Toll-like receptor gene variants and bacterial vaginosis among HIV-1 infected and uninfected African women

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Abstract

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Bacterial vaginosis (BV) is a common vaginal syndrome associated with altered microflora that increases the risk of preterm delivery and acquisition of sexually transmitted diseases. The cause of BV is unknown although toll-like receptors (TLRs) that initiate the innate immune response may be important. We evaluated associations between TLR SNPs and BV among HIV-1 infected and uninfected African women. Logistic regression and a GEE model were used to assess associations between SNPs in *TLRs 2-4*, *7-9* and BV as classified by Nugent's criteria. Among HIV-1 uninfected women, *TLR7* rs5743737, *TLR7* rs1634323 and *TLR2* rs1898830 were associated with a decreased risk of BV while *TLR7* rs179012 was associated with an increased risk. TLR2 SNPs rs1898830 and rs3804099 were associated with a decreased risk of BV among HIV-1 infected women. Our findings indicate that HIV-1 infection may modify the association of TLR variants with risk for BV.

BACKGROUND

Epidemiology and disease impact

Bacterial vaginosis (BV) is a complex microbial imbalance of the vaginal flora and considered the most common bacterial infection among women of childbearing age. BV is associated with significant health consequences including increased risk of pregnancy loss and preterm delivery, pelvic inflammatory disease, and post surgical infections[1, 2]. Furthermore, BV is associated with an increased risk of acquiring a number of sexually transmitted infections (STIs) including gonorrhea, chlamydia, HSV-2 and HIV-1[3-5]. BV has been linked to increased viral shedding of both HIV-1 and herpes simplex virus type 2 (HSV-2), which could contribute to an increased risk of transmission of these STIs to male partners [5, 6]. Specifically, women with BV are three times more likely to transmit HIV-1 to their male partner as women without BV [7]. The population attributable risk of BV for HIV-1 and HSV-2 is approximately 15-20%, indicating that prevention and treatment of BV could significantly help reduce the burden of these STIs [3, 6, 7]. Risk factors for developing BV include douching, increased number of sexual partners, infrequent condom use, being of African descent, and HIV-1 infection [8-11]. Epidemiological studies have estimated the overall prevalence of BV in the United States at 30% [12]. African women have a higher prevalence of BV with estimates up to 55% and one study showing that 95% of women in a Ugandan cohort developed BV at least once during two years of follow up [13-15].

Symptoms, Diagnosis & Treatment

Symptoms of BV include vaginal discharge and odor. These symptoms are nonspecific and many women with BV do not report having any symptoms, making BV diagnosis more difficult [12, 16]. In a clinical setting, BV can be diagnosed when three of the following Amsel's criteria are met: 1) vaginal pH >4.5; 2) white adherent discharge; 3)

fishy odor upon the addition of 10% potassium hydroxide; 4) epithelial cells with gram-variable bacteria attached to their surface (clue cells)[17]. However, since BV is asymptomatic in many women, laboratory rather than clinical testing is considered the gold standard for diagnosis. Specifically, the Nugent's criteria uses Gram staining of vaginal smears and scoring samples based on presence or absence of large Gram-positive rods (e.g. lactobacillus), small Gram-negative to gram-variable rods (e.g. *G. vaginalis*), and curved gram-negative rods. Nugent's criteria is scored on a scale of 0-10: BV is indicated by a score of 7 to 10, intermediate flora is a score of 4 to 6, and scores of 0 to 3 indicate normal vaginal flora [18].

Antibiotics can be used to treat BV although recurrence is high even after the recommended regimen; one study showed that almost 60% of women treated for BV had a recurrence within 12 months [19].

Pathogenesis

Healthy vaginal bacterial flora is dominated by *Lactobacillus* spp that offer protection against potential pathogens. Vaginal lactobacilli can produce lactic acid, hydrogen peroxide and bacteriocins, all of which contribute to suppression of endogenous pathogens [20]. Reduction of lactobacilli and replacement with a diverse mix of anaerobic bacteria is characteristic of BV [2]. The Gram-variable microorganism *Gardnerella vaginalis* is highly associated with BV occurrence and is considered to be the primary pathogen that flourishes as lactobacilli numbers decrease[2]. *G. vaginalis* is immotile and forms a biofilm on the vaginal epithelium that provides a scaffolding to which other bacteria can adhere [2, 21-23]. Biofilms may contribute significantly to persistent BV infection since antibiotics are not as effective against bacteria organized in biofilms[24]. Although *G. vaginalis* is highly associated with BV, it is not a specific marker because it can also be found in the vagina of women without BV. Other bacteria, particularly *Atopobium vaginae,* have also been

associated with BV[2]. Importantly, it is not understood whether overgrowth of *G.vaginalis* and other pathogenic bacteria or loss of lactobacilli from the vaginal microflora plays a primary causal role in the development of BV [25].

Despite its high prevalence and significant sequelae, the pathogenesis of BV has not been entirely elucidated, impeding development of effective treatment and prevention interventions. Several theories have emerged to explain the cause of BV and in particular the decrease in lactobacilli flora. A disturbance of the vaginal flora caused by douching or antibiotic treatment, bacteriophages (viruses that infect bacteria), or changes in circulating hormones could initiate a decrease in the lactobacilli population, making women more prone to the overgrowth of BV associated bacteria [2, 26]. The innate immune system also may be an important mediator of BV incidence and persistence.

Toll-like receptors (TLRs) are a critical component of the innate immune system that recognize pathogen-associated molecular patterns (PAMPs). Pathogen recognition by TLRs initiates the activation of inflammatory cytokines, type 1 interferon (IFN) and chemokines [25, 27]. There are 10 identified TLRs in humans that recognize bacterial, viral, fungal, and parasitic molecular patterns (Table 1); TLRs are located on antigen presenting cells, fibroblasts, and epithelial cells[25]. TLRs 1-9 are constitutively expressed in epithelial cells of the female genital tract [28, 29].

Table 1. Known TLR PAMP and species recognition and location within the cell (Adapted from Kawai et. al., 2011) [30]

Toll-like Receptor	PAMPs	Pathogen type	TLR cellular location
TLR1	Lipoproteins	Bacteria	Cell surface
TLR2	Lipoproteins Structural protein Mannan tGPI-mutin	Bacteria Virus Fungus Parasite	Cell surface
TLR3	dsRNA	Bacteria	Intracellular vesicles
TLR4	LPS Structural protein Mannan glycoinositolphospholipids	Bacteria Virus Fungus Parasite	Cell surface
TLR5	Flagellin	Bacteria	Cell surface
TLR6	Lipoproteins zymosan, β-glucan	Bacteria Fungus	Cell surface
TLR7	ssRNA	Bacteria Virus Fungus	Intracellular vesicles
TLR8	ssRNA	Virus	Intracellular vesicles
TLR9	CpG DNA Hemozoin	Bacteria Virus Fungus Parasite	Intracellular vesicles
TLR11	Profilin-like molecule	Parasite	Cell surface Intracellular vesicles

Both *in-vitro* and *in-vivo* studies have suggested that TLRs are important in the immune response to BV although which TLRs and the exact mechanism through which they contribute to BV development has yet to be determined.

In healthy vaginas, Lactobacilli may selectively inhibit the inflammatory response in a similar way to commensal bacteria of the gastrointestinal tract [31]. A recent study showed that probiotic Lactobacilli suppressed expression of inflammatory genes along the nuclear factor-kappa β (NFκB) signaling pathway including TLR2 and TLR6 but may induce IL1-α and IL-1β possibly via an alternate signaling pathway to NFκB, indicating selective inhibition of a inflammatory response [32]. Low levels of cytokines and inflammation characteristic of healthy vaginas could help foster beneficial flora [31, 33, 34]. Furthermore, an *in-vitro* study showed that cell lines from the lower female genital tract had less diverse and lower levels of cytokines, including IL-8, macrophage colony-stimulating factor (M-CSF) and transforming growth factor β (TGFβ) than the upper genital tract, suggesting that the former ismay be more tolerant of beneficial bacterial flora that can, in turn, provide protection against pathogens [31, 35].

In contrast, women with BV have been shown to have increased levels of inflammatory cytokines in the genital tract and TLRs have been identified as one potential pathway for this cytokine response [36-40]. Specifically, a TLR2-mediated immune response has been detected in human monocytic cells after *in vitro* exposure to cervicovaginal lavages from women with BV [39]. A separate study found that the pro-inflammatory response, particularly increases in IL-8, to short chain fatty acids (produced by BV-associated pathogens) in peripheral blood mononuclear cells (PBMCs) and neutrophils was enhanced by TLR2 and TLR7 ligands [41].

One hypothesis regarding the innate immune system's contribution to the development of BV is that healthy vaginal flora may serve to modestly activate TLRs which in turn inhibit BV-associated bacteria [25]. This theory hypothesizes that inhibition of TLRs with a consequent reduced innate host response, could allow BV-associated pathogens to proliferate in the genital tract [25]. In support of this hypothesis, unsaturated fatty acids, which can be produced by BV associated bacteria, have been shown to mediate *in vitro* inhibition of TLR2 and TLR4 activation in murine cells [42]; however other studies have

found TLRs mediate a host response to BV [39, 41]. Furthermore, fatty acids that do inhibit TLR2 and TLR4 have been found to be of dietary origin and not produced by BV-associated bacteria [42]. Thus, the causal link between inhibition of TLR responses and proliferation of BV-associated bacteria is still unclear. Longitudinal clinical studies may help clarify whether suppression of the host innate response is a critical event that leads to proliferation of BV-associated bacteria.

While studies suggest that TLRs may be important in the host response to BV, it is unclear what role the innate immune system plays in the shift from healthy flora to flora characteristic of bacterial vaginosis. One avenue to further substantiate the role of TLRs in innate immune response to BV is to evaluate association of TLR polymorphisms to BV susceptibility and outcomes. Several studies have assessed polymorphisms in the TLR genes and their association with BV. Notably, a recent study of single nucleotide polymorphisms (SNPs) in the genes for TLR1, TLR2, TLR4, and TLR9 were found to be associated with an increased risk of BV among HIV-1 infected African-American adolescents [43]. Prior to these findings, several studies were unable to find a significant association between BV occurrence and TLR polymorphisms [44-46]. However, *TLR1* and *TLR4* polymorphisms have also been specifically associated with levels of the BV-associated pathogens *Atopobium vaginae and Gardnerella vaginalis,* respectively [44, 46]. In this study we aimed to test the hypothesis that SNPs in *TLR2*, *TLR3*, *TLR4*, *TLR7*, *TLR8*, *TLR9* and associated pathway genes *MYD88, TIRAP and ACAA1* are associated with BV occurrence in longitudinal follow-up of HIV-1 infected and uninfected African women.

METHODS

Study population

Randomized Control Trial

Participants were from a cohort of African HIV-1 serodiscordant couples recruited for a randomized control trial (RCT) that took place at 14 sites across seven Southern and East

African countries: Uganda, Kenya, Rwanda, Tanzania, Zambia, Botswana, and South Africa. This RCT (the Partners in Prevention HSV/HIV Transmission Study) recruited approximately 3,400 heterosexual couples in which one partner was HIV-1 seropositive and dually infected with herpes simplex virus type 2 (HSV-2) with CD4 count ≥ 250 cells/mm^3 and the other partner was HIV-1 seronegative. The study evaluated the efficacy of HSV-2 suppression with acyclovir provided to the HIV-1/HSV-2 infected partner to prevent HIV-1 transmission from them to their HIV-1 uninfected sexual partner. Couples were followed for up to 24 months and provided with HIV-1 prevention services including counseling, sexually transmitted infection (STI) assessment and treatment, and free condoms HIV-1 infected individuals whose disease progressed to meeting local criteria for antiretroviral treatment (ART) were provided this through the study[47].

Nested case control study

A nested case-control study had been previously performed among participants in the Partners in Prevention HSV/HIV Transmission Study to evaluate TLR associations with HIV-1 outcomes in 847 HIV-1 exposed seronegative (HESN) and their HIV-1 infected partners. Couples with HIV-1 transmission were matched to couples without transmission based on factors that contribute most strongly to HIV-1 exposure [48] HESN individuals with the highest levels of HIV-1 exposure were also included (R. Mackelprang and J. Lingappa, manuscript submitted). In this context, genotyping of TLRs and other host genes was then performed on this subset of participants including HIV-1 uninfected, HIV-1 seroprevalent and HIV-1 seroconverting participants (Figure 1).

BV Analysis

This analysis included 392 women who had both TLR genotyping data and BV assessed during the RCT.

Human subjects research was approved at the University of Washington, all affiliated institutions, and at each local study site. Participants provided written informed consent.

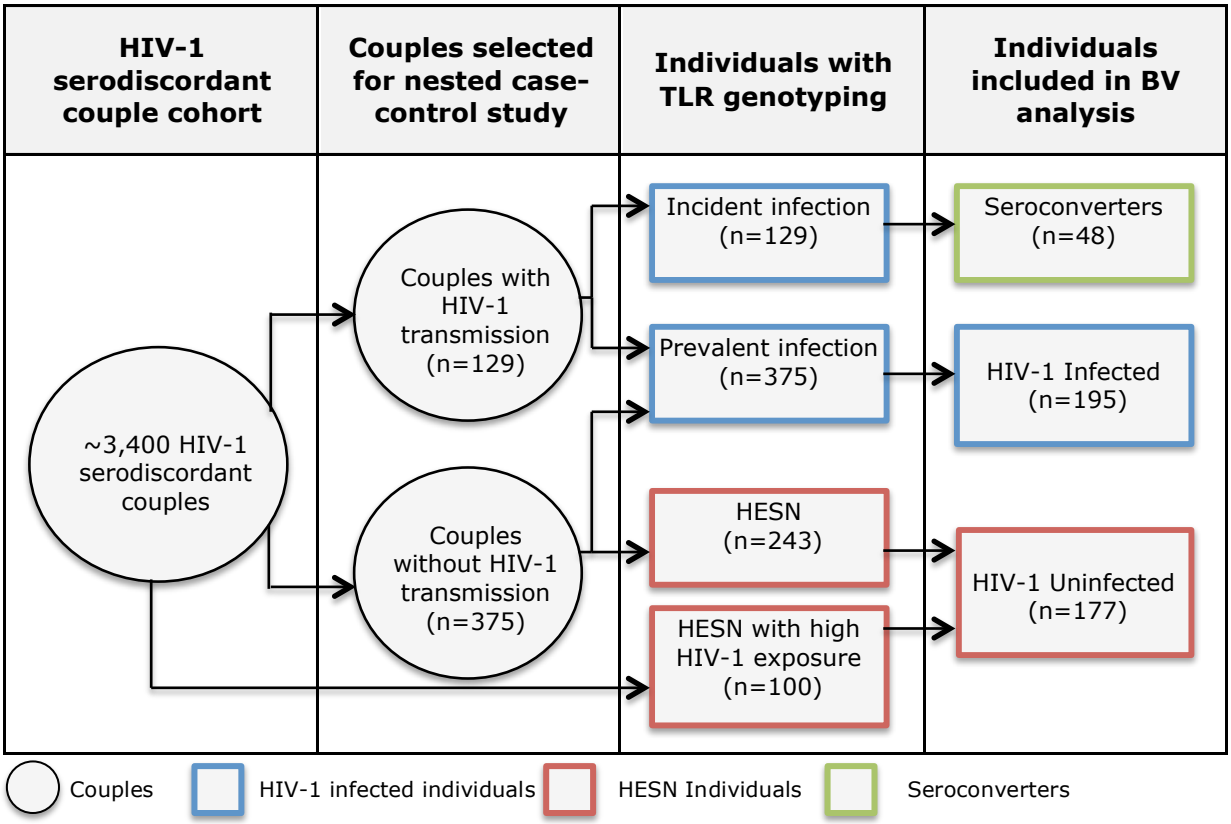


Figure 1. Sample selection for nested case-control study of HIV-1 infected cases and HESN controls from HIV-1 serodiscordant couples and subsequent selection of individuals with BV previously assessed for current analysis.

HIV-1 detection and classification

Participants in the HSV-HIV Transmission Study were screened for HIV-1 prior to enrollment using an HIV-1 enzyme immunoassay (EIA) as described elsewhere [49]. At the time of enrollment, 195 women ultimately included in this analysis were HIV-1 infected; 177 women were HIV-1 uninfected. HIV-1 uninfected participants received HIV-1 testing at

quarterly study visits and 48 acquired HIV-1 during follow-up. In our analyses HIV-1 status for these seroconverters was determined at the time of first BV diagnosis: five women seroconverted before BV diagnosis (classified as HIV-1 infected), 27 seroconverted after BV diagnosis (classified as HIV-1 uninfected) and 16 did not develop BV (classified as HIV-1 infected) (Table 2).

Table 2. HIV-1 classification for seroconverters included in this analysis based on date of seroconversion and date of first BV diagnosis

N	HIV-1 Seroconversion date relative to BV diagnosis date:	HIV-1 classification in this analysis:
5	Before BV diagnosis	HIV-1 Infected
27	After BV diagnosis	HIV-1 Uninfected
16	No BV diagnosis	HIV-1 Infected

Bacterial Vaginosis evaluation

During the HSV/HIV Transmission Study, vaginal swabs were collected at enrollment and quarterly follow-up visits. BV classification was determined by Nugent's criteria, as described above.

Vaginal swabs collected from participants were rolled onto glass slides, dried, and methanol fixed at the respective study site. Gram staining and evaluation were performed for all slides at the Center for Microbiology Research Laboratory at the Kenya Medical Research Institute (KEMRI), with all slides double-read by two technologists [7].

Recognizing the reduced sample size associated with women who had normal flora, in this study women were classified as having BV with a Nugent's score of 7 to 10 and lacking BV with a Nugent's score of 0 to 6. However, a secondary analysis also evaluated

the impact on effect sizes when the comparison group was limited to those with normal flora (Nugent score of 0 to 3)

TLR selection and genotyping

Venous blood archived during the RCT was used to provide isolated DNA via the Puregene DNA purification system (Qiagen, Valencia, CA). TLR SNPs were genotyped using the Illumina Custom Oligo Polled Assay (OPA). Genotyping was completed for 124 SNPs in *TLR2* (n=9), *TLR3* (n=13), *TLR4* (n=22), *TLR7* (n=40), *TLR8* (n=25), *TLR9* (n=3), *MYD88* (n=4) and *TIRAP* (n=8); 117 of these are haplotype-tagging SNPs that were chosen to represent common variation across the genes. Haplotypes were inferred from the Yoruba HapMap population and SNPs tagging each haplotype with >5% frequency were selected as tagSNPs (Figure 2) (R. Mackelprang and J. Lingappa, manuscript submitted). The remaining 7 SNPs are candidate SNPs that have previously been associated with BV or HIV outcomes (Table 3).

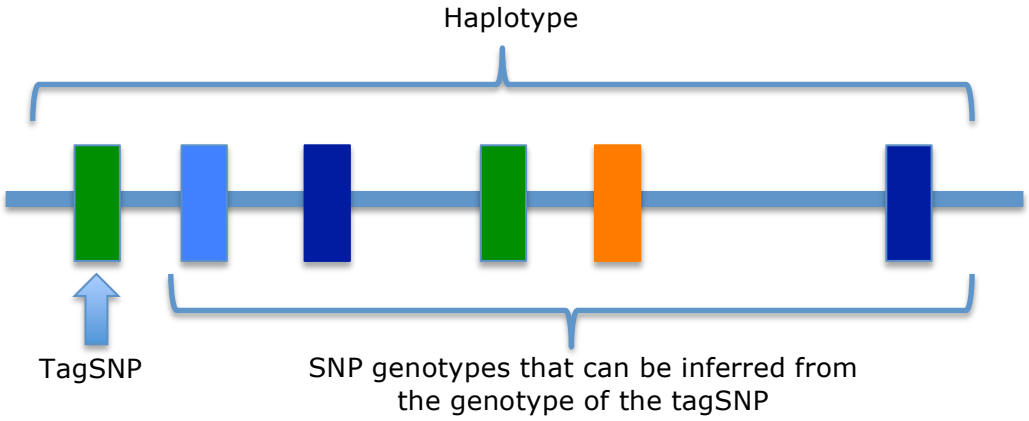


Figure 2. Schematic for haplotype tagging SNP. Haplotypes are defined by the non-random association of sets of SNPs in a specific population and commonly interpreted as evidence of ancestral linkage of SNPs within that population. By genotyping a single tagSNP, the genotypes of the remaining SNPs in that haplotype may be inferred based on that non-random association.

SNPs were excluded from all downstream analyses if the call rate was <95% (n=7), the minor allele frequency (MAF) was <5% (n=19), or if Hardy-Weinberg Equilibrium was not met (n=0). A total of 25 SNPs were excluded (24 tagSNPs and 1 candidate SNP) for a total of 99 SNPs included in the analyses.

Table 3. Candidate SNPs included in the analysis.

rs Number	Position	Gene	Gene Region	Amino Acid Change	Chromosome	Major Allele	Minor Allele	Reason for Candidate Inclusion
rs1898830	154827902	*TLR2*	Intron	Not applicable	4	A	G	Associated with BV (OR = 1.86) based on Amsel criteria [43]
rs3804099	154844105	*TLR2*	Synonymous	N199N	4	C	T	Associated with difference in HIV set-point (prevalent cases): mean difference = -0.42 [b]
rs3804100	154844858	*TLR2*	Synonymous	S450S	4	T	C	Associated with difference in HIV set-point (all cases): mean difference = 0.34 [b]
rs4986791	119515422	*TLR4*	Missense	T399I	9	C	T	Associated with BV (OR = 1.34) based on Nugent & Amsel criteria[a] [43]
rs179012	12811482	*TLR9*	Intron	Not applicable	X	A	G	Associated with difference in HIV set-point (all cases): Mean difference = -0.37 [b]
rs187084	52236070	*TLR9*	Upstream (5') of gene	Not applicable	3	A	G	Associated with BV (OR = 1.52) based on Nugent & Amsel criteria [43]
rs352140	52231736	*TLR9*	Synonymous	P545P	3	C	T	Associated with BV (OR = 1.24) based on Nugent & Amsel criteria [43]

a. rs4986790 was associated with BV risk
b. R. Mackelprang and J. Lingappa, manuscript submitted

Sample exclusions

Genotyping data was available for 392 women who had BV assessed during follow-up. Women were excluded from downstream analyses if their reported sex did not match genotypic sex as determined by heterozygosity of X chromosome SNPs included in genotyping (n=9), if they exhibited genotypic missingness >10% (n=4), or exhibited relatedness to participants as determined by Identity by State (IBS) >95% (n=7). A total of 20 women were excluded for a total of 372 women included in the analyses.

Statistical Analysis

1. Logistic Regression Model

Analysis of TLR polymorphisms association with BV was performed using logistic regression and stratified by HIV-1 status at the time of BV diagnosis –essentially comparing women who never had BV to those who ever had BV. The decision to stratify by HIV-1 was based on the fact that HIV-1 has been shown to interact with TLRs during the HIV-1 infection process implying that HIV-1 itself could modify the effect of TLRs on BV[50].

BV cases were women who had any visit with BV (Nugent's score = 7-10) at any point during follow-up; controls had normal or intermediate flora (Nugent's score = 0-6) for all follow-up visits. Logistic regression models were adjusted for population stratification using three EIGENSTRAT eigenvectors from principal component decomposition performed in a previous GWAS in this study population [51, 52]. Likelihood ratio testing was used to determine the ability of the logistic regression model to fit the data using genotype for each SNP. We report likelihood ratio testing p-values for all logistic regression analyses. Confidence intervals are calculated using Wald tests, therefore they may not reflect the significance of the p-value.

2. Generalized Estimating Equation (GEE) Model

A second analysis of TLR polymorphisms association with BV was performed using a GEE model to utilize the correlation of BV data across multiple visits for each woman. The GEE model allows for the assessment of the probability of BV at any visit. GEE models were also stratified by HIV-1 status at the time of BV diagnosis and adjusted for principal components.

Both logistic regression and GEE models were adjusted only for principal components. No other adjustments were made given that factors directly affecting the risk of BV would not also affect the genotype of an individual; as such, there are no true confounders in the model that require adjustment [53]. Furthermore, factors that may be in the causal pathway between TLR variation and BV occurrence will not be adjusted for in this analysis. For example, if TLR gene variants affect HIV-1 viral load (effect mediator), which also affects susceptibility to BV, controlling for viral load would bias the results of the effect of TLR on BV towards the null (Figure 3). Instead, we are interested in the *total effect* of TLR gene variants on BV occurrence, including effects through mediating pathways, so potential mediators will not be adjusted for in this analysis [54].

Figure 3. Schematic of TLR SNP association with BV including potential effect mediators (ex. HIV-1 viral load) that lie within the causal pathway.

TagSNP associations were corrected for multiple comparisons using a Bonferroni adjustment based on N=99 SNP comparisons: at the $\alpha = 0.05$ level, adjusted p-values ≤

0.0005 were considered significant. All analyses assumed a dominant model of inheritance and were performed in R utilizing the GenABEL and geepack packages[55, 56].

RESULTS

Study participants

Among 372 women included in the analyses, 216 (58%) were classified as HIV-1 infected (Table 4); 165 (76%) HIV-1 infected women were classified as BV cases with at least one BV visit and 51 (24%) were classified as controls with no BV during follow-up. HIV-1 infected BV controls were more likely than cases to be East African (84% versus 69%) and have higher CD4+ count at study enrollment (520 versus 434 cells/mm^3). HIV-1 infected cases and controls were similar in age (29 years), had similar plasma HIV-1 RNA (4.7 versus 4.4 $\log_{10}$ at enrollment) and HSV-2 prevalence (99% versus 92%). Participants who were HIV-1 infected upon enrollment of the HSV/HIV Prevention Study were randomized to acyclovir at similar rates among BV cases and controls (51% versus 50%).

The remaining 156 (42%) women were classified as HIV-1 uninfected; 105 (67%) of which were classified as BV cases and 51 (33%) as BV controls. More HIV-1 uninfected cases than controls were East African (86% versus 78%) although cases and controls were of similar age (27 versus 28) and had similar prevalence of HSV-2 (92% versus 90%). Forty-eight women acquired HIV-1 during follow-up; 35 (73%) seroconverters had BV during follow-up, 27 (56%) had BV before HIV-1 seroconversion and 8 (17%) developed BV after HIV-1 seroconversion.

Table 4. Description of study participants by HIV and BV status.

	HIV-1 Infected[b] *(n =216)*		*HIV-1 Uninfected (n =156)*		*Total* (n =372)
	BV (n=165)	***No BV n=(51)***	***BV (n=105)***	***No BV (n=51)***	
Age, years	29 (24, 34)	29 (25, 33)	27 (22, 31)	28 (24, 33)	28 (24, 33)
East African	114 (69%)	43 (84%)	90 (86%)	40 (78%)	287 (77%)
CD4+ Count (enrollment;cells/mm^3)	434 (330, 575)	520 (408, 755)	-	-	445 (334, 604)
Plasma HIV-1 RNA (enrollment; log_{10})	4.7 (4.0, 5.1)	4.4 (3.9, 4.9)	-	-	4.7 (4.0, 5.0)
Plasma HIV-1 RNA (set point; log_{10})	4.7 (4.2, 5.1)	4.5 (3.9, 5.0)	-	-	4.6 (4.0, 5.1)
Randomized to Acyclovir[c]	80 (51%)	19 (50%)	-	-	99 (51%)
HSV-2 Infected[b]	163 (99%)	47 (92%)	97 (92%)	46 (90%)	353 (95%)
Follow-up time, days	714 (450, 738)	546 (446, 720)	696 (494, 816)	545 (322, 720)	638 (450, 726)

a. Numbers (%) are provided for categorical variables and medians (inter-quartile ranges) are provided for continuous variables
b. HIV-1, HSV-2 infected prior to first BV diagnosis
c. Randomization for participants who entered RCT as HIV-1 infected only

TLR associations with BV

Logistic regression model results

Among HIV-1 infected individuals, the candidate SNP *TLR2* 816 C/T (rs3804099) was associated with a reduced risk of having BV during follow-up (Odds Ratio [OR] = 0.81, 95% Confidence Interval (CI): 0.21, 3.17; p = 0.01); this is a synonymous SNP located in exon 3 of the *TLR2* gene (Table 5).

Among HIV-1 uninfected individuals, two *TLR7* tagSNPs were found to be associated with a reduced risk of BV and a *TLR7* candidate SNP was associated with an increased risk of BV. The intronic tagSNP *TLR7* rs5743737 was associated with a decrease in BV risk and retained significance after Bonferroni correction (OR= 0.32, 95% CI: 0.28, 0.38; $p_{corrected}$=0.005). Among women with the *TLR7* rs5743737 SNP variant, 30% developed BV compared to 74% of women with the wild-type allele (Figure 4). Similarly, the intronic

tagSNP *TLR7* rs1634323 was associated with a decreased risk of BV and retained significance after Bonferroni correction (OR= 0.41, 95% CI: 0.33, 0.51; $p_{corrected}$ =0.01); 42% of women with the *TLR7* rs1634323 SNP variant developed BV compared to 75% with the wild-type allele. The intronic candidate SNP *TLR7* rs179012, previously associated with a decrease in HIV-1 plasma RNA set-point, was found to be associated with an increased risk of BV (OR=1.65, 95% CI: 0.23, 11.78; p = 0.04). Among women with the TLR7 rs179012 SNP variant, 78% developed BV during follow-up compared to 62% of women who carried the wild-type allele.

TLR7 rs5743737 and *TLR7* rs1634323 are in moderate linkage disequilibrium (R^2=0.61) but neither are in linkage disequilibrium with *TLR7* rs179012 (both R^2<0.001) (Figure 5)

Table 5. Associations between TLR SNPs and BV occurrence as assessed by logistic regression

			Number of BV cases by genotype(% of all cases)			
Gene Variant	**Cases**	**MAF**	***WT***	**SNP**		
***TLR2* - rs1898830 Intron (A/G) [Candidate SNP]**		G	*AA*	*AG/GG*	*OR*	*LR p-value*
	All	0.09	227 (74%)	43 (65%)	0.75	0.14
	HIV-1 Infected		139 (79%)	26 (65%)	0.72	0.09
	HIV-1 Uninfected		88 (68%)	17 (65%)	0.78	0.46
***TLR2* - rs3804099 Exon 3 (C/T) Synonymous [Candidate SNP]**		T	*CC*	*CT/TT*		
	All	0.29	**148 (78%)**	**122 (67%)**	**0.76**	**0.02**
	HIV-1 Infected		**94 (84%)**	**71 (68%)**	**0.81**	**0.01**
	HIV-1 Uninfected		54 (70%)	51 (65%)	0.76	0.54
***TLR7* - rs179012 Intron (A/G) [Candidate SNP]**		G	*AA*	*AG/GG*		
	All	0.22	165 (73%)	105 (72%)	1.01	0.77
	HIV-1 Infected		102 (82%)	63 (68%)	0.76	0.10
	HIV-1 Uninfected		**63 (62%)**	**42 (78%)**	**1.65**	**0.03**
***TLR7* - rs1634323 Intron (A/G) [TagSNP]**		G	*AA*	*AG/GG*		
	All	0.13	211 (75%)	59 (65%)	0.71	0.09
	HIV-1 Infected		122 (75%)	43 (81%)	1.28	0.21
	HIV-1 Uninfected		**89 (75%)**	**16 (42%)**	**0.41**	**0.0001***
***TLR7* - rs5743737 Intron (A/G) [TagSNP]**		G	*AA*	*AG/GG*		
	All	0.08	236 (75%)	34 (58%)	0.52	0.006
	HIV-1 Infected		138 (77%)	27 (75%)	0.86	0.92
	HIV-1 Uninfected		**98 (74%)**	**7 (30%)**	**0.32**	**$5x10^{-5}$ ***

** Retain significance after Bonferonni correction.*

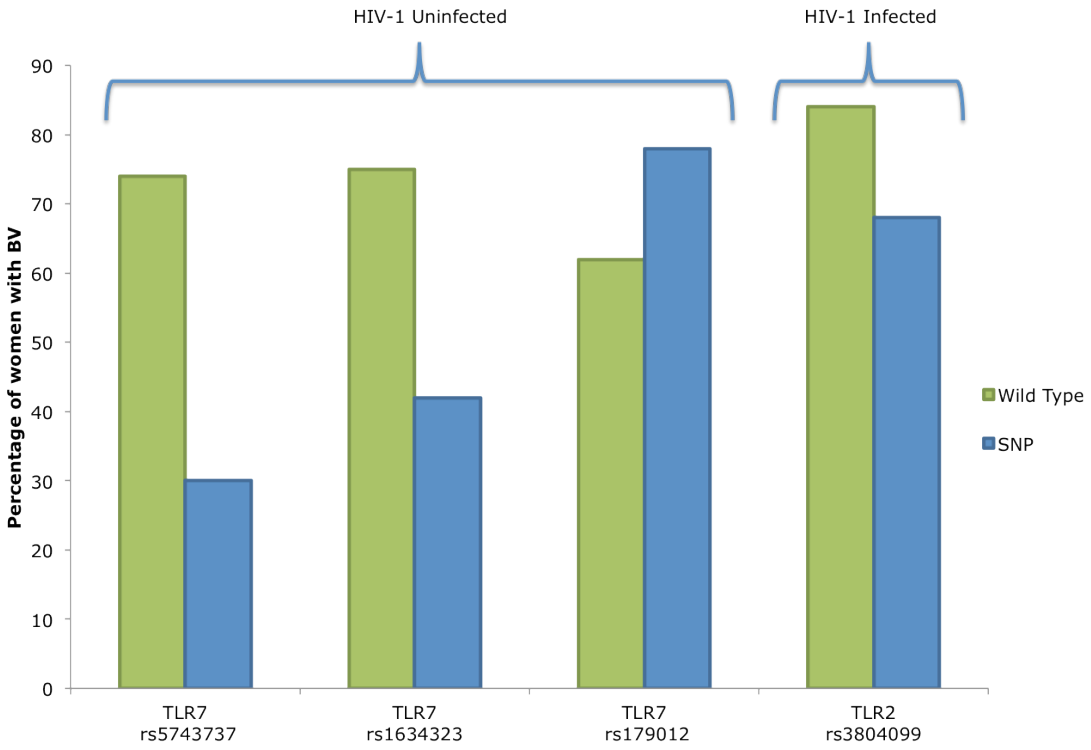


Figure 4. Percentage of women with BV by SNP status. Data shown for SNPs found to be significant in the Logistic Regression model.

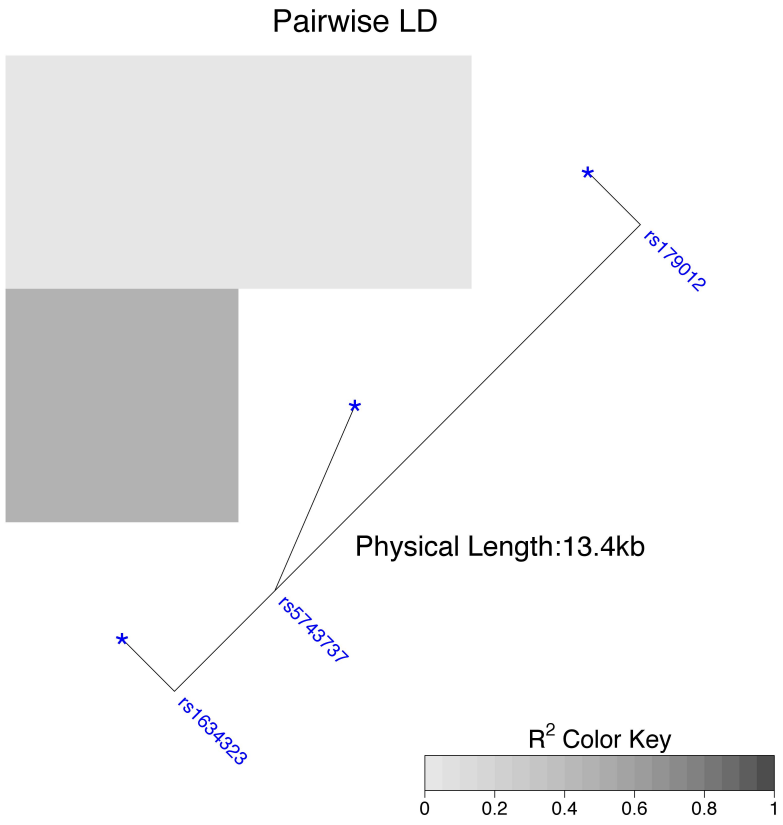


Figure 5. Pairwise linkage disequilbrium (R^2) for TLR7 SNPs found to be significant in logistic regression analysis of TLR associations with BV. $R^2 = 1$ indicates that one loci perfectly correlates with the other loci.

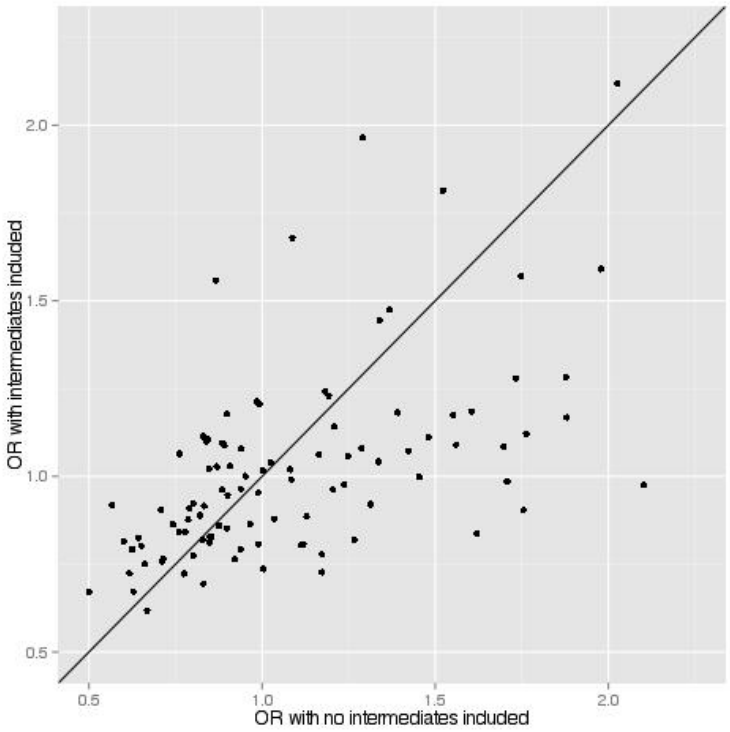
A secondary analysis using logistic regression evaluated the impact on effect sizes when the BV control group was limited to those with normal flora at all visits (Nugent score of 0 to 3). Odds Ratios were similar for the primary analysis including controls with intermediate and normal flora (Nugent score of 0 to 6) and the analysis utilizing controls with only normal flora (Table 6; Figure 6). Significance levels between analyses differed, likely due to fewer women included in the secondary analysis of women with only normal flora (Table 7). Nearly half of all women included as controls in the primary analysis were excluded in the secondary analysis because they had at least one visit with intermediate flora.

Table 6. TLR associations with BV assessed by logistic regression; OR for analysis where controls included women with intermediate vaginal flora versus OR for analysis where controls included women with only normal vaginal flora.

Gene Variant	*Results including intermediates*		*Results omitting intermediates*	
	OR	***p-value[a]***	***OR***	***p-value[a]***
HIV-1 Infected				
TLR2 - rs3804099 Exon 3 (C/T) Synonymous [Candidate SNP]	0.81	0.01	0.85	0.08
HIV-1 Uninfected				
TLR7 - rs5743737 Intron (A/G) [TagSNP]	0.32	$5x10^{-5}$[b]	0.35	0.001
TLR7 - rs1634323 Intron (A/G) [TagSNP]	0.41	0.0001[b]	0.51	0.003
TLR7 - rs179012 Intron (A/G) [Candidate SNP]	1.65	0.04	1.70	0.03

a. Unadjusted p-values reported. p-values <0.05 considered significant for candidate SNPs; p-values<0.0005 considered significant for tagSNPs.
b. Retain significance after Bonferroni correction.

A. HIV-1 infected



B. HIV-1 uninfected

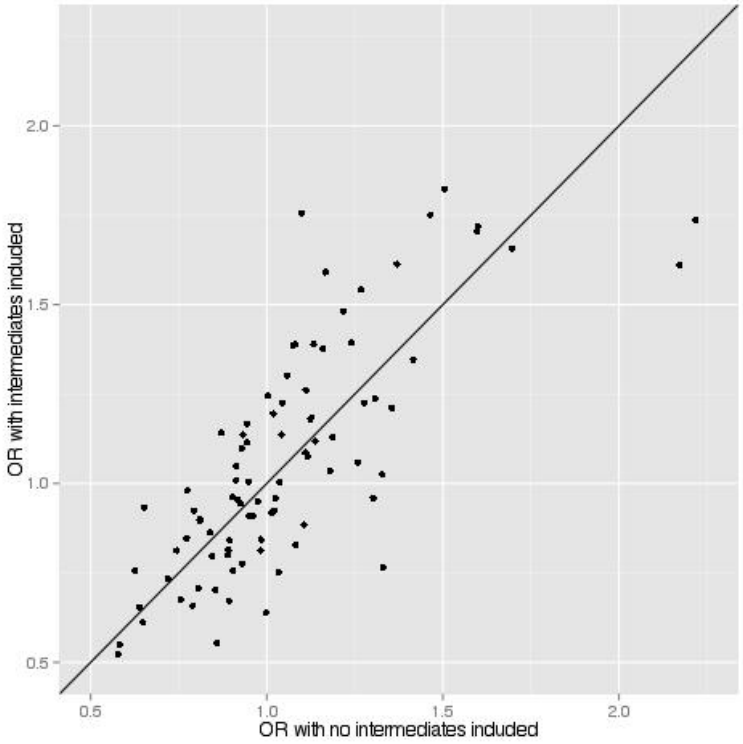


Figure 6. Odds ratio from logistic regression where controls included only women with normal vaginal flora versus odds ratio from logistic regression where controls included women with intermediate vaginal flora for A. HIV-1 infected individuals and B. HIV-1 uninfected individuals.

Table 7. Number of women included as controls with either normal or intermediate vaginal flora in the primary analysis and controls with only normal vaginal flora included in the secondary analysis.

	HIV-1 Infected	HIV-1 Uninfected	All Women
BV Controls - Primary analysis (Nugent's score 0-6)	51	51	102
BV Controls - Secondary analysis (Nugent's score 0-3)	22	32	48

GEE model results

The candidate SNP *TLR2* 15607 A/G (rs1898830) was found to be associated with a reduced risk of having BV in both HIV-1 infected (OR = 0.56, 95% CI: 0.35, 0.93; p = 0.02) and uninfected (OR = 0.41, 95% CI: 0.19, 0.87; p =0.02) women (Table 8). This *TLR2* SNP was previously associated with an increased risk of BV in African-Americans [43]. Among all women with this *TLR2* SNP variant, 27% of all follow-up visits were classified as BV compared to 41% of all visits for the women carrying the wild-type allele (OR=0.53, 95% CI: 0.35, 0.79; p=0.002) (Figure 7). *TLR2* 15607 A/G (rs1898830) is in low linkage disequilibrium with *TLR2* 816 C/T (rs3804099) that was associated with reduced risk of BV in the logistic regression model (R^2=0.35). The intronic candidate SNP *TLR7* rs179012 was found to be associated with an increased risk of BV (OR=1.70, 95% CI: 1.01, 2.87; p = 0.05) among HIV-1 uninfected women. Among all women with this *TLR7* SNP variant, 38%

of all follow-up visits were classified as BV compared to 31% of all visits for the women carrying the wild-type allele.

Table 8. Associations between TLR SNPs and BV incidence as assessed by GEE Model

Gene Variant	Cases	MAF	Number of BV visits by genotype (% of all visits)			
			WT	**SNP**		
***TLR2* - rs1898830 Intron (A/G) [Candidate SNP]**		G	*AA*	*AG/GG*	*OR*	*p-value*[a]
	All	0.09	**727 (41%)**	**109 (27%)**	**0.53**	**0.002**
	HIV-1 Infected		**461 (45%)**	**74 (31%)**	**0.56**	**0.02**
	HIV-1 Uninfected		**266 (36%)**	**35 (21%)**	**0.41**	**0.02**
***TLR7* - rs179012 Intron (A/G) [Candidate SNP]**		G	*AA*	*AG/GG*		
	All	0.22	**484 (37%)**	**352 (42%)**	**1.41**	**0.04**
	HIV-1 Infected		308 (42%)	227 (40%)	1.21	0.36
	HIV-1 Uninfected		**176 (31%)**	**125 (38%)**	**1.70**	**0.05**

a. Unadjusted p-values reported. p-values <0.05 considered significant for candidate SNPs; p-values<0.0005 considered significant for tagSNPs.

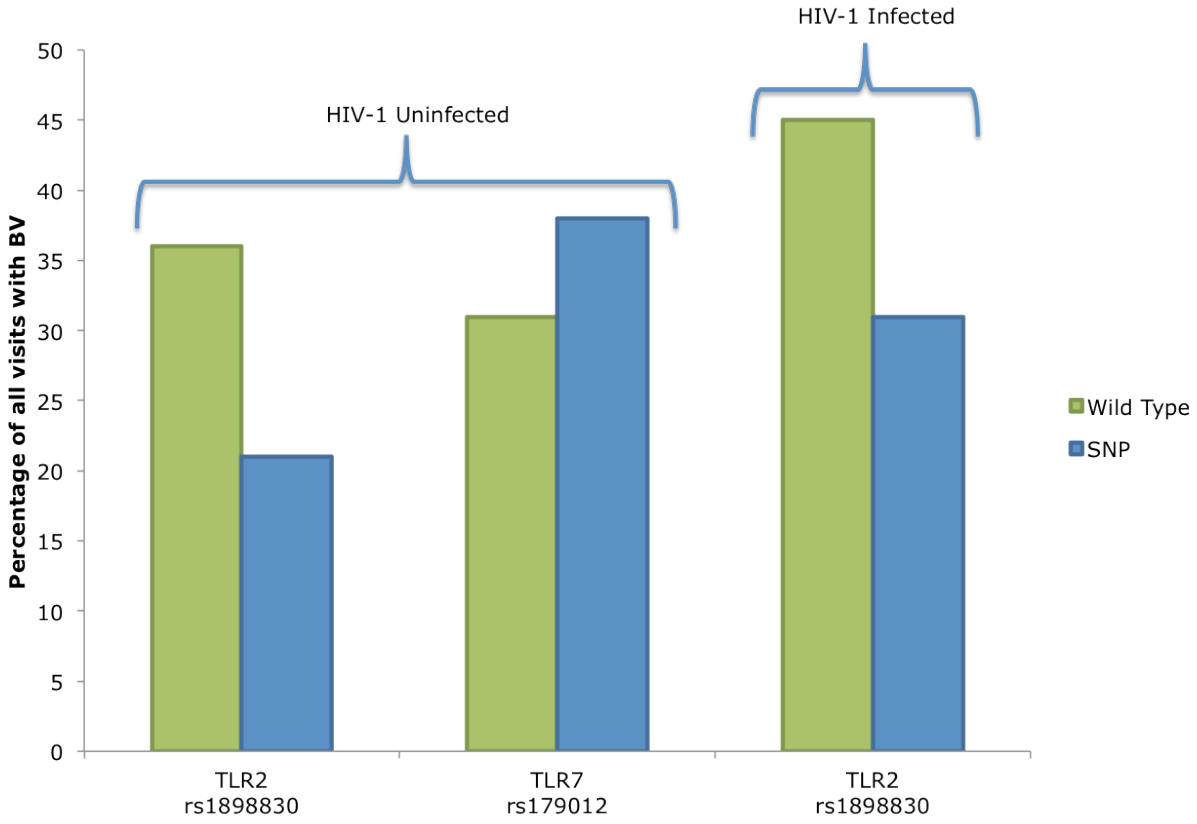


Figure 7. Percentage of total follow-up visits classified as BV by SNP status. Data shown for SNPs found to be significant in the GEE model.

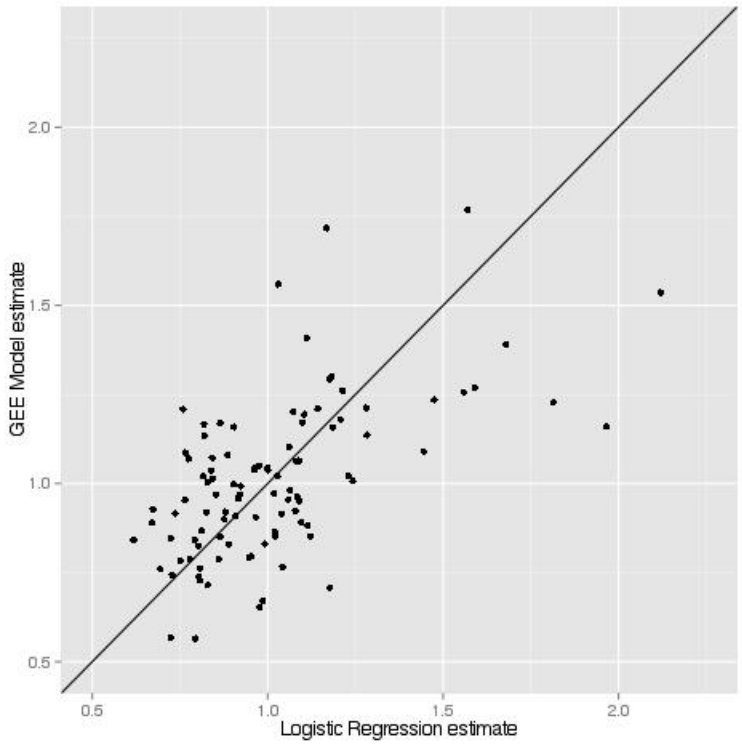
Results from the logistic regression and GEE models yielded similar risk estimates although the levels of significance varied between models (Table 9; Figure 8).

Table 9. Associations between TLR SNPs and BV as assessed by logistic regression and GEE models

Gene Variant	*Logistic Regression Results*		*GEE Model Results*	
	OR	***p-value*[a]**	***HR***	***p-value*[a]**
HIV-1 Infected				
TLR2 - rs1898830 Intron (A/G) [Candidate SNP]	0.72	0.09	**0.56**	**0.02**
TLR2 - rs3804099 Exon 3 (C/T) Synonymous [Candidate SNP]	**0.81**	**0.01**	0.87	0.48
HIV-1 Uninfected				
TLR7 - rs5743737 Intron (A/G) [TagSNP]	**0.32**	**$5x10^{-5}$**[b]	0.28	0.01
TLR7 - rs1634323 Intron (A/G) [TagSNP]	**0.41**	**0.0001**[b]	0.54	0.07
TLR7 - rs179012 Intron (A/G) [Candidate SNP]	**1.65**	**0.04**	**1.70**	**0.05**
TLR2 - rs1898830 Intron (A/G) [Candidate SNP]	0.78	0.46	**0.41**	**0.02**

a. Unadjusted p-values reported. p-values <0.05 considered significant for candidate SNPs; p-values<0.0005 considered significant for tagSNPs.
b. Retain significance after Bonferroni correction.

A. HIV-1 infected



B. HIV-1 uninfected

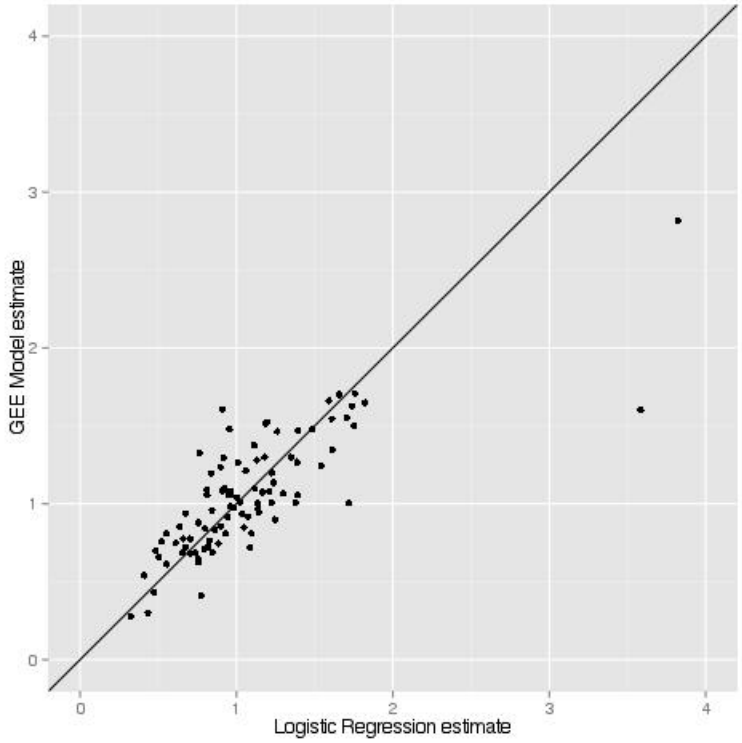


Figure 8. Risk estimate from logistic regression versus risk estimate from GEE model for A. HIV-1 infected individuals and B. HIV-1 uninfected individuals. SNPs that were found to be significant were clustered along the line.

DISCUSSION

Although the role of the innate immune system in the development and persistence of BV has not been entirely elucidated, TLRs have been identified as potentially important mediators. Genetic polymorphisms in the TLR genes could confer altered risk of BV but studies designed to assess this relationship have been limited and inconclusive. Our study is the first aimed to look at the association between TLR SNPs and BV in African women, who generally have high rates of BV, likely contributing to increased HIV-1 transmission and susceptibility. Furthermore, our study cohort allows us to evaluate the effect of TLR polymorphisms in the context of African women with and without HIV-1 infection and compare findings to a recent study evaluating TLR SNPs in HIV-1 infected African-Americans.

Our study found that SNPs in *TLR2* and *TLR7* may contribute to BV development in both HIV-1 infected and uninfected women and that these effects may be modified by HIV-1 status. In HIV-1 uninfected but not HIV-1 infected women, three *TLR7* SNPs were found to be associated with BV development. Two haplotype tagging *TLR7* SNPs were associated with a significant reduction in BV during study follow-up. *TLR7* rs5743737 was associated with ~70% reduction in risk of BV during follow-up (logistic regression: OR=0.32; $p_{corrected}$=0.005). Similarly, *TLR7* rs1634323 was associated with ~60% reduction in risk of BV during follow-up (logistic regression: OR=0.41; $p_{corrected}$=0.01). The third *TLR7* SNP rs179012 was associated with an increased risk of BV development (logistic regression: OR=1.65; p=0.04; GEE model: OR=1.70, 95% CI: 1.01, 2.87; p = 0.05). The *TLR7* variant (rs179012) was previously associated with lower plasma HIV-1 set-point in both seroprevalent and incident individuals in the same cohort (R. Mackelprang and J. Lingappa, manuscript submitted). Notably, no previous genetic epidemiology studies have evaluated the effect of TLR7 on BV development.

Differential associations of *TLR7* SNPs and BV in HIV-1 infected and uninfected individuals may be due to HIV-1 effects on endosomal and phagosomal pathways. Although typically associated with recognition of viral ssRNA, limited reports have found that TLR7 can recognize bacterial ssRNA in intracellular vesicles and induce interferon production [57-59]. Disruption of the phagosomal maturation pathway could inhibit this interferon response since TLR7-dependent bacterial recognition occurs late in the maturation pathway of these organelles [58]; HIV-1 has been shown to interfere with phagosome-lysosome fusion and recent studies have shown that HIV-1 trafficking to early endosomes could interfere with TLR7-mediated IFN- α response [50, 60]. If our hypothesis that TLR7 is associated with protection from BV in HIV-1 uninfected women is correct, then fact that HIV-1 disrupts TLR7-mediated immune response could explain the increased prevalence of BV in the context of HIV-1 infection (Figure 9).

Although the TLR7 agonist gardiquimod has been shown to have an *in vitro* anti-HIV effect through inhibition of virus production, likely through IFN- α induction [59], no studies have evaluated the impact of TLR7 agonists on development of BV. Based on our study results, we hypothesize that increased TLR7 activation will inhibit the growth of BV-associated bacteria while TLR inhibition would increase BV incidence [25].

Among HIV-1 infected women, the synonymous *TLR2* SNP 816 C/T located in exon 3 (rs3804099) was associated with a decreased risk of BV development during study follow-up (logistic regression: OR=0.81; p=0.01). The *TLR2* 816 C/T SNP was previously associated with a lower plasma HIV-1 set-point in seroprevalent African individuals from the same cohort (R. Mackelprang and J. Lingappa, manuscript submitted). Other studies have found that low CD4+ cell count is associated with an increased risk of BV [61, 62]; the association between low CD4+ cell count and high HIV-1 viral load [63] supports the idea that a TLR polymorphism that contributes to a decreased HIV-1 viral load, and hence a higher CD4+ count, could also be protective against BV. It should be noted, however, that

one study found HIV-1 infected East African women with BV had higher CD4+ cell count than women without BV [64]. Better understanding of the relationship between HIV-1 viral load and CD4+ cell count with BV is needed to understand how TLRs ultimately affect the development of BV.

Our data also show that the intronic *TLR2* SNP 15607 A/G (rs1898830) was associated with a decreased risk of BV in both HIV-1 infected and uninfected individuals (GEE model: OR=0.53, 95% CI: 0.35, 0.79; p=0.002). Other studies have supported the idea that TLR2 is an important mediator in the host response to BV [38, 39]. For example, *TLR2* 15607 A/G was found among African-American HIV-1 infected adolescents to be associated with a nearly two-fold increased risk of BV based on Amsel criteria, a clinical scoring system for BV [43]. The discrepancy in the risk estimates may be related to the effect of *TLR2* 15607 A/G on HSV-2 shedding; a study found that *TLR2* 15607 A/G was associated with both increased HSV-2 shedding and lesions in HIV-1 uninfected Caucasians [65]. The cohort in the Royse, et al. study had low levels of HSV-2 infection (11%) while nearly 95% of our study participants were HSV-2 infected. The difference in HSV-2 status between the two study cohorts may also contribute to our inability to confirm other TLR SNP associations that Royse et al. found to be significant. The interactions between TLR2, HSV-2, HIV-1 and BV are complex and warrant further research to understand the underlying biological mechanism that is at play (Figure 9).

There are several limitations to our study. First, study participants were selected by convenience sampling from two previous studies where data on BV occurrence and TLR genotyping existed. This method of sampling limits the generalizability of our findings and may introduce bias in our analyses. In particular, HIV-1 uninfected participants were HIV exposed seronegative, and could be expected to have a different immune profile than unexposed women. The high prevalence of HSV-2 in our study participants could also contribute to results that cannot be generalized to the general population. Although generalizability may be poor, this study includes women who are at high risk of BV - HIV-1

and HSV-2 infected, African women - and for whom studies aiming to understand the pathology and/or treatment of BV is critically important. Next, it is important to consider that tagSNPs used in this study are not necessarily causal SNPs and the biological mechanism underlying this association with BV cannot be defined based on this analysis. Further studies should be conducted that aim to understand the functionality of the SNP on the receptor signaling and cytokine release and to validate its effect on BV occurrence.

Our findings indicate that TLR gene variants may differentially contribute to BV occurrence in HIV-1 infected and uninfected women. Specifically, TLR2 may be important in providing protection against BV in both HIV-1 infected and uninfected women but more research is needed to understand the complex contribution of TLR2 to the immune response to BV, HSV-2 and HIV-1. Furthermore, TLR7 gene variants appear to be important in providing protection against BV in HIV uninfected women. These findings suggest that TLR7 could provide a potential target for treatment or prevention of BV.

Confirmatory research to understand how these TLR gene variants contribute to the development of BV will help provide information that can be used for prevention strategies or therapeutics. The significant health sequelae of BV and its contribution to the burden of STIs warrant research that can lead to a reduction of BV in women around the globe.

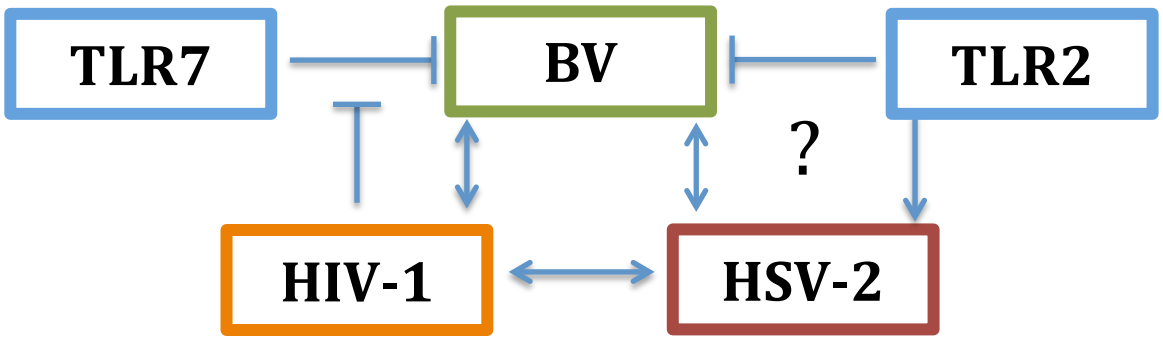


Figure 9. Schematic of the interactions between TLRs, BV, HIV-1 and HSV-2

Ethical Analysis

Secondary genetic research uses previously collected data to assess additional genetic associations which may be different than those outlined in the original research questions. Regulation of secondary research is limited beyond de-identification of samples and observance of any explicit restrictions outlined in the original informed consent, making it a popular mechanism to generate more research from data already available [66]. We have now seen how harms can be caused by secondary genetic research; notably, the Havasupai Indians in Arizona felt wronged when genetic samples originally collected to study diabetes were used to assess genetic susceptibility to schizophrenia, suggested inbreeding, and made claims about their people's origins [67]. Furthermore, research has shown that secondary genetic analyses, even when following regulation for use of genetic samples, can produce results that are potentially harmful or stigmatizing to a population[66]. These concerns may be heightened in low-income, or historically disadvantaged, populations or within developing nations where researchers from more affluent countries are performing the research. In particular, research that has focused on African populations has been criticized as career enhancers for researchers in affluent countries without considerable benefit to African people. In order to protect less affluent populations from potential harm and lack of benefit, some scholars and political advocates have introduced the concept of genomic sovereignty[68-70]. Genomic sovereignty is the idea that a person, group or nation can own their genetic material and control how it is disseminated and used [70].

Declaration of genomic sovereignty could prevent potentially harmful results of secondary genetic analyses by embargoing genetic material from developing countries to more affluent countries. Limiting access to a nation's genetic materials would certainly be a way to protect from harms as a result of secondary research. However, at the same time, a nation declaring genomic sovereignty is only addressing the issue of ownership, insufficient to truly advance equitable and beneficial research that will reduce disparities[68]. This

analysis explores the potential consequences of genomic sovereignty and the obligations of researchers from developed countries to offer the same benefits that genomic sovereignty aims to provide, particularly in the context of secondary genetic research.

Consequences of declaring genomic sovereignty

Declaration of genomic sovereignty would give developing countries greater leverage to set terms under which foreign researchers would be required to abide. This could ultimately mean less genetic research as many developing countries lack the infrastructure and technology to perform effective genetic studies without the support of more affluent countries. Controlling how genomic material can be used, both in primary and secondary genetic research, means that African leaders could dictate that research be done only on diseases that are most debilitating to the African people. More highly focused research could provide increased direct benefit to the populations that have had genomic sovereignty declared. While there may be more power to control what genetic research is being conducted, overall research may be diminished, as affluent researchers may no longer have incentive or access to conduct research in these populations. Decreased research and strained relationships between researchers in developing and developed countries is far more harmful to the former than the latter. The intent to help more African people by conducting more targeted research could be all but lost in declaring genomic sovereignty if not managed well.

Conversely, maintaining the status quo of genetic research would perpetuate a trend where research may happen in developing countries but at the expense of the needs of African countries. The African people and researchers would not receive the same direct benefit as they would if Africans entirely dictated the terms of the research; it may not be relevant or provide investment in capacity building. Health disparities would continue to grow if the research was not aimed at reducing the disease burden of the African people and instead followed the research and professional agenda of researchers from affluent

countries. Ultimately, when considering consequences alone, it does not appear to be advantageous for developing countries to either adopt genomic sovereignty or not. Further ethical guidance is needed to find a more just way forward.

Obligations of researchers from developed countries

As outlined by de Vries and Pepper (2012), genomic sovereignty is insufficient to ensure just and equitable genetic research [68]. Other values and processes must be enacted to ensure that all stakeholders are receiving equal benefits and harms. As such, researchers from developed countries have an obligation to ensure the principles of equity, beneficence and social justice [71]. A responsible researcher from a developed country utilizing African genomic information is obligated to increase partnership with African researchers, aid in capacity building, and promote good science to help solve health problems of the African people. Researchers from developed countries who aim to achieve these three goals can ease the sentiment that genomic sovereignty is a necessity to protect African people.

First, research should not simply be used for career advancements by researchers from affluent countries; African researchers should be given the opportunity to partner with researchers from other countries to benefit both parties in terms of insights and knowledge exchange. Increased partnership will also allow for a prioritization by African researchers to ensure the research being conducted is for the benefit of local health problems. Next, African countries need the technology and infrastructure to support genomic research in order to have more control over their genomic samples and how they are utilized. Researchers from developed countries should be enabling genetic research to be done in the place where the genomic samples are originated. Countries that possess the scientific infrastructure will have increased control over genetic samples and more direct regulation of secondary uses of samples and data. Lastly, research using human samples cannot be done for the sake of publication or recognition; it must aim to benefit the research participants

and the people who are affected by the disease being studied. Conducting genetic research that allows for innovations related to prevention or treatment of diseases ensures that participation in research is not trivial. Furthermore, good science in developing countries may not only require the study of health problems of most concern, it may require ancillary care for study participants. If immediate health concerns of study participants can be addressed in a feasible manner, there is an obligation of researchers to ensure this benefit [72].

Conclusion

Genomic sovereignty has emerged as a way for developing countries to protect their genomic material and ensure research that benefits African people. While genomic sovereignty might provide a way to control the terms of genetic research, it may not necessarily provide the most benefit to the people. However, researchers from developed countries can aid in reaching the goals of genomic sovereignty by increasing partnership, assisting in capacity building and performing research that is for the benefit of Africans.

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were determined through the UW CFAR Retrovirology lab (UW CFAR supported by NIH P30 AI027757-25).

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