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The Contribution of Cervicovaginal Infections to the Immunomodulatory Effects of Hormonal Contraception

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ABSTRACT Particular types of hormonal contraceptives (HCs) and genital tract infections have been independently associated with risk of HIV-1 acquisition. We examined whether immunity in women using injectable depot medroxyprogesterone acetate (DMPA), combined oral contraceptives (COC), or no HCs differs by the presence of cervicovaginal infections. Immune mediators were quantified in cervical swabs from 832 HIV-uninfected reproductive-age Ugandans and Zimbabweans. Bacterial infections and HIV were diagnosed by PCR, genital herpes serostatus by enzyme-linked immunosorbent assay (ELISA), altered microflora by Nugent score, and *Trichomonas vaginalis* and *Candida albicans* infection by wet mount. Generalized linear models utilizing Box-Cox-Power transformation examined associations between levels of mediators, infection status, and HCs. In no-HC users, *T. vaginalis* was associated with broadest spectrum of aberrant immunity (higher interleukin 1β [IL-1β], IL-8, macrophage inflammatory protein 3α [MIP-3α], β-defensin 2 [BD2], and IL-1 receptor antigen [IL-1RA]). In women with a normal Nugent score and no genital infection, compared to the no-HC group, COC users showed higher levels of IL-1β, IL-6, IL-8, and IL-1RA, while DMPA users showed higher levels of RANTES and lower levels of BD2, both associated with HIV seroconversion. These effects of COC were blunted in the presence of gonorrhea, chlamydia, trichomoniasis, candidiasis, and an abnormal Nugent score; however, RANTES was increased among COC users with herpes, chlamydia, and abnormal Nugent scores. The effect of DMPA was exacerbated by lower levels of IL-1RA in gonorrhea, chlamydia, or herpes, SLPI in gonorrhea, and IL-1β, MIP-3α, and IL-1RA/IL1β ratio in trichomoniasis. Thus, the effects of HC on cervical immunity depend on the genital tract microenvironment, and a weakened mucosal barrier against HIV may be a combined resultant of genital tract infections and HC use.

IMPORTANCE In this article, we show that in young reproductive-age women most vulnerable to HIV, hormonal contraceptives are associated with altered cervical immunity in a manner dependent on the presence of genital tract infections. Through altered immunity, hormones may predispose women to bacterial and viral pathogens; conversely, a preexisting specific infection or disturbed vaginal microbiota may suppress the immune activation by levonorgestrel or exacerbate the suppressed immunity by DMPA, thus increasing HIV risk by their cumulative action. Clinical studies assessing the effects of contraception on HIV susceptibility and mucosal immunity may generate disparate results in populations that differ by microbiota background or prevalence of undiagnosed genital tract infections. A high prevalence of asymptomatic infections among HC users that remain undiagnosed and untreated raises even more concerns in light of their combined effects on biomarkers of HIV risk. The molecular mechanisms of the vaginal microbiome's simultaneous interactions with hormones and HIV remain to be elucidated.

Most commonly used hormonal contraceptives (HCs), including the progestin injectable depot medroxyprogesterone acetate (DMPA [also known as Depo-Provera]), injected every 3 months, and combined estrogen-progestin oral contraceptives (COC), as well as pregnancy have been variably associated with increased risk of human immunodeficiency virus (HIV) and other sexually transmitted infections (1–7). In addition, a large epidemiological study, supplemented with molecular analysis of transmitting HIV, suggested that women using certain types of

HCs may confer a higher risk of HIV transmission to their male partner (5). A recent study showed that the progestin levonorgestrel may decrease the clearance of high-risk human papillomavirus (HPV) infection and possibly increase acquisition (8), thus suggesting a potential impact of HCs on cervical immunity going beyond the risk of HIV. While experts continue to debate the validity of the epidemiological associations, to which populations they may apply, and how to balance the risk of HCs versus risks of pregnancy, over 150 million women continue using hormonal

contraceptives. In the African epicenter of the epidemic, the United Nations (UN) estimates that 29% of all women using a modern contraceptive method use DMPA (9), and the rates are especially high in eastern and South Africa, where the epidemic is most severe.

To date, it remains unclear whether there are biological grounds for the disparate epidemiological data on the effect of hormonal contraceptives on HIV acquisition. A reasonable biological explanation would be that microbial factors not taken into consideration by published epidemiological studies invert, suppress, or amplify hormonal influences on the cervicovaginal mucosal immune barrier.

To address this question, we focused our attention on genital inflammation because of its known role as a risk factor of HIV acquisition (10) and on the uterine cervix as a major contributor to the cervicovaginal secretory mucosal barrier (11–13). Many cytokines and other innate immunity mediators secreted by the cervix have been implicated in HIV immunopathogenesis through host cell activation or direct effects on the viral replication cycle (8, 14). Although it has been shown that female genital tract inflammation and immunity are hormonally regulated and linked to menstrual cycle phase (15–20) and to cervical ectopy, which is also hormonally regulated (15), limited clinical data exist on the effect of hormonal contraception on cervical immunity (21, 22). A small study (15 DMPA users, 18 levonorgestrel intrauterine device users, and 23 controls) limited to women with no sexually transmitted infections (STIs) showed significant gene upregulation of pathways of inflammation and immunity in the cervical transformation zone associated with progestin use (23). Analysis of cervicovaginal lavage specimens and paired serum samples from 18 DMPA users, 14 COC users, and 21 control women measured only interferon alpha (IFN-α) and showed reduced local and systemic levels with DMPA use (24). Another relatively small study of HIV-uninfected women, in which most women (>64%) belonged to the special category of HIV-discordant couples, only 16 women used oral contraceptives, and 41 used DMPA versus 171 in the no-HC group, and where STIs showed imbalanced distribution, mostly recorded in women who did not use HCs, found increased cervical levels of some but not other antibacterial cationic peptides in DMPA users (25). Earlier we utilized a much bigger study of 832 women from the Hormonal Contraception and Risk of HIV (HC-HIV) cohort to test the association between HCs, cervical immunity, and risk of HIV acquisition. We found that elevated cervical levels of the chemokine RANTES (*r*egulated upon *a*ctivation, *n*ormal *T*-cell *e*xpressed, and *s*ecreted) was associated with higher risk of HIV seroconversion within the next 3 months and that cervical levels of RANTES were also higher in the DMPA users ($n = 307$) compared to those who did not use HCs ($n = 226$) or those who used COC ($n = 299$). In addition, DMPA users had lower levels of β-defensin 2 (BD2), and COC users had higher levels of most inflammatory proteins measured (26). However, in our published analyses of the HC-HIV cohort, we did not assess immunity in association with concurrent bacterial, fungal, or viral cervicovaginal infections (CVIs) and altered microflora.

Reproductive tract infections and microbiome disturbances, and especially the syndrome clinically diagnosed as bacterial vaginosis (BV), are recognized as proinflammatory risk factors for HIV acquisition and transmission (27–29). However, studies that have attempted to establish cytokine signatures of STIs and vagi-

nal microbiota have not specifically elucidated how microbial factors contribute to the altered cervical barrier in women using hormonal contraceptives (30, 31). We hypothesized that cervicovaginal pathogens (e.g., *Trichomonas vaginalis*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, genital herpesvirus 2 [HSV-2], and *Candida albicans*) and disturbances in the normal vaginal microbiota that are often undiagnosed, not treated, and especially common and hazardous in women at risk of HIV (32–35) may alter or contribute to the effects of hormonal contraceptives on cervical immunity. To test this hypothesis we performed secondary analysis of the HC-HIV cohort, which provided both immunologic and microbiologic data. We assessed the combined effects of cervicovaginal infections (CVIs) and hormonal contraceptives on levels of immune mediators chosen based on their proven biological significance (36–39) and abundant production by the cervicovaginal epithelium (11, 40–44), as well as based on their reliable measurement in human cervicovaginal secretions (36, 37, 45–47). The following 10 proteins were selected to represent five major classes of immunoinflammatory mediators: (i) the cytokines interleukin-1β (IL-1β) and IL-6, (ii) chemokines XCL8 (IL-8), CCL20 (macrophage inflammatory protein 3α [MIP-3α]), and CCL5 (RANTES), (iii) vasoactive mediators and adhesion molecules acting downstream from cytokine and chemokine activation, including vascular endothelial growth factor (VEGF) and soluble intercellular adhesion molecule 1 (sICAM-1 [CD54]), (iv) the anti-inflammatory cytokine antagonist IL-1 receptor antagonist (IL-1RA), and (v) the antibacterial and antiviral proteins secretory leukocyte protease inhibitor (SLPI) and BD2.

RESULTS

Prevalence and distribution of lab-confirmed CVIs. In this study, controls (633 women who remained HIV negative) were matched to cases (199 women who later HIV seroconverted) by a composite STI variable (bacterial vaginosis [BV] and chlamydia- and/or gonorrhea-positive status). As expected from this matched design driven by the high rate of STIs associated with HIV, the overall prevalence of lab-confirmed CVIs was high (86%) in our nested cohort (Table 1). The distribution of CVIs differed by HC use. The COC and DMPA users had lower rates of lab-confirmed CVIs (84.3% and 84.4%, respectively) compared to the no-HC group (91%) ($P = 0.046$). The same differences by HC use were observed among the nonpregnant women alone ($P = 0.043$), suggesting that the imbalanced distribution was not driven by the higher rates of pregnancy, which expectedly occurred among the no-HC users and is considered a risk factor for CVIs. The differences could not be explained by the prevalence of unprotected sex acts, since the prevalence of unprotected sex and the number of unprotected sexual acts were highest in COC users, followed by DMPA users and no-HC users ($P < 0.001$) (Fig. 1A). On the other hand, the higher rate of CVIs among the no-HC group appeared driven by HSV (which was not used in the matching composite variable in our cohort): 69% (133/473) of no-HC users were HSV$^+$ compared with 58% (168/473) and 57% (172/473) in the COC and DMPA groups, respectively ($P = 0.01$).

The laboratory analysis showed a high degree of overlapping CVIs. Importantly more than half of our study cohort (473/832 [57%]) was herpes positive by serology, and over one-third was positive for BV (265/832 [32%]) and these two CVIs had an imbalanced distribution among the other CVIs, as shown in Table 2 (for the nonpregnant women only). The herpes-positive cases

TABLE 1 Distribution of CVIs stratified by hormonal contraception use, pregnancy, and breastfeeding

	No. (%) of CVIs in treatment group[a]									
	COC		DMPA		No-HC		Total			P
Group	CVI+	CVI−	CVI+	CVI−	CVI+	CVI−	CVI+	CVI−	n[b]	value
Nonpregnant and nonbreastfeeding	229 (84.50)	42 (15.50)	187 (84.23)	35 (15.77)	144 (92.31)	12 (7.69)	560 (86.29)	89 (13.71)	649	0.043
Nonpregnant and breastfeeding	11 (78.57)	3 (21.43)	67 (85.90)	11 (14.10)	35 (92.11)	3 (7.89)	113 (78.29)	17 (13.08)	130	0.400
Pregnant	7 (87.50)	1 (12.50)	1 (50.00)	1 (50.00)	25 (83.33)	5 (16.67)	33 (82.50)	7 (17.50)	40	0.446
Total n	247 (84.30)	46 (15.70)	255 (84.44)	47 (15.56)	204 (91.07)	20 (8.93)	706 (86.20)	113 (13.80)	819	0.046

[a] The CVIs include *T. vaginalis*, *N. gonorrhoeae*, *C. trachomatis*, *C. albicans*, HSV-2, and abnormal microflora by Nugent score. COC, combined estrogen-progestin oral contraceptive (levonorgestrel); DMPA, injectable progestin (Depo-Provera); no-HC, no hormonal contraceptives.
[b] Thirteen women were excluded from the analysis due to insufficient lab test data to classify their CVI status.

were evenly distributed among women with chlamydiasis, candidiasis, or BV but significantly ($P < 0.05$) more common among women positive for *T. vaginalis* infection or gonorrhea (74% versus 59% among the *T. vaginalis* infection- or gonorrhea-positive versus negative). The BV-positive status (Nugent score of 7 to 10) was evenly distributed, except among women positive for candidiasis, where BV was significantly ($P < 0.05$) less common (14% among candidiasis-positive versus 39% among candidiasis-negative women), which was expected based on previously reported inverse relationship between BV and candidiasis in the overall HC-HIV study (48).
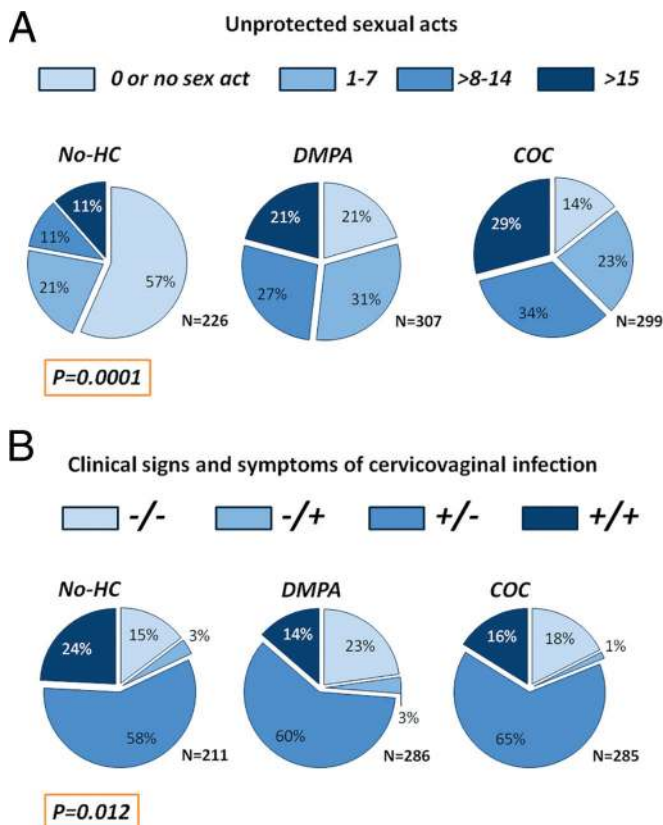

FIG 1 Distribution of number of sexual acts (A) and clinical signs and symptoms of cervicovaginal infections (B) among women who chose not to use hormonal contraception (no-HC) and women with majority use of combined oral contraceptives (COC) or DMPA. *P* values indicate differences among the HC groups.

Distribution of clinical signs and symptoms. The prevalence of clinical signs and symptoms of CVIs were also differentially distributed among the various contraceptive methods ($P = 0.012$) (Fig. 1B). This analysis excluded 50 women who answered some questions about symptoms with "don't know" or for whom no assessment was done due to menses. The greatest proportion of women had asymptomatic clinically manifested signs of infections (61%), which were most prevalent among the COC users (65%), followed by the DMPA (60%) and no-HC users (58%). In contrast, symptomatic presence of signs of CVIs was more common in the no-HC group than in the COC and DMPA groups (24% versus 16% and 14%, respectively; $P = 0.009$).

To investigate the potential impact of the uneven distribution of symptoms on our immunologic analyses, we compared levels of immune mediators between women with lab-confirmed CVIs who did or did not report symptoms. No significant differences were found except for BD2 (higher in symptomatic women [$P < 0.01$]) and SLPI (lower in symptomatic women [$P = 0.05$]) (see Table S1 in the supplemental material).

Cervical immunity by CVI status and HC use. To control for the uneven distribution for overlapping infections, signs, and symptoms, we assessed the differences in cervical immune mediators by CVI status and HC use using a multivariable analysis adjusting for overlapping individual CVIs and for clinical signs and symptoms of CVI via generalized linear models.

The concentrations of each biomarker in no-HC users stratified by CVI are shown in Table S2 in the supplemental material. To illustrate the combined effect of CVIs and HCs, we used the average of the CVI-free no-HC group as a baseline and calculated differences between this baseline and each combination of CVI with no-HC, DMPA, or COC (Fig. 2).

First assessed were differences between no HCs and the two types of HCs within each CVI group (see Table S3 in the supplemental material). In the CVI-free group (normal Nugent score of <4 and lab-confirmed negative results for chlamydia, gonorrhea, herpes, or *T. vaginalis* and *Candida* infection), COC users compared to no-HC users showed higher levels of IL-1β ($P < 0.001$), IL-6 ($P < 0.01$), and IL-8 and IL-1RA ($P < 0.05$). In the CVI-free group, compared to the no-HC group, DMPA users showed higher RANTES and lower BD2 levels ($P < 0.05$).

The effects of COC were different within the CVI-positive groups. The above immunostimulatory effects of COC use observed compared to those of no HC use among the CVI-free women were blunted or even reversed in the presence of most CVIs, with a few exceptions. Compared to the no-HC group, IL-1β was significantly increased by COC use only within the BV+

TABLE 2 Distribution of HSV-2 and BV infections overlapping with other CVIs among all nonpregnant women

	No. (%) of CVIs overlapping with HSV-2 and BV[a]											Total n
	T. vaginalis infection		Chlamydia		Candidiasis		Nugent score		Gonorrhea			(% [n = 791])
Parameter	−	+	−	+	−	+	<7	>7	−	+		
HSV-2												
−	**300.00 (40.54)**	**11.00 (25.58)**	301.00 (40.08)	10.00 (31.25)	270.00 (39.53)	41.00 (40.59)	203.00 (42.56)	93.0 (35.5)	**299.00 (40.57)**	**12.00 (26.09)**		473.00 (59.8)
+	**440.00 (59.46)**	**32.00 (74.42)**	450.00 (59.92)	22.00 (68.75)	413.00 (60.47)	60.00 (59.41)	274.00 (57.44)	169.0 (64.5)	**438.00 (59.43)**	**34.00 (73.91)**		311.00 (39.32)
Nugent score												
<7	459.00 (65.01)	22.00 (56.41)	464.00 (65.08)	17.00 (51.52)	**399.00 (61.29)**	**82.00 (86.32)**	481.00 (100)	0 (0)	455.00 (64.91)	26.00 (57.78)		481.00 (60.81)
7–10	247.00 (34.99)	17.00 (43.59)	249.00 (34.92)	16.00 (48.48)	**252.00 (38.71)**	**13.00 (13.68)**	0 (0)	265.0 (100)	246.00 (35.09)	19.00 (42.22)		265.00 (33.5)

[a] The CVIs include T. vaginalis, N. gonorrhoeae, C. trachomatis, and C. albicans. The boldface values show significantly imbalanced distributions ($P \leq 0.05$).

group ($P < 0.001$), IL-6 and IL-8 were increased only within the BV$^+$ ($P < 0.001$) and HSV-2$^+$ groups ($P < 0.001$ and $P = 0.01$, respectively), and in addition, in these two groups, COC use showed higher levels of MIP-3α ($P < 0.001$), VEGF ($P = 0.04$ for herpes and $P < 0.001$ for BV), and SLPI ($P < 0.001$). MIP-3α and SLPI were also increased ($P < 0.001$) by COC use within the candidiasis-positive group, and BD2 was decreased in the candidiasis-positive ($0 = 0.04$) and HSV-2$^+$ ($P < 0.001$) groups. Levels of IL-1β ($P = 0.01$) and IL-8 ($P = 0.04$) were even decreased in COC users with $T.$ $vaginalis$ infection. COC use failed to increase IL-1RA compared to no-HC use in any of the CVI$^+$ groups, and in addition, the anti-inflammatory ratio of IL-1RA to IL-1β was decreased by COC use in women with $T.$ $vaginalis$ infection, BV ($P < 0.001$), gonorrhea ($P = 0.01$), and herpes ($P = 0.04$).

Similarly, the effects of DMPA use were influenced by the presence of CVIs. RANTES remained significantly increased by DMPA use only in women positive for herpes, BV ($P < 0.001$), or candidiasis ($P = 0.04$) and with an abnormal Nugent score of 4 to 6 ($P = 0.05$). Lower BD2 remained associated with DMPA use only if positive for herpes, $T.$ $vaginalis$ infection ($P < 0.001$), or chlamydia ($P = 0.01$). The immunosuppressive effect of DMPA was exacerbated by additional lower levels of IL-1RA in women with gonorrhea ($P = 0.01$), chlamydia ($P = 0.05$), or HSV-2 ($P < 0.001$), SLPI in women with gonorrhea ($P = 0.05$), or IL-1β, MIP-3α, and IL-1RA/IL-1β ratio in those with $T.$ $vaginalis$ infection ($P < 0.001$).

We next examined differences by CVI status within each HC-use stratum (Fig. 2; see Table S4 in the supplemental material). In the no-HC group, $T.$ $vaginalis$ infection was the only CVI to show significant changes detectable by increased levels of multiple immune mediators, including IL-1β ($P < 0.001$), BD2 ($P = 0.01$), IL-8, IL-1RA ($P = 0.02$), and MIP-3α ($P = 0.04$). Of the other CVIs lab confirmed within the no-HC group, only gonorrhea and chlamydia showed an immunostimulatory effect limited to increased IL-1β levels ($P < 0.001$) or IL-1RA ($P < 0.001$), respectively.

Within the COC user group, increased levels were seen only for RANTES by herpes, intermediary Nugent score, and BV and BD2 by $T.$ $vaginalis$ infection ($P = 0.01$). In contrast, chlamydia decreased IL-1β levels ($P = 0.03$), herpes decreased IL-8 ($P = 0.05$) and VEGF ($P = 0.02$) levels, gonorrhea ($P = 0.03$) and candidiasis ($P = 0.04$) decreased VEGF, and an intermediary Nugent score was associated with the broadest immunosuppressive effect demonstrated by decreased levels of IL-1RA, IL-6, IL-8, VEGF ($P < 0.001$), IL-1β ($P = 0.01$), MIP-3α (0.02), and SLPI ($P = 0.05$).

Within the DMPA user group, gonorrhea was associated with decreased SLPI ($P = 0.04$) and IL-1RA ($P = 0.02$), chlamydia with reduced levels of IL-8 ($P = 0.05$) and VEGF ($P = 0.02$) but increased levels of RANTES ($P = 0.01$), $T.$ $vaginalis$ infection with decreased levels of SLPI ($P = 0.02$), and herpes (0.04), candidiasis, and BV ($P = 0.01$) with increased levels of BD2.

DISCUSSION

The HC-HIV study had previously found that women who used DMPA, but not those who used COC, were at significantly increased risk of HIV acquisition compared to women not using hormonal contraception (1, 7), and we had shown that this risk may be mediated by differential effects of DMPA and COC on cervical innate immunity (22). More specifically, in the nested
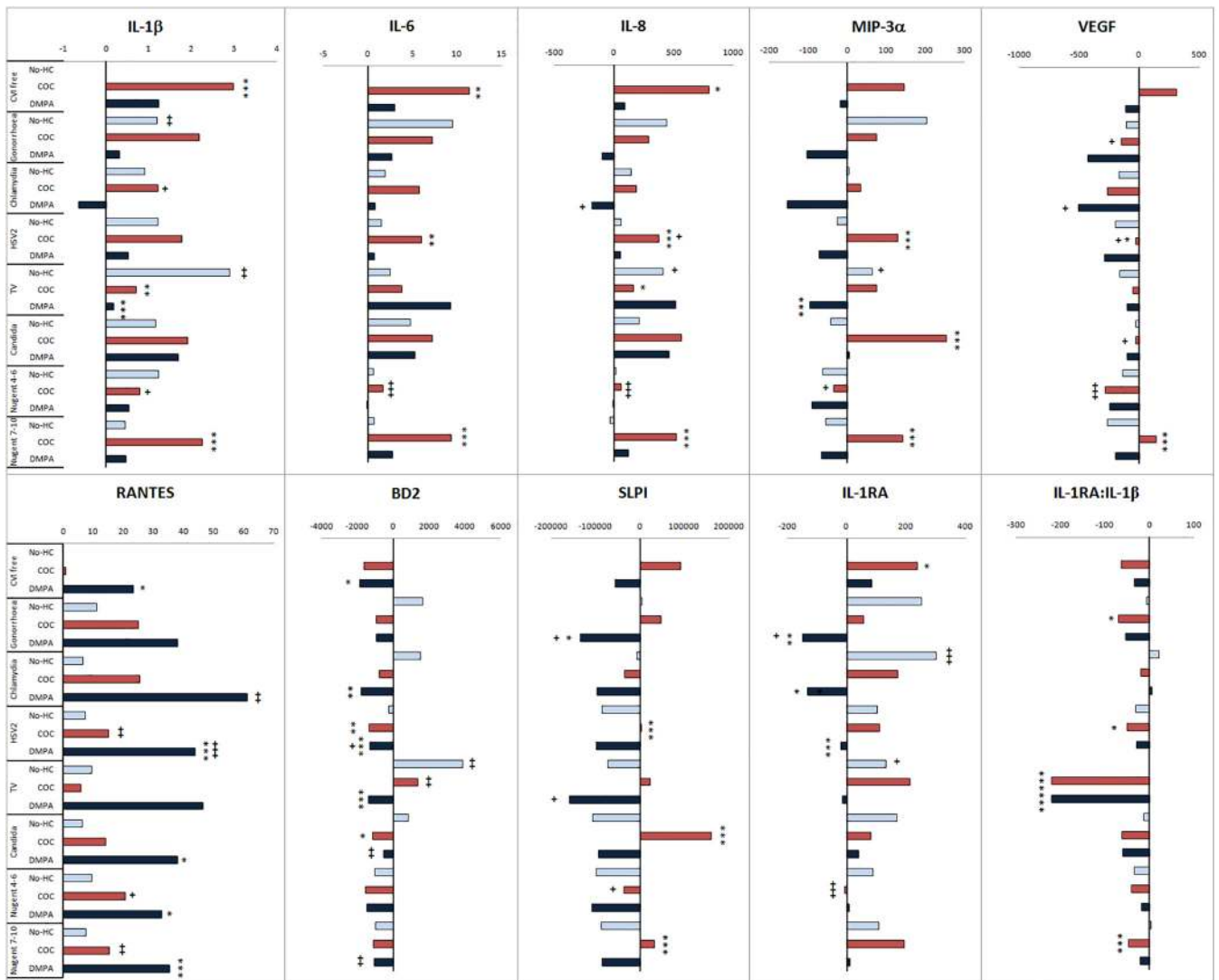
FIG 2 Combined effects of cervicovaginal infections (CVIs) and hormonal contraception (HC) on markers of cervical immunity. Women were stratified by CVI status and HC use within each CVI stratum, and levels of immune biomarkers were compared by multivariable analysis via generalized linear models and Wilcoxon tests after adjustment for other individual CVIs. Bars represent the differences between the average concentrations calculated for each of the combined CVI-plus-HC category listed on the left and the average concentration calculated for the physiologic CVI-free no-HC baseline (15 women who were infection free, had a normal Nugent score, and who did not use any HC). Consequently, the CVI-free, no-HC baseline average was set to 0 in each bar plot. *P* values with asterisks (*, *P* < 0.05; **, *P* < 0.01; and ***, *P* < 0.001) signify differences between no-HC and combined oral contraceptive (COC) or DMPA users within each CVI stratum. *P* values with plus signs (**+,** *P* < 0.05; **++,** *P* < 0.01; and **+++,** *P* < 0.001) signify differences between each CVI-positive group and the CVI-free group matched by HC (e.g., no-HC, COC, and DMPA). The *P* values for the two comparisons and the number of women in each group are shown in Tables S1 and S2 in the supplemental material.

cohort of 199 HIV seroconverters and 633 controls, HIV serocon-version was associated with preceding higher levels of RANTES and BD2 and lower levels of SLPI (22). We now demonstrate that cervicovaginal infections modified these three markers of HIV seroconversion risk in a manner dependent on HC use. Of special concern is our new finding that even though COC use had no effect on RANTES in CVI-free women, RANTES was increased among the COC users in association with herpes and abnormal vaginal microbiota (Nugent score of ≥4), both highly prevalent in women at HIV risk. Thus, dependent on the CVI and microbiota status, COC use may convey risk of HIV. RANTES was increased by DMPA in CVI-free women, but this effect was amplified by some CVIs, such as chlamydia and herpes. SLPI, which is a major

antibacterial and antiviral effector in the cervicovaginal environ-ment, previously shown to be reduced by BV and *T. vaginalis* infection (49), was decreased by *T. vaginalis* infection and gonor-rhea among the DMPA users and among the COC users—by intermediary abnormal microbiobiota (Nugent score of 4 to 6). The third marker of HIV seroconversion risk, decreased BD2, was increased among no-HC and COC users by *T. vaginalis* infection and among DMPA users by herpes, candidiasis, and BV. Thus, the differential effects of COC and DMPA on the risk of HIV acquisi-tion may be explained by immune factors potentiated by popula-tion differences in the prevalence of abnormal microbiota and bacterial, viral, or protozoan sexually transmitted infections.

In addition to RANTES, SLPI, and BD2, which were associated

with subsequent HIV seroconversion and altered by DMPA or DMPA when combined with specific CVIs but also similarly by COC use only when combined with certain CVIs, the profiles of other proinflammatory mediators differed by HC use. Overall, COC increased levels of the proinflammatory mediators IL-1β, IL-6, and IL-8, while DMPA did not change or decreased their levels when combined with CVIs (e.g., chlamydia). In CVI-free women, COC but not DMPA use was associated with higher levels of IL-1β, IL-6, and IL-8. In agreement with our findings, an experimental study showed upregulation of IL-6 in ectocervical and vaginal epithelial cells when treated with COC but not DMPA (50). Another in-vitro study showed that a DMPA dose of 10^{-7} M induced downregulation of a broad spectrum of immune mediators in peripheral blood mononuclear cells (24). In the same study, IL-6 was suppressed by DMPA but only at a very high dose of 10^{-6} M, which may not be maintained at the cervical tissue level.

The biological grounds for differences observed in the effects of DMPA and COC can be attributed to differential regulation of inflammatory pathways by different progestins like DMPA and levonorgestrel (24), as well as to differences between progestins alone and progestins combined with estrogens. In another experimental study, natural estrogen, as well as natural progesterone combined with estrogen, but not progesterone alone decreased BD2 expression by vaginal epithelial cells *in vitro* (51). In contrast, in our study, DMPA, which represents progestin action alone, suppressed BD2 levels, while COC, which is an estrogen-progestin combination, did not suppress BD2 unless combined with herpes or candidiasis. These differences between the natural hormones and DMPA and COC can be explained by the fact that synthetic progestins, unlike the natural reproductive hormones, promiscuously bind to multiple steroid receptors (52–54), leading to activation of different transcription factors and cofactors leading to transactivation or repression of a myriad of immune response genes (55–57).

We found that in comparison to CVI-free women, women positive for BV and herpes experienced broader proinflammatory effects with COC use, including higher levels of IL-6, IL-8, and/or IL-1β, and in addition, higher levels of MIP-3α and VEGF and lower values of the anti-inflammatory IL-1RA/IL-1β ratio. These changes may lead to increased risk of HIV due to inflammatory tissue damage and HIV host cell activation. Higher cervicovaginal levels of IL-1β, IL-6, and IL-8 and lower levels of IL-1RA correlated with cervicovaginal epithelial tissue damage and inflammatory infiltration in a recent prospective randomized trial assessing vaginal mucosal safety of cellulose sulfate, nonoxynol-9, and the universal hydroxyethyl cellulose (HEC) placebo (58). Preexisting high cervicovaginal levels of IL-1β and IL-8 predicted tenofovir gel failure to prevent HIV in a case-control study of HIV seroconverters and HIV-negative controls (59). The recent CAPRISA 004 trial showed that the effectiveness of the tenofovir gel, the first vaginal microbicide to show promise for HIV prevention, was diminished by preceding innate immune activation measurable not only at the cervical but also at the systemic level (60). Increased IL-6 was among the systemic markers predicting HIV seroconversion in the CAPRISA trials (60). Finally, IL-1β, IL-6, and IL-8 were inversely correlated with systemic CD4 counts in cervicovaginal specimens from women with acute HIV infection (61).

Some aspects of the innate immune barrier amplified by COC and DMPA in uninfected women (e.g., higher IL-8 and IL-1RA by COC use and higher RANTES by DMPA use) may also be protective against some forms of BV or BV persistence. In the HC-HIV study, both DMPA and COC were associated with a reduced prevalence of BV (6, 48, 62). The reduction in BV with HC use was also seen in the Mombasa sex worker study (6).

On the other hand, once established, the abnormal intermediary vaginal microbiota was associated with a broadly suppressed cervical innate immunity among the COC users in our study (lower levels of IL-1β, IL-6, IL-8, MIP-3α, VEGF, SLPI, and IL-1RA). This immunosuppressed state in the presence of intermediary vaginal microbiota is especially concerning since this condition of the vaginal microenvironment is not routinely diagnosed and treated, and it may facilitate the survival of other sexually transmitted pathogens, especially in women using COC. In addition, other aspects of innate and acquired immunity not measured in our study can be suppressed by HCs. In murine models of STIs, progesterone suppressed Th17 cell responses to gonorrhea, shifting the balance to immune tolerance (63), and estradiol downregulated Th17 responses to *C. albicans* infection (64). In our study, COC use was associated with suppressed BD2 among the women positive for candidiasis and BD2 is essential for antifungal defense and for killing *C. albicans* in particular (65). Suppressed immune responses to *C. albicans* infection may explain why COC use was associated with more candidiasis in the HC-HIV study (48).

In conclusion, using clinical specimens that were obtained from women attending reproductive health clinics in Uganda and Zimbabwe, we showed that cervicovaginal pathogens and altered vaginal microbiota contribute to the differences in the effects of HCs on the cervical mucosal immune environment in HIV-negative women. The high prevalence of asymptomatic infections especially among COC and DMPA users that remain likely undiagnosed and untreated raises even more concerns in light of their combined effects on cervical immunity and biomarkers associated with risk of HIV. A deeper understanding of the pathogen-HC interactions may provide further insights for development of targeted interventions based on HC use and CVI status to improve reproductive health in women. Awareness of this fact can facilitate the design of clinical trials and meta-analyses to better define the role of different types of HCs on HIV risk. HCs can alter the risk of HIV acquisition and transmission differently based upon the background differences in concurrent CVIs. Epidemiological studies assessing the effect of HCs on mucosal immunity may generate different results in populations that differ by CVI prevalence or microbiota characteristics. In the future, these findings should be accounted for when assessing the risk of HIV acquisition/transmission and designing multipurpose technologies to better prevent pregnancy and HIV acquisition in women.

MATERIALS AND METHODS

Study design. For this nested study, we utilized samples and data from 18- to 35-year-old HIV-uninfected participants in the HC-HIV study ($n = 823$) enrolled from family planning clinics in Uganda and Zimbabwe (7). The nested study included 199 women (51 Ugandan and 148 Zimbabwean) who became HIV infected, sampled at the study visit just prior to the one at which HIV seroconversion was documented (median of 3 months). These samples were matched with samples from 633 controls (160 Ugandan and 473 Zimbabwean), who remained HIV uninfected during a 6-month follow-up. The population characteristics of the cohort studied here are presented in detail elsewhere (66). Briefly, contraceptive group designation was based on the primary contraceptive method

women used during the time between their previous study visit and the selected visit. Women in the non-hormonal contraceptive (no-HC) group used only condoms or no contraception. Women who chose to use hormonal contraceptives received from study clinicians either DMPA (150 mg injected every 3 months) or COC (30 μg ethinyl estradiol [EE] and 150 μg levonorgestrel). All women were asked to abstain from sexual intercourse 48 h prior to cervical swab specimen collection. No specimens were collected during menstrual bleeding, and friable cervix/visible blood was rarely recorded during swab collection.

Cases and controls were matched by study site, age, a composite sexually transmitted infection (STI) variable (see below), and time in study, with up to 4 matched controls for each case. The composite STI variable was set to 1 if a participant was diagnosed with *C. trachomatis*, *N. gonorrhoeae* (both confirmed by PCR), or had bacterial vaginosis (BV) at either the visit at which HIV infection was detected or at the previous visit (the last HIV-negative visit). The composite STI variable was set to 0 for women who tested negative for all 3 of these conditions at both visits. The composite STI variable was chosen to control for exposures rather than to detect interactions among specific pathogens.

Ethics statement. The study was carried in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). The parent HC-HIV study was carried with subjects' informed consent and Institutional Review Board approval for human subject research at participating institutions in the United States and Africa. The biomarker sub study protocol received a nonhuman subject determination (use of deidentified data) from the Office of International Research Ethics at FHI 360 and the Institutional Review Board at Brigham and Women's Hospital.

Clinical and laboratory diagnosis of infection. A CVI-free status was defined as having no laboratory-diagnosed infection. Laboratory diagnosis of CVIs included PCR (Roche Amplicor) for *C. trachomatis* and *N. gonorrhoeae*, antibody enzyme-linked immunosorbent assay (ELISA) for herpes simplex virus 2 (HSV-2), wet mount for *T. vaginalis* and *Candida*, and Nugent scoring for BV. HIV-negative status was ascertained by PCR.

Clinical signs of CVIs were defined as positive (+) when any one of the following findings was recorded by clinicians on a physical exam: inflammation or ulcers of the vulva; yellow/green, white/creamy/grey, or mixed vaginal discharge; positive whiff test; presence of clue cells; abnormal vaginal epithelium; abnormal cervical epithelium; or yellow/green cervical mucus.

Information on symptoms of CVIs were obtained through a structured interview during which subjects were asked if they had abnormal vaginal discharge, genital itching, lower abdominal pain, or pain during sex and were given the options to answer with "yes," "no," or "don't know." Symptoms were defined as positive (+) when abnormal vaginal discharge, genital itching, lower abdominal pain, or pain during sex was reported. Bleeding between periods was not considered a symptom of CVI. For this analysis, only those who answered "no" to all of the above were considered symptom free (those who answered "don't know" were excluded).

Biomarkers of cervical immunity. For biomarkers of cervical immunity, we utilized cervical Dacron swabs, which were collected in Amplicor lysis buffer (Roche Diagnostics) and processed as previously described (67).

Eight biomarkers (IL-1β, IL-1RA, IL-6, IL-8, RANTES, MIP-3α, VEGF, and sICAM-1) were measured simultaneously using the Meso Scale Discovery (MSD) multiplex platform and Sector Imager 2400 (MSD, Gaithersburg, MD). This MSD detection platform has been validated for accuracy and precision of cytokine recovery using international standards by comparisons with traditional ELISA (68) and has shown high clinical content validity for all eight biomarkers in large clinical cohorts (69–78). The MSD 8-plex was custom designed and optimized to allow detection of each biomarker within the linearity concentration range of the eluted cervical swab samples. SLPI and BD2 were measured by ELISA (Quantikine human SLPI assay from R&D Systems, Minneap-

olis, MN, and human β-defensin 2 assay from Phoenix Pharmaceuticals, Inc., Burlingame, CA).

Each sample was tested in duplicate, and the average value was normalized to average milligram total protein concentration obtained from duplicate measurement using the Pierce bicinchoninic acid (BCA) protein assay (Fisher Scientific, Pittsburgh, PA). The ELISA and BCA assays were read using a Victor2 reader (PerkinElmer, Boston, MA). The percentages of coefficient of variation (CV) of duplicate values obtained by this method were <10%. A quality control sample pool that showed values within the linearity range was split into aliquots, and one aliquot was tested on each assay plate showing interplate variation of <25% for all immunoassays and proteins. Spiking of the Amplicor lysis buffer and diluent with known concentrations of the test proteins confirmed no assay interferences at the chosen lowest sample dilutions (2-fold for the MSD 8-plex, 80-fold for SLPI, and 25-fold for BD2). All samples showed values above the low limits of detection (LLD) for each assay 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 pg/ml; and sICAM-1, 3.6 pg/ml. For SLPI, the LLD was 0.46 ng/ml, and for BD2 the LLD was 12.2 pg/ml.

Statistical analysis. The Box-Cox power transformation approach was used to transform biomarker concentrations into normal distributions for statistical modeling analyses. Descriptive statistics, including medians and ranges were used to summarize biomarker levels. The associations between levels of mediators and CVIs, clinical signs and symptoms of CVIs, and HC use were evaluated by the Wilcoxon tests and Kruskal-Wallis tests. Fisher's exact test was used to evaluate the association between CVIs, HC use, and women's characteristics. Bivariate and multivariable analyses controlling for other CVIs via generalized linear models were used to examine the effects of individual CVIs and HC use on levels of mediators. This study was based on a secondary data analysis to explore the impact of CVIs on the relationship between HC use and cervical safety/immunity biomarkers. Due to the *post hoc* nature of the analysis power calculation were not applicable (79). Statistical analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00221-15/-/DCSupplemental.

Table S1, DOCX file, 0.01 MB.
Table S2, DOCX file, 0.01 MB.
Table S3, DOCX file, 0.01 MB.
Table S4, DOCX file, 0.02 MB.

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