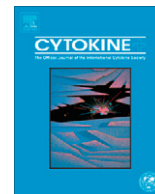




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## Baseline variation and associations between subject characteristics and five cytokine biomarkers of vaginal safety among healthy non-pregnant women in microbicide trials

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### ABSTRACT

Interleukins (IL)-8, IL-1 $\alpha$ , IL-1 $\beta$ , and IL-1 receptor antagonist (IL-1RA) have emerged as indicators of vaginal inflammation and HIV-1 transmission risk. We provide values and factors of normal variation of these immune mediators in premenopausal women to allow their wider clinical application as biomarkers of vaginal health. Cross-sectional analyses (Kruskal–Wallis and Wilcoxon exact tests) of cytokine concentrations in relation to sociodemographic variables and Nugent score were performed on baseline (prior to product) cervicovaginal lavage from two Phase I randomized microbicide trials. All women in the analysis had regular menstrual cycles, 72 h abstinence, normal blood and Pap tests, and absence of genitourinary infections, study-relevant allergies, antibiotics use and history of substance abuse. Cytokine norms were defined as the values among those with Nugent score <4. Among women with normal Nugent score ( $n = 92$ ), IL-8 and IL-1 $\beta$  were lowest in those using abstinence as compared to hormonal contraceptives or male/female sterilization as their primary method for birth control. No difference was found by age, prior pregnancy, or education, and also by race after controlling for contraceptive method. Women with abnormal (>7) and borderline (4–6) Nugent scores had elevated IL-1 $\alpha$  and/or IL-1 $\beta$  although their IL-1RA-to-IL-1( $\alpha + \beta$ ) ratio remained within the normal range due to higher IL-1RA. Women with borderline Nugent scores had IL-8 levels above the normal range. IL-8 and the IL-1RA-to-IL-1 ratio can be used as independent biomarkers of vaginal immune balance. More studies must determine the role of sexual activity, contraceptive method, and borderline Nugent scores, which normally are not exclusion criteria for enrollment in microbicide trials but may affect product tolerability and HIV-1 risk due to the aberrant cytokine levels.

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### 1. Introduction

Despite improved access to prevention programs and effective therapies and the global decline in HIV incidence since 1999, in 2009 there were still 2.6 million new HIV infections and 1.8 million HIV-related deaths [1]. Globally, an estimated 31.4–35.3 million people were living with HIV in 2009 (14.8–17.2 million women) and the vast majority unaware of their status [1]. Recent estimates suggest that scaling up currently available prevention methods (condoms and abstinence) in 125 low- and middle-income countries would avert only half (28 million) of the new sexually trans-

mitted infections projected to occur in the next 10 years [2]. Thus new modes of prevention are desperately needed and, after many years of development efforts and recent promising results from the tenofovir trial in Africa [3], vaginal/topical anti-HIV microbicides are emerging as a viable and highly desired alternative.

To be effective, a topical microbicide must avoid mucosal inflammation, a known risk factor for HIV-1 transmission, and preserve the natural immune barrier against STIs [4–6]. Therefore vaginal cytokine mediators of inflammation have been proposed as objective measures of mucosal safety of topical microbicides [6–8].

The proinflammatory interleukins IL-1 $\alpha$ , IL-1 $\beta$  and IL-8 and the antiinflammatory protein IL-1 receptor antagonist (RA) have emerged as biomarkers of vaginal epithelial/mucosal damage and inflammation with key roles in HIV-1 transmission [4,9–12]. Membrane-damaged epithelial cells release intracellular stores of IL-1 $\alpha$  and IL-1 $\beta$  [13], which weaken the natural anti-HIV mucosal

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barrier via inducing an inflammatory reaction, attracting and activating HIV-1-susceptible CD4 positive cells and enhancing the viral replication in acutely or latently infected cells [9]. Both IL1 $\alpha$  and IL1 $\beta$  bind to and internalize the membrane receptor IL-1RI on virtually any cell type leading to activation of NF- $\kappa$ B and other proinflammatory signal transduction pathways and consequently the expression of a myriad of inflammatory genes [14]. However, upon membrane damage epithelial cells also release IL-1RA [13], which occupies the same IL-1RI but does not activate the cells and hence attenuates the inflammatory response [15]. Therefore a lowered vaginal mucosal ratio of IL-1RA/IL-1( $\alpha + \beta$ ) has been considered as an additional biomarker of risk of HIV infection [10,13,16,17]. IL-1 $\beta$  up-regulates IL-1RA synthesis under conditions of inflammation but it also augments its own production and the expression of proinflammatory genes including IL-8 by the human vaginal and cervical epithelial cells [13,15,18,19]. IL-8 recruits CD4 + HIV-1 host cells and neutrophils driving inflammatory tissue damage [20,21]. IL-8 also stimulates HIV-1 replication in macrophages and T cells [22]. Consistent with these biological functions, increased levels of IL-1 $\beta$  and IL-8 have been associated with HIV-1 shedding in vaginal secretions [11,23,24].

A number of animal and human studies have produced evidence that levels of IL-1 $\alpha$ , IL-1 $\beta$ , IL-8, and IL-1RA in cervicovaginal lavage (CVL) positively correlate with histopathology and clinical signs of vaginal inflammation [4,13,25–28]. IL-1 $\alpha$ , IL-1 $\beta$ , IL-8 but not IL-1RA increased and the IL-1RA-to-IL-1 ratio decreased in CVLs after repeated vaginal use of Nonoxynol-9 (N-9)-based contraceptives and other irritating products [10,13]. These cytokine findings were associated with NF- $\kappa$ B activation in the vaginal epithelium which altogether may underlie the inflammatory damage that has caused N-9 to fail as a microbicide candidate [5,13,29].

Furthermore, the interest in using IL-1 $\alpha$ , IL-1 $\beta$ , IL-1RA, and IL-8 as biomarkers of vaginal health has been supported by many studies showing that they are reproducibly detectable in human CVL by traditional ELISA [8,10,12,30–33] and remain stable in the CVL matrix for at least 24 h at temperatures up to 37 °C without addition of protease inhibitors [8]. Because of the strong biologic rationale of using cytokines as biomarkers of HIV transmission risk, steps have been taken toward establishing interlaboratory standards of their measurement in vaginal fluid [34]. However, despite all these favorable reports, gaps and weaknesses of data available about the normal variation of IL-1 $\alpha$ , IL-1 $\beta$ , IL-8, IL-1RA and the IL1RA/IL-1 ratio in the vaginal secretions of healthy nonpregnant premenopausal women have hampered their wider clinical application. Information on numeric values of cytokine concentration ranges along with recommended dilution within assay linearity and factors of physiologic variation is needed for the establishment of valid normative ranges of these biomarkers. Only one study on a small number of healthy women ( $n = 10$ ) has published numeric values of median and range of all five of these biomarkers in 10 ml saline CVL (the most common mode of vaginal cytokine sampling to date) along with information on sample dilution [13]. Several other studies of healthy nonpregnant women have provided numeric values of median and/or ranges of IL-1 $\beta$  ( $n = 60$ , [35]) and IL-8 ( $n = 24$ , [36] and  $n = 60$  [35]) in 10 ml CVL. Other studies provided baseline mean or mean/inter-quartile ranges or confidence intervals for IL8, IL-1 $\alpha$  or IL-1 $\beta$  in less common volumes of CVL (3 mL [37], 5 mL [38] or 20 mL [39]). Only one study looked at correlation of IL-1 $\beta$  and IL-8 with age in healthy women ( $n = 55$ ), although the age range was not defined [31]. In all these studies race and method of birth control remained undefined as potential factors of normative variation. Moreover, most published reports lack technical details, e.g. sample dilution, assay linearity and reproducibility, which are essential for establishing CLIA standards for cytokine evaluation.

To address some of these gaps we conducted a secondary analysis pooling baseline sociodemographic data and measurements of IL-1 $\alpha$ , IL-1 $\beta$ , IL-1RA, the IL-1RA-to-IL-1( $\alpha + \beta$ ) ratio, and IL-8, collected under standardized operational procedures from two 14-day randomized Phase I studies of candidate microbicides, providing reference values for what we consider norm in these trials.

## 2. Methods

### 2.1. Clinical study protocols and population

The Phase I studies, reported in detail elsewhere [32,40], enrolled healthy female volunteers 18–50 years old, with regular menstrual cycles, and not at risk for pregnancy, who provided signed informed consent in five clinical centers: CONRAD Clinical Research Center at the Eastern Virginia Medical School (Norfolk, VA, USA), the Magee-Women's Hospital (Pittsburgh, PA, USA), University of Cincinnati (Cincinnati, OH, USA), Johns Hopkins (Baltimore, MD, USA), and PROFAMILIA (Santo Domingo, Dominican Republic). CVLs for cytokine assessments were collected at the four US centers. The studies were IRB approved at each clinical and laboratory site in accordance with the Declaration of Helsinki. Volunteers were ineligible if they had a hysterectomy, were breastfeeding or within 2 months of last pregnancy outcome, were allergic to the study products, N-9 or latex or had prior exposure to the study gel. Participants had to have no genitourinary or sexually transmitted infection, including bacterial vaginosis (BV) by Amsel criteria [41], normal blood counts and blood chemistry ( $\pm 20\%$ ), no recent antibiotics use (within 2 weeks) and no drug or alcohol abuse. Samples for Nugent scoring were collected at baseline, but results were not obtained immediately and were not used to exclude women. Women were excluded if they were diagnosed with BV based on clinical (Amsel's) criteria. All participants were instructed to abstain from vaginal intercourse for 72 h prior to baseline collection. The 72 h interval was part of the primary study design aimed at reducing baseline variability due to presence of semen constituents, conservatively chosen based on data for vaginal clearance of semen antigens within 24–48 h [42,43] or findings of vaginal trauma after intercourse [44].

The secondary cytokine analysis, reported here, is based on all participants for whom both Nugent score and cytokine data were available at baseline (before product). For two women who participated in both studies one set of baseline values was randomly removed. The demographic characteristics of this population are shown in Table 1.

To assess the effects of contraceptive method on the normal vaginal cytokine baseline, participants were grouped based on self-reported contraceptive method used for at least 2 weeks prior to study enrollment: (1) protected from pregnancy by sexual abstinence only, (2) hormonal contraceptives or (3) male/female sterilization. Three subjects were excluded from this part of the analysis because their method could not be categorized into one of these groups.

### 2.2. Sample collection and processing

All baseline CVLs were collected between day 5 and 13 of the menstrual cycle. Following placement of a speculum, the vaginal walls and cervix were washed with 10 ml of normal non-buffered saline, using a syringe. Special care was taken to avoid trauma to the epithelium and to direct the stream of saline away from the cervical os. The fluid was collected in conical polypropylene tubes and centrifuged for 10 min at 1000g to remove cells and debris. The supernatant was frozen at  $-70^{\circ}\text{C}$  and transported to the Labora-

**Table 1**

Demographics of study population (all healthy participants with baseline cytokine and Nugent score ( $n = 109$ )).

| Demographics                      |                         |                  |
|-----------------------------------|-------------------------|------------------|
| Age (in years)                    | Mean (SD)               | 28.7 (7.2)       |
|                                   | Median (Range)          | 28.0 (18.0–49.0) |
| Education: school years completed | Mean (SD)               | 14.7 (2.6)       |
|                                   | Median (Range)          | 14.0 (9.0–22.0)  |
| Ethnicity [ $n$ (%)]              | Hispanic/Latina         | 1 (0.9)          |
|                                   | Not Hispanic/Latina     | 108 (99.1)       |
| Race [ $n$ (%)]                   | Black                   | 23 (21.1)        |
|                                   | White                   | 84 (77.1)        |
|                                   | Mixed Race              | 2 (1.8)          |
| Partner status [ $n$ (%)]         | Living with partner     | 60 (55.0)        |
|                                   | Not living with partner | 29 (26.6)        |
|                                   | No partner              | 20 (18.3)        |
| Nugent score [ $n$ (%)]           | Normal (<4)             | 92 (84.4)        |
|                                   | Borderline (4–6)        | 7 (6.4)          |
|                                   | Abnormal (>7)           | 10 (9.2)         |

tory of Genital Tract Biology at Brigham and Women's Hospital for cytokine analysis.

A vaginal smear was obtained for Gram stain from the vaginal wall, air dried on a glass slide and transported to the Magee-Women's Research Institute according to standard procedures for Nugent scoring [45]. The "Nugent score" is an accepted scoring system using microscopic examination of a Gram stained vaginal smear for determining BV. A score of 4–6 is considered an intermediate score and 7–10 is consistent with BV. The positive clinical diagnosis of BV was defined as the presence of three of the four following Amsel's criteria: (1) characteristic discharge, (2) whiff test positive, (3) vaginal pH exceeding 4.5, (4) presence of clue cells on wet mount (greater than 20% of vaginal epithelial cells).

### 2.3. Cytokine measurement

Standardized preanalytic, analytic and postanalytic procedures were developed for the measurement of cytokines in CVL by ELISA and carried out in the Brigham and Women's Laboratory of Genital Tract Biology, which is accredited for this type of analysis by the College of American Pathologists. Before cytokine analysis each

sample was thawed once for aliquoting. The bench time for CVL processing at room temperature was controlled and did not exceed 1.5 h. Aliquots were kept in sealed Micronic microtubes (USA Scientific Inc., Ocala, FL) to improve storage stability and allow quick multi-channel processing during immunoassays. Each cytokine was analyzed in individual previously non-thawed aliquots. Chemiluminescence-based QuantiGlo IL-1 $\beta$  and photometric Quantikine IL-1RA ELISA kits were purchased from R&D Systems, Minneapolis, MN. Photometric Endogen IL-1 $\alpha$  and IL-8 ELISA kits were obtained from Pierce, now part of Thermo Scientific (Rockford, IL). Relative luminescence units (RLU) or optical densities (OD) were read using a Multilabel Microplate Counter Victor 2 (Perkin Elmer Life Sciences, Boston, MA). The readings were converted to concentrations by linear interpolation from calibrator curves using WorkOut Version 1.5 Wallace Software (DAZDAQ Ltd.).

It is important to note that IL-1 $\alpha$  and IL-1 $\beta$  are synthesized as larger precursors, which are processed to mature fragments; however, while IL-1 $\beta$  requires enzymatic cleavage to become biologically active, both the precursor and the mature IL-1 $\alpha$  forms are biologically active [15]. The ELISA assays used in this study measured both IL-1 $\alpha$  forms and the active IL-1 $\beta$  form (manufacturer information).

The IL-1 $\alpha$ , IL-1 $\beta$  and IL-8 assays were manufacturer-calibrated against WHO/NSBC standards with the following conversion indexes: 1 pg Endogen Pierce IL-1 $\alpha$  = 0.25 NIBSC 86/632 Units; 1 pg QuantiGlo IL-1 $\beta$  = 0.1128 NIBSC 86/680 Units; 1 pg Endogen Pierce IL-8 = 0.0013 NIBSC 89/520 Units. The Quantikine IL-1RA assay has been calibrated against highly purified *E. coli*-expressed recombinant IL-1RA.

All CVL samples were tested in duplicates. A quality control CVL pool was generated and split samples from the same pool were tested in each plate to assess inter-assay variability by calculating coefficients of variation (CV%) =  $100 \times \text{standard deviation} / \text{average}$  of all plate runs of each cytokine. The following precision parameters and cut-off levels were established for reproducibly quantifiable CVL cytokine levels: IL-1 $\alpha$  > 20.48 pg/ml (CV < 20%), IL-1 $\beta$  > 10 pg/ml (CV < 10%, dilution factor 2), IL-8 > 256 pg/ml (CV < 22%, dilution factor 10), and IL-1RA > 4690 pg/ml (CV < 13%, dilution factor 100). These cut-off levels are consistent with biologically significant ED<sub>50</sub> values established for each cytokine *in vitro*. To measure the homeostatic balance between the antagonist and

**Table 2**

Baseline cytokine data by categorized baseline Nugent scores ( $n = 109$ ).

| Characteristic                          | Normal (<4)          | Borderline (4–6)             | Abnormal (> 7)               | <i>p</i> -Values <sup>a</sup> |
|-----------------------------------------|----------------------|------------------------------|------------------------------|-------------------------------|
| IL-1 $\alpha$ <sup>b</sup> (pg/ml)      |                      |                              |                              | 0.0800                        |
| Mean (SD)                               | 30.0 (36.0)          | 58.3 (66.4)                  | 92.0 <sup>f</sup> (110.0)    |                               |
| Median (Range)                          | 18.1 (0.0–267.1)     | 20.5 (10.4–171.1)            | 31.7 (13.9–306.4)            |                               |
| IL-1 $\beta$ <sup>c</sup> (pg/ml)       |                      |                              |                              | 0.0022                        |
| Mean (SD)                               | 68.3 (261.0)         | 98.3 <sup>f</sup> (95.3)     | 298.3 <sup>f</sup> (558.6)   |                               |
| Median (Range)                          | 18.7 (0.0–2423.0)    | 62.6 (4.9–287.3)             | 113.0 (6.1–1847.8)           |                               |
| IL-1RA <sup>d</sup> (ng/ml)             |                      |                              |                              | 0.0010                        |
| Mean (SD)                               | 133767 (129737)      | 232274 <sup>f</sup> (139531) | 301880 <sup>f</sup> (232995) |                               |
| Median (Range)                          | 108013 (853–1046382) | 193541 (108674–520643)       | 226749 (78052–756466)        |                               |
| IL-8 <sup>e</sup> (pg/ml)               |                      |                              |                              | 0.0645                        |
| Mean (SD)                               | 890.2 (1289.1)       | 2845.0 <sup>f</sup> (2891.1) | 1531.7 (2131.6)              |                               |
| Median (Range)                          | 460.0 (0.0–7591.0)   | 1994.5 (193.0–8159.5)        | 589.2 (21.3–6420.4)          |                               |
| IL-1RA:IL-1( $\alpha$ + $\beta$ ) ratio |                      |                              |                              | 0.6121                        |
| Mean (SD)                               | 3898 (5232)          | 2518 (1932)                  | 2592 (3073)                  |                               |
| Median (Range)                          | 2191 (50–28300)      | 2013 (574–5225)              | 1654 (271–10681)             |                               |
| Participants [ $n$ ]                    | 92                   | 7                            | 10                           |                               |

<sup>a</sup> Two-tailed *p*-values based on Monte Carlo estimates for the Kruskal–Wallis exact test.

<sup>b</sup> *p*-Values: normal vs. borderline 0.2919; normal vs. abnormal 0.0351; abnormal vs. borderline 0.6618.

<sup>c</sup> *p*-Values: normal vs. borderline 0.0220; normal vs. abnormal 0.0053; abnormal vs. borderline 0.5947.

<sup>d</sup> *p*-Values: normal vs. borderline 0.0130; normal vs. abnormal 0.0030; abnormal vs. borderline 0.6618.

<sup>e</sup> *p*-Values: normal vs. borderline 0.0202; normal vs. abnormal 0.5340; abnormal vs. borderline 0.2278.

<sup>f</sup> Significantly different from normal by above criteria.

**Table 3**Baseline cytokine data by primary contraceptive method used for at least 2 weeks prior to enrollment by participants with normal baseline Nugent scores ( $n = 89$ )<sup>a</sup>.

| Characteristic                  | Hormonal               | Male/female sterilization | Abstinence as only method | <i>p</i> -Value <sup>b</sup> |
|---------------------------------|------------------------|---------------------------|---------------------------|------------------------------|
| IL-1 $\alpha$ pg/ml             |                        |                           |                           | 0.8833                       |
| Mean (SD)                       | 29.9 (38.1)            | 28.7 (26.4)               | 26.0 (22.0)               |                              |
| Median (Range)                  | 19.0 (3.9–267.1)       | 17.7 (2.8–108.8)          | 18.2 (0.0–76.8)           |                              |
| IL-1 $\beta$ pg/ml              |                        |                           |                           | 0.0063                       |
| Mean (SD)                       | 103.2 (337.1)          | 26.6 (22.9)               | 13.0 (17.4)               |                              |
| Median (Range)                  | 22.0 (0.0–2423.00)     | 20.7 (0.0–79.1)           | 7.3 (0.0–64.5)            |                              |
| IL-1RA pg/ml                    |                        |                           |                           | 0.1865                       |
| Mean (SD)                       | 154905 (156942)        | 107556 (62098)            | 97739 (76663)             |                              |
| Median (Range)                  | 117399 (14568–1046382) | 108013 (853–267509)       | 62128 (16758–274022)      |                              |
| IL-8 <sup>d</sup> pg/ml         |                        |                           |                           | 0.0003                       |
| Mean (SD)                       | 1052.0 (1509.8)        | 1143.3 (974.4)            | 334.2 (584.1)             |                              |
| Median (Range)                  | 547.7 (22.2–7591.0)    | 836.0 (0.0–3702.2)        | 159 (0.0–2632.6)          |                              |
| IL-1RA:IL-1( $\alpha + \beta$ ) |                        |                           |                           | 0.5724                       |
| Mean (SD)                       | 3514 (4412)            | 2488 (2240)               | 5275 (7024)               |                              |
| Median (Range)                  | 2191 (50–28300)        | 1912 (305–9122)           | 2933 (346–27930)          |                              |
| Participants [n]                | 54                     | 16                        | 19                        |                              |

<sup>a</sup> One participant enrolled as “abstinence” but used oral contraceptive up until time of study is re-categorized as hormonal group; one participant using no method and two participants using condoms are excluded from this analysis.

<sup>b</sup> Two-tailed *p*-values based on Monte Carlo estimates for the Kruskal–Wallis exact test.

<sup>c</sup> *p*-Values: hormonal vs. male/female sterilization 0.6255; hormonal vs. abstinence as only method 0.0015; abstinence as only method vs. male/female sterilization 0.0311.

<sup>d</sup> *p*-Values: hormonal vs. male/female sterilization 0.1870; hormonal vs. abstinence as only method 0.0006; abstinence as only method vs. male/female sterilization 0.0002.

agonists of the IL-1 receptor we calculated the ratio of IL-1RA: IL-1 (sum of IL-1 $\alpha$  and IL-1 $\beta$ ).

#### 2.4. Statistical analysis

Nonparametric methods were chosen because cytokine levels were not normally distributed. Initial analysis compared levels measured in baseline CVLs from all participants in the two studies and then assessed associations between Nugent score and each cytokine ( $n = 109$ ). Analysis of factors affecting the normal baseline Nugent scores ( $n = 92$ ). Wilcoxon (two levels in baseline characteristics) and Kruskal–Wallis (more than two levels) exact tests were applied using Monte Carlo estimates (two-tailed *p*-values). If tests among three or more levels showed a *p*-value  $\leq 0.10$  (two-tailed), then pair-wise comparisons were performed to isolate the sources of differences. In addition, two-way Analyses of Variance (ANOVAs) were conducted on log-transformed cytokine values (to normalize the distribution) to assess the effect of race controlling for contraceptive method and vice versa. Given the exploratory nature of this study (secondary analysis not powered to detect specific differences) statistical tests should be considered hypothesis-

generating; no adjustments to alpha levels were made for multiple analysis. All analyzes were conducted using The SAS<sup>®</sup> System v. 9.2.

### 3. Results

No differences in the baseline cytokine concentrations were found between the two Phase I studies, supporting the decision to pool the data (not shown). Analyses did not control for center to avoid obscuring associations with demographic characteristics of interest.

The categorized Nugent score was associated with significant differences in cytokine levels (Table 2). Compared to those with normal Nugent scores ( $<4$ ), women with abnormal ( $\geq 7$ ) Nugent scores showed higher levels of IL-1 $\alpha$ , IL-1 $\beta$  and IL-1RA ( $p < 0.0351$ , 0.0053 and 0.0030, respectively), and women with borderline (4–6) Nugent scores showed higher levels of IL-1 $\beta$ , IL-1RA, and IL-8 ( $p = 0.0220$ , 0.0130, and 0.0202, respectively). The IL-1RA:IL1( $\alpha + \beta$ ) ratio showed no significant differences between these groups. To characterize factors affecting the normal cytokine

**Table 4**Baseline cytokine data by race in all healthy participants with normal baseline Nugent scores ( $n = 92$ ).

| Characteristic                  | White                  | Black/mixed race    | <i>p</i> -Value <sup>a</sup> | <i>p</i> -Value <sup>b</sup> |
|---------------------------------|------------------------|---------------------|------------------------------|------------------------------|
| IL-1 $\alpha$ pg/ml             |                        |                     | 0.1032                       | 0.6085                       |
| Mean (SD)                       | 31.1 (37.6)            | 25.4 (28.9)         |                              |                              |
| Median (Range)                  | 20.1 (0.0–267.1)       | 13.6 (2.3–108.8)    |                              |                              |
| IL-1 $\beta$ pg/ml              |                        |                     | 0.0113                       | 0.0627                       |
| Mean (SD)                       | 81.8 (289.7)           | 12.5 (12.5)         |                              |                              |
| Median (Range)                  | 20.4 (0.0–2423.0)      | 8.4 (0.0–43.1)      |                              |                              |
| IL-1RA pg/ml                    |                        |                     | 0.6464                       | 0.3327                       |
| Mean (SD)                       | 139814 (140212)        | 108922 (69760)      |                              |                              |
| Median (Range)                  | 108013 (14569–1046382) | 113680 (853–270031) |                              |                              |
| IL-8 pg/ml                      |                        |                     | 0.0100                       | 0.0980                       |
| Mean (SD)                       | 1030.2 (1397.9)        | 315.1 (262.5)       |                              |                              |
| Median (Range)                  | 533.9 (0.0–7591.0)     | 272.8 (0.0–871.8)   |                              |                              |
| IL-1RA:IL-1( $\alpha + \beta$ ) |                        |                     | 0.2522                       | 0.7630                       |
| Mean (SD)                       | 3736 (5188)            | 4565 (5510)         |                              |                              |
| Median (Range)                  | 2068 (50–28300)        | 3036 (305–24139)    |                              |                              |
| Participants [n]                | 74                     | 18                  |                              |                              |

<sup>a</sup> Two-tailed *p*-values based on Monte Carlo estimates for the Wilcoxon two-sample exact test.

<sup>b</sup> Two-tailed *p*-values based on two-way ANOVA on log-transformed cytokine values controlling for contraceptive method prior to enrollment.

**Table 5**

Correlation of baseline cytokine concentrations with age in all healthy participants with normal baseline Nugent scores ( $n = 92$ ).

| Characteristic                        | Spearman correlation coefficient | <i>p</i> -Values <sup>a</sup> |
|---------------------------------------|----------------------------------|-------------------------------|
| IL-1 $\alpha$                         | 0.0354                           | 0.7375                        |
| IL-1 $\beta$                          | 0.0022                           | 0.9833                        |
| IL-1RA                                | –.0338                           | 0.7491                        |
| IL-8                                  | 0.0258                           | 0.8072                        |
| IL-1RA:IL-1( $\alpha + \beta$ ) ratio | –.0943                           | 0.3714                        |

<sup>a</sup> Two-tailed *p*-values computed by treating  $t = ((\sqrt{n-2}) \times \text{corr}) / (\sqrt{1 - \text{corr}^2})$  as coming from a *t* distribution with  $n-2$  degree of freedom, where *n* is sample size and corr is the Spearman correlation.

baseline among healthy individuals, the remaining analyzes were conducted only on women with normal Nugent scores.

The three groups classified by contraceptive method showed significant differences in IL-1 $\beta$  and IL-8, with lowest values in the abstinence group (Table 3). IL-1 $\beta$  was significantly lower in the abstinence group as compared to both the sterilization ( $p = 0.0311$ ) and hormonal contraception groups ( $p = 0.0015$ ). The significance was even stronger for IL-8 values ( $p = 0.0002$  and  $0.0006$ , respectively). Controlling for race to address confounding between race and contraception method did not change these results. No association was found between IL-1RA-to-IL-1( $\alpha + \beta$ ) ratio and contraceptive method.

Baseline levels of IL-1 $\beta$  and IL-8 were lower in Black/mixed race women as compared to White women by initial analysis; however, these differences were eliminated after statistically controlling for contraceptive method (Table 4).

No associations were found between cytokine baselines and age (Table 5) or education ( $\leq$ high school versus  $>$  high school) and prior pregnancy (data not shown).

#### 4. Discussion

Our study provides normative ranges for levels of IL-1 $\alpha$ , IL-1 $\beta$ , IL-1RA, the ratio of IL-1RA to cumulative IL-1 $\alpha$  and  $\beta$ , and IL-8 in vaginal secretions sampled between days 5–13 of the menstrual cycle in healthy non-menopausal non-pregnant women sexually abstinent for at least 72 h. No linear correlation with age was found in the range of 18–49 years. This finding was in agreement with the only previous report on age as a factor of vaginal cytokine variation [31]. The inter-quartile or 95% confidence intervals reported previously for these biomarkers in 10 ml CVL from healthy non-pregnant women [13,35,36] coincide with the normal ranges determined in our study (Table 2). Although numerous other studies have measured cytokines in cervicovaginal lavage secretions to assess the immune status of pregnant women or women with BV, HIV, and other STIs, not all are cited here because they were not designed to establish the physiologic variation of cytokines in healthy nonpregnant individuals or did not provide baseline numeric values that could be used as a basis for comparison with the ranges established in our study. Our investigation showed that among the factors typically recorded and studied in healthy participants of microbicide safety trials, Nugent score and mode of contraception had a significant effect on the cytokine baseline.

Our study participants were bacterial vaginosis (BV) negative by Amsel criteria and had no clinical signs of vaginitis or vaginosis such as discharge, odor, pain, clue cells etc. although 16% had abnormal or borderline Nugent scores at enrollment. Both the abnormal and borderline Nugent scores were associated with higher IL-1 $\beta$ ; however, abnormal Nugent score was also associated with higher IL-1 $\alpha$  while borderline Nugent score was also associated with higher IL-8 levels. Despite the fact that in our study the number of women with Nugent score  $>4$  was small, our findings sup-

port previously published data and underscore the importance of assessing borderline Nugent score and associated intermediary vaginal flora as independent factors of microbicide trial outcome and HIV infection risk. In a nested case-control study, women with borderline Nugent score were slightly more likely to seroconvert than participants with normal Nugent score [46]. Moreover, a recent study showed that clinically diagnosed BV and intermediate flora were independently associated with HIV acquisition after controlling for demographic and behavioral covariates and other reproductive tract infections [47]. Our findings of higher IL-1 $\beta$  and IL-8 baseline in association with borderline Nugent scores in the absence of clinical vaginitis and vaginosis suggest a plausible mechanism facilitating HIV acquisition in women with intermediate flora via upregulation of cytokine-driven HIV replication. The potential impact of borderline Nugent scores on the mucosal responses to vaginal microbicides warrants further studies.

In our study, abnormal and borderline Nugent scores in otherwise healthy women were also associated with an increase of anti-inflammatory IL-1RA, which may be a physiologic response to increased IL-1 aimed at maintaining a balanced IL-RA-to-IL-1( $\alpha + \beta$ ) ratio. Interestingly, IL-8 was significantly elevated in women with borderline Nugent score despite the fact that their IL-1RA-to-IL-1( $\alpha + \beta$ ) ratios were within the normal baseline range (Table 2). Since none of the women in our study had clinically diagnosed vaginitis or vaginosis (only women negative for BV by Amsel criteria were enrolled in the study), it is possible that an elevated IL-8 combined with a compensated IL-1RA-to-IL-1 ratio may represent an early sign of subclinical bacterial imbalance while a clinically significant BV may better correlate with an imbalanced IL-1RA-to-IL-1 ratio as suggested by a study in pregnant women [48]. Because our study only enrolled women negative for BV by Amsel criteria and hence the number of women with abnormal and intermediary Nugent scores was relatively low (Table 1), future studies focusing on women with clinical BV should address the importance of the IL-1ra-to-IL-1 ratio as a biomarker of BV. Similarly to the effect of subclinical abnormal microflora, contraceptive method affected IL-1 $\beta$  and IL-8 but not IL-1RA and the IL-1RA-to-IL-1 ratio in the healthy population of women (Table 3). These findings suggest that IL-8 and the IL-1RA:IL-1( $\alpha + \beta$ ) ratio may be independent variables of cytokine imbalance in the vaginal environment and that IL-8 may be increased independently from the IL-1 cytokine family in the absence of clinically significant symptoms. The normal IL-1RA/IL-1( $\alpha + \beta$ ) baseline measured in our study showed wide (several log) margins of variation, which may reflect physiologic conditions or genetic polymorphisms known to regulate levels of IL-1RA and other members of the IL-1 family [49]. On the other hand, other studies have shown that a significant decrease in the anti-inflammatory IL-1RA to IL-1( $\alpha + \beta$ ) ratio was induced by Nonoxynol-9 in vaginal epithelial cells [13,50,51] and in CVLs after vaginal application of N-9 or irritating doses of lime juice and those changes were accompanied by significantly higher IL-8 and inflammatory cell influx [10,13]. Thus, a significant drop of the IL-1RA-to-IL-1( $\alpha + \beta$ ) ratio from its subject-specific baseline might be indicative of symptomatic inflammatory conditions or advanced mucosal damage. Future clinical studies should explore the value of this ratio as a biomarker of vaginal health.

Our analysis of cytokine baseline also brings up the importance of sexual abstinence versus other contraceptive methods as factors with a significant impact on the vaginal immune balance. Our data showed significantly lowered proinflammatory cytokines in women abstaining from intercourse for at least 2 weeks as compared to those relying on hormonal contraceptives or surgical sterilization as a primary birth control method. These data support the hypothesis that sexual intercourse and/or method of contraception may predispose to mucosal damage and increased HIV risk by

modifying the physiologic baseline of proinflammatory cytokines, e.g. IL-1 $\beta$  and IL-8. The hypothesis needs to be addressed by future studies. Unplanned exploratory analyses from the Nigerian arm of the cellulose sulfate (CS) trial showed that CS might have lowered risk for gonorrhea and chlamydial infection in women who had above median coital frequency at baseline and above median average of number partners during follow-up [52]. Thus it has been proposed that it may be beneficial for effectiveness trials to have two cohorts of women with one being less sexually active [52,53].

Unfortunately, most microbicide safety trials like ours and most effectiveness trials assess sexual activity via participant self-report rather than via objective measures of semen exposure [54]. The prostate specific antigen (PSA) test has been recommended as a sensitive method for assessing recent unprotected intercourse (within 24 h) but it is cleared quickly from the vaginal secretions following intercourse and on the other hand ectopic prostatic tissue has been reported in human uterine cervix and vagina [55–57] and therefore should be interpreted with caution. Also, semen biomarkers cannot be used to ascertain lack of condom-protected intercourse. Substantial discrepancies have been reported between self-reported sexual abstinence and positive results from PSA measurement in vaginal swab elutions [42]. Our data demonstrating a significant difference in proinflammatory cytokine levels between subjects who reported sexual abstinence versus alternative methods of contraception stress the importance of improved monitoring of the frequency of sexual intercourse and method of contraception in future microbicide trials using objectively measurable biomarkers.

Another plausible explanation for baseline differences attributable to method of contraception could be interference of hormonal level fluctuations with pathways that regulate vaginal mucosal cytokines. Our study lacks data in this respect as the baseline sampling occurred between menstrual cycle days 5 and 13 (to allow for product application prior to menses) thus avoiding the menstrual phases previously linked to spikes in proinflammatory cytokines in cervical mucus or vaginal lavage [23,58]. Future studies need to address the effects of physiologic and pharmacologic hormonal fluctuations on the vaginal cytokine baseline.

Level of education and past pregnancy had also no significant effect on the cytokine values of healthy non-pregnant recently abstinent women of reproductive age. Our study found also no differences by race after controlling for method of contraception, although it has to be noted that no Asian women were represented and the effect of Hispanic ethnicity could not be evaluated (Table 1). The role of genetic, racial and geographic factors in the cytokine homeostasis has been studied mostly in pregnant women [59–63]. A recent study in pregnant women concluded that cervical IL-1 $\alpha$  and IL-6 levels were significantly different between black and white women without BV but not with BV [61]. Allelic polymorphisms have been linked to increased production of IL-1, IL-1RA and other cytokines and cytokine receptors with possible implications for HIV infection and transmission risk [17]. Our study found differences between black and white women, which however were not present after controlling for differences in contraceptive method, thus suggesting that some racial differences may be behavioral (e.g. contraceptive choice) rather than genetic. Socio-economic status may also underlie interracial and interethnic differences in immune mediator levels through impact of stress, malnutrition, and obesity [64]. These factors remain to be studied in relation to the vaginal cytokine network.

Neuroendocrine mechanisms related to pregnancy, lactation, menopause, circadian cycle, and physical exercise may influence the cytokine baseline in healthy women [8]. Most of these factors have not been studied with respect to normal cytokine variation in the vaginal mucosal environment. Pregnancy, lactation and menopause were exclusion criteria in the Phase I studies used here

so they could not be addressed in our analysis. More studies are needed to elucidate the role of these factors in the cytokine network in relation to the effectiveness of topical microbicides and the female genital tract susceptibility to HIV infection.

## Disclosure statement

None of the authors have any conflict of interest with this study.

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