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ORIGINAL ARTICLE

Genital inflammatory status and the innate immune response to contraceptive initiation

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Abstract

Problem: Data on the effects of contraceptives on female genital tract (FGT) immune mediators are inconsistent, possibly in part due to pre-existing conditions that influence immune mediator changes in response to contraceptive initiation.

Methods: This study included 161 South African women randomised to injectable depot medroxyprogesterone acetate (DMPA-IM), copper intrauterine device (IUD), or levonorgestrel (LNG) implant in the Evidence for Contraceptive Options and HIV Outcomes (ECHO) trial. We measured thirteen cytokines and antimicrobial peptides previously associated with HIV acquisition in vaginal swabs using Luminex and ELISA, before, and at 1 and 3 months after contraceptive initiation. Women were grouped according to an overall baseline inflammatory profile. We evaluated modification of the relationships between contraceptives and immune mediators by baseline inflammation, demographic, and clinical factors.

Results: Overall, LNG implant and copper IUD initiation were associated with increases in inflammatory cytokines, while no changes were observed following DMPA-IM initiation. However, when stratifying by baseline inflammatory profile,

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women with low baseline inflammation in all groups experienced significant increases in inflammatory cytokines, while those with a high baseline inflammatory profile experienced no change or decreases in inflammatory cytokines.

Conclusion: We conclude that pre-contraceptive initiation immune profile modifies the effect of contraceptives on the FGT innate immune response.

KEYWORDS
contraception, copper, cytokines, female, inflammation, intrauterine devices, levonorgestrel, medroxyprogesterone acetate

1 | INTRODUCTION

Contraceptive use is critical to reduce maternal and infant mortality and morbidity[1] and to empower women. Injectables and implants are widely used methods of contraception and account for over half of all modern contraceptive use in Sub-Saharan Africa.[2] The use of hormonal contraceptive methods, particularly the 3-month 150 mg depot medroxyprogesterone acetate intramuscular injection (DMPA-IM), overlaps with high rates of HIV and other sexually transmitted infections (STIs) among young women in Sub-Saharan Africa.[3,4] Some observational studies had suggested that DMPA-IM was associated with up to 40–50% increased risk of HIV,[4,5] but the Evidence for Contraceptive Options and HIV Outcomes (ECHO) randomized clinical trial found no statistically significant overall differences in HIV acquisition between women using DMPA-IM, copper IUD or LNG implant.[6] However, the study was powered to detect ≥ 50% difference in HIV acquisition between randomized groups and the trial design precluded the ability to provide information on differences in HIV risk between the randomized methods and no method or condom use only.[7] It therefore remains unknown whether these contraceptives increase HIV risk relative to other forms of contraception or no contraception. Ultimately, the impact of contraceptives on female genital tract (FGT) immunology in ways that may increase the risk of STIs, including HIV, is not well understood.

Although FGT inflammation is critical for defence against pathogens, women with increased immune mediators have been shown to have higher risk of STI and HIV acquisition,[8] likely due to reduced epithelial barrier integrity and recruitment and activation of HIV target cells. Additionally, sustained reproductive tract inflammation may be associated with infertility, endometriosis and increased risk of preterm labour.[9–11]

Different contraceptives appear to affect FGT inflammatory profiles in unique ways and published data are inconsistent for injectables,[12–14] with some studies demonstrating elevated inflammatory cytokines, while others show decreases or no differences among DMPA-IM users and control groups. Changes in inflammatory profiles remain largely unexplored for long-acting reversible contraceptive (LARC) methods, such as LNG implants and copper IUDs. However, use of copper IUDs has been associated with increased bacterial vaginosis (BV),[15] a known cause of FGT inflammation.[16]

A recent study found DMPA-IM to be associated with increased molecular pathways of inflammation in the vaginal mucosa of *Lactobacillus*-dominant women, while no differences were seen in non-*Lactobacillus* dominant women.[17] This suggests that pre-existing differences in FGT immune factors may modify the biological effects of contraceptives and thus may explain discrepant results reported in prior studies.

The aims of this study were to evaluate changes in immune mediators among 161 South African women following randomization to DMPA-IM, copper IUD, and LNG implant, and to determine the influence of demographic and biological factors on these changes.

2 | METHODS

2.1 | Study participants

The Evidence for Contraceptive Options and HIV Outcomes (ECHO) trial was a randomised multi-centre trial conducted in 12 research sites in South Africa, Kenya, Zambia and 'Eswatini. Participants were enrolled between December 2015 and September 2017. In brief, women who were not pregnant, HIV-seronegative, aged 16–35 years, seeking effective contraception, without medical contraindications to the trial contraceptive methods, reported not using injectable, intrauterine or implantable contraception for the previous 6 months and reported being sexually active, were eligible. The study design and primary results have been reported previously.[6] Participants at two ECHO trial sites, Setshaba Research Centre (SRC) in Tshwane (n = 52) and MatCH Research Unit (MRU) in eThekwini (n = 109), South Africa, were invited to participate in a biological mechanisms sub-study. The University of the Witwatersrand and University of Cape Town Human Research Ethics Committees approved this study and all participants provided written informed consent. Approximately equal numbers of women in each group agreed to specimen collection including 51 in the copper IUD group, 52 in the DMPA-IM group, and 58 in the LNG implant group for a total of 161 participants. Demographic, behavioural, and clinical data were collected on standardized case report forms in the parent ECHO trial. Cervical ectopy was estimated by clinicians using unaided visual inspection during speculum

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examination and rated in the following categories: 0–25%, 26–50%, 51–75% and 76–100%.

2.1.1 | Specimen collection

Lateral vaginal wall swab samples were collected between June and December 2017 for cytokine, secretory leukocyte protease inhibitor (SLPI), human beta-defensin (HBD)-1 and –2, prostate specific antigen (PSA) measurement and STI testing. Specimens were collected at baseline (immediately before contraceptive method initiation), month 1 (M1), and month 3 (M3) for immune marker assessment at near peak (M1) and near trough (M3, before DMPA re-administration) MPA concentrations. All samples were collected by placing Dacron swabs on the lateral vaginal wall and rotating 360 degrees. Swabs for immune mediator analysis were stored in cryovials at -80°C for a median time of 23 months (range: 19–26 months) before further processing and testing.

2.1.2 | Lateral vaginal wall swab processing

Frozen lateral vaginal wall swabs were thawed on ice overnight at 4^0C. The following day, 1 ml of phosphate buffered saline (PBS; Sigma-Aldrich, P5493) was added to each tube; tubes were vortexed for 60 s at a low speed and subsequently incubated at 4^0C for 1 h. Excess mucus was scraped off on the inner wall of the tubes and each tube was vortexed for another 30 s at a low speed. Prior to immune marker measurements, the supernatants were transferred into filter centrifuge tubes (Corning® Costar® Spin-X® tubes Sigma-Aldrich, CLS8160) and centrifuged for 10 min at 4000 Relative Centrifugal Force (RCF). Filters were removed and the supernatants vortexed for 10 s at a low speed. The tubes were kept on dry ice throughout processing.

2.1.3 | Cytokine, antimicrobial peptide and prostate specific antigen measurement

Macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4), MIP-3α (CCL20), interferon gamma-induced protein (IP)-10 (CXCL10), regulated on activation, normal T cell expressed and secreted (RANTES; CCL5), interleukin (IL)-6, IL-8, IL-1β and tumour necrosis factor (TNF)-α, interferon (IFN)-α, and SLPI were measured using a customized Human Magnetic Luminex Screening Assays (R&D Systems, Minneapolis, USA. Lot L128368, Catalogue number: LXSAHM-02; LXSAHM-09). Each of the selected mediators has previously been found to be associated with changes in HIV and/or SIV infection risk.[8,14,18,19] Participant samples were randomly assigned to plates and cytokines were measured in duplicate with means used for statistical analysis. All timepoints from the same participants were run on the same plates. Data were generated using a Bio-Rad Bio-Plex® 200 system with Bio-Plex Manager Software 6.1 (Bio-Rad, Hercules, CA) as described previously.[8] Human Defensin Beta 1 and 2 (HBD-1/2) were measured using Enzyme Linked Immunosorbent Assay (ELISA) kits from Novus Biologicals (Product no. NBP2-67933 and NBP2-77363, respectively). Reagent preparation and assay procedures were conducted according to the manufacturer's instructions and a 2-fold sample dilution was used. Samples were analysed on an ELISA plate reader (Spectramax 250) at 450nm. HBD-1 and 2 were measured singly due to limited sample volume. For all immune mediators, concentrations that were below the assay lower limit of detection were reported as the mid-point between the lowest concentration measured and zero. PSA, as a measure of recent unprotected vaginal sex, was measured using Human Kallikrein 3/PSA Quantikine ELISA (R&D Systems, USA).

2.1.4 | STI testing

Lateral vaginal wall swab samples were thawed overnight on ice at 4°C. The following day, swabs were eluted in 1 ml phosphate buffered saline (PBS) and 250μl of sample was transferred to labelled sample tubes. DNA was extracted using the Roche Nucleic acid Kit 1 (Cat. No. 03730964001) and the MagNa Pure Compact Instrument (Product no. 03731146001) and 100μl of the extracted DNA was stored at -20°C until testing. STI testing was conducted using the STD Direct Flow Chip Kit (Master Diagnostica®–Ref: MAD-003938M-HS12). The extracted DNA samples were thawed on ice until fully resuspended. DNA was amplified using multiplex PCR followed by hybridisation according to the manufacturer's instructions for the detection of multiple STI causing organisms including *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium* and *hominis*, *Ureaplasma urealyticum/parvum*, Herpes Simplex Virus (HSV)-1 and 2 and *Treponema pallidum*. The results were analysed using hybriSoft analysis software (Master Diagnostica®). HSV-2 serology was conducted at Bio Analytical Research Corporation South Africa (BARC SA).[6] Vaginal swabs were also examined for the presence of *T. vaginalis* as described by Schirm *et al*.[20] In brief, primers and JOE-labelled probe targeting *T. vaginalis*-specific 2-kb repeated sequence was employed using ViiA 7 Real-Time PCR System (ThermoFisher Scientific). All samples were processed in duplicate to determine the mean cycle threshold (Ct) value. Samples with Ct values of less than 40 were considered positive. Wells with no DNA served as no template controls, and standard curves using serial dilutions of *T. vaginalis* genomic DNA were included.

2.1.5 | Data analysis

Statistical analyses were performed using STATA (Statacorp, USA), GraphPad Prism (GraphPad Software, USA) and SAS (SAS Institute Inc., USA). Immune mediators with Spearman rho score <.8 for duplicate measures (MIP-1ß) and those that were undetectable in ≥40% of samples (MIP-1ß and RANTES) were excluded from analysis (Table S1). A composite inflammation variable was generated using factor analysis of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) and chemokines (IL-8, MIP-1α, IP-10, MIP-3α) to group women according to baseline inflammation. Women with factor scores greater than or equal to the median were categorized as high inflammation; those with factors scores below the median were categorized as low inflammation. Immune mediator concentration changes from baseline to M1 and M3 within each contraceptive group and within group stratified by baseline inflammatory profile, were analysed using Wilcoxon signed rank test

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and Mann-Whitney U test, respectively. Differences in fold changes between the contraceptive methods at M1 and M3 post contraceptive initiation were assessed using Mann-Whitney U test. *P*-values were adjusted for multiple comparisons using a false discovery rate (FDR) step down procedure.[21] For multivariable analyses, immune mediator concentrations were transformed using Box-cox power transformation and analysed using generalized linear models. All analyses were stratified by baseline inflammatory profile; site and age were included as covariates in all models. In addition, baseline demographic and clinical variables including body mass index (BMI, >30 vs. ≤30), PSA (detected/not detected), clinical STI signs (yes/no where yes included vaginal or cervical ulcer or discharge, or cervical motion tenderness), cervical ectopy (yes/no), active STI (positive/negative for any of *C. trachomatis*, *M. genitalium*, *N. gonorrhoeae*, *T. vaginalis* and HSV-2) and HSV-2 serostatus (positive/negative) were evaluated as potential effect modifiers in all models; subsequent analyses were stratified for variables with interaction terms with *P*-values <.1. Confounders were identified from models evaluating immune mediator changes from baseline to M3; variables were evaluated through forward selection and retained if their inclusion resulted in a >10% change in the effect estimate (Table S2).

3 | RESULTS

Demographic and behavioural characteristics were balanced across randomized contraceptive groups at baseline (Table 1). Refusal rates were low, supporting the idea that women did not self-select based on any potential confounding variable. Generally, baseline FGT immune mediator concentrations were higher in women aged 15–24 years ($n = 101$) compared to women ≥25 years ($n = 60$), except for HBD-1 (Table S3). Six of eleven immune mediators included in this analysis, namely, TNF-α ($P = .044$), IL-6 ($P = .015$), IL-1β ($P = .001$), IL-8 ($P = .004$), MIP-1α ($P = .017$) and SLPI ($P = .034$) were statistically significantly higher in younger women. Additionally, concentrations of IL-1β ($P = .019$) and IL-8 ($P = .028$) were significantly higher in women with an active STI at baseline compared to women who did not have an STI, and HBD-2 ($P = .048$) concentrations were lower in women who were HSV-2 seropositive. Women enrolled at the SRC site in Tshwane had higher concentrations of IL-6 ($P = .003$) and MIP-3α ($P = .039$) at baseline compared to women enrolled at the MRU site in eThekwini (Table S3).

3.1 | Changes in immune mediators following contraceptive initiation

3.1.1 | Copper IUD

At M1 following copper IUD insertion, concentrations of IL-6, IL-1β, IL-8, MIP-1α and IP-10 were significantly raised from baseline. However, these changes did not remain significant after adjusting for multiple comparisons (Figure 1A). In multivariable models, concentrations of IL-6 were significantly increased at M1 but no significant changes were observed at M3 in bivariable or multivariable models (Table S4).

3.1.2 | DMPA-IM

No significant changes in immune mediator concentrations were observed following DMPA-IM initiation in bivariable (Figure 1B) or multivariable models (Table S4).

3.1.3 | LNG implant

At M1 post-LNG implant insertion, no statistically significant changes in immune mediator concentrations from baseline were evident (Figure 1C). However, at M3, TNF-α, IP-10, MIP-3α and SLPI concentrations were significantly increased; MIP-3α remained increased after adjustment in multivariable models (Table S4).

While LNG implant use was associated with more significant changes in immune mediator concentrations compared to copper IUD use, the overall magnitude of change was greater in copper IUD users (Figure S1).

3.2 | Baseline FGT immune profiles modify the effects of contraceptives on immune mediator concentrations

Baseline inflammation, active STI, body mass index (BMI) and cervical ectopy were identified as significant modifiers of the effects of contraceptives on immune mediator changes in multivariable models. Overall, women with low baseline inflammation experienced greater increases in immune mediator concentrations compared to those with high baseline inflammation (Figure 2; Table 2). In contrast, women with high baseline FGT inflammation, experienced minimal changes or decreases in immune mediator concentrations following contraceptive initiation (Figure 2; Table 2).

3.2.1 | Copper IUD

In copper IUD users, changes in TNF-α, IL-8 and MIP-1α between baseline and M3 were significantly greater in women with low versus those with high baseline inflammation (Figure 2A). In multivariable models, IL-6, IL-1β, IL-8, and IP-10 were significantly raised in women with low baseline inflammation at M1 and M3 relative to baseline. Additionally, MIP-1α followed the same pattern at M3 only (Table 2). No significant changes were observed among women who had high baseline inflammation.

3.2.2 | DMPA-IM

In DMPA-IM users, changes in IL-8, MIP-1α and IP-10 between baseline and M1 were significantly greater in women with low compared to high baseline inflammation (Figure 2B). TNF-α, IL-6, IL-1β, IL-8, MIP-3α and SLPI followed the same pattern at M3 (Figure 2B). In

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TABLE 1 Baseline demographic, behavioural and clinical characteristics of study participants randomized by contraceptive method

	Copper IUD (*n*=51)	DMPA-IM (*n*=52)	LNG Implant (*n*=58)	Total (*n*=161)	*P*-value
	n (%)	*n* (%)	*n* (%)	*n* (%)	
Site					
Setshaba Research Centre (*n*=52)	17	15	20	52	
MatCH Research Unit (*n*=109)	34	37	38	109	
Age (in years)					
Median (range)	23 (18–33)	23 (18–33)	23 (18–33)	23 (18–33)	.794
Marital status					
Married (monogamous)	0 (0)	0 (0)	1 (2)	1 (1)	.677
Never married	51 (100)	52 (100)	57 (98)	160 (99)	
Highest level of education					
Attended post-secondary school	14 (28)	12 (23)	10 (17)	36 (22)	.981
Secondary school, complete	13 (26)	16 (31)	25 (43)	54 (34)	
Secondary school, not complete	24 (47)	24 (46)	23 (40)	71 (44)	
Perlvic exam assessment					
Abnormal	7 (14)	5 (10)	5 (9)	17 (11)	.424
Vaginal discharge	6 (86)	3 (60)	5 (100)	14 (82)	.743
Mucopurulent cervical discharge	0 (0)	2 (40)	1 (20)	3 (18)	.368
Other	1 (14)	1 (20)	0 (0)	2 (12)	.669
Previous contraceptive use					
None	2 (34)	0 (0)	1 (2)	3 (2)	.506
IUD	0 (0)	0 (0)	0 (0)	0 (0)	N/A
Implant	2 (4)	2 (4)	2 (3)	6 (4)	1
DMPA	30 (59)	26 (50)	32 (55)	88 (55)	.774
NET-En	15 (29)	12 (23)	11 (19)	38 (24)	.217
Oral contraceptives	5 (10)	2 (4)	3 (5)	10 (6)	.432
Male/female condoms	35 (69)	35 (67)	38 (66)	108 (67)	.761
STI prevalence					
Neisseria gonorrhoeae	2 (4)	2 (4)	3 (5)	7 (4)	.929
Chlamydia trachomatis	7 (14)	8 (15)	16 (28)	31 (19)	.131
Trichomonas vaginalis	16 (31)	12 (23)	19 (33)	47 (29)	.495
Mycoplasma genitalium	1 (2)	2 (4)	4 (7)	7 (4)	.444
HSV-2-shedding	3 (6)	4 (8)	2 (3)	9 (6)	.624
HSV-2-serology	14 (27)	21 (40)	18 (31)	53 (33)	.351

Abbreviations: IUD, intrauterine device; DMPA-IM, 150 mg intramuscular depot medroxyprogesterone acetate; LNG, levonorgestrel; BMI, body mass index; HSV-2, herpes simplex virus type 2; PSA, prostate specific antigen; N/A, not available.

multivariable models, women with low baseline inflammation experienced significant increases in IL-8 and MIP-3α at M3 and in MIP-1α at both M1 and M3, relative to baseline (Table 2). In the high baseline inflammation group, significant decreases in MIP-1α and IP-10 were observed at M3, and SLPI was significantly decreased at M1 relative to baseline (Table 2).

3.2.3 | LNG implant

In LNG implant users, changes in IL-1β, IL-8, MIP-1α and MIP-3α between baseline and M3 were significantly greater in women with low compared to high baseline inflammation (Figure 2C). In multivariable models, women with low baseline inflammation experienced

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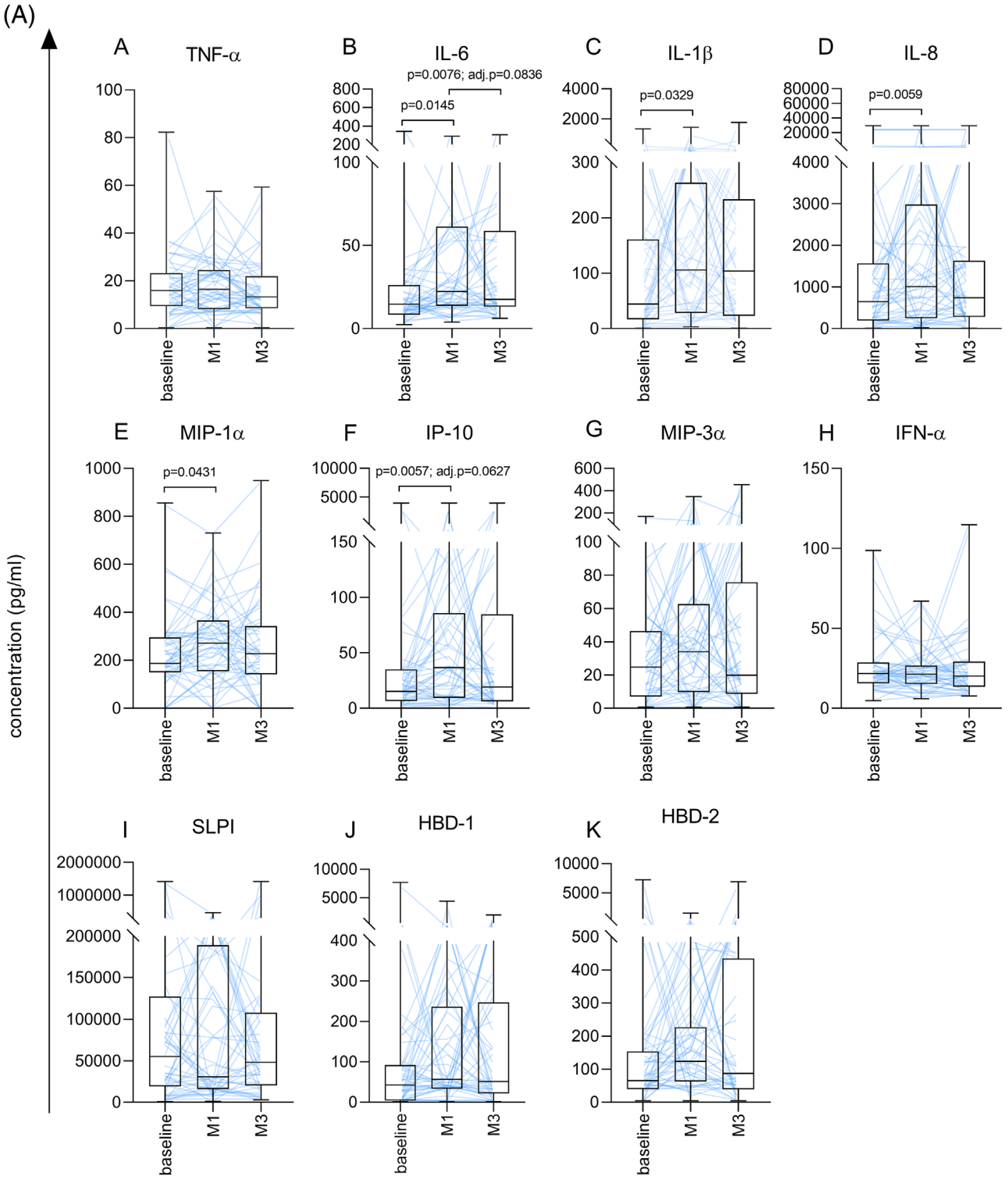

FIGURE 1 (A-K). Changes in genital immune mediator concentrations (pg/ml) over time. Boxplots representing mean immune mediator concentrations at baseline, 1 month (m1) and 3 months (m3) following contraceptive initiation in women using (A), copper IUD ($n = 51$), (B), DMPA-IM ($n = 52$) and (C), LNG implant ($n = 58$). Error bars indicate the ranges. Each coloured line represents one woman and immune mediator changes over time. The colour of the lines is based on the contraceptive methods used (blue, copper IUD; pink, DMPA-IM; green, LNG implant). Samples were run in duplicate. Wilcoxon signed rank test was used for comparisons and *P*-values were adjusted for multiple comparisons using a false discovery rate step-down procedure. Abbreviations: IUD, intrauterine device; DMPA-IM, 150 mg intramuscular depot medroxyprogesterone acetate; LNG, levonorgestrel; M1, 1-month post contraceptive initiation; M3, 3 months post contraceptive initiation; TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, interferon-alpha, SLPI, secretory leukocyte protease inhibitor; HBD, human beta defensin. * Indicates $P < .05$ after adjusting for multiple comparisons

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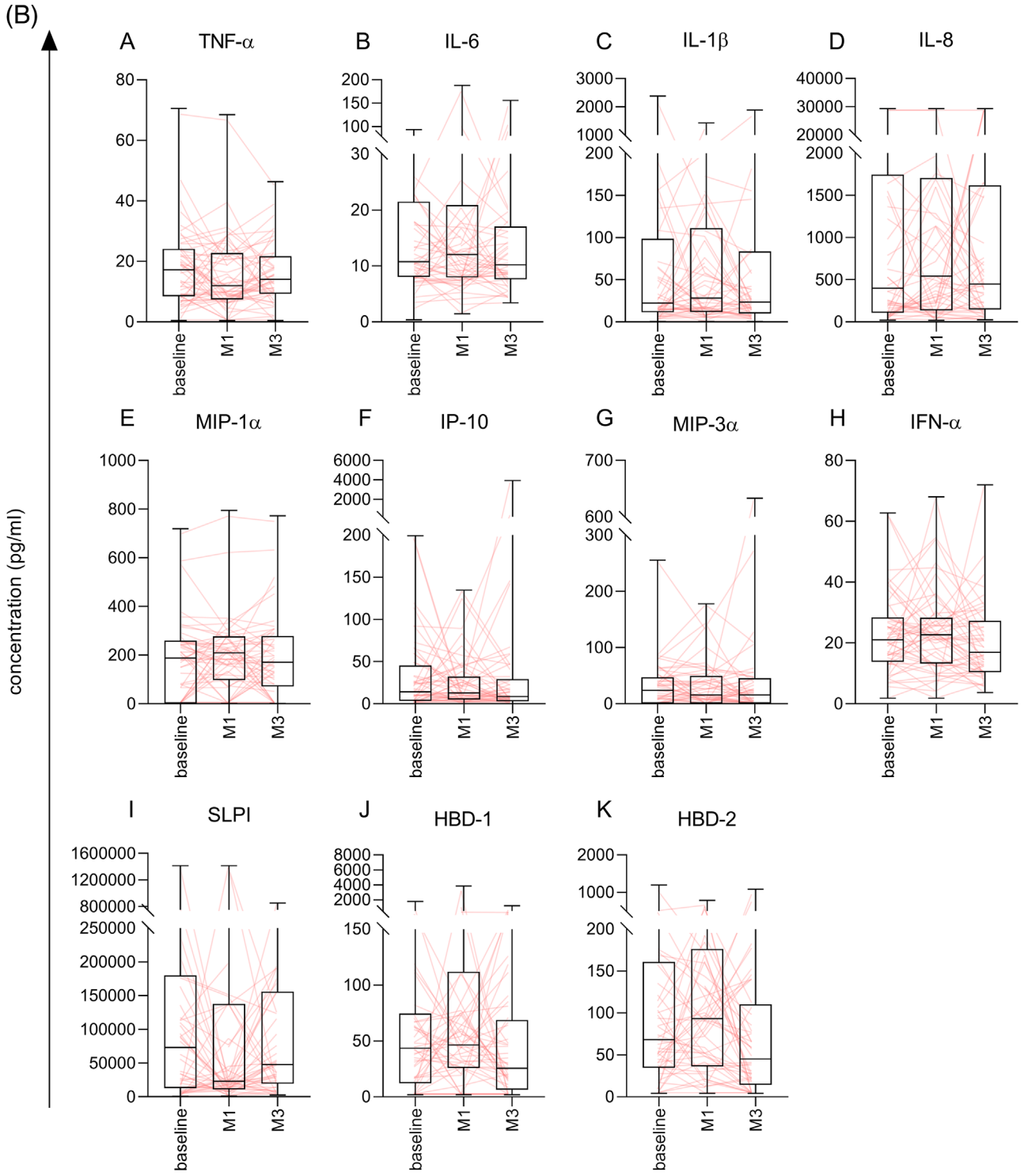

FIGURE 1 Continued

significant increases in IL-6 at M1 and M3 and in IL-8, IP-10, MIP-3α and SLPI at M3 relative to baseline. In contrast, LNG implant use was associated with decreased IL-1β and IFN-α concentrations at M1 and M3 relative to baseline in women with high baseline inflammation (Table 2).

4 | DISCUSSION

In South Africa, adolescent girls and young women are at a high risk of STIs and HIV infection. They also need safe and effective contraceptive methods to prevent unplanned pregnancies and as a tool of

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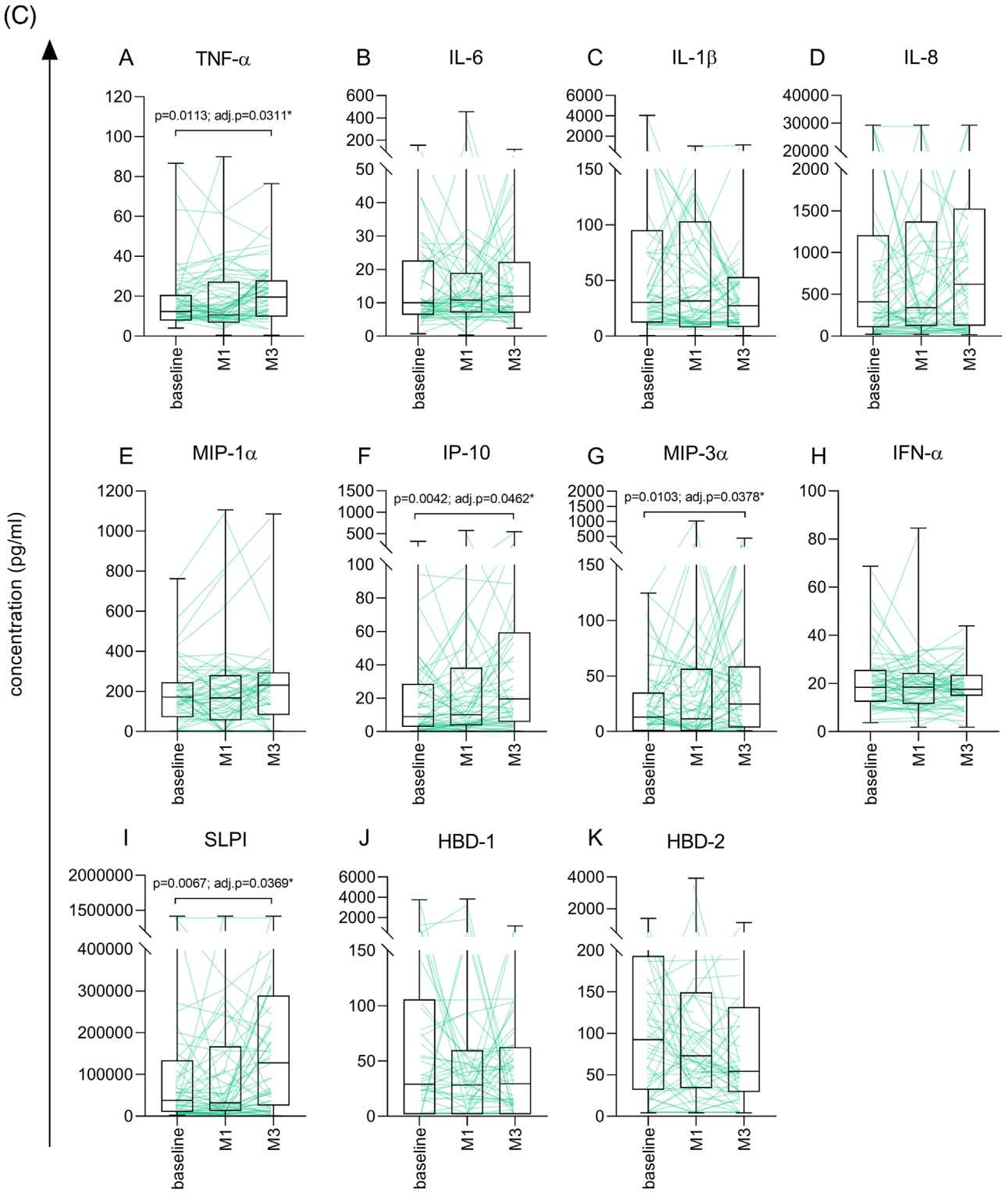

FIGURE 1 Continued

empowerment. While highly effective contraceptive methods are critical for improving women's health, their impact on FGT immunity, and consequential effects on STI/HIV risk, have been widely debated. In this study, we assessed the effects of DMPA-IM, copper IUD and LNG implant on FGT immune mediators among South African women participating in the ECHO clinical trial.

Overall, LNG implant use was associated with increases in several inflammatory cytokines and SLPI at M3 following contraceptive initiation, while women using the copper IUD had increased IL-6 at M1, and minimal changes were observed in the DMPA-IM arm. However, stratification of women according to baseline FGT inflammation revealed opposing effects of these contraceptives on immune

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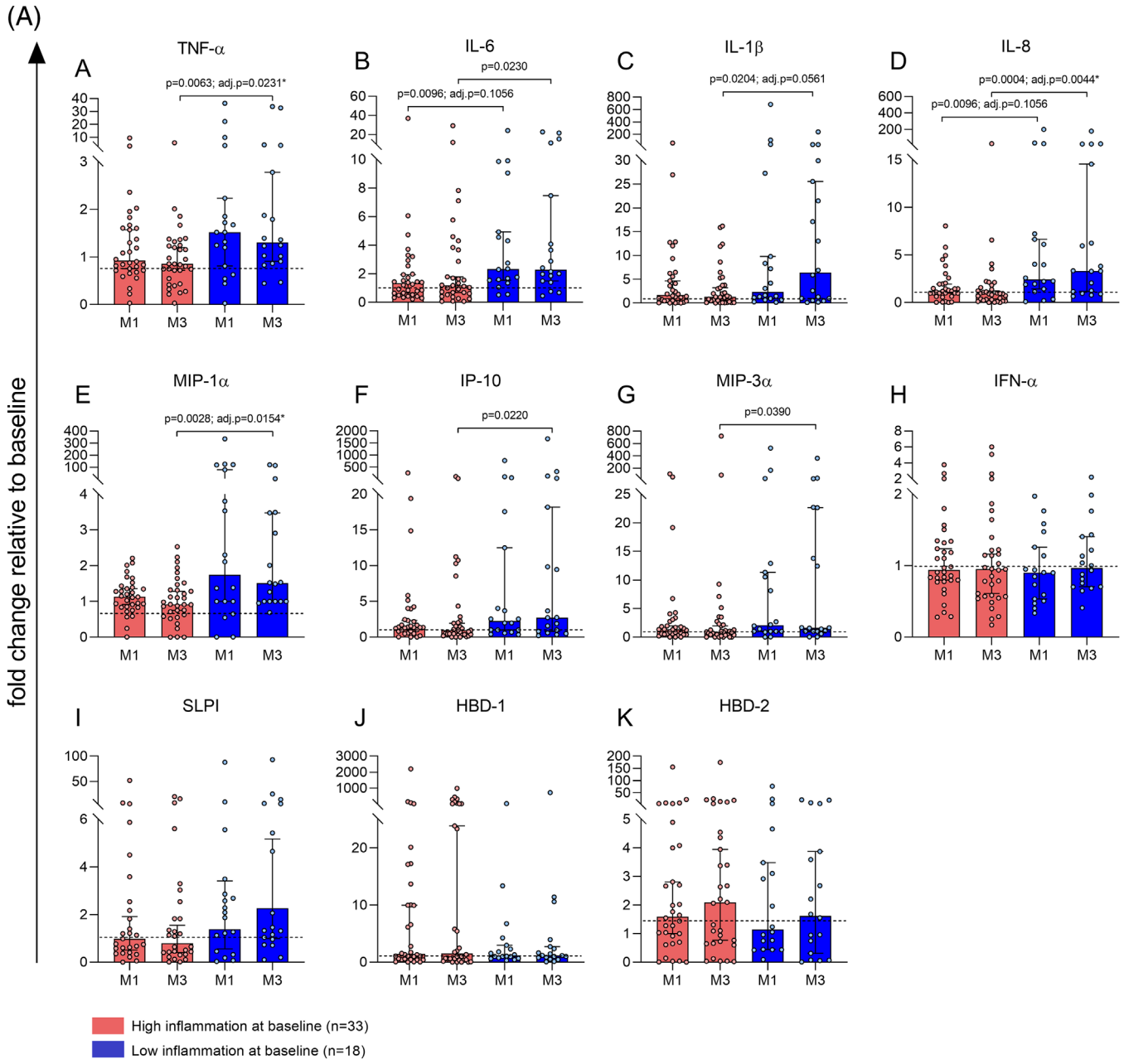

FIGURE 2 (A-K). Fold changes of genital immune mediator concentrations relative to baseline. Bar graphs representing the fold change of immune mediator concentrations at 1-month (M1) and 3 months (M3) following contraceptive initiation in women using (A), the copper IUD ($n = 51$), (B), DMPA-IM ($n = 52$) and (C), the LNG implant ($n = 58$) in women with high (pink) and low (blue) baseline inflammation. Women were grouped into high (pink) and low (blue) baseline inflammation using overall factor scores at baseline. Samples were run in duplicate. The immune mediators TNF-α, IL-8, MIP-1α, IL-6, IP-10, IL-1β and MIP-3α were included in the factor analysis. All pro-inflammatory cytokines and chemokines were loaded onto the same factor and scores were generated to represent overall level of inflammation. Error bars indicate the 95% confidence intervals. Mann-Whitney U test was used for comparisons and *P* values were adjusted for multiple comparisons using a false discovery rate step-down procedure. Abbreviations: IUD, intrauterine device; DMPA-IM, 150 mg intramuscular depot medroxyprogesterone acetate; LNG, levonorgestrel; M1, 1-month post contraceptive initiation; M3, 3 months post contraceptive initiation; TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, interferon-alpha, SLPI, secretory leukocyte protease inhibitor; HBD, human beta defensin. * Indicates $P < .05$ after adjusting for multiple comparisons

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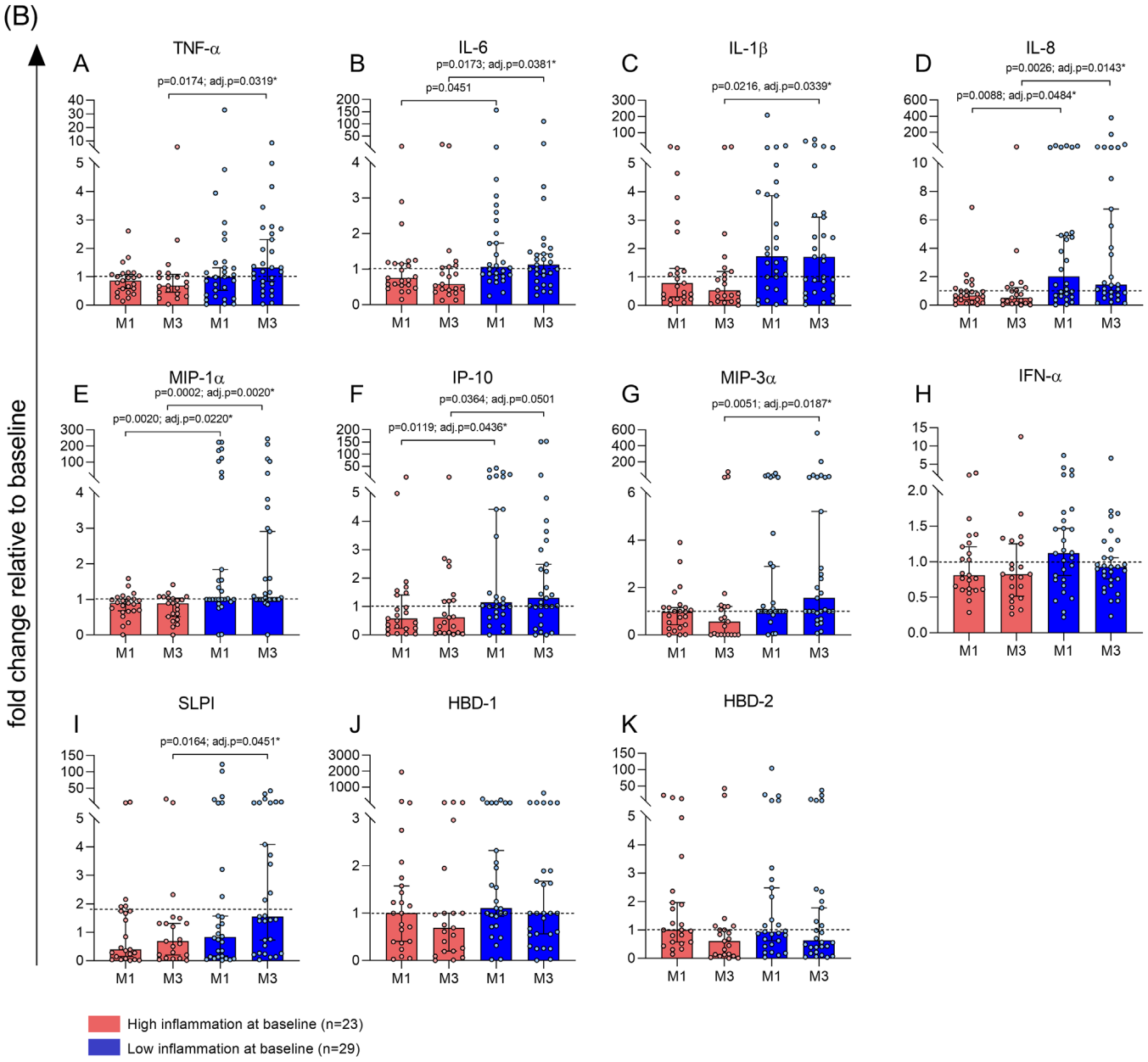

FIGURE 2 Continued

mediators, dependent on pre-existing inflammatory profile. In each group, women with low inflammation status at baseline experienced significant increases in immune mediators by M1 and/or M3, while women with high baseline inflammation experienced no significant changes in the copper IUD group and marginally significant decreases in several immune mediators following contraceptive use in the DMPA-IM and LNG implant group. These findings suggest that pre-existing differences in local inflammatory profiles, that may be mediated by genetic or environmental factors, modify the effects of contraceptives on FGT innate immune responses. The mechanisms underlying these changes remain to be determined, however, it is unlikely that STI incidence and/or changes in sexual behaviour explain the findings as our analyses were adjusted for these confounders. Our results offer a possible explanation for the contradictory findings of previous studies investigating FGT cytokine changes among women using injectable contraceptives, with some showing elevated inflammatory profiles and others showing reduced inflammation.[12–14,19,22,23] Importantly, each of the immune mediators measured in this study has been previously linked to changes in SIV or HIV infection risk.[8,18,14] Elevated concentrations of inflammatory cytokines in the FGT may reduce mucosal barrier integrity,[24] leaving the FGT prone to invasion by pathogenic microorganisms, including HIV and several other STI-causing agents. FGT inflammation is also associated with recruitment and activation of HIV target cells and direct promotion of HIV replication.[24–26]

In line with our results, a recent study explored the effects of DMPA-IM on the activation of HIV cellular targets and, importantly,

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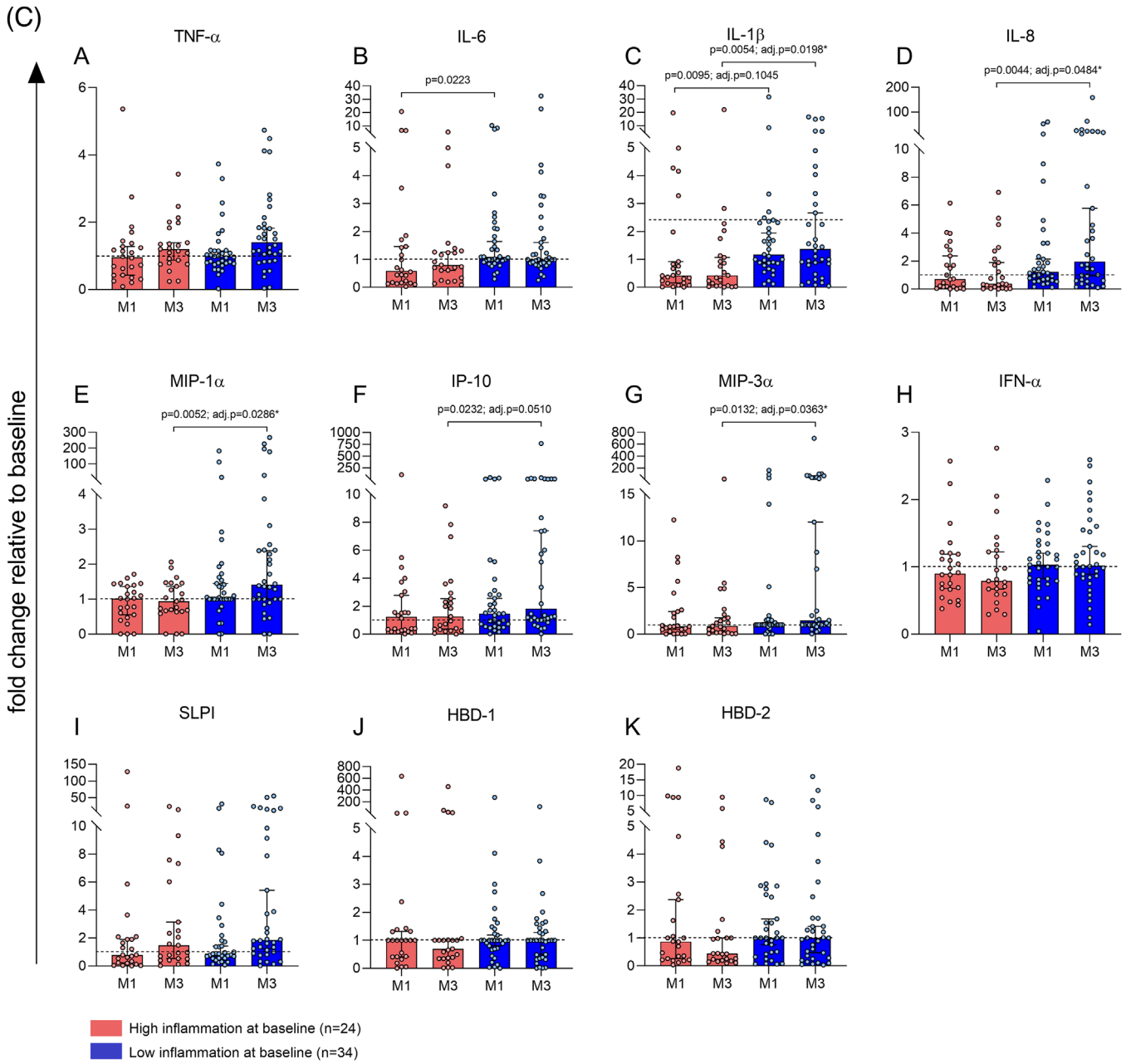

FIGURE 2 Continued

inflammation in sex workers versus non-sex workers.[27] Involvement in sex work has been associated with an immune tolerance phenotype due to constant exposure of the FGT to antigens. DMPA-IM use, however, was associated with increased T cell activation and inflammation in sex workers to the same level that the authors observed in non-sex workers using DMPA-IM.[27] Thus, sex workers who had lower immune activation profiles at baseline experienced greater increases in immune mediators following DMPA-IM initiation compared to non-sex workers, which is in line with our study.

There are several factors that may impact an individual's FGT inflammation status prior to contraceptive initiation. Out of the eleven immune mediators measured in this study, nine were elevated in younger women compared to older women at baseline. Previous studies have reported on the inverse relationship between age and genital inflammation[8] but the causes are not well understood. Although STIs were associated with elevated levels of some immune mediators at baseline, STI prevalence did not differ between age groups (data not shown) and BMI, cycle phase, presence of PSA, clinical signs as well as cervical ectopy were not associated with immune mediator levels. Thus, it is unlikely that differences in these factors explain the difference in immune mediator levels observed between age groups. One hypothesis is that the reproductive tract of young women represents a naïve reactive state that is anatomically immature and lacks tolerance to sex and male semen, in turn leading to increased genital

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TABLE 2 Changes in immune mediator concentrations from baseline to months 1 and 3 (M1 and M3) by contraceptive method stratified by baseline inflammation and adjusted for selected confounders[a]

	Copper IUD (*n*=51)		DMPA-IM (*n*=52)		LNG implant (*n*=58)	
	P-value	*P*-value	*P*-value	*P*-value	*P*-value	*P*-value
High baseline inflammation						
Immune mediator	**M1**	**M3**	**M1**	**M3**	**M1**	**M3**
TNF-α	.976	.078	.069	.053	.388	.944
IL-6	.251	.428	.310	.366	.177	.259
IL-1β	.643	.823	.538	.178	.066	.022
IL-8	.481	.070	.064	.075	.222	0.102
MIP-1α	.661	.298	.063	.032	.184	.067
IP-10	.531	.671	.057	.030	.446	.855
MIP-3α	.872	.649	.054	.160	.580	.539
IFN-α	.893	.914	.277	.328	.297	.043
SLPI	.901	.252	.046	.111	.279	.671
HBD-1	.074	.156	.810	.282	.705	.421
HBD-2	.521	.264	.248	.109	.850	.439
Low baseline inflammation						
Immune mediator	**M1**	**M3**	**M1**	**M3**	**M1**	**M3**
TNF-α	.148	.081	.481	.444	.690	.176
IL-6	<.001	<.001	.094	.290	.027	.039
IL-1β	.017	.012	.221	.231	.395	.534
IL-8	.010	<.001	.062	.014	.176	.018
MIP-1α	.066	.011	.006	.002	.669	.086
IP-10	.018	.006	.088	.510	.115	.002
MIP-3α	.074	.059	.350	.011	.996	.004
IFN-α	.181	.978	.346	.508	.796	.512
SLPI	.553	.075	.793	.166	.673	.024
HBD-1	.073	.193	.292	.282	.324	.421
HBD-2	.188	.960	.559	.457	.827	.760

significant decrease

significant increase

Abbreviations: IUD, intrauterine device; DMPA-IM, 150 mg intramuscular depot medroxyprogesterone acetate; M1; 1-month following contraceptive initiation; M3, 3 months following contraceptive initiation; LNG, levonorgestrel; TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, interferon-alpha.

Note: Participants who didn't receive the study contraceptive to which they were randomized are excluded from analyses.

[1]Box-cox power transformation is applied to each cytokine concentration for analyses.

[2]*P*-value is computed from T test after a generalized linear model is fit for each cytokine.

[3]If a variable of interest has a significant modification effect on a cytokine (*P*<.1), the model for that cytokine will stratify on the variable and adjusted with site and dichotomous age group.

Baseline inflammation is stratified on regardless.

[a]All the models are always adjusted with study site and dichotomous age group (under 25 or otherwise). The confounders are selected forward into the model as covariates from BMI, PSA, clinical signs, ectopy, active STI, and HSV-2 seropositivity. Variables with at least 10% contribution to the standardized coefficient will be retained in the model.

inflammation and increased risk for STI and HIV infection. Women from SRC in Tshwane also tended to have higher concentrations of genital immune mediators compared to women from MaTCH in eThekwini prior to contraceptive initiation. The vaginal microbiome is closely linked to genital inflammation[28] and the vaginal microbial composition has been found to differ by geographical location.[29] A *Lactobacillus*-dominant FGT microbiome is associated with low levels of inflammation and protection against HIV, while women with non-*Lactobacillus* dominant microbiota have increased inflammation and HIV risk.[28,30–32] Other environmental factors that may directly or

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indirectly influence local immune mediator levels include STIs, sexual behaviour patterns, such as the number of partners,[33] vaginal insertion and/or hygiene practices,[34] cigarette smoking[35] and Vitamin D levels.[36]

Other possible factors affecting immune mediators include pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domain or NOD-like receptors (NLRs) that recognize and bind foreign structures known as pathogen-associated molecular patterns (PAMPs) that are present on microbial cell surfaces, resulting in the stimulation of a pro-inflammatory immune response. Mouse models have demonstrated that expression levels of these PRRs have the potential to impact immune responsiveness and overall inflammatory profiles[37]. Interestingly, it has also been shown that some TLR genes are cyclically expressed throughout the menstrual cycle, with expression levels highest during the secretory phase[38,39].

Host genetics may also play a role, and, although evidence of a possible relationship between host genetics and immune mediator levels in the FGT is scarce, some studies have identified gene polymorphisms that modulate inflammatory response in the FGT.[40,41] For example, polymorphisms in the TLR4 gene (896 A>G polymorphisms) may lead to a subdued inflammatory response to lipopolysaccharide (LPS) from *G. vaginalis* among pregnant women.[40] Additionally, a polymorphism in the TNF-α gene (TNFA-208G>A) was associated with TNF-α concentrations in BV positive women.[41] It is thus possible that host genetics may modulate immune responses to vaginal pathogens and thus impact genital inflammation.

Sex steroid hormones are inherently linked to the regulation of microbial populations in the FGT.[42] While endogenous oestrogen and its synthetic analogue estradiol have previously been associated with an increased abundance of beneficial *Lactobacillus* species,[43] the findings of previous studies on the effects of progestin-only contraceptives, such as DMPA-IM, on vaginal microbial communities have been inconsistent.[22,44–50] The injectable has also been linked to altered cell-mediated immune responses, which in turn increase a woman's susceptibility to BV and STIs, including HIV. Recently, it has been demonstrated that effects of DMPA-IM on genital inflammatory pathways may be modified by the FGT microbiome. In a study by Noël-Romas *et al*., serum-MPA levels positively correlated with evidence of inflammation in the vaginal mucosal fluid of women with a *Lactobacillus*-dominant microbiome, but not in women with a non-*Lactobacillus* dominant microbiome. Additionally, while women with a *Lactobacillus*-dominant microbiome using DMPA-IM had a >3-fold increased risk of HIV acquisition, increased HIV risk was not observed in women who had a non-*Lactobacillus* dominant microbiome. Therefore, it is possible that DMPA-IM only increases inflammatory responses among women who do not already have high levels of inflammation.

Copper IUD use has been associated with increased microbial diversity and BV,[15,51] providing a possible explanation for the observed increased inflammation. While the mechanisms underlying the association between copper IUD use and BV are not fully established, the presence of a foreign body in the uterus and vagina may facilitate increased growth of anaerobic bacteria associated with BV.[51] Another possible explanation is that copper has been found to have antimicrobial activity[52] and may differentially impact different bacterial taxa to favour non-optimal species. Additionally, copper IUD use is frequently accompanied by a temporary increase in volume and duration of menses.[53,54] It has previously been shown that the relative abundance of *G. vaginalis* and *Lactobacillus* species fluctuate throughout a woman's normal menstrual cycle, with menses associated with increased *G. vaginalis* and decreased *Lactobacillus* species.[55] An extension of menses in copper IUD users could possibly result in an environment which facilitates increased growth of *G. vaginalis* which may persist to the point of dysbiosis in some women.[55,56]

Reports on the inflammatory potential of the LNG implant are sparse. While its use was associated with increases in several immune mediators in this study, Achilles *et al*. found no changes in cervical or systemic HIV target cell populations, cytokines and soluble mediators in women using the LNG implant for up to 6 months following contraceptive initiation.[57]

Another progestin-only implant, the etonogestrel implant, however, has previously been linked to higher levels of CD4+ T cells expressing the HIV co-receptor CCR5, as well as the soluble lymphocyte activation marker (sCD40L) after 3 months of contraceptive initiation.[58] Interestingly, scD40L, among other immune mediators, was associated with an increase in HIV risk in the CAPRISA 002 trial.[18] While this demonstrates that implants have been linked to changes in the genital immune environment, ultimately, we do not understand the mechanisms behind this possible relationship and our findings remain surprising.

Among women who had high inflammation at baseline, DMPA-IM and LNG implant were associated with decreases in immune mediators that were not observed in the copper IUD arm. In has been suggested that progestin-only contraceptives such as DMPA-IM may have anti-inflammatory effects by binding to the glucocorticoid receptor.[59] It is possible that this causes decreased inflammatory responses among women with high inflammation at baseline. On the other hand, contraceptive induced immunosuppression among individuals with low inflammatory profiles may increase susceptibility to STIs or BV and resulting inflammation. It is also possible that these contraceptives have multiple, divergent effects on immune pathways and the microbiota, dependent on factors that we were not able to assess in this study, and that are important to investigate in future studies.

4.1 | Limitations

Sex hormones, particularly oestradiol and progesterone, are also involved in immune regulation in the FGT by maintaining the balance between protection from pathogens while simultaneously allowing for reproductive functions.[60] The different levels of endogenous hormones during various menstrual cycle phases may significantly affect FGT inflammation.[42,61–64] In the future, we will be evaluating baseline levels of and changes in both endogenous and exogenous among the women participating in this study. It will also be useful to explore whether changes in the microbiome may explain immune mediator changes in these women.

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The significantly greater increases in women with low baseline inflammation were accompanied by marginally significant decreases in several immune mediators in women with high baseline inflammation in the DMPA-IM and LNG implant arm, suggesting the possibility that immune mediator concentrations naturally regressed to a mean value when women were grouped according to their baseline inflammatory profiles. In the copper IUD group, however, no diminution in immune mediator concentrations was observed across the timepoints in women with high baseline inflammation. Additionally, the absolute concentrations of three out of the five significantly raised immune mediators went above their respective baseline means at month 3 following contraceptive initiation (data not shown). Taken together, it is thus unlikely that the changes observed can be attributed to immune mediator levels regressing to a mean level of genital inflammation, particularly in the copper IUD group. Women with high baseline inflammation in the DMPA-IM and LNG implant group, however, experienced some marginally significant decreases in immune mediators and therefore we cannot disregard the possibility that the differential changes we observed among women with different baseline inflammatory profiles may in part be due to concentrations regressing to the mean.

5 | CONCLUSION

The substantial differences in immune mediator changes between women with higher versus lower levels of genital inflammation prior to contraceptive initiation may have contributed to the emergence of contradictory reports on the inflammatory potential of certain contraceptives, particularly for DMPA-IM. It is, therefore, important to gain a better understanding of the factors that influence immune changes in response to contraceptive use at an individual level to determine the impact of contraceptives on reproductive health, including STI and HIV acquisition risk more broadly. It will also be important to evaluate whether the effects of contraceptives on innate immune mediators are sustained or enhanced over longer periods of time during contraceptive use. Continued research to understand these effects is critical for safe contraceptive use and to inform novel contraceptive development.

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CONFLICT OF INTEREST

There is no competing interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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