CSF, IL-10, IL-12(p40), IP-10 and TNF- α compared to controls. After controlling for multiple comparisons, IL-10 and IP-10 were significantly elevated in susceptible cases compared to susceptible controls (Figure 1b).

In multivariate logistic regression models among HIV-1 susceptible cases and controls, considering IL-10 and IP-10 separately and controlling for other predictors of HIV-1 transmission, risk of HIV-1 acquisition in HIV-1 susceptible cases remained significantly higher among those with elevated IL-10 (adjusted odds ratio [adjOR] 2.15 per 1 \log_{10} increase, $p < 0.001$) and elevated IP-10 (adjOR 6.62 per 1 \log_{10} increase, $p < 0.001$, Table 3) concentrations, compared with susceptible controls.. IL-10 and IP-10 concentrations were moderately correlated (Spearman's $\rho = 0.41$, $p < 0.001$, Figure 2a). However, in a multivariate logistic regression model that included both IL-10 and IP-10, both cytokines remained significantly associated with HIV-1 acquisition in HIV-1 susceptible partners (adj OR 1.61 per 1 \log_{10} increase, $p = 0.04$ and adj OR 4.51 per 1 \log_{10} increase, $p = 0.002$).

In HIV-1 infected partners, IL-10 and IP-10 concentrations were mildly correlated (Spearman's $\rho = 0.31$, $p < 0.001$, Figure 2b). In multivariate logistic regression models for HIV-1 infected partners, adjusted for covariates and considering each cytokine separately, HIV-1 transmission risk was significantly associated with higher IL-10 concentrations (adjOR 2.04 per 1 \log_{10} increase, $p = 0.007$) but not concentrations of IP-10 (adjOR 1.85 per 1 \log_{10} increase, $p = 0.2$).

Results were similar in an adjusted model containing both IL-10 and IP-10. However, in an adjusted multivariate model, that included both IL-10 and IP-10 and other covariates except for plasma HIV-1 RNA concentrations, HIV-1 transmission was found to be significantly associated with both IL-10 (adjOR 2.49 per 1 log₁₀ increase, 95% confidence interval [CI] 1.49-4.15, p<0.001) and IP-10 (adjOR 3.09 per 1 log₁₀ increase, 95% CI 1.41-6.79, p=0.005), suggesting higher plasma HIV-1 RNA concentrations explained some of the effect of these cytokines, particularly IP-10.

Within the partnerships, concentrations of IL-10 were weakly, but statistically significantly, correlated between the two members (Spearman's rho=0.17, p<0.001), but concentrations of IP-10 were not significantly correlated (Spearman's rho=0.01, p=0.7). In a final multivariate logistic regression model including concentrations of IL-10 and IP-10 for both partners and other covariates, IP-10 concentrations in the HIV-1 susceptible partner were significantly associated with HIV-1 risk (adjOR 4.76 per log₁₀ increase, 95% CI 1.85-12.23, p=0.001) as were IL-10 concentrations in HIV-1 infected partners (adjOR 1.87 per log₁₀ increase, 95% CI 1.08-3.23, p=0.02).

Discussion

In this analysis of the relationship between immune activation and risk of HIV-1 transmission among HIV-1 serodiscordant couples, systemic immune activation was measured by a panel of cytokines on pre-seroconversion samples to assess differences between both partners in couples among whom HIV-1 transmission occurred and those among whom transmission did not occur. A unique aspect of our design was that we were able to simultaneously assess immune activation in both partners among the pre-seroconversion visits transmitting couples and compare those results to both partners in couples in which the HIV-1 susceptible partner remained uninfected. We found statistically significant differences in the individual cytokines IL-

10 and IP-10 after controlling for multiple comparisons. Concentrations of systemic IL-10 and IP-10 were higher among HIV-1 infected partners who transmitted and their HIV-1 seroconverting partners than among the HIV-1 infected and uninfected partners in couples which did not transmit, suggesting potentially important parallels in drivers of immune activation risk for HIV-1 susceptibility and infectiousness. This is the first study of HIV-1 serodiscordant couples to show a similar association between cytokine concentrations and HIV-1 transmission risk in each partner.

For persons at risk for HIV-1, immune activation may contribute to HIV-1 acquisition risk by the dysregulation of cytokines involved in promoting an anti-viral response. For persons with HIV-1 infection, immune activation has been associated with increases in viral replication and viral shedding at mucosal sites [71, 73, 75] which may suggest increased infectiousness and risk for onward transmission of HIV-1. IL-10 (interleukin 10) is an immunomodulatory cytokine and is involved in the inhibition of inflammatory response and cytokine production. It has been shown in multiple studies to be associated with inhibition of T cell proliferation [160, 161] and enhanced activation of natural killer cells [162, 163]. Our findings suggest that the inhibitory activities of IL-10 may limit the immune response necessary to prevent HIV-1 transmission. IP-10 (interferon γ -induced protein 10) is a C-X-C chemokine associated with T cell migration to sites of inflammation [164]. In HIV-1 infection, IP-10 is an early marker of disease progression [165, 166] and associated with increased HIV-1 shedding from the vaginal mucosa [167]. Although IP-10 was not found to be significantly associated with HIV-1 transmission in the primary multivariate models, when excluding plasma HIV-1 RNA in the adjusted model, IP-10 was found to be associated with HIV-1 transmission, suggesting it may be associated with the increased viral load that drives HIV-1 transmission risk. The role of IP-10 in HIV-1 susceptibility is poorly understood, although there is evidence that it increases inoculum through stimulating viral replication at the time of exposure and potentially has a role in viral entry [168]. However, this

hypothesis would need to be further investigated in other cohorts. One recent study of immune activation and HIV-1 susceptibility found a non-statistically significant elevated plasma IL-10 concentrations in high risk African women that acquired HIV-1 compared to those that did not seroconvert [169]. That study also found significant associations between HIV-1 acquisition and TNF- α , IL-2, IL-7 and IL-12p70; those cytokines were not statistically significantly greater in our HIV-1 susceptibility analyses, although mean concentrations were higher in cases compared to controls.

We did not conduct longitudinal cytokine measurements to make inferences about the chronicity of immune activation in persons who acquired or transmitted HIV-1. However, we measured cytokine concentrations at the last study visit at which initially HIV-1 uninfected subjects were known to be HIV-1 uninfected, so our results directly assess immune activation prior to HIV-1 acquisition. In addition, we did not assess the cause of immune activation in our population. Immune activation can be the result of fixed or modifiable factors, including systemic and genital infections, hormonal fluctuations and genetic characteristics. Infections with ulcerative and non-ulcerative STIs are associated with immune activation in genital mucosa and increased HIV-1 susceptibility [65, 170-172]. Our study used serum cytokine measures rather than from genital secretions, but systemic infections, such as parasitic infections and tuberculosis, increase immune activation [62, 173, 174] and may be related to increase HIV-1 susceptibility and transmission. Immune activation at the site of HIV-1 acquisition may be more specific than systemic infection. Future research should focus on identifying pathogens or other factors associated with immune activation, including assessing cytokine concentrations in mucosal samples.

In conclusion, we found that significant differences in immune function were present between couples who transmitted HIV-1 compared to those without HIV-1 transmission and notable parallels in specific cytokines – IL-10 and IP-10 – were associated with both HIV-1 susceptibility

and infectiousness in HIV-1 transmitting couples. Defining the role of immune activation on transmission of HIV-1 remains a critical step in understanding HIV-1 pathogenesis. The results of our study can be applied to future research of the role of immune response on HIV-1 transmission and the development of an HIV-1 vaccine.

Acknowledgments

We gratefully acknowledge the invaluable contributions of the HIV-1 serodiscordant couples who participated in this study.

Partners in Prevention HSV/HIV Transmission Study Team:

University of Washington Coordinating Center, Seattle, USA: Connie Celum (principal investigator), Anna Wald (protocol co-chair), Jairam Lingappa (medical director), Amalia Margaret, James P. Hughes (protocol statisticians), Lawrence Corey, Jared M. Baeten, M. Juliana McElrath (co-investigators)

Study sites and site principal investigators:

Cape Town, South Africa (University of Cape Town): David Coetzee; **Eldoret**, Kenya (Moi University, Indiana University): Kenneth Fife, Edwin Were; **Gaborone**, Botswana (Botswana Harvard Partnership): Max Essex, Joseph Makhema; **Kampala**, Uganda (Infectious Disease Institute, Makerere University): Elly Katabira, Allan Ronald; **Kisumu**, Kenya (Kenya Medical Research Institute, University of California San Francisco): Elizabeth Bukusi, Craig Cohen; **Kitwe**, Zambia (Rwanda Zambia HIV Research Group, and Emory University): Susan Allen, William Kanweka; **Moshi**, Tanzania (Kilimanjaro Christian Medical College, Harvard University): Saidi Kapiga, Rachel Manongi; **Nairobi**, Kenya (University of Nairobi, University of Washington): Carey Farquhar, Grace John-Stewart, James Kiarie; **Ndola**, Zambia (Rwanda Zambia HIV Research Group, and Emory University): Susan Allen, Mubiana Inambao; **Orange Farm**, South Africa (Reproductive Health Research Unit, University of the Witwatersrand): Sinead Delany-Moretlwe, Helen Rees; **Soweto**, South Africa (Perinatal HIV Research Unit, University of the Witwatersrand): Guy de Bruyn, Glenda Gray, James McIntyre; **Thika**, Kenya (University of Nairobi, University of Washington): Nelly Rwamba Mugo

Data management was provided by DF/Net Research, Inc. (Seattle, USA) and site laboratory oversight was provided by Contract Lab Services (University of the Witwatersrand, Johannesburg, South Africa).

Table 8. Characteristics of the immune activation nested case-control cohort

	Number (%) or median (IQR)	
	Couples with HIV-1 acquisition, N=120	Couples without HIV-1 acquisition, N=321
<i>Couple Characteristics</i>		
Female gender, HIV-1 infected partner	60 (50.0%)	114 (35.5%)
East African (vs. southern African)	79 (65.8%)	219 (68.2%)
Married/living with HIV-1 infected partner	112 (93.3%)	299 (93.2%)
Number of children within partnership	1 (0-2)	1 (0-2)
Unprotected sex at visit selected for cytokine testing	50 (41.7%)	61 (19.0%)
<i>Characteristics of HIV-1 susceptible partner</i>		
Age, in years	29 (24-37)	33 (28-41)
Any syndromic diagnosis of genital tract infection*	18 (15.0%)	15 (4.7%)
<i>Characteristics of HIV-1 infected partner</i>		
Age, in years	30 (26-35)	33 (27-39)
Any syndromic diagnosis of genital tract infection*	14 (11.8%)	35 (10.9%)
HIV-1 plasma viral load, log ₁₀ copies/mL	4.9 (4.3-5.3)	4.0 (3.3-4.8)

*Includes *urethritis, cervicitis, vaginitis, genital ulcer disease, pelvic inflammatory disease, herpes simplex virus and lymphogranuloma venereum*

IQR=interquartile range

Table 9. Mean (range) log₁₀ concentration of cytokine analytes

Analyte	HIV-1 susceptible partners				HIV-1 infected partners			
	Seroconverters, N=120	Non-seroconverters, N=321	Unadjusted p-value	Adjusted p-value**	Transmitters, N=120	Non-transmitters, N=321	Unadjusted p-value	Adjusted p-value**
EGF	2.14 (0.18-3.15)	2.23 (0.18-3.13)	0.1	0.3	2.14 (0.18-3.15)	2.23 (0.18-3.13)	0.1	0.3
Eotaxin	1.93 (0.18-3.18)	1.98 (0.65-2.66)	0.3	0.9	1.93 (0.18-3.18)	1.98 (0.65-2.66)	0.2	0.99
Fractalkine	1.07 (0.18-3.41)	1.02 (0.18-3.54)	0.3	0.99	1.07 (0.18-3.41)	1.02 (0.18-3.54)	0.6	0.99
G-CSF	1.64 (0.52-2.52)	1.56 (0.18-2.84)	0.003	0.1	1.64 (0.52-2.52)	1.56 (0.18-2.84)	0.01	0.1
GM-CSF	0.82 (0.18-3.49)	0.8 (0.18-3.43)	0.3	0.99	0.82 (0.18-3.49)	0.8 (0.18-3.43)	0.8	0.99
IFN γ	1.13 (0.18- 3.4)	1.16 (0.18-3.27)	0.02	0.8	1.13 (0.18- 3.4)	1.16 (0.18-3.27)	0.7	0.99
IL-10	0.98 (0.18-2.39)	0.76 (0.18-2.52)	<0.001	0.001	0.98 (0.18-2.39)	0.76 (0.18-2.52)	<0.001	0.003
IL-12 (p40)	0.92 (0.18-3.29)	0.75 (0.18-3.13)	0.01	0.4	0.92 (0.18-3.29)	0.75 (0.18-3.13)	0.03	0.3
IL-12 (p70)	0.49 (0.18-2.92)	0.56 (0.18-3.72)	0.03	0.2	0.49 (0.18-2.92)	0.56 (0.18-3.72)	0.3	0.99
IL-13	0.32 (0.18-2.36)	0.3 (0.18-3.02)	0.09	0.8	0.32 (0.18-2.36)	0.3 (0.18-3.02)	0.7	0.99
IL-17	0.47 (0.18-2.54)	0.52 (0.18-2.61)	0.08	0.9	0.47 (0.18-2.54)	0.52 (0.18-2.61)	0.4	0.99
IL-1 α	0.29 (0.18- 2.9)	0.3 (0.18-2.45)	0.9	0.99	0.29 (0.18- 2.9)	0.3 (0.18-2.45)	0.9	0.99
IL-1 β	0.34 (0.18-2.97)	0.28 (0.18-2.68)	0.3	0.99	0.34 (0.18-2.97)	0.28 (0.18-2.68)	0.2	0.99
IL-1R α	0.58 (0.18-3.66)	0.56 (0.18-3.27)	0.4	0.99	0.58 (0.18-3.66)	0.56 (0.18-3.27)	0.8	0.99
IL-2	0.35 (0.18- 2.8)	0.34 (0.18-2.78)	0.05	0.9	0.35 (0.18- 2.8)	0.34 (0.18-2.78)	0.8	0.99
IL-4	0.31 (0.18-3.17)	0.23 (0.18-2.73)	0.1	0.8	0.31 (0.18-3.17)	0.23 (0.18-2.73)	0.1	0.8
IL-5	0.33 (0.18- 1.7)	0.27 (0.18-2.02)	0.2	0.9	0.33 (0.18- 1.7)	0.27 (0.18-2.02)	0.06	0.9
IL-6	0.5 (0.18-2.52)	0.48 (0.18-3.44)	0.3	0.99	0.5 (0.18-2.52)	0.48 (0.18-3.44)	0.6	0.99
IL-7	0.42 (0.18-2.31)	0.38 (0.18-2.31)	0.1	0.99	0.42 (0.18-2.31)	0.38 (0.18-2.31)	0.3	0.8
IL-8	1.61 (0.18- 4)	1.62 (0.41- 4)	0.8	0.99	1.61 (0.18- 4)	1.62 (0.41- 4)	0.9	0.99
IP-10	2.81 (2.25-3.71)	2.68 (1.94-3.52)	<0.001	<0.001	2.81 (2.25-3.71)	2.68 (1.94-3.52)	<0.001	0.001
MCP-1	2.43 (1.4-3.67)	2.45 (1.25-3.74)	0.8	0.99	2.43 (1.4-3.67)	2.45 (1.25-3.74)	0.6	0.99
MIP-1 α	1.72 (0.18-3.71)	1.82 (0.18- 4)	0.7	0.99	1.72 (0.18-3.71)	1.82 (0.18- 4)	0.3	0.99
MIP-1 β	1.88 (0.74-3.12)	1.85 (0.18-3.16)	0.7	0.99	1.88 (0.74-3.12)	1.85 (0.18-3.16)	0.4	0.99
RANTES	3.16 (0.83- 3.7)	3.13 (1.59- 4)	0.3	0.99	3.16 (0.83- 3.7)	3.13 (1.59- 4)	0.3	0.99
scd40l	2.55 (0.18-3.06)	2.62 (0.18-3.22)	0.3	0.9	2.55 (0.18-3.06)	2.62 (0.18-3.22)	0.1	0.99
TGF- α	0.83 (0.18-1.82)	0.79 (0.18-2.28)	0.2	0.7	0.83 (0.18-1.82)	0.79 (0.18-2.28)	0.2	0.05
TNF- α	1.12 (0.18-2.49)	1 (0.18-2.59)	0.03	0.99	1.12 (0.18-2.49)	1 (0.18-2.59)	0.002	0.4
VEGF	2.06 (0.18-3.32)	2.2 (0.18-3.47)	0.7	0.99	2.06 (0.18-3.32)	2.2 (0.18-3.47)	0.1	0.8

*P-value estimated from two-sided Student's T-test comparing mean difference in mean concentrations between seroconverters and non-seroconverters

**P-value adjusted for multiple comparisons using permutation t-test for means

Table 10. Logistic regression models associating cytokine concentrations with HIV-1 risk

	Adjusted, individual cytokine models				Adjusted, HIV-1 susceptible partner only	Adjusted, HIV-1 infected partner only	Adjusted, both partners*
	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)
<i>HIV-1 susceptible partner</i>							
IL-10, per 1 log ₁₀ increase	2.15 (1.43-3.23)				1.61 (1.03-2.54)		1.55 (0.97-2.48)
IP-10, per 1 log ₁₀ increase		6.62 (2.78-15.78)			4.51 (1.77-11.48)		4.76 (1.85-12.23)
<i>HIV-1 infected partner</i>							
IL-10, per 1 log ₁₀ increase			2.04 (1.21-3.44)			1.96 (1.16-3.32)	1.87 (1.08-3.23)
IP-10, per 1 log ₁₀ increase				1.85 (0.79-4.33)		1.59 (0.67-3.75)	1.74 (0.71-4.25)

All models adjusted for gender of HIV-1 infected partner, plasma HIV-1 RNA concentration for the HIV-1 infected partner (log₁₀ copies/mL), report of unprotected sex within the partnership (yes/no), and any STI diagnosis in either the HIV-1 susceptible or infected partner

*In this model with IL-10 and IP-10 in both HIV-1 susceptible and infected partners, the adjusted ORs for the covariates were: female gender of HIV-1 infected partner (adjOR 1.74, 95% CI 1.04-2.93, p=0.04), plasma HIV-1 RNA (adjOR 1.93 per 1 log₁₀ increase, 95% CI 1.42-2.63, p<0.001), unprotected sex (adjOR 3.18, 95% CI 1.85-5.44, p<0.001), STI in the HIV-1 susceptible partner (adjOR 2.17, 95% CI 0.88-5.33, p=0.09), and STI in the HIV-1 infected partner (adjOR 0.97, 95% CI 0.45-2.09, p=0.9)

Figure legends**Figure 6. Mean log₁₀ concentration of cases and controls**

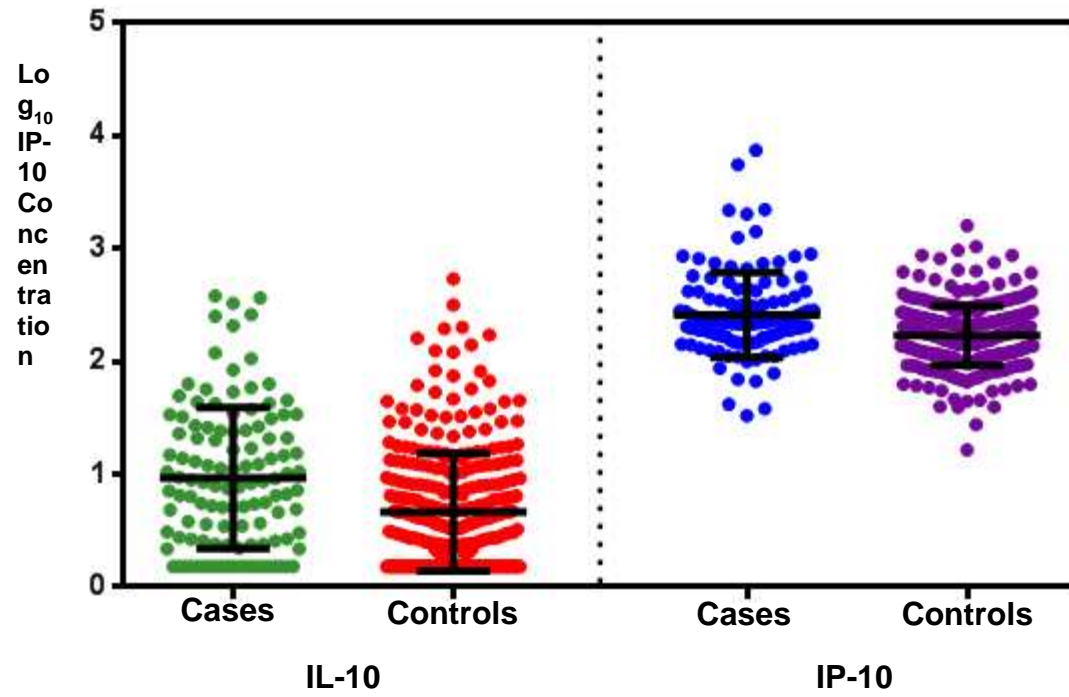
The mean distribution of log₁₀ concentration for IL-10 and IP-10 for a) HIV-1 susceptible cases and controls and b) HIV-1 infected cases and controls. Individual log₁₀ cytokine concentrations plotted with mean (middle bar) and standard deviation (top and bottom bars).

Figure 7. Correlation of IL-10 and IP-10 log₁₀ concentrations

Scatterplot and regression line with Spearman's rho and p-values for a) IL-10 and IP-10 log₁₀ concentrations in HIV-1 susceptible partners; b) IL-10 and IP-10 log₁₀ concentrations in HIV-1 infected partners; c) IL-10 log₁₀ concentrations within serodiscordant couples, and d) IP-10 log₁₀ concentrations within serodiscordant couples

Figure 6.

a. Mean IL-10 and IP-10 log₁₀ concentration for HIV-1 susceptible partners



b. Mean IL-10 and IP-10 log₁₀ concentration for HIV-1 infected partners

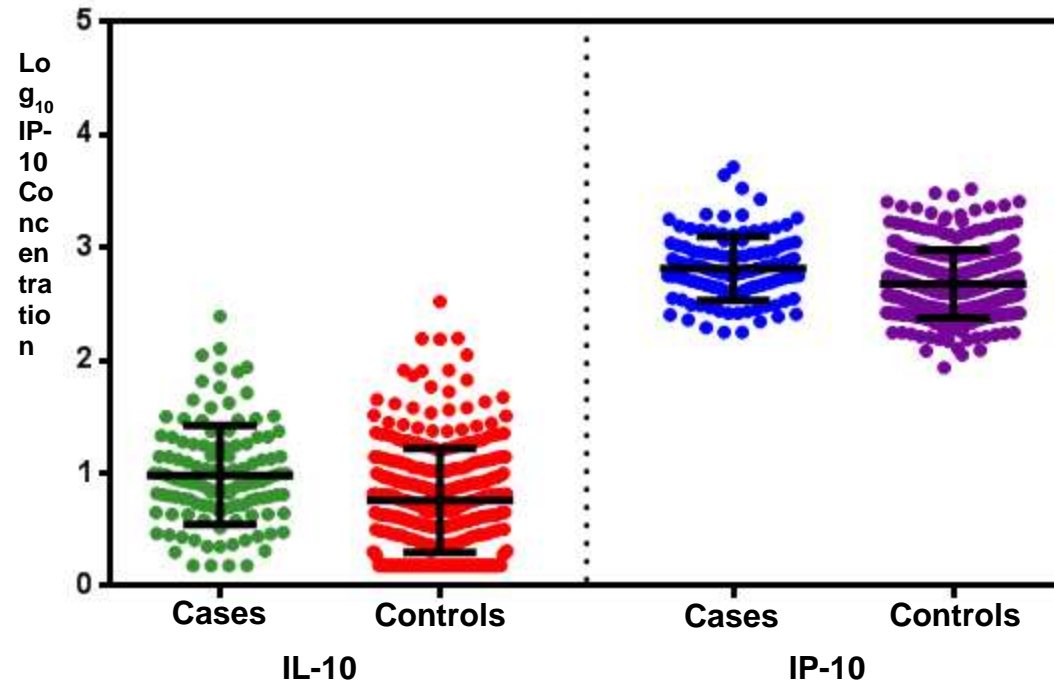
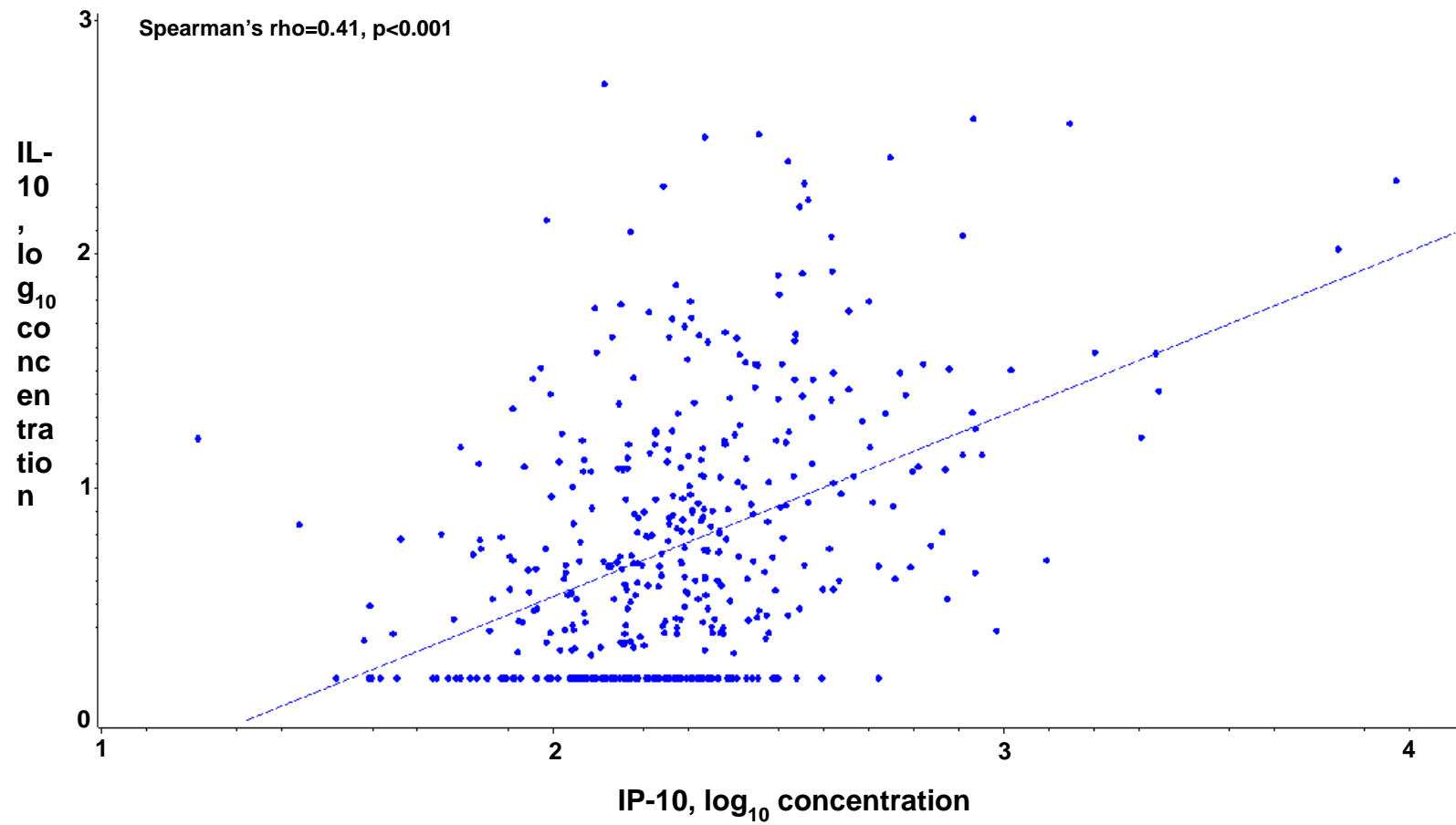
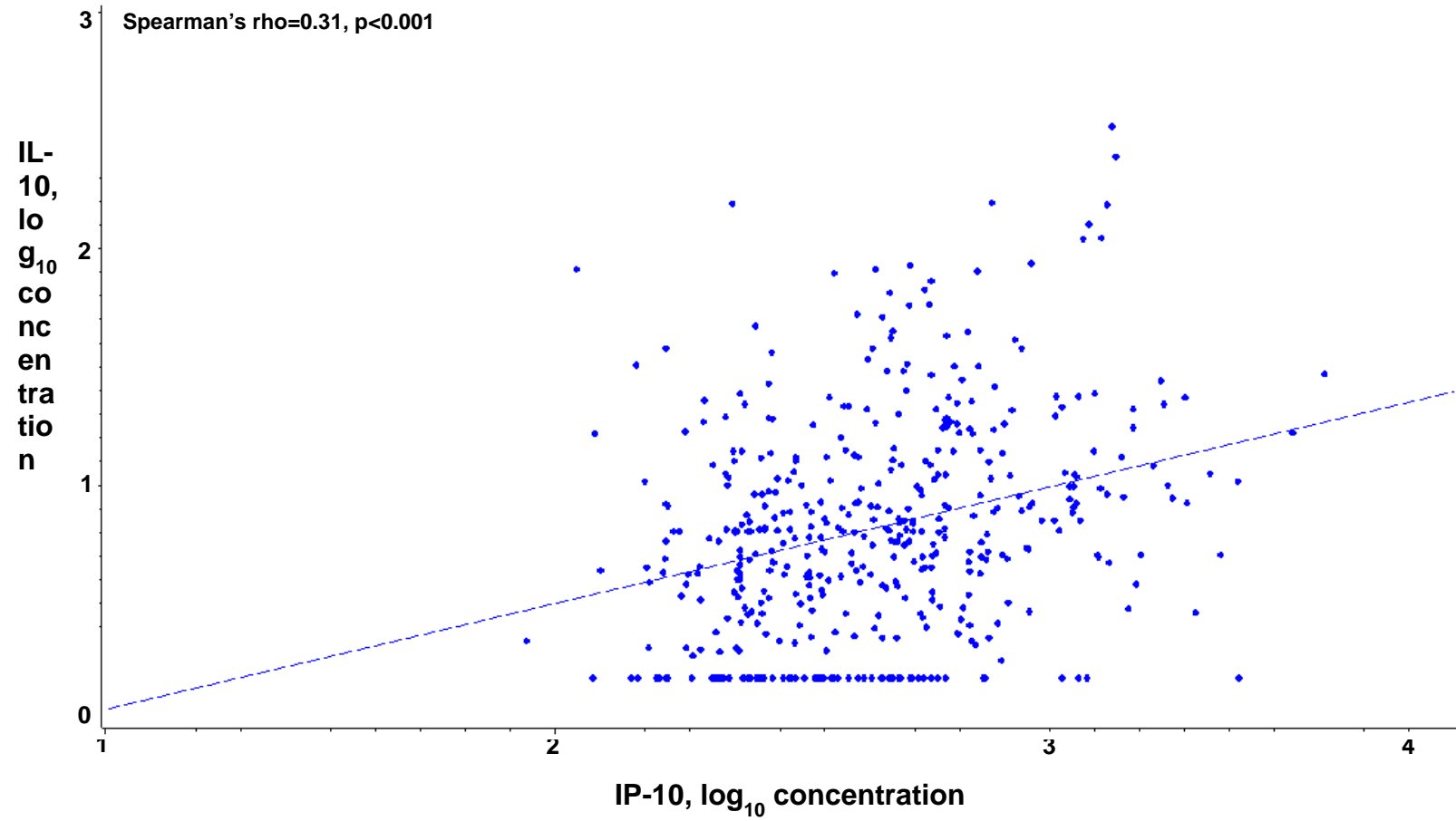


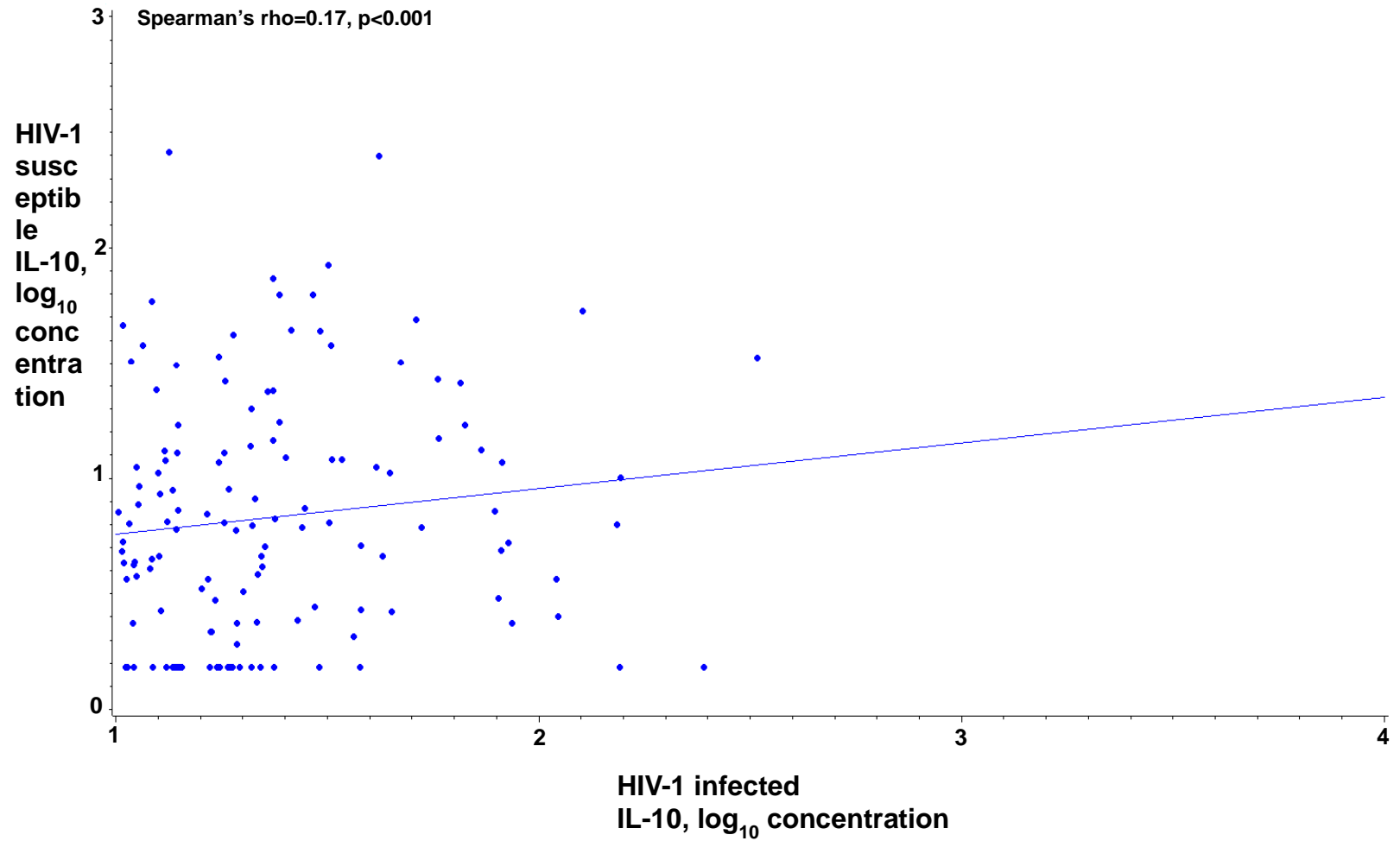
Figure 7.

a. Correlation of IL-10 and IP-10 log₁₀ concentrations within HIV-1 susceptible partners

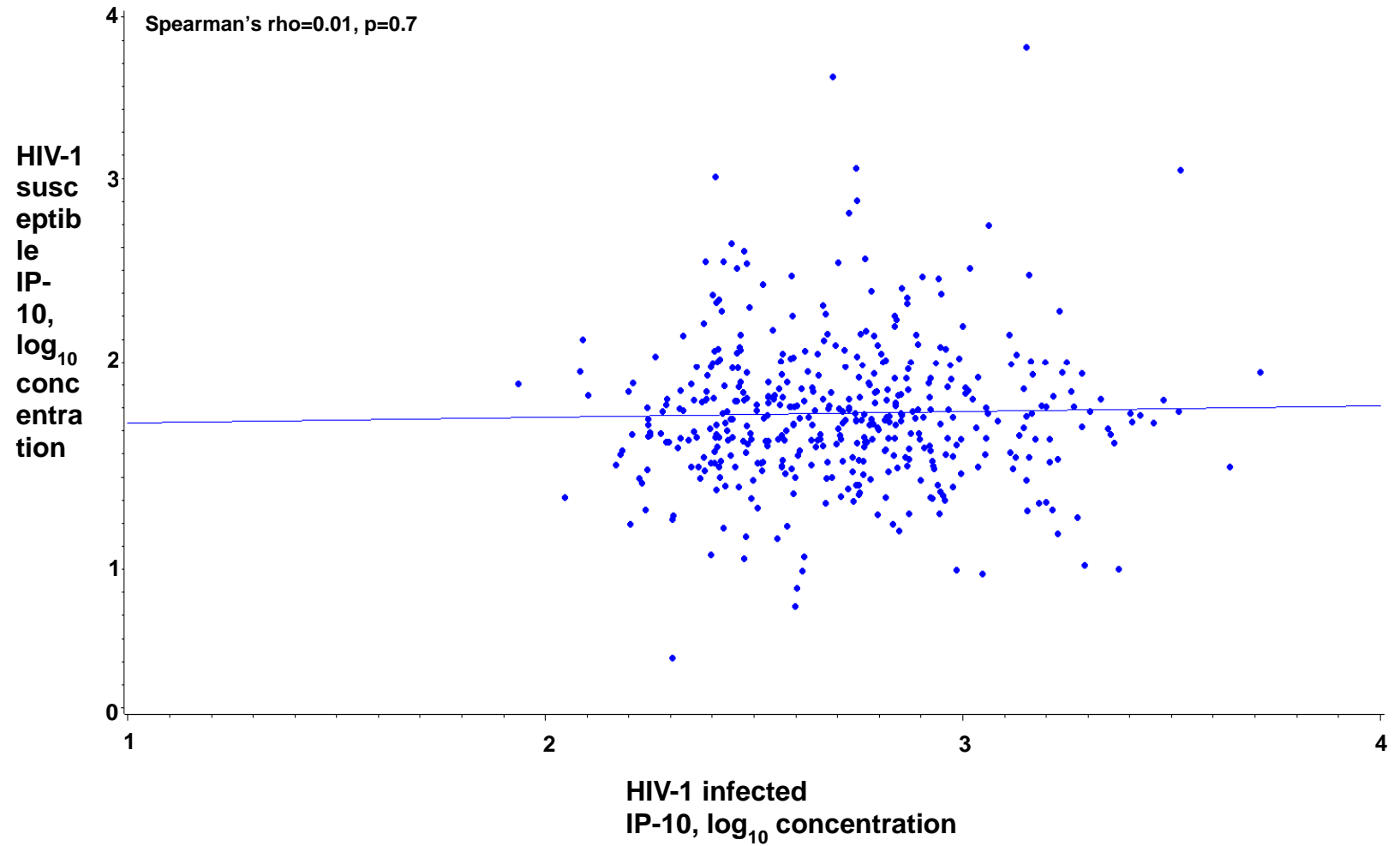


b. Correlation of IL-10 and IP-10 \log_{10} concentrations within HIV-1 infected partners



c. Correlation of IL-10 \log_{10} concentrations between HIV-1 serodiscordant partners

d. Correlation of IP-10 \log_{10} concentrations between HIV-1 serodiscordant partners



Chapter 6: Conclusion

Developing new strategies in prevention, including biomedical interventions and effective vaccines, requires a broad understanding of HIV-1 clinical characteristics and viral processes in both HIV-1 infected and susceptible persons. The populations used for the presented studies include the largest and most geographically diverse cohort of HIV-1 serodiscordant couples accumulated, comprising extensive longitudinal clinical data and biologic specimens. Genetic and epidemiologically confirmed linkage of HIV-1 infection within couples provides the most unbiased and direct observation of transmission events for analysis. The data presented in this dissertation fill important knowledge gaps related to HIV-1 infectiousness and transmission risk.

Interpretation of Findings

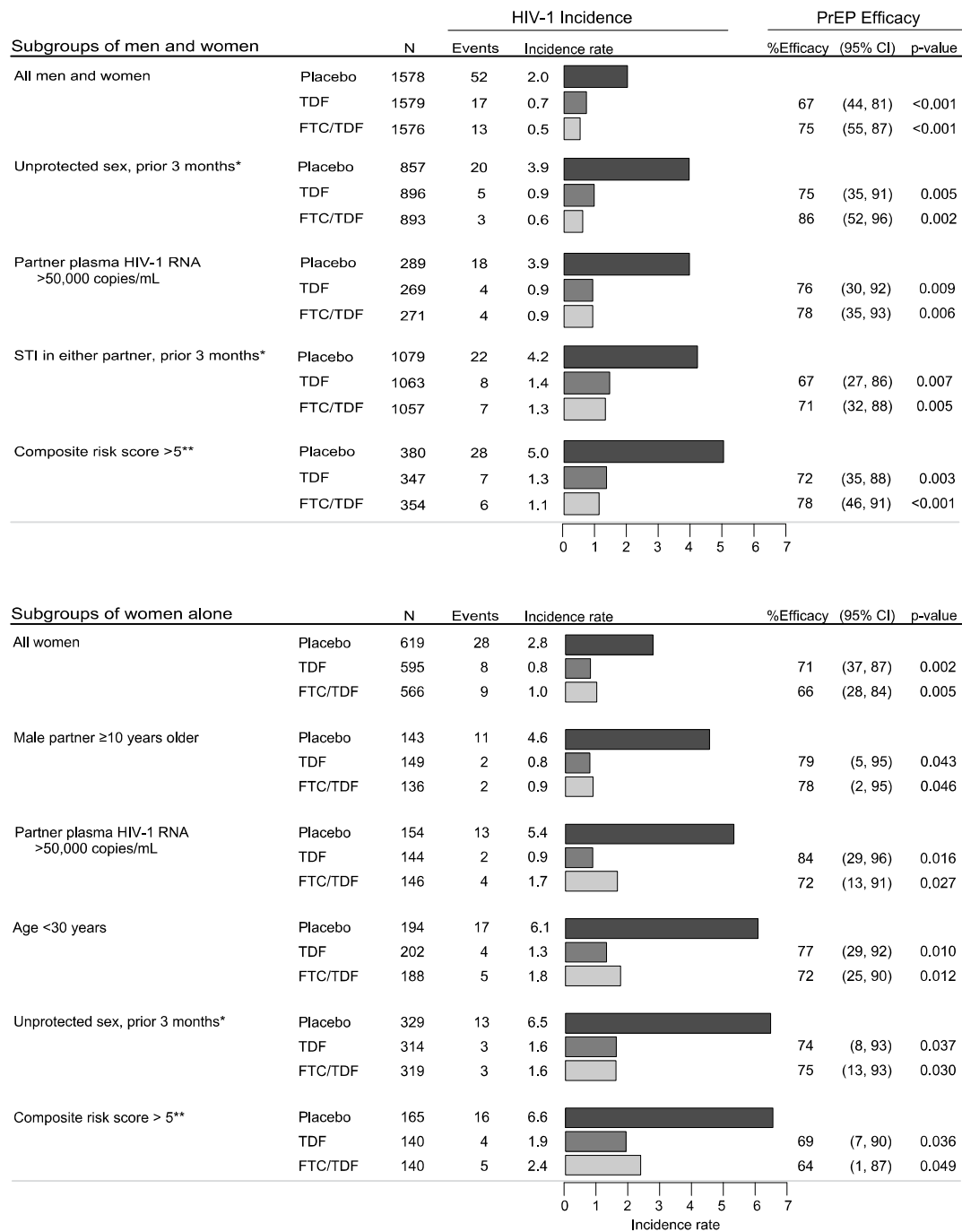
Chapter 2: HIV-1 Transmission Risk Score. To maximize resources, there is a critical need to efficiently identify subpopulations at highest risk for targeted HIV-1 prevention. In Chapter 2, we showed that a discrete combination of clinical and behavioral characteristics can define highest-risk HIV-1 serodiscordant couples. The well-established predictors included in our risk score can be easily measured, and the worksheet is feasible for implementation in a clinical or field setting. The risk score has robust predictive ability, shown in both internal and external validation. Additionally, in settings where plasma HIV-1 RNA is not available, the risk score can be used without laboratory measurement. Our findings are specific to defining HIV-1 transmission risk in heterosexual HIV-1 serodiscordant couples in sub-Saharan Africa. However, the methods used to develop the risk score can be applied to other populations (e.g. men who have sex with men) and risk measurement (e.g. treatment adherence).

Originally developed as a tool for defining high risk couples for enrollment in HIV-1 prevention studies, the risk score has implications for broad use in multiple settings, including roll-out of prevention programs. The risk score allows for flexibility in defining high-risk couples based on the risk group cutoff. In our study, we provided the example of a risk score of ≥ 6 as the cutoff

for high risk, with an incidence of 8.3 per 100 person-years. However, a risk score ≥ 5 predicts and incidence rate of more than 5 per 100 person-years and allows for a less conservative definition of high risk. In a demonstration project (The Partners Demonstration Project) of pre-exposure prophylaxis (PrEP) among HIV-1 serodiscordant couples, a risk score of ≥ 5 has been included in the enrollment criteria. The implementation of the risk score worksheet is being studied for feasibility in clinical research settings.

The risk score has also been used to assess the efficacy of PrEP in subgroups of high-risk couples. The initial findings from the Partners PrEP Study showed significant efficacy of both tenofovir (TDF) (67% efficacy, 95%CI 44-81%, $p < 0.001$) and combination tenofovir/emtricitabine (TDF/FTC) (75% efficacy, 95%CI 55-87%, $p < 0.001$) compared to placebo [175]. PrEP was also found to be effective in three other studies: TDF2 (heterosexual men and women in Botswana) , iPrEx (men who have sex with men in multiple settings) and the Bangkok Tenofovir Study (injecting drug users) [176-178]. However, two studies of PrEP in high risk women, Fem-PrEP and VOICE, were stopped completely or in part due to futility [179, 180], suggesting that PrEP might not be effective in higher risk populations. We applied the risk score to the Partner PrEP study population and assessed TDF and TDF/FTC in a high-risk subgroup of couples. We found PrEP remained significantly associated with a reduction in HIV-1 transmission, with efficacy comparable to the general population [181]. Additional analyses of subgroup populations found the composite risk score to have comparable PrEP efficacy with other measures of HIV-1 transmission risk, including among high-risk women (Figure 8) [182].

Figure 8. HIV-1 incidence and PrEP efficacy overall and among higher-risk subgroups



Chapter 3: Unreported ART Use. Of the 3,371 HIV-1 infected participants with plasma HIV-1 RNA available at enrollment in the Partners in Prevention HSV/HIV Transmission Study, nearly a quarter (23.1%) had a plasma HIV-1 RNA <2000 copies/mL. We tested serum for six antiretroviral drugs and found that 22% had evidence of ART use, including almost half (46%) of those with undetectable HIV-1 plasma RNA. It is important to note that the prevalence of ART detected at enrollment was only 5% of the entire cohort and this finding did not have a significant impact on study results.

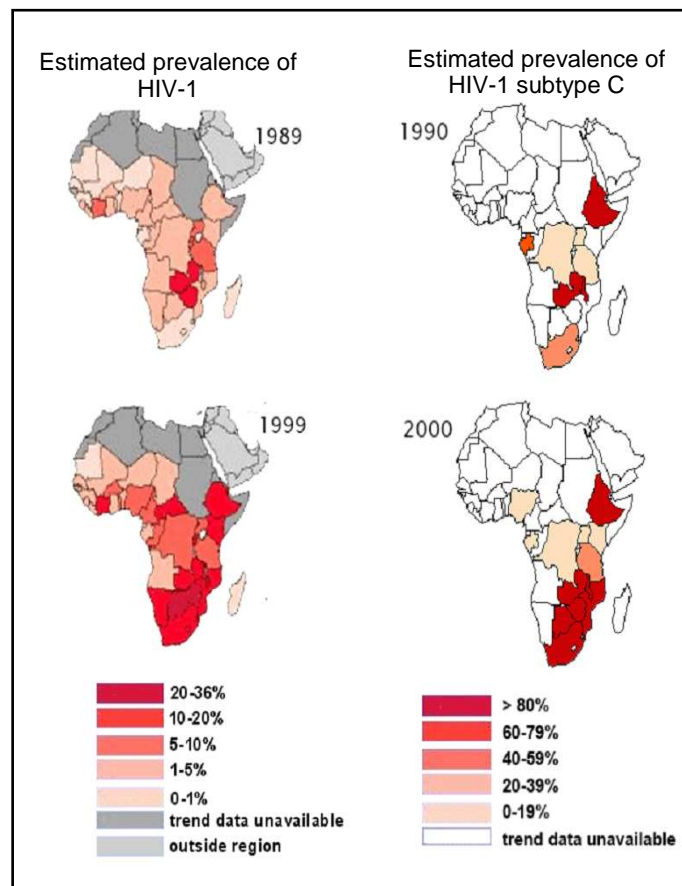
Although ART use was an exclusion criteria for study enrollment, these participants did not disclose having used ART. We do not have enough information to know whether the ART use occurred at or before the time of study enrollment, so we cannot speculate as to whether the underreporting was deliberate or if the participant had discontinued ART before screening. The screening process only included a question about current ART use and did not ask participants about any history of ART use. Although viral load testing is expensive and may not be available at study sites, our findings suggest that it may be important to measure plasma HIV-1 RNA at study enrollment if the study may be confounded or biased by ART use. Also, it may be important to establish whether participants are ART-naïve or have a history of ART that could have residual effects on viral load.

Chapter 4: HIV-1 Subtype and Transmission. We found that HIV-1 subtype is regionally distributed in sub-Saharan Africa: subtypes A and D most common in eastern Africa and subtype C most common in southern African, as previously reported in epidemiological literature on HIV-1 infection. In a nested case-control analysis, we did not find an association between subtype C and increased risk of HIV-1 transmission compared to subtypes A or D. The results remained consistent in a sensitivity analysis of longitudinal data from a case-cohort design. Additionally, we did not find subtype-related differences in plasma or genital HIV-1 RNA levels.

This study is the first to assess subtype in a large, geographically diverse population with multiple subtypes available for comparing HIV-1 transmission among genetically-linked serodiscordant couples. Our results provide a better understanding of HIV-1 virology and are important in the development of an effective vaccine. An ideal candidate vaccine would provide cross-clade immunity, but one of the challenges of developing a broad-spectrum homologous vaccine has been differential vaccine protection due to genetic differences in viral strain [183, 184]. Our findings suggest that transmissibility differences of subtypes may not pose as big a threat to the identification of a cross-reactive vaccine. However, further research needs to be done to confirm these results, including other settings such as different geographic regions with different subtype distributions, acute phase of primary infection, and non-heterosexual modes of transmission.

From an epidemiological perspective, identifying the factors related to the rapid expansion of subtype C in remains an important research question. The hypothesis that subtype C is associated with increased transmission is related to the observed correlation between HIV-1 prevalence and increase in the spread of subtype over time (Figure 11) [185]. We proposed

Figure 9. HIV-1 prevalence and subtype C distribution over time



several alternative reasons for the rapid explosion of subtype C in southern Africa, including found effect, behavioral differences in sexual networks where subtype is more prevalent, and slower disease progression allowing for more transmission opportunities over time. However, further investigation will be necessary to understand the dominance of subtype C and its impact on the HIV-1 epidemic.

Chapter 5: Immune Activation and HIV-1 Transmission. Using a panel of cytokines, we assessed the relationship between immune activation and HIV-1 acquisition and transmission. We found elevated levels of the cytokines IL-10 and IP-10 to be associated with both HIV-1 seroconversion and infectiousness. This parallel result found in both HIV-1 infected and uninfected partners is interesting, because it suggests potentially multiple mechanisms for the associated cytokines in increasing transmission risk. The findings also suggest that a common pathogen may be present in both partners that increase HIV-1 transmission risk through an inflammatory response. We did not find sexually transmitted infections to be a modifier of this association. We were limited by the lack of mucosal samples for our study and could not conduct immunological testing on genital secretions to determine if localized inflammatory response was associated with greater HIV-1 transmission. However, systemic coinfections that increase immune activation may be associated with HIV-1 replication and viral load and possible onward transmission. For example, IL-10 is associated with Helminth infections, and deworming programs have been shown to decrease HIV viral load and slow disease progression [186, 187]. Unfortunately, we do not have sufficient data on non-mucosal pathogens to investigate the specific cause of the increases in IL-10 and IP-10 concentrations. Further research into pathogens or other causes of elevated cytokines is needed to better understand the relationship between immune activation and both HIV-1 acquisition and transmission.

Viral load and HIV-1 prevention

A theme found throughout the studies discussed in this dissertation is the importance of viral load in defining HIV-1 transmission risk and identifying biologic pathways for increased HIV-1 infectiousness. In Chapter 2, we present a risk score developed using viral load as a primary predictor for HIV-1 transmission. We also provide a second risk score without viral load that, although effective in settings where viral load is not available, has more limited predictive ability and reduced sensitivity in identifying highest risk subgroups compared to the full risk score. Additionally, in our analysis of unreported ART use (Chapter 3), we conclude that measuring viral load is important in targeted research and intervention programs aimed at maximizing prevention resources.

The risk score utilized for enrolling high-risk couples in the Partners Demonstration Project includes viral load measurement, and a study outcome is the feasibility of measuring viral load in a clinical setting with limited resources. There is concern that measuring viral load in resource-limited settings is not practical and far too costly to implement on a wide scale [188]. However, studies have shown that PrEP is more cost-effective when used for targeted prevention in high risk populations [189]. Advances in PCR technology, including blood spot testing and real-time PCR, have shortened testing time, eased specimen transport and reduced the cost per viral load test [190]. New, sophisticated methods for measuring viral load rapidly and at lower cost are currently being developed in the hope that viral load testing will become a standard of care and prevention in all settings [191, 192]. Recently released guidelines on the use of ART for treatment and prevention includes recommendations for viral load monitoring for ART treatment failure, which could increase transmission risk [193], and a report from Medecins Sans Frontieres strongly encourages more effort to overcome barriers to implementing viral load testing as standard of care [194]. Viral load data are important in refining prevention programs targeted at populations at greatest risk for HIV-1 transmission.

Biologic factors can increase HIV-1 transmission risk, either directly or indirectly through increasing viral load. We assessed two distinct biologic characteristics, subtype and immune activation, to determine associations with HIV-1 transmission. In Chapter 4, our primary interest was in determining whether subtype C was directly associated with increased HIV-1 transmission. In a secondary study, we also looked at whether individuals with subtype C were more likely to have higher setpoint viral loads, thus higher infectiousness and increased transmission risk. In all analyses, we did not find subtype C to be associated with increased HIV-1 transmission or increased viral load. In our analysis of immune activation and HIV-1 transmission (Chapter 5), we looked at whether mean differences in cytokines were associated with differential HIV-1 transmission, either by directly impacting infectivity or through mediation of viral load. We found that elevated IL-10 and IP-10 concentrations were associated with greater HIV-1 transmission and that immune response may contribute to viral replication and increased transmission risk. Viral load remains important in measuring infectiousness but also provides some insight into the biological mechanisms that contribute to the heterogeneity of infectiousness.

Methodology

The studies presented here employed multiple statistical methods to assess correlates of HIV-1 transmission risk and provide unbiased, controlled results. Risk factors and biologic correlates of HIV-1 transmission in longitudinal studies can be a challenge to analyze due to missing data, repeated measures and highly correlated data. Additionally, the ability to detect association with transmission risk may be limited by study design or definitions of covariates. We selected robust statistical methods that could handle the multiple complexities of our studies (Table 12).

Table 11. Select statistical methods

Statistical method	Chapter	Purpose	Description
Signal detection ROC analysis	2	Creating categorical covariates	Selects optimal cut-off points for categorizing continuous data; useful for highly correlated or interactive predictors
Akaike Information Criterion (AIC) with stepwise selection	2	Selecting final prediction model	Best prediction model determined by standard stepwise procedure, and final model selected by lowest AIC for goodness of fit
10-fold cross validation of area under the curve (AUC)	2	Internal validation of prediction model	10 subsets of data are sampled, each with an individual AUC to compare to the AUC of the final prediction model
Multiple imputation for missing data	4	Imputes missing data	Plausible values are substituted for missing data in multiple imputed datasets that are analyzed and compiled into single parameter estimates
Case-cohort analysis with weighted estimates	4	Conduct prospective cohort analyses using nested case-control data	Uses nested case-control data to create a cohort, where the controls are weighted to the inverse of the sample fraction
Generalized Estimating Equations (GEE)	4	Analysis of correlated data	Analysis of longitudinal/repeated measures data in which the outcomes are correlated
Permutation for multiple comparisons	5	Control for multiple comparison problem	Randomly reassigns outcome to observations multiple times and calculates a distribution of estimates

Prediction model. In Chapter 2, we developed a prediction model to create a risk score for identifying couples at highest risk for HIV-1 transmission using a multi-step procedure. For efficiency and feasibility in clinical or field setting, we categorized all continuous variables (age, plasma HIV-1 RNA and number of children) using signal detection ROC analysis to create a simple scorecard for transmission risk. Standard categorizations for these variables are arbitrary and may not be optimal cutoff for creating risk groups. For example, in HIV-1 literature, age is commonly categorized as 18-24, 25-34, 35-44 and 45+. However, based on our recursive partitioning, we determined that the more appropriate cutoff for the youngest age group was 21 years. Based on this, we found that the subgroup of HIV-1 uninfected partners less than 20 years of age to be a substantial predictor of transmission risk, thus increasing the robustness of our model. Our prediction model was selected using stepwise Cox proportional hazards methods to select appropriate risk factors. To avoid over- or under-fitting of the model,

the final prediction model was determined by the lowest Akaike Information Criterion ($AIC = 2K - 2\log(L)$) as a measure of goodness of fit. Finally, we chose to use a 10-fold cross validation method for internal validation of the area under the curve (AUC). Our data did not contain a large number of seroconverters per risk group, and the 10-fold cross validation produces estimates with lower variance compared to other validation methods when the sample size is smaller [195].

Missing data. Participant study visits include the collection of self-reported information, clinical observations and laboratory test results, and data can be missing from visits for a variety of reasons. Complete case analysis, where only available data is used, is often acceptable with missing study data. However, if reasons for missing data are related to other analysis covariates, complete case analysis can lead to biased results. In the HIV-1 subtype and transmission analysis (Chapter 4), we did not have subtype data from HIV-1 infected participants where the plasma HIV-1 RNA level was too low for viral amplification. We were concerned that if subtype was associated with viral replication, we would bias the results by excluding participants without subtype (and therefore, low viral load). We performed multiple imputations to account for missing subtype data in our logistic regression. Using Markov chain Monte Carlo methods, we imputed missing subtypes with information from the following covariates: study region, gender, number of children, education, age, married/cohabiting couple, partnership duration, sexually transmitted infections, reported unprotected sex, plasma HIV-1 RNA and male circumcision.

Permutation for multiple comparisons. Most common methods for handling multiple comparison problems assume independence of the comparisons. The cytokines used as a marker for immune activation (Chapter 5) are highly correlated, and standard methods may be overly

conservative. Permutation tests do not make assumptions about correlated data and provide adjusted p-values for several types of tests, including the t-tests used in our analysis. The data were resampled 20,000 times with the outcome (seroconversion) redistributed among the cases and controls, allowing for the correlated cytokine data to remain intact within each participant. The p-values of the original estimates were adjusted using the distribution of estimates from the multiple permutations.

Conclusion

Data from HIV-1 serodiscordant couples studies offer an important opportunity to explore correlates of infectiousness and risk factors for transmission. In a collection of studies of HIV-1 transmission, we have shown that understanding HIV-1 infectiousness requires a broad spectrum approach, including clinical, virologic and immunologic data from both the HIV-1 transmitter and HIV-1 seroconverter. We have described the application of a diverse collection of analytical methods for assessing HIV-1 infectiousness and describing HIV-1 transmission in serodiscordant couples. Ongoing research of HIV-1 pathogenesis and the development of novel prevention strategies require a multidisciplinary approach to effectively convert our understanding of infectiousness and transmission into successful interventions. The recent development of highly effective prevention strategies, including ART for prevention and PrEP, has renewed the urgency to refine methods for implementing these prevention programs to maximize effect and reduce burden in resource limited settings. We have provided data with potential applications for developing more effective prevention programs and understanding biologic pathways for the development of new biomedical interventions.

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