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Changes in vaginal microbiota and immune mediators in HIV-1-seronegative Kenyan women initiating depot medroxyprogesterone acetate

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Abstract

Background—Depot-medroxyprogesterone acetate (DMPA) is associated with HIV acquisition. We studied changes in vaginal microbiota and inflammatory milieu after DMPA initiation.

Methods—In a cohort of HIV-negative Kenyan women, we collected monthly vaginal swabs over 1 year pre- and post-DMPA. Using quantitative PCR, we compared quantities of *Lactobacillus crispatus, L. jensenii, L. iners*, *Gardnerella vaginalis*, and total bacterial load (16S

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rRNA gene levels). Six vaginal immune mediators were measured with ELISA. Trends in detection and quantity of bacteria were estimated by logistic and linear mixed-effects regression.

Results—From 2010-2012, 15 HIV-seronegative women initiated DMPA, contributing 85 visits (median 6 visits/woman (range 3-8)). The median time of DMPA-exposed follow-up was 8.4 months (range 1.5-11.6). Seven women (46%) had bacterial vaginosis (BV) within 70 days before DMPA start. *Lactobacillus iners* was detected in 13 women (87%) prior to DMPA start, but other lactobacilli were rarely detected. *Gardnerella vaginalis* declined by 0.21 $\log_{10}$ copies/swab per month after DMPA exposure (p=0.01). Total bacterial load declined by 0.08 $\log_{10}$ copies/swab per month of DMPA (p=0.02). Sustained declines in interleukin (IL)-6 (p=0.03), IL-8 (p=0.04) and IL-1 receptor antagonist (p<0.001) were also noted. Nine women (60%) had *L. crispatus* detected post-DMPA; which was significantly correlated with reduced IL-6 (p=<0.01) and IL-8 (p=0.02).

Conclusion—Initiation of DMPA led to sustained shifts in vaginal bacterial concentrations and levels of inflammatory mediators. Further studies are warranted to outline components of the vaginal microbiota influenced by DMPA use, and impact on HIV susceptibility.

Keywords

Bacterial vaginosis; BV; vaginal microbiota; depot-medroxyprogesterone actetate; DMPA; Depo-Provera; *Lactobacillus*; *Gardnerella vaginalis*; HIV-1 susceptibility; IL-6; IL-8; IL-1 receptor antagonist; IL-1ra

Introduction

Hormonal contraception, especially progesterone-based contraception such as depot-medroxyprogesterone acetate (DMPA), has been associated with human immunodeficiency virus-1 (HIV) acquisition in multiple observational cohort studies of high-risk women in sub-Saharan Africa.[1-4] DMPA is the cornerstone of many family planning programs, especially in Africa, as it is requires no daily adherence, preserves women's contraceptive privacy, and can be distributed without a sophisticated health care infrastructure. Effects of DMPA on HIV susceptibility and transmission may result from behavioral, biological, or a combination of effects. Given the urgency of the HIV epidemic among women of reproductive age in sub-Saharan Africa, where 7,000 young women are infected each week, the association of DMPA and HIV acquisition requires urgent investigation and is a priority issue in women's reproductive health today.[5]

A potential biological explanation of the increased HIV risk observed among DMPA users is that DMPA may affect the composition of the vaginal microbiota in ways that increase HIV susceptibility. In fact, DMPA has long been observed to reduce the incidence of bacterial vaginosis (BV),[6-9] a syndrome of overgrowth of anaerobic vaginal bacteria instead of *Lactobacillus* species-dominant microbiota typically associated with vaginal health. Reductions in BV from DMPA use would be expected to reduce HIV risk, since BV may be a modifiable risk factor for HIV acquisition,[10,11] and affects at least 20-40% of women in sub-Saharan Africa,[9,12] where women are disproportionately affected by HIV.[13] Yet while DMPA use may lead to fewer women with BV, there are plausible mechanisms by which DMPA's effect on vaginal microbiota could lead to increased HIV acquisition risk. For

example, DMPA use might change the vaginal microbiota so that BV is reduced, but does not result in a *Lactobacillus* species-rich vaginal bacterial community that is associated with the lowest HIV risk.[14] DMPA, which binds avidly to glucocorticoid receptors, may also change the inflammatory environment surrounding vaginal bacteria.

Investigating the influence of DMPA on vaginal microbial communities could elucidate mechanisms by which DMPA use could augment mucosal HIV susceptibility.[15] The objective of this study was to explore changes in key vaginal bacteria (*Lactobacillus crispatus, L. jensenii, L. iners* and *Gardnerella vaginalis*) after DMPA initiation. Due to DMPA's known immunomodulatory effects, we also studied innate immune mediators that could be impacted by DMPA.

Methods

Study population

The Mombasa Cohort prospectively follows Kenyan women at risk for HIV acquisition from transactional sex. To characterize changes in the quantities of key vaginal bacterial species before and after DMPA use, we identified HIV-seronegative women initiating DMPA between May 2010 – June 2012. All women with DMPA initiation were included if they met the following criteria: a 70-day window unexposed to DMPA; at least one specimen collected 3 months before starting DMPA; and at least 2 specimens after DMPA initiation. Up to 8 specimens were tested per woman, and specimens were tested up to 12 months after DMPA initiation. This study was approved by institutional review boards of University of Washington, Fred Hutchinson Cancer Research Center and University of Nairobi/Kenyatta National Hospital. Written informed consent was obtained from all participants.

Study procedures

Women returned for monthly visits, and completed a questionnaire at every visit assessing HIV risk behaviors and contraceptive use; visits were rescheduled if women were menstruating. Clinical examinations, STI and HIV testing were performed monthly; vaginal fluid was collected for wet mount and Gram staining. Vaginal swabs for analysis of microbiota were collected by swabbing the fornices and vaginal walls with dry Spin-Eze™ polyester push-off swabs (Fitzco, Spring Park, MN) which were frozen immediately, and cryopreserved at −70 C. *Lactobacillus* culture was performed. Cytokine swabs were collected by rolling a polyester swab across the vaginal wall and freezing in 1 mL of media (70% RPMI, 20% fetal calf serum and 10% dimethyl sulfoxide). Antibiotic dispensing was recorded at each visit; exposure was defined as any antibiotic dispensed in the 30 days prior to sample collection.

Laboratory methods

Chlamydia trachomatis and *Neisseria gonorrhoeae* were tested quarterly using molecular methods (APTIMA Combo-2, Hologic/Gen-Probe, San Diego, CA); *Lactobacillus* cultures were performed monthly. Vaginal saline wet mount was examined microscopically for presence of motile trichomonads and fungal elements. A drop of 10% potassium hydroxide

was added to the slide and evaluated for presence of yeast buds or hyphae. HIV testing was performed by enzyme-linked immunosorbent assay (ELISA) as previously described.[16] Gram stains of vaginal discharge were evaluated according to Nugent's methods, with Nugent score ≥ 7 considered a diagnosis of BV.[17]

For quantification of vaginal microbiota, each sample was prepared by adding 500 μL filtered saline to the dry swab in tube, which was vortex-mixed for 1 minute and spun at 14,000 xg to pellet cells. Supernatant was removed, then DNA was extracted from pelleted material using the BiOstic Bacteremia DNA Isolation Kit (Mobio, Carlsbad, CA),[18] eluted in 120 μl buffer and 3 μl of DNA used for each qPCR assay. To assess contamination, sham digests from swabs without human contact were processed in parallel. DNA samples were tested for PCR inhibition as described previously,[19] defined as delay in the threshold cycle by ≥2 cycles compared to no-template controls.

Using previously described techniques,[20,21] qPCR assays were used to detect and quantify the vaginal bacterial species *L. crispatus*, *L. jensenii*, *L. iners*, and *G. vaginalis*, relying upon a probe-based assay with 16S rRNA gene-specific primers, and a taxon-directed hydrolysis probe (Taqman format). Total bacterial load was assessed by the qPCR detection of the 16S rRNA gene in bacteria using broad range bacterial primers.[22] Core reagents were from Life Technologies (Carlsbad, CA) and master mixes contained buffer A (1 mM), deoxynucleotide triphosphates (1 mM), magnesium (3 mM), AmpErase uracil-N-glycosylase (0.05 U) and AmpliTaq Gold polymerase (0.6-0.9U) per reaction. Primers were added at 0.8 μM per reaction and final probe concentration was 150-300 nM. Assays underwent 45 cycles of amplification on the StepOnePlus thermal cycler (Life Technologies). Specificity and sensitivity were defined as described previously.[20] Plasmid standards were run in duplicate from 10^7 to 2.5 copies, and values are reported as 16S rRNA gene copies/swab.

Commercial ELISA kits (IL-8 [Life Technologies], IL-6, Interferon-γ- inducible protein 10 (IP-10), IL-1 receptor antagonist (IL-1ra), regulated on activation, normal T-cell expressed and secreted (RANTES), secretory leukocyte protease inhibitor (SLPI) [all R&D Systems, Minneapolis, MN]) were used to determine quantities of inflammatory mediators. Vaginal swabs were thawed and immediately loaded on plates for analysis. Fifty μL were analyzed for IL-8, 100μL for all other kits. Kit standards were diluted in serial 3:1 dilutions covering the suggested linearity range, using calibrator diluents provided, and analyzed in duplicate on separate plates to create standard curves. Samples above the range of the standard curve were diluted using reagent-specific diluents and re-run. IL-6, IL-8, IP-10 and RANTES were tested without dilution, IL-1ra was tested at 1000x dilution, SLPI was tested at minimum 20x dilution. Optical density at 450 nm was measured using a SpectraMax M2 microplate reader and SoftMax Pro software (Molecular Devices, Sunnyvale, CA). All results were analyzed using a log-log plot, except IL-8, which was analyzed using four-parameter logistic regression.

Statistical analysis

We used data from before and after DMPA initiation, so each woman served as her own control. Bacterial and inflammatory mediator levels were treated as detectable (yes/no)

binary variables if greater than 30% of the data were below limits of detection. Trends in detection and $\log_{10}$ quantity of bacteria were estimated by logistic and linear mixed-effects regression models respectively, with random intercepts and with random slopes (for linear models only) addressing right-censoring under the assumption of continued trends per subject. Due to both right and left censoring of inflammatory mediator levels, tobit models were used, with intercept-only random effects. Contraceptive method is self-reported at enrollment and each follow-up visit. DMPA start date was computed as the midpoint between the date of the last visit of DMPA-nonuse and the first visit of reported DMPA use. Analysis time is centered on this start date estimate. Bacterial levels, when not detected, were assigned half the detection limit. Tobit models were also run to assess sensitivity to this "imputation" of values below detection. Data from clinical correlates including vaginal discharge, pH and Nugent score were included from all visits pre and post DMPA, including visits where vaginal swabs were not collected.

In addition to an unadjusted model, we adjusted estimates of changes in bacteria levels and inflammatory mediators, or changes in odds of detection, on/off DMPA by including pre-specified potential confounding factors that may change in women following DMPA initiation. We investigated antibiotic use, vaginal washing, new STI, age, number of partners and sexual activity in the previous week, condom use (100% v. intermittent v. never) and parity, in that order. Adding each variable to the model cumulatively, variables that changed estimates by greater than 10% were retained in adjusted models. Antibiotic exposure in past 30 days was assessed for effect modification in all models; metronidazole exposure was assessed separately to account for its differing effects on vaginal *Lactobacillus* colonization.[23,24]

Results

Baseline characteristics

Fifteen women met inclusion criteria, contributing 85 vaginal swab samples. The median length of DMPA-unexposed follow up was 42 days (minimum [min] 7, maximum [max] 117). The median length of DMPA-exposed follow-up was 8.4 months (min-max 1.5-11.6); the median number of samples analyzed per woman was 6 (min-max 3-8). Baseline characteristics of these women are summarized in Table 1. Seven (47%) of the women had BV (Nugent score ≥7) within 70 days of their last pre-DMPA visit, and of those, 5 (33%) had BV at the visit immediately pre-DMPA. Fourteen of the women reported last menstrual periods of a median of 4.4 weeks (interquartile range [IQR] 1.7, 6.4) prior to their last pre-DMPA visit. One woman reported not menstruating for 30 weeks at baseline. No woman tested positive for *Trichomonas vaginalis*, *Chlamydia trachomatis*, or *Neisseria gonorrhoeae* at the pre-DMPA visit. After initiation, STIs remained rare; 2 women were diagnosed with *C. trachomatis*. At baseline, two women had been exposed to antibiotics in the 30 days prior to study entry. During follow-up, 4 women were exposed to antibiotics, affecting 8 specimens, and no woman had metronidazole exposure within 30 days before specimen collection.

Baseline bacteria quantities

We studied changes in 4 vaginal bacteria: *Lactobacillus crispatus* and *Lactobacillus jensenii*, two components of a healthy vaginal microbiome; *Gardnerella vaginalis*, an anaerobe typically linked to BV; and *Lactobacillus iners*, a species which is a component of both healthy and abnormal vaginal communities. Baseline bacteria quantities, measured in 16s rRNA gene copies/swab at the last pre-DMPA visit, are summarized in Table 2. *Lactobacillus crispatus* and *L. jensenii* were below detection for the majority of samples, with detection in only 2 (13%) and 1 (7%) of the 15 women at their last pre-DMPA visit sample. The majority of women had detectable levels of *L. iners* and *G. vaginalis* at baseline, with a median of 7.7 $\log_{10}$ copies/swab (IQR: 5.8, 8.6) and 7.8 $\log_{10}$ copies/swab (IQR: 6.7, 8.2) respectively.

Change in bacteria over time

Quantities of *G. vaginalis* and total vaginal bacterial load declined markedly after DMPA initiation (p=0.01, p=0.02, respectively) (Table 2, Figure 1). For *G. vaginalis*, this translated to a nearly 2-log decline over 12 months of followup. *L. iners* remained highly prevalent with no significant changes observed after DMPA initiation. Among the two species rarely detected at baseline, *L. crispatus* and *L. jensenii*, we found no change in the odds of detection of either species *a*fter initiation of DMPA. There was also no change in the likelihood of detection of *Lactobacillus* by culture before compared to after DMPA initiation (odds ratio (OR) for *Lactobacillus* detection by culture for each additional month on DMPA =1.013, 95% CI: 0.877, 1.168; p=0.87).

We modeled trends in bacterial species over time, adjusting for potential confounding factors. The model estimates for lactobacilli time trends were not substantially influenced by potential confounding factors. The adjusted model for *G. vaginalis* incorporated vaginal washing and recent antibiotic use, and continued to show that quantities decreased over time (p=0.04). These relationships were preserved after assessing for effect modification with recent antibiotic use, with no significant interaction between time trends and antibiotic use observed (data not shown). Total bacterial load also declined over time (p=0.02) and this decline was sustained after adjustment for multiple comparisons (p=0.04).

Clinical correlates with changes in vaginal microbiota

Nugent scores were compared by linear spline, stratified by BV within 70 days of the last pre-DMPA sample. There was no significant association between DMPA exposure and Nugent score, with an estimated 0.03 Nugent score units per month decrease following initiation among subjects without BV exposure pre-DMPA (change – 0.027; 95% CI: 0.12, 0.068; p-value = 0.58 and an increase among subjects with BV exposure pre-DMPA (0.018; 95% CI -0.12, 0.16; p-value = 0.80).

No clinical correlates of vaginal health were significantly associated with DMPA use, including vaginal pH, Nugent score, self-report of abnormal vaginal discharge, or clinician observation of abnormal vaginal discharge (data not shown).

Baseline inflammatory mediator levels

We measured inflammatory mediators IL-6, IL-8, IL-1ra, IP-10, and SLPI, and RANTES at the same timepoints pre- and post-DMPA initiation. Three women in the microbiota analysis did not have specimens for analysis. Of the remaining 12 women, at baseline, RANTES was not detected in 5 women and was detected below laboratory standard for the remaining 7 women. The other inflammatory mediators were detected in all women, and are described in Table 3 and supplemental Table 1.

Changes in inflammatory mediators levels over time

Quantities of 3 inflammatory mediators decreased significantly over time after DMPA initiation. The slope of decline was as follows: for $\log_{10}$ IL-6 levels, slope -0.07 (p=0.03), $\log_{10}$ IL-8 levels, slope -0.06 (p=0.04), $\log_{10}$ IL-1ra levels, slope -0.04 (p<0.001) (Table 3). We looked for possible confounding factors among our pre-specified mediators of the relationship between inflammation and DMPA use. No factors altered the strong association between DMPA initiation and decline in IL-1ra; after adjusting for vaginal washing, the relationship between IL-6 and DMPA was slightly attenuated (slope -0.06, p=0.04); although a trend persisted, DMPA no longer significantly affected IL-8 levels after adjustment for vaginal washing (slope -0.05, p=0.07). Figure 2 displays individual inflammatory mediator quantities over time.

Since there was high diversity of vaginal microbiota among women studied, we examined bacterial species and immune factors to see if there were associations. We found that *L. crispatus* was consistently inversely associated with immune factors IL1-ra, IL-6 and IL-8, and *G. vaginalis* was consistently positively associated with IL-8 (Supplemental Figure 1).

Discussion

This unique prospective cohort study demonstrated that initiation of DMPA led to significant changes in vaginal microbiota. The quantity of *G. vaginalis* declined by almost 100-fold after initiation of DMPA, and this decline was sustained during long-term DMPA use. Total vaginal bacterial load also declined with DMPA use. There were marked decreases in pro-inflammatory mediators IL-6, IL-8 and regulatory protein IL-1ra following DMPA initiation, suggesting that either DMPA exposure or the observed microbiota shifts precipitated changes in the immune environment of the vagina.

Prior studies have found decreased incidence of BV among users of DMPA in both developed and developing country settings.[7,8,25,26] Our results, showing decline in *G. vaginalis*, a BV-associated species, expand on this observation. *Gardnerella vaginalis* is nearly universally associated with BV, and the reduction in this species documented in our cohort may help to explain the decreased incidence of BV among users of DMPA. Studies that quantify vaginal microbiota daily have noted that menses coincide with transient increase in *G. vaginalis*, which requires iron for growth.[21,27] Since DMPA induces amenorrhea with long-term use, lack of menstrual blood could provide a mechanism for long-term reductions in *G. vaginalis* or reduced incidence of BV.[21] However, amenorrhea

would not explain the relatively rapid shift seen in our study, as these women would be unlikely to experience amenorrhea in the first months of using DMPA.

Despite reductions in *G. vaginalis*, we did not observe increases in *L. crispatus* or *L. jensenii* among study participants. Mitchell *et al* reported, similarly, that among 32 US women, there was a 50% reduction in culture detection of H_20_2-producing lactobacilli after one year of DMPA exposure.[28] This further illustrates that while DMPA use may reduce BV, it does not increase *Lactobacillus* colonization. A *Lactobacillus*-dominated vaginal microbiota may be protective against HIV,[29-31] but our understanding of protection offered by lactobacilli is limited by the lack of application of molecular methods to understand specifically which lactobacilli are most beneficial. Studies are underway to determine if specific anaerobic species associated with BV are linked to HIV susceptibility, which would aid in the interpretation of our observations of low *Lactobacillus* colonization in these women.

Our cohort had high rates of *L. iners*, a species that is not associated with protection; *L. iners* is linked with higher baseline pH levels in the vagina, disrupted microbiota, and intermediate Nugent scores.[32,33] *Lactobacillus iners* is globally common in both healthy and dysbiotic vaginal states,[12,34,35] and appears to differentially express genomic elements in vaginal dysbiotic and healthy states.[36] *Lactobacillus iners* quantities did not change with DMPA use in our cohort. Understanding why *L. iners* remained unaffected by DMPA, determining whether *L. iners* dominates the microbiota of DMPA users, and determining the role of DMPA in hormonal regulation of *L. iners* gene expression would represent major advances in understanding how the predominant vaginal microbiota of African women may be influenced by reproductive hormones and contraception.

We observed significant declines over time in inflammatory mediators IL-6 and IL-8 after initiation of DMPA. Higher levels of *L. crispatus* were associated with lower levels of IL-6 and IL-8. Previous studies have also noted lower IL-8 levels in women with the highest levels of *L. crispatus* in the vagina.[37] A possible mechanism for this effect was suggested by a study showing that medroxyprogesterone acetate (MPA) acts on glucocorticoid receptors *in vitro* to reduce pro-inflammatory gene expression in cervical epithelial cells, which include reductions in mRNA expression of the genes encoding IL-6, IL-8 and RANTES.[38] This explanation for our findings makes more sense than an upstream explanation for this effect, which would suppose that a reduction in pro-inflammatory mediators is caused by fewer immune cells present. In fact, some studies have shown increased numbers of immune cells, including HIV target cells such as T cells, macrophages and CCR5+ cells, in the vaginal epithelium of women initiating DMPA,[39,40] although there is little accompanying description of the vaginal microbiota. In one study, both microbiota and target cells were assessed, and CCR5+ target cells were not increased in DMPA users, while lactobacillus colonization declined among DMPA users.[28] Our understanding of the inflammatory effects of DMPA will remain limited until microbiota can also be studied as a major modifier of local immunity.

Our observation of IL-1ra decline with DMPA use is intriguing. A statistically significant reduction in IL-1ra among DMPA users was also seen in a large cohort study in Africa assessing HIV acquisition, although there was no difference in IL-1ra levels among women

who acquired HIV compared with women who remained HIV-negative.[41] A study of US women noted that those with vaginal microbiota dominated by *L. iners* or *G. vaginalis* had substantially higher levels of IL-1ra than women with microbiota dominated by *L. crispatus.*[42] Reduction in IL-1ra may be a reaction to a reduction in overall inflammation, or may be a specific transcriptional effect of DMPA that is not linked to other inflammatory activity.

Our study had several strengths. We used comprehensively collected longitudinal clinical data to conduct this analysis, which allowed us to observe changes in individual women. Further, our relatively long period of follow up (median 8 months) allowed for measurement of trends over time. Our study population of African urban women represents the population in which heterosexual HIV acquisition risk is highest, and in which prior studies have indicated increases in HIV acquisition among DMPA users.[2] DMPA users have been noted to have increases in sexually transmitted infections (STIs) including chlamydia,[6-8] possibly due to behavior changes after starting contraception. Women in our study had similar sexual behavior and condom use before and after starting DMPA, and we collected detailed data on these behaviors, allowing us to attempt to control for them. Women also received regular screening and treatment for STIs, which allowed for more focused observation of the vaginal microbiota with less interference from asymptomatic or untreated STIs. Bacterial vaginosis was treated only if women were symptomatic, as there is currently no indication for treatment of asymptomatic BV in non-pregnant women.

This study was limited by the small number of women investigated. Although we did not have a control group, our measurements pre- and post- DMPA allow each woman to serve as her own control. We studied 4 species of bacteria in the vagina, but were unable to include most BV-associated bacteria because of budgetary constraints. Information about vaginal bleeding post-DMPA was imprecise, and we were unable to examine the role of vaginal bleeding and menstruation on changes in the microbiota and inflammatory markers. Our findings in these women engaging in transactional sex in an African city may not be representative of women in other settings.

In conclusion, using DMPA appears to have caused important, lasting alterations in vaginal microbiota, and was significantly associated with a large reduction in the quantity of *G. vaginalis* and inflammatory mediators IL-6, IL-8 and IL-1ra. At present, there are insufficient data to make definitive statements about how these changes would influence HIV susceptibility; but these findings are intriguing, and highlight the need for further research to define quantitative changes in components of the full vaginal microbiota that could be related to HIV acquisition and transmission risk. Further investigation of microbiome and mucosal immunological changes with DMPA may help to elucidate the multifactorial influences that this hormonal contraceptive exerts on the vaginal immune environment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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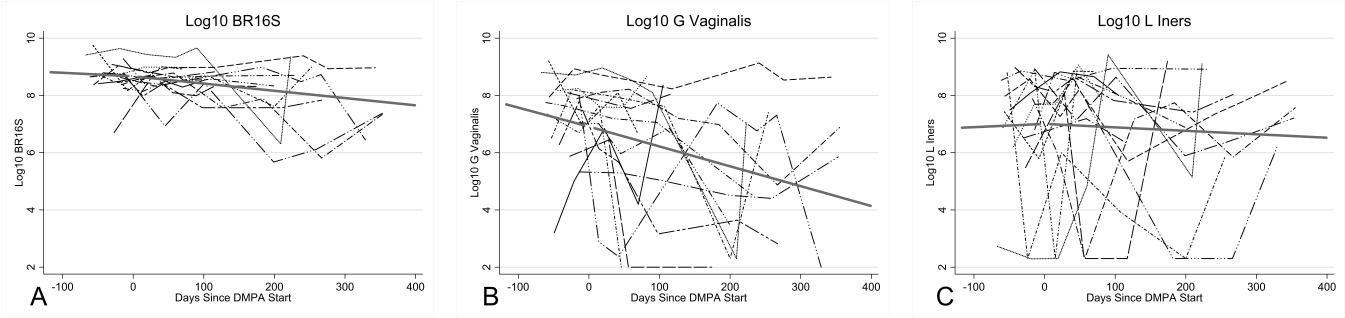

Figure 1.

Quantities of vaginal bacteria over time, for all subjects, with depot-medroxyprogesterone acetate (DMPA)-fitted trend lines pre- and post-DMPA. A, Bacterial load by broad-range 16S rRNA gene qPCR. B, *Gardnerella vaginalis.* C, *Lactobacillus iners*. Bacterial quantities were $\log_{10}$-transformed. Values below the lower limit of detection were assigned a value of half the lower limit of detection. P-values and estimates were calculated using linear mixed effects models with linear splines pre- and post-DMPA initiation (random intercepts and random slopes post DMPA-initiation).

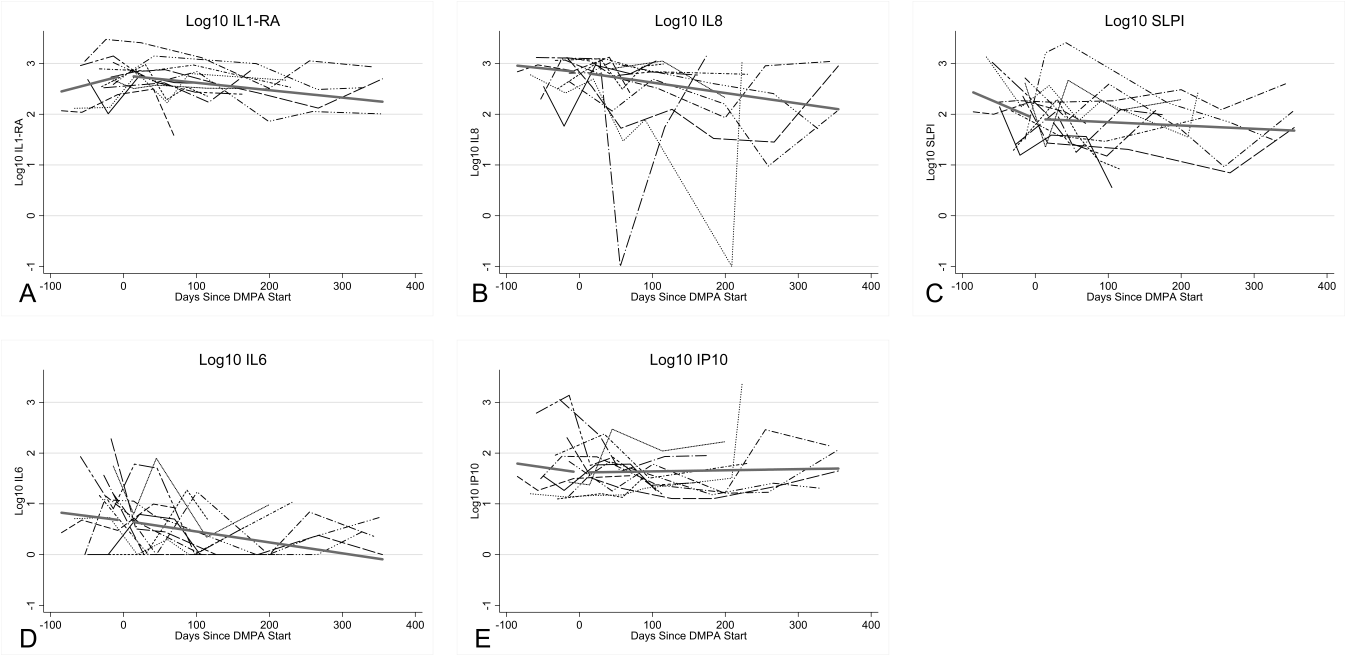

Figure 2.

Levels of inflammatory mediators for all subjects over time with depot-medroxyprogesterone acetate (DMPA)-fitted trend lines pre- and post-DMPA. A: Interleukin (IL)-1 receptor antagonist. B, IL-8. C, secretory leukocyte protease inhibitor (SLPI). D, il-6. E, Interferon-γ-inducible protein 10 (IP-10). P-values and estimates were calculated using linear mixed effects models with linear splines pre- and post-DMPA initiation (random slopes post DMPA-initiation).

Table 1

Baseline characteristics of 15 women starting DMPA.

Characteristic	Median (IQR) or N(%)
Age at pre-DMPA visit	33 (25, 38)
Years of education at enrollment	12 (6, 12)
Ever married at enrollment	7 (47)
Workplace at enrollment: bar/restaurant/guesthouse	9 (60)
Workplace at enrollment: nightclub	3 (20)
Workplace at enrollment: home-based*	1 (7)
Number sex partners in last week at pre-DMPA visit	2 (1, 3)
Number of new sex partners in last week at pre-DMPA visit	0 (0, 4)
Number of sex acts without condom in last week at pre-DMPA visit	0 (0, 2)
Weeks since last menstrual period at last pre-DMPA visit¥	4.4 (1.7, 6.4)
Ever pregnant at enrollment	14 (93)
Number of pregnancies among those ever pregnant at enrollment	2.5 (1,3)
Reported any vaginal washing activity in past week at last pre-DMPA visit	9 (60)
Any antibiotics prescribed at or before last pre-DMPA visit€	2 (13)
Self-reported abnormal discharge at *any* pre-DMPA visit£	4 (27)
Clinician reported abnormal discharge at last pre-DMPA visit	6 (40)
Contraceptive method: condoms only at last pre-DMPA visit	15 (100)
Any BV (Nugent score ≥ 7) in last 70 days at last pre-DMPA visit	7 (47)
BV (Nugent score ≥ 7) at last pre-DMPA visit	5 (33)
Vaginal pH at last pre-DMPA visit	4.7 (4.4, 5.3)

IQR= interquartile range, N=number of respondents, DMPA=depot-medroxyprogesterone acetate, BV=bacterial vaginosis

*The remaining two women responded “Other” when asked about workplace.

¥Missing for one woman.

€Missing for four women.

£Zero women reported abnormal discharge at the last pre-DMPA visit.

Table 2

Bacterial levels detected in each vaginal swab (16S rRNA gene copies/swab) at the pre-DMPA visit; and change over time in bacteria levels after DMPA initiation.

	N (%) above detection	Median (IQR), log_{10} scale *	Estimate (Slope post-DMPA) ¥	(95% CI)		p-value	Adjusted p-value **
Detect *L. crispatus* (OR)	2 (13)	-	1.20	0.96	1.49	0.11	0.18
Detect *L. jensenii* (OR)	1 (7)	-	0.99	0.79	1.24	0.93	0.93
Log_{10}*L. iners*	13 (87)	7.7 (5.8, 8.6)	−0.04	−0.2	0.13	0.66	0.83
Log_{10}*G. vaginalis*	15 (100)	7.8 (6.7, 8.2)	−0.21	−0.37	−0.05	0.01	0.04
Log_{10} Total bacterial load (BR16s)	15(100)	8.7 (8.4, 9.0)	−0.08	−0.14	−0.01	0.02	0.04

N = participants, IQR = Interquartile Range, LLOD = Lower limit of detection, OR = Odds ratio, CI = Confidence interval; p-values and estimates are by linear mixed effects models with linear splines pre- and post-DMPA initiation (random intercepts and random slopes post DMPA-initiation).

Nine of the 15 women (60%) had detectable *L. crispatus* levels at some time during follow up.

* Median (IQR) only reported for those with greater than 50% above detection.

** Adjusted using Simes' method.

¥ Estimated multiplicative change per month in Odds Ratio of detection or arithmetic change per month in log_{10} levels following estimated date of DMPA initiation, accounting for variability between subjects in slopes.

Table 3

Baseline levels and estimates of change over time in inflammatory mediator levels after DMPA initiation.

	Baseline level, Median (IQR), log_{10} scale*	Estimate (Slope post-DMPA)¥	(95% CI)		p-value	Adjusted p-value*
Log_{10} IL-6	1.1 (0.6, 1.4)	−0.07	−0.12	−0.01	0.03	0.08
Log_{10} IL-8	2.9 (2.5, 3.1)	−0.06	−0.12	−0.00	0.04	0.08
Log_{10} IL1-ra	2.6 (2.5, 3)	−0.04	−0.07	−0.02	<0.001	0.001
Log_{10} IP-10	1.7 (1.2, 2.1)	0.01	−0.03	0.04	0.71	0.71
Detect RANTES	-	1.07	0.85	1.35	0.56	0.67
Log_{10} SLPI	2.2 (1.5, 2.4)	−0.02	−0.06	0.02	0.35	0.53

Units for IL-6, IL-8, IP-10, RANTES: pg/ml. Units for SLPI, IL1-ra: ng/ml.

CI = Confidence Interval; p-values and estimates are by linear mixed effects models with linear splines pre- and post-DMPA initiation (random slopes post DMPA-initiation)

*adjusted for multiple comparisons using Simes' method.

¥Estimated multiplicative change per month in Odds Ratio of detection or arithmetic change per month in log_{10} levels following estimated date of DMPA initiation, accounting for variability between subjects in slopes.