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Association of interleukin-1β and interleukin-1 receptor antagonist polymorphisms with bacterial vaginosis in non-pregnant Italian women

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Bacterial vaginosis (BV) is the most prevalent alteration of vaginal microflora worldwide. BV is a polymicrobial disorder, and its etiology is elusive. Factors predisposing to this recurrent condition are not fully characterized. We aimed to investigate whether interleukin-1β (IL-1β) and IL-1 receptor antagonist (IL-1ra) polymorphisms are associated with BV in non-pregnant white Italian women. Genomic DNA was obtained from 164 BV positive, and 406 control women. Two diallelic polymorphisms in the IL-1β gene (*IL-1B*) representing C/T base transitions at −511 and +3954 positions and a variable number tandem repeats (VNTR) in intron 2 of the IL-1ra gene (*IL-1RN*) were assessed. We demonstrated that women who were homozygous for −511 CC or +3954 TT of the *IL-1B* gene were at increased risk for BV with an odds ratio (OR) = 1.5 [95% confidence interval (CI) = 1.03–2.14, *P* = 0.032], and OR = 2.8 (95% CI = 1.37–5.88, *P* = 0.004), respectively. The haplotype −511/+3954 T-C was protective for BV, with an OR = 0.7 (95% CI = 0.49–0.90, *P* = 0.009). The *IL-1RN* VNTR genotype was not associated with BV, although the rare allele 3 showed a trend towards protection (*P* = 0.049). These data show that host genetic variants at the IL-1β locus predispose to BV among Caucasian non-pregnant women. Further studies will determine whether these genetic polymorphisms modulate the risk for BV recurrence, and/or BV associated severe adverse outcomes as preterm birth and human immunodeficiency virus transmission.

Key words: bacterial vaginosis/*IL-1* gene polymorphisms/*IL-1* haplotype/IL-1 receptor antagonist polymorphism/vaginal flora

Introduction

Bacterial vaginosis (BV) is the most prevalent vaginal disorder in reproductive age women (Eschenbach, 1993; Cauci *et al.*, 2002a). BV is associated with several adverse outcomes, including increased susceptibility to human immunodeficiency virus (HIV) and herpes simplex virus type 2 (HSV-2) infections (Sewankambo *et al.*, 1997; Cherpes *et al.*, 2005; Schwebbe, 2005), upper genital tract infections (Ness *et al.*, 2005), endometritis, post-surgical infections (Guaschino *et al.*, 2002), urinary tract infections and adverse pregnancy outcomes such as spontaneous abortion, preterm delivery and low birthweight (Hillier *et al.*, 1995; Goldenberg *et al.*, 2005). BV is a complex polymicrobial disorder characterized by a decreased lactobacilli flora and a largely increased colonization of several facultative and/or strictly anaerobic micro-organisms, mainly *Gardnerella vaginalis*, *Prevotella* spp., *Bacteroides* spp., *Mobiluncus* spp., Gram-positive cocci and genital mycoplasmas (*Mycoplasma hominis* and *Ureaplasma urealyticum*) (Eschenbach, 1993; Thorsen *et al.*, 1998). BV has recently been defined as a microbial/mucosal immunity disorder (Cauci, 2004; Romero *et al.*, 2004; Culhane *et al.*, 2006). Factors predisposing to BV remain only partially defined; in particular, reasons for ethnic disparities in BV prevalence and for the high recurrence rate of BV are unknown (Pereira *et al.*, 2005; Schwebbe, 2005; Sobel, 2005; Marrazzo, 2006). An indicator of factor(s) predisposing to BV includes the observation that BV positive women show elevated concentrations of vaginal interleukin-1β (IL-1β) (Cauci *et al.*, 2002b, 2003; Cauci, 2004). IL-1β is a well-known proinflammatory cytokine, a key mediator of the host immune response to invading micro-organisms (Dinarello, 1996). This cytokine is able to induce the secretion of several inflammatory factors by female reproductive tract epithelial cells, including IL-8, tumour necrosis factor-α, IL-6 and granulocyte-macrophage colony-stimulating factor (Fortunato *et al.*, 1996; Wira *et al.*, 2005). IL-1β binds the IL-1 receptor on the surface of a variety of cells and initiates a cascade of events leading to recruitment and activation of macrophages and neutrophils. The activity of IL-1β is modulated by a competitive receptor antagonist (IL-1ra). One study reported that a disproportionate increase in vaginal levels of IL-1β over IL-1ra in women with altered bacterial flora in mid-trimester gestation was associated with subsequent spontaneous preterm delivery (Genc *et al.*, 2004b).

The genes of IL-1β (*IL-1B*) and IL-1ra (*IL-1RN*) are located close to each other on chromosome 2 in humans (Webb *et al.*, 1986). Polymorphisms in these genes correlate with altered protein expression

in vitro (Pociot *et al.*, 1992; Hernandez-Guerrero *et al.*, 2003) and *in vivo* (Dennis *et al.*, 2004; Hall *et al.*, 2004). Specific polymorphisms in genes encoding cytokines have been associated with an increased risk for, and worsened outcomes in many human conditions, including infectious and inflammatory diseases (periodontal disease, gastric cancer, hepatocellular carcinoma, rheumatoid arthritis and Alzheimer's disease) (El-Omar *et al.*, 2000; Licastro *et al.*, 2004; Pawlik *et al.*, 2005; Hirankarn *et al.*, 2006; Lopez *et al.*, 2005). Several polymorphisms in the *IL-1B* gene have been described. Among these are single-nucleotide polymorphisms (SNPs) located at position −511 in the promoter region (di Giovine *et al.*, 1992) and another SNP located at position +3954 in exon 5 (TaqI restriction site polymorphism designated as +3953 in the first studies) (Pociot *et al.*, 1992). The *IL-1RN* gene has a penta-allelic polymorphic site in intron 2, containing a variable number of 86 bp identical tandem repeats [Variable number tandem repeat (VNTR)] (Tarlow *et al.*, 1993).

Recently, a study performed on mostly black pregnant women found that women with BV were less likely to be homozygous for the minor allele (C; $P = 0.04$) at site +3954 *IL-1B*, whereas no significant findings were noted for the −511 *IL-1B* polymorphic site (Goepfert *et al.*, 2005). According to these authors, these relationships did not seem to differ by race. Studies of associations between polymorphisms in immune system-related genes may be confounded by allele frequency differences between ethnic groups under examination (Pillay *et al.*, 2000; Tishkoff and Williams, 2002). Notably, ethnic differences exist both in the prevalence of infections and in the extent of immune responses to infection (Nguyen *et al.*, 2004; Goldenberg *et al.*, 2005; Menon *et al.*, 2006a). BV is 2- to 3-fold more frequent in African-American women than in white non-Hispanic women in the USA (Goldenberg *et al.*, 2005; Pereira *et al.*, 2005).

Our study determined the genotype frequencies of −511 and +3954 SNPs of *IL-1B*, and intron 2 VNTR of *IL-1RN* in white Italian women, a relatively homogeneous ethnic group. We examined whether the polymorphisms of these genes are associated with the occurrence of BV in non-pregnant women.

Materials and methods

Subjects and biological samples

Women were recruited during routine gynaecological examination to perform Papanicolau test in Trieste, Italy. All women were enrolled after informed consent according to the Institutional Ethics Committees. Inclusion criteria were: non-pregnant Caucasian women without severe medical illnesses, no antibiotic use in the last 2 weeks and ≥18 years of age. Exclusion criteria were: presence of vaginal bleeding (because it can confound vaginal flora assessment), yeast vaginitis (by clinical signs), *Trichomonas vaginalis* (evaluated on a wet smear and/or Pap smear exam), *Neisseria gonnorrhoeae* (based on clinical criteria confirmed by swab culture Thayer–Martin medium) and *Chlamydia trachomatis* (by ligase chain reaction). Rates of sexually transmitted infections (STIs) in this mostly middle class population as determined in parallel studies are very low (e.g. the frequency of *T. vaginalis* is <2%, Guaschino S, unpublished). As a routine practice, at the time of scheduling of the Pap test, patients were requested to refrain from sexual intercourse and from any vaginal treatment for 3 days prior to their checkups. At the visit, the provider further requested the date of the last sexual intercourse. Women who had intercourse in the previous 3 days or who had sperm detected in the Gram-stain smear were excluded from the study. Cases and controls were matched by age and menopausal status.

Women with BV and healthy controls were clinically recruited using Amsel's criteria (Amsel *et al.*, 1983). Vaginal specimens were collected by sterile cotton swabs. One swab was used to create an air-dried vaginal smear that was Gram stained and scored according to Nugent *et al.* (1991). For inclusion in the BV positive group, a Nugent score of 7 or greater was used. For inclusion in the healthy control group a Nugent score from 0 to 3 was used. Women with intermediate flora by Gram staining were excluded from the study. No women with BV, using Amsel's criteria, had a Nugent score of 0–3, and no women classified as healthy by Amsel's criteria had a Nugent score of 7–10. A second vaginal swab was squeezed into 1 ml of sterile saline. Samples were centrifuged at 700g for 3 min at 4°C and then frozen at −80°C from the time of collection until analysis. Personnel assessing Nugent scores and genetic polymorphisms were blinded to clinical findings. A subset of 200 Gram-stained slides were read by a second microscopist blinded to first readings. Concordance rate between slide evaluations was more than 96%.

IL-1B and IL-1RN gene polymorphism identification

Genomic DNA was extracted from the swab pellet fraction according to the standard proteinase-K digestion and ethanol extraction method. The extracted DNA was then stored at −20°C until further analysis.

To examine the −511 SNP, the promoter region of *IL-1B* was amplified by PCR, using the primers 5′-TGGCATTTGATCTGGTTCATC-3′ and 5′-GTTTAGGAATCTTCCCACTT-3′ (di Giovine *et al.*, 1992). The protocol included 35 cycles at 94°C for 1 min, 55°C for 30 s and 72°C for 30 s and a final extension at 72°C of 5 min. At the end of the procedure, the amplicons were digested with AvaI at 37°C for 3 h. Fragments were analysed after electrophoresis on 10% acrylamide gels and visualized with ethidium bromide. This gave products of 190 and 114 bp (C allele) or 304 bp (T allele).

To determine the +3954 SNP, the polymorphic region containing the TaqI restriction site was amplified using the following primers: 5′-GTTGTCATCAGACTTTGACC-3′ and 5′-TTCAGTTCATATGGACCAGA-3′ (Bioque *et al.*, 1995). The 249 bp products were digested with TaqI at 65°C for 1 h, resulting in fragments that either remained intact (T allele) or were digested into two fragments of 135 and 114 bp (C allele). The restriction fragments were analysed by electrophoresis on 10% acrylamide gels and visualized with ethidium bromide.

The *IL-1RN* intron 2 VNTR polymorphism was analysed using 5′-CTCAGCAACACTCCTAT-3′ and 5′-TCCTGGTCTGCAGGTAA-3′ as primers (Tarlow *et al.*, 1993). The PCR products of 410 bp (allele 1 = 4 repeats of the 86 bp region), 240 bp (allele 2 = 2 repeats), 500 bp (allele 3 = 5 repeats), 325 bp (allele 4 = 3 repeats) and 595 bp (allele 5 = 6 repeats) were analysed by electrophoresis on 8% acrylamide gel stained with ethidium bromide.

Statistical analysis

Differences in clinical characteristics between cases and controls were tested using Statistical Package for Social Sciences (SPSS). The genotype frequencies for each polymorphism were compared between cases and controls, using, Pearson's chi-square or Fischer's exact test as appropriate (also using SPSS). The OR and the 95% confidence interval (CI) were calculated to evaluate the genotype effects of each genotype against all others resulting in 2×2 comparisons. This approach allowed testing both dominant and recessive genetic models of risk. The *P*-values for $2 \times n$ tests for each genotype were obtained using the program R X C based on the Metropolis algorithm (Raymond and Rousset, 1995), and available at http://www.marksgeneticsoftware.net/rxc.htm. Testing for Hardy–Weinberg equilibrium (HWE) and allele frequency differences were performed using tools for population genetic analysis (TFPGA) at the same website and based on the algorithms of Raymond and Rousset (1995). When there were deviations from HWE, we tested for genotype association as recommended by Sasieni (1997).

Genetic effects were also assessed using logistic regression, including clinical variables that differed between cases and controls to assess whether genetic effects are still significant after controlling for other significant variables. Logistic regression was performed with Stata®, version 9 (StataCorp 2005). Haplotype frequencies were estimated with the program PowerMarker (Liu and Muse, 2005), which uses an expectation maximization (EM) algorithm (Zhao and Sham, 2002). Haplotypes estimated to be below 3% in frequency were not reported. Differences in the haplotype distributions between cases and controls were determined by haplotype trend regression using this software (Zaykin *et al.*, 2002). The OR and CI were obtained for each haplotype in relation to BV status using the Stata® software package. The reference distribution for which all the other haplotypes were compared against was the most common haplotype C-C.

To account for multiple testing issues, a false discovery rate (FDR) cut-off value was calculated for each test performed and any *P*-value that was less

than this cut-off is considered significant. The FDR cut-off is calculated by first ranking all *P*-values from smallest to largest and then applying the formula $(i/m)q^*$, where i is the rank of the given *P*-value, m is the number of tests performed and q^* is the proportion of false positives that is acceptable. We set q^* to 0.1.

Results

Of 602 subjects enrolled, 32 had intermediate vaginal flora by Gram staining (12 were initially classified as BV and 20 as healthy by Amsel's criteria) and were excluded from this study. The final analysis was performed on 570 women, including 164 (28.8%) women with BV and 406 (71.2%) healthy controls. Demographic and behavioural characteristics are presented in Table I. Mean age of the study population was 37.1 ± 11.2 years, 88.8% (506/570) were premenopausal women, 11.2% (64/570) were post-menopausal women and 54.8% (311/568) were nulliparous. Cases and controls did not differ by age, and menopausal status. BV positive women were less likely to use oral contraceptives or condoms, but more likely to use coitus interruptus, free intercourse, intrauterine device (IUD) or surgical sterilization than healthy controls. In addition, BV positive women were more likely to be smokers, and less likely to be nulliparous than controls.

Study women were genotyped for the three gene loci (*IL-1B* in position −511 and +3954; and *IL-1RN* intron 2 VNTR). Figure 1 shows representative ethidium bromide stained electrophoresis gels for −511, +3954 *IL-1B*, and VNTR *IL-1RN* polymorphisms assessment. One datum was not available for +3954 genotype, and one for *IL-1RN* genotype. Neither case nor control samples deviated from HWE at site −511, but cases deviated at +3954 of the *IL-1B* gene ($P = 0.001$). Both cases and controls deviated from HWE for the VNTR in *IL-1RN* ($P = 0.001$ for cases and $P < 0.001$ for controls).

Examination of the distribution of −511 *IL-1B* genotypes in the 570 study subjects revealed that 44.2% were homozygous CC, 44.2% heterozygous CT and 11.6% homozygous TT (Table II). Subjects with BV were more likely to be homozygous for C allele at position -511 than healthy women, OR = 1.488 (95% CI = 1.033–2.141), $P = 0.032$. The two groups also differed significantly in allele frequency ($P = 0.021$). Allele and genotype distribution of *IL-1B* +3954 gene polymorphism are shown in Table III. The homozygous CC of *IL-1B* +3954 was the most common genotype in our sample of Italian women (62.9%), the second was the CT genotype (31.6%), followed by the TT genotype (5.4%). The differences between BV positive women and healthy cases concerned only the TT homozygous genotype. Women with BV exhibited a significantly higher frequency of the TT genotype (9.8% versus 3.7%, $P = 0.004$). This finding resulted in an increased risk of BV for TT homozygous carriers, with an OR of 2.837 (95% CI = 1.368–5.884).

Clinical variables that might impact the association with genotype were assessed using logistic regression. The variables that were included in the model were oral contraceptive use, parity and smoking. Two models were assessed; one consisting of +3954 *IL-1B* with the clinical variables and the other model consisting of −511 *IL-1B* with the same variables. For these models, the baseline clinical variables were oral contraceptive use, nulliparity and non-smoking. The baseline +3954 *IL-1B* genotypes were CC and CT, and the baseline −511 *IL-1B* genotypes were CT and TT. These analyses revealed that individuals with BV were significantly more likely to have the homozygous −511 CC genotype than healthy women, OR = 1.579 (95% CI = 1.005–2.480), $P = 0.047$. Similar results were seen for *IL-1B* +3954 TT with OR = 3.210 (95% CI = 1.362–7.564), $P = 0.008$. In both of the logistic regression analyses, the genetic effects were still significant with OR comparable to those without the clinical variables included.

For the *IL-1RN* VNTR polymorphism, only three alleles were observed in our study; alleles 4 and 5 were not detected. In our population, the *IL-1RN**1 allele frequency was 71.7%, the second commonest allele, *IL-1RN**2, had an allele frequency of 25.9% and *IL-1RN**3 had a frequency of 2.4% (Table IV). The same proportion of alleles was observed in women with BV and healthy controls ($P = 0.116$). The overall distribution of *IL-1RN* genotypes between BV cases and controls did not differ significantly ($P = 0.344$). However, we observed a trend towards decreased frequencies of cumulative 1/3, 2/3 and 3/3 genotypes in BV positive women (1.8%) compared with healthy women (5.7%), OR = 0.312 (95% CI = 0.092–1.055, $P = 0.048$), suggesting a dominant effect of the allele 3. In addition, allele 3 tended to be less common in BV positive women (0.9%) compared with healthy women (3.0%, $P = 0.049$).

Haplotype analyses were performed using all three sites as they are relatively close on the chromosome and may interact with each other; additionally the two sites in the *IL-1B* gene were examined separately

Table I. Demographic and behavioural characteristics of 570 study women

Characteristics	All women $n = 570$[a]	BV positive $n = 164$	Healthy controls $n = 406$	*P*-value	FDR cut-off
Age (years)	37.1 ± 11.15[b]	37.9 ± 10.38[b]	36.7 ± 11.45[b]	0.089	0.053
Premenopausal women	506 (88.8%)[c]	147 (89.6%)[c]	359 (88.4%)[c]	0.770	0.092
Nulliparity	311 (54.75%)[c]	74 (45.7%)[c]	237 (58.4%)[c]	0.007	0.018
Sexual debut <18 years	121/404 (27.2%)[c,d]	35/108 (32.4%)[c,d]	86/296 (29.0%)[c,d]	0.540	0.087
Oral contraceptive use	136/500 (27.2%)[c,e]	26/145 (17.9%)[c,e]	110/355 (31.0%)[c,e]	0.003	0.011
Coitus interruptus	130/500 (26.0%)[c,e]	50/145 (34.5%)[c,e]	80/355 (22.5%)[c,e]	0.007	0.021
Condom	125/500 (25.0%)[c,e]	17/145 (11.7%)[c,e]	108/355 (30.4%)[c,e]	<0.001	0.003
No sexual activity[f]	49/500 (9.8%)[c,e]	19/145 (13.1%)[c,e]	30/355 (8.5%)[c,e]	0.135	0.058
Free intercourse	43/500 (8.6%)[c,e]	20/145 (13.8%)[c,e]	23/355 (6.5%)[c,e]	0.013	0.029
Intrauterine device	14/500 (2.8%)[c,e]	9/145 (6.2%)[c,e]	5/355 (1.4%)[c,e]	0.006	0.016
Surgical sterilization[c,e]	6/500 (12.0%)[c,e]	5/145 (3.4%)[c,e]	1/355 (0.28%)[c,e]	0.009	0.024
Smokers	139/446 (31.2%)[c,g]	53/120 (44.2%)[c,g]	86/326 (26.4%)[c,g]	0.001	0.008
10 or more cigarettes per day	54/446 (12.1%)[c,g]	26/120 (21.7%)[c,g]	28/326 (8.6%)[c,g]	<0.001	0.005

BV, bacterial vaginosis; FDR, false discovery rate.
[a]Some data of study women were missing, thus percentages were calculated from subjects with completed data for that question.
[b]Average ± standard deviation.
[c]Number (%).
[d]Age of sexual debut was available for 404 women, 108 BV positive and 296 healthy controls.
[e]Contraception data were available for 500 premenopausal women, 145 BV positive and 355 healthy controls.
[f]Virgins or women not having sexual intercourse in the last 3 months.
[g]Smoking data were available for 446 women, 120 BV positive and 326 healthy controls.

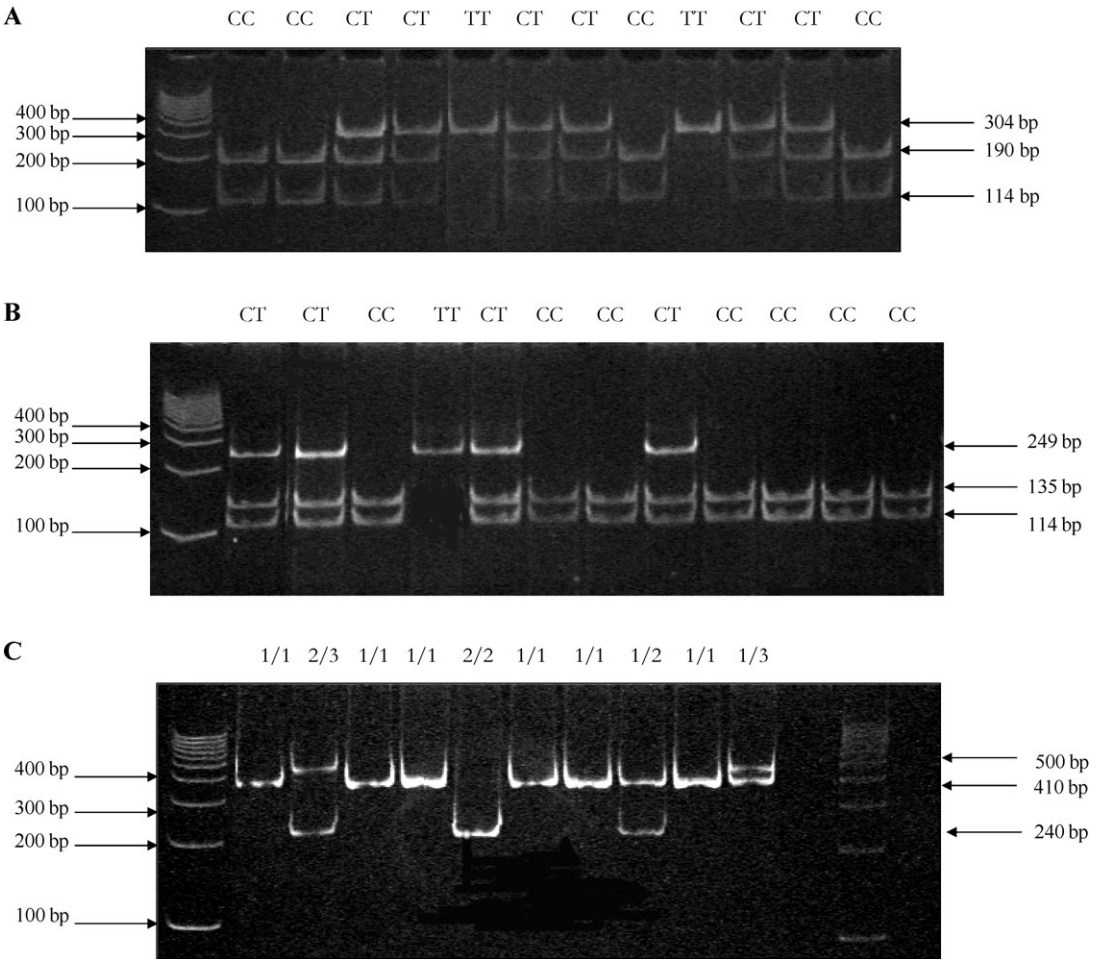

Figure 1. Representative electrophoresis ethidium bromide stained gels for -511 *IL-1B* (**A**), +3954 *IL-1B* (**B**) and VNTR intron 2 *IL-1RN* alleles (**C**).

(Table V). In neither case did the haplotype frequencies differ significantly between the groups, although the −511/ + 3954 haplotype was almost significant ($P = 0.071$)**.** The haplotype T-C was in lower frequency in women with BV and the OR for this haplotype relative to the C-C haplotype was also significant, OR = 0.663 (0.486–0.904), $P = 0.009$. This suggests that the haplotype T-C is conferring a protective effect.

Discussion

BV is present in 10–20% of white non-Hispanic women and in 30–50% of black women (Eschenbach, 1993; Hillier *et al.*, 1995; Cauci *et al.*, 2002a; Culhane *et al.*, 2006). It is as yet an unresolved question whether differences of BV prevalence in white and black women are caused primarily by environmental/behavioural factors only, and/or whether different genetic backgrounds determine the risk of BV acquisition and recurrence (Russell *et al.*, 2005; Sobel, 2005; Marrazzo, 2006). In general, BV associated alterations of the vaginal mucosal system are only partially understood (Imseis *et al.*, 1997; Cauci *et al.*, 2002b, c, 2003; Cauci, 2004; Russell *et al.*, 2005).

Our study was performed to evaluate the SNP polymorphisms at positions −511 and +3954 of the *IL-1B* gene, and the intron 2 VNTR polymorphism of the *IL-1RN* gene in non-pregnant Italian women in relation to occurrence of BV. A similar investigation has

Table II. Genotype frequencies of interleukin-1β gene (*IL-1B*) promoter at position -511 in Italian female population, comparison of BV positive and healthy control women

	All subjects ($n = 570$)	Women with BV ($n = 164$)	Healthy women ($n = 406$)	OR (95% CI)	*P*-value	FDR cut-off
IL-1B promoter genotype (−511)						
CC	252 (44.2%)	84 (51.2%)	168 (41.4%)	1.488 (1.033–2.141)	0.032	0.037
CT	252 (44.2%)	66 (40.2%)	186 (45.8%)	0.797 (0.551–1.151)	0.266	0.074
TT	66 (11.6%)	14 (8.5%)	52 (12.8%)	0.635 (0.342–1.181)	0.149	0.061
					0.069	0.045
IL-1B promoter allele						
Allele C	756 (66.3%)	234 (71.3%)	522 (64.3%)		0.021	0.034
Allele T	384 (33.7%)	94 (28.7%)	290 (35.7%)			

OR, odds ratio.

Table III. Genotype frequencies of *IL-1B* exon 5 at position +3954 in Italian female population, comparison of BV positive and healthy control women

	All subjects ($n = 569$)	Women with BV ($n = 163$)	Healthy women ($n = 406$)	OR (95% CI)	*P*-value	FDR cut-off
IL-1B exon 5 genotype (+3954)						
CC	358 (62.9%)	104 (63.8%)	254 (62.6%)	1.055 (0.723–1.539)	0.782	0.095
CT	180 (31.6%)	43 (26.4%)	137 (33.7%)	0.704 (0.469–1.054)	0.088	0.050
TT	31 (5.4%)	16 (9.8%)	15 (3.7%)	2.837 (1.368–5.884)	0.004	0.013
					0.013	0.032
IL-1B exon 5 allele						
Allele C	896 (78.7%)	251 (77.0%)	645 (79.4%)		0.393	0.079
Allele T	242 (21.3%)	75 (23.0%)	167 (20.6%)			

been performed on US black and white pregnant women by Goepfert *et al.* (2005); however, that study examined, by allele-specific PCR, polymorphisms at positions −511 and +3954 of the *IL-1B* gene and the VNTR of *IL-1RN* was not studied. A limitation of that study was that it did not consider the different genetic profiles of black and white populations separately, thereby overlapping racial groups. We avoided this potential confounder by considering Italian women only, a homogeneous white ethnic group.

We demonstrated that women homozygous for the genotype −511 CC and +3954 TT of *IL-1B* gene had a 1.5 and 2.8-fold, respectively, greater risk of BV ($P = 0.032$ and $P = 0.004$, respectively). These results were robust to the inclusion of clinical variables that were significantly different between cases and controls. Our study was the first to show that a specific haplotype of the *IL-1B* the −511/+3954 T-C was protective against BV (OR = 0.66, 95% CI = 0.49–0.90). The effect of haplotype variation is particularly important as it has recently been demonstrated that single SNPs alone do not provide unambiguous information about *IL-1B* transcriptional regulation (Chen *et al.*, 2006). Additionally, we demonstrated that the *IL-1RN* had no influence on BV, with the exception of a tendency for protective effects of the rare allele *IL-1RN**3 ($P = 0.049$). We caution that this variant deviated from HWE in both cases and controls and may not therefore represent a valid comparison. Explanations of this deviation from HWE include population stratification and genotyping error. The former is not likely given the nature of the sampled population and the lack of deviations at the other markers. However, genotyping cannot be eliminated as a possibility at present.

The *IL-1B* (−511) has been studied in several pathological conditions (Hefler *et al.*, 2002). Of note, homozygosity for *IL-1B* −511 CC was associated with recurrent pregnancy loss (Wang *et al.*, 2006). A recent study demonstrated that the eradication rate of *Helicobacter pylori* in patients with *IL-1B* −511 CC was lower compared with CT and TT genotypes (Sugimoto *et al.*, 2006). *IL-1B* −511 CC genotype was also significantly associated with chronic hepatitis B virus infection (Hirankarn *et al.*, 2006). Studies of LPS stimulation of cells *in vitro* and investigations in patients have demonstrated increased production of IL-1β when the T allele is present (−511) (Hall *et al.*, 2004; Chan *et al.*, 2006). Consequently, the C allele (−511) in the promoter region should confer a limited ability to raise IL-1β levels in response to stimuli.

The +3954 genotype TT has been associated with a wide array of diseases (periodontitis, rheumatoid arthritis and Alzheimer's disease) (Licastro *et al.*, 2004; Lopez *et al.*, 2005; Pawlik *et al.*, 2005). The findings that BV and severe periodontitis are associated with the same polymorphism are of particular interest as some studies demonstrated an association between BV and periodontal diseases (Oittinen *et al.*, 2005).

According to some authors, the polymorphism at +3954 in exon 5 of the *IL-1B* gene contributes to an increase in IL-1β protein secretion (Pociot *et al.*, 1992; Hernandez-Guerrero *et al.*, 2003; Hollegaard and Bidwell, 2006). This observation supports the hypothesis that women carrying hyper-responsive proinflammatory cytokine genes may overrespond to vaginal infection secreting higher amounts of cytokines, which may lead to pathology. However, *in vitro* findings of increased IL-1β production associated with the T (+3954) allele were not confirmed by *in vivo* findings performed in human subjects, showing a reduced inflammatory reaction in allele T carriers (Dennis *et al.*, 2004). It remains controversial what effects an amino acid substitution in the sequence of IL-1β protein can elicit. Contradictory results could derive from the influence of the other variants in linkage with this site,

Table IV. Genotype frequencies of interleukin-1 receptor antagonist gene (*IL-1RN*) in Italian female population, comparison of BV positive and healthy control women

	All subjects ($n = 569$)	Women with BV ($n = 163$)	Healthy women ($n = 406$)	OR (95% CI)	*P*-value	FDR cut-off
IL-1RN variable number tandem repeats genotype						
1/1	318 (55.9%)	98 (60.1%)	220 (54.2%)	1.275 (0.881–1.844)	0.197	0.066
1/2	161 (28.3%)	42 (25.8%)	119 (29.3%)	0.837 (0.555–1.263)	0.396	0.082
1/3	19 (3.3%)	3 (1.8%)	16 (3.9%)	0.457 (0.131–1.590)	0.207	0.068
2/2	64 (11.2%)	20 (12.3%)	44 (10.8%)	1.151 (0.655–2.020)	0.625	0.089
2/3	6 (1.1%)	0 (0%)	6 (1.5%)		0.190	0.063
3/3	1 (0.2%)	0 (0%)	1 (0.2%)		1.000	0.100
1/3, 2/3 and 3/3	26 (4.6%)	3 (1.8%)	23 (5.7%)	0.312 (0.092–1.055)	0.048	0.039
					0.344	0.076
IL-1RN allele						
Allele 1	816 (71.7%)	241 (73.9%)	575 (70.8%)		0.116[a]	0.055
Allele 2	295 (25.9%)	82 (25.2%)	213 (26.2%)			
Allele 3	27 (2.4%)	3 (0.9%)	24 (3.0%)		0.049[b]	0.042

[a]Comparison of all three alleles.
[b]Comparison of allele 3 versus others.

Table V. Haplotype frequencies in women with BV and those without BV

	Haplotype frequency		OR (95% CI)	*P*-value	FDR cut-off
	Women with BV ($n = 163$)	Healthy women ($n = 406$)			
Haplotype (three sites) (−511, +3954, *IL-1RN*)					
C-C-1	0.413	0.353			
C-T-1	0.149	0.148			
T-C-1	0.139	0.180			
C-C-2	0.119	0.100			
T-C-2	0.095	0.134			
T-T-1	0.038	0.027			
				0.210	0.071
Haplotype (two sites) (−511, +3954)					
C-C	0.532	0.473	1.0 (reference)		
T-C	0.238	0.321	0.663 (0.486–0.904)	0.009	0.026
C-T	0.181	0.169	0.956 (0.671–1.362)	0.803	0.097
T-T	0.049	0.036	1.225 (0.648–2.314)	0.532	0.084
				0.071	0.047

which could be assessed by *IL-1* haplotype, and/or by tissue-specific effects, and/or depend upon the context of the stimulation (different micro-organisms could modulate IL-1β in different ways).

It is interesting to note that the *IL-1RN* genotype was not associated with BV in our study. This finding confirms a previous investigation performed in 5 BV positive and 87 control white pregnant women performed by Genc *et al.* (2004a). The *IL1RN**2 allele has a lower frequency in the black as compared with the white population (Pillay *et al.*, 2000; Nguyen *et al.*, 2004; Genc *et al.*, 2004a), thus it is a potential candidate to account for differences in innate immunity responses between white and black ethnic groups. The *IL1RN**2 has been implicated in increased inflammatory responses, and it has been associated with several pathological conditions (El-Omar *et al.*, 2000; Gerber *et al.*, 2005).

Overall, our findings suggest that the association of the *IL-1* genotype with BV does not go in the direction of an enhanced inflammatory response genetic background, but towards a decreased *IL-1* stimulation. Immunity to infections at mucosal sites is likely modulated by subtle tuning of cytokine levels, a partially diminished IL-1β rise could hamper defenses against BV-associated micro-organisms. We could infer that the CC (−511) and TT (+3954) genotypes of the *IL-1B* gene interact to reduce IL-1β levels and/or limit the amplification of the IL-1β proinflammatory signals. This is consistent with our observation that the −511/+3954 T-C haplotype appears to be protective against BV. Notably, this fits with the fact that BV is not typically considered an inflammatory condition. BV positive women do not show increased vaginal neutrophil number in comparison to healthy women, although IL-1β levels are increased (Cauci *et al.*, 2003). The reduced proinflammatory cascade could derive both by host genetic factors and/or by microbial effects (Cauci *et al.*, 2002c; Cauci, 2004).

In our study, we found that contraceptive and behaviour factors differ between BV positive and healthy women. The negative association between BV and oral contraceptive pill and condom use (Shoubnikova *et al.*, 1997; Calzolari *et al.*, 2000) and the positive association between BV and IUD use (Calzolari *et al.*, 2000) are well documented in the literature. In addition, the association of smoking with BV was found in several studies (Hellberg *et al.*, 2000). In our present study, we observed that contraceptive and behavioural factors do not modulate the *IL-1* genotype association with BV.

Our findings of genetic predispositions for BV in non-pregnant women have potentially several implications. The high recurrence rate of BV observed in some women as well as the severe adverse outcomes associated with BV could be modulated by specific genetic factors. On the basis of the consideration that BV affects many million women worldwide and that BV-associated adverse outcomes are severe conditions as preterm birth, low birth weight and HIV transmission, genetic studies appear greatly important in this field (Menon *et al.*, 2006b).

A limit of our study is that we could not assess the role of the *IL-1* genetic variation on gene expression or gene product levels. A strength of our study is a well-characterized racially homogeneous European population. On the other hand, this may limit the ability to generalize our findings to other ethnic groups.

Further research into factors that increase women's susceptibility to BV will help to inform the design of vaginal microbicides/immune modulators and other BV prevention/cure interventions.

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