INFLAMMATION OF INNATE IMMUNE RESPONSES IN THE FEMALE GENITAL TRACT: ASSOCIATION WITH USE OF INJECTABLE PROGESTIN-ONLY CONTRACEPTION, REPRODUCTIVE TRACT INFECTIONS AND RISK OF HIV ACQUISITION

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ABSTRACT

Jennifer Caraway Deese: Inflammation of Innate Immune Responses in the Female Genital
Tract: Association with Sse of Injectable Progestin-Only Contraception, Reproductive Tract
Infections and Risk of HIV Acquisition
(Under the direction of William Miller)

The successes and failures of many HIV prevention trials, including those of microbicides, antiretrovirals and vaccines, have led to a renewed interest in the biological mechanisms at the site of HIV infection. In women, the anatomical site of exposure and first infection is most often the vaginal and cervical mucosa.[1] Well accepted biological risk factors for HIV infection in women include mucosal disruption; immune factors, including the availability of CD4+/CCR5+ cells types; sexually transmitted infections (STI) and disturbances in the vaginal biome (e.g., bacterial vaginosis (BV)).[2] More recently, the profile of innate immune biomarkers – including cytokines, chemokines and antibacterial proteins – and associated levels of cellular activation have become a focus of interest as a potential mechanism of increased HIV risk.

The results of prior studies in this area are varied, and additional research is needed. Potential reasons for the variability in results includes use of different specimen collection methods, methodological and analytical differences, statistical consequences (the increased probability of finding significant, yet spurious, associations in the case of measurement of multiple outcomes and multiple hypothesis testing) and differential selection of endpoints. Stored specimens from FEM-PrEP trial participants offered an opportunity to overcome some of the aforementioned challenges in studying the relationship between inflammatory cytokines, chemokines and antibacterial proteins with HIV and associated risk factors.

FEM-PrEP was a Phase III, randomized, double-blind, placebo-controlled effectiveness and safety trial to assess the role of emtricitabine/tenofovir disoproxil fumarate (FTC/TDF, i.e., Truvada) in preventing HIV acquisition in women. FEM-PrEP was conducted in Bondo, Kenya; Pretoria and Bloemfontein, South Africa; and Arusha, Tanzania.[3] The trial enrolled HIV-negative women between the ages of 18-35 who met medical and behavioral eligibility criteria including being at high risk of HIV. Using stored specimens from the trial we estimated innate immune biomarker concentrations among Kenyan and South African women at high risk of HIV infection in the absence of known risk factors, and explored the association of those risk factors on biomarker concentrations, and analyzed elevated innate immune biomarker concentrations as a risk factor for HIV infection in a longitudinal analysis

To my father, mother, sister and daughter who have provided constant love and support.
To Lut Van Damme, dear friend and mentor.

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BV   bacterial vaginosis

DMPA   depot medroxyprogesterone acetate

FTC/TDF   emtricitabine/tenofovir disoproxil fumarate

GM-CSF   granulocyte-macrophage colony-stimulating factor

HIV   human immunodeficiency virus

IVF   intermediate vaginal flora

IL   interleukin

IP   Interferon gamma-induced protein

IPC   injectable progestin-only contraception

MIP   macrophage inflammatory protein

NET-EN   norethisterone oenanthate

PSA   prostate specific antigen

RANTES   regulated and normal T cell expressed and secreted

RTI   reproductive tract infection

SLPI   secretory leukocyte peptidase inhibitor

STI   sexually transmitted infection

TFV   tenofovir

TFV-DP   tenofovir diphosphate

TNF   tumor necrosis factor

CHAPTER 1: SPECIFIC AIMS

The successes and failures of many HIV prevention trials, including those of microbicides, antiretrovirals and vaccines, have led to a renewed interest in the biological mechanisms at the site of HIV infection. In women, the anatomical site of exposure and first infection is most often the vaginal and cervical mucosa.[1] Well accepted biological risk factors for HIV infection in women include mucosal disruption, which may occur via physical or chemical disturbance; immune factors, including the availability of CD4+/CCR5+ cells types; sexually transmitted infections (STI) and disturbances in the vaginal biome such as with bacterial vaginosis (BV).[2] More recently, the profile of innate immune biomarkers – including cytokines, chemokines and antibacterial proteins – and associated levels of cellular activation have become a focus of interest as a potential mechanism of increased HIV risk.

The results of many studies of the relationship between innate immune biomarkers and HIV risk are quite varied, and additional research is needed. There are a plethora of potential reasons for the variable results including, but not limited to, 1) specimen variability (differences in collection timing, preparation and storage), 2) use of different assays, 3) methodological differences including varying exposure and outcome definitions and differential control of confounders, 4) statistical consequences – the increased probability of finding significant, yet spurious, associations in the case of measurement of multiple outcomes and multiple hypothesis testing and 5) a large number of innate immune biomarkers that are costly to measure resulting in differential selection of endpoints by different study teams.

Stored specimens from FEM-PrEP trial participants offered an opportunity to overcome some of the aforementioned challenges in studying the relationship between inflammatory cytokines, chemokines and antibacterial proteins and HIV.[3] FEM-PrEP was a Phase III,

randomized, double-blind, placebo-controlled effectiveness and safety trial to assess the role of emtricitabine/tenofovir disoproxil fumarate (FTC/TDF, i.e., Truvada) in preventing HIV acquisition in women. FEM-PrEP was conducted in Bondo, Kenya; Pretoria and Bloemfontein, South Africa; and Arusha, Tanzania. The trial enrolled HIV-negative women between the ages of 18-35 who met medical and behavioral eligibility criteria including being at high risk of HIV. Using stored specimens from the trial we estimated innate immune biomarker concentrations among Kenyan and South African women at high risk of HIV infection in the absence of known risk factors for HIV, and explored the association of those risk factors on biomarker concentrations, and analyzed elevated innate immune biomarker concentrations as a risk factor for HIV infection in a longitudinal analysis.

The innate immune markers selected for this analysis were informed by prior research which demonstrated variability in concentrations of these markers to be associated with injectable progestin-only contraceptive (IPC) use and with risk of HIV infection.[4, 5] This research was designed to further elucidate whether there are significant differences in genital tract innate immune profiles among women with different risk factors for HIV infection (i.e., IPC use and reproductive tract infections (RTI)) and to determine if higher concentrations of innate immune biomarkers are associated with increased risk of HIV infection. The results of this research will provide insight into potential mechanisms of increased risk of HIV infection among women using IPC and may be useful in informing safety evaluations of existing and future HIV prevention products and other medical technologies.

AIM 1: Cross sectional analysis of the association between selected immune biomarkers, injectable progestin-only contraception and RTIs

Hypotheses:

Primary exposure:

Depot medroxyprogesterone acetate (DMPA) use for ≥ 3 months: We hypothesized that women who were using DMPA ≥ 3 months would have higher median concentrations of all measured

cytokines and lower median secretory leukocyte peptidase inhibitor (SLPI) concentration as compared to the reference population.

Secondary exposures:

1. Norethisterone oenanthate (NET-EN) use ≥3 months: We did not anticipate any differences in exposure and outcome variables among users of NET-EN as compared to the reference population.

2. Gonorrhea: We hypothesized that women with *Neisseria gonorrhoeae* (NG) infection would have higher median concentrations of all measured cytokines and lower median SLPI concentration as compared to the reference population.

3. Chlamydia: We hypothesized that women with *Chlamydia trachomatis* (CT) infection would have higher median concentrations of all measured cytokines and lower median SLPI concentration as compared to the reference population.

4. Trichomoniasis and BV: We hypothesized that women with *Trichomonas vaginalis* (TV) infection and BV would have higher median concentrations of all measured cytokines and lower median SLPI concentration as compared to the reference population.

5. Intermediate vaginal flora (Nugent score 4-6): We hypothesized that women with intermediate vaginal flora (IVF) would have higher median concentrations of all measured cytokines and lower median SLPI concentration as compared to the reference population.

6. BV (Nugent score 7-10): We hypothesized that women with BV would have higher median concentrations of all measured cytokines and lower median SLPI concentration as compared to the reference population.

Overview: Selected innate immune biomarker concentrations (cytokines: macrophage inflammatory protein (MIP)-1α, MIP-1β, interleukin (IL)-6, IL-8, IL-1α, IL-1β, interferon gamma-induced protein (IP)-10, regulated and normal T cell expressed and secreted (RANTES), granulocyte macrophage colony-stimulating factor (GM-CSF) and antibacterial protein: SLPI) were estimated in cervical swab eluents (originally obtained for baseline CT/NG testing) from

Kenyan and South African women. We analyzed the association between the selected biomarkers and IPC use (including both DMPA and NET-EN) and RTIs (including NG, CT, TV, IVF and BV). We also evaluated differences in markers by age and study site.

AIM 2: Case-cohort analysis of innate immune inflammation as a risk factor for HIV acquisition

Hypothesis: We hypothesized that overall innate immune inflammation would be positively associated with the risk of HIV infection.

Overview: A case-cohort study was done to analyze the association between selected innate immune biomarker concentrations (cytokines: MIP-1α, MIP-1β, IL-6, IL-8, IL-1α, IL-1β, IP-10, GM-CSF and RANTES and antibacterial protein: SLPI) and risk of HIV infection among a high-risk population of Kenyan and South African women. Cases are all individuals who became HIV infected between enrollment in the clinical trial through 60 weeks of follow-up. Fifteen percent of the total enrolled cohort was selected as a sub-cohort for independent analyses by FHI 360 and were used for this analysis.

CHAPTER 2: BACKGROUND AND SIGNIFICANCE

Globally, women continue to experience a disproportionate burden of HIV compared to men.[6] In sub-Saharan Africa, the epicenter of the AIDS epidemic, women account for 58% of total HIV infections, and among women, young women (aged15-24) experience the highest rates of HIV infection. While dramatic gains have been made over the last decade in reducing HIV incidence in many countries, including among women, through increased delivery of prevention programs, antiretrovirals to HIV infected individuals which reduces transmission, implementation of adult male circumcision programs and other interventions, the total number of new HIV infections remains unacceptably high.

The underlying cause of higher HIV incidence rates among women as compared to men are a result of a complex set of social, behavioral and biological determinants that vary by region, and within regions, by populations defined by those determinants. These determinants range from socioeconomic and gender inequalities to biological differences in men and women that render women more susceptible to infection. Understanding the determinants of disease – at all levels – is necessary for designing optimal public health interventions.

In recent years, a number of large clinical trials have been done to evaluate the effectiveness of numerous novel biomedical interventions – including, but not limited to, topical microbicides with various mechanisms of action, vaccines, treatment as prevention (TasP) and oral pre-exposure prophylaxis (PrEP) – for the prevention of HIV acquisition. While some of these trials were successful in identifying new approaches to reducing rates of HIV transmission, many trials were unsuccessful.[3, 7-13] Nonetheless, the "failed" trials have provided new insight into biological (and behavioral) factors that may influence population effectiveness of biologically efficacious interventions. Innate immune inflammation in the female

genital compartment is one of the potential modifiers of intervention effectiveness identified in prior trials.[5, 14]

Sexual HIV transmission in women and innate immunity

The majority of sexual HIV transmissions in women are believed to occur in the lower reproductive tract which includes the ectocervix and vagina.[1, 15] Well accepted vaginal/cervical biological risk factors for HIV infection include mucosal disruption, which may occur via physical or chemical disturbance; immune factors, including the availability of target cell types; genital co-infections (which likely increase risk through the aforementioned and additional mechanisms); and disturbances in the vaginal biome such as with BV.[2]

Successful establishment of HIV infection requires transmission across the multi-layered squamous epithelium of the vaginal mucosa, infection of innate immune cells, which likely first include Langerhans' cells (LC) and macrophages, which stimulates an influx of CD4+ T cells, the primary cell target of HIV. These target cells are recruited to the focal area of infection by secretion of cytokines – chemical messengers that function at mucosal surfaces to regulate differentiation, maturation and recruitment of lymphocytes - by infected innate immune cells[16] It is hypothesized that rapid recruitment of target cells to the site of exposure, accompanied by a slower response by more effective immune mechanisms (e.g., natural killer cell, humoral immune responses) may facilitate, rather than inhibit, HIV infection in a process that has been described as "too little too late".[1]

Expanding on this theory, several recent studies have begun to evaluate the association of various innate immune markers and potential risk factors for HIV infection – including, but not limited to, age, contraceptive use, prevalent STI and vaginal discharge.[4, 17, 18] In addition, a few studies have also looked at the levels of innate immune factors as independent risk factors for HIV acquisition.[5, 18, 19]

Injectable progestin-only contraception, HIV and innate immune markers

The impact of IPC on HIV acquisition risk remains unclear.[20, 21] DMPA has long been associated with an increased risk of HIV infection in observational epidemiological studies and most recently, in two meta-analyses, one which pooled individual participant data from >37,000 women.[22-26] However, other studies, some of which were included in the aforementioned meta-analysis, have found no such significant association.[21, 27-31] While the recent meta-analyses provide robust evidence, the inability to definitively untangle contraceptive use from associated risk behaviors (e.g., condom use) using observational methods has left open the possibility of unmeasured confounding. As such, a large randomized controlled trial on the topic is currently in planning.[32] The association of NET-EN use and HIV risk is also unclear, but has received much less attention.[23, 29, 31, 33, 34] The potential increased risk of HIV infection among DMPA users has been attributed to multiple potential causes including behavioral differences in DMPA vs. non-DMPA users, vaginal thinning, modification of RTI risk and both mucosal innate immune inflammation and suppression which are illustrated in Figure 2.1.[26, 35]

The majority of studies on the immunomodulatory effects of DMPA have been conducted in vitro using both human and mouse cell lines, and in vivo in macaques, with a predominant focus on IFN-γ production, establishment of HIV infection, post-infection viral load, viral diversity and antibody production. These studies are by necessity among small numbers of subjects with limited statistical power.[36-41] The relevance of in vitro and macaque studies to in vivo human responses is difficult to assess. Moreover, most studies that have been done in humans have evaluated immune responses in circulating plasmacytoid dendritic cells (pDC) and activated T cells in peripheral blood mononuclear cells and several studies have shown divergent innate immune responses in the systemic vs. female genital compartments.[36, 42]

Data from studies which analyze locally relevant specimens from the human female genital tract are limited. A recently published study by Guthrie which focused on cationic

polypeptides with reported antiviral activity found that DMPA users has significantly higher mean levels of human neutrophil peptides (HNP) 1-3 and human cathelicidin-related antimicrobial peptide 18 (LL-37) and lactoferrin as compared to non-users of HC.[43] The authors suggest that the seemingly contradictory finding of increased levels of proteins with antiviral activity among DMPA users could be explained by the unknown cumulative effects of these markers, and their complex interactions with other unmeasured markers. Another large study among 832 women in Zimbabwe and Uganda found that DMPA users had significantly higher RANTES and lower beta-defensin 2 (BD-2) as compared to non-HC users.[4] Research to better understand the biological associations of the innate immune system and HIV at the site of exposure and infection are needed to clarify the observed association of higher rates of HIV acquisition among DMPA users. If DMPA users are definitively found to have a higher increased biological risk of HIV infection, data from such biological studies could be used in future safety evaluations HIV prevention technologies and new and existing contraceptive methods.[35]

Reproductive tract infections, HIV and innate immune markers

Mucosal disruption is a well-accepted mechanism of increased risk of HIV infection among individuals with STI, particularly ulcerative STIs.[2] Other mechanisms include both immune changes and alterations of the genital microbiome. Recent work undertaken in South African HIV-uninfected women to characterize innate immune marker concentrations found unique cytokine signatures for different RTIs. Importantly, only 19% of women who had ≥ 1 RTI were symptomatic, in a setting in which syndromic diagnosis is the predominant approach to STI management, leaving the majority of women with RTI – and therefore at increased risk of HIV infection – undiagnosed. Further characterization of innate immune characteristics among women with RTI could be used in the development of less expensive screening tools for use in settings where pathogen-specific tests are cost-prohibitive and would provide more basic insight into the risk of HIV infection among women with RTI.

Innate immune markers and HIV acquisition – preclinical evidence

Published reviews of early biological events during HIV infection and studies in macaques provide perhaps the clearest picture on the relationship between innate immune activation of cytokines and HIV. Macaque models provide the advantage of known exposure dose and time and the ability to take strategically timed pre- and post-infection specimens. Though the model has many advantages over human studies, it is important to acknowledge that it does not perfectly represent human infection – significantly higher virus doses have often been used as compared to levels women are exposed to during vaginal sex, cell-free virus is used for inoculation in macaques whereas cell-free and cell-associated virus exposure occurs in humans, macaques are often treated with DMPA to thin the vaginal epithelium (which, as described above, may also cause other unknown physiologic and immunologic changes) and the natural vaginal biome differs between macaques and humans.[1]

The hypothesis of inflammatory cytokine and chemokine elevation as a risk factor for HIV infection is well grounded in basic biology and supportive preclinical studies.[1, 19, 44, 45] Prior to HIV exposure, a limited number of activated CD4+ T cells, the principal target cell-type of early HIV-1 infection, are readily available at the mucosal surface. They exist primarily in low density underneath the epithelium and in deeper sub-mucosal layers (Figure 2.2).[1] Macaque studies have demonstrated that simian immunodeficiency virus (SIV) exploits the innate immune system to increase the availability of target cells.[1, 19] Upon exposure to HIV, the mucosa immediately engages in signaling activities that serve to both inhibit and propagate infection. Signaling begins with epithelial cell expression of the chemokine MIP-3α resulting in the recruitment of pDC. The pDCs express MIP-1α and MIP-1β which in turn recruit CD4+ T cells, part of the innate immune system ordinarily central to an effective immune response, but in the case of HIV, also the principal target of early infection.[1, 19, 46] As a result, the initial immune response likely provides some level of local viral control (e.g., MIP-1α and MIP-1β block CCR5 receptors on CD4+ T cells thereby preventing attachment of HIV), though, coupled with an

adaptive immune response that is too late, insufficient to prevent spread to distant mucosal and lymphoid tissues.[44]

By extension of the aforementioned biology, numerous conditions which cause an increase in the inflammatory status of the genital tract could therefore theoretically increase susceptibility to HIV infection including, but not limited to, STIs, altered vaginal flora, sexual behavior and contraceptive use.

Innate immune markers and HIV acquisition – clinical evidence

Data from CAPRISA 004, a randomized controlled trial conducted in Durban, South Africa to evaluate the effectiveness of tenofovir vaginal gel for HIV prevention, when used before and after sex, suggest that genital tract elevation of MIP-1α and MIP-1β are associated with increased risk of HIV infection (after adjustment for age and tenofovir gel use).[5] Among women who became HIV infected during the trial, there was a statistically significant correlation in pre- and post-HIV infection cytokine elevation after adjusting for multiple comparisons (IL-1α, IL-1β, IL-6, TNF-α, MIP-1β, MCP-1 and IL-7). In a crude analysis not adjusted for tenofovir gel use, the incidence rate ratio (IRR) for women with and without increased cytokine levels was 2.1 (95% CI 0.9-5.3). In addition, the data suggest that elevated cytokines may modify the effectiveness of tenofovir gel (IR 0.0 vs. 18.8). These sub-analyses however were limited by small sample sizes, notably with cell sizes of zero in some cases.

In a separate analysis using plasma samples from the same population, a variety of immune markers were measured in a case control analysis including 13 cytokines (GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13 and TNF-α).[14] TNF-α, IL-2, IL-7 and IL-12p70 were all significantly associated with HIV risk when comparing cases to controls. There were significant limitations to the design of this study however, including a case: control ratio <1, control selection based on a self-reported sexual behavior variable which could plausibly be related to exposure, different selection criteria for cases and controls (leading to substantial demographic and behavioral differences in cases and controls), lack of explanation

of confounder selection and possible unmeasured confounding (e.g., no data on STIs were included in the final model). Although the results are interesting, the design limitations of this study are substantial and additional research is necessary to confirm the results.

A well designed, large secondary analysis of women in Zimbabwe and Uganda who participated in a trial designed to compare the effect of DMPA, combined oral contraceptives (COC) and non-hormonal contraceptives on HIV acquisition provide additional insight into inflammation and HIV risk.[4] 199 women who became HIV infected during the study were matched to 633 uninfected women on the visit immediately preceding seroconversion (scheduled visits occurred every three months), study site, age and STI status (positive defined as having CT, NG and/or BV). Overall, a statistically significant increase in RANTES and decrease in SLPI were associated with HIV seroconversion at the next visit. Notably, this is the previously described study which also found an increase in RANTES and lower BD-2 among DMPA users.

Summary

In summary, a better understanding of the innate immune factors that are associated with known and potential risk factors for HIV infection, such as DMPA use and RTIs, as well as innate immune inflammation that may exist in the absence of such risk factors, will improve our understanding of the determinants of HIV infection at the site of exposure. A complete understanding of such determinants could be useful in multiple ways, including but potentially not limited to, 1) the safety evaluation of existing and novel technologies that may have an impact on the local immune environment, 2) in designing better screening tools for RTI detection, and thereby identify individuals in need of treatment, and 3) to understand any modification of the effectiveness of novel HIV prevention technologies by the innate environment.

Table 2.1 Names and functions of selected cytokines [1, 46, 47]

Cytokine	Function
Inflammatory	
IL-1α	A member of the interleukin 1 cytokine family. Involved in various immune responses, inflammatory responses and hematopoiesis. Produced by monocytes and macrophages in response to cell injury and induced apoptosis.
IL-1β	A member of the interleukin 1 cytokine family. Mediator of the inflammatory response, involved in numerous cellular activities including cellular proliferation, differentiation and apoptosis.
IL-6	Functions in inflammation and the maturation of B cells, recruits neutrophils, dendritic cells, macrophages and lymphocytes.
Chemokines	
IP-10	A member of the CXC subfamily and ligand for the receptor CXCR3. Binding of this protein to CXCR3 results in pleiotropic effects, including stimulation of monocytes, natural killer and T-cell migration, and modulation of adhesion molecule expression.
MIP-1α/CCL3	Important in the early inflammatory response for activation and recruitment of multiple innate and adaptive effector cells to the site of virus entry. Plays a role in inflammatory responses through binding to the receptors CCR1, CCR4 and CCR5. Polymorphisms at this locus are associated with susceptibility and resistance to HIV-1 infection. Expressed by plasmacytoid dendritic cells.
MIP-1β/CCL4	Expressed by plasmacytoid dendritic cells, recruits CCR5+ CD4 T cells and blocks the CCR5 co-receptor on CD4+ T cells.
IL-8	Secreted by multiple cell types and a major mediator of the inflammatory response through recruitment of neutrophils, dendritic cells, macrophages and lymphocytes.
RANTES	Displays chemotactic activity for blood monocytes, memory T helper cells and eosinophils, is one of the major HIV-suppressive factors produced by CD8+ cells as a natural ligand for the chemokine receptor CCR5 which thereby suppresses in vitro replication of the R5 strains of HIV-1.
Hematopoietic	
GM-CSF	Stimulates growth and differentiation of hematopoietic precursor cells from various lineages, including granulocytes, macrophages, eosinophils and erythrocytes.

Figure 2.1 Biological mechanisms that may contribute to an increased risk of HIV acquisition

and transmission among women using hormonal contraceptives such as DMPA
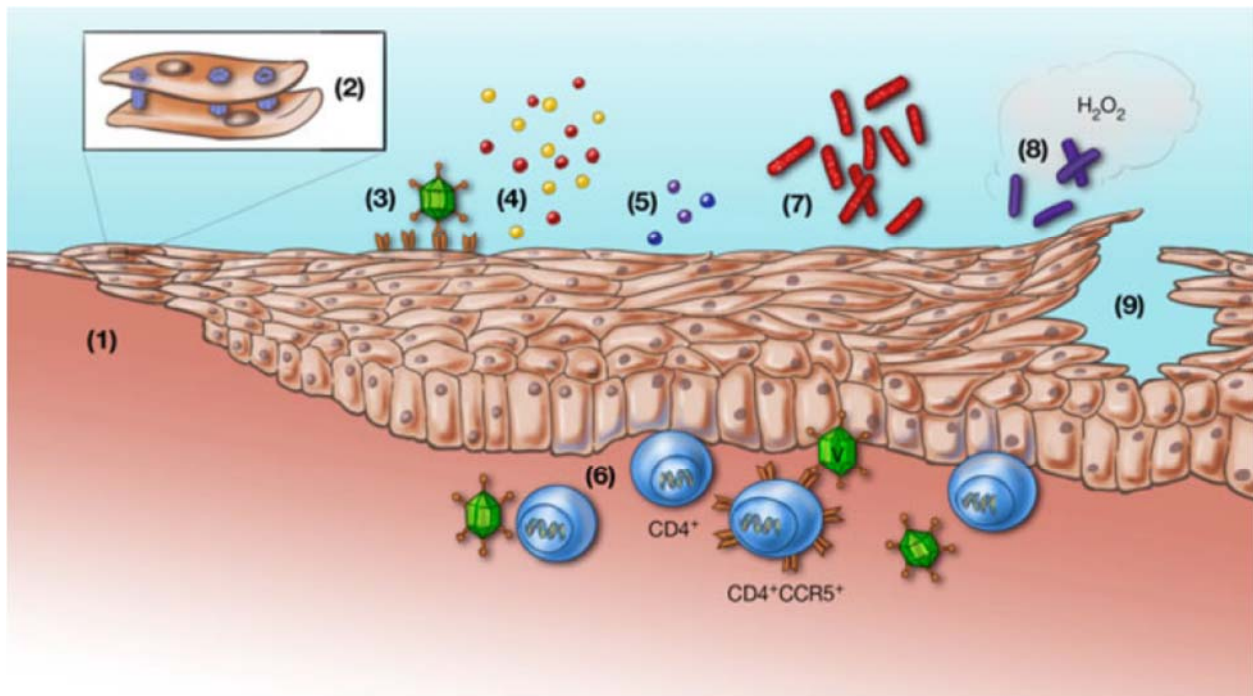
Figure and text taken directly from Murphy 2014

(1) Thinning of the epithelial barrier
(2) Disruption of intracellular junctional complexes
(3) Upregulation of Trojan horse receptors that capture HIV particles
(4) Increased secretion of inflammatory mediators that recruit or activate immune target cells and/or facilitate HIV replication
(5) Decreased secretion of antimicrobial peptides (e.g., SLPI, defensins) that contribute to soluble host defense
(6) Increases CCR5 expression on CD4+ cells rendering the cells more susceptible to HIV
(7) Increased BV-associated bacteria
(8) Decreased hydrogen peroxide producing lactobacilli
(9) Increased genital herpes shedding or clinical lesions with associated disruption of the epithelial barrier

Figure 2.2 Inflammation, innate immunity, mucosal epithelial signaling and target cell availability

at mucosal front lines
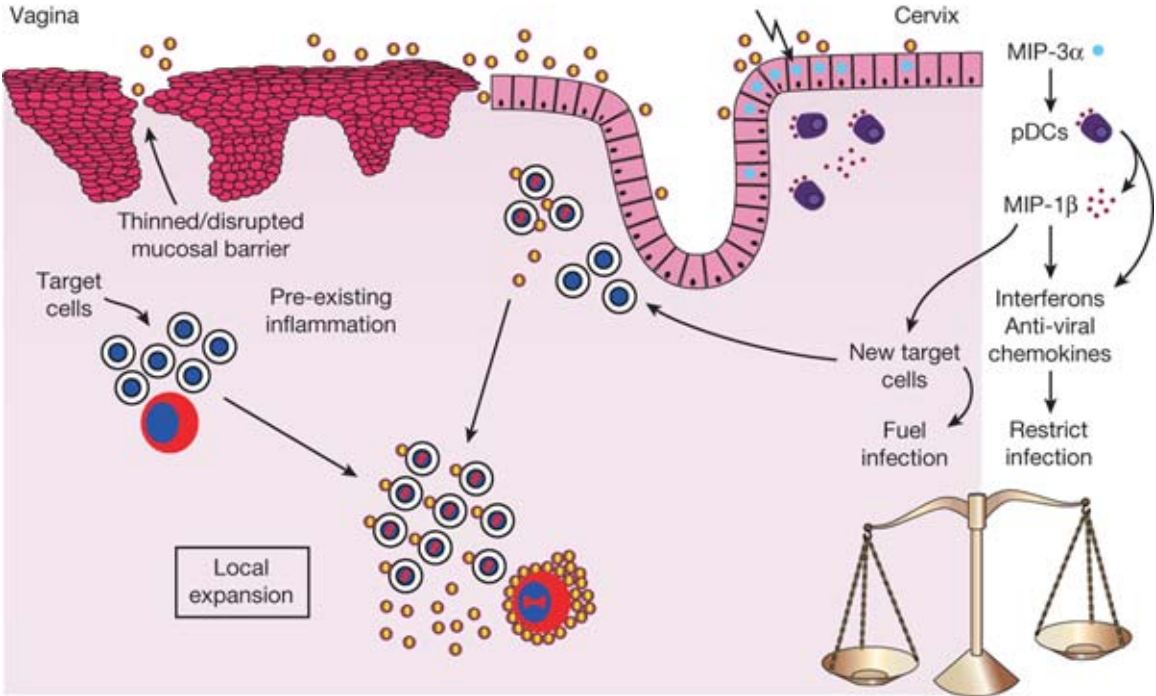
Figure taken directly from Haase 2010.

CHAPTER 3: RESEARCH DESIGN AND METHODS

Aim 1: Cross sectional analysis of selected innate immune biomarkers and their association with progestin-only contraceptive use and reproductive tract infections among high risk population

Study Design

A cross-sectional design was used to estimate innate immune biomarker concentrations (cytokines: MIP-1α, MIP-1β, IL-6, IL-8, IP-10, IL-1α, IL-1β and RANTES and antibacterial protein: SLPI) among a selection of women categorized into single risk-factor exposure groups. The large size of the population screened for the FEM-PrEP trial provided a unique opportunity to evaluate associations between selected exposures and outcomes in subpopulations of participants without other potential confounding factors. Because genital samples were only uniformly collected at screening, only a cross-sectional analysis was possible for this aim. While this information does not provide evidence on the causal nature of the relationship between these exposures and the selected innate immune markers, they provide additional data for hypothesis generation in this data-limited area of HIV prevention.

Study Population

Women who were screened for the FEM-PrEP clinical trial in the Bondo, Kenya and Pretoria, South Africa sites and met the following criteria were eligible for sampling:

- Not menstruating (genital specimens not collected from menstruating women)

- Had valid screening HIV test results

- Had valid screening STI results (including NG, CT and TV)

- Had valid screening Nugent score results

- Was using DMPA, NET-EN, a non-hormonal contraceptive method or no contraceptive method at screening

  - If using DMPA or NET-EN at screening, she must have been using the method for ≥3 months

A total of 508 women were selected for this analysis, among whom 376 had stored specimens available for analysis. Women without and with characteristics suggested by previous research to be covariates of innate immune biomarker concentrations and HIV risk were selected (i.e., women using DMPA or NET-EN, and women with a STI or BV). This approach allowed for direct comparison of the Kenya and South Africa immune profiles in the absence of potentially important confounding factors and provided a reference population to which 'risk-factor' populations could be compared to analyze the association between innate immune elevation and various risk factors for HIV.

Exposure Assessment

A reference population of 99 women who reported not currently using HC and who were NG/CT/TV negative and had normal vaginal flora by Nugent score (hereafter referred to as RTI negative) was identified for comparison to seven 'risk-groups'.

Seven 'risk-groups' were defined as follows:

1) DMPA (n=75) - women who self-reported having used DMPA within the past three months and who were RTI negative

2) NET-EN (n=37) - women who self-reported having used NET-EN within the past three months and who were RTI negative

3) NG (n=15) – women who were positive for *Neisseria gonorrhoeae* by laboratory testing, self-reported no use of HC and were otherwise RTI negative

4) CT (n=22) - women who were positive for Chlamydia trachomatis by laboratory testing, self-reported no use of HC and were otherwise RTI negative

5) TV + BV (n=27) - women who were positive for *Trichomonas vaginalis* by laboratory testing and had bacterial vaginosis by Nugent score, self-reported no use of HC and were otherwise RTI negative

6) IVF (n=28) – women who had IVF by Nugent score, self-reported no use of HC and were otherwise RTI negative

7) BV (n=73) - women who had BV by Nugent score, self-reported no use of HC and were otherwise RTI negative

Notes:

- *There were an insufficient number of participants with Trichmoniasis and no other covariates (n=3) for analysis of TV as a single exposure. The majority of participants with Trichomoniasis infection at baseline had comorbid BV, therefore, only a combined category of Trichomoniasis + BV could be analyzed.*

- *Syphilis testing was done at baseline in the trial; however, low prevalence precluded analysis of syphilis as a single exposure group.*

Age was self-reported by participants at baseline. Participants were required to use an effective contraceptive method (defined as oral contraceptive pills (OC), injectables [DMPA or NET-EN], implant, intrauterine device (IUD) or have been previously sterilized) to meet trial eligibility criteria; the most frequently used contraceptive methods (i.e., OCs, injectables) were provided at the study sites. Contraceptive use was documented at baseline and during follow-up visits; injection dates were recorded for injectable methods. RTIs, including NG, CT, TV, BV and syphilis and were diagnosed by laboratory testing and treated at baseline. All data were recorded on standard trial case report forms.

Outcome Assessment

The following immune markers were quantified in stored endocervical specimens: MIP-1α, MIP-1β, IL-6, IL-8, IL-1α, IL-1β, IP-10, RANTES, GM-CSF and SLPI.

Confounder Assessment

Prostate-specific antigen (PSA) testing was done to control for recent unprotected semen exposure. PSA testing was conducted using the Seratec © PSA semiquant assay (Seratec Diagnostica, Göttingen, Germany). Sex without a condom in the past seven days was self-reported by participants. Candida was detected using wet mount and microscopy.

Statistical Analysis

Reliability and reproducibility of cytokine and SLPI readings were assessed using Spearman's rank correlation. Cytokine and SLPI concentrations were calculated as the mean of duplicate samples from the same plate. For cases in which one observation was detectable and the second observation was below the limit of detection, the observed concentration was used. For cases in which both observations were below the lower limit of detection, the lower limit of detection was imputed. The associations between PSA detection and exposure and outcome variables were assessed using Fisher's exact and Wilcoxon-Mann-Whitney tests. Exposure-outcome bivariate associations were assessed using Wilcoxon-Mann-Whitney tests and general linear models, controlling for PSA detection (as a marker of recent semen exposure), self-reported sex without a condom in the past seven days and the presence of candida. All analyses were done using SAS® software version 9.3 (SAS Institute, Cary, NC).[48]

Aim 2: Case-cohort analysis of selected immune biomarkers as risk factors for HIV infection

Study Design

A case-cohort design was employed for Aim 2 to analyze the association of selected innate immune biomarkers and risk of HIV infection. The cost of conducting cytokine analyses on specimens of the entire cohort was cost-prohibitive; therefore, feasible design approaches for this aim were a case-cohort or case-control design. A case-cohort design was selected over the case-control design as more extensive potential confounder information is available from a

pre-existing case-cohort sample. All cases and a 15% sub-cohort of the entire study population were previously selected to analyze the association between HIV infection and Truvada (tenofovir and emtricitabine) plasma and intracellular drug levels. Use of this existing case-cohort sample provided the opportunity to control for time-varying Truvada exposure. In addition, a comprehensive review of the literature suggested the following variables as potential confounders of the effect of innate immune elevation on HIV risk: age, DMPA use, RTI and site (as a proxy variable for local STI/HIV prevalence). The relationships between the exposure, outcome and covariates is presented in Figure 3.1 in the form of a Directed Acyclic Graph.

Study Population

Women who were enrolled in the FEM-PrEP clinical trial in the Bondo, Kenya and Pretoria, South Africa sites and met the following criteria were eligible for inclusion:

- Did not have a positive HIV RNA or DNA PCR result on the enrollment sample

- *Sub-cohort*: A 15% random sample of the entire enrolled study cohort

- *Cases*: all participants with incident HIV infection defined as having initial and confirmatory HIV positive antibody results according to the trial testing algorithm between enrollment and 60 weeks of follow-up and having been confirmed as an incident HIV case according to confirmatory testing at the Institute of Tropical Medicine in Belgium

Exposure Assessment

The following immune markers were quantified in stored endocervical specimens: MIP-1α, MIP-1β, IL-6, IL-8, IL-1α, IL-1β, IP-10, RANTES, GM-CSF and SLPI.

Outcome Assessment

HIV testing was conducted every four weeks from trial enrollment through 60 weeks of follow-up using a standardized rapid test algorithm; results were recorded on standardized case report forms. Confirmatory testing was done at the Institute of Tropical Medicine in Belgium on specimens from women who had positive antibody results according to the rapid algorithm.

Time to HIV infection was defined as the time between enrollment and date of collection of the

first specimen which was HIV RNA and/or DNA positive.

Statistical Analysis

Reliability and reproducibility of cytokine and SLPI readings were assessed using

Spearman's rank correlation. Cytokine concentrations were calculated as the mean of duplicate

samples from the same plate, the mean of duplicate samples from separate plates if one

observation was undetectable on the plate with duplicate specimens, the observed

concentration if only 1/3 readings was detectable and the median value between zero and the

lowest observed standard if all three observations were undetectable. SLPI concentrations were

calculated as the mean of duplicate readings; if both observations were below the limit of

detection, the midpoint between zero and the lower limit of detection was imputed, if both

observations were above the limit of detection, the upper limit of detection was imputed.

Cytokines and SLPI were analyzed in multivariate models as: 1) ten dichotomous variables (one

per marker), and 2) a single dichotomous summary variable of inflammation (cytokines) and a

separate dichotomous variable for SLPI (< or ≥ the median concentration). For the former,

individual marker concentrations were dichotomously coded as < or ≥ the median concentration.

For the latter, an intermediate summary variable was set equal to the number of cytokines ≥ the

median concentration (range 0-9) per woman. The median of the intermediate summary

variable was then defined as the cut point for the final dichotomous inflammation variable.

Missing covariate data was handled as follows: 1) RTI/STI pelvic exam data were used to

impute missing results based on syndromic management assessment, 2) contraceptive method

was carried forward from the previous non-missing visit, 3) FTC/TDF use was carried forward

from the previous non-missing visit. FTC/TDF use was coded as no/low among all women in the

placebo arm.

Bivariate associations between immune markers and HIV infection were assessed using

Wilcoxon-Mann-Whitney tests. A Cox-proportional hazard model with pseudo-likelihood and

robust variance was used to model the association between immune marker concentrations and risk of HIV infection over time.[49-51] Age and RTI/STI were fixed at baseline whereas contraceptive method and FTC/TDF use were time-varying. Covariates were evaluated as potential effect measure modifiers with retention of covariates pre-specified for likelihood ratio tests resulting in p<0.20. Covariates that resulted in >10% change in the hazard ratio through backwards selection were retained in the final model. All analyses were done using SAS® software version 9.3 (SAS Institute, Cary, NC).[48]

Laboratory Methods

The laboratory methods described here, with the exception of TFV and TFV-DP detection which are only applicable to Aim 2, are applicable to both Aims 1 and 2.

Sterile synthetic swabs were collected and eluted in 1000-1200μL phosphate buffered saline (PBS) and the volume which remained after NG/CT testing was stored at -70^{0}C during the FEM-PrEP trial (between May 2009 and November 2010). Cytokine and SLPI testing was conducted on these stored aliquots between July 2012 and April 2013. Cytokine concentrations were measured using MilliplexTM Human Cytokine kits (Millipore, MA, USA) on a Bio-PlexTM Suspension Array Reader (Bio-Rad Laboratories Inc®, CA, USA) according to a written standard operating procedure in accordance with manufacturer instructions; specimens were pre-filtered by centrifugation using 0.2μm cellulose acetate filters (Sigma-Aldrich, USA) to ensure mucus did not interfere with the assay and were tested in duplicate on the same plate. Each sample was also tested on a second plate in order to evaluate inter-assay reproducibility. SLPI concentrations were measured in duplicate using the Quantikine Human SLPI Immunoassay kit from R&D Systems (Minneapolis, MN) by adapting the manufacturer's instructions for vaginal specimens (1:400 in calibrator diluent RDT). Optical densities were read at 450 nm with a second reference filter of 570 nm using a Biotek ELx800 reader (Vermont, USA) and KCJunior Software. PSA testing was performed using the Seratec © PSA semiquant assay (Seratec Diagnostica, Göttingen, Germany). A volume of 120μL of filtrated swab elute

was used for testing according to the manufacturer's instructions with specimens classified as negative, low positive or positive.

RTI testing was done during the trial; CT/NG testing was done using polymerase chain reaction (PCR) on the Roche Cobas Amplicor platform, TV was detected using wet mount microscopy and BV visualized using Gram staining and Nugent scoring. Tenofovir (TFV) and tenofovir diphosphate (TFV-DP) were measured in plasma and upper layer packed cells using protein precipitation and LC-MS/MS detection using methods that have been described.[3, 52]

Tables and Figures

Figure 3.1 Directed Acyclic Graph, Aim 2
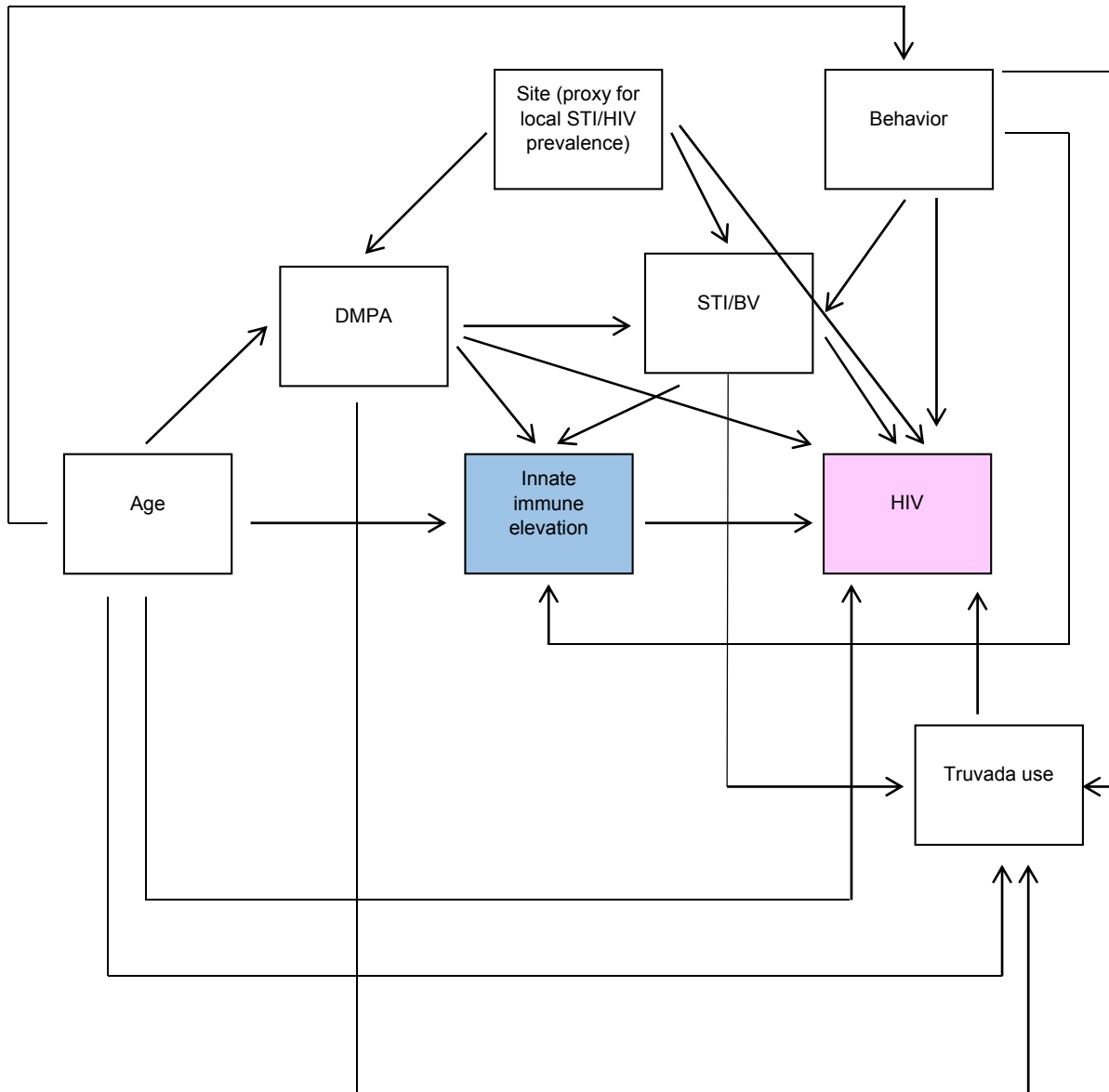
Table 3.1 Determination of minimally sufficient adjustment set, *Aim 2*

Path[1]	Type[2]	Status[3]	Possible adjustment set[4]
IIE → HIV	C	O	
IIE ← Age → HIV	NC	O	A
IIE ← Age → DMPA → HIV	NC	O	A or D
IIE ← Age → DMPA ← Site → HIV	NC	B	
IIE ← Age → DMPA → STI → HIV	NC	O	A or D or S
IIE ← Age → DMPA → STI ← Site → HIV	NC	B	
IIE ← Age → DMPA ← Site → STI → HIV	NC	B	
IIE ← Age → Behavior → STI → HIV	NC	O	A or B or SB
IIE ← DMPA → Age → HIV	NC	O	A or D
IIE ← DMPA → Age → Behavior → HIV	NC	O	A or D or B
IIE ← DMPA → HIV	NC	O	D
IIE ← DMPA ← Site → HIV	NC	O	D or S
IIE ← DMPA ← Site → STI → HIV	NC	O	D or S or SB
IIE ← DMPA → STI → HIV	NC	O	D or SB
IIE ← DMPA → STI ← Site → HIV	NC	B	
IIE ← DMPA → Site → STI ← Behavior → HIV	NC	B	
IIE ← DMPA → STI ← Behavior → HIV	NC	B	
IIE ← DMPA → Age → Behavior → STI → HIV	NC	O	D or A or B or SB
IIE ← STI → HIV	NC	O	SB
IIE ← STI ← Behavior → HIV	NC	O	B or SB
IIE ← STI ← Site → DMPA ← Age → Behavior → HIV	NC	B	
IIE ← STI → DMPA ← Age → Behavior → HIV	NC	B	
IIE ← STI → DMPA ← Age → HIV	NC	B	
IIE ← STI ← Site → HIV	NC	O	SB or S
IIE ← Behavior → Truvada use → HIV	NC	O	B or T
IIE ← Behavior → STI/BV → Truvada use → HIV	NC	O	B or SB or T
IIE ← Behavior → STI/BV ← Site → DMPA ← Age → Truvada Use → HIV	NC	B	
IIE ← Behavior → STI/BV ← DMPA ← Age → Truvada use → HIV	NC	B	
IIE ← STI/BV ← Behavior → Truvada use → HIV	NC	O	SB or B or T
IIE ← STI/BV ← Site → DMPA ← Age → Truvada use → HIV	NC	B	
IIE ← DMPA ← Age → Truvada use → HIV	NC	O	D or A or T
IIE ← DMPA ← Site → STI/BV ← Behavior → Truvada use → HIV	NC	B	
IIE ← DMPA ← STI/BV ← Behavior → Truvada use → HIV	NC	O	D or SB or B or T
IIE ← Age → Truvada use → HIV	NC	O	A or T

[1] IIE = innate immune elevation
[2] C=causal, NC=non-causal
[3] O=open, B=blocked
[4] A=age, D=DMPA, S=site, B=behavior, SB=STI/BV

CHAPTER 4: INJECTABLE PROGESTIN-ONLY CONTRACEPTION IS ASSOCIATED WITH INCREASED LEVELS OF PRO-INFLAMMATORY CYTOKINES IN THE FEMALE GENITAL TRACT

OVERVIEW

Problem

Genital inflammatory changes may be a mechanism of increased HIV risk among injectable progestin-only contraception (IPC) users.

Method of Study

We conducted a cross-sectional analysis of 376 Kenyan and South African women. Genital cytokines and secretory leukocyte peptidase inhibitor concentrations in a reference population were compared to IPC users and women with RTI.

Results

No significant variability in marker concentrations was observed by age or site. Depot medroxyprogesterone acetate (DMPA) users had significantly higher MIP-1α, MIP-1β, IL-6, IL-8, IP-10 and RANTES concentrations. Norethisterone oenanthate (NET-EN) users had significantly higher IL-6, IL-8 and RANTES concentrations. Women with sexually transmitted infections had variable inflammation, and women with BV exhibited a mixed phenotype of up and downregulation.

Conclusions

The finding of substantial mucosal inflammation among DMPA users provides evidence which, combined with the results of prior studies, suggests that DMPA may create an immune environment conducive to HIV target cell recruitment and inhibitory for antiviral activity.

INTRODUCTION

The successes and failures of many HIV prevention trials, including those of microbicides, antiretrovirals and vaccines, have led to great interest in the biology of HIV transmission. In heterosexual women, the anatomical site of exposure and first infection is most often the vaginal and cervical mucosa.[1] Well-accepted biological cervicovaginal risk factors for HIV infection include mucosal epithelial disruption, which may occur via physical or chemical disturbance; immune factors, including the availability of CCR5+/CD4+ target T cells; sexually transmitted infections (STI); and disturbances in the vaginal microbiota, such as bacterial vaginosis (BV).[2] Depot medroxyprogesterone acetate (DMPA), a progestin-only contraceptive injectable (IPC), has also been associated with altered inflammatory responses and increased HIV risk although epidemiological studies remain inconclusive.[22, 23]

Female genital mucosal inflammation, characterized as increased inflammatory cytokines, decreased antibacterial proteins and/or increased target cell activation, has been associated both directly and indirectly with increased risk of HIV infection.[4, 53] Several immune markers have been evaluated with variable associations with potential risk factors of interest (i.e., race, RTIs, age, cervical ectopy, menstrual phase, pregnancy, breastfeeding and hormonal contraceptive use).[17, 18, 54, 55] Evaluations of the impact of RTIs on female genital cytokine profiles have been limited by use of specimens from previously completed clinical trials which lacked available data on potentially important confounders. Furthermore, the impact of hormonal contraception (HC) on these inflammatory markers is unclear. We examined cytokine and secretory leukocyte peptidase inhibitor (SLPI) profiles among women using IPC, including DMPA and norethisterone oenanthate (NET-EN) – a two-monthly injectable progestin, and women with RTIs. In this study, we compared these results to a reference group of women not using HC and who were free of common RTIs.

METHODS

Design

We conducted a cross-sectional study to evaluate the association between genital mucosal inflammatory markers, IPC use and RTIs among women screened for the FEM-PrEP trial in Pretoria, South Africa and Bondo, Kenya. FEM-PrEP was a Phase III, randomized, double-blind, placebo-controlled effectiveness and safety trial to assess the role of emtricitabine/tenofovir disoproxil fumarate (FTC/TDF, i.e., Truvada) in preventing HIV acquisition in women conducted in Bondo, Kenya; Pretoria and Bloemfontein, South Africa; and Arusha, Tanzania.[3] The FEM-PrEP trial uniformly collected and stored endocervical swab specimens at the screening visit. Due to the large trial size, we were able to select sub-populations with single risk factors for comparisons; all 'risk-groups' were compared to a reference population of women who were not using HC, were STI negative and had normal vaginal flora.

Study population

Women who were screened for the FEM-PrEP trial in Bondo, Kenya and Pretoria, South Africa were eligible for selection if they had valid screening HIV and RTI test results (including Neisseria gonorrhoeae (NG), Chlamydia trachomatis (CT), Trichomonas vaginalis (TV), syphilis and BV by Nugent score) and were using DMPA, norethisterone oenanthate (NET-EN), a non-hormonal contraceptive method or no contraceptive method. All women who had only one of the aforementioned potential risk factors for HIV (i.e., use of IPC or a single RTI [with the exception of women with TV infection who predominantly had concurrent BV]), and a reference group of women were selected yielding a total 376 participants for analysis as follows: 1) reference population (no current HC use, RTI negative) n=99, for comparison to the following 'risk-groups': 2a) DMPA group (used DMPA within the past three months, RTI negative) n=75, 2b) NET-EN group (used NET-EN within the past three months, RTI negative) n=37, 2c) women with NG (no current HC use and otherwise RTI negative) n=15, 2d) women with CT (no current HC use and

otherwise RTI negative) n=22, 2e) women with both TV and BV (no current HC use and

otherwise RTI negative) n=27, 2f) women with intermediate flora (Nugent score 4-6, no current

HC use and otherwise RTI negative) n=28, and 2g) women with BV (Nugent score 7-10, no

current HC use and otherwise RTI negative) n=73 (Figure 4.1). Although syphilis testing was

done at baseline, the low prevalence precluded analysis among women with syphilis.

Laboratory methods

The following immune markers were quantified: macrophage inflammatory protein (MIP)-

1α, MIP-1β, IL-6, IL-8, IL-1α, IL-1β, IP-10, RANTES, granulocyte macrophage colony-

stimulating factor (GM-CSF) and SLPI. Prostate-specific antigen (PSA) testing was also done to

control for recent semen exposure in statistical analyses.[56]

Sterile synthetic swabs were collected and eluted in 1000-1200μL phosphate buffered

saline (PBS) and the volume which remained after NG/CT testing was stored at -70^0C during

the FEM-PrEP trial (between May 2009 and November 2010). Cytokine, SLPI and PSA testing

were conducted on the stored aliquots between July 2012 and April 2013. Cytokine

concentrations were measured using MilliplexTM Human Cytokine kits (Millipore, MA, USA) on

a Bio-PlexTM Suspension Array Reader (Bio-Rad Laboratories Inc®, CA, USA) according to a

written standard operating procedure in accordance with manufacturer instructions; specimens

were pre-filtered by centrifugation using 0.2μm cellulose acetate filters (Sigma-Aldrich, USA) to

ensure mucus did not interfere with the assay and were tested in duplicate on the same plate.

SLPI concentrations were measured in duplicate using the Quantikine Human SLPI

Immunoassay kit from R&D Systems (Minneapolis, MN) by adapting the manufacturer's

instructions for vaginal specimens (1:400 in calibrator diluent RDT). Optical densities were read

at 450 nm with a second reference filter of 570 nm using a Biotek ELx800 reader (Vermont,

USA) and KCJunior Software. PSA testing was performed using the Seratec © PSA semiquant

assay (Seratec Diagnostica, Göttingen, Germany). A volume of 120μL of filtrated swab elute

was used for testing according to the manufacturer's instructions with specimens classified as

negative, low positive or positive. RTI testing was done during the trial; NG/CT testing was done using polymerase chain reaction (PCR) on the Roche Cobas Amplicor platform, TV was detected using wet mount microscopy and BV visualized using Gram staining and Nugent scoring.

Statistical analysis

Reliability and reproducibility of cytokine and SLPI readings were assessed using Spearman's rank correlation. Cytokine and SLPI concentrations were calculated as the mean of duplicate samples from the same plate. For cases in which one observation was detectable and the second observation was undetectable, the observed concentration was used. For cases in which both observations were undetectable, the lower limit of detection was imputed. The associations between PSA detection, as a potential confounder, and exposure and outcome variables were assessed using Fisher's exact and Wilcoxon-Mann-Whitney tests. Exposure-outcome bivariate associations were assessed using Wilcoxon-Mann-Whitney tests and general linear models, controlling for PSA detection (as a marker of recent semen exposure), self-reported sex without a condom in the past seven days and the presence of candida. All analyses were done using SAS® software version 9.3 (SAS Institute, Cary, NC).[48]

This research was approved by the FHI 360 Protection of Human Subjects Committee and the Office of Human Research Ethics at the University of North Carolina at Chapel Hill. Trial participants included this analysis provided informed consent during the FEM-PrEP study for future research on stored specimens.

RESULTS

Among the 376 non-pregnant, HIV negative women in this analysis, the median age was 24 years (range 18-35). Women in Bondo were somewhat older than women in Pretoria (median 25 vs. 22 years, respectively) and had fewer years of education (median 8 vs. 12). Women were more likely to use condoms with other partners (i.e., sex with partners other than who the woman considered to be her primary sex partner) vs. primary partners in both sites

(Table 4.1), although women in Bondo used condoms less with either partner type as compared to women from Pretoria (primary partner mean percentage condom use in the last 7 days 27% vs. 40% and 'other' partner mean percentage condom use in the last 7 days 52% vs. 68%, respectively). Contraceptive use was generally low at the baseline visit (by study design), with over half of the population reporting no use of contraception. With the exception of TV and BV, RTIs were more prevalent among women in Pretoria. Herpes simplex virus type 2 (HSV-2) testing was only conducted on a subset of women in the parent clinical trial; among women in this analysis, HSV-2 data were available for 142 (36%) women from the Pretoria site with 85 (60%) of those women testing seronegative and 57 (40%) testing seropositive.

Cytokines and SLPI detection

Spearman correlation between intra-plate cytokine concentration readings was generally high with all correlation coefficients ≥0.96 with the exception of MIP-1α (0.84). The validity of extrapolated readings was evaluated separately; extrapolated readings remained significantly correlated (p≤0.05) for IL-6, IL-8, IL-1β and RANTES (ρ range: 0.85-0.95) although correlation was considerably lower for MIP-1α, MIP-1β, IL-1α and IP-10. Cytokine concentrations that were below the limit of detection ranged from <1% to 15%. Only one observation for one marker was above the limit of detection in this analysis (IL-8). GM-CSF was not included in any analyses due to a large number of observations below the detectable range (36%). Correlation between duplicate SLPI readings was high (ρ 0.99) and most observations were within the limits of detection (98.4%).

PSA detection

We measured PSA to control for contamination with semen. Three-quarters of specimens were PSA negative (75%); 15% were classified as positive and 10% as low positive indicating that these women had likely engaged in unprotected sexual intercourse within 48 hours of sample collection.[14, 15] In bivariable analyses, women who were using DMPA (p=0.004), had BV (p<0.001) or had any STI or BV (p=0.006) were significantly more likely to be

PSA positive. IL-6 (p=0.026), IL-8 (p=0.018) and IL-1β (p=0.042) concentrations were

significantly lower, and SLPI (p=0.020) concentrations were significantly higher, in specimens

classified as PSA positive. In subsequent multivariable analyses, we therefore adjusted for PSA

as a potential confounder with PSA coded dichotomously (low positive and positive collapsed

into a single positive category).

Association of inflammatory markers with age and study site

Cytokine and SLPI profiles in the reference population of 99 women reporting no HC

use, and who were NG, CT and TV negative and with normal vaginal flora, did not vary

significantly when stratified by site or age group (Table 4.2).

Association of inflammatory markers with injectable progestin-only contraceptive use

DMPA users had the highest number of elevated genital inflammatory cytokines;

concentrations of MIP-1α, MIP-1β, IL-6, IL-8, IP-10 and RANTES were significantly elevated as

compared to the reference group of women reporting no HC use and who were RTI negative

(Table 2, Figures 4.2, 4.3, 4.4). Similarly, IL-6, IL-8 and RANTES concentrations were

significantly higher in NET-En users as compared to the reference population. Adjustment for

PSA, self-reported sex without a condom in the past seven days and the presence of candida

had little effect (data not shown).

Association of inflammatory markers with reproductive tract infections

Women with STIs or BV generally had less inflammation compared to DMPA users

(Table 4.2). Women with NG had significantly higher IL-1α and lower SLPI concentrations, and

women with CT had significantly higher IP-10 and RANTES concentrations, as compared to the

reference group. Women who had both TV and had BV had significantly elevated IL-8, IL-1α

and IL-1β and significantly lower SLPI concentrations. Similarly, women with only BV had

significantly elevated IL-1α and IL-1β concentrations, as well as significantly lower MIP-1α, MIP-

1β and IP-10 concentrations. Women with intermediate flora (Nugent score 4-6) also had

significantly elevated IL-1α and IL-1β, as well as IL-6 and IL-8 concentrations. Adjustment for

PSA, self-reported sex without a condom in the past seven days and the presence of candida had little effect (data not shown).

Sensitivity analysis

In a sensitivity analysis using available HSV-2 data, there were no significant differences in any of the measured immune markers between HSV-2 positive vs. HSV-2 negative women in the reference group. Furthermore, the proportion of women who were HSV-2 positive did not differ when comparing women between the reference and exposure groups, which suggests the results may not be confounded by HSV-2 status.

DISCUSSION

The impact of IPC on HIV acquisition risk remains unclear. DMPA has been associated with an increased risk of HIV infection in observational epidemiological studies and most recently, in two meta-analyses, one which pooled individual participant data from >37,000 women.[22, 23] However, other studies, some of which were included in the aforementioned meta-analysis, have found no such association.[28-31, 57] Association of NET-EN use and HIV risk is also unclear, but has received much less attention.[29, 31, 33, 34]

In this evaluation, we controlled for many potential confounders, including RTIs, HIV positivity, pregnancy and recent exposure to semen. We observed significant increases in IL-6, IL-8 and RANTES among both DMPA and NET-EN users, compared to women without RTIs or IPC use. DMPA users also had significantly higher concentrations of MIP-1α, MIP-1β and IP-10. The consistent findings among DMPA and NET-EN users suggest high-dose progestin exposure is associated with an increase in pro-inflammatory cytokines. These results are consistent with a large study of hormonal contraception and HIV in Uganda and Zimbabwe which found significant elevation of RANTES among women using DMPA in endocervical specimens.[4] Increased concentrations of MIP-1α, MIP-1β, IL-8 and IP-10, which were significantly elevated in DMPA-using women in our study, have similarly been associated with increased risk of HIV acquisition.[53]

Perhaps surprisingly, women with RTIs had fewer median increases in inflammatory cytokines as compared to women using DMPA. Elevated RANTES and IP-10 concentrations among CT infected women and IL-1α among NG infected women has been observed previously.[55] With BV, some markers of inflammation were increased and others decreased as previously reported.[55, 58] The marked difference in the distributions among women with both TV and BV, as compared to the reference population (Figures 4.3, 4.4, 4.5), suggests a potential synergistic force on inflammation, and suppression of SLPI, among women with both STIs and BV. A confirmed profile of inflammatory markers associated with RTIs could be used in the development of a cost-effective screening tool to improve RTI detection in high prevalence settings that rely on syndromic management.

The lack of complete HSV-2 data among women in this study many have affected the results. However, a recent paper by Masson et al which sought to characterize innate immune profiles of women with STIs found no association between HSV-2 status as measured by antibody detection in serum or active infection with viral shedding.[55] Further, due to the cross-sectional design, a causal relationship between the exposures and observed increase in inflammatory markers cannot be established. However, the strict inclusion criteria and participant sampling approach utilized, along with measurement of PSA to control for recent sexual activity, methodologically controls for many potential confounders including pregnancy, comorbid RTIs and HIV.

In summary, IPC use was associated with increased markers of inflammation in the female genital tract. Such inflammation offers biological plausibility for increased risk of HIV acquisition, an ongoing concern for users of these agents. Given the gravity of this problem, a randomized clinical trial to define the role of hormonal contraception in HIV acquisition has been planned.[32]

Figure 4.1 Flow diagram of participant selection
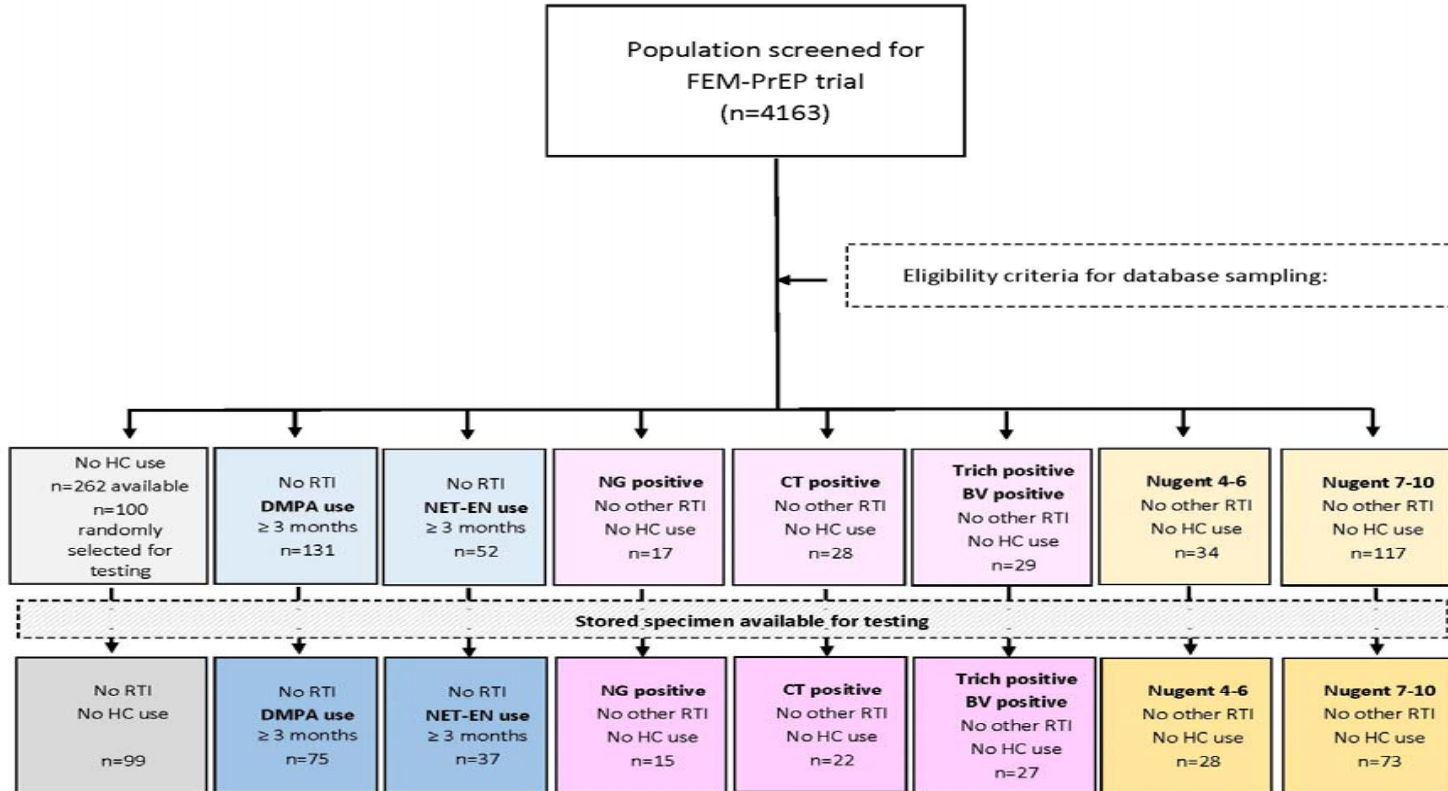
*RTI includes *Neisseria gonorrhoeae* (NG), *Chlamydia trachomatis* (CT), *Trichomonas vaginalis* (TV), syphilis and bacterial vaginosis by Nugent score

Table 4.1 Study population characteristics

Table I. Study Population Characteristics

Characteristic	Bondo N (%) (N=206)	Pretoria N (%) (N=170)	Total N (%) (N=376)
Age			
18-24	90 (44)	112 (66)	202 (54)
25-29	51 (25)	32 (19)	83 (22)
30-35	65 (32)	26 (15)	91 (24)
Sexual behavior			
Has primary partner	200 (97)	169 (99)	369 (98)
Has other partners	105 (51)	30 (18)	135 (36)
Contraceptive method			
Depot medroxyprogesterone acetate (DMPA)	62 (30)	13 (8)	75 (20)
Norethisterone enanthate (NET-EN)	0 (0)	37 (22)	37 (10)
Condoms	19 (9)	7 (4)	26 (7)
Female sterilization	10 (5)	2 (1)	12 (3)
Other[1]	4 (2)	0 (0)	4 (1)
None	111 (54)	111 (65)	222 (59)
Reproductive tract infections			
Chlamydia	1 (<1)	21 (12)	22 (6)
Gonorrhea	3 (1)	12 (7)	15 (4)
Trichomoniasis	22 (11)	5 (3)	27 (7)
Syphilis[2]	0 (0)	4(2)	4 (1)
Bacterial vaginosis[3]	79 (38)	21 (12)	100 (27)
Intermediate vaginal flora[4]	11 (5)	17 (10)	28 (7)
Candida	18 (9)	18 (15)	47 (13)
Herpes simplex virus type 2[5]	---	57 (40%)	---

[1] Two participants reported calendar method, two reported herbal
[2] Syphilis results were not available for 15 participants (6 in Pretoria, 9 in Bondo)
[3] Bacterial vaginosis defined as Nugent Score 7-10
[4] Intermediate vaginal flora defined as Nugent Score 4-6
[5] Herpes simplex virus type 2 results only available for 36% of women (n=142) from the Pretoria site

Table 4.2 Genital protein concentrations (median (IQR), pg/mL) by subgroups

Table 2. Genital protein concentrations (median (IQR), pg/mL) by subgroup								
	Pro-inflammatory							
Characteristics	MIP-1α	MIP-1β	IL-6	IL-8	IL-1α	IL-1β	IP-10	RANTES
Women without selected covariates (reference population)								
Total (n=99)	9	15	3	250	27	3	33	2
	(3,27)	(4,33)	(2,9)	(92,650)	(11,65)	(1, 6)	(10,116)	(1,5)
By Site Bondo, Kenya (n=50)	8	8	3	138	26	3	31	2
	(3,25)	(3,24)	(3,9)	(52,460)	(11,73)	(2,4)	(14,80)	(1,2)
Pretoria, South Africa (n=49)	11	18	3	465	23	2	38	3
	(5,29)	(4,52)	(2,9)	(141,928)	(12,58)	(1,7)	(8,244)	(1,14)
By Age 18-24 (n=60)	8	10	2	156	22	3	26	2
	(3,24)	(3,37)	(2,6)	(77,530)	(11,45)	(1,4)	(9,107)	(1,5)
Age 25-29 (n=17)	17	18	7	491	28	3	66	2
	(11,26)	(16,23))	(2,12)	(166,739)	(20,77)	(1,13)	(21,133)	(2,3)
Age 30-35 (n=22) (ref)	10	11	4	412	30	3	44	2
	(3,29)	(4,35)	(2,19)	(118, 974)	(10,90)	(1,7)	(2,86)	(2,4)
Covariate category (comparator populations)								
DMPA use ≥ 3 months (n=75)	**24****	**20***	**13****	**617****	30	3	**86****	**9****
	(7, 56)	**(10, 51)**	**(4, 32)**	**(248, 1511)**	(14, 69)	(1, 19)	**(33, 236)**	**(4, 17)**
NET-EN use ≥ 3 months (n=37)	19	20	**13***	**632***	28	3	38	**8***
	(8, 34)	(10, 33)	**(4, 24)**	**(370, 1013)**	(14, 65)	(1, 11)	(13, 293)	**(2, 17)**
Gonorrhea (n=15)	8	11	4	364	**45***	2	18	2
	(5, 38)	(3, 56)	(1, 8)	(127, 1661)	**(30, 225)**	(1, 25)	(6, 115)	(1, 7)
Chlamydia (n=22)	12	14	9	427	37	2	**82***	**6***
	(6, 19)	(7, 25)	(3, 13)	(174, 770)	(9, 171)	(1, 7)	**(25, 380)**	**(2, 12)**
Trichomoniasis + BV (n=27)	7	7	4	**749****	**283****	**39****	26	2
	(3, 15)	(4, 16)	(2, 10)	**(419, 3208)**	**(68, 590)**	**(8, 138)**	(19, 40)	(1, 8)
Intermediate flora[2] (n=28)	11	15	**10***	**595***	**73****	**10***	42	2
	(2, 26)	(3, 36)	**(3, 37)**	**(268, 1408)**	**(41, 213)**	**(1, 110)**	(8, 92)	(1, 16)
Bacterial vaginosis[2] (n=73)	**4***	**6***	5	442	**95****	**9****	**14***	2
	(2, 17)	**(3, 17)**	(3, 12)	(65, 1186)	**(58, 266)**	**(3, 31)**	**(2, 49)**	(1, 4)

* Wilcoxon-Mann-Whitney test significant as compared to the reference population at p≤0.05

** Wilcoxon-Mann-Whitney test significant as compared to the reference population at p≤0.001

[1] Sample sizes for SLPI analyses varied slightly from cytokine analyses due to SLPI assay failures as follows: DMPA=71, BV=72, ≥ 1 STI and/or BV=136

[2] Intermediate flora defined as Nugent Score 4-6; bacterial vaginosis defined as Nugent Score 7-10

Figure 4.2 Inflammatory marker concentrations in reference group and IPC users
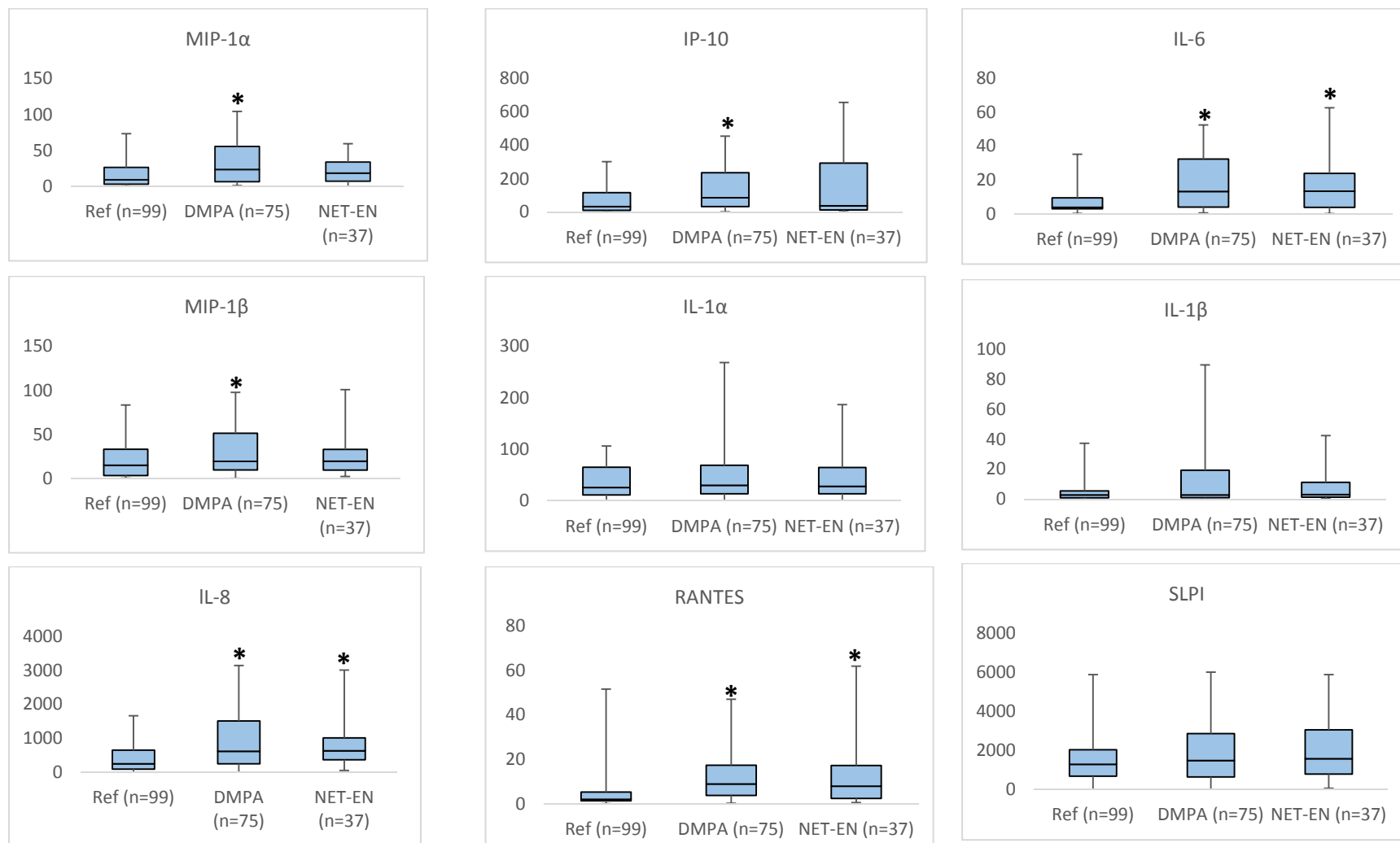
y-axis is concentration of marker in pg/mL. Top whisker of all plots only extends to the 90th percentile to make central data visible.
*Wilcoxon-Mann-Whitney test significant as compared to the reference population at p≤0.05

Figure 4.3 Total inflammatory markers concentrations by 'risk group'
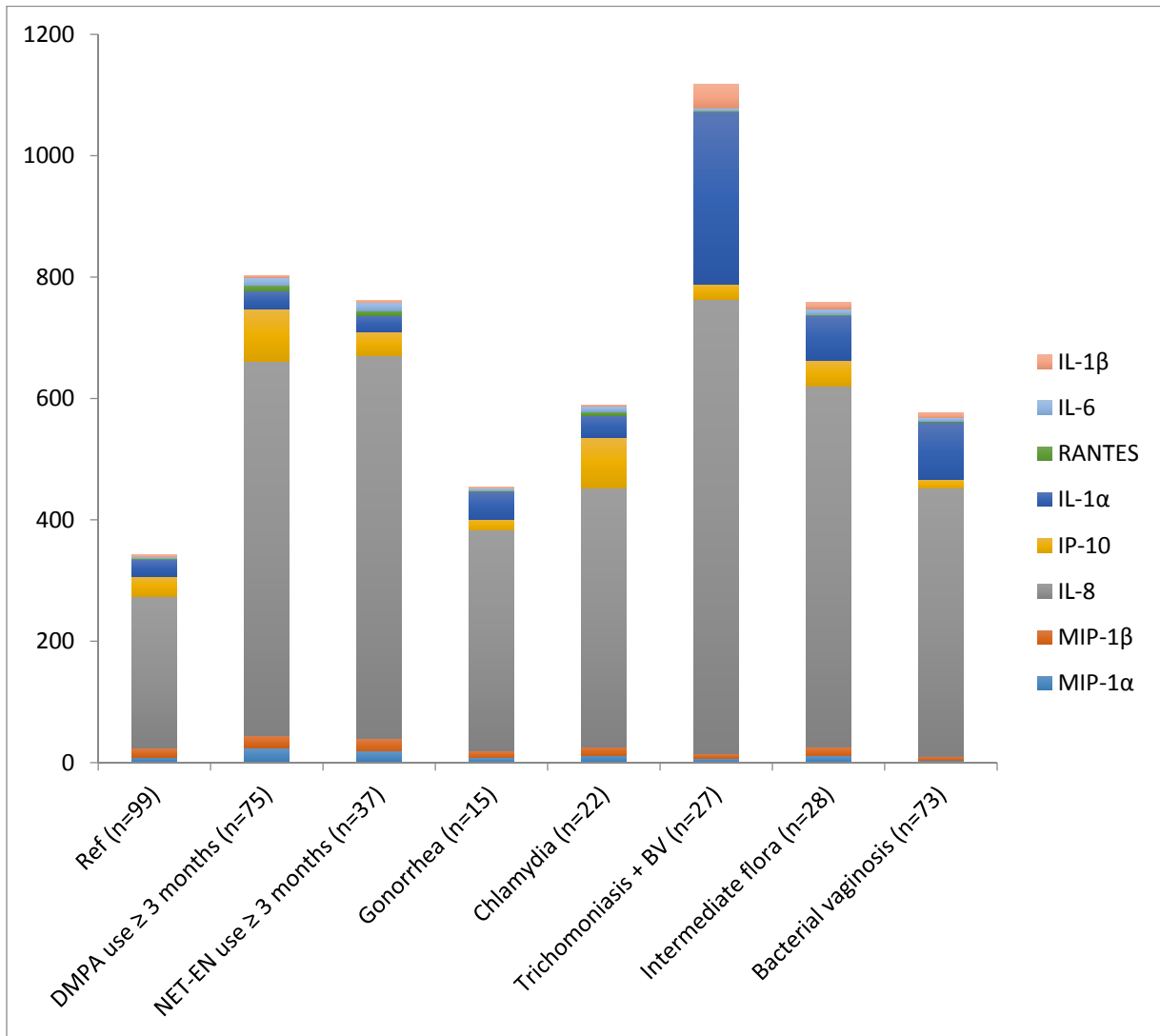
Figure 4.4 Total inflammatory markers concentrations by 'risk group', removing IL-8
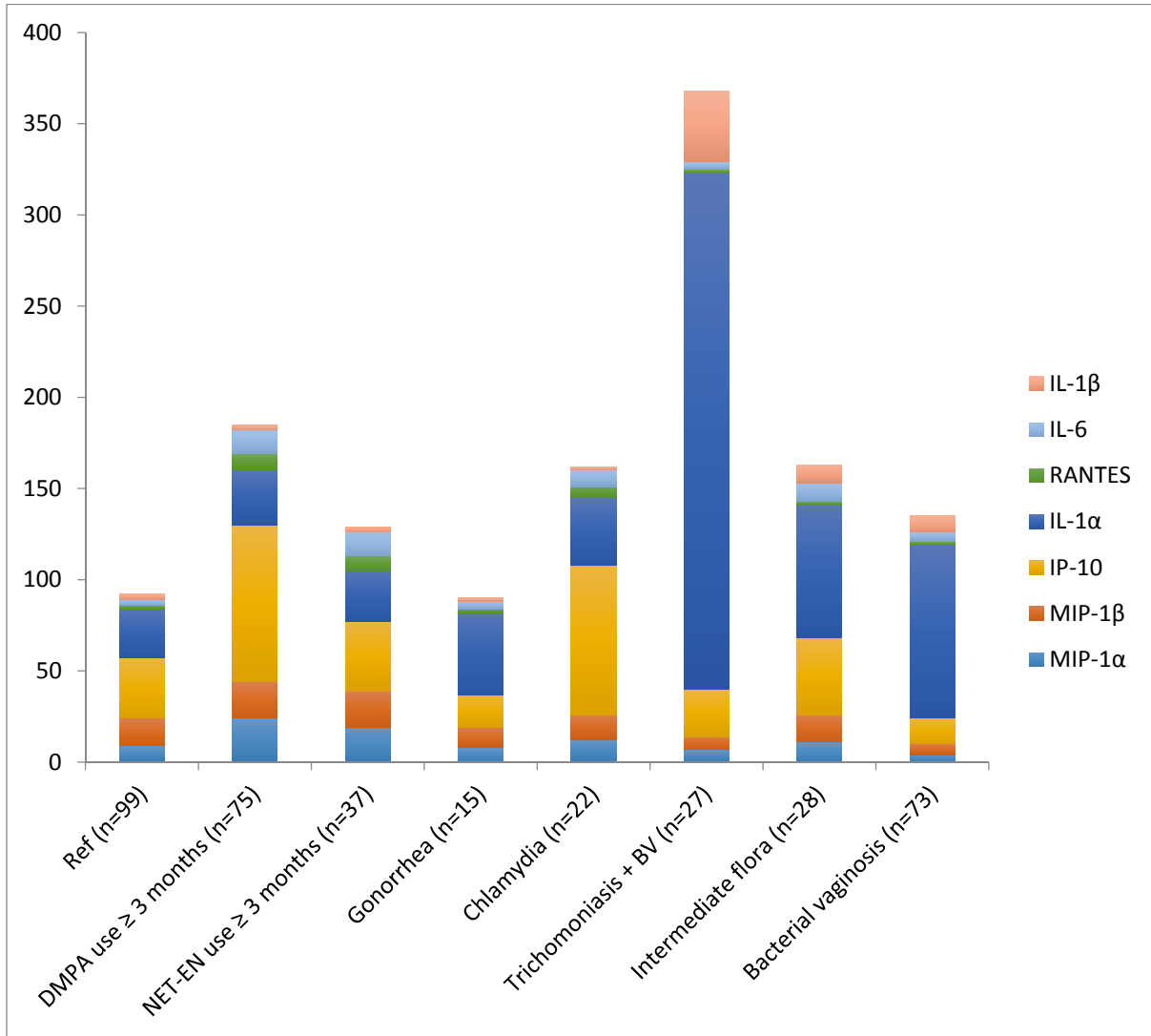
Figure 4.5 Total SLPI concentrations by 'risk group'
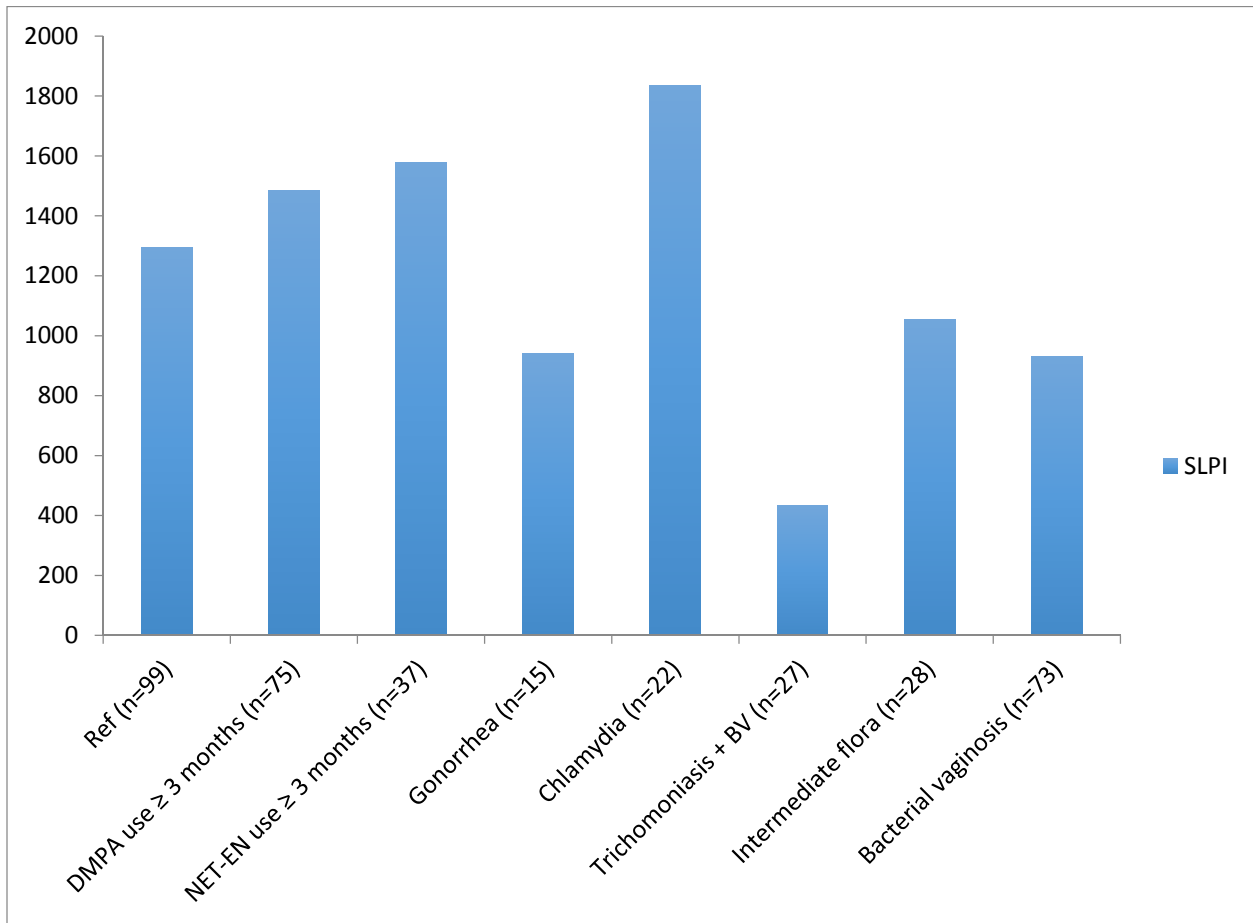
CHAPTER 5: INNATE IMMUNITY MARKERS ASSOCIATED WITH HIV ACQUISITION AMONG WOMEN AT HIGH RISK

OVERVIEW

Problem

Women with genital tract inflammation may be at increased risk of HIV infection independent of other known risk factors for HIV acquisition.

Method of Study

We conducted a case-cohort study to analyze the association between innate immune factors in the female genital tract and HIV acquisition. Women in Kenya and South Africa who became HIV infected between enrollment and 60 weeks of follow-up during a clinical trial of Truvada for HIV prevention were included as cases. A random sub-cohort of women in the clinical trial was selected from individuals who were HIV RNA and DNA PCR negative at enrollment and completed at least one follow-up visit. A Cox model with pseudo likelihood and robust variance estimation were used to assess the association of innate immune factors and HIV acquisition.

Results

Higher IL-1β and RANTES concentrations were associated with HIV acquisition when including women from both the Truvada and placebo arms of the clinical trial. When restricting the analysis to women in the placebo group, higher MIP-1α and IL-8 concentrations were also associated with HIV acquisition. In a model using a composite variable for innate immune inflammation, no association was observed. Coding of innate immunity markers had considerable influence over effect estimates in sensitivity analyses.

Conclusions

Increased HIV acquisition among women with higher baseline concentrations of several pro-inflammatory cytokines suggests local innate immune inflammation may be a mechanism of increased risk of HIV acquisition. Understanding the association between innate immunity and HIV acquisition is important for the rationale design of biomedical technologies – including both microbicides and vaccines – for HIV prevention.

INTRODUCTION

Many biological risk factors for HIV acquisition, including mucosal epithelial disruption, the availability of CCR5+/CD4+ target cells, sexually transmitted infections, disturbances in the vaginal microbiota, such as bacterial vaginosis (BV), are well characterized and accepted.[59, 60] However, the association between innate immune factors and HIV acquisition remains unclear. Variations in innate immune factor concentrations in the female genital tract have been associated with HIV acquisition. In a trial in South Africa, women with increased MIP-1α, MIP-1β, IP-10 and IL-8 concentrations experienced higher rates of HIV acquisition; this finding was also observed within the active trial arm (tenofovir gel) suggesting that local inflammation may reduce the effectiveness of tenofovir gel for HIV prevention.[53] Women who seroconverted during a multi-center African trial were more likely to have detectable HβD-2 concentrations and greater E. coli bactericidal activity compared to women who remained HIV negative, and in a separate study among women in Zimbabwe and Uganda, higher RANTES concentrations and lower SLPI concentrations were associated with HIV seroconversion.[4, 61]

We evaluated the effect of innate immune concentrations on HIV acquisition using stored specimens collected during FEM-PrEP, a randomized trial of Truvada for prevention of HIV acquisition among high-risk women. Understanding the association between innate immunity and HIV acquisition is important in the rationale design of biomedical technologies for HIV prevention, and for both understanding the results of clinical trials of biomedical HIV prevention products and their potential impact on the HIV epidemic.

METHODS

Ethics statement

This research was approved by the FHI 360 Protection of Human Subjects Committee and the Office of Human Research Ethics at the University of North Carolina at Chapel Hill. Trial participants included this analysis provided informed consent during the FEM-PrEP clinical trial for future research on stored specimens.

Study design and population

This prospective case-cohort analysis used data from the FEM-PrEP clinical trial, which has been described elsewhere.[3] In brief, FEM-PrEP was a randomized, double-blind, placebo-controlled trial of once-daily tenofovir disproxil fumarate-emtricitabine [TDF/FTC (marketed as Truvada); Gilead Sciences, Foster City, CA] designed to assess the effectiveness and safety of TDF/FTC as pre-exposure prophylaxis (PrEP) for HIV prevention among high-risk women in Africa.

Selection of the subcohort

We randomly selected ~15% of enrolled women stratified by site from clinical study sites that contributed at least one incident HIV infection during the clinical trial into a sub-cohort independent of case status for an analysis of Truvada drug exposure and HIV acquisition. However, the Bloemfontein, South Africa site did not store baseline genital specimens from participants, which were used to define our exposures, and was therefore excluded from this analysis. Women were eligible for selection into the sub-cohort if they were HIV RNA and DNA PCR negative at enrollment and completed at least one follow-up visit, yielding a sub-cohort of 200 women. Among these 200 women, 164 had stored specimens available for exposure assessment including 6 cases (Figure 5.1).

Selection of cases

All women from the Bondo, Kenya and Pretoria, South Africa sites who were HIV RNA and DNA PCR negative at enrollment, completed at least one follow-up visit and became HIV infected between enrollment and 60 weeks of follow-up were included as cases, yielding a total of 68 incident HIV infections. Among these 68 cases, 47 women had stored specimens available for exposure assessment.

Ascertainment of exposure

The following immune markers were quantified in stored endocervical specimens: macrophage inflammatory protein (MIP)-1α, MIP-1β, interleukin (IL)-6, IL-8, IL-1α, IL-1β,

interferon gamma induced-protein (IP)-10, regulated on activation normal T cell expressed and secreted (RANTES), granulocyte macrophage colony-stimulating factor (GM-CSF) and secretory leukocyte peptidase inhibitor (SLPI).[4, 5, 42]

Ascertainment of covariates

The following covariates were identified using directed acyclic graphs: age, injectable contraception use, reproductive tract/sexually transmitted infections (RTI/STI), presence of prostate specific antigen (PSA) (marker of recent unprotected sexual exposure) and TDF/FTC use.[62, 63] Age was self-reported by participants at baseline. Participants were required to use an effective contraceptive method (defined as oral contraceptive pills (OC), injectables [DMPA or NET-EN], implant, IUD or have been previously sterilized) to meet trial eligibility criteria; the most frequently used contraceptive methods (i.e., OCs, injectables) were provided at the study sites. Contraceptive use was documented at baseline and during follow-up visits; injection dates were recorded for injectable methods. RTI/STI, including bacterial vaginosis (BV), Chlamydia trachomatis (CT), Neisseria gonorrhoeae (NG), Trichomonas vaginalis (TV) and syphilis and were diagnosed by laboratory testing and treated at baseline (subsequent RTI/STI infection was managed via syndromic diagnosis and treatment). Age, contraceptive method and RTI/STI were recorded on standard trial case report forms. Concentrations of tenofovir (TFV) in plasma and intracellular tenofovir diphosphate (TVF-DP) in upper layer packed cells were analyzed in stored specimens to assess TDF/FTC use.[11] TFV and TVF-DP concentrations were used to create a 5-level adherence variable in consultation with the study pharmacologist, and collapsed into three levels for this analysis (i.e., 'none/low' [TFV-DP <10,000 femtomoles/mL and TFV undetectable], 'good/excellent' [TFV-DP>100,000 femtomoles/mL and TFV>10 ng/mL] and 'some' [TFV-DP and/or TFV values in between the two prior specified indices]).

Ascertainment of incident HIV infection

HIV testing was conducted every four weeks from trial enrollment through 60 weeks of follow-up using a standardized rapid test algorithm; results were recorded on standardized case report forms. Confirmatory testing was done at the Institute of Tropical Medicine in Belgium on specimens from women who had positive antibody results. Time to HIV infection was defined as the time between enrollment and date of collection of the first specimen which was HIV RNA and/or DNA positive.

Laboratory methods

Sterile synthetic swabs were collected and eluted in 1000-1200µL phosphate buffered saline (PBS) and the volume which remained after NG/CT testing was stored at -70^0C during the FEM-PrEP trial (between May 2009 and November 2010). Cytokine and SLPI testing was conducted on these stored aliquots between July 2012 and April 2013. Cytokine concentrations were measured using MilliplexTM Human Cytokine kits (Millipore, MA, USA) on a Bio-PlexTM Suspension Array Reader (Bio-Rad Laboratories Inc®, CA, USA) according to a written standard operating procedure in accordance with manufacturer instructions; specimens were pre-filtered by centrifugation using 0.2µm cellulose acetate filters (Sigma-Aldrich, USA) to ensure mucus did not interfere with the assay and were tested in duplicate on the same plate. Each sample was also tested on a second plate in order to evaluate inter-assay reproducibility. SLPI concentrations were measured in duplicate using the Quantikine Human SLPI Immunoassay kit from R&D Systems (Minneapolis, MN) by adapting the manufacturer's instructions for vaginal specimens (1:400 in calibrator diluent RDT). Optical densities were read at 450 nm with a second reference filter of 570 nm using a Biotek ELx800 reader (Vermont, USA) and KCJunior Software. PSA testing was performed using the Seratec © PSA semiquant assay (Seratec Diagnostica, Göttingen, Germany). A volume of 120µL of filtrated swab elute was used for testing according to the manufacturer's instructions with specimens classified as negative, low positive or positive.

RTI/STI testing was done during the trial; CT/NG testing was done using polymerase chain reaction (PCR) on the Roche Cobas Amplicor platform, TV was detected using wet mount microscopy and BV visualized using Gram staining and Nugent scoring. TFV and TVF-DP were measured in plasma and upper layer packed cells using protein precipitation and LC-MS/MS detection using methods that have been described previously.[3, 52]

Statistical analysis

Reliability and reproducibility of cytokine and SLPI readings were assessed using Spearman's rank correlation. Cytokine concentrations were calculated as the mean of duplicate samples from the same plate, the mean of duplicate samples from separate plates if one observation was undetectable on the plate with duplicate specimens, the observed concentration if only 1/3 readings was detectable and the median value between zero and the lowest observed standard if all three observations were undetectable. SLPI concentrations were calculated as the mean of duplicate readings; if both observations were below the limit of detection, the midpoint between zero and the lower limit of detection was imputed, if both observations were above the limit of detection, the upper limit of detection was imputed.

Cytokines and SLPI were analyzed in multivariable models as: 1) continuous variables, and 2) a single dichotomous composite variable of inflammation (cytokines) and a separate dichotomous variable for SLPI (< or ≥ the median concentration). For the composite variable, an intermediate variable was set equal to the number of cytokines ≥ the median concentration (range 0-9) per woman. The median of the intermediate variable was defined as the cut point for the final composite inflammation variable. Exploratory analyses were done to evaluate the influence of exposure coding.

Missing covariate data was handled as follows: 1) RTI/STI pelvic exam data were used to impute missing results based on syndromic management assessment, 2) contraceptive method was carried forward from the previous non-missing visit and 3) TDF/FTC use was

carried forward from the previous non-missing visit. TDF/FTC use was coded as none/low among all women in the placebo arm.

Bivariable associations between immune factors and HIV acquisition were assessed using Wilcoxon-Mann-Whitney tests. A Cox-proportional hazard model with pseudo-likelihood and robust variance was used to model the association between immune marker concentrations and risk of HIV infection over time.[49-51] Age and RTI/STI were fixed at baseline whereas contraceptive method and TDF/FTC use were time-varying. Covariates were evaluated as potential effect measure modifiers with retention of covariates pre-specified for likelihood ratio tests resulting in p<0.20. Potential confounders that resulted in >10% change in the hazard ratio through backwards selection were retained in the final model. All analyses were done using SAS® software version 9.3 (SAS Institute, Cary, NC).[48]

RESULTS

Most women in this study were young; over half were ≤24 years old. Baseline RTI/STI laboratory results were available for 97% of women; 3/11 women with missing RTI/STI results had pelvic exam data, all of which were normal, and were classified as RTI/STI negative. RTI/STI prevalence was high – 14% of women had at least one STI (NG, CT and/or TV) and 45% of women had BV resulting in 52% of the population having ≥1 RTI/STI at baseline. Due to trial eligibility criteria, all women at baseline were using an effective method of contraception, with the majority (66%) using injectables (among those 68% DMPA and 32% NET-EN), 26% using oral contraceptives and the remaining 8% having been sterilized or using the IUD or implant (Table 5.1). Nearly all women (98%) reported continued use of effective contraception throughout follow-up, with DMPA being reported during 43%, NET-EN during 20% and other methods during 35% of follow-up time.

As has been reported before, TDF/FTC use was low in the trial and similarly in this case-cohort population; 50% of follow-up visit intervals among sub-cohort women in the active arm were classified as no/low TDF/FTC use, 26% as some use and 24% as good/excellent use.

Immune markers were reliably detected in stored endocervical specimens and intra- and inter-plate correlation between observations was high (intra-plate ρ range 0.89-0.99, inter-plate ρ range 0.70-0.99).

Younger age, TDF/FTC concentrations consistent with none/low (vs. some and good/excellent) use and higher IL-1α concentrations were significantly associated with case status (Table 5.1).

In multivariable modeling of innate immune factors as continuous exposure variables, none were significantly associated with HIV acquisition defined as p≤0.05 (Table 5.2). However, increased IL-1β and RANTES were associated with HIV acquisition among women who were PSA positive at baseline. When restricting the analysis population to women in the placebo arm only, MIP-1β and IL-8 were also significantly associated with HIV acquisition among women who were PSA positive at baseline (data not shown). No association was observed between inflammation and risk of HIV acquisition when modeling the composite inflammation variable (Table 5.3).

Sensitivity analyses were done to explore the influence of variable coding on effect estimates (Figures 5.2, 5.3). Notably, variations of exposure variable coding had varying influence – both in estimate directionality and statistical significance - on the primary effect estimates (Figures 5.2, 5.3).

DISCUSSION

Use of a case-cohort study design within a completed large HIV prevention trial provided the opportunity to assess the association of innate immune factors and HIV acquisition in vivo among a large sample of women. The selection of innate immune markers evaluated in this study was informed by prior research and the finding of increased HIV acquisition among women with higher concentrations of RANTES, IL-1β, MIP-1β and IL-8 is biologically plausible. RANTES has both protective and inflammatory functions in HIV infection, by binding the CCR5 HIV co-receptor while also functioning as a chemoattractant for target CD4+ T cells. Higher

RANTES concentrations have been shown to correlate with increased numbers of HIV-1 targets cells in the genital mucosa.[64] A recent study among women in Uganda and Zimbabwe similarly found higher RANTES to be associated with HIV acquisition.[4] IL-1β is one of two members of the IL-1 cytokine family which activates the NF-kβ pathway resulting in activation of numerous pro-inflammatory cytokines and increases in IL-1β have been associated as a potential mechanism of increased HIV risk observed in women with BV.[65, 66] Both MIP-1β and IL-8 are chemoattractant of CCR5+ CD4+ T cells and other cell types (i.e., dendritic cells, macrophages) susceptible to HIV infection, respectively.[64, 67] Elevated levels of MIP-1β and IL-8 were associated with increased HIV acquisition among women in South Africa.[53] We did not observe an association between concentrations of SLPI and IP-10 and HIV acquisition which has been observed in other studies.[4, 53] This may be due to insufficient power in our study or due to different immune factor measurement methods across studies.

The consistent restriction of significant findings to women who were PSA positive at baseline may be explained by PSA positivity as an indicator of future HIV exposure. Women who were PSA positive had recent unprotected sexual exposure and may represent a population of women more likely to have had unprotected sexual exposure, a requirement for HIV acquisition, during the trial.

The influence of exposure coding on point estimates and statistical significance is an important finding. Most published research in the area of innate immunity and HIV risk report results from analyses using a single exposure classification scheme and there is inconsistency in classification schemes across studies which limits comparability of results. Establishment of a minimal set of standard exposure classification criteria would be useful for advancing our understanding of female innate immunity and HIV acquisition.

While an original 15% (n=300) subcohort was selected for multiple exposure-outcome assessments using FEM-PrEP trial data, genital specimens were only available for 164 women from the selected cohort for this analysis, yielding an 8% person-sample (11% person-year

sample) from the original cohort. It is possible that the study was underpowered to detect any true effects that were not significant in this study. In addition, exposure misclassification may have occurred. Genital specimens were only systematically collected at baseline in the parent trial. While one study has suggested intra-person stability of relative cytokine concentrations over time, the results must be confirmed.[68]

In summary, we found that baseline inflammation of several pro-inflammatory cytokines was associated with HIV acquisition among women in the FEM-PrEP trial. These data combined with the results of other recent clinical studies suggest increased innate immune activity may increase women's risk of HIV acquisition independent of other known risk factors.

Tables and Figures

Figure 5.1 Flow diagram of participant selection

```
                    ┌─────────────────────────────────┐
                    │ Participants from four study     │
                    │ sites participating in the       │
                    │ FEM-PrEP clinical trial          │
                    │ (n=2120)                          │
                    └─────────────────────────────────┘
                                    │
                                    ▼
                    ┌─────────────────────────────────┐
                    │ Eligible cohort                   │
                    │ (HIV RNA and DNA PCR negative at  │
                    │ enrollment, made one post-        │
                    │ enrollment study visit, from Bondo│
                    │ or Pretoria site)                 │
                    │ (n=720 in Bondo, 750 in Pretoria) │
                    └─────────────────────────────────┘
              Cases          │           Sub-cohort
        ┌──────────────┴──────────────────┐
        ▼                                  ▼
┌──────────────────────┐    ┌──────────────────────────┐
│ Women who became HIV │    │ A randomly selected sub-  │
│ infected during      │    │ cohort of participants    │
│ regular follow-up    │    │ (stratified by site)      │
│ (n=68)               │    │ (n=200, 9 of which are    │
│                      │    │ cases)                    │
└──────────────────────┘    └──────────────────────────┘
        │                                  │
        ▼                                  ▼
┌──────────────────────┐    ┌──────────────────────────┐
│ Participants with    │    │ Participants with         │
│ endocervical swab    │    │ endocervical swab         │
│ specimens available  │    │ specimens available for   │
│ for testing          │    │ testing                   │
│ (n=47)               │    │ (n=164, 6 of which are    │
│                      │    │ cases)                    │
└──────────────────────┘    └──────────────────────────┘
```
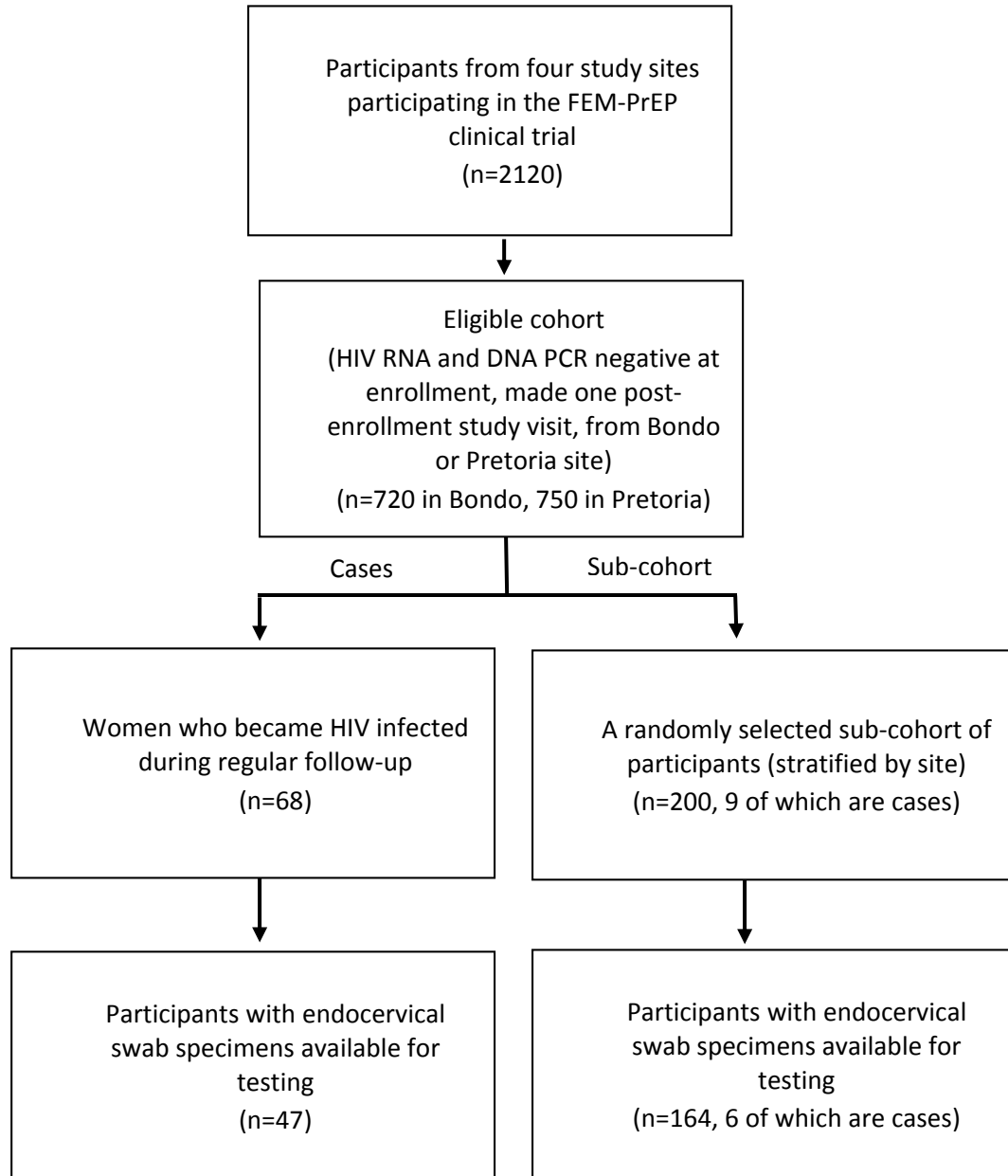
Table 5.1 Characteristics of cases and non-cases

Characteristic	Cases (n=47)	Non-cases (n=158)	p[†]	Full FEM-PrEP cohort[‡] (n=2120)
Total	47 (23)	158 (77)	---	
Bondo, Kenya, n (%)	24 (22)	87 (78)	0.62	739 (35)
Pretoria, South Africa, n (%)	23 (24)	71 (76)		764 (36)
Age, median (range)	23 (18-33)	25 (18-35)	0.03	23 (18-35)
Contraceptive method at baseline, n (%)				
Oral contraceptive pills	16 (34)	38 (24)	0.17	638 (30)
Depot medroxyprogesterone acetate (DMPA)	20 (43)	72 (46)	0.72	820 (39)
Norethisterone enanthate (NET-EN)	10 (21)	34 (22)	0.97	581 (27)
Female sterilization	1 (2)	5 (3)	1.00	39 (2)
Implant	0 (0)	8 (5)	0.20	34 (2)
IUD	0 (0)	1 (1)	1.00	8 (<1)
Contraceptive method during follow-up, n (%) [δ]				
Depot medroxyprogesterone acetate (DMPA)	130 (44)	758 (44)	0.66	N/A
Norethisterone enanthate (NET-EN)	52 (18)	341 (20)	0.32	N/A
Other or none	114 (39)	625 (36)	Ref	N/A
Reproductive tract infections at baseline, n (%)				
Chlamydia	6 (13)	11 (7)	0.23	108/1887 (6)
Gonorrhea	4 (9)	4 (3)	0.08	264/1887 (14)
Trichomoniasis	2 (4)	3 (2)	0.33	109/1893 (6)
Syphilis	2 (4)	4 (3)	0.62	36/2118 (2)
Bacterial vaginosis [ɸ]	26 (58)	67 (45)	0.13	785/1877 (42)
≥1 STI and/or BV [κ]	28 (61)	74 (50)	0.18	999 (47)
Truvada adherence during follow-up [δ,ˆ]				
None/low	67 (39)	449 (52)	Ref	NA
Some	59 (34)	210 (24)	<0.01	NA
Good/excellent	46 (27)	203 (24)	0.05	NA
Cytokine concentration, median (pg/mL)				
MIP-1α	13.3	11.6	0.94	NA
MIP-1β	17.3	18.9	0.93	NA
IL-6	12.3	8.7	0.52	NA
IL-8	475.6	385.5	0.33	NA
IP-10	37.7	26.4	0.31	NA
IL-1α	93.7	52.0	0.02	NA
IL-1β	8.2	4.9	0.08	NA
RANTES	3.9	2.6	0.39	NA
GMCSF	1.6	1.6	0.84	NA
SLPI (pg/mL)	951.3	1033.4	0.68	NA

| Person-years of follow-up (total) | 159 | --- | 1407 |

Figure 5.2 Influence of variable (continuous) coding on hazard ratios
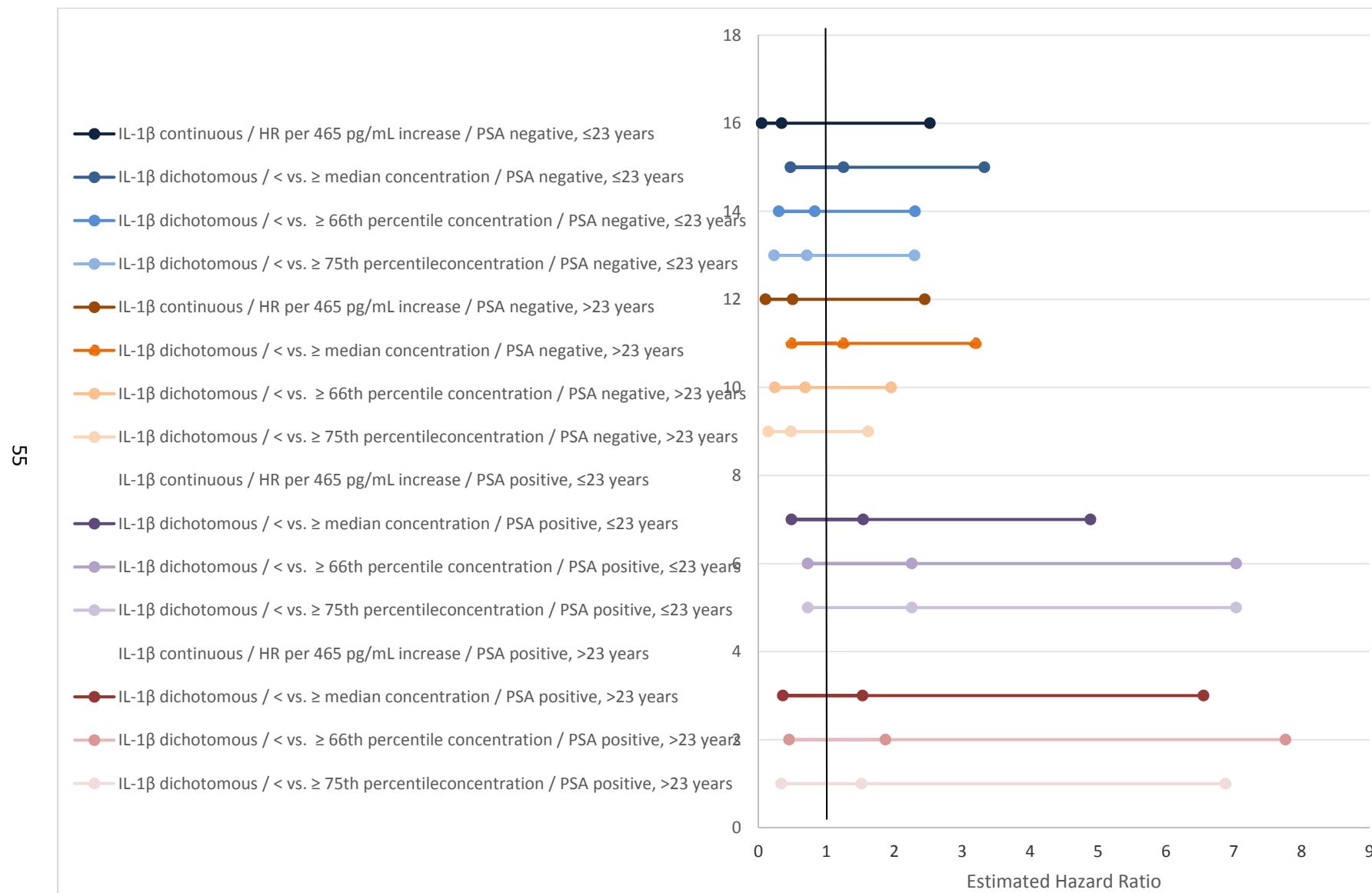
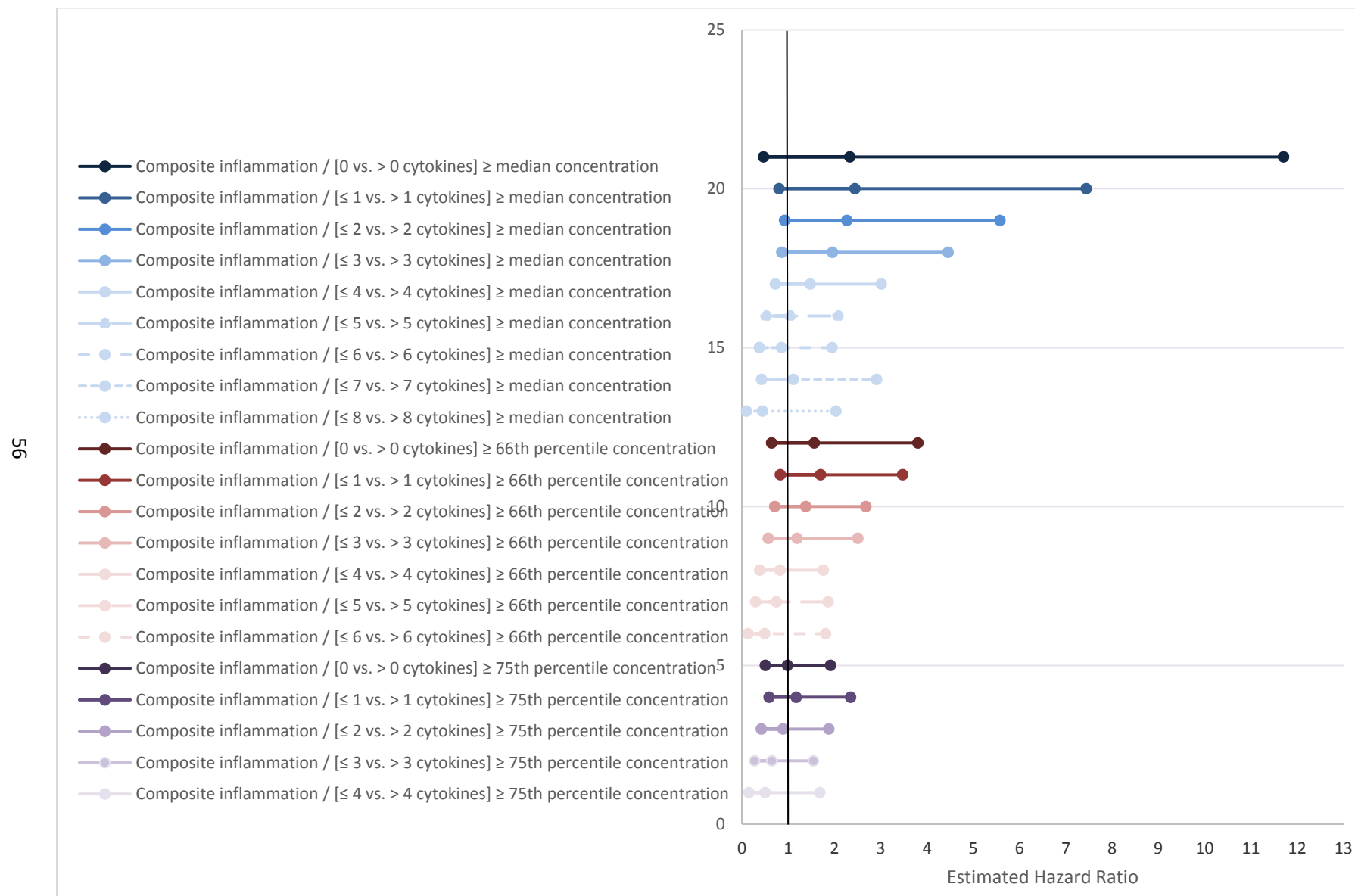Figure 5.3 Influence of variable (composite) coding on hazard ratios

Table 5.2 Risk of HIV acquisition by baseline genital immune marker concentrations

(continuous)

	Bivariable analysis		Multivariable analysis[‡]	
	HR (95% CI)[†]	P	HR (95% CI)[†]	P
MIP-1α[1]	0.37 (0.08, 1.76)	0.21	---	---
None/low adherence	---	---	0.84 (0.56, 1.27)	
Some adherence	---	---	0.01 (0.00, 2.81)	0.40
Good/excellent adherence	---	---	0.27 (0.01, 12.05)	
MIP-1β[2]	0.37 (0.02, 7.65)	0.52	---	---
Negative RTI/STI	---	---	0.11 (0.01, 1.63)	0.11
Positive RTI/STI	---	---	4.82 (0.06, 424.01)	
IL-6[3]	0.81 (0.56, 1.15)	0.23	---	---
IL-8[3]	1.02 (0.85, 1.22)	0.87	---	---
Negative PSA	---	---	0.83 (0.63, 1.08)	0.17
Positive PSA	---	---	1.38 (0.98, 2.00)	
IL-1α[4]	0.98 (0.83, 1.16)	0.82		
None/low adherence	---	---	0.88 (0.71, 1.09)	
Some adherence	---	---	1.52 (0.93, 2.49)	0.24
Good/excellent adherence	---	---	1.04 (0.16, 6.89)	
IL-1β[3]	1.03 (0.86, 1.25)	0.75		
Negative PSA, ≤ 23 years	---	---	0.34 (0.05, 2.52)	
Negative PSA, >23 years	---	---	0.50 (0.10, 2.45)	
Positive PSA, ≤ 23 years	---	---	75.06 (25.64, 219.76)	0.39
Positive PSA, >23 years	---	---	111.06 (3.99, 3090.70)	
IP-10[5]	0.96 (0.75, 1.23)	0.75	---	---
None/low adherence	---	---	1.00 (0.81. 1.24)	
Some adherence	---	---	0.06 (0.01, 0.65)	1.00
Good/excellent adherence	---	---	1.20 (0.67, 2.15)	
RANTES[3]	1.34 (0.74, 2.41)	0.33	---	---
Negative PSA, negative RTI/STI	---	---	0.61 (0.18, 2.00)	
Negative PSA, positive RTI/STI	---	---	1.32 (0.54, 3.22)	0.41
Positive PSA, negative RTI/STI	---	---	0.84 (0.26, 2.74)	
Positive PSA, positive RTI/STI	---	---	1.84 (1.55, 2.19)	
GMCSF[6]	0.94 (0.73, 1.20)	0.60	0.90 (0.70, 1.16)	0.46
SLPI[3]	1.00 (0.89, 1.13)	0.95	---	---

[†]HRs per 20% increase in concentration
[‡] HRs presented by level of interaction term where interaction term likelihood ratio test p<0.20
[^] Positive NG, CT, TV and/or BV results
[1] Model controlling for baseline RTI/STI, use of injectable contraception and age
[2] Model controlling for age
[3] No covariates retained in model
[4] Model controlling for use of injectable contraception and age
[5] Model controlling for use of injectable contraception
[6] Model controlling for baseline RTI/STI

Table 5.3 Risk of HIV acquisition by baseline genital immune marker concentrations (composite)

	Bivariable analysis		Multivariable analysis[‡]	
	HR (95% CI)†	P	HR (95% CI)†	P
Inflammation^	0.92 (0.49, 1.72)	0.76	---	---
Truvada use (no/low)	---	---	1.25 (0.57, 2.76)	
Truvada use (some)	---	---	0.32 (0.05, 1.92)	0.58
Truvada use (good/excellent)	---	---	1.89 (0.16, 21.87)	
SLPI	1.00 (0.89, 1.13)	0.95	1.037 (0.92, 1.17)	0.57

†HRs per 20% increase in concentration
‡ HRs presented by level of interaction term where interaction term likelihood ratio test p<0.20. Model controlling for baseline RTI/STI and age.
^ Inflammation classified as ≥ 5 inflammatory cytokines (MIP-1α, MIP-1β, IL-8, IP-10, IL-1α, RANTES, IL-6, IL-1β, GMCSF) above the median concentration

CHAPTER 6: DISCUSSION

Aim 1: Cross sectional analysis of the association between selected immune biomarkers, injectable progestin-only contraception and RTIs

Summary of Findings

No significant variability in marker concentrations was observed by age or site. DMPA users had significantly higher MIP-1α, MIP-1β, IL-6, IL-8, IP-10 and RANTES concentrations. NET-EN users had significantly higher IL-6, IL-8 and RANTES concentrations. Women with STIs had variable inflammation, and women with BV exhibited a mixed phenotype of up and downregulation.

Interpretation

A similar marker profile in DMPA and NET-EN users suggest IPC may induce changes in the innate immune environment of the female genital tract. Numerous immunologic studies have shown RANTES to be both an effective blocker of the HIV CCR5 co-receptor, yet a potent chemoattractant of CD4+ T cells, the primary cell target of HIV. The consistent finding of RANTES elevation among DMPA users in this analysis and in that of Morrison et. al., combined with the aforementioned immunological evidence, suggests RANTES elevation as a potential mechanism of the increased rate of HIV acquisition among DMPA users observed in epidemiological studies. Women using DMPA and NET-EN did not have any significant decrease in SLPI levels suggesting that the potential increased risk of HIV infection in IPC users is not due to a decrease in this protective protein. However, the innate immune system is complex and interactive, and no single marker likely functions in isolation to alter HIV risk. We also found DMPA use to be associated with increased MIP-1α, MIP-1βIL-6, IL-8 and IP-10, all of which were associated with significantly increased risk of HIV infection in the CAPRISA 004

study. The collective results of this, and the aforementioned studies, suggests elevated inflammatory cytokine concentrations to be one potential mechanism of the observed increased risk of HIV infection among DMPA users. Because NET-EN is rarely used outside of South Africa, globally only a small proportion of women using hormonal contraception utilize this method. However, within South Africa, which is also the epicenter of the HIV epidemic, NET-EN is widely used. The similar profile of elevated cytokines in DMPA and NET-EN users suggests that further research is needed to more broadly clarify any potential association between IPC and HIV risk. Lastly, data from several other studies demonstrates a potential modification of the effects of exogenous hormones on the innate immune environment, and inversely, a modification of RTI risk in the presence of exogenous hormone use. While the magnitude of these effects is unclear, our data suggest that IPC use and RTIs are also both independently associated with changes to the innate immune environment.

Limitations

Due to the cross-sectional nature of our study design, causality of any of the exposures on the measured immune markers cannot be established. However, the strict inclusion criteria and participant sampling approach utilized, along with measurement of PSA to control for recent sexual activity, methodologically controls for many potential confounders including pregnancy, comorbid RTIs and HIV. The lack of complete HSV-2 data among women in this study may have affected the results. However, a recent paper by Masson et al which sought to characterize innate immune profiles of women with STIs found no association between HSV-2 status as measured by antibody detection in serum or active infection with viral shedding.[55]Similarly, the lack of available data on infections with other pathogens (e.g., human papillomavirus, *Mycoplasma genitalium*) may have affected our results. We were only able to measure a selection of cytokines and SLPI, and do not have data on other markers of the local immune response such as total lymphocytes, markers of cellular activation, immunoglobulins and other proteins that may be of relevance (e.g., IFN-α, IFN-β, IFN-γ, IL-1R,

IL-1Ra, human α- and β-defensins), though the panel of selected cytokines was informed by the results of previous studies.[2, 4, 14, 16, 69-73] Although cytokines are important biomarkers of immune processes in the female genital tract, particularly inflammation, different results between studies emphasize the need for the development of standardized approaches to marker selection, testing methods, specimen collection methods and storage conditions and confounder data collection to allow for better comparability of results across studies.

Future research directions

A longitudinal study is needed to better determine if the observed associations with IPC use are causal in nature. We are currently preparing a research proposal that will utilize stringent laboratory and behavioral baseline evaluations, inclusion criteria and visit timing (in regard to hormonal cycling) to better study the effects of HC use on the innate immune environment.

Aim 2: Case-cohort analysis of innate immune inflammation as a risk factor for HIV acquisition

Summary of Findings

In bivariate analysis, younger age, FTC/TDF concentrations consistent with no/low (vs. some and good/excellent) use and higher IL-1α concentrations were significantly associated with incident HIV infection. In multivariate modeling using the single cytokine inflammation variable, none of the selected immune markers were significantly associated with risk of HIV infection. Injectable contraceptive use was removed from the final model according to previously defined criteria; adherence to FTC/TDF was retained as an interaction term. Younger women (<23 years old) had lower risk of HIV infection as compared to older women (≥23 years old) (despite a marginal p-value (0.07), age exerted a substantial influence on the primary exposure effect estimates and was retained in the final model). Women with baseline STI and/or BV had a 74% greater risk of infection than women with no STI/BV at baseline (again, result was not

significant at p≤0.05 but variable had a substantial influence on effect estimates and was retained). When modeling using ten dichotomous exposure variables (i.e., < vs. ≥ median), IP-10 was significantly associated with risk of HIV infection as was baseline STI/BV. Age remained marginally significant. However, when modeling using continuous exposure variables, RANTES was significantly associated with risk of HIV infection.

Interpretation and Public Health Significance

The overall lack of observed association between the selected markers and HIV acquisition may be attributable to study design limitations. The association between age and baseline STI/BV infection and HIV risk, and the overall prevalence of STI/BV in this cohort, are notable. Young women remain at unacceptably high risk of HIV infection, and both age and prevalent RTI/STI may be effective tools for identifying those who would benefit most from targeted HIV prevention efforts. The field of innate immunity and HIV risk is in need of standardized methods for measuring and analyzing immune markers.

The association between younger age and increased risk of HIV infection has been repeatedly observed among young women in sub-Saharan Africa, and has been reported from the parent FEM-PrEP trial.[8, 74-76] More recent data from a large prevention trial suggest that this risk has not declined in recent years and that young women remain at unacceptably high risk.[12] Moreover, the finding of substantial RTI prevalence at baseline reveals a substantial burden of treatable disease in this population. Notably all women with an RTI diagnosis received treatment during the trial; therefore, the observed increased risk among these women was unlikely due to any biological effect of the baseline RTI, but rather subsequent risk behavior, which may have included repeat infection. This finding suggests that prevalent RTI may be used as an indicator of future HIV risk. Currently, most public health programs in sub-Saharan Africa rely on syndromic diagnosis, which has been documented to substantially underestimate infection. Use of more sensitive RTI screening tools could potentially reduce HIV incidence by multiple mechanisms, both through effective identification and treatment of active STIs which

likely increase the biological risk of HIV infection, as well as through identification of individuals who may be at increased risk of future HIV exposure and infection for targeted prevention intervention.

Limitations

While an original 15% (n=300) subcohort was selected for multiple exposure-outcome assessments using FEM-PrEP trial data, genital specimens were only available for 164 women from the selected cohort for this analysis, yielding an 8% person-sample (11% person-year sample) from the original cohort. It is possible that the study was underpowered to detect any true effects that were not observed. In addition, exposure misclassification may have occurred. Genital specimens were only systematically collected at baseline in the parent trial. While one study has suggested intra-person stability of relative cytokine concentrations over time, that study was also limited by small sample size; the true nature of cytokine stability over time remains unclear and further research is necessary for meaningful use of these markers in analyses of HIV acquisition.

Future research directions

All studies conducted in vivo in women on the subject of innate immune factors and HIV risk have been conducted as secondary analyses of studies not designed to answer the research questions. The limitations of this approach include distant timing between marker measurement and study endpoints (i.e., HIV infection or censoring) and inadequate collection of, or delineation of, potentially important confounder data. While studies with HIV infection endpoints are costly and a study designed to look at immune factors and incident HIV infection would not likely be funded, a sub-study designed to answer these questions adjoined to a larger HIV study would be a viable and cost-effective method to more definitively address these questions. In addition, data in this area are not sufficiently robust (in terms of sample size and control of covariates that influence markers) to as yet define the optimal coding scheme, and as such, there is substantial inconsistency in classification schemes across studies which limits

comparability of results. Establishment of a minimal set of exposure measurement classification criteria would be useful for advancing our understanding of female innate immunity and HIV acquisition. In the interim, researchers should evaluate the influence of coding schemes on effect estimates in sensitivity analyses of their results.

APPENDIX 1: STANDARD OPERATING PROCEDURE: HUMAN CYTOKINE MAGNETIC BEAD PLATE ASSAY

1 General information

1.1 Aim and application

The test is used to simultaneously detect multiple selected cytokines in vaginal biological samples.

1.2 Principle

MILLIPLEX MAP is based on the Luminex® xMAP® technology which is capable of performing a variety of bioassays including immunoassays on the surface of fluorescent-coded magnetic beads known as MagPlex TM-C microspheres.

- Luminex uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of two dyes, 100 distinctly colored bead sets can be created, each of which is coated with a specific capture antibody.
- After an analyte from a test sample is captured by the bead, a biotinylated detection
- antibody is introduced. The reaction mixture is then incubated with Streptavidin PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- The microspheres are allowed to pass rapidly through a laser, which excites the internal dyes marking the microsphere set. A second laser excites PE, the fluorescent dye on the reporter molecule.
- Finally, high-speed digital-signal processors identify each individual microsphere and quantify the result of its bioassay, based on fluorescent reporter signals.

The capability of adding multiple conjugated beads to each sample results in the ability to obtain multiple results from each sample. Open-architecture xMAP technology enables multiplexing of many types of bioassays reducing time, labor and costs over traditional methods.

1.3 Reagents required not provided in kit
- Diluted Phosphate buffered saline (PBS; # 8582680)
  - Weighing 0.96 g PBS and 7.65g NaCl.
  - Dissolve in 1L MBW.
  - Sterilize by filtration.
  - Store at 2 - 8°C.
- Bleach (10%)
- Isopropanol (70%)
- BioRad Bio-Plex Validation Kit (catalogue # 171-203001)
- BioRad Bio-Plex Calibration Beads set (catalogue # 171-203060
- BioRad Bio-Plex Sheath Fluid (catalogue # 171-000055)

1.4 Apparatus, equipment and materials
- Micro-pipettes (200;1000 µl)
- Multichannel pipettes (50; 300 µl)
- Filter tips (200;1000 µl)
- Spin-X Centrifuge Filter Tube

- Plate sealers
- Duran Bottle 1L
- Biological safety cabinet Class II
- Refrigerator (2-8°C)
- Freezer (-80°C ± 5°C)
- Disposable Gloves
- Lab Coats + sleeve protectors
- Racks for cryotubes
- Vortex
- Eppendorf tubes, 1.5ml conical
- Falcon tubes (50 ml)
- Reagent reservoirs
- Aluminum foil
- Absorbent pads or paper towels
- Waste receptacle
- Plate shaker
- BioRad Bio-Plex 200 System and Bio-Plex Manager Software
- Hand held magnetic block

1.5 Samples for analysis
- Vaginal secretions collected by using dry synthetic swabs eluted in diluted PBS and stored at -80°C ± 5°C.

- **RAYON®** *Swabs*
  - Thaw the swabs samples overnight at 2-8°C on ice.
  - Determine the correct number of 2.0 ml screw-cap Sarstedt tubes needed. Label two tubes and one Spin-X filter tube for each patient sample and place in rack.
  - Pipette 1.2 ml PBS buffer directly onto the swab inside its cover using a sterile disposable pipette.
  - Vortex for 15 sec.
  - Remove the diluted PBS again with the disposable pipette and transfer it into the Eppendorf tube.
  - Pipette 750 µl sample over into Spin-X filter tube and close the cap.
  - Centrifuge the Spin-X filter tube at maximum speed for 10 min in a microcentrifuge.
  - Load the remaining volume of the samples on the corresponding Spin-X filter and centrifuge at maximum speed for 10 min.
  - Take out the filter from the Spin-X filter tube and discard.
  - Pipette 100 µl of the sample in the second labeled Eppendorf tube.
  - Store both samples tubes (Spin-X tube and the Eppendorf tube) at -80°C.

3 Definitions and abbreviations
- PBS   : Phosphate buffered saline
- NaCl   : Sodium Chloride
- MBW   : Molecular Biology grade Water
- BVK   : Biological safety cabinet
- MLT   : medical laboratory technologist
- SOP   : Standard Operating Procedure
- MFI   : Median Fluorescent Intensity
- 5PL   : 5-parameter logistic
- Std : standard

4 Method

4.1 Sample Preparation

Preparation of endocervical swab PBS eluted samples:
- Thaw samples on ice in an icebox at 2-8°C.
- Samples to be kept on ice during processing.
- In a BVK, vortex samples and transfer a maximum of 750 µl to a labeled Corning Spin-X Centrifuge Filter tube.
- Centrifuge at 13,000 rpm for 10 minutes.
- Transfer 100 µl aliquots to separate, labeled sterile Eppendorf tube and retain remaining volume in Spin-X Centrifuge filter tube (filter is discarded).
- Store at -80°C ± 5°C.
- Avoid multiple (>2) freeze/thaw cycles.

4.2 Kit Storage Conditions

- Recommended storage for kit components is 2-8°C.
- Once the standards and controls have been reconstituted, immediately transfer contents into polypropylene vials. DO NOT STORE RECONSITUTED STANDARDS OR CONTROLS IN GLASS VIALS. For long-term storage, freeze reconstituted standards and controls at ≤-20°C. Avoid multiple (>2) freeze thaw cycles.
- DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin.

4.3 Technical Guidelines

To obtain reliable and reproducible results, the operator should carefully read the entire kit protocol and this SOP and fully understand all aspects of each assay step before running the assay. The following notes should be reviewed and understood before the assay is set-up.

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not use beyond the expiration date on the label.
- Do not mix or substitute reagents with those from other lots or sources.
- The Antibody-Immobilized Beads are light sensitive and must be protected from light at all times. Cover the assay plate containing beads with opaque plate lid or
- aluminum foil during all incubation steps.
- It is important to allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Wash Buffer provided.
- After hydration, all standards and controls must be transferred to polypropylene tubes.
- The standards prepared by serial dilution must be used within 1 hour of preparation.
- Discard any unused standards except the standard stock which may be stored at ≤ -20°C for 1 month and at ≤ -80°C for greater than one month.
- If samples fall outside the dynamic range of the assay, further dilute the samples with the appropriate diluent and repeat the assay.
- Any unused mixed Antibody-Immobilized Beads may be stored in the Bead Mix bottle at 2-8°C for up to one month.

- During the preparation of the standard curve, make certain to mix the higher concentration well before making the next dilution. Use a new tip with each dilution.
- The plate should be read immediately after the assay is finished. If, however, the plate cannot be read immediately, seal the plate, cover with aluminum foil or an opaque lid, and store the plate at 2-8°C for up to 24 hours. Prior to reading, agitate the plate on the plate shaker at room temperature for 10 minutes. Delay in reading a plate may result in decreased sensitivity for some analytes.
- The titer plate shaker should be set at a speed to provide maximum orbital mixing without splashing of liquid outside the wells. For the recommended plate shaker, this would be a setting of 5-7, which is approximately 500-800 rpm.
- Ensure that the needle probe is clean. This may be achieved by sonication and/or alcohol flushes.
- Vortex all reagents well before adding to plate.

4.4 Preparation of reagents:

A. Preparation of Individual Vials of Beads.
- For individual vials of beads, vortex each antibody-bead vial for 2 minutes.
  - ➢ *If vortexing 2-3 vials of beads simultaneously, vortex each individual vial for a few seconds just before addition of beads to the Mixing Bottle.*

- Add 60 μL from each of the 9 antibody bead vials to the Mixing Bottle and bring final volume to 3.0 mL with Bead Diluent by adding 2,460 μL bead diluent.

List of beads used for testing:

Beads	
44_Anti Human IL-1α Bead	74_Anti-Human RANTES Bead
63_Anti-Human IL-8 Bead	46_Anti-Human IL-1β Bead
65_Anti-Human IP-10 Bead	57_Anti-Human IL-6 Bead
72_Anti-Human MIP-1α Bead	20_Anti-Human GM-CSF Bead
73_Anti-Human MIP-1β Bead	

B. Preparation of Quality Controls
- Before use, reconstitute Quality Control 1 and Quality Control 2 with 250 μL deionized water.
- Invert the vial several times to mix and vortex.
- Allow the vial to sit for 5-10 minutes and then transfer the controls to appropriately labeled polypropylene microfuge tubes.

 Unused portion may be stored at -20°C for up to one month.

C. Preparation of Wash Buffer
- Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution.
- Dilute 30 mL of 10X Wash Buffer with 270 mL deionized water.

 Store unused portion at 2-8°C for up to one month.

D. Preparation of Human Cytokine Standard

- Prior to use, reconstitute the Human Cytokine Standard with 250µL deionized water to give a 10,000 pg/mL concentration of standard for all analytes.
- Invert the vial several times to mix.
- Vortex the vial for 10 seconds.
- Allow the vial to sit for 5-10 minutes and then transfer the standard to an appropriately labeled polypropylene microfuge tube.

  ➢ This will be used as the 10,000 pg/mL standard; the unused portion may be stored at -20°C ± 5°C for up to one month.

- Label five polypropylene microfuge tubes 2,000, 400, 80, 16, and 3.2 pg/mL.
- Add 200 µL of Assay Buffer to each of the five tubes.
  ➢ Prepare serial dilutions by adding 50 µL of the 10,000 pg/mL reconstituted standard to the 2,000 pg/mL tube,
  ➢ mix well and transfer 50 µL of the 2,000 pg/mL standard to the 400 pg/mL tube,
  ➢ mix well and transfer 50 µL of the 400 pg/mL standard to the 80 pg/mL tube,
  ➢ mix well and transfer 50 µL of the 80 pg/mL standard to 16 pg/mL tube,
  ➢ mix well and transfer 50µL of the 16 pg/mL standard to the 3.2 pg/mL tube and mix well.
- The 0 pg/mL standard (Background) will be Assay Buffer.

Scheme:

Standard Concentration (pg/mL)	Volume of Deionized Water to Add	Volume of Standard to Add
10,000	250 µL	0

Standard Concentration (pg/mL)	Volume of Assay Buffer to Add	Volume of Standard to Add
2,000	200 µL	50 µL of 10,000 pg/mL
400	200 µL	50 µL of 2000 pg/mL
80	200 µL	50 µL of 400 pg/mL
16	200 µL	50 µL of 80 pg/mL
3.2	200 µL	50 µL of 16 pg/mL
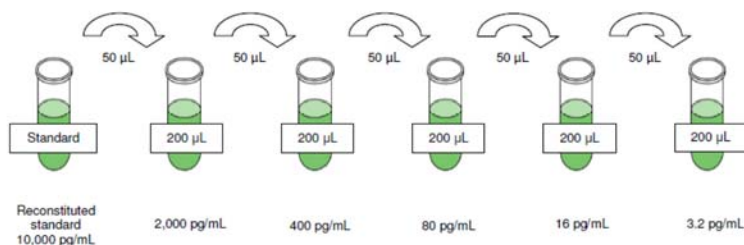
4.5 Immunoassay Procedure

Prior to beginning this assay, it is imperative to read this SOP completely and to thoroughly understand the Technical Guidelines.

- Allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- Diagram the placement of Standards [0 (Background), 3.2, 16, 80, 400, 2,000, and 10,000 pg/mL], Controls 1 and 2, and samples on Well Map Worksheet in a vertical configuration.
  - ➢ *It is recommended to run the assay in duplicate.*

- Thaw the testing samples <u>OVERNIGHT</u> at 2-8°C on ice.
- Fill a <u>non-testing plate</u> with the samples by adding 100 µl of each sample into the appropriate wells of the empty microtiter plate. Seal the plate with a plate sealer and store in the refrigerator at 2-8°C until transfer of the samples into the cytokine testing plate.
- Start the following preparations:
  - Preparation of Individual Vials of Beads (see instructions above).
  - Preparation of Quality Controls (see instructions above).
  - Preparation of Wash Buffer (see instructions above).
  - Preparation of Human Cytokine Standard (see instructions above).

- Add 200 µL of Wash Buffer into each well of the cytokine testing plate (plate from the kit). Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
- Decant Wash Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
- Add 25 µL of Assay Buffer to the sample wells of the cytokine testing plate.
- Add 25 µL of diluted PBS to the background, standards, and control wells of the cytokine testing plate. Seal the plate with a plate sealer..
- Bring out the non-testing plate from the refrigerator.
- Add 100 µL of each Standard or Control into the appropriate wells of the non-testing plate. Assay Buffer should be used for 0 pg/mL standard (Background).
- Using a multichannel, transfer 25 µl of the samples from the non-testing plate into the cytokine testing according to the worksheets.
  - ➢ Mix the samples thoroughly by aspirating up and down with the pipette 5 times.
- Vortex the Mixing Bottle for 2 minutes and then add 25 µL of the Mixed Beads to each well of the cytokine testing plate.

  - ➢ *During addition of Beads, tilt trough containing beads periodically while adding to wells to prevent settling of beads. Beads are light sensitive, turn off lights as possible.)*
  - ➢ *Once beads are added to the plate wells the plate must be protected from light as much as possible.*

- Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation (600 rpm) on a plate shaker for 16 hours at 2-8°C (cold room).

  - ➢ *An overnight incubation may improve assay sensitivity for some analytes.*

- Unwrap the plate from foil and wash plate following instructions listed below:

  - Rest plate on hand-held magnet for 60 seconds to allow complete settling of magnetic beads. Cover plate with aluminum foil during settling of beads.
  - **<u>Plate remains attached on the hand-held magnet:</u>** remove foil and sealer from the plate.

- Remove well contents by gently decanting the plate in a waste receptacle.
- Gently tap on absorbent pads to remove residual liquid.
- Remove plate from the hand-held magnet.
- Add 200 µL of Wash Buffer to each well.
- Seal, cover with foil and incubate with agitation on a plate shaker at 600 rpm for 30 seconds at room temperature (20-25°C).
- Reattach plate to the hand-held magnet for 60 seconds and repeat wash step once.

- Add 25 µL of Detection Antibodies into each well. (Note: Allow the Detection Antibodies to warm to room temperature prior to addition.)
- Seal, cover with foil and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C). **DO NOT ASPIRATE AFTER INCUBATION.**
- Add 25 µL Streptavidin-Phycoerythrin to each well containing the 25 µL of Detection Antibodies.
- Seal, cover with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).
- Unwrap the plate from foil and wash plate following instructions listed below:

  - Rest plate on hand-held magnet for 60 seconds to allow complete settling of magnetic beads. Cover plate with aluminum foil during settling of beads.
  - **Plate remains attached on the hand-held magnet:** remove foil and sealer from the plate.
  - Remove well contents by gently decanting the plate in a waste receptacle.
  - Gently tap on absorbent pads to remove residual liquid.
  - Remove plate from the hand-held magnet.
  - Add 200 µL of Wash Buffer to each well.
  - Seal, cover with foil and incubate with agitation on a plate shaker at 600 rpm for 30 seconds at room temperature (20-25°C).
  - Reattach plate to the hand-held magnet for 60 seconds and repeat wash step once.

- Add 150 µL of Sheath Fluid (or Drive Fluid if using MAGPIX®) to all wells. Resuspend the beads on a plate shaker for 10 minutes.
  - *IF plate cannot be processed immediately, no Sheath Fluid is added.* Store plate sealed and wrapped in aluminum foil at *2-8°C.*
  - *Resuspend the beads on a plate shaker for 10 minutes before running the plate on the Bioplex array reader.*

- Run plate on Bio-Plex 200 system.
- Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic (5PL) method for calculating cytokine/chemokines concentrations in samples.

4.6 Bio-Plex 200 System start up

Machine may be started up while plate is on shaker after adding the Detection Antibodies to the wells. Start up takes approximately 10 minutes.

1. Empty waste bottle. Recap the bottle and close firmly.
2. Fill sheath fluid bottle to fill line if needed. Recap the bottle and close firmly.

3. Turn on Bio-Plex 200 system via the two switches at the backside of the instrument. Switch on the PC.
4. Login to computer and start software. Click "Start Up" and follow on screen instructions; by filling the 70% isopropanol and deionized water reservoirs to run the start up procedure. Eject plate holder, insert calibration plate and click "OK".
5. While start up runs, open a new experiment by clicking top left blank template icon.
   - Select "2. Select Analytes" and choose all analytes to be analyzed. Confirm the regions for each analyte using the bead bottles (see list of beads to be tested above_open file: FEM-PrEP HCYTOMAG with Rantes).
   - Select "3. Format Plate" and set up plate to match worksheet including blanks, standards, controls and samples.
   - Select "4. Enter Standards Info" and enter description and concentrations of standards.
     - Ensure "same concentration values for all analytes" box is checked.
     - Ensure regression type is "logistic – 5PL".
     - Enter "pg/ml" in concentration units field.
   - Not necessary to add any information to "5. Enter Control Info" screen.
   - Select "6. Enter Sample Info" and enter or copy from excel lab numbers of samples into description field. Dilution column should all be 1.0.
     - Review the lab numbers to ensure no transcription errors are present if manually entered.
   - Select "7 Run Protocol": Enter "50" in "Beads per region" field. Click "Advanced Settings" and enter "100 µL" in "sample volume" field and click "override gates" box. Enter "4500" in low box and "20,000" in high box. Thick: Autosave after run. Be sure to save the protocol set-up to the hard drive.

4.6.1 Alternative for programming a new run

1. Select "Open Protocol": Open a previously programmed template with the same testing format.
2. Select "6: Enter Sample Info" and enter or copy from excel lab numbers of samples into description field. Dilution column should all be 1.0.
   - Review the lab numbers to ensure no transcription errors are present.
3. Select "7 Run Protocol". Check the following:
   - "50" in "Beads per region" field.
   - Click "Advanced Settings": "100 µL" in "sample size" field.
   - Click "override gates" box: "4500" in low box and "20,000" in high box and then confirm with "Yes".
4. Tick " Rerun/Recovery Mode.
5. Tick all boxes in the "WELL" field.
6. Save as new protocol.
7. Click START to initiate the run.

4.7 Optics Warm Up

To ensure accurate and reproducible results, the optics (i.e., the lasers) in the Bio-Plex array reader must warm up for at least 30 minutes prior to calibration and reading assays. Optics warm up begins when you first turn on the array reader.

4.8 Calibration

Before calibrating, make sure that optics warm up is complete.
- Remove calibration beads from refrigerator and confirm these are within the expiry window.
- Vortex red and green top calibration beads for 2 minutes.
- Select "Calibrate" in top right corner and follow on screen instructions.
- Add 5 drops of each calibration bead to the calibration plate.
- Add distilled water to the calibration plate.

➢ *The Bio-Plex array reader should be calibrated each day after the start up procedure is complete and the optics have warmed up. Also, the array reader must be re-calibrated if the instrument temperature changes by more than 2º C during the course of the day. The array reader includes a temperature gauge; if the temperature changes by more than 2º C, a message box will prompt you to recalibrate.*

4.9 Plate analysis

- Insert test plate into machine. Remove adhesive plate cover after placing plate in holder.
- Click "start". Run will take 45-60 minutes.
- Once the run is complete, evaluate standard curves, quality controls and select output data.

4.9.1 Evaluate standard curve
- Choose red check box icon and select columns for data export including: Type, Well, Outlier, Description, FI, FI-background, Std Dev, and %CV, Obs conc, Observed concentration x 100.
- Ensure "save settings" is clicked.
- Click "show replicates" icon (automatic output is average of replicate observations).
- Review "observed concentration x 100" column. The values should be close to 100%. (Note: MIP-1α often plateaus).
- Modify the standard curve calculation by checking "outlier" as needed to exclude selected observations from the calculation. Exclude as few values as necessary.
- View standard curve by clicking on "Standard Curve" on left side of screen.
- Repeat steps for each analyte.
- Save the results file to the hard drive.
- Eject plate from Bio-Plex 200 system and attach used plate to magnet, allow to stand for 60 seconds and dump contents. Store plate wrapped in aluminum foil at 2-8°C overnight.
- Perform shut down procedure after last run.

4.9.2 Review quality control data
Compare quality control values against the ranges provided in the kit. Note any values that are out of range when sending results to FHI 360.

4.9.3 Export data

Export to Excel and select "multiple analyte" and save to hard drive.

4.9.4 <u>Wash between plates</u>

To wash the fluidics lines between each plate reading to prevent traces of sample or other debris from building up inside the system.

- *This actions needs to be done before each new plate reading is started.*
- Click "Wash Between Plates" button and follow on screen instructions; by filling the 10% isopropanol and deionized water reservoirs to run the procedure. Eject plate holder, insert (MCV) calibration plate and click "OK".

4.10 Shut down procedure

- Click "Shut down" and follow on screen instructions; by filling the 10% bleach and deionized water reservoirs to run the shut down procedure. Eject plate holder, insert calibration plate and click "OK".
- Turn off Bio-Plex 200 system (two switches) and switch off the PC.
- Empty waste bottle. Recap the bottle and close firmly.
- Fill sheath fluid bottle to fill line if needed. Recap the bottle and close firmly.
- Wash calibration plate (i.e. MVC plate) by running water over plate at an angle.
- Dry plate and return to storage compartment of the Bio-Plex 200 system.

5 Quality control

5.1 Internal quality control
- Send standard curve data to Lindi Roberts for quality assurance review.

9 Reporting
- All raw data is send to Jen Deese (FHI) for further analysis.

10 Storage of samples
- Aliquots of PBS are stored at -80°C ± 5°C as long as needed for the study.

11 Training
- The trainee follows the complete procedures with an experienced person and then makes an analysis on a blind panel.

14 Safety and environment
- See WebIso: safety and environment

15 Attachments and forms for completion
- Worksheet Human Cytokine/Chemokine Magnetic Bead Assay
- MILLIPLEX MAP Kit: Human Cytokine/Chemokine Magnetic Bead Panel

16 Revision

Changes with respect to the previous published version:	New Sop

17 Approval and distribution

	Name and function
Initiated by:	Jen Deese Lindi Roberts Saïd Abdellati
Approved by:	
Manual distribution:	

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