ORIGINAL ARTICLE

Inflammatory cytokine biomarkers of asymptomatic sexually transmitted infections and vaginal dysbiosis: a multicentre validation study

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ABSTRACT

Objectives Vaginal dysbiosis and STIs are important drivers of the HIV epidemic and reproductive complications. These conditions remain prevalent, partly because most cases are asymptomatic. We have shown that inflammatory cytokines interleukin (IL)-1α, IL-1β and interferon-γ-induced protein (IP)-10 are biomarkers for detecting asymptomatic STIs and vaginal dysbiosis (bacterial vaginosis (BV) or intermediate microbiota). This study aimed to validate the performance of these biomarkers in African women recruited regardless of symptoms.

Methods IL-1α, IL-1β and IP-10 were measured in menstrual cup secretions, endocervical, lateral vaginal wall and vulvovaginal swabs from 550 women from Pretoria, Soweto and Cape Town, South Africa and Bondo, Kenya using Luminex and ELISA. STIs were assessed by PCR, BV by Nugent scoring and vaginal microbiota by 16S rRNA sequencing.

Results Across four study populations and four types of genital specimens, the performance of IL-1α, IL-1β and IP-10 for identification of women with STIs, BV or intermediate microbiota was consistent. Of the genital samples assessed, biomarkers measured in lateral vaginal wall swabs performed best, correctly classifying 76%(95% CI 70% to 81%) of women according to STI, BV or intermediate microbiota status (sensitivity 77%, specificity 71%) and were more accurate than clinical symptoms (sensitivity 41%, specificity 57%) (p=0.0003). Women incorrectly classified as STI/BV positive using the biomarkers had more abundant dysbiosis-associated bacteria, including *Prevotella bivia* and *Gardnerella* sp, detected by 16S rRNA sequencing, but not Nugent scoring. Including vaginal pH with the cytokine biomarkers improved the accuracy of the test (82% (95% CI 75% to 88%) correctly classified), although pH alone had poor specificity (61%).

Conclusions An inexpensive, point-of-care screening test including IL-1α, IL-1β and IP-10 (and potentially pH) could be used in resource-limited settings to identify women with asymptomatic STIs and dysbiosis. These women could then be referred for aetiological testing, followed by specific treatment.

INTRODUCTION

STIs, bacterial vaginosis (BV) and the inflammation caused by these conditions are associated with increased susceptibility to HIV acquisition and reproductive complications.[1–4] Recent studies have highlighted the importance of the vaginal microbiome in HIV transmission, with increased abundance of *Prevotella bivia*, *Gardnerella* sp and other anaerobes associated with greater risk of HIV infection.[5 6] Several laboratory and relatively rapid nucleic acid amplification tests for common STIs that offer accurate diagnosis have been developed, while Nugent scoring is the gold standard for BV diagnosis.[7] However, the expense and technical requirements of these tests limit their use in resource-limited settings.[8–10] In addition, antigen-detecting point-of-care (POC) tests for STIs generally have inconsistent predictive value and are more accurate in symptomatic women.[11 12] Therefore, STIs and BV are managed according to clinical signs and symptoms in these settings, rather than by aetiology, due to cost or poor test performance.[8] Although syndromic management appears to be effective for managing curable ulcerative STIs, such as chancroid and syphilis,[13] women who have BV or discharge-causing STIs (eg, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, *Mycoplasma genitalium*) are frequently asymptomatic and would thus not seek healthcare and/or receive treatment.[1] These asymptomatic infections are, however, associated with similar levels of genital inflammatory markers, and therefore equivalent HIV risk, as symptomatic disease.[1 3] The prevalence of these infections/conditions remains unacceptably high, particularly in resource-limited settings, and there is an urgent need to change current STI and vaginal dysbiosis (BV and intermediate microbiota) management strategies for women, especially in regions of high HIV prevalence.

We aim to develop an inexpensive POC biomarkers test to identify asymptomatic women with discharge-causing STIs and vaginal dysbiosis. Offering this test to women attending family planning, antenatal and primary healthcare clinics would enable the detection of a large number of women with asymptomatic STIs and dysbiosis,

thereby improving current management by identifying infections that are currently being missed. In a cohort of women from Durban, South Africa,[14] we found that two inflammatory cytokines (interleukin (IL)-1β and interferon-γ-induced protein (IP)-10), measured in cervicovaginal lavages, correctly classified 75% of women according to STI/BV status (sensitivity 72% and specificity 81%). Including a third cytokine, IL-1α, improved the fit of the model and marginally improved the accuracy.[14] While these results were encouraging, we sought to evaluate the utility of these biomarkers in the broader African community and in different genital sample types. In this study, we validated the cytokines in >500 women enrolled in two studies (Women's Initiative in Sexual Health (WISH)[15] and the FEM-PrEP clinical trial[16]) conducted in four different regions of sub-Saharan Africa, comparing their performance with clinical signs and/or symptoms of STIs and vaginal dysbiosis. In addition, we investigated biomarker performance across four genital sample types.

METHODS

Participants

This study included sexually experienced HIV-negative young women, regardless of symptoms, from the WISH study in Soweto (n=139) and Cape Town (n=147), South Africa.[15] Additionally, we conducted a secondary analysis of FEM-PrEP clinical trial data,[16 17] which included 264 HIV-uninfected women (aged 18–35 years) from Bondo, Kenya (n=144) and Pretoria, South Africa (n=120). Women ≥18 years provided written informed consent, those <18 years provided assent and parental consent from parents/guardians.

Biomarker measurement

Three genital samples were collected during WISH: (1) disposable menstrual cups (MC; Softcup, Evofem, USA) (n=285); (2) vulvovaginal swabs (VS) (n=281); and (3) lateral vaginal wall swabs (LWS) (n=274) (online supplementary methods). IP-10, IL-1α and IL-1β were measured in swab samples by ELISA (R&D Systems, USA) and in MC samples by Luminex (Bio-Rad Laboratories, USA).[18] LWS pH was measured using colour-fixed indicator strips with a range of 3.6–6.1 (0.3–0.5 increments; Macherey-Nagel, Germany). Prostate specific antigen (PSA) was measured in LWS using Human Kallikrein 3/PSA ELISA kits (R&D Systems). Blood endogenous hormone and cotinine concentrations were measured using electrochemiluminescence immunoassays (Cobas, Roche Diagnostics, USA) and ELISA (Sigma-Aldrich, USA), respectively.

During the FEM-PrEP trial, endocervical swabs (ES) (online supplementary methods) were collected for IP-10, IL-1α and IL-1β measurement using Milliplex Human Cytokine kits (Millipore, USA).[17] PSA testing was performed using Seratec PSA Semiquant assays (Seratec Diagnostica, Germany).

STI and BV diagnosis

In WISH, PCR was used to identify STIs (*C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, *M. genitalium*, herpes simplex virus (HSV) 1/2, *Treponema pallidum* and *Haemophilus ducreyi*) in VS samples.[15] LWS were collected for BV assessment by Nugent scoring. Blood was collected for HIV testing (Determine HIV-1/2 Ag/Ab Combo, Alere, USA) and HSV-2 serology (HerpeSelect HSV-2 ELISA, Focus Diagnostics, USA). Human papilloma virus (HPV) was genotyped using Roche Linear Array HPV Genotyping kits (Roche, USA). A subset of women (n=36) was questioned on symptoms of STIs/BV (abnormal vaginal discharge/odour, genital irritation, dyspareunia, bleeding after sex, genital sores, rash, enlarged inguinal lymph nodes). 16S rRNA amplicon sequencing of LWS was performed using an Illumina MiSeq platform (online supplementary methods).[19]

FEM-PrEP participants were tested for *N. gonorrhoeae* and *C. trachomatis* by PCR (Roche Cobas Amplicor, Roche, Switzerland), *T. vaginalis* and fungal hyphae by wet mount microscopy, and BV by Nugent scoring.[17] Clinical signs of an STI or BV (abnormal discharge, inflammation, tenderness, vesicles, ulceration) were documented by the clinician.

Statistical analyses

Statistical analyses were performed using STATAV.11 (StataCorp, USA), R and GraphPad Prism V.6.0c (GraphPad, USA). Logistic regression with postestimation classification was used to evaluate the predictive value of $\log_{10}$-transformed IP-10, IL-1α and IL-1β and other variables. The Youden Index was used to determine probability cut-offs for each model, assuming that sensitivity and specificity are of equal importance. The likelihood ratio (LR) test was used to compare nested models (online supplementary methods).

RESULTS

Performance of cytokine biomarkers in different cohorts

Of the women in the WISH study (Cape Town and Soweto, South Africa),[15] 206/286 (72%) had a discharge-causing STI (*C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, *M. genitalium*) or vaginal dysbiosis (BV/intermediate microbiota). In all genital samples collected, despite being from distinct locations in the lower reproductive tract, elevated IL-1α and IL-1β and reduced IP-10 together were consistently predictive of an STI or dysbiosis (figure 1), correctly classifying 76%, 76% and 73% of women for LWS, MC and VS, respectively (table 1). Although LWS and MCs performed similarly, LWS are easier to collect and process and would likely be a more feasible method of sample collection for a POC test than MCs.

Of the 264 women in the FEM-PrEP substudy (Bondo, Kenya and Pretoria, South Africa),[17] 165/264 (63%) had a laboratory-diagnosed discharge-causing STI or vaginal dysbiosis (27 *T. vaginalis*; 15 *N. gonorrhoeae*; 22 *C. trachomatis*; 100 BV (Nugent score 7–10); 28 intermediate microbiota (Nugent score 4–6)). IL-1α, IL-1β and IP-10 concentrations in ES correctly classified 73% of women according to STI/vaginal dysbiosis status, with 70% sensitivity and 79% specificity (table 1).

When comparing models including only two cytokines, we found that IL-1α+IP-10 performed better in FEM-PrEP, whereas IL-1β+IP-10 performed better in WISH (online supplementary table 1). In WISH, the model including all three cytokines significantly improved the fit compared with IL-1α+IP-10 (LR χ^2: 22.9, p<0.0001), whereas in FEM-PrEP, the model including all three cytokines significantly improved the fit compared with IL-1β+IP-10 (LR χ^2: 35.71, p<0.0001). Although IL-1α and IL-1β are members of the same family, across the two cohorts, inclusion of both performs better than choosing one.

Cytokine biomarker performance compared with clinical signs or symptoms

The performance of the cytokine biomarkers was compared with the current standard of care in South Africa, syndromic management. In WISH participants for whom symptoms were recorded (n=36), symptoms detected 41% of those with laboratory-diagnosed STIs or dysbiosis, with a specificity of only 57% (table 1). IL-1α, IL-1β and IP-10 measured in LWS correctly classified more women than clinical symptoms (Fischer's exact p=0.0003).

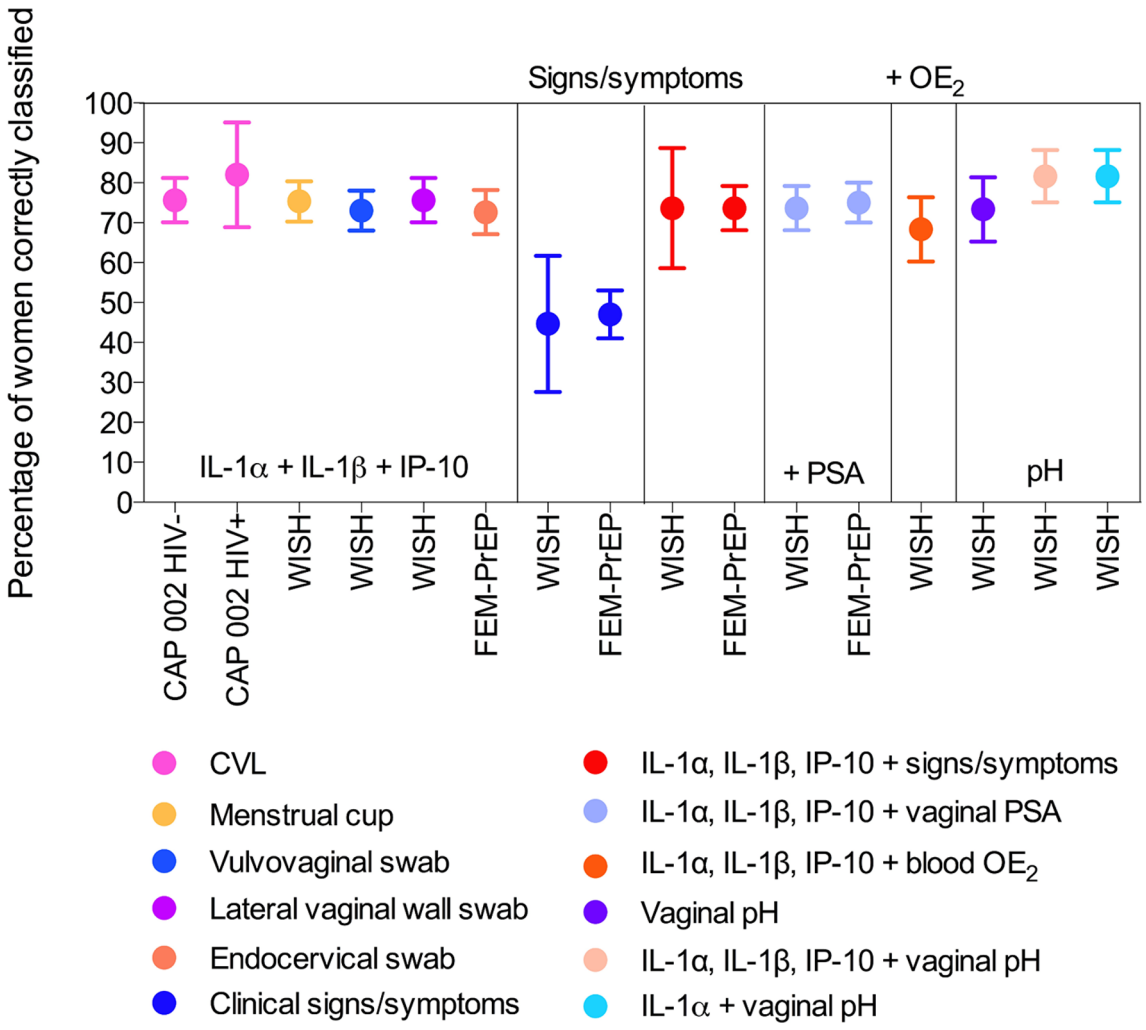

Figure 1 Percentage of women correctly classified using different models in different cohorts and using different genital samples. Logistic regression with postestimation classification was used to classify women according to continuous (cytokines) and ordinal (pH) variables. Dots indicate the percentage of women correctly classified using each model and error bars indicate the 95% CIs. The accuracy of IL-1α, IL-1β and IP-10 concentrations in different sample types collected in different studies is shown, followed by clinical signs or symptoms, IL-1α, IL-1β and IP-10 together with either signs/symptoms, vaginal PSA concentrations, blood oestradiol (OE_2) concentrations or vaginal pH. The predictive value of vaginal pH alone is shown, as well as vaginal pH together with IL-1α only. CVL, cervicovaginal lavage; IL, interleukin; IP, interferon-γ-induced protein; PSA, prostate specific antigen; WISH, Women's Initiative in Sexual Health.

Including clinical symptoms in the biomarkers model did not improve the accuracy or fit (LR χ^2: 0.14, p=0.711). Similarly, in FEM-PrEP, the three biomarkers performed better than clinical signs (Fischer's exact p<0.0001). However, including clinical signs in the cytokine model marginally improved the accuracy (74% vs 73% correctly classified; table 1), but not the fit (LR χ^2: 0.01, p=0.938).

Drivers of incorrect classification using the cytokine model

Since multiple factors may influence vaginal cytokine concentrations, we evaluated potential confounders in the WISH cohort (table 2). HSV-2 DNA positivity, HPV infection and vulvovaginal candidiasis (VVC) were not associated with false positive results. In WISH, detection of PSA, a semen marker, was not associated with incorrect classification, and adjusting for PSA did not influence the accuracy of the biomarkers model. However, in FEM-PrEP, adjusting for PSA in the model marginally increased the accuracy (online supplementary table 1). Although false negative women had lower blood oestradiol concentrations than true positive women (p=0.02), oestradiol concentrations were not associated with cytokine concentrations and oestradiol inclusion in the biomarkers model did not improve the fit (LR χ^2: 1.61, p=0.204) or accuracy (online supplementary table 1).

In WISH, STI/BV-negative women who were incorrectly classified as positive had higher vaginal pH than those correctly classified as negative (table 2), suggesting altered vaginal microbiota (not detected by Nugent scoring) that may influence genital cytokine concentrations. Thus, we used 16S rRNA microbiome data to evaluate the bacteria associated with each of the cytokine groupings (online supplementary figure 1).[19] False positive women identified by the biomarkers model had increased relative abundance of several organisms associated with dysbiosis and BV, including *P. bivia* and *Gardnerella* (figure 2).

Vaginal pH improved the performance of the cytokine model

As vaginal pH differed between false positive and true negative women, we investigated whether addition of pH to the cytokine model improved the performance. Although vaginal pH alone correctly classified 74% of women according to STI/dysbiosis status (table 1), the specificity was lower than the cytokine model

Table 1 Performance of IL-1α, IL-1β and IP-10 biomarkers of STIs and vaginal dysbiosis

Cohort	Biomarker	Sample	Probability cut-off	Model classification	True STI*/BV diagnosis (n)		Sensitivity	Specificity	PPV	NPV	Correctly classified
					Pos	Neg	% (95% CI)	% (95% CI)	% (95% CI)	% (95% CI)	(%)
WISH	IL-1α+IL-1β+IP-10	MC	0.7703	Pos	148	12	72 (65 to 78)	85 (75 to 92)	93 (87 to 96)	54 (45 to 63)	76 (70–80)
				Neg	58	68					
	IL-1α+IL-1β+IP-10	VS	0.7089	Pos	148	21	73 (67 to 79)	73 (62 to 83)	88 (82 to 92)	52 (42 to 61)	73 (68–78)
				Neg	54	58					
	IL-1α+IL-1β+IP-10	LWS	0.7287	Pos	154	22	77 (71 to 83)	71 (59 to 81)	88 (82 to 92)	54 (44 to 64)	76 (70–81)
				Neg	45	53					
	Clinical symptoms	NA	NA	Pos	12	3	41 (24 to 61)	57 (18 to 90)	80 (52 to 96)	19 (5 to 42)	44 (28–62)
				Neg	17	4					
	IL-1α+IL-1β+IP-10+clinical symptoms	LWS	0.8174	Pos	20	1	69 (49 to 85)	86 (42 to 100)	95 (76 to 100)	40 (16 to 68)	72 (55–86)
				Neg	9	6					
	pH	LWS	0.7660 (pH 4.7)	Pos	83	11	77 (68 to 84)	61 (41 to 78)	88 (80 to 94)	40 (26 to 57)	74 (65–81)
				Neg	25	17					
	IL-1α+IL-1β+IP-10+pH	LWS	0.7012	Pos	94	10	87 (79 to 93)	64 (44 to 81)	90 (83 to 95)	56 (38 to 74)	82 (75–88)
				Neg	14	18					
	IL-1α+pH	LWS	0.7286	Pos	93	9	86 (78 to 92)	68 (48 to 84)	91 (84 to 96)	56 (38 to 73)	82 (75–88)
				Neg	15	19					
FEM-PrEP	IL-1α+IL-1β+IP-10	ES	0.6632	Pos	115	21	70 (62 to 77)	79 (69 to 86)	85 (77 to 90)	61 (52 to 69)	73 (67–78)
				Neg	50	78					
	Clinical signs	NA	NA	Pos	47	22	29 (22 to 36)	78 (68 to 86)	68 (56 to 79)	39 (33 to 47)	47 (41–53)
				Neg	118	77					
	IL-1α+IL-1β+IP-10+clinical signs	ES	0.6677	Pos	115	19	70 (62 to 77)	81 (72 to 88)	86 (79 to 91)	62 (53 to 70)	74 (68–79)
				Neg	50	80					

**Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium* and *Trichomonas vaginalis* were assessed by nucleic acid amplification tests (NAAT) in WISH. *C. trachomatis* and *N. gonorrhoeae* were assessed by NAATs and *T. vaginalis* by wet mount microscopy in FEM-PrEP.

BV, bacterial vaginosis; ES, endocervical swab; IL, interleukin; IP, interferon-γ-induced protein; LWS, lateral vaginal wall swab; MC, menstrual cup; NA, not applicable; Neg, negative; NPV, negative predictive value; Pos, positive; PPV, positive predictive value; VS, vulvovaginal swab; WISH, Women's Initiative in Sexual Health.

Table 2 Drivers of incorrect classification in the WISH cohort

	Total	True negative	False positive	P values	True positive	False negative	P values
	n/total (%)	n/total (%)	n/total (%)	(TN vs FP)	n/total (%)	n/total (%)	(TP vs FN)
Chlamydia trachomatis PCR+	84/274 (30.7)	0/53 (0)	0/22 (0)	1.00	61/154 (39.6)	23/45 (51.1)	0.18
Neisseria gonorrhoeae PCR+	23/274 (8.4)	0/53 (0)	0/22 (0)	1.00	15/154 (9.7)	8/45 (17.8)	0.18
Trichomonas vaginalis PCR+	16/274 (5.8)	0/53 (0)	0/22 (0)	1.00	13/154 (8.4)	3/45 (6.7)	1.00
Mycoplasma genitalium PCR+	11/274 (4.0)	0/53 (0)	0/22 (0)	1.00	7/154 (4.6)	4/45 (8.9)	0.54
Bacterial vaginosis (Nugent 7–10)	128/274 (46.7)	0/53 (0)	0/22 (0)	1.00	**113/154 (73.4)**	**15/45 (33.3)**	**<0.0001**
Intermediate microbiota (Nugent 4–6)	40/274 (14.6)	0/53 (0)	0/22 (0)	1.00	34/154 (22.1)	6/45 (13.3)	0.29
Herpes simplex virus type 2 PCR+	8/274 (2.9)	0/53 (0)	0/22 (0)	1.00	8/154 (5.2)	0/45 (0)	0.20
Herpes simplex virus type 1 PCR+	0/274 (0)	0/53 (0)	0/22 (0)	1.00	0/154 (0)	0/45 (0)	1.00
Haemophilus ducreyi PCR+	0/274 (0)	0/53 (0)	0/22 (0)	1.00	0/154 (0)	0/45 (0)	1.00
Treponema pallidum PCR+	0/274 (0)	0/53 (0)	0/22 (0)	1.00	0/154 (0)	0/45 (0)	1.00
Human papilloma virus PCR+	151/229 (65.9)	21/45 (46.7)	9/19 (47.4)	1.00	97/129 (75.2)	24/36 (66.7)	0.39
High-risk HPV*	130/229 (56.8)	17/45 (37.8)	7/19 (36.8)	1.00	85/129 (65.9)	21/36 (58.3)	1.00
Low-risk HPV**	100/229 (43.7)	14/45 (31.1)	7/19 (36.8)	0.77	62/129 (48.1)	17/36 (47.2)	1.00
Candidiasis	27/274 (9.9)	7/53 (13.2)	3/22 (13.6)	1.00	11/154 (7.1)	6/45 (13.3)	0.22
Using DMPA	34/248 (13.7)	5/37 (13.5)	2/19 (10.5)	1.00	22/149 (14.8)	5/43 (11.6)	0.80
Using Nur-Isterate	117/248 (47.2)	16/37 (43.2)	13/19 (68.4)	0.09	67/149 (45.0)	21/43 (48.8)	0.73
PSA positive	70/272 (25.7)	11/52 (21.2)	4/22 (18.2)	1.00	45/153 (29.4)	10/45 (22.2)	0.45
Residence (number of Capetonians/total (%))	144/274 (52.6)	18/53 (34.0)	13/22 (59.1)	0.07	86/154 (55.2)	27/45 (60.0)	0.73
Cotinine detection	27/144 (18.8)	2/18 (11.1)	0/13 (0)	0.50	21/86 (24.4)	4/27 (14.8)	0.43
Vaginal product insertion	23/213 (10.8)	4/41 (9.8)	2/16 (12.5)	1.00	13/119 (10.9)	4/37 (10.8)	1.00
Blood contamination	29/274 (10.6)	2/53 (3.8)	2/22 (9.1)	0.58	19/154 (12.3)	6/45 (13.3)	0.80
	Median (IQR)	**Median (IQR)**	**Median (IQR)**		**Median (IQR)**	**Median (IQR)**	
Age	18 (17–20)	18 (17–20)	17.5 (17–19.75)	0.68	18 (17–20)	19 (18–20)	0.57
Vaginal pH	4.7 (4.4–5.3)	**4.1 (4.1–4.4)**	**4.7 (4.1–5.1)**	**0.02**	**5.0 (4.7–5.3)**	**4.7 (4.1–4.9)**	**0.0005**
Nugent score	5 (0–9)	0 (0–0.5)	0 (0–1)	0.16	**8 (6–9)**	**2 (0–7.5)**	**<0.0001**
Oestradiol (pmol/L)	72.1 (53.5–103.2)	81.0 (61.7–98.9)	71.4 (56.9–82.4)	0.35	**79.6 (52.9–125.1)**	**63.9 (46.8–71.0)**	**0.02**
Progesterone (nmol/L)	0.7 (0.4–1.2)	1.0 (0.5–1.3)	1.0 (0.6–1.6)	0.70	0.7 (0.4–1.2)	0.6 (0.4–1.1)	0.47
Luteinising hormone (IU/L)	4.4 (2.7–6.3)	4.2 (1.8–9.4)	4.3 (1.8–6.4)	0.73	4.5 (3.2–6.4)	3.7 (2.4–5.1)	0.26
Testosterone (nmol/L)	1.0 (0.3–1.4)	1.2 (0.3–1.5)	1.7 (1.1–1.8)	0.33	0.9 (0.3–1.2)	1.0 (0.7–1.4)	0.13

Values in bold indicate statistically significant differences between true negative and false positive women and between true positive and false negative women (p<0.05).
DMPA, depot medroxyprogesterone acetate; FN, false negative (STI and dysbiosis positive by PCR and Nugent scoring but grouped as negative using the biomarkers); FP, false positive (STI and dysbiosis negative by PCR and Nugent scoring but grouped as positive using the biomarkers); HPV, human papilloma virus; PSA, prostate specific antigen; TN, true negative (STI and dysbiosis negative by PCR and Nugent scoring and grouped as negative using the biomarkers); TP, true positive (STI and dysbiosis positive by PCR and Nugent scoring and grouped as positive using the biomarkers model); WISH, Women's Initiative in Sexual Health.

(61% vs 71%). Inclusion of vaginal pH in the cytokine model improved the accuracy, correctly classifying a greater proportion of women (82%) compared with the model including only cytokines (76%; figure 1). Including vaginal pH in the model also reduced the number of cytokines needed to achieve a similar predictive value as the model including three cytokines, with IL-1α and vaginal pH together correctly classifying 82% of women (table 1).

Cytokine model detected vaginal dysbiosis more accurately than STIs

While 88% (113/128) of the women with BV and 85% (34/40) of the women with intermediate microbiota were identified using the cytokine model, the model identified significantly more women with STIs than clinical symptoms (77/109 (71%) vs 5/14 (36%), p=0.0145). We evaluated whether excluding intermediate microbiota as an endpoint influenced model performance and found that IL-1α, IL-1β and IP-10 correctly classified 72% of women according to STI and BV status only (online supplementary table 1). Inclusion of VVC together with STIs, BV and intermediate microbiota as an endpoint in the model reduced the accuracy (70% correctly classified). Additionally, as under current guidelines, only women with STIs, and symptomatic BV and VVC would receive treatment, we evaluated the predictive value of the biomarkers for identifying these women and found that the cytokines correctly classified 78% of women (online supplementary table 1).

DISCUSSION

We validated IL-1α, IL-1β and IP-10 biomarkers of STIs and dysbiosis in >500 women residing in four regions in Africa and in four genital sample types, confirming consistent performance. LWS was the best sample type for biomarker measurement, based on the accuracy for identifying STIs/BV (76% of women correctly classified) and ease of collection. Inclusion of vaginal pH may refine this tool and the model including pH and IL-1α correctly classified 82% of women. Compared with the current standard of care, syndromic management, women with genital conditions were identified using the biomarkers with significantly higher sensitivity and specificity.

An inexpensive, POC biomarkers triage test for identifying women with asymptomatic STIs and dysbiosis could be used in family planning, antenatal and primary healthcare clinics to screen women who are not necessarily seeking healthcare for

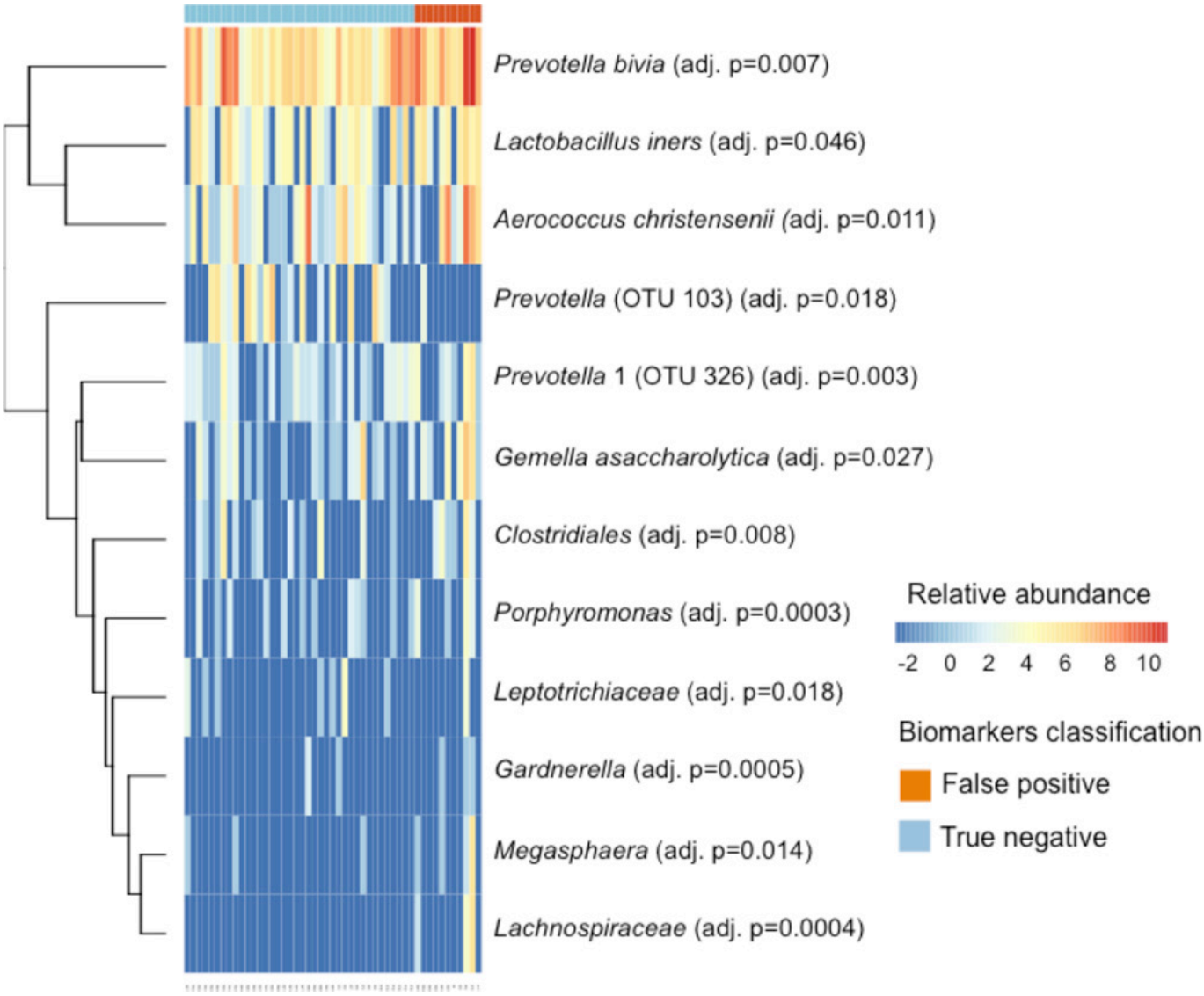

Figure 2 Bacteria that were significantly differentially abundant between a subset of false positive (n=11) and true negative (n=38) women. Differential abundance testing was used to determine the bacteria that differed significantly between false positive women (STI/bacterial vaginosis (BV) negative by PCR and Nugent scoring but grouped as positive using interleukin (IL)-1α, IL-1β and interferon-γ-induced protein (IP)-10) and true negative women (STI/BV negative by PCR and Nugent scoring and grouped as negative using the biomarkers). Differences in microbial composition between groups were assessed using metagenomeSeq's MRfulltable function with a custom filter to determine significance: merged taxa were deemed significantly different if they exhibited a fold change (beta coefficient) of ≥1.25, had an adjusted p value of ≤0.05 and if at least one of the two groups being compared had ≥20% of samples with the given operational taxonomic unit/taxa or the Fisher's exact test result was significant after multiple testing correction. False positive women identified by the biomarkers model had increased relative abundance of dysbiotic and BV-associated organisms, including *Prevotella bivia*, *Aerococcus christensenii*, *Gemella asaccharolytica*, *Clostridiales*, *Porphyromonas*, *Leptotrichiaceae*, *Gardnerella*, *Megasphaera* and *Lachnospiraceae,* while the abundance of *Prevotella* OTU 103 was lower in false positives compared with true negatives.

genital infections. Although only a small proportion of women access healthcare specifically for STI/BV treatment, many reproductive-aged, sexually active women in resource-limited settings use hormonal contraception (~34% in South Africa).[20] Offering this test to women visiting family planning facilities could potentially identify a large pool of women with asymptomatic genital infections. These women could be triaged for further testing to determine the underlying aetiology of their inflammation. While women with confirmed asymptomatic STIs could be given specific antibiotic treatment, management of women identified as having BV or intermediate microbiota is more complex.[21] Antibiotic treatment of symptomatic BV is associated with high recurrence rates,[22] and under current guidelines, asymptomatic BV or intermediate microbiota would not normally be treated.[8] Additionally, vaginal dysbiosis may resolve in some women without treatment.[23] However, it is nonetheless critical to develop cost-effective tools to identify women with asymptomatic BV and intermediate microbiota as they have high levels of genital inflammatory markers and are at high risk of HIV infection, acquisition of other STIs, HPV persistence and reproductive complications.[4 5 17 19 24 25] Improved management strategies for women with inflammatory BV and intermediate microbiota are urgently needed, including improved therapies (such as adjunctive symbiotic treatment or biofilm disruption), behavioural modification recommendations, HPV vaccination and antiretroviral pre-exposure prophylaxis programmes. Screening

reproductive-aged women with this test prior to expensive aetiological testing would result in a large reduction in the proportion of women requiring aetiological testing at the population level, with potentially substantial cost savings. The inexpensive nature of this test would also allow for routine monitoring of women over time, during family planning visits, for example, or monitoring of pregnant women identified as high risk.

Genital inflammatory cytokine responses may be influenced by factors other than discharge-causing STIs, BV and intermediate microbiota, although we focused here on these conditions as they are frequently asymptomatic and symptoms tend to be non-specific when present. We thus investigated the impact of potentially confounding factors on participant classification using the biomarkers model.[17 24 26 27] HSV-2, HPV, age, VVC, hormonal contraception, vaginal product use and blood contamination did not influence model accuracy. Although inclusion of PSA as a marker of semen exposure did not influence the predictive value of the biomarkers tool in the WISH and discovery studies,[14] PSA marginally improved the accuracy of the FEM-PrEP model, suggesting some effect. Blood oestradiol concentrations were lower in women who had an STI/dysbiosis but were incorrectly classified as negative, compared with women correctly classified as positive. Although oestradiol was not associated with changes in cytokine concentrations, it can modulate inflammatory responses[26] and may, thus, have influenced participant classification. A limitation of this study is that we did not evaluate the impact of HIV, parasitic infections, anti-inflammatory, antibiotic and antiretroviral use, and pregnancy, which could influence genital inflammatory status and hence biomarker accuracy. A limitation of the biomarkers is that 23% of the women with an STI, BV or intermediate microbiota were not identified. These women had low levels of genital inflammation and may have recently acquired these infections, or have resolving or less severe infections. Additionally, we found that these cytokines are not accurate biomarkers of VVC, which is also associated with increased risk of HIV acquisition.[28]

Exclusion of women with intermediate microbiota or asymptomatic BV as endpoints in the model did not materially influence the predictive value. Women diagnosed with intermediate microbiota would not normally receive antibiotic treatment and there are currently no treatment guidelines for women with asymptomatic BV. However, these women had high levels of inflammation in FEM-PrEP,[17] which suggests that treatment or closer monitoring of women with intermediate microbiota and asymptomatic BV should be considered. Interestingly, the effects of STIs or dysbiosis on cytokine profiles were cohort dependant, with chlamydia and gonorrhoea in the discovery cohort (Durban, South Africa),[27] BV in the WISH cohort (Cape Town and Soweto, South Africa)[18] and intermediate microbiota in FEM-PrEP (Bondo, Kenya and Pretoria, South Africa)[17] associated with the highest cumulative genital inflammatory cytokine concentrations within each cohort. Thus, in WISH and FEM-PrEP, the biomarkers model was more accurate when used to predict vaginal dysbiosis only, suggesting that the biomarkers only detect STIs that cause significant inflammatory cytokine responses.

Women who were STI, BV and intermediate microbiota negative by laboratory PCR tests and Nugent scoring, but classified as positive using the biomarkers model (false positive), had higher vaginal pH and increased abundance of dysbiotic bacteria (identified by 16S rRNA sequencing) compared with true negatives. It is thus possible that these bacteria are not always detected by Nugent scoring. Several taxa that were more abundant in false positive women were also significantly more abundant in women with genital inflammation.[24] This suggests that some women have genital inflammation caused by these bacteria that are not necessarily detected by Nugent scoring and could also benefit from antibiotic treatment, and that the accuracy of the model would be higher than 76% if specific dysbiotic bacteria were included as endpoints.

Vaginal pH, historically used for detecting BV (but not predictive of an STI), was marginally less accurate than the cytokine model (74% vs 76%) and had lower specificity (61% vs 71%). However, addition of pH to one inflammatory cytokine (IL-1α or IL-1β) had a better predictive value than all three cytokines in combination. Although vaginal pH detects altered vaginal microbiota, which was very prevalent in the WISH cohort, the cytokine biomarkers detect both women with BV and inflammatory STIs[14] and those with the highest levels of genital inflammation, who may be at increased risk of HIV.[3] Therefore, the combination of an inflammatory marker and pH may be effective at identifying genital infections/conditions in general.

The cytokine model that we validated here represents a significant improvement on the current standard of care for identifying STIs and BV in resource-limited settings. Similar POC tests have already been developed that measure IL-6 and IP-10, demonstrating the technical feasibility of this approach.[29 30] Until accurate, inexpensive, rapid POC tests are developed, this POC test may prove useful to decrease the prevalence of these genital conditions. Given the important link between genital inflammation and HIV risk, this biomarker test could impact incidence of HIV infection and reproductive complications in regions where syndromic management is practised.

Key messages

- Interleukin (IL)-1α, IL-1β and interferon-γ-induced protein-10 consistently identified women with STIs/dysbiosis across four populations and different types of genital specimens, performing better than clinical signs and symptoms.
- Including vaginal pH with the cytokine biomarkers improved the accuracy of the test, although pH alone had poor specificity.
- An inexpensive, biomarkers point-of-care test could be used in family planning and primary healthcare clinics as a triage test to identify women with asymptomatic STIs/dysbiosis.
- Women identified using this test could subsequently be referred for more costly aetiological testing to identify the cause of their inflammation, followed by specific treatment.

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Contributors LM conceptualised the study, generated some of the data, analysed the data and wrote the manuscript. SB managed the WISH cohort, generated and analysed some of the data and wrote the manuscript. JD, KL and SA generated and analysed some of the data and contributed to manuscript preparation. SD assisted with the management of the WISH cohort, processed clinical samples, generated some of the data and contributed to manuscript preparation. HG and SZJ assisted with the management of the WISH cohort, processed the clinical samples and contributed to manuscript preparation. ALW, TC and HJ supervised the acquisition and analysis of some of the data and contributed to manuscript preparation. FL analysed the data and contributed to manuscript preparation. LVD and KA managed the FEM-PrEP trial, collected clinical data and contributed to manuscript preparation. LGB, GG and JD managed the clinical sites for the WISH study, collected some of the clinical data and contributed to manuscript preparation. JASP conceptualised the study, managed the WISH cohort, supervised the acquisition and analysis of some of the data and contributed to manuscript preparation.

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Competing interests JASP and LM, together with the University of Cape Town, have submitted a Patent application for IP-10 and IL-1α/β use for diagnosing an inflammatory condition in the female genital tract likely caused by an STI or BV.

Patient consent Not required.

Ethics approval The University of Witwatersrand (M1307) and the University of Cape Town (267/2013) Human Research Ethics Committees and the FHI 360 Protection of Human Subjects Committee (10015) approved this study.

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