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Toll-like receptor variants and cervical *Atopobium vaginae* infection in women with pelvic inflammatory disease

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Abstract

Problem—Toll-like (TLR) receptor genetic variants have been implicated in bacterial vaginosis (BV). We determined if TLR variants are associated with fastidious BV-associated microbes that are linked with infertility following pelvic inflammatory disease (PID).

Method of Study—*Sneathia spp.*, *Atopobium vaginae*, BVAB1 and *Ureaplasma urealyticum* were measured in 250 women from the PID Evaluation and Clinical Health (PEACH) study. Relative risk (RR) and 95% confidence intervals (CI) were calculated adjusting for chlamydia and gonorrhea. Principal component analysis was used to adjust for population stratification. A false discovery rate q-value of 0.05 was significant.

Results—TLR2-1733C>A (p=0.003) and TLR2-616A>G (p=0.004) were associated with cervical *A. vaginae*. TLR2-1733C>A and TLR6-438C>T were associated with *A. vaginae* detection in the endometrium, but this was not significant after adjustment for multiple comparisons (FDR q-value=0.06).

Conclusions—Host gene variants in TLR2 signaling pathways were modestly associated with cervical *A. vaginae* in women with clinical PID.

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Keywords

Bacterial vaginosis; Inflammation; pelvic inflammatory disease

Introduction

Bacterial vaginosis (BV), an alteration of the vaginal flora, has been linked to pelvic inflammatory disease (PID).¹ Anaerobic and facultative aerobic bacteria including *Gardnerella vaginalis*, *Mycoplasma hominis*, *Prevotella spp.*, anaerobic gram-positive cocci, and *Ureaplasma urealyticum* are associated with this polymicrobial syndrome.² Studies using cultivation independent nucleic acid amplification of DNA from vaginal swabs, have identified novel bacteria associated with BV including *Atopobium vaginae*, *Megasphaera spp.*, *Sneathia spp.* (previously *Leptotrichia*), and three species in the *Clostridiales* order named bacterial vaginosis associated bacteria (BVAB)-1, BVAB-2, and *Mageeibacillus indolicus* (formerly BVAB-3).^{3, 4} The PID Evaluation and Clinical Health (PEACH) study found that fastidious BV-associated bacteria (*Atopobium vaginae*, *Sneathia spp.*, and BVAB-1), determined by 16s PCR, at the cervix and/or endometrium in women with clinical PID is associated with persistent endometritis, recurrent PID and infertility, independent of chlamydia and gonorrhea.⁵ However, the mechanisms leading to acquisition, upper genital tract ascension and subsequent sequelae remain unclear.

Toll-like receptors (TLR) are part of the innate immune system and eliminate pathogens through nuclear factor kappa-b (NF- κ B) signaling.⁶ Genetic variants in TLR genes can have direct or indirect biological consequences through overt or inadequate inflammatory response, possibly influencing disease progression. TLR2 (forms a heterodimer with TLR1 and TLR6) and TLR4 are expressed throughout the female reproductive tract.⁶ Variants in these gene pathways have been implicated in BV⁷⁻⁹ and PID,¹⁰ possibly through excessive inflammatory responses. Suppression of TLR regulatory function has also been hypothesized to contribute to the development of BV.¹¹ A PEACH sub-study has shown that TLR2 variants are associated with BV and endometrial colonization of facultative and anaerobic bacteria, isolated by culture, in African American women.¹² Altered TLR signaling may increase susceptibility to upper genital tract infection with BV associated bacteria.¹¹ However, individual fastidious BV-associated bacteria, associated with reproductive morbidity,⁵ were not examined.

Our objective was to determine if TLR variants are associated with fastidious BV-associated bacteria in women with clinical PID. As factors that promote bacterial ascension to the upper genital tract are poorly understood, we further determined if TLR variants increased endometrial infection with fastidious bacteria. *Sneathia spp.*, *A. vaginae*, and BVAB1 were selected because of associations with PID and subsequent infertility,⁵ which suggest these microbes may lead to upper genital tract pathology. Fredricks et al., have shown that BVAB-1 is highly specific to BV while *A. vaginae* has high sensitivity and moderate specificity, although this varies by population.³ *Atopobium vaginae* results in strong inflammatory and innate immune responses, while *Sneathia* and BVAB-1 are associated with BV-related symptoms and inflammation.¹³ Cervical *G. vaginalis* was measured at baseline in

PEACH but data was only available for half of the women in our cohort and thus was excluded.

Materials and Methods

This was a cross-sectional analysis nested within the PID Evaluation and Clinical Health (PEACH) study, a randomized clinical trial comparing inpatient and outpatient treatment in preventing long-term complications in 831 women with clinical PID.¹⁴ Women between the ages of 14 and 37 years with clinically suspected PID (pelvic pain less than 30 days duration, pelvic organ tenderness, and leukorrhea, mucopurulent cervicitis, or untreated cervicitis) were recruited between March 1996 and February 1999 from emergency departments, OB/GYN clinics, STD clinics, and private practices at thirteen U.S. clinical sites. All women provided informed consent and were randomized to inpatient or outpatient cefoxitin and doxycycline treatment. The University of Pittsburgh Institutional Review Board approved the study.

As part of the PEACH protocol, gynecological examinations were performed at baseline and 5 and 30 days post-treatment. Vaginal smears were gram stained for BV diagnosis in a central laboratory by Nugent criteria. Endometrial biopsy specimens underwent histological examination and endometritis was determined by at least five neutrophils in the endometrial surface epithelium in the absence of menstrual endometrium and/or at least two plasma cells in the endometrial stroma.¹⁵ Chlamydial polymerase chain reaction (PCR, Roche Diagnostics) and gonococcal culture were performed on endocervical swabs.¹⁴ Specimens were stored and later used to test for *M. genitalium*, using a microwell-plate-based PCR assay (MgPa-IMW) targeting the MgPa gene. Frozen specimens stored in -80°C freezers were thawed, purified using the MasterPure DNA purification kit for patient specimens (Epicentre) and tested by species-specific PCR assays for *Sneathia sanguinegens*, *Sneathia amnionii*, *Atopobium vaginae*, and BVAB1.⁵ For *Ureaplasma urealyticum*, PCR assays were performed using a modification of the procedure of Xiao et al.¹⁶, which has been described previously.⁵

A total of 290 women from PEACH had previously been genotyped for TLR variants¹⁰ to examine associations with chlamydial-PID. All women who had data on *C. trachomatis* infection and had stored buffy coats (n=237) or serum samples (n=53) available were genotyped. There were no differences in genotype frequencies between sample types. A total of 205 women of self-reported non-Hispanic black race, 51 women of non-Hispanic white race and 34 women of “other” race/ethnicity were included in the study. This racial/ethnic distribution is the same as the original PEACH study. For each TLR gene, HapMap (<http://hapmap.ncbi.nlm.nih.gov/>) was used to identify SNPs with a minor allele frequency ≥ 0.05 and blocks of linkage disequilibrium were identified. A total of 10 tagging SNPs (tag a particular haplotype in a gene region) from TLR1 (rs5743618, rs4833095), TLR2 (rs3804099, rs11938228, rs1898830), TLR6 (rs1039559, rs5743810, rs3775073) and TLR4 (rs4986790, rs4986791) were genotyped by fluorescence polarization.¹⁷ PCR reactions included 2.5µl of 1X PCR buffer (Invitrogen), 1.0µl of MgCl₂, 4µl of dNTPs, 1.5µl of each primer, 0.1µl of Taq polymerase (Invitrogen), and 13.4µl of dH₂O. PCR products were resolved by electrophoresis (3% agar gel) and visualized under UV light after ethidium

bromide staining. Genotypes were assigned by direct comparison to controls of sequence confirmed genotypes, and a 5% random resample was included for consistency of the genotyping. All SNPs were tested for deviations from Hardy-Weinberg equilibrium. Table 1 shows allele frequencies of each TLR variant. Among the 290 women with genotype data, a total of 250 also had data on fastidious BV-associated microbes for the current investigation which was approved by the Texas A&M University Institutional Review Board.

Relative risk (RR), 95% confidence intervals (CI) and p-values were estimated using modified Poisson regression with robust error estimates.¹⁸ We identified *a priori* genotypes that may be associated with genital infections. For example, the TLR1 Asn248Ser CC genotype is more frequent in pregnant women with *A. vaginae*,⁷ the TLR1 Ser602Ile TT genotype is associated with *C. trachomatis*,¹⁰ the TLR2 -616A>G AA genotype is more frequent in women with PID and BV,¹² and the TLR2 Asn199 CC genotype is more frequent in BV among HIV positive women.¹⁹ One of our objectives was to determine if these potential “risk” genotypes are associated with fastidious genital microorganisms. However, many were more frequent in our population (i.e. they are not the minor genotype). This is likely due to our cohort being primarily of black race. Indeed, many functional TLR genotype frequencies significantly differ by race/ethnicity where a minor genotype in those of white race may be the most common genotype in those of black race.²⁰ For consistency across all analyses, homozygous genotypes with the lowest frequency were collapsed with heterozygotes and used as the reference category (AA vs. AG + GG). STIs were examined as potential confounders, as some are associated with TLR variants as well as BV-associated bacteria.^{5, 10} Other clinical and demographic factors were either not associated with BV-associated bacteria or TLR genetic variants and were not considered confounders. We ultimately adjusted for *C. trachomatis* and *N. gonorrhoeae*. Principal component analysis was used to correct for population stratification. Principal components are specific to each marker’s frequency across ancestral populations and overcomes well known limitations of adjusting for self-reported race.²¹ A False Discovery Rate (FDR) q-value of 0.05 indicated statistical significance after correction for multiple comparisons.²² Q-values are adjusted p-values calculated using an optimised FDR approach.

We used multifactor dimensionality reduction (MDR), which collapses high-dimensional genetic data allowing for detection of gene-gene interactions in smaller sample sizes, even in genes without independent main effects.²³ This is a non-parametric and genetic model free method.²³ Model fit is determined by cross-validation consistency (CV, ranges from 1–10) calculated using training and testing datasets. Prediction error was estimated using 10-fold cross-validation. This procedure is performed ten times, using different random number seeds, to reduce the chance of observing spurious results due to chance divisions of the data. For our analysis, a Monte Carlo *p*-value, derived from permutations, of < 0.01 was significant. MDR models were race-adjusted.

Results

Approximately 64% of women were <25 years of age, 70.7% were black, 42.2% had less than a high school education, 88.7% were single, 54.8% did not douche, 55.0% were non-smokers and 69.5% did not report illicit drug use. These characteristics are similar to the

PEACH cohort, except our sub-study had higher rates of chlamydia (Table 2). Overall, *Sneathia spp.* were detected in cervix and/or endometrium of 65.9% of women with clinical PID, *U. urealyticum* in 28.7%, *A. vaginae* in 81.7%, and BVAB-1 in 60.8%. *Chlamydia trachomatis* (36.5%), *N. gonorrhoeae* (27.7%) and *M. genitalium* (12.1%) were also common.

Women with *A. vaginae* detected at the cervix and/or endometrium were significantly (chi-square $p < 0.05$) more likely to be black (74.6% vs. 52.0%), single (90.0% vs. 72%), have chlamydia (40.0% vs. 16.0%) and have gonorrhea (30.0% vs. 14%; $p = 0.05$) compared to women without *A. vaginae*. Women with *Sneathia spp.* were more likely to be black (79.0% vs. 55.0%), single (92.0% vs. 81.0%), use drugs (35.0% vs. 21.0%), and have gonorrhea (35.0% vs. 13.0%). Only black race significantly differed between women with and without BVAB1 (78.0% vs. 60%) and there were no differences found for women with or without *U. urealyticum*.

Associations between TLR genotypes and BV-associated bacteria (*Sneathia spp.*, *A. vaginae*, BVAB-1), and *U. urealyticum* detected at the cervix are presented in Table 3. After adjustments and correction for type 1 error, the CC genotype for TLR2 -1733C>A (RR_{adj} 1.4, 95% CI 1.2–1.7) and the AA genotype for TLR2 -616A>G (RR_{adj} 1.4, 95% CI 1.2–1.7) were associated with cervical *A. vaginae*.

When we examined endometrial infection similar trends were observed (Table 4). However, there were no significant associations after adjustment for multiple comparisons, although the CC genotype for TLR2 -1733C>A (RR_{adj} 1.5, 1.2–2.0) and the TT genotype for TLR 6 -438C>T (1.6, 1.2–2.2) were marginally associated with endometrial *A. vaginae* (FDR q -value=0.06).

As an exploratory analysis, we examined if TLR variants were associated with ascending *A. vaginae* infection. First, we identified all women with *A. vaginae* at the cervix only (absence of endometrial infection) at baseline ($n=76$). We then examined associations between TLR variants and women with cervical or endometrial *A. vaginae* at 30-days follow-up compared to women who cleared infection at follow-up. Among women with baseline *A. vaginae* at the cervix only, TLR4 -787A>G (RR_{adj} 3.6, 95% CI 1.2–10.9; $p=0.02$) and TLR2 -616A>G (RR_{adj} 5.7, 95% CI 1.2–26.0; $p=0.02$) were associated with cervical infection at 30-days follow-up. Furthermore, TLR2 -616A>G (RR_{adj} 1.8, 95% CI 1.1–3.0; $p=0.01$) was associated with endometrial *A. vaginae* infection at 30-days follow-up. When we compared endometrial *A. vaginae* at follow-up to women who remained positive at the cervix only at follow-up, TLR2 -616A>G was no longer statistically significant (RR_{adj} 1.5, 95% CI 0.9–2.8; $p=0.1$).

Lastly, we explored gene-gene interactions for *A. vaginae* using MDR. The best fit model for cervical *A. vaginae* displayed a consistent (CV 9/10) moderate synergistic interaction between TLR1 Asn248Ser and TLR2 Asn199. The highest risk group were women who carried the GG genotype for TLR 1 Asn248Ser and the CC or CT genotype for TLR2 Asn199. However, this model had low sensitivity (60.8%) and specificity (65.3%). A less consistent (CV 6/10) strong synergistic interaction was identified between TLR1 Asn248Ser,

TLR2 -1733C>A and TLR4-385G>A, with the highest risk group being women with the GG, CC and GG genotypes respectively. This model had moderate sensitivity (79.5%) and low specificity (55%). For endometrial *A. vaginae* there was an inconsistent (CV 6/10) interaction between TLR2 -616A>G and TLR6 -438C>T. The highest risk group were women who carried the TT genotype for TLR2 -616A>G and the TT genotype for TLR6 -438C>T (sensitivity 89%; specificity 30%).

Discussion

We have previously shown that TLR2 genetic variants are associated with BV and colonization of facultative and anaerobic bacteria, isolated by culture, in the endometrium.¹² This suggested that TLR2 variants may increase susceptibility to vaginal flora alterations and presence of anaerobic bacteria in the endometrium. However, there is an increasing appreciation for the role of fastidious BV-associated bacteria in PID and subsequent morbidity.^{5, 24} We found that TLR2 variants are associated with cervical *A. vaginae*. Our findings are significant as women with PID who test positive for fastidious BV-associated bacteria at the cervix and/or endometrium have an increased risk of endometritis, recurrent PID and infertility independent of *N. gonorrhoeae* and *C. trachomatis*.⁵ This is consistent with the hypothesis that host factors may alter immune function in the genital tract to enhance or reduce bacterial overgrowth.^{11, 13} However, over 70% of our population was of black race. The vaginal microbiome²⁵ and TLR allele frequencies²⁰ vary by race. Thus, our results are likely only generalizable to black women with symptomatic clinical PID.

Genetic variants in TLR genes are linked to BV, *G. vaginalis* and *A. vaginae* in a handful of studies.⁷⁻⁹ In a study of 144 non-Hispanic white pregnant women, Verstraeten et al., found that TLR1 Asn248Ser CC genotype was associated with vaginal carriage of *A. vaginae*.⁷ In our study, Asn248Ser was associated with cervical *A. vaginae* but not after correction for multiple comparisons. TLR1 Asn248Ser is a functional variant and increases Pam3CSK4 induced tumor necrosis factor alpha and interleukin 12 in peripheral blood mononuclear cells and monocyte derived dendritic cells.²⁶ Interestingly, Asn248Ser is associated with *C. trachomatis*, upper genital tract infection and reduced pregnancy rates in women with PID.¹⁰

After correction for multiple comparisons, TLR2 variants (-1733C>A and -616A>G) were associated with cervical *A. vaginae*. TLR2 -1733C>A and TLR6 -438C>T were marginally associated with endometrial *A. vaginae* after correction for multiple comparisons. Thus, variants in the TLR2 pathway may increase susceptibility to lower genital tract infection with *A. vaginae*. Larger studies are needed to determine if TLR variants influence ascension to the upper genital tract. When we examined women with baseline *A. vaginae* at the cervix only, TLR2 -616A>G was associated with endometrial *A. vaginae* at 30-days follow-up compared to women who cleared infection. However, no associations were found when we compared women who remained positive at the cervix only to those with ascending infection.

Atopobium vaginae results in a strong pro-inflammatory response, can activate NF- κ B in cervicovaginal epithelial cells^{27, 28} and induces vaginal epithelial cell cytokine secretion via TLR2.²⁹ When challenged with persistent infection with BV-associated bacteria, genetically

susceptible women may be at increased risk for excessive TLR signaling. This is consistent with findings that BV-associated bacteria at the cervix and/or endometrium increase the risk of persistent endometritis and infertility.⁵ However, the exact function of TLR2 -1733C>A and -616A>G are not known. Minor homozygotes and heterozygotes for both variants have fluctuations (lower or higher) in sputum neutrophils, eosinophils, and macrophages and disease progression in obstructive pulmonary disease patients.³⁰ The TLR2 -616A>G minor allele increases shedding of genital herpes simplex virus type 2.³¹ This variant is also associated with lower T-regulatory cells (Tregs) in cord blood from neonates with atopic mothers but higher Tregs in mothers without atopy.³² TLR2 -616A>G could impact immunomodulatory function, possibly enhancing bacterial overgrowth. Our previous study found that TLR2 -616A>G was associated with BV diagnosed by Nugent's criteria and endometrial infection with facultative and anaerobic gram-negative bacteria.¹²

Further research is needed to understand the mechanisms that may lead to upper genital tract ascension and pathology following infection with BV-associated bacteria. This is particularly important as *A. vaginae*, *Sneathia* and BVAB1 do not respond to treatment for PID with cefoxitin and doxycycline.⁵ This cross-sectional analysis is unable to make any definitive conclusions about the exact biological mechanisms that link genetic variants to increased presence of BV-associated bacteria. Furthermore, effect sizes were relatively moderate which may suggest that environmental factors play a larger role in the presence of cervical BV-associated bacteria in women with clinical PID. Epistasis (gene-gene interaction) has been suggested to have larger effects on complex diseases than single gene variants.²³ However we did not consistently find any strong synergistic relationships following the MDR analysis.

We focused on only a few select fastidious bacteria and did not utilize high-throughput sequencing and do not have data on bacterial burden. However, these bacteria have been previously linked to PID-associated sequelae⁵ and have moderate to high sensitivity for BV diagnosis.³ Although implicated in BV pathogenesis, only a subset of women in PEACH had data on *G. vaginalis*. However, *G. vaginalis* was not independently associated with PID in the Gyn Infections Follow-through (GIFT) study which prospectively followed 1,199 at high risk for STIs.³³ A FDR q-value of 0.05 indicated significance, which means that 5% of tests with a q-value of 0.05 or less (<1% of overall tests) may be false positives. We did not have data on immune biomarkers which is needed to understand if genetic variants alter genital tract inflammation. It is possible that transcervical sampling of the endometrium can result in contamination of endometrial biopsy specimens. However, any contamination would not be expected to differ by TLR variant and is unlikely to be a major source of bias. All women in our study had clinical PID but our objective was to determine if TLR variants are associated with cervical or endometrial infection with BV-associated bacteria. This is important as not all women with cervical infection will develop upper genital tract infection. Furthermore, our ability to examine endometrial infection is a strength of our cohort.

Larger studies are needed to confirm our results and determine if TLR2 variants mediate pathology following infection with fastidious bacteria. Prospective studies of women at high risk for STI's who have not yet developed PID may provide insight into whether TLR variants, in combination with environmental factors, increase the risk of lower genital

bacterial infection and/or upper genital tract ascension. Overall, women who carry variants in the TLR2 signaling pathway may have a modest increased risk BV-associated bacteria in the cervix and endometrium.

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Table 1

Genes, SNPs and genotype frequencies

Gene, SNP reference	rs number	Function	Allele frequency	Genotype, frequency
TLR1 Ser602Ile	rs5743618	Missense	G (0.25)	GG (0.11)
			T (0.75)	GT (0.28)
				TT (0.60)
TLR1 Asn248Ser	rs4833095	Missense	T (0.34)	TT (0.17)
			C (0.66)	CT (0.35)
				CC (0.49)
TLR2 Asn199	rs3804099	Synonymous codon	C (0.59)	CC (0.38)
			T (0.41)	CT (0.41)
				TT (0.20)
TLR2 -1733C>A	rs11938228	Intron variant	C (0.79)	CC (0.64)
			A (0.21)	AC (0.31)
				AA (0.06)
TLR2 -616A>G	rs1898830	Intron variant	A (0.81)	AA (0.67)
			G (0.19)	AG (0.30)
				GG (0.04)
TLR6: -438C>T	rs1039559	Intron variant	T (0.74)	TT (0.55)
			C (0.26)	CT (0.38)
				CC (0.04)
TLR6 Ser249Pro	rs5743810	Missense	C (0.84)	CC (0.73)
			T (0.16)	CT (0.22)
				TT (0.05)
TLR6 Lys421	rs3775073	Synonymous codon	G (0.56)	GG (0.34)
			A (0.44)	AG (0.45)
				AA (0.21)
TLR4 -385G>A	rs5030728	Intron variant	G (0.56)	GG (0.64)
			A (0.20)	AG (0.32)
				AA (0.03)
TLR4 -787A>G	rs1927911	Intron variant	T (0.50)	TT (0.39)
			C (0.50)	CT (0.39)
				CC (0.30)

Table 2

Baseline characteristics of the PEACH study and genetic sub-study

Variable	PEACH cohort N=831 N(%)	Genetic sub-cohort N=250 N(%)
Age		
<19	209 (25.2)	68(27.3)
19–24	333 (40.1)	91(36.6)
25+	289 (34.8)	90 (36.1)
Race		
NH-Black	621 (74.7)	176 (70.7)
NH-White	133 (16.0)	43 (17.3)
Other	77 (9.3)	30 (12.1)
Education		
Less HS	319 (38.5)	105 (42.2)
HS or GED	310 (37.4)	90 (36.1)
Some college	200 (24.1)	54 (21.6)
Marital Status		
Single	678 (89.7)	204 (88.7)
Married	78 (10.3)	26 (11.3)
<i>C. trachomatis</i>		
Cervical	179 (22.0)	90 (36.1)
Endometrial	64 (9.2)	31 (13.1)
Cervical and/or endometrial	181 (25.4)	91 (36.5)
<i>N. gonorrhoeae</i>		
Cervical	175 (21.5)	59 (23.9)
Endometrial	79 (14.1)	32 (16.1)
Cervical and/or endometrial	176 (28.1)	59 (27.7)
<i>M. genitalium</i>		
Cervical	74 (10.9)	22 (9.2)
Endometrial	50 (8.2)	16 (7.0)
Cervical and/or endometrial	88 (15.0)	27(12.1)
BV		
Normal	139 (18.6)	41 (17.5)
Intermediate	168 (22.4)	60 (25.5)
BV	440 (58.9)	134 (57.0)
Cervical <i>G. vaginalis</i>	115 (33.0)	61 (39.1)
Endometritis		
No	343 (54.5)	109 (51.7)

Variable	PEACH cohort N=831 N(%)	Genetic sub-cohort N=250 N(%)
Yes	311 (47.6)	102 (48.3)
<i>Sneathia spp.</i>		
Cervical and/or endometrial		159(65.9)
Cervical	N/A	141(58.3)
Endometrial		98(42.1)
<i>U. urealyticum</i>		
Cervical and/or endometrial		66(28.7)
Cervical	N/A	61(25.2)
Endometrial		39(16.7)
<i>A. vaginae</i>		
Cervical and/or endometrial		201(81.7)
Cervical	N/A	179(73.7)
Endometrial		110(47.0)
BVAB1		
Cervical and/or endometrial		144(60.8)
Cervical	N/A	112(46.1)
Endometrial		82(35.0)
Douched		
No	461 (55.8)	159(54.8)
Yes	365 (44.2)	131(45.2)
Smoker		
No	448 (54.2)	137 (55.0)
Yes	378 (45.8)	112 (45.0)
Drug user		
No	592 (71.7)	173 (69.5)
Yes	234 (28.3)	76 (30.5)

Table 3

Association between TLR variants and cervical BV-associated bacteria and *U. urealyticum* at baseline

Variant	Cervical BV-associated microbes				Cervical <i>U. urealyticum</i>			
	Sneathia spp.		<i>A. vaginae</i>		BVAB1		<i>U. urealyticum</i>	
	RR _{adj.} (95% CI)	p	RR _{adj.} (95% CI)	p	RR _{adj.} (95% CI)	p	RR _{adj.} (95% CI)	p
TLR1: Ser602Ile								
GG + GT	–	–	–	–	–	–	–	–
TT	1.4, 1.0–1.9	0.06	1.3, 1.0–1.6	0.08	1.3, 0.8–2.1	0.22	0.6, 0.3–1.3	0.22
TLR1: Asn248Ser								
AA + AG	–	–	–	–	–	–	–	–
GG	1.1, 0.8–1.6	0.45	1.4, 1.1–1.8	0.01*	1.2, 0.8–1.9	0.40	0.9, 0.5–2.0	0.88
TLR2: Asn199								
TT + CT	–	–	–	–	–	–	–	–
CC	1.0, 0.7–1.4	0.89	1.2, 0.9–1.6	0.15	0.8, 0.5–1.3	0.39	0.8, 0.4–1.7	0.55
TLR2: –1733C>A								
AA + AC (ref)	–	–	–	–	–	–	–	–
CC	1.3, 1.0–1.8	0.05	1.4, 1.2–1.7	0.003**	1.0, 0.7–1.6	0.83	0.9, 0.5–1.7	0.76
TLR2: –616A>G								
GG+AG (ref)	–	–	–	–	–	–	–	–
AA	1.2, 0.9–1.6	0.33	1.4, 1.2–1.7	0.004**	0.9, 0.6–1.4	0.73	0.7, 0.4–1.5	0.39
TLR6: –438C>T								
CC+TC (ref)	–	–	–	–	–	–	–	–
TT	1.3, 0.9–1.7	0.16	1.3, 1.1–1.6	0.01*	1.0, 0.6–1.5	0.94	0.7, 0.4–1.5	0.40
TLR6: Ser249Pro								
TT + CT (ref)	–	–	–	–	–	–	–	–
CC	1.0, 0.7–1.5	0.90	1.2, 0.9–1.5	0.14	0.7, 0.4–1.2	0.18	0.6, 0.3–1.2	0.14

Variant	Cervical BV-associated microbes						Cervical U. urealyticum	
	Sneathia spp.			A. vaginae			U. urealyticum	
	RR _{adj.} (95% CI)	p	RR _{adj.} (95% CI)	p	RR _{adj.} (95% CI)	p	RR _{adj.} (95% CI)	p
TLR6: Lys421								
AA + AG (ref)	–	–	–	–	–	–	–	–
GG	1.0, 0.7–1.5	0.85	1.2, 0.9–1.6	0.18	0.9, 0.5–1.6	0.79	1.0, 0.4–2.3	0.97
TLR4: –385G>A								
AA + AG (ref)	–	–	–	–	–	–	–	–
GG	1.2, 0.9–1.6	0.14	1.3, 1.1–1.6	0.01*	1.1, 0.7–1.6	0.69	0.7, 0.4–1.2	0.22
TLR4: –787A>G								
CC + CT (ref)	–	–	–	–	–	–	–	–
TT	1.2, 0.9–1.6	0.23	1.2, 1.1–1.5	0.04*	1.0, 0.7–1.5	0.99	1.8, 0.4–1.6	0.50

Relative risk, 95% confidence intervals and p-values were calculated using Poisson Regression with robust errors measurements. All models are adjusted for population stratification. Additionally, models are adjusted for chlamydia and gonorrhea.

* Significant at p<0.05;

** FDR q-value >0.05

Table 4

Association between TLR variants and endometrial BV-associated bacteria and *U. urealyticum* at baseline

Variant	Endometrial BV-associated microbes				Endometrial <i>U. urealyticum</i>			
	Sneathia spp.	A. vaginae	BVAB1	U. urealyticum	RR _{adj.} (95% CI)	p	RR _{adj.} (95% CI)	p
TLR1: Ser602Ile								
GG + GT	–	–	–	–	–	–	–	–
TT	1.4, 0.9–2.3	0.13	1.2, 0.8–1.7	0.48	1.5, 0.8–2.6	0.16	0.5, 0.1–1.9	0.28
TLR1: Asn248Ser								
AA + AG	–	–	–	–	–	–	–	–
GG	1.1, 0.7–1.8	0.61	1.6, 1.1–2.4	0.02	1.3, 0.7–2.2	0.36	1.1, 0.2–4.5	0.94
TLR2: Asn199								
TT + CT	–	–	–	–	–	–	–	–
CC	1.0, 0.6–1.6	0.88	1.2, 0.9–1.6	0.27	0.7, 0.4–1.3	0.29	1.5, 0.2–11.1	0.66
TLR2: –1733C>A								
AA + AC (ref)	–	–	–	–	–	–	–	–
CC	1.3, 0.9–1.9	0.22	1.5, 1.2–2.0	0.009**	1.1, 0.7–1.8	0.75	0.7, 0.2–2.0	0.48
TLR2: –616A>G								
GG+AG (ref)	–	–	–	–	–	–	–	–
AA	1.0, 0.8–1.5	0.94	1.5, 1.1–2.1	0.01*	1.0, 0.6–1.6	0.86	0.6, 0.2–2.2	0.44
TLR6: –438C>T								
CC+TC (ref)	–	–	–	–	–	–	–	–
TT	1.4, 0.9–2.1	0.12	1.6, 1.2–2.2	0.009**	1.0, 0.6–1.6	0.97	2.3, 0.5–10.2	0.24
TLR6: Ser249Pro								
TT + CT (ref)	–	–	–	–	–	–	–	–
CC	1.1, 0.6–2.0	0.65	1.2, 0.9–1.7	0.20	0.6, 0.4–0.9	0.04*	0.8, 0.1–4.4	0.79

Variant	Endometrial BV-associated microbes						Endometrial U. urealyticum		
	Sneathia spp.			A. vaginae			BVAB1		
	RR _{adj.} (95% CI)	p		RR _{adj.} (95% CI)	p		RR _{adj.} (95% CI)	p	RR _{adj.} (95% CI)
TLR6: Lys421									
AA + AG (ref)	–			–			–		–
GG	1.1, 0.7–1.9	0.62		1.2, 0.8–1.8	0.37		1.0, 0.6–1.9	0.91	1.9, 0.3–13.1
TLR4: –385G>A									
AA + AG (ref)	–			–			–		–
GG	1.3, 0.9–1.8	0.17		1.4, 1.0–1.8	0.03*		1.1, 0.7–1.6	0.73	1.7, 0.5–5.9
TLR4: –787A>G									
CC + CT (ref)	–			–			–		–
TT	1.2, 0.8–1.8	0.43		1.3, 1.0–1.7	0.09		1.0, 0.6–1.6	0.88	0.3, 0.1–1.8

Relative risk, 95% confidence intervals and p-values were calculated using Poisson Regression with robust errors measurements. All models are adjusted for population stratification. Additionally, models are adjusted for chlamydia and gonorrhea.

* Significant at p<0.05;