Cervical Inflammation and Immunity Associated With Hormonal Contraception, Pregnancy, and HIV-1 Seroconversion

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Objective: Hormonal contraception (HC), younger age, and pregnancy have been associated with increased HIV risk in some studies. We sought to elucidate the biological mechanisms for these associations.

Design: Case–control selection of specimens from a large, prospective, clinical study.

Methods: We enrolled and followed 4531 HIV-negative women from Uganda and Zimbabwe using either the injectable depo-medroxyprogesterone acetate (DMPA), combined oral contraception, or no HC (NH). Innate immunity mediators were measured in cervical samples collected from women at their visit before HIV seroconversion (n = 199) and matched visits from women remaining HIV uninfected (n = 633). Generalized linear models were applied after Box–Cox power transformation.

Results: Higher RANTES and lower secretory leukocyte protease inhibitor (SLPI) levels were associated with HIV seroconversion. DMPA users had higher RANTES and lower BD-2 levels. Most inflammation-promoting and/or inflammation-inducible mediators were higher [interleukin (IL)-1β, IL-6, IL-8, MIP-3α, vascular endothelial growth factor, and SLPI], and the protective BD-2 and IL-1RA:IL-1β ratio were lower among combined oral contraception users. Pregnant women showed a similar cervical immunity status (higher IL-1β, IL-6, IL-8, vascular endothelial growth factor, SLPI, and IL-1RA; lower IL-1RA:IL-1β). Age <25 years was associated with lower SLPI, IL-8, MIP-3α but higher IL-1RA:IL-1β. Zimbabwean women (with higher HIV seroconversion rates) had overall higher pro-inflammatory and lower anti-inflammatory protein levels than Ugandan women.

Conclusions: HC use, pregnancy, and young age alter cervical immunity in different ways known to increase risk of HIV, for example, through increased levels of pro-inflammatory cytokines or decreased levels of SLPI. Higher levels of RANTES may be one factor underlying a possible association between DMPA use and risk of HIV acquisition.

Key Words: HIV-1, hormonal contraception, cervical inflammation, immunity, pregnancy

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INTRODUCTION

Hormonal contraception (HC), including the 3-monthly progestin injectable depo-medroxyprogesterone acetate (DMPA) and combined oral contraceptives (COCs), and pregnancy have been associated with increased HIV acquisition and other sexually transmitted infections (STIs) in some studies,[1–6] but not others.[7–9] One prospective study supplemented with molecular analysis of transmitted HIV indicated that HC might also contribute to higher risk of HIV transmission to the male partner.[5]

Although the evidence remains inconclusive,[10,11] over 150 million women worldwide use HC. The most commonly

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C.M. and R.F. have contributed equally. Designed the study: C.S.M., R.F., C.M., P.C., and G.D. Analyzed the HC-HIV data: P.C., C.K., C.S.M., R.F., C.M., and G.D; Conducted the measurements of biomarkers: R.F. Wrote the first draft of the article: C.S.M., R.F., and P.C. Contributed to the writing of the article: C.M., G.D., C.K., T.C., and R.S. Agree with the article's results and conclusions: C.S.M., R.F., C.M., P.C., C.K., T.C., R.S., and G.D.

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used contraceptive in sub-Saharan African countries is DMPA. In February 2012, the World Health Organization issued a technical statement that "women at high risk of HIV can continue to use all existing hormonal contraceptive methods without restriction…" but "Because of the inconclusive nature of the body of evidence on progestogen-only injectable contraception and risk of HIV acquisition, women using progestogen-only injectable contraception should be strongly advised to also always use condoms, male or female, and other preventive measures."[11] Unfortunately, many women have limited control over condom use and thus are unable to adopt the World Health Organization recommendation.

The HC-HIV study was the largest prospective study specifically designed to examine the association between HC and HIV.[1] It was a multicenter cohort study in Uganda, and Zimbabwe, with over 4500 women followed quarterly for up to 24 months or until HIV acquisition.[9] The study found that women who used DMPA, but not COCs, were at significantly increased risk of HIV acquisition compared with women not using HC.[1] Data and stored samples from this study were available for more in-depth evaluation and were used in the analysis reported here.

Biomarkers of cervical inflammation and immunity are influenced by the hormonal changes associated with the menstrual cycle[12–16]; however, limited data exist on how HC and pregnancy affect them. The stored HC-HIV study samples provided a rare opportunity to assess innate immunity factors that may predispose to HIV acquisition in the context of hormonal contraceptives. The objectives of this study were 3-fold: (1) to characterize normative baseline levels of cervical mucosal immunity mediators in HIV-uninfected women, (2) to evaluate their association with the risk of HIV acquisition, and (3) to assess their association with HC and pregnancy. Understanding how HC interacts with immunity, and especially the local mucosal environment, to affect HIV acquisition would allow for more effective contraceptive guidelines and public health policies.

METHODS

Study Population and Variables

Data and samples were from a longitudinal study (HC-HIV study) of HC—either DMPA (150 mg, every 3 months) or COC (30 μg ethinylestradiol and 150 μg levonorgestrel)—and HIV acquisition.[9] We enrolled 4531 HIV-uninfected women ages 18–35 years from family planning clinics in Uganda and Zimbabwe.[9] Contraceptive group was assigned based on the primary contraceptive method women used during the time between their previous study visit and the selected visit. Women in the nonhormonal group used only condoms or no contraception. Participants were seen every 12 weeks for up to 24 months. We identified 199 women with incident HIV infection as cases (51 Ugandan and 148 Zimbabwean) and matched 633 HIV-uninfected controls (up to 4 controls per case) on study site, age, a composite STI variable (presence of *Chlamydia trachomatis* or *Neisseria gonorrhoeae* infection or bacterial vaginosis), and time in study.[17] Samples were taken from the visit immediately before HIV seroconversion (on average of 12 weeks before seroconversion) or the matched visit for uninfected controls.

The study protocol received a nonhuman subject determination (use of de-identified data) from the Office of International Research Ethics at FHI 360 and the Institutional Review Board at Brigham and Women's Hospital.

Sample Collection and Processing

This study used residual endocervical swab specimens collected in Uganda and Zimbabwe to determine infection by *C. trachomatis* and *N. gonorrhoeae*, and stored frozen after the STI diagnosis was completed.[18] Women were asked to abstain from sexual intercourse 48 hours before specimen collection. No specimens were collected during menstrual bleeding, and friable cervix/visible blood was rarely recorded during swab collection. The swab collection followed the Roche Diagnostics protocol. In brief, mucus and loose cellular material was first removed from the ectocervix with one of the large swabs provided in the Roche Amplicor packet (Roche Diagnostics, Indianapolis, IN). A second swab was inserted into the endocervical canal, gently rotated 3–5 seconds, and withdrawn avoiding contact with vaginal surfaces. The swab was placed in 1 mL Amplicor lysis medium and after agitation discarded along with any excess mucus in the specimen. The lysis buffer contained proteinase K among other enzymes intended to degrade any residual mucus to provide access to bacterial DNA in the sample. After removal of the swab, each sample was mixed with 1 mL diluent (Roche Diagnostics) and processed for STI diagnosis per manufacturer's instruction. Leftovers of the Amplicor-extracted solutions were shipped on ice to Johns Hopkins Bloomberg School of Public Health (Baltimore, MD) for storage at −80°C until shipped to the Laboratory of Genital Tract Biology, Brigham and Women's Hospital, they were aliquoted and stored at −80°C in air-tight coded micronic tubes (USA Scientific Inc., Ocala, FL) until analyzed.

Measurement of Biomarkers

Interleukin (IL)-1β, IL-1RA, IL-6, IL-8, RANTES, MIP-3α, vascular endothelial growth factor (VEGF), and soluble ICAM-1 were measured in samples diluted 2-fold using a custom-designed Meso Scale Discovery multiplex and Sector Imager 2400 (Meso Scale Discovery, Gaithersburg, MD). The multiplex was optimized to detect each biomarker within the linearity concentration range of the cervical swab elutions. The lower limits of detection (LLD) were as follows: IL-1β = 1.2 pg/mL, IL-1RA = 0.16 ng/mL, IL-6 = 1.7 pg/mL, IL-8 = 0.8 pg/mL, RANTES = 1.8 pg/mL, MIP-3α = 16.4 pg/mL, VEGF = 0.12 ng/mL, and sICAM-1 = 3.6 pg/mL, secretory leukocyte protease inhibitor (SLPI) (LLD = 0.46 ng/mL, minimum dilution 80-fold) and BD-2 (LLD = 12.2 pg/mL, minimum dilution 25-fold) were measured by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, and Phoenix Pharmaceuticals, Burlingame, CA, respectively). All samples showed values above the detection range. Each sample was tested in duplicate and normalized to average milligram total protein concentration obtained from duplicate measurement using the BCA protein assay. Enzyme-linked immunosorbent assay and BCA assays

were read using Victor2 (Perkin Elmer, Boston, MA). The coefficient of variation of duplicate values obtained by this method was <10%. A quality control sample was split and tested on each assay plate showing interplate variation of <25%. The Amplicor lysis buffer and diluent were spiked with known concentrations of the test proteins, and no significant interference with the immunoassay detection was confirmed. Although the use of the Amplicor lysis buffer precluded removal of cells by centrifugation of the original cervical swab extracts, the contribution of cellular content was minimized because of the gentle noninvasive fashion of collecting the swabs after the removal of mucus and loose cellular material. Moreover, the normalization to total protein levels allowed comparisons of the total levels of immune mediators available in the cervical compartment of each subject, and the potential effects of random variation in cell numbers were diminished by the large sample size of the study.

Statistical Analysis

We compared demographic characteristics among HC exposure groups using Cochran–Mantel–Haenszel or Fisher exact tests. Descriptive statistics including medians and interquartile ranges were used to summarize nontransformed biomarker levels for each characteristic and to compare the differences among HC group using the Wilcoxon–Mann–Whitney test. Biomarker concentrations were not normally distributed; to allow for better comparison of biomarker data among HC exposure groups, we used Box–Cox power transformation[19] to make the data more normal and used generalized multivariable linear models to incorporate covariates into the analysis. Covariates evaluated for their impact on biomarker levels included HIV status, HC, age, country, pregnancy, breastfeeding, number of sexual partners, number of unprotected sexual acts, current STIs/RTIs [bacterial vaginosis (BV), candidiasis, chlamydia, gonorrhea, herpes simplex virus-2 infection, trichomoniasis], STI signs and symptoms, and vaginal practices. P values <0.05 were considered statistically significant. Statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC).

RESULTS

NH and DMPA users were more prevalent in Zimbabwe, whereas COC users were more prevalent in Uganda (Table 1). As expected, pregnancy was most prevalent among the NH group and breastfeeding was most prevalent in DMPA users. NH users were least likely to report unprotected sex but had multiple sex partners and were STI/RTI-infected more often than the other groups. Hormonal contraceptive use was similarly distributed among cases and controls. However, these data do not contradict the major findings of the HC-HIV study where higher risk of HIV among DMPA users was found, because not all HC-HIV study participants (rather only cases and matched controls) were included in the present biomarker substudy and only crude results related to the HC and HIV acquisition relationship are presented in Table 1.

We found differences in biomarker concentrations between women becoming HIV infected versus those remaining uninfected by contraceptive method, age, and geographic location (Table 2). In contrast to women remaining HIV uninfected, women becoming HIV infected had higher levels of RANTES and BD-2 and lower levels of SLPI.

COC use compared with NH use and pregnancy compared with no pregnancy were associated with higher pro-inflammatory and lower anti-inflammatory mediator levels despite lower prevalence of STI/RTIs. Compared with NH users, COC users had higher levels of 6 of 10 inflammation-associated proteins measured (pro-inflammatory IL-1β, IL-6, IL-8, MIP-3α, VEGF, and anti-inflammatory SLPI) but lower levels of anti-inflammatory/protective IL-1RA:IL-1β ratio and BD-2. Pregnancy was associated with a similar pattern—higher levels of all pro-inflammatory markers except MIP-3α, and a lower IL-1RA:IL-1β ratio; however, unlike COC, pregnancy was associated with significantly higher levels of the anti-inflammatory mediator IL-1RA (suggesting that the decrease in the IL-1RA:IL-1β ratio was because of disproportional elevation in IL-1β) and no significant difference in SLPI or BD-2 levels.

In marked contrast to COCs, DMPA was associated with higher RANTES and ICAM-1 and lower IL-1RA levels. The only similarity between DMPA and COC users was a lower BD-2 level compared with the NH group.

Breastfeeding women had lower levels of most pro-inflammatory mediators (IL-1β, IL-6, IL-8, and MIP-3α) and lower IL-1RA and SLPI than non-breastfeeding women (Table 2). Younger women (<25 years) had lower levels of IL-8, MIP-3α, and SLPI compared with older women. Compared with Ugandans, Zimbabwean women had higher IL-6, IL-8, RANTES, MIP-3α, ICAM-1, and SLPI and lower IL-1RA, IL-1RA:IL-1β, and BD-2 levels.

Differences in levels of cervical immunity biomarkers (in pg/mL adjusted to mg/mL of total protein) were examined in multivariable modeling controlling for behavioral risk factors and STI/RTIs (see Methods). These analyses confirmed that HIV seroconversion was associated with higher RANTES and lower SLPI levels (Table 3). Also, with the exception of RANTES and ICAM-1, all pro-inflammatory or inflammation-induced mediators (IL-1β, IL-6, IL-8, MIP-3α, VEGF, and SLPI) were higher in COC users, and the protective anti-inflammatory IL-1RA:IL-1β ratio and BD-2 were decreased in the COC group. Again, DMPA use was associated with increased RANTES and reduced BD-2 levels while the profile of immuno-inflammatory mediators in pregnancy was similar to that of COC use. Multivariable modeling also confirmed the age-related and geographic differences (Table 3).

To further investigate the relationship between HC and pregnancy and immuno-inflammatory mediators, we considered the subset of 718 specimens that could be categorized into mutually exclusive contraceptive/pregnancy groups: COC use but not pregnant/breastfeeding, DMPA use but not pregnant/breastfeeding, pregnant but no HC use or breastfeeding, and breastfeeding but no HC use or pregnancy, each compared with no HC use, and not pregnant/breastfeeding. We found very similar results to those reported in Table 3 with the exception that COC use was positively associated with IL-1RA (0.02, 95% CI: 0.00 to 0.03), DMPA use was positively associated with ICAM-1 (0.78, 95% CI: 0.27 to 1.29), pregnancy was no longer significantly associated with

TABLE 1. Participants' Characteristics by Hormonal Contraceptive Use

Characteristics	Majority COC (n = 299) n (%)	Majority DMPA (n = 307) n (%)	Majority NH (n = 226) n (%)	Total (n = 832) n (%)	P*†
HIV status					
Becoming HIV+	61 (20)	74 (24)	64 (28)	199 (24)	
Remaining HIV−	238 (80)	233 (76)	162 (72)	633 (76)	0.109
Country					
Zimbabwe	64 (21)	236 (77)	150 (66)	621 (75)	
Uganda	235 (79)	71 (23)	76 (34)	211 (25)	0.003
Age at screening, yrs					
18–24	164 (55)	172 (56)	121 (54)	457 (55)	
25–35	135 (45)	135 (44)	105 (46)	375 (45)	0.85
At visit before HIV seroconversion or matched visit for HIV uninfected					
Pregnant	8 (3)	2 (1)	31 (14)	41 (5)	<0.001
Breastfeeding	14 (5)	80 (26)	39 (17)	133 (16)	<0.001
≥2 sexual partners	7 (2)	7 (2)	11 (5)	25 (3)	0.158
No unprotected sex or no sex acts	43 (14)	63 (21)	128 (57)	234 (28)	<0.001
Herpes simplex virus-2 seropositive	172 (58)	172 (56)	148 (66)	492 (60)	0.051
Any RTI/STI positive	233 (80)	230 (76)	193 (87)	656 (80)	0.007
No. unprotected acts					
15+	87 (29)	64 (21)	26 (12)	177 (21)	
8–14	100 (33)	84 (27)	24 (11)	208 (25)	
1–7	69 (23)	96 (31)	48 (21)	213 (26)	
0 (all acts protected by condoms or no sex acts)	43 (14)	63 (21)	128 (57)	234 (28)	<0.0001

RTI/STI, current reproductive tract or sexually transmitted or infections (bacterial vaginosis, candidiasis, chlamydia, gonorrhea, genital herpes, trichomoniasis).
*Cochran–Mantel–Haenszel test.
†Fisher exact test for categorical variables; the comparison of *P* value shows differences between the COC, DMPA, and NH.

SLPI (2.32, 95% CI: −0.69 to 5.34), and breastfeeding no longer significantly associated with IL-6 (0.12, 95% CI: −0.03 to 0.28) or MIP-3α (−0.02, 95% CI: −0.13 to 0.10) but significantly associated with VEGF (0.06, 95% CI: 0.01 to 0.10) and IL-1RA (0.02, 95% CI: 0.00 to 0.04).

Because we had previously found strong modification by age (18–24 vs. ≥25 years) on the HC-HIV acquisition relationship,[1] we examined whether age significantly modified levels of immuno-inflammatory mediators among hormonal contraceptive users. The only significant interaction we found was for RANTES. The difference in RANTES between COC and NH users was greater among younger than older women (0.32, 95% CI: 0.08 to 0.57). SLPI was reduced (but not statistically significantly) between younger COC (−1.19, 95% CI: −3.06 to 0.67) and DMPA (−1.31, 95% CI: −3.10 to 0.48) users.

Of the 832 participants, 764 (91.8%) reported the same contraceptive method throughout the risk period (from the visit before seroconversion to the seroconversion visit). The relationship between HC use and cervical immunity biomarkers recalculated for these 764 women was similar to the original results except that for COC users IL-1RA became statistically significant (0.01, 95% CI: 0.00 to 0.02) (Table 3).

DISCUSSION

The HC-HIV study provided a valuable opportunity to assess immune factors that may predispose to HIV acquisition in hormonal contraceptive users. Data exist on differences between women who were already HIV infected compared with HIV-uninfected women, but only a few studies have examined a limited number of innate immune differences, for example, NK cells[19] or bactericidal activity,[20] between HIV-uninfected women who later acquired HIV versus those remaining HIV uninfected.

It is important to note that all 10 inflammation-associated innate immunity proteins we assessed can be induced by inflammatory conditions as part of the protective role of inflammation in clearing bacterial and viral infections. However, while 7 of those proteins (IL-1β, IL-6, IL-8, RANTES, MIP-3α, ICAM-1, and VEGF) can initiate and/or perpetuate inflammatory tissue damage, the other 3 (IL-1RA, BD-2, and SLPI) are regarded as purely protective because they attempt to counterbalance the inflammatory reaction to avoid excessive inflammatory tissue damage (IL-1RA and SLPI) and/or have direct microbicidal properties (SLPI and BD-2). Thus, the fact that Zimbabwean women had a higher rate of HIV seroconversion despite relatively higher SLPI levels as compared with Ugandan women may at first seem paradoxical; however, the higher SLPI levels in these women are consistent with the overall heightened inflammatory state with higher levels of IL-6, IL-8, RANTES, MIP-3α, and ICAM-1 combined with lower levels of IL-1RA, IL-RA: IL-1β ratio, and BD-2 suggesting a deficiency in counterbalancing the inflammatory tissue reaction, which logically puts them at higher risk of HIV despite higher SLPI. Our data

TABLE 2. Protein Levels (Median, 25th–75th Interquartile Range) by Subgroups Compared by Wilcoxon–Mann–Whitney Test

	Pro-inflammatory Biomarkers*					
Characteristics	**IL-1ß**	**IL-6**	**IL-8**	**RANTES**	**MIP-3α**	**ICAM-1**
All Women	5.1 (3–9)	20.1 (12–35)	845.5 (388–1750)	42.6 (19–100)	359.3 (194–718)	530.2 (274–806)
HIV Status						
Becoming HIV+	5.0 (3–8)	18.1 (13–33)	686.7 (380–1564)	63.2 (27–117)†	329.2 (205–645)	533.7 (272–843)
Remaining HIV–	5.1 (3–9)	20.7 (12–37)	881.1 (397–1798)	37.8 (18–95)	372.5 (193–730)	529.2 (274–795)
Country						
Zimbabwe	5.1 (3–8)	21.2 (13–37)†	914.0 (477–1773)†	49.8 (23–119)‡	403.9 (208–782)‡	553.3 (304–858)‡
Uganda	5.0 (3–9)	16.9 (11–32)	600.4 (264–1670)	26.4 (14–65)	302.3 (163–525)	430.1 (214–720)
Age, yrs						
18–24	4.9 (3–9)	18.8 (12–35)	713.0 (360–1749)§	40.4 (19–102)	326.7 (185–635)†	535.8 (269–793)
25–35	5.2 (3–8)	21.4 (14–35)	929.7 (477–1751)	43.6 (20–95)	434.8 (209–786)	519.8 (280–823)
Contraception‖						
COC	6.0 (3–10)†	24.4 (15–44)‡	1034.3 (540–2373)‡	33.5 (18–81)	514.8 (250–975)‡	490.7 (292–767)
DMPA	4.5 (3–7)	18.3 (12–32)	725.5 (325–1477)	74.2 (32–145)‡	315.0 (169–572)	604.2 (296–967)†
Nonhormonal	5.1 (3–9)	18.1 (12–30)	719.7 (348–1564)	31.3 (15–67)	309.4 (185–609)	490.9 (250–768)
Pregnant						
Yes	8.4 (4–15)‡	29.7 (16–54)†	1603.6 (748–3469)‡	21.7 (15–32)‡	435.4 (205–771)	379.5 (211–613)§
No	5.0 (3–8)	19.7 (12–34)	812.2 (382–1670)	45.5 (19–102)	358.1 (193–708)	536.4 (278–820)
Breastfeeding						
Yes	4.1 (3–6)§	17.1 (11–26)†	705.0 (413–1138)§	55.6 (23–120)	283.9 (175–474)‡	557.7 (268–858)
No	5.2 (3–9)	21.0 (13–38)	892.0 (384–1891)	40.2 (19–98)	402.2 (205–754)	521.6 (277–798)

	Pro-inflammatory Biomarkers*	Anti-inflammatory and Microbicidal Biomarkers*				
Characteristics	**VEGF**	**IL-1RA**	**Ratio IL-1RA: IL-1ß**	**SLPI**	**β-Defensin-2**	**Total Protein (mg/mL)**
All Women	1331.5 (958–1943)	968.9 (711–1245)	188.2 (126–268)	165,447 (61,235–373,282)	1489.8 (485–4359)	0.6 (0.5–0.8)
HIV Status						
Becoming HIV+	1302.5 (907–1764)	952.1 (719–1212)	204.6 (128–273)	126,691 (45,708–291,764)‡	1881.5 (843–5935)†	0.7 (0.5–0.8)
Remaining HIV–	1351.4 (970–2021)	971.7 (706–1258)	183.2 (126–266)	182,937 (65,173–401,951)	1386.5 (435–3712)	0.6 (0.5–0.8)
Country						
Zimbabwe	1335.2 (993–1908)	941.6 (681–1234)†	183.1 (122–261)†	198,977 (73,716–420,564)‡	1393.5 (469–3980)§	0.6 (0.5–0.8)§
Uganda	1300.1 (882–2084)	1012.4 (779–1323)	209.4 (131–327)	111,161 (43,425–241,100)	1795.1 (572–5414)	0.6 (0.5–0.8)
Age, yrs						
18–24	1317.9 (915–2005)	984.8 (707–1258)	193.5 (126–281)	154,654 (58,335–346,684)§	1449.7 (523–4079)	0.6 (0.5–0.8)
25–35	1363.7 (992–1886)	942.6 (713–1238)	181.0 (124–259)	183,277 (63,841–418,135)	1508.2 (453–4851)	0.6 (0.5–0.8)
Contraception‖						
COC	1446.4 (1050–2241)†	1013.6 (764–1290)	159.3 (116–258)†	273,670 (105,432–520,063)‡	1483.0 (533–3552)†	0.6 (0.5–0.8)
DMPA	1227.0 (878–1745)	868.0 (642–1216)§	200.7 (135–271)	123,537 (44,700–300,730)	1157.9 (407–3556)‡	0.7 (0.5–0.9)‡
Nonhormonal	1266.1 (968–1854)	981.7 (728–1241)	200.0 (124–288)	145,876 (55,088–311,595)	2232.7 (592–7365)	0.6 (0.5–0.8)
Pregnant						
Yes	2279.6 (1042–2861)†	1068.9 (832–1645)†	132.9 (91–233)§	212,120 (87,132–472,264)	2155.3 (481–6410)	0.6 (0.5–0.8)
No	1325.5 (956–1888)	964.0 (701–1240)	189.4 (127–269)	157,933 (59,795–370,555)	1468.2 (486–4282)	0.6 (0.5–0.8)
Breastfeeding						
Yes	1298.4 (917–1802)	841.9 (638–1148)†	197.9 (137–267)	99,238 (42,950–193,867)‡	1393.5 (392–5514)	0.7 (0.5–0.8)
No	1358.3 (964–2018)	981.2 (721–1273)	182.7 (124–269)	188,482 (68,467–412,418)	1491.9 (523–4262)	0.6 (0.5–0.8)

*Concentration values for each biomarker are presented in pg/mL adjusted to mg/mL total protein.
†$P < 0.01$.
‡$P < 0.001$.
§$P < 0.05$.
‖Nonhormonal is the reference comparison group.

TABLE 3. Summary of Final Multivariable Models for Cervical Immunity Biomarkers*

Characteristics	Differences in Mean Levels of Pro-inflammatory Markers (95% CI)†				
	IL-1ß	IL-6	IL-8	RANTES	MIP-3α
HIV status					
Becoming HIV+ (ref: remaining negative)	0.00 (−0.07 to 0.08)	−0.00 (−0.08 to 0.06)‡	−0.17 (−0.57 to 0.23)	0.25 (0.06 to 0.44)§	−0.00 (−0.05 to 0.05)
Country					
Zimbabwe (ref: Uganda)	0.03 (−0.05 to 0.10)	0.11 (0.04 to 0.18)‖	0.59 (0.19 to 0.98)‖	0.56 (0.40 to 0.73)¶	0.12 (0.06 to 0.17)¶
Age at screening					
18–24 yrs (ref: 25–35 yrs)	−0.06 (−0.13 to 0.01)	−0.05 (−0.11 to 0.02)	−0.47 (−0.82 to −0.13)‖	0.05 (−0.10 to 0.21)	−0.05 (−0.10 to −0.01)§
Majority HC use					
COC (ref: NH)	0.12 (0.02 to 0.21)§	0.18 (0.09 to 0.26)¶	1.05 (0.60 to 1.50)¶	0.08 (−0.11 to 0.27)	0.12 (0.06 to 0.18)¶
DMPA (ref: NH)	−0.03 (−0.13 to 0.06)	0.04 (−0.04 to 0.12)	0.17 (−0.27 to 0.62)	0.64 (0.44 to 0.84)¶	−0.03 (−0.08 to 0.03)
At visit before HIV seroconversion or matched for HIV-uninfected controls					
Pregnant (ref: nonpregnant)	0.31 (0.15 to 0.47)¶	0.31 (0.16 to 0.46)¶	1.73 (0.90 to 2.55)¶	−0.26 (−0.57 to 0.06)	0.05 (−0.05 to 0.16)
Breastfeeding (ref: non-breastfeeding)	−0.05 (−0.14 to 0.05)	−0.09 (−0.17 to −0.00)§	−0.04 (−0.52 to 0.45)	−0.04 (−0.26 to 0.18)	−0.07 (−0.14 to −0.01)§

Characteristics	Differences in Mean Levels of Pro-inflammatory Markers (95% CI)†		Differences in Mean Levels of Anti-Inflammatory and Microbicidal Biomarkers			
	ICAM-1	VEGF	IL-1RA	Ratio IL-RA:IL-1ß	SLPI	β-Defensin-2
HIV status						
Becoming HIV+ (ref: remaining negative)	0.15 (−0.27 to 0.57)	−0.01 (−0.03 to 0.02)	0.00 (−0.01 to 0.01)‡	0.08 (−0.25 to 0.41)	−1.49 (−2.80 to −0.18)§	0.14 (−0.01 to 0.28)
Country						
Zimbabwe (ref: Uganda)	0.77 (0.32 to 1.21)¶	0.01 (−0.02 to 0.03)	−0.02 (−0.02 to −0.01)‖	−0.44 (−0.77 to −0.10)§	3.80 (2.54 to 5.06)¶	−0.18 (−0.32 to −0.04)§
Age at screening						
18–24 yrs (ref: 25–35 yrs)	−0.02 (−0.39 to 0.35)	−0.02 (−0.04 to 0.01)	0.00 (−0.01 to 0.01)	0.30 (0.02 to 0.58)§	−1.24 (−2.37 to −0.12)§	−0.03 (−0.16 to 0.09)
Majority HC use						
COC (ref: NH)	−0.21 (−0.73 to 0.32)	0.04 (0.01 to 0.06)‖	0.01 (<−0.01 to 0.02)	−0.47 (−0.84 to −0.11)§	3.34 (1.90 to 4.77)¶	−0.21 (−0.37 to −0.05)‖
DMPA (ref: NH)	0.31 (−0.19 to 0.82)	−0.00 (−0.03 to 0.02)	−0.01 (−0.02 to <0.01)	−0.13 (−0.50 to 0.24)	−0.34 (−1.75 to 1.08)	−0.27 (−0.43 to −0.11)¶
At visit before HIV seroconversion or matched for HIV-uninfected controls						
Pregnant (ref: nonpregnant)	−0.73 (1.61 to 0.14)	0.09 (0.04 to 0.13)¶	0.02 (0.01 to 0.04)§	−0.97 (−1.59 to −0.34)‖	2.73 (0.14 to 5.33)§	−0.05 (−0.35 to 0.24)
Breastfeeding (ref: non-breastfeeding)	−0.26 (−0.77 to 0.24)	−0.00 (−0.03 to 0.02)	−0.01 (−0.02 to 0.01)	0.05 (−0.35 to 0.45)	−3.21 (−4.74 to −1.68)¶	−0.02 (−0.19 to 0.15)

*Based on Box–Cox transformation, $Y' = (Y^{\lambda} - 1)/\lambda$, that makes the original biomarker measures, Y, into the data Y' and more normally distributed. Backward selection was used. HIV status, country, age, and contraceptive use were forced into the model; the model adjusted for number of sexual partners and unprotected sex acts, current sexually transmitted or reproductive tract infections (STI/RTI), signs and symptoms of STI/RTI, and vaginal hygiene practices.

†The difference of concentration levels for each biomarker are transformed based on Box–Cox transformation.

‡"0.00" refers to having a small positive difference (eg, 0.002) from the reference group; "−0.00" refers to having a small negative difference (eg, −0.003) from the reference group.

§$P < 0.05$.

‖$P < 0.01$.

¶$P < 0.001$.

support the notion that HIV risk can be increased by multiple separate mechanisms, decompensated inflammation being one of them, and immune suppression exemplified by SLPI decrease being another.

The 10 chosen proteins comply with criteria for biomarkers based on: (1) biological significance—a significant correlation with inflammatory and infectious conditions in the female reproductive tract[21,22]; (2) relevance to the epithelial barrier function—abundant expression by human cervicovaginal epithelial cells in a steady state or in response to inflammatory stimuli[23–27]; (3) reliable measurement in human cervicovaginal secretions[21,22,28]; (4) detectability in the dacron swabs used in this study;[29] and (5) ex vivo stability in the context of cervicovaginal secretions at variable storage temperatures.[21]

Two biomarkers—higher RANTES and lower SLPI—were predictive of HIV seroconversion after controlling for multiple known risk factors. Elevated RANTES was associated with Zimbabwe site—the site with higher HIV seroconversion rates—and DMPA use, also a risk factor for HIV in the HC-HIV study. Lower SLPI was associated with younger age and breastfeeding.

RANTES plays a dual role in HIV infection. It binds CCR5, a coreceptor for HIV, and may competitively inhibit viral entry; however, it also recruits HIV target cells ($CD4^+$ T cells, monocytes, and dendritic cells) to mucosal sites of injury or infection[30] and is elevated in vaginal secretions of women at highest risk of HIV infection.[31] Besides RANTES, the increase of ICAM-1 in DMPA users can facilitate

leukocyte traffic to the mucosal site. In agreement with our findings, DMPA upregulates RANTES in vaginal epithelial cell lines,[32] and a single DMPA injection causes influx of vaginal immune cells.[33] Thus, an increase of cervical RANTES and ICAM-1 presents a plausible biological mechanism for increased HIV acquisition risk among DMPA users. An alternative would be that RANTES and ICAM-1 are increased as an early viral stress response[25] because of sensing viruses before HIV seroconversion.

The immune mediator negatively associated with HIV seroconversion in our study, SLPI, was lower in the DMPA users (although not significantly so). SLPI was lower in breastfeeding and younger women suggesting they might be at higher HIV risk based on a compromised cervical barrier. This agrees with previous findings that SLPI, in both oral and vaginal secretions, seems to limit HIV transmission.[34–37] SLPI levels are reduced in women with BV[31] and *Trichomonas vaginalis* infection[25,38]; both infections have been associated with increased risks of HIV acquisition.[39] In adolescent girls, lower SLPI was associated with HC and BV.[38] In our study, SLPI remained negatively associated with HIV seroconversion even after adjustment for the presence of STI/RTIs.

Most previous epidemiological studies suggest that COCs do not increase risk of HIV acquisition.[40] However, like DMPA, COCs affected the cervical immune environment but in a manner suggesting a pro-inflammatory mucosal state: pro-inflammatory cytokines and chemokines except RANTES and ICAM-1 were higher in COC than in NH users. SLPI, which can be upregulated by inflammatory conditions,[27] was increased in agreement with the pro-inflammatory cytokine activation. We saw a similar profile in pregnant and breastfeeding women, and because both pregnancy and breastfeeding are progesterone-dominated states, our data are consistent with the hypothesis that a progestogen-dominated state influences the local cervicovaginal immune environment. While higher SLPI levels may be regarded as a compensatory reaction and may be protective, the overall pro-inflammatory activation and especially the increase of IL-1β, IL-6, IL-8, and MIP-3α in the vaginal mucosa has been associated with increased numbers of activated immune cells,[26] which facilitate dissemination of the initial population of founder viruses,[41] and with higher vaginal HIV load.[42,43] This heightened pro-inflammatory profile could explain the 2-fold increased risk of HIV transmission in serodiscordant couples during pregnancy[3] and suggests that similar mechanisms could underlie HIV transmission risk in COC users.

The geographic differences in our study provided additional support for the role of immuno-inflammatory mediators in HIV acquisition risk. Similar to COC users and pregnant women, Zimbabwean women had higher pro-inflammatory and lower anti-inflammatory mediator levels than Ugandan women. This heightened inflammatory profile may contribute to the higher HIV infection rates among Zimbabwean women in this cohort.[9] Increased numbers of activated mucosal T cells and reduced SLPI have been described for Kenyan compared with American women.[44] The authors hypothesized that these differences could partially explain higher HIV incidence in the Kenyan women, although it is unclear whether these immune differences are because of genetic, geosocial, or local comorbidity variations.

Few previous studies have examined the relationship between inflammatory markers and COC use and pregnancy. A small study of 16 women using COCs, intravaginal rings, or no HC found that the cervicovaginal lavage (CVL) fluid from HC users inhibited in vitro herpes simplex virus infection to a lesser degree (36%) than CVLs from normally cycling women (57%).[45] However, few significant differences in levels of immuno-inflammatory mediators were found between the small COC, pregnant, and normally cycling subgroups.[45] A study of 70 pregnant and 35 nonpregnant women found that CVL from pregnant women had significantly greater *Escherichia coli* bactericidal activity, higher concentrations of pro-inflammatory cytokines, and lower levels of beta defensins than nonpregnant controls.[46] The authors hypothesized that the observed changes during pregnancy might protect against colonization and ascending infection. However, these same changes might put women at higher risk of HIV infection because of increased recruitment and activation of target cells. In the HC-HIV study, we found no significant increase in HIV acquisition among COC users[1,9] or pregnant women.[47] Perhaps, the high levels of SLPI, an innate virucidal peptide, in COC users and during pregnancy counterbalanced the increase of pro-inflammatory mediators. The mucosal inflammatory state might still lead to increased transmission risk to male partners among HIV-infected women.

Our study has numerous strengths. First, we had large numbers of cervical specimens from women with documented HIV seroconversion and from matched uninfected women. Second, we had fairly even distribution of women using DMPA, COCs, and no HC. Third, HC and pregnancy were measured in a standardized manner across sites. Finally, our laboratory measures were reliable. All protein analyses were performed under strict standardized procedures in a highly experienced laboratory with documented quality assurance measures. We used a state-of-the-art electrochemiluminescence multiplex platform with high accuracy and precision.[48,49] Biomarker levels in the cervical swab specimens were normalized to total protein levels—a widely accepted method for mucosal secretion assessment.[12,29]

Our study also had limitations. We did not measure immuno-mediators in systemic circulation and cannot evaluate the role of systemic immunity in HIV acquisition. We also had limited information on the vaginal microbiome, although we used a standardized Nugent score to measure BV. The analysis is limited by its cross-sectional nature. As evidenced in Table 1, there were differences in sexual practices and condom use among the 3 contraceptive groups. However, a number of differences that were measured were controlled for (eg, number of unprotected sex acts) in the multivariate analysis presented in Table 3. Although cases seroconverted at the next study visit, we only analyzed biomarkers from the single preceding visit. We are currently conducting a longitudinal analysis using the same study cohorts.

In conclusion, we found that contraceptive method, pregnancy, and lactation affect the cervical immune environment, each in a manner consistent with inflammation-facilitated risk of HIV acquisition. Differences in background levels of inflammation-associated mediators between countries/geographic regions and between young and older women may add to their susceptibility to acquire and transmit HIV infection.

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