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The Effect of Contraception on Genital Cytokines in Women Randomized to Copper Intrauterine Device, Depot Medroxyprogesterone Acetate, or Levonorgestrel Implant

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Background. The ECHO trial randomized women to intramuscular depot medroxyprogesterone acetate (DMPA-IM), levonorgestrel implant (LNG-implant), or copper intrauterine device (Cu-IUD). In a substudy of the ECHO trial, we tested the hypothesis that contraceptives influence genital inflammation by comparing cervicovaginal cytokine changes following contraception initiation. In addition, we compared cytokine profiles in women who acquired HIV (cases) versus those remaining HIV negative (controls).

Methods. Women (n = 251) from South Africa and Kenya were included. Twenty-seven cervicovaginal cytokines were measured by Luminex at baseline, and 1 and 6 months after contraceptive iTanko et alnitiation. In addition, cytokines were measured preseroconversion in HIV cases (n = 25) and controls (n = 100).

Results. At 6 months after contraceptive initiation, women using Cu-IUD had increased concentrations of 25/27 cytokines compared to their respective baseline concentrations. In contrast, women initiating DMPA-IM and LNG-implant did not experience changes in cervicovaginal cytokines. Preseroconversion concentrations of IL-1β, IL-6, and TNF-α, previously associated with HIV risk, correlated with increased HIV risk in a logistic regression analysis, although not significantly after correcting for multiple comparisons. Adjusting for contraceptive arm did not alter these results.

Conclusions. Although Cu-IUD use broadly increased cervicovaginal cytokine concentrations at 6 months postinsertion, these inflammatory changes were found not to be a significant driver of HIV risk.

Clinical Trials Registration. NCT02550067.

Keywords. reproductive tract; DMPA-IM; LNG-implant; Cu-IUD; inflammation.

Over 800 million women of reproductive age use modern contraceptive methods [1], including long-acting progestin-only injectables, implants, and intrauterine devices (IUDs) [2, 3] In sub-Saharan Africa, the most commonly used contraceptives are the intramuscular injectable depot medroxyprogesterone acetate (DMPA-IM) and the progestin-only levonorgestrel (LNG) implant, which both suppress ovulation, cause cervical mucus thickening, and endometrial atrophy [2, 4]. These contraceptives are highly effective in preventing unintended pregnancies [5]. However, the observed link between DMPA-IM use and increased human immunodeficiency virus (HIV) risk warranted urgent attention [6–8]. The Evidence for Contraceptive Options and HIV Outcomes (ECHO) trial was conducted to compare the relative HIV incidence rates in women randomly assigned to receive DMPA-IM, LNG-implant, and copper IUD (Cu-IUD) [9]. This found no significant difference in incidence of HIV acquisition among the contraceptive methods tested.

Prior to the ECHO trial, pointing to mechanisms underlying contraceptive impact on HIV risk, several observational studies showed that some hormonal contraceptives (primarily

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DMPA-IM but also combined oral contraceptives) induce genital inflammation, evidenced by increased production of inflammatory cytokines and chemokines [10, 11], decreased antiviral immunity or increased mucosal HIV target cell frequencies [12–14], and disruption/thinning of the squamous epithelial barrier in the lower reproductive tract [15, 16]. Others have demonstrated an inflammatory response to the Cu-IUD in the lower genital tract and endometrium, possibly directed at Cu [17, 18]. In this mucosal substudy within the ECHO trial, we hypothesized that these contraceptive methods would differentially influence the genital immune environment, with a focus on inflammation. Thus, we assessed longitudinal cytokine profiles in matched cervicovaginal samples collected before, and at 1 and 6 months after contraception initiation. We further compared cervicovaginal cytokine profiles in preseroconversion samples from women who subsequently acquired HIV (cases) and those who remained HIV negative (controls) to evaluate cytokine association with HIV risk.

METHODS

Study Participants

Of the 12 study sites of the ECHO trial, 3 sites with capacity for genital sample collection and storage were selected for this mucosal substudy. The ECHO trial was an open-label randomized trial assessing the incidence of HIV among women assigned to use DMPA-IM, LNG-implant, or the Cu-IUD (Clinicaltrials.gov NCT02550067) [9]. The parent trial enrolled women between December 2015 and September 2017 who were aged 16–35 years, HIV negative, sexually active, desiring effective contraception, and willing to be randomized to 1 of 3 study contraceptive methods. Women were excluded if they reported having used injectable contraception (DMPA or norethisterone enanthate), an implant, or IUD in the last 6 months, or if they had any medical contraindication to the study contraceptive methods [9]. Ethical approvals for this nested substudy were obtained from the Human Research Ethics Committees at the University of Cape Town (HREC 371/2015), University of Witwatersrand (HREC PRC 141112), Kenya Medical Research Institute (KEMRI; SERU/CMR/P0014/3109), University of Washington (STUDY00000261), and FHI360 (523201). All participants provided written informed consent.

Design of Pre-/Post-Contraceptive–Initiation Substudy

Following ethics approvals, we consecutively offered enrolment into this pre-/post-contraceptive–initiation mucosal substudy to all women participating in the ECHO trial at Wits Reproductive Health and HIV Institute (Wits RHI; Johannesburg, South Africa; n = 109), Desmond Tutu Health Foundation Emavundleni (Cape Town, South Africa; n = 151), and KEMRI (Kisumu, Kenya; n = 170). Approximately 20 women per arm per study site were randomly selected and included if they had initiated their randomized contraceptive method and used it for at least the first month.

Design of Case-Control Substudy

Available cervicovaginal secretions (CVS) samples from all women across the 3 study sites who subsequently acquired HIV were included for a case-control study of cytokines and HIV risk. To ensure sufficient statistical power of the study, a random selection of women who did not seroconvert were selected as controls at a ratio of 4 controls to 1 case, matched on study site, visit, and age. Samples from the visit prior to HIV seroconversion (median of 6 months; interquartile range [IQR], 3.0–12.0 months) were assayed. For controls, the sample from the same time period (median, 6 months; IQR, 3.0–12.0 months) in follow-up was selected. Baseline samples for all women in the case-control study were also included, if available.

Mucosal Sample Collection and Processing

CVS were collected from study participants using a disposable menstrual cup (Softcup; Evofem), placed by the participant or study clinician and worn for approximately 1 hour prior to the collection of other mucosal samples. Upon removal, the menstrual cup was immediately placed into a sterile 50-mL Falcon tube and transported on ice to the laboratory within 6 hours of collection. At the laboratory, the tube was centrifuged at 1500 rpm for 10 minutes at 4°C, after which the menstrual cup was carefully removed and discarded. The tube containing the secretion was weighed and the volume of the secreted fluid calculated (weight of tube with secretion – weight of empty tube). The secretion was diluted 5-fold with sterile phosphate buffer saline (Sigma-Aldrich) and stored in cryovials at –80°C until use [19, 20].

Diagnosis of Sexually Transmitted Infections and Bacterial Vaginosis

Testing for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* was performed at screening using endocervical swabs and treatment was provided upon etiologic diagnosis or when a woman presented with symptoms, according to national algorithms in each country at the time. Treatment for sexually transmitted infections (STIs) was provided on site as soon as possible after diagnosis and that for infections diagnosed via laboratory testing on screening samples; this often resulted in treatment being delivered at the enrolment visit. For *C. trachomatis*/*N. gonorrhoeae* testing, GeneXpert Instrument Systems platform (Cepheid) with the Abbott Real Time PCR assay (Abbott Molecular) were used at Wits RHI and Emavundleni sites, while the Panther System (Hologic) was used at the KEMRI site. Herpes simplex virus type 2 (HSV-2) serology was performed for HSV-2 gG2 IgG using the enzyme-linked immunosorbent assay (EIA; HerpeSelect, Focus Diagnostics) at baseline for all 3 sites [9]. For HSV-2 seropositive and indeterminate EIA results, confirmatory testing was performed via western blot at the University of Washington Virology laboratory. For bacterial vaginosis (BV)

diagnosis, lateral vaginal swabs were rolled onto a glass slide, fixed, and kept at room temperature. Nugent scoring was conducted by the National Institute for Communicable Diseases laboratory in Johannesburg, South Africa [21]. BV was treated syndromically, as per national guidelines in each participating country.

Measurement of Cytokines

CVS were assayed using Luminex (Bio-Plex Pro Human cytokine 27-plex; Bio-Rad Laboratories) to measure the concentrations of 27 cytokines, including inflammatory cytokines (interleukin 1β [IL-1β], IL-6, IL-12p70, tumor necrosis factor-α [TNF-α]); chemokines (IL-8, Eotaxin, IFN-γ inducible protein-10 [IP-10], monocyte chemoattractant protein-1 [MCP-1], macrophage inflammatory protein-1α [MIP-1α], MIP-1β, regulated on activation, normal T-cell expressed and secreted [RANTES]); adaptive cytokines (IL-2, IL-4, IL-5, IL-13, IL-15, IL-17A, interferon-γ [IFN-γ]); growth factors (IL-7, IL-9, platelet-derived growth factor-BB [PDGF-BB], fibroblast growth factor [FGF]-basic, granulocyte-colony stimulating factor [G-CSF], granulocyte-macrophage colony stimulating factor [GM-CSF], vascular endothelial growth factor [VEGF]); and regulatory cytokines (IL-1 receptor agonist [IL-1RA], IL-10), according to the manufacturer's protocol. CVS samples were thawed and filtered using SPIN-X 0.2-μm cellulose acetate filters (Sigma). A total of 1046 CVS samples (both pre- and postcontraceptive and HIV case-control samples) were run on 14 plates over 10 days. To control for inter- and intraplate variation, 5 CVS samples were run in duplicate on each plate (intraplate controls) and a panel of 5 samples were run on all 14 plates (interplate controls; Supplementary Table 1). To enable comparison across plates, matched CVS samples from baseline and 1 month after contraceptive initiation were first assayed on the same plate, and then samples from 6 months after contraceptive initiation with their corresponding baseline samples and case-control samples were assayed on the same plate. Thus, the results generated from 1- and 6-month post-contraceptive–initiation samples are not represented on the same figures. The lower limit of detection for cytokines ranged from 1.4 to 92.6 pg/mL. Data were collected using a Bio-Plex Suspension Array Reader (Bio-Rad Laboratories). A Bio-Plex manager software (version 4) was used to analyze the data and a 5-parameter logistic regression formula was used to calculate cytokine concentrations from standard curves. Cytokine concentrations below the lower limit of detection of the assay were reported as the midpoint between zero and the lowest concentration measured. Cytokines that were undetectable in > 40% of samples assayed were excluded for all analyses.

Statistical Analyses

Unsupervised hierarchical clustering from $\log_{10}$-transformed cytokine data was performed to group women according to the relatedness of their cytokine expression profiles using the R package "ComplexHeatmap" [22]. The difference in cytokine concentration between contraceptive arms was determined using the R package "vegan" [23] by permutational multivariate analysis of variance (PERMANOVA) using euclidean distance matrices and the adonis function. A sparse partial least-squares discriminant analysis (sPLSDA) was used to determine cytokine profiles that differ between contraceptive groups using the R package "mixOmics" [24]. The Wilcoxon signed rank test was used for paired samples in the pre-/post-contraception–initiation analyses. The relationship between contraception and HIV acquisition risk was assessed in an intention-to-treat and per-protocol analyses. Both analyses gave similar results. For HIV cases and controls, differences in baseline characteristics were evaluated using the Fisher exact, χ^2, and Mann-Whitney *U* tests, while differences in cytokine levels were determined using the Mann-Whitney *U* test (for unpaired samples). Multivariate logistic regression analyses were used to determine the association between HIV acquisition and cytokine concentrations and adjustment for contraceptive arm. Intraplate correlations were determined by the nonparametric Spearman rank correlation test. A false discovery rate step-down procedure was used to adjust *P* values to decrease false-positive results due to multiple comparisons [25]. *P* values < .05 were considered significant. GraphPad Prism version 8.3 (GraphPad software) and R version 3.6 (R Core Team) were used for statistical analyses and data visualization.

RESULTS

Comparison of CVS Cytokine Concentrations Before and After Contraceptive Initiation

To compare CVS cytokine changes associated with contraceptive initiation, a total of 149 women with samples available at baseline, and 1 month and 6 months postinitiation were included. Five women did not have samples at 1 month. Of these 149 women, 52/149 were randomized to the Cu-IUD arm, 47/149 to the DMPA-IM arm, and 50/149 to the LNG-implant arm (Supplementary Figure 1A). None of the participants switched contraceptive arms prior to the 6-month visit. At baseline, no significant differences in demographics, sexual risk behaviors, and STI prevalence were evident among women randomized to different contraceptive arms (Table 1). No significant differences in baseline CVS cytokine concentrations were noted across the 3 contraceptive arms (Supplementary Table 2).

At 1 month after contraceptive initiation, no significant changes in CVS cytokine concentrations were found compared to their respective baseline samples in any of the contraceptive arms (Figure 1). In contrast, at 6 months after contraceptive initiation, major shifts in CVS cytokine profiles were noted in women randomized to the Cu-IUD arm, with 25/27 of the cytokines being significantly elevated compared to matched baseline samples, after

Table 1. Baseline Characteristics of Participants in the Pre-/Post-Contraceptive–Initiation Study

Characteristic	Randomization Arm		
	Cu-IUD	DMPA-IM	LNG-implant
n	52	47	50
Age, y, median (25th–75th percentile)	24 (19.3–28.8)	24 (21.0–28.0)	23 (20.8–26.0)
BMI, kg/m^2, median (25th–75th percentile)	24 (21.2–30.3)	24 (21.7–28.7)	23 (20.4–29.7)
Genital infection, n (%)			
Chlamydia trachomatis	14 (26.9)	7 (14.9)	5 (10.0)
Neisseria gonorrhoeae	4 (7.7)	3 (6.4)	2 (4.0)
Bacterial vaginosis			
Negative, Nugent 0–3	25 (48.1)	32 (68.1)	27 (54.0)
Intermediate, Nugent 4–6	8 (15.4)	5 (10.6)	6 (12.0)
Positive, Nugent 7–10	19 (36.5)	10 (21.3)	17 (34.0)
Herpes simplex virus type 2			
Negative	23 (44.2)	15 (31.9)	21 (42.0)
Indeterminate	5 (9.6)	6 (12.8)	7 (14.0)
Positive	24 (46.2)	26 (55.3)	21 (42.0)
Demographics/sexual risk behavior, n (%)			
Condomless sex in previous 3 mo	37 (71.2)	33 (70.2)	36 (72.0)
Condom usage with last vaginal sex among women who had sex	22 (42.3)	25 (53.2)	23 (46.0)
Vaginal bleeding during sex	8 (15.4)	6 (12.8)	7 (14.0)
>1 partner in previous 3 mo	9 (17.3)	5 (10.6)	3 (6.0)
Marital status			
Never married	37 (71.2)	32 (68.1)	31 (62.0)
Married	15 (28.8)	15 (31.9)	19 (38.0)
Education			
No schooling	1 (1.9)	0 (0.0)	0 (0.0)
Primary school education	16 (30.8)	11 (23.4)	8 (16.0)
Secondary education	30 (57.7)	34 (72.3)	37 (74.0)
Tertiary education	5 (9.6)	2 (4.3)	5 (10.0)

No record of an HSV-2, chlamydia, and gonorrhea tests was reported for 1 woman randomized to LNG-implant. No BMI measurement was available for 1 woman randomized to DMPA-IM arm. Twenty-five women included in this table were part of the case-control study (2 cases and 23 controls).

Abbreviations: BMI, body mass index; Cu-IUD, copper intrauterine device; DMPA-IM, intramuscular depot medroxyprogesterone acetate; LNG, levonorgestrel.

adjusting for multiple comparisons (Figure 2). Cytokines that were significantly elevated in the Cu-IUD arm included all 4 of the inflammatory cytokines that were measured (IL-1β, IL-6, IL-12p70, TNF-α), all 7 chemokines (IL-8, Eotaxin, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES), 6/7 adaptive cytokines (IL-2, IL-4, IL-5, IL-13, IL-15, IL-17A, but not IFN-γ), 6/7 growth factors (IL-7, IL-9, FGF-basic, G-CSF, GM-CSF, VEGF, but not PDGF-BB), and 1/2 regulatory cytokines (IL-10 but not IL-1RA; Figure 2). Of these, MIP-1α (median, 21.4-fold increase; IQR, 1.3–59.6; adjusted $P = .0002$), MIP-1β (7.9-fold increase; IQR, 1.3–22.2; adjusted $P = .0003$), IL-6 (10.1-fold increase; IQR, 1.5–83.6; adjusted $P = .0003$), MCP-1 (8.6-fold increase; IQR, 2.2–15.4; adjusted $P = .002$), and IL-1β (8.4-fold increase; IQR, 1.1–31.0; adjusted $P = .0002$) were the most strongly upregulated. While the regulatory cytokine IL-10 was moderately elevated in women using Cu-IUD (1.4-fold increased; IQR, 1.0–3.4; adjusted $P = .001$), IL-1RA concentrations were suppressed (0.8-fold decrease; IQR, 0.5–1.1; adjusted $P = .02$), suggesting dysregulation of the cervicovaginal inflammatory environment. Changes in CVS cytokine profiles were not observed in women randomized to the LNG-implant or DMPA-IM arms, compared to baseline.

Unsupervised hierarchical clustering of the 6-month cytokine concentrations showed that women in the Cu-IUD arm clustered distinctly from those in the DMPA-IM and LNG-implant arms (Figure 3A). In a sparse partial least squares discriminant analysis (sPLSDA), differences in cytokine profiles between the 3 contraceptive groups were significant (PERMANOVA $P = .001$; Figure 3B). Women randomized to the Cu-IUD arm had the majority of CVS cytokines (24/27) negatively loaded on X variate 1 in the sPLSDA, compared to DMPA-IM and LNG-implant initiation, which clustered distinctly, associated with only IL-1RA or the combination of IL-8 and VEGF loadings on X variate 1, respectively (Figure 3C). This suggests a differential effect of contraceptives on cytokines.

Evaluation of CVS Cytokine Profiles Prior to HIV Seroconversion

Twenty-five women acquired HIV during follow-up in this mucosal substudy. CVS cytokine profiles in preseroconversion samples from these women were compared to 100 women who did not seroconvert (Supplementary Figure 1B). Two cases switched contraceptive arms prior to the preseroconversion sample and 2 controls switched contraceptive arms at the matched time

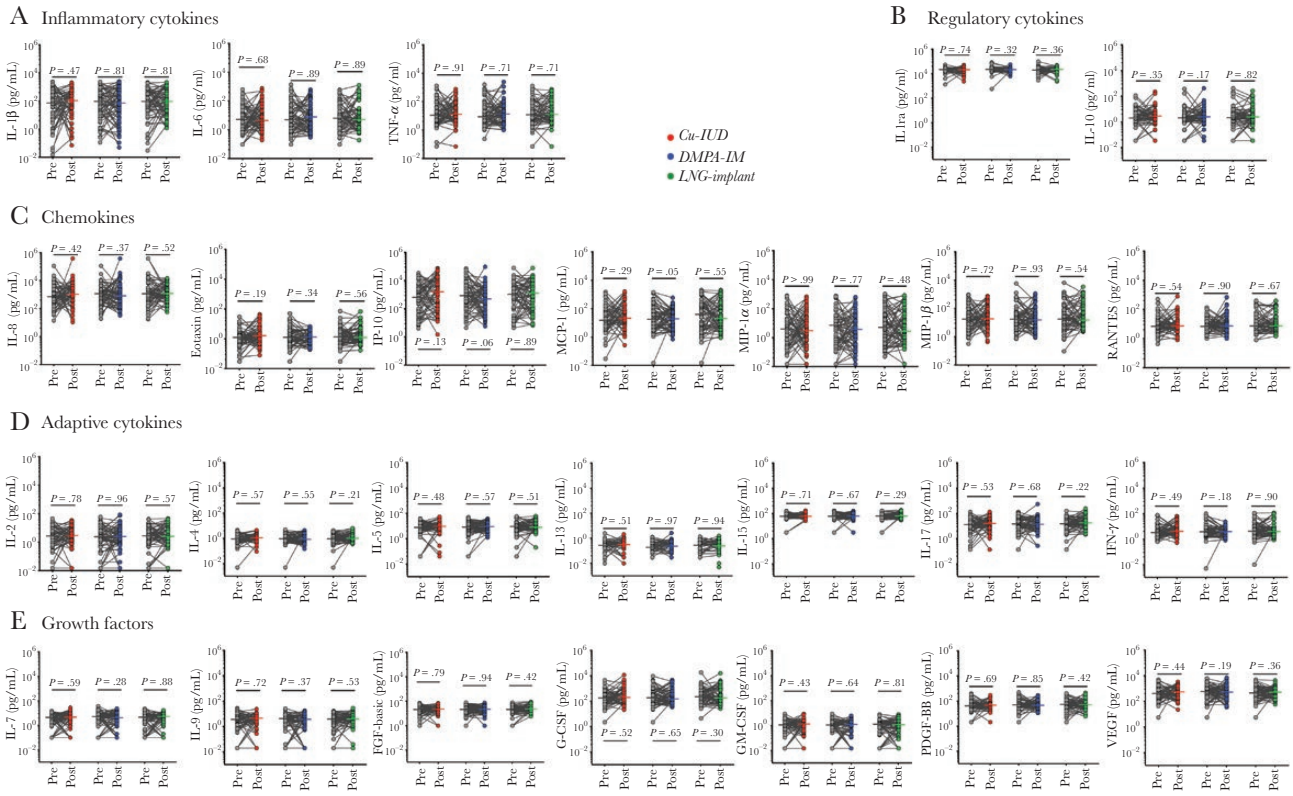

Figure 1. *A–E*, Effect of 1-month contraceptive use on cervicovaginal cytokines. Concentrations of each cytokine before (Pre) and 1 month after (Post) contraceptive initiation. Grey dots indicate precontraceptive cytokine concentrations. Cu-IUD (n = 60), DMPA-IM (n = 67), and LNG-implant (n = 63) users are shown in red, blue, and green, respectively. Horizontal lines indicate the median. Statistical significance was calculated using a Wilcoxon signed rank for paired samples with adjustment for multiple comparisons using the false discovery rate step-down procedure. A *P* value < .05 was considered significant. Abbreviations: Cu-IUD, copper intrauterine device; DMPA-IM, intramuscular injectable depot medroxyprogesterone acetate; FGF, fibroblast growth factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN-γ, interferon-γ; IL, interleukin; IL-1ra, IL-1 receptor agonist; IP-10, IFN-γ inducible protein-10; LNG, levonorgestrel; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; PDGF-BB, platelet-derived growth factor-BB; RANTES, regulated on activation, normal T-cell expressed and secreted; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

point (either to DMPA-IM or no contraceptive). No differences in baseline demographics, sexual risk behaviors, and genital infections were noted between HIV cases and controls (Table 2). Compared to controls, women who subsequently acquired HIV had higher concentrations of 12/27 cytokines across various functional classes in CVS samples collected preseroconversion in an intention-to-treat analysis(Table 3). These included IL-1β, IL-6, TNF-α, Eotaxin, RANTES, IL-17, GM-CSF, IL-15, IL-2, IL-4, IL-5, and FGF-basic. However, none of these differences remained significant after adjusting for multiple comparisons.

Logistic regression analysis also suggested that preseroconversion concentrations of several cytokines were positively associated with subsequent HIV acquisition in the intention-to-treat analysis, including IL-1β (odds ratio [OR], 1.59; 95% confidence interval [CI], 1.06–2.53; unadjusted $P = .035$), IL-6 (OR, 1.58; 95% CI, 1.04–2.43; unadjusted $P = .034$), TNF-α (OR, 2.15; 95% CI, 1.15–4.16; unadjusted $P = .019$), Eotaxin (OR, 3.19; 95% CI, 1.04–10.65; unadjusted $P = .048$), IL-2 (OR, 2.32; 95% CI, 1.11–5.29; unadjusted $P = .034$), IL-4 (OR, 3.35; 95% CI, 1.18–10.44; unadjusted $P = .028$), IL-5 (OR, 3.01; 95% CI, 1.14–8.80; unadjusted $P = .033$), IL-17 (OR, 2.59; 95% CI, 1.25–5.87; unadjusted $P = .015$), FGF-basic (OR, 3.48; 95% CI, 1.16–11.49; unadjusted $P = .032$), and GM-CSF (OR, 2.45; 95% CI, 1.08–6.47; unadjusted $P = .048$; Table 4). None of these associations remained significant after correcting for multiple comparisons. Controlling for contraceptive arm in the model did not have a substantial impact on these associations.

DISCUSSION

Access to safe and effective contraception has brought substantial improvements to maternal and child health [26]. Despite these important public health gains for women and their children, certain contraceptive methods have been associated with increased genital inflammation, raising concerns about unintended consequences for disease susceptibility, including a small risk for acquisition of HIV and other STIs [10, 27, 28]. In this mucosal substudy within the ECHO trial, we show that women randomized to the Cu-IUD experienced significant and broad increases in cervicovaginal cytokine concentrations 6 months after Cu-IUD insertion while those using DMPA-IM

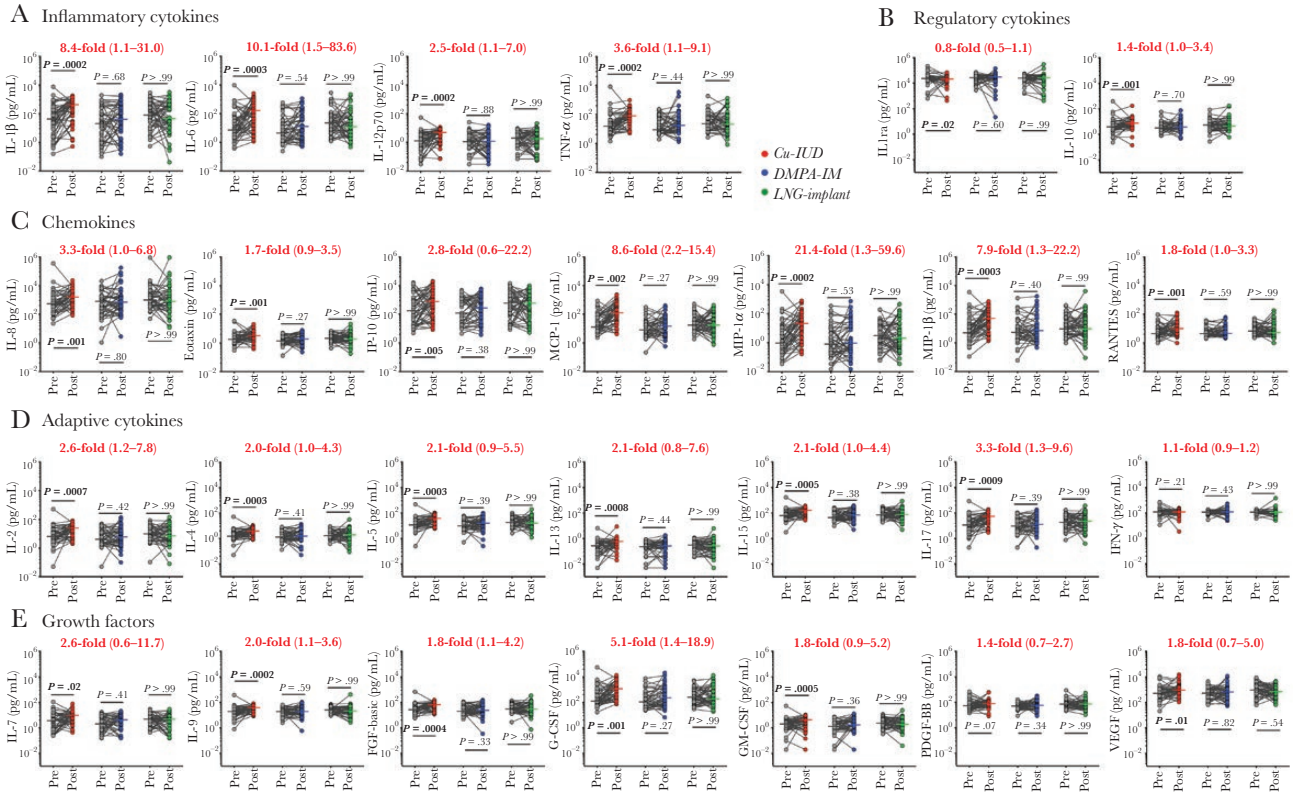

Figure 2. *A–E*, Effect of 6-months contraceptive use on cervicovaginal cytokines. Concentrations of each cytokine before and 6 months after contraceptive initiation. Grey dots indicate precontraceptive cytokine concentrations. Cu-IUD (n = 52), DMPA-IM (n = 47), and LNG-implant (n = 50) users are shown in red, blue, and green, respectively. Horizontal lines indicate the median. Median fold change and interquartile range is represented in red for the Cu-IUD arm. Statistical significance was calculated using a Wilcoxon signed rank for paired samples with adjustment for multiple comparisons using the false discovery rate step-down procedure. A *P* value < .05 was considered significant. Abbreviations: Cu-IUD, copper intrauterine device; DMPA-IM, intramuscular injectable depot medroxyprogesterone acetate; FGF, fibroblast growth factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN-γ, interferon-γ; IL, interleukin; IL-1ra, IL-1 receptor agonist; IP-10, IFN-γ inducible protein-10; LNG, levonorgestrel; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; PDGF-BB, platelet-derived growth factor-BB; RANTES, regulated on activation, normal T-cell expressed and secreted; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

and LNG-implant did not. In addition, although women who subsequently acquired HIV during follow-up had higher CVS concentrations of several inflammatory cytokines and chemokines (including IL-1β, IL-6, TNF-α, Eotaxin, IL-2, IL-4, IL-5, IL-17, GM-CSF, and FGF-basic) compared to women who remained HIV negative, this was not significant after correcting for multiple comparisons. Correcting for contraceptive arm did not significantly impact the relationship between CVS cytokines and HIV risk, after adjusting for multiple comparisons. This is consistent with the finding that contraceptive arm was not a driver of HIV risk in the ECHO trial, despite being associated with inflammatory changes in the pre-/post-contraceptive–initiation analysis. Combined with data from the parent ECHO trial results, our findings suggest that the broad changes in genital cytokine profiles associated with Cu-IUD insertion were either of insufficient magnitude, duration, or nature to impact HIV risk significantly over the other 2 study arms.

Previous observational studies, including those from our group, suggested that DMPA-IM use either increased or decreased CVS cytokine concentrations [10, 11, 29–31]. In this substudy from the ECHO trial, comparing before contraceptive initiation to after initiation CVS cytokine concentrations in women randomized to contraceptives, we did not find any change in cytokines in women randomized to DMPA-IM or LNG-implant in the 6 months after contraceptive initiation.

Insertion of Cu-IUDs has been demonstrated to have a significant histological, biochemical, and inflammatory immune impact on the female reproductive tract [32–34]. Early studies, focusing on tissues collected from the upper reproductive tract, suggested that inflammatory changes were restricted to the uterus and endometrium [32–34]. It is now clear that these changes are also easily detectable in cervicovaginal secretions collected in the lower reproductive tract [35, 36]. While we did not detect inflammatory changes at 1 month after Cu-IUD insertion across our 3 study sites, another substudy within the ECHO trial that recruited from different clinical sites in South Africa (Pretoria and Durban) found elevated cytokines at 1 month after Cu-IUD insertion [37]. Others have similarly reported increased inflammatory biomarkers as early as 2–4 weeks

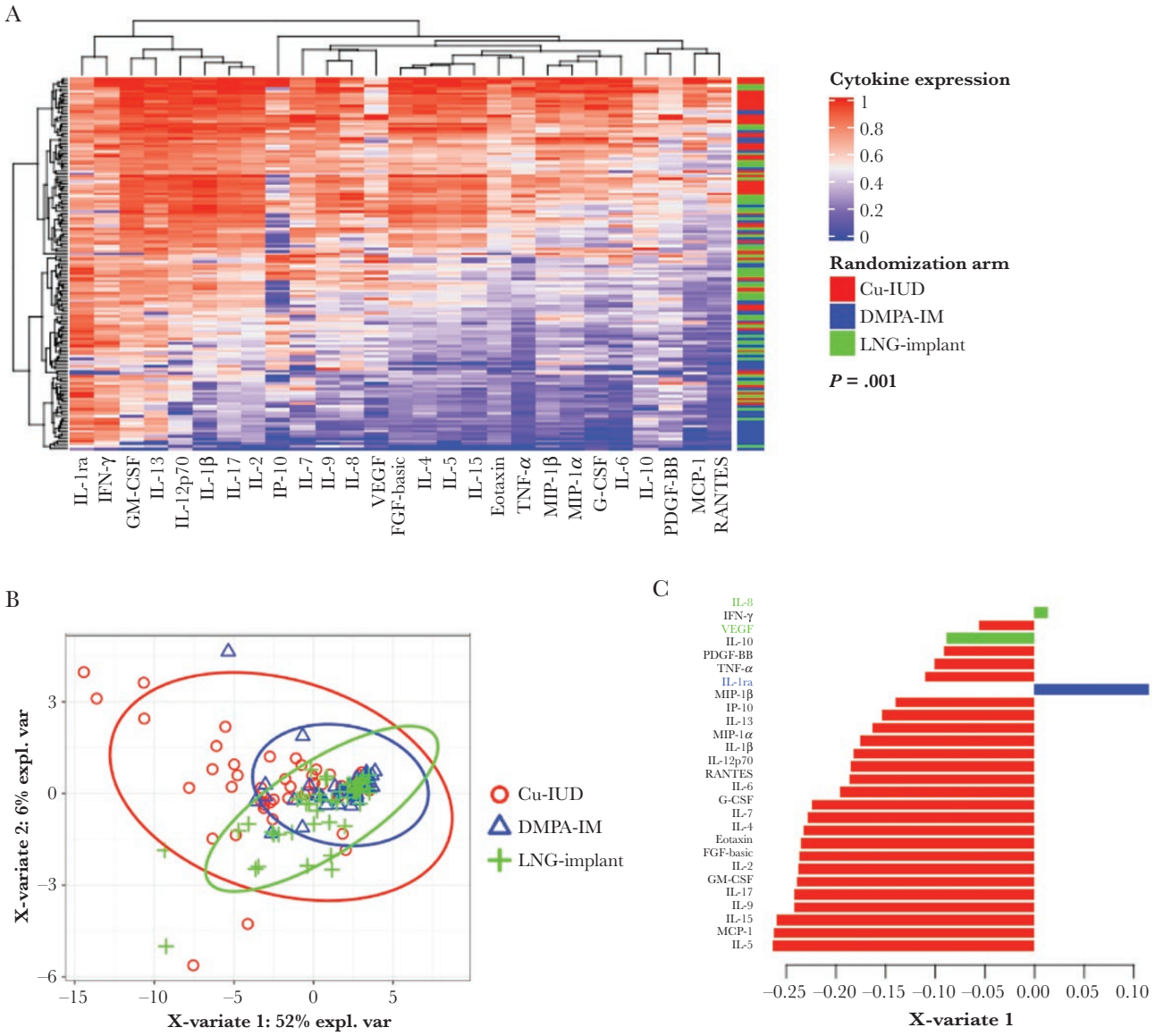

Figure 3. Visualization of clustering of cytokines 6 months after contraceptive initiation. *A*, Unsupervised hierarchical clustering showing the variation in cytokine concentrations in individual women and clustering women according to the similarities of their cytokine expression profiles and their contraceptive method 6 months after initiation of contraceptive. Cytokine concentrations are indicated using a color scale that ranges from blue (low) through white to red (high). The dendrogram above the heat map indicates degrees of relatedness between cytokine profiles within the study participants. The dendrogram on the left of the heat map depicts relationships between the cytokine expression levels across all the study participants. *P* value for statistical significance was generated using PERMANOVA. *B*, sPLSDA of cytokines 6 months after contraceptive initiation. *C*, Barplots indicate the loadings of X-variate 1 from the sPLSDA analysis. The absolute value and the negative or positive sign represent the importance of the cytokine and the correlations between the variables, respectively. Red, blue, and green represent Cu-IUD (n = 52), DMPA-IM (n = 47), and LNG-implant (n = 50), respectively. Abbreviations: Cu-IUD, copper intrauterine device; DMPA-IM, intramuscular injectable depot medroxyprogesterone acetate; expl. var, explained variation; FGF, fibroblast growth factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN-γ, interferon-γ; IL, interleukin; IL-1ra, IL-1 receptor agonist; IP-10, IFN-γ inducible protein-10; LNG, levonorgestrel; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; PDGF-BB, platelet-derived growth factor-BB; RANTES, regulated on activation, normal T-cell expressed and secreted; sPLSDA, sparse partial least-squares discriminant analysis; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

after Cu-IUD insertion [35, 36]. The reason for the delayed inflammatory signatures in the Cu-IUD arm across our sites is unclear, although it would be important to determine whether this enhanced genital inflammation associated with insertion of the Cu-IUD is sustained beyond 6 months or transient, to fully understand the relationship between Cu-IUD, inflammation, and HIV risk.

While it is tempting to assume that cytokines measured in cervicovaginal fluid originate from the lower reproductive tract, it is likely the Cu-IUD induces cytokine production by cells in both the upper and lower reproductive tract [38]. It is also clear from the earliest studies that both anatomical and inflammatory changes observed following insertion of a Cu-IUD occur in the upper reproductive tract, because the device is intrauterine

Table 2. Baseline Characteristics of Participants in the Case-Control Study

Characteristic	HIV Cases	Controls	*P* Value
n	25	100	
Age, y, median (25th–75th percentile)	22 (20.0–26.5)	23 (20.0–26.0)	.78[a]
BMI, kg/m^2, median (25th–75th percentile)	26 (21.1–27.9)	26 (22.8–30.8)	.22[a]
Time point for sample collection prior to HIV seroconversion, mo, median (25th–75th percentile)	6 (3.0–12.0)	6 (3.0–12.0)	>.99[a]
Genital infection, n (%)			
Chlamydia trachomatis	8 (32.0)	20 (20.4)	.28[b]
Neisseria gonorrhoeae	3 (12.0)	6 (6.1)	.39[b]
Herpes simplex virus type 2			
Negative	5 (20.0)	42 (42.9)	.11[c]
Indeterminate	4 (16.0)	11 (11.2)	
Positive	16 (64.0)	45 (45.9)	
Demographics/sexual risk behaviors, n (%)			
Condomless sex in previous 3 mo	20 (80.0)	61 (61.0)	.10[b]
Condom usage with last vaginal sex among women who had sex	12 (52.2)	50 (55.0)	.82[b]
Vaginal bleeding during sex	2 (11.1)	3 (4.8)	.31[b]
>1 partner in previous 3 mo	3 (12.0)	4 (4.0)	.14[b]
Marital status			
Never married	16 (64.0)	75 (75.0)	.32[b]
Married	9 (36.0)	25 (25.0)	
Education			
Primary school education	7 (28.0)	18 (18.0)	.27[b]
Secondary and tertiary education	18 (18.0)	82 (82.0)	

No record of an HSV-2, chlamydia, and gonorrhea tests was reported for 2 women in the control group. *P* value < .05 is considered significant. Cases are HIV seroconverters and controls are HIV nonseroconverters.

Abbreviations: BMI, body mass index; HIV, human immunodeficiency virus; HSV, herpes simplex virus.

[a]Mann-Whitney *t* test.

[b]Fisher exact test.

[c]χ^2 test.

[32–34]. Because CVS collected in menstrual cups likely reflects a mixture of fluids from both the upper and lower reproductive tracts, the location of inflammation within the female genital tract is important to consider in trying to understand the relationship between inflammation and HIV risk, particularly because the dogma for decades has been that HIV infects target cells located in the lower reproductive tract of women [39], although this has recently been disputed [40].

Increases in cervicovaginal cytokine concentrations have also been linked with disrupted epithelial barrier integrity [41, 42], which increases HIV risk. BV and some STIs are thought to concurrently disrupt mucosal epithelial barrier integrity and induce inflammatory cytokines [43–45]. Vaginal microbial communities dominated by certain *Lactobacillus* spp. have been associated with improved epithelial barrier integrity, while those dominated by *Gardnerella vaginalis*, common in BV, have been associated with compromised epithelial barrier function, linked to their production of soluble metabolites that inhibit wound healing [44]. Women using a Cu-IUD have been shown to be more susceptible to BV, *Candida,* and *Mycoplasma* infections [46, 47], suggesting that differences in the vaginal bacteriome and mycobiome of women randomized to the Cu-IUD in this trial need to be determined urgently. Indeed, women in the Cu-IUD arm in this mucosal substudy did have marked changes in vaginal microbiota compared to their respective baseline samples and the other contraceptive arms [48].

Our study had some limitations. The ECHO trial did not include a no contraceptive group, so we were not able to compare CVS cytokine changes over 6 months in noncontracepting versus contracepting women. We did not determine long-term persistence of CVS cytokine concentrations. In addition, we could not perform longitudinal analyses using a generalized estimating equation model with repeated measures due to our assaying schema and lack of sufficient matching Luminex data with all time points. The case-control analysis was limited by the small number of women who HIV seroconverted in this substudy, representing only a subset of women (3/12 clinical sites) who participated in the ECHO trial. Future research is needed to confirm our findings in the full ECHO cohort by increasing the sample size and/or including a noncontraceptive control group. Finally, many factors that were not measured in this substudy may influence genital inflammation, including behavioral factors (condom use, partner HIV status) and infections with common STIs (chlamydia, gonorrhea, HSV-2, and trichomoniasis [49, 50]).

Our findings from this mucosal mechanisms substudy within the first large, randomized, and longitudinal trial of contraceptive methods in a setting of high HIV prevalence, that

Table 3. Cytokine Concentrations of HIV Cases and Controls in an Intention-to-Treat Analysis

Cytokine	HIV Cases (n = 25)		Controls (n = 100)		Unadjusted *P* Value	Adjusted *P* Value
	Median	IQR	Median	IQR		
Inflammatory						
IL-1β	260.7	45.2–1005. 0	52.0	5.7–454.4	.03	.09
IL-6	93. 5	3.6–399.0	11.4	2.6–82.0	.04	.09
IL-12p70	3.5	0.7–6.6	1.8	0.6–4.3	.09	.13
TNF-α	59. 3	11.0–151.0	15.1	6.6–67. 4	.02	.16
Chemokines						
IL-8	1783.0	356.5–3985.0	533.3	206.8–2121.0	.07	.14
Eotaxin	2.6	1.5–4.4	1.6	1.1–3.3	.02	.20
IP-10	252.9	54.6–3122.0	141.6	20.8–1156.0	.14	.19
MCP-1	26.9	8.0–81.1	14.7	5.0–61.3	.16	.21
MIP-1α	3.2	0.8–28.3	1.6	0.3–16.7	.18	.22
MIP-1β	14.5	3.5–149.8	8.1	2.7–32.7	.21	.23
RANTES	8.2	4.3–19.3	4.7	3.4–9.7	.02	.13
Adaptive						
IL-2	14.5	4.5–36.1	5.5	2.6–21.0	.03	.08
IL-4	3.0	1.4–5.0	1.4	1.0–3.8	.03	.08
IL-5	26.2	11.0–46.5	13.5	6.9–34.3	.02	.11
IL-13	0.4	0.3–0.6	0.2	0.1–0.4	.07	.12
IL-15	97.8	51.0–204.9	57.3	34.9–140.5	.02	.11
IL-17	39.1	17.1–81.1	12.2	5.5–47.7	.01	.22
IFN-γ	121.2	88.8–134.6	109.4	84.9–127.2	.43	.45
Growth factors						
IL-7	5.4	2.3–12.5	3.5	0.6–7.7	.10	.14
IL-9	28.5	16.2–42.4	18.7	11.4–38.5	.07	.13
FGF-basic	44.7	21.4–92.4	22.5	15.0–57.4	.02	.10
G-CSF	414.0	102.1–1307.0	166.2	55.0–863.9	.20	.23
GM-CSF	3.43	1.57–5.71	1.7	0.8– 4.4	.04	.08
PDGF-BB	63.5	32.6–103.2	50.5	35.3–74.5	.41	.44
VEGF	919.2	505.2–1752.0	717.4	263.9–1241.0	.06	.13
Regulatory						
IL-1RA	25 029.0	19 944.0–34 390.0	23 765.0	16 291.0–31 878.0	.51	.51
IL-10	6.3	2.2–11.6	3.5	2.2–6.9	.07	.12

Cases are HIV seroconverters and controls are HIV nonseroconverters. *P* values were calculated using the nonparametric Mann-Whitney *t* test before adjusting for multiple comparisons using the false discovery rate. *P* value < .05 is considered significant.

Abbreviations: FGF, fibroblast growth factor; G-CSF, granulocyte-colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL, interleukin; IP-10, IFN-γ inducible protein-10; IQR, interquartile range; MCP-1, monocyte chemoattractant protein-1; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T cell expressed and secreted; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

includes 3 clinical trial sites from South Africa and Kenya, importantly demonstrates that the Cu-IUD is significantly more inflammatory than hormonal contraceptives (DMPA-IM and LNG-implant), although the Cu-IUD was included as the nonhormonal inert alternative. Despite being a strong influence on cervicovaginal inflammation, Cu-IUD did not increase HIV acquisition risk more than DMPA-IM or LNG-implant in women randomized to this study arm. While this supports the overall finding of the ECHO trial, our results suggest that hormonal and nonhormonal contraceptives likely influence immune mechanisms and HIV risk in the female genital tract distinctly and that "one mechanism does not explain all."

In conclusion, we found that Cu-IUD, but not DMPA-IM nor LNG-implant, broadly increased concentrations of cervicovaginal inflammatory, chemotactic, and adaptive cytokines and growth factors. Furthermore, we found that CVS cytokines were not significantly associated with HIV acquisition in the HIV case-control analysis. We conclude that genital inflammation caused by insertion of the Cu-IUD was not sufficient to increase HIV risk compared to DMPA-IM and LNG-implant in the ECHO trial. While genital inflammation induced by Cu-IUD did not appear to significantly impact HIV risk in this substudy, these effects may indicate the potential for susceptibility to other pathogens that warrant further investigation.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of

Table 4. Relationship Between HIV Seroconversion and Cytokine Concentrations in an Intention-to-Treat Analysis

Cytokine	Unadjusted OR (95% CI)	*P* Value	Adjusted for Contraceptive Arm OR (95% CI)	*P* Value
Inflammatory				
IL-1β	1.59 (1.06–2.53)	.035	1.55 (.99–2.55)	.068
IL-6	1.58 (1.04–2.43)	.034	1.53 (.97–2.46)	.071
IL-12p70	2.05 (.96–4.83)	.078	1.83 (.81–4.57)	.168
TNF-α	2.15 (1.15–4.16)	.019	2.08 (1.06–4.22)	.035
Chemokines				
IL-8	1.53 (.90–2.62)	.114	1.51 (.86–2.63)	.142
Eotaxin	3.19 (1.04–10.65)	.048	2.83 (.84–10.34)	.100
IP-10	1.34 (.88–2.07)	.174	1.32 (.85–2.05)	.220
MCP-1	1.32 (.78–2.21)	.284	1.19 (.66–2.10)	.560
MIP-1α	1.29 (.87–1.94)	.207	1.23 (.80–1.89)	.341
MIP-1β	1.38 (.84–2.26)	.197	1.29 (.76–2.18)	.335
RANTES	2.12 (.90–4.98)	.081	1.93 (.78–4.68)	.146
Adaptive				
IL-2	2.32 (1.11–5.29)	.034	2.20 (1.01–5.34)	.062
IL-4	3.35 (1.18–10.44)	.028	3.10 (1.00–10.60)	.058
IL-5	3.01 (1.14–8.80)	.033	2.82 (.98–9.01)	.065
IL-13	1.68 (.80–3.99)	.199	1.49 (.69–3.60)	.336
IL-15	2.99 (1.00–9.73)	.057	2.73 (.82–9.86)	.110
IL-17	2.59 (1.25–5.87)	.015	2.55 (1.16–6.12)	.026
IFN-γ	1.66 (.21–14.02)	.627	1.31 (.16–11.42)	.800
Growth factors				
IL-7	2.07 (.99–4.71)	.065	1.92 (.88–4.48)	.111
IL-9	2.85 (.87–10.50)	.096	2.50 (.71–9.84)	.166
FGF-basic	3.48 (1.16–11.49)	.032	3.26 (.98–12.01)	.063
G-CSF	1.41 (.80–2.50)	.229	1.30 (.70–2.42)	.399
GM-CSF	2.45 (1.08–6.47)	.048	2.23 (.96–6.12)	.089
PDGF-BB	1.75 (.45–6.34)	.398	1.41 (.34–5.36)	.620
VEGF	2.44 (.93–6.94)	.079	2.23 (.83–6.47)	.123
Regulatory				
IL-1RA	1.34 (.50–4.42)	.593	1.34 (.49–4.50)	.601
IL-10	2.06 (.94–4.67)	.073	1.88 (.84–4.32)	.124

Logistic regression was performed and the *P* values shown are unadjusted for multiple comparisons; *P* value < .05 is considered significant.

Abbreviations: CI, confidence interval; FGF, fibroblast growth factor; G-CSF, granulocyte-colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL, interleukin; IP-10, IFN-γ inducible protein-10; MCP-1, monocyte chemoattractant protein-1; OR, odds ratio; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T cell expressed and secreted; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

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Data availability. Access to the data from this ancillary study of the ECHO Study may be requested through submission of a research concept to: rheffron@uw.edu. The concept must include the research question, data requested, analytic methods, and steps taken to ensure ethical use of the data. Access will be granted if the concept is evaluated to have scientific merit and if sufficient data protections are in place. As of the time of publication, data access applications are in process with the governing institutional review boards of the ECHO Study to make deidentified data from the primary ECHO dataset publicly available.

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