Microbiota unbalance in relation to high-risk human papillomavirus cervical infection

Juan Facundo Gomez Cherey ,[1] Sandra Noemi Payalef,[2,3] Laura Fleider,[1] Ana Paula Reyes,[2,3] Verónica Andrea Maldonado,[1] Mirta Olga Losada,[2,3] Xin Chen,[4] Lucia Helena Cardinal,[5] Youxiang Wang,[4] Silvio Alejandro Tatti,[1] Beatriz Elizabeth Perazzi[2,3]

For numbered affiliations see end of article.

Correspondence to
Juan Facundo Gomez Cherey, Programa de Diagnóstico, Terapéutica y Vacunación del Tracto Genital Inferior, Universidad de Buenos Aires Facultad de Medicina, Ciudad Autónoma de Buenos Aires, Argentina; facundo8617@gmail.com

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ABSTRACT

Objectives To assess vaginal dysfunction using basic vaginal states and the presence of lactobacillary microbiota in patients with human papillomavirus (HPV) infection with no squamous intra-epithelial lesions (SIL), with low-grade squamous intra-epithelial lesions (L-SIL), and with high-grade squamous intra-epithelial lesions (H-SIL) or squamous cell carcinoma compared with a control group (HPV-negative); to establish the prevalence of bacterial vaginosis, candidiasis, and trichomoniasis in the different age groups; and to characterize the species of lactobacilli according to the type of lesion.

Methods A cross-sectional study was carried out of patients who underwent clinical examination and collection of vaginal fornixes to study basic vaginal states and culture. Species identification of lactobacilli was performed by mass spectrometry. The results were analyzed using the χ^2 and Fisher's tests; $p<0.05$ was considered significant. High-risk viral types were determined using a multiplex real-time polymerase chain reaction test.

Results A total of 741 patients were analyzed and divided into three age groups: Group 1 aged 18–24 years (n=138), Group 2 aged 25–50 years (n=456), and Group 3 aged >50 years (n=147). All groups were further divided into an HPV-negative (control) group and an HPV-positive group without lesions, with L-SIL, or with H-SIL/squamous cell carcinoma. The prevalence of unbalanced basic vaginal states in patients with H-SIL/squamous cell carcinoma was 72.7% (p=0.03) in Group 1, 53.1% (p=0.05) in Group 2, and no cases of unbalance were detected in Group 3. The prevalence of bacterial vaginosis in women with H-SIL/squamous cell carcinoma in Group 1 was 54.5% and in Group 2 was 43.7%. Patients with H-SIL/squamous cell carcinoma had a prevalence of 21.4% of *Lactobacillus crispatus*, 42.9% of *L. jensenii*, and 14.3% of *L. iners*.

Conclusions A greater unbalance of vaginal microbiota was observed in patients with SIL, especially in those with H-SIL/squamous cell carcinoma. In this group, an increase in *L. jensenii* and *L. iners* compared with control was found. *L. crispatus* had a similar prevalence to the control group. It is important to characterize the lactobacilli species since the unbalance alters the vaginal microenvironment and acts as a co-factor in the persistence of HPV infection.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The state of the gastrointestinal microbiome may influence cancer development.

WHAT THIS STUDY ADDS

⇒ An unbalanced vaginal microbiota and low prevalence of *L. crispatus* were observed in patients with a high-grade squamous intra-epithelial lesion (H-SIL) or squamous cell carcinoma.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ It is important to characterize lactobacilli species because its change may be a co-factor in the persistence of HPV.

INTRODUCTION

The vaginal microbiota is a complex system which co-exists in permanent harmony forming an ecosystem. Lactobacilli are the most abundant vaginal bacteria in women of reproductive age. Glycogen deposits in the vaginal epithelium are used by them in anaerobic glycolysis, which results in lactic acid production that reduces vaginal pH and protects against potentially pathogenic micro-organisms.[1] Recently, molecular methods have demonstrated the presence of *Lactobacillus crispatus*, *L. gasseri*, *L. jensenii*, and *L. iners* as the most common bacteria.[2] The vaginal microbiome constantly changes over the course of life. It is therefore admissible to recognize the influence of estrogenic origin on vaginal colonization, with a predominance of *Lactobacillus* spp.[3 4]

There are several factors that can influence the composition of the vaginal microbiome, which are divided into non-modifiable (ethnicity, age, menstrual cycle) and modifiable (use of different contraceptive methods, sexual behavior, douching, smoking, and diet).[5] Healthy vaginal states are associated with low bacterial diversity. Many, but not all, community state types of healthy women of reproductive age are dominated by *Lactobacillus* species, which remain stable but not permanent (the community composition changes during menstruation).[6] A vaginal microbiome dominated by lactobacilli prevents lower genital tract infections such as bacterial vaginosis and the acquisition of sexually transmitted infections such as human papillomavirus (HPV).[7 8] However, recent evidence suggests that the state of the microbiome may influence the development of cancer, a situation that has

already been characterized for the gastrointestinal microbiome.[9] HPV infection and development of squamous intra-epithelial lesions (SIL) may additionally be influenced by the chemical structure of the lactic acid molecule itself. Lactic acid is a chiral compound with a D- and L-isomer, with the former being predominantly produced by *L. jensenii*, *L. crispatus*, and *L. gasseri*. However, the L-isomer of lactic acid is produced by the vaginal epithelium, *L. iners*, and various anaerobes associated with dysbiosis.[10]

HPV viral oncogenesis is a clear example of the association between gynecological tumors and microbiome unbalance. Moscicki *et al* reported the incidence of HPV infection in over 50% of women aged <30 years in a longitudinal study with 3 years of follow-up.[11] Mitra *et al* showed that women with a *Lactobacillus*-dominant microbiome at baseline are more likely to have regressive disease at 12 months.[12] Silva *et al* reported that women with HPV infection had increased *L. gasseri* and *Gardnerella vaginalis* content, while balanced microbiomes have a higher proportion of *L. crispatus*.[13 14]

The objectives of this study were (1) to assess vaginal dysfunction using basic vaginal states by the Balance of the Vaginal Content (BAVACO) methodology and the presence of lactobacillary microbiota in patients with HPV infection with no SIL, L-SIL and H-SIL/squamous cell carcinoma compared with a control group; (2) to establish the prevalence of bacterial vaginosis, candidiasis, and trichomoniasis in the different groups according to lesion type; and (3) to characterize the species of lactobacilli according to the lesion type.

METHODS

In this consecutive prospective descriptive cross-sectional study, a total of 741 patients aged 18–85 years with onset of sexual intercourse were examined. They were treated between October 2015 and June 2021. The study population was classified into three groups according to age: Group 1, 18–24 years; Group 2, 25–50 years; and Group 3, >50 years. Each group was sub-divided into HPV-negative patients (control group), HPV-positive without lesions, with L-SIL, and with H-SIL/squamous cell carcinoma. The exclusion criteria were: patients who had received antibiotics within 15 days prior to the examination, pregnancy, genital malformations, patients undergoing treatment with corticosteroids or chemotherapy, non-initiated sexual intercourse, and sexual intercourse 48 hours prior to the study.

After signing the informed consent form, data were collected to complete the gynecological record. In addition, medical and epidemiological data of interest were collected. Samples were taken for cervical, exocervical, and endocervical cytology. After the application of acetic acid, colposcopy was performed at 16× magnification. The data collected were catalogued according to the International Federation for Colposcopy and Cervical Pathology classification (2011 version).[15]

Papanicolaou (Pap) smears were stained and reported according to the Bethesda classification (2014 version).[16] Histological specimens were reported according to the Lower Anogenital Squamous Terminology (LAST) Project.[17]

All patients underwent clinical examination and collection of vaginal fornixes for microbiological study by conventional methodology and the study of the basic vaginal state (BVS) by BAVACO methodology. The time between taking the sample and processing it was 30 min or less. The microbiological study of the vaginal contents included the following examinations:

1. Gram and prolonged May–Grunwald Giemsa (MGG) stained smears to visualize bacterial microbiota and to visualize *Trichomonas vaginalis* and the vaginal inflammatory reaction, respectively, using an optical microscope with magnification 400× and 1000×.
2. Wet smear examination with 1 mL of physiological solution to visualize the vaginal inflammatory reaction, yeasts, and *T. vaginalis* using an optical microscope with magnification 400×.
3. Determination of the pH of the vaginal secretion with pH indicator strips.
4. Wet smear examination with 1 mL 10% potassium hydroxide and the fishy odor test.
5. Liquid culture medium (modified thioglycolate) for *T. vaginalis,* with incubation for 7 days at 37°C in an atmosphere of 5% carbon dioxide.[18 19]
6. Columbia agar base culture with 5% human blood to cultivate vaginal microbiota including *Lactobacillus* species and Man Rogosa agar to cultivate some *Lactobacillus* species with 48-hour incubation at 37°C in an atmosphere of 5% carbon dioxide, preserving the sample in Stuart's medium.

Candidiasis was detected by wet smear examination with physiological solution and 10% potassium hydroxide and by culture on Sabouraud agar and blood agar. Sabouraud agar was cultured for 48 hours at 28°C in an ambient atmosphere. The investigation of *T. vaginalis* was carried out by direct microscopic observation with Phisical Solution (PS), prolonged MGG staining, and culture on modified thioglycolate.[18 19]

The diagnosis of bacterial vaginosis was made using Nugent's criteria.[20] The study of BAVACO included the morphological analysis of the vaginal content according to the relationship between the numerical value and the vaginal inflammatory reaction, identifying five basic vaginal states: normal microbiota (I), normal microbiota plus inflammatory reaction (II), intermediate microbiota (III), bacterial vaginosis (IV), and non-specific microbial vaginitis (V).[21]

For the isolation of the different species of lacobacilli, blood agar and Man Rogosa agar were used and identification was performed by BD Bruker MALDI-TOF mass spectrometry (Matrix Assisted Laser Desorption/Ionization, with Time-of-Flight ion detector), using a database that included more than 90 species of lactobacilli and considering a score ≥1.7 for species level, validated against 16S rRNA gene sequencing (reference method).[22–24] For the detection of high-risk HPV (hr-HPV), a multiplex real-time polymerase chain reaction test, AmpFire Multiplex HPV assays, was performed on the patient samples (Catalog number: MHPVF1618-100), which detects the presence of 15 types of hr-HPV and simultaneously HPV-16 and 18 in one reaction plus an internal control (human β-globin gene) with isothermal fluorescent detection in real time. Probes specific for HPV-16, HPV-18, hr-HPV non-16/18 (31, 33, 35, 39, 45, 51, 52, 52, 53, 56, 58, 59, 66, 68), and the internal control were labeled with CY5, ROX, FAM and HEX, respectively. The lack of an exponential amplification curve in the HEX channel was interpreted as an invalid result.[25]

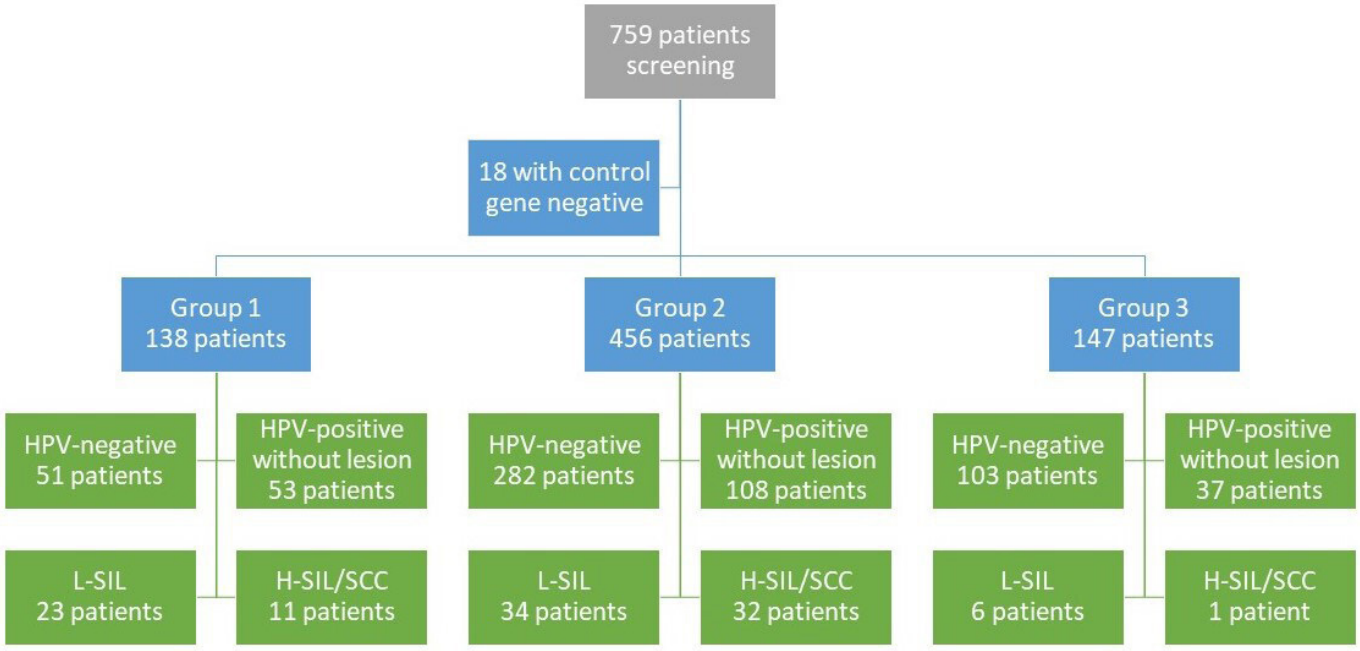

Figure 1 Flowchart of patients included in the study.

Statistical analysis

To compare the prevalence of the basic vaginal state and lactobacilli species, candidiasis, bacterial vaginosis, and trichomoniasis in the different age groups and sub-groups according to the intra-epithelial lesion found in the patient, the $\chi 2$ test, odds ratio (OR) and Fisher's test were used. A p value <0.05 was considered significant. Statistical analysis was performed with Epi Data version 7.2.2.6 (Centers for Disease Control and Prevention, US Department of Health & Human Services).

RESULTS

A total of 741 patients were studied and were divided into three groups according to age: 18–24 years (n=138), 25–50 years (n=456), and >50 years (n=147). Samples with no amplification of the control gene in the HR-HPV test were excluded (18 patients) (Figure 1). The prevalence of basic vaginal state of vaginal content unbalance (III, IV and V) in Group 1 was as follows: HPV-negative 35.3% (18/51), HPV-positive without lesions 35.8% (19/53; OR 1.02, p=0.56), L-SIL 52.2% (12/23; OR 2.0, p=0.13), and H-SIL/squamous cell carcinoma 72.7% (8/11; OR 4.89, p=0.03) (Table 1).

The prevalence of basic vaginal state of vaginal content unbalance (III, IV and V) in Group 2 was as follows: HPV-negative 35.5% (100/282), HPV-positive without lesions 35.2% (38/108; OR 0.99, p=0.53), L-SIL 55.9% (19/34; OR 2.28, p=0.01), and H-SIL/squamous cell carcinoma 53.1% (17/32; OR 2.63, p=0.05) (Table 1). The prevalence of basic vaginal state of vaginal content unbalance (III, IV and V) in Group 3 was as follows: HPV-negative 20.4% (21/103), HPV-positive without a lesion 24.3% (9/37; p=0.37), L-SIL 16.7%

Table 1 Frequency of basic vaginal states of vaginal content unbalance in all patients according to type of lesion

		HPV-positive with no lesion		LSIL		HSIL/SCC		HPV-negative (control)	
Age group	**Basic vaginal state (BVS)**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
Group 1: 18–24 years	Vaginal content unbalance (III–IV–V)	19	35.8	12	52.2	8	72.7	18	35.3
	Normal microbiota (I–II)	34	64.2	11	47.8	3	27.3	33	64.7
	Total	53	100	23	100	11	100	51	100
Group 2: 25–50 years	Vaginal content unbalance (III–IV–V)	38	35.2	19	55.9	17	53.1	100	35.5
	Normal microbiota (I–II)	70	64.8	15	44.1	15	46.9	182	64.5
	Total	108	100	34	100	32	100	282	100
Group 3: >50 years	Vaginal content unbalance (III–IV–V)	9	24.3	1	16.7	0	0	21	20.4
	Normal microbiota (I–II)	28	75.7	5	83.3	1	100	82	79.6
	Total	37	100	6	100	1	100	103	100

HPV, human papillomavirus; HSIL, high-grade squamous intra-epithelial lesions; LSIL, low-grade squamous intra-epithelial lesions; SCC, squamous cell carcinoma.

Table 2 Prevalence of bacterial vaginosis, candidiasis, and trichomoniasis in all groups according to type of squamous intra-epithelial lesion

Group 1: 18–24 years	**Vaginal pathogen**	**HPV-positive with no lesion (n=53)**		**L-SIL (n=23)**		**H-SIL/SCC (n=11)**		**HPV-negative (control) (n=51)**	
		N	**%**	**N**	**%**	**N**	**%**	**N**	**%**
	Bacterial vaginosis	18	34.0	10	43.5	6	54.5	14	27.5
	Candidiasis	15	28.3	4	17.4	0	0	13	25.5
	Trichomoniasis	0	0	0	0	1	9.1	3	5.9
Group 2: 25–50 years	**Vaginal pathogen**	**HPV-positive with no lesion (n=108)**		**LSIL (n=34)**		**HSIL/SCC (n=32)**		**HPV-negative (control) (n=282)**	
		n	**%**	**n**	**%**	**n**	**%**	**n**	**%**
	Bacterial vaginosis	27	25	18	52.9	14	43.7	85	30.1
	Candidiasis	28	25.9	7	20.6	4	12.5	61	21.6
	Trichomoniasis	3	2.8	4	11.8	0	0	10	3.5
Group 3: >50 years	**Vaginal pathogen**	**HPV-positive with no lesion (n=37)**		**L-SIL (n=6)**		**H-SIL/SCC (n=1)**		**HPV-negative (control) (n=103)**	
		n	**%**	**n**	**%**	**n**	**%**	**n**	**%**
	Bacterial vaginosis	7	18.9	1	16.7	0	0	14	13.6
	Candidiasis	1	2.7	1	16.7	0	0	8	7.8
	Trichomoniasis	0	0	0	0	0	0	2	1.9

HPV, human papillomavirus; HSIL, high-grade squamous intra-epithelial lesions; LSIL, low-grade squamous intra-epithelial lesions; SCC, squamous cell carcinoma.

(1/6; p=0.66) (Table 1). No cases of unbalance were detected in the H-SIL/squamous cell carcinoma sub-group.

Analysis of the prevalence of bacterial vaginosis in Group 1 was as follows: HPV-negative 27.5% (14/51), HPV-positive without lesions 34.0% (18/53; OR 1.35, p=0.24), L-SIL 43.5% (10/23; OR 2.03, p=0.09), H-SIL/squamous cell carcinoma 54.5% (6/11; OR 3.17, p=0.05), in Group 2 the prevalence of bacterial vaginosis was HPV-negative 30.1% (85/282), HPV-positive without lesions 25% (27/108; OR 0.69, p=0.07), L-SIL 52.9% (18/34; OR 2.58, p=0.01), H-SIL/squamous cell carcinoma 43.7% (14/32; OR 1.78, p=0.06), and in Group 3 it was HPV-negative 13.6% (14/103), HPV-positive without lesions 18.9% (7/37; OR 1.43, p=0.24), L-SIL 16.7% (1/6; OR 1.53, p=0.34), and no cases of bacterial vaginosis were detected in patients with H-SIL/squamous cell carcinoma (Table 2).

The prevalence of candidiasis in the three groups was as follows: Group 1: HPV-negative 25.5% (13/51), HPV-positive without lesion 28.3% (15/53; OR 1.15, p=0.38), L-SIL 17.4% (4/23; OR 0.62, p=0.23), and no cases of candidiasis infection in patients with H-SIL/squamous cell carcinoma; Group 2: HPV-negative 21.8% (61/282), HPV-positive without lesions 25.9% (28/108; OR 1.25, p=0.19), L-SIL 20.6% (7/34; OR 0.93, p=0.45), H-SIL/squamous cell carcinoma 12.5% (4/32; OR 0.51, p=0.11); Group 3: HPV-negative 7.8% (8/103), HPV-positive without lesions 2.7% (1/37; OR 0.32, p=0.15), L-SIL 16.7% (1/6; OR 2.3, p=0.25), and no cases of candidiasis infection in patients with H-SIL/squamous cell carcinoma (Table 2).

The prevalence of trichomoniasis in Group 1 was as follows: HPV-negative 5.9% (3/51) and H-SIL/squamous cell carcinoma 9.1% (1/11; OR 1.16, p=0.34). No cases of trichomoniasis infection were detected in HPV-positive patients without lesions and L-SIL. In Group 2 the prevalence of HPV-negative was 2.6% (10/282), HPV-positive without lesions 2.8% (3/108; OR 0.77, p=0.37), L-SIL 11.8% (4/34; OR 3.6, p=0.05), and no cases of trichomoniasis infection in patients with H-SIL/squamous cell carcinoma. The patients in Group 3 were HPV-negative 1.9% (2/103). No cases of trichomonas infection were detected in the other groups studied (Table 2).

Lactobacillary microbiota was detected in 479 of 741 samples (82 in Group 1, 283 in Group 2, and 114 in Group 3), of which 253 cultivable lactobacilli could be characterized. The frequency of the different species of lactobacilli found in the study patients according to the type of HPV lesion is shown in Table 3. *L. jensenii* had a prevalence of 42.9% in patients with H-SIL/squamous cell carcinoma compared with 24.8% in the control group (OR 2.27, p=0.08), *L. iners* had a prevalence of 14.3% in patients with H-SIL/squamous cell carcinoma compared with 4.1% in the control group (OR 3.86, p=0.09), and *L. crispatus* had a prevalence of 21.4% in patients with H-SIL/squamous cell carcinoma compared with 23.4% in the control group (OR 0.89, p=0.45).

DISCUSSION

Summary of main results

A greater unbalance of the vaginal microbiota was observed in patients with SIL in Groups 1 and 2 than in controls, especially in

Table 3 Frequency of the different species of lactobacilli found in study patients according to type of HPV-related lesion

	HPV-positive with no visible lesion		L-SIL		H-SIL/SCC		HPV-negative (control)	
Species of lactobacilli	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
L. crispatus	19	25	7	38.9	3	21.4	34	23.4
L. gasseri	29	38.2	6	33.3	3	21.4	64	44.1
L. jensenii	20	26.3	4	22.2	6	42.9	36	24.8
L. iners	5	6.6	0	0	2	14.3	6	4.1
L. vaginalis	0	0	0	0	0	0	4	2.9
L. salivarius	2	2.6	0	0	0	0	1	0.7
L. fermentum	0	0	1	5.6	0	0	0	0
L. paracasei	1	1.3	0	0	0	0	0	0
Total	76	100	18	100	14	100	145	100

HPV, human papillomavirus; H-SIL, high-grade squamous intra-epithelial lesions; L-SIL, low-grade squamous intra-epithelial lesions; SCC, squamous cell carcinoma.

the H-SIL/squamous cell carcinoma group. In our population, bacterial vaginosis was more prevalent than candidiasis or trichomoniasis in all groups. Patients with H-SIL/squamous cell carcinoma had a bacterial vaginosis prevalence of 54.5% in Group 1 and 43.7% in Group 2. In patients with H-SIL/squamous cell carcinoma there was an increase in *L. jensenii* and *L. iners* compared with the control group (species that have a less protective role in vaginal dysfunction). *L. crispatus* had a similar prevalence to the control group.

Results in the context of published literature

The main factor in cervical carcinogenesis is HPV infection, and the microbiome that accompanies HPV infection is one of the most important co-factors in cell transformation. Host defense mechanisms, including immune mediators in the female genital tract microenvironment, play a role in the clearance and persistence of HPV and the risk of developing cervical cancer.[26] The composition of the microbiota is influenced by numerous factors, with ethnicity being the main intrinsic factor associated with the microbiome composition.[10] Age, an intrinsic host factor, is associated with the risk of acquiring HPV infection. HPV is most prevalent among adolescents and young adults between 15 and 25 years of age, and then is increased by a lack of immune responses and squamous metaplasia because the basal cells are more susceptible to HPV infection associated with dysbiosis.[27]

Cervical cancer is a long-standing disease, and there are stages prior to its development during which the conditions of the cervical and vaginal environment are modified, such as vaginal acidity and the cytokine pattern that transitions from a pro-inflammatory pattern to a state of local immunosuppression.[28] Women with dysbiosis can develop chronic inflammation, which may be an important factor for cancer development in different tissue types, including cervical tissue.[29]

In our study, 54.2% of HPV-16-infected patients in Group 1 had associated bacterial vaginosis while, in Groups 2 and 3, HPV-16 was associated with bacterial vaginosis in 34.7% and 22.2% of patients, respectively (data not shown). These findings agree with those of Lewis *et al*, who describe the relationship between bacterial vaginosis and HPV as consistent with longitudinal studies demonstrating a greater association between the pathogen and an increased incidence of infection and decreased clearance of HPV.[30] Bacterial vaginosis, likely due to HPV infection, can alter the vaginal mucosal metabolism (including biogenic amines, glutathione, and lipid-related metabolites), host immunity, or both, resulting in changes in the structure of the vaginal ecosystem.[31–33] Lee *et al* reported that HPV-infected vaginal epithelia show a decrease in glycogen production, which is a source of energy for lactobacilli and responsible for the decrease in vaginal pH.[34] Audirac *et al* showed by metagenomics that patients with cervical carcinoma had a higher predominance of anaerobic microbiota compared with negative controls, who had a higher predominance of lactobacillary microbiota.[35]

In our study, patients with H-SIL/squamous cell carcinoma had a high prevalence of bacterial vaginal unbalance in Group 1 of 72.7% and in Group 2 of 53.1%. Similar results were described by Mitra *et al* who described a decrease in lactobacillary microbiota in 33% of women with H-SIL and in 40% of those with invasive carcinoma.[36] When a depletion in *Lactobacillus* is observed in the H-SIL group, enrichment in the bacteria associated with bacterial vaginosis is found.[37 38] Likewise, Gillet *et al* found that the presence of bacterial vaginosis in a patient increases the risk of having a SIL (OR 1.51, 95% CI 1.24 to 1.83; p<0.05). In group 3, due to the small number of patients with L-SIL and H-SIL/squamous cell carcinoma, the same findings as in Groups 1 and 2 in relation to vaginal microbiota were not observed.[14]

In our study, patients with H-SIL/squamous cell carcinoma had a high prevalence of bacterial vaginosis (54.5% in Group 1 and 43.7% in Group 2) and a low prevalence of candidiasis (0% and 12.5%, respectively). This decrease in the prevalence of candidiasis observed in women with H-SIL/squamous cell carcinoma may be due to the decreased lactobacillary microbiota.[39] However, the growth of the *Candida* complex is a difficult topic to understand.

In our study, 9.1% of patients with H-SIL/squamous cell carcinoma in Group 1 had associated trichomoniasis but, in Groups 2 and 3, we did not detect any cases of trichomoniasis in the H-SIL/squamous cell carcinoma sub-group. Kalia *et al* reported that, after

colonization, *T. vaginalis* modifies the vaginal environment including a fall in lactobacilli and pH >4.5, which supports its further growth and proliferation. This might be due to *T. vaginalis*-mediated lactobacilli phagocytosis which destabilizes and challenges the host protective environment.[5] However, the exact mechanism behind this is puzzling and has not been clearly elucidated.

E5 protein is essential for HPV replication, but this molecule has a very sensitive low pH. Lactic acid is a chiral compound with a D- and L-isomer, the D-isomer being predominantly produced by *L. crispatus, L. jensenii*, and *L. gasseri*. However, the L isomer is produced by *L. iners,* the vaginal epithelium per se, and anaerobic bacteria.[38] The H-SIL/squamous cell carcinoma sub-group showed a decrease in the prevalence of *L. crispatus* (21.4%), in agreement with that described by Gao *et al* for the Asian population and by Dareng *et al* for the African population.[8 40] Likewise, in our study we observed a high prevalence of *L. jensenii* (42.9%) and *L. iners* (14.3%) compared with the control group (24.8% and 4.1%, respectively), which would predispose these patients to vaginal dysfunction. These findings are in agreement with those described in Korea by Oh *et al* who found a predominance of *Atopobium vaginae, G. vaginalis*, and *L. iners* and a decrease in *L. crispatus* associated with an increased risk of SIL.[13] Alimena *et al* and Usyk *et al* report that SIL has also been associated with increased microbial diversity. The other species contributing to microbial diversity and the risk of SIL include *Atopobium vaginae*, *G. vaginalis*, *Fusobacterium* spp, and *Sneathia* spp. In particular, *Sneathia* spp are present in many women from a number of different countries with HPV infection and SIL.[41 42] Likewise, Clarke *et al* reported that increased pH was associated with an increased risk of HPV infection, since it was observed that *L. crispatus* is able to acidify the vaginal environment at pH <4.0, while others such as *L. gasseri* reach a higher pH ranging from 4.4 to 5.0.[36 43]

Strengths and weaknesses

One of the strengths of our study is the large sample size which enabled us to establish statistically significant associations between H-SIL/squamous cell carcinoma and vaginal dysbiosis. The BAVACO methodology allowed us to generate a complete evaluation of the genital tract based only on the morphological analysis, which is low cost and comparable to metagenomic findings. The identification of vaginal lactobacilli by means of MALDI-TOF methodology is important because not all the lactobacilli species have the same protective characteristics. The limitations of the study are related to it being a cross-sectional study, which does not establish strength of association. We should have carried out a longitudinal study, but it is highly questionable from an ethical point of view to leave a pre-neoplastic lesion to its natural evolution (risk of malignant transformation).

Implications for future practice and research

In our study, patients with H-SIL/squamous cell carcinoma showed a high prevalence of vaginal content unbalance. This finding suggests that the decrease in lactobacilli may correlate with the presence of significant disease. These results are important for clinical practice because they emphasize the role of protective lactobacilli microbiota in preventing the progress to H-SIL/squamous cell carcinoma. Also, this microbiota might act as an early biomarker to identify higher risk patients. Finally, the microbiota can possibly be used as a modifiable factor through the use of pre- or pro-biotics, in order to prevent progression to H-SIL/squamous cell carcinoma.

CONCLUSIONS

This study shows that it is important to characterize lactobacilli species, since the increase in non-protective species in concordance with the increase in bacterial vaginosis in patients with H-SIL/squamous cell carcinoma may alter the vaginal microenvironment and act as potential co-factors in the persistence of HPV infection and increase the risk of acquiring other sexually transmitted infections.

Author affiliations
[1]Programa de Diagnóstico, Terapéutica y Vacunación del Tracto Genital Inferior, Universidad de Buenos Aires Facultad de Medicina, Ciudad Autónoma de Buenos Aires, Argentina
[2]Departamento de Bioquímica Clínica - Laboratorio de Bacteriología Clínica, Universidad de Buenos Aires Facultad de Farmacia y Bioquimica, Ciudad Autónoma de Buenos Aires, Argentina
[3]Instituto de Fisiopatología y Bioquímica Clínica (INFIBIOC), Universidad de Buenos Aires Facultad de Farmacia y Bioquimica, Buenos Aires, Argentina
[4]Atila BioSystems, Mountain View, California, USA
[5]Departamento de Patología, Universidad de Buenos Aires Facultad de Medicina, Ciudad Autónoma de Buenos Aires, Argentina

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ORCID iD
Juan Facundo Gomez Cherey http://orcid.org/0000-0003-0107-2834

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