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Cervical carcinogenesis, bacterial vaginosis, HPV-mRNA test and relapse of CIN2+ after loop electrosurgical excision procedure (LEEP)

A. FREGA[1], G. LAVINI[1], A. GUARINO[1], V. GIOVANALE[1], A. LUKIC[1], P. BIANCHI[1], G. COZZA[1], R. MARZIANI[1], A. D'AMBROSIO[2], D. FRENCH[3], D. CASERTA[1]

[1]Department of Medical and Surgical Science and Translation Medicine, Sant'Andrea Hospital, School of Medicine and Psychology, Sapienza University of Rome, Rome, Italy
[2]Multifactorial Disease and Complex Phenotype Research Area, Bambino Gesù Children's Hospital IRCCS, Rome, Italy
[3]Department of Clinical and Molecular Medicine, Sant'Andrea Hospital, School of Medicine and Psychology, Sapienza University of Rome, Italy

Abstract. – OBJECTIVE: The aim of the study was to evaluate the relationship between bacterial vaginosis (BV) and relapse of cervical intraepithelial neoplasia grade 2 or more (CIN2+) after Loop electrosurgical excision procedure (LEEP).

PATIENTS AND METHODS: One hundred four patients who underwent LEEP for CIN2+ were followed up every six months for three years. Fifty-three were negative for BV and fifty-one were positive. Each clinical control included Pap test, colposcopy, Amsel criteria test, HPV-DNA, and HPV-mRNA test.

RESULTS: Patients' age, presence of BV, positivity to HPV-DNA and HPV-mRNA tests were analyzed. The average age of patients was 42.5 ± 8.92 years (median: 42.5; range from 27 to 58 years). The minimum follow-up was 6 months and maximum 36 months (average: 22.8 ± 4.53; median: 24). The 10% of the patients with HPV-mRNA test negative had relapsed, compared to 45% of patients with HPV-mRNA test positive. Among the 53 patients without BV the 20% had relapsed compared with 23% of 51 patients with diagnosis of BV.

CONCLUSIONS: There is no evidence for higher percentage of relapse in patients with BV, submitted to excisional procedure for CIN2+ associated to HPV-m-RNA test positivity. There is only a correlation among BV and relapse of CIN2+ lesions after LEEP.

Key Words
Cervical intraepithelial neoplasia (CIN), Loop Electrosurgical Excision Procedure (LEEP), Bacterial vaginosis, HPV-mRNA, Relapse.

Introduction

Bacterial vaginosis (BV) is a common vaginal disorder during reproductive's age women, particularly in those with age between 25-30 years old. It is not an inflammatory process, but rather to an alteration of the normal quantitative and polymicrobial vaginal ecosystem. BV is characterized by a reduction of vaginal lactobacilli and an overgrowth of other (facultative) anaerobic bacteria[1], such as *Gardnerella vaginalis*, present in 95% of cases of BV, *Mobiluncus, Mycoplasma, Bacteroides, Prevotella, Peptostreptococcus, Fusobacterium, Porphyromonas,* and *Senathia*[2,3].

BV can be diagnosed by Amsel criteria, modified Amsel criteria, Nugent's scoring system or Papanicolaou-stained vaginal smears[4]. BV is characterized by a gray/white discharge, malodorous "fishy" discharge upon adding 10% potassium hydroxide, high vaginal pH (>4.5) and the presence of clue cells[5]. Several papers suggested that BV may be involved in cervical carcinogenesis[6] and can be associated to high susceptibility to Human Papilloma Virus (HPV) acquisition and a lower immunocompetence for clearance HPV infection because BV can destroy innate vaginal defenses[7].

In literature, the association between BV and the onset of cervical intraepithelial neoplasia (CIN) has been deeply investigated, while no researches have been performed on the role of BV in relapse of cervical intraepithelial neoplasia grade 2 or more (CIN2+) after Loop Electrosurgical Excision Procedure (LEEP), as far as we know. The aim of the study was to evaluate the relationship between BV and relapse of CIN2+ after LEEP.

Patients and Methods

From January 2010 to September 2015, 104 out of 9830 patients have been selected among those who attended to Department of Medical and Surgical Science and Translational Medicine, Sant'Andrea Hospital, School of Medicine and Psychology,

Corresponding Author: Antonio Frega, MD; e-mail: antonio.frega@uniroma1.it

Sapienza University of Rome. Fifty-three out of 104 patients resulted BV negative and 51 BV positive. All the patients were positive to HPV-DNA test and they underwent to a *LEEP* for CIN2+. The study was reviewed and approved by the Institutional Review Board (Prot. CE 1591/13). All the women who had been previously treated by LEEP and all study participants were provided informed written consent. The study was conducted in accordance to the Helsinki Declaration. Exclusion criteria were the presence of risk factors for preneoplastic cervix injuries such as smoke, HIV positivity, menopause, use of progestogens, immunosuppression, and pregnancy.

The Loop Electrosurgical Excision Procedure (LEEP) was performed by Ellmann Surgitron 120 IEC, Dual Radio Frequency equipment, with diathermic loops of different measures: small, 10 x 10 mm; middle-size, 15 x 12 mm; large, 20 x 15 mm; basing on the localization of the lesions and on the dimension of the cervix.

The sample was stained in 10% formalin and analyzed for the histological diagnosis by skilled pathologists. Every sample was doubled checked by two different pathologists who agree with the diagnosis.

All patients performed Pap test. The cytological findings were formulated according to Bethesda System (2001). Women with anomalous cytological results: Atypical squamous cells of undetermined significance (ASC-US); Atypical squamous cells – cannot exclude HSIL (ASC-H)

Low grade squamous intraepithelial lesion (LSIL); High grade squamous intraepithelial lesion (HSIL); Atypical Glandular Cells not otherwise specified (AGC-NOS) Atypical Glandular Cells, suspicious for AIS or cancer (AGC-neoplastic) underwent to a colposcopy performed by a Colposcope Zeiss T 50 (Carl Zeiss, Inc., Jena, Germany).

The colposcopy was considered satisfactory if the squamous-columnar junction (SCJ) has been entirely identified. The colposcopy findings were reported according to the International Classification Colposcopic IFCPC 2012. All colposcopic direct biopsies were performed in areas that showed a higher degree of atypia. All the patients were revalued every 6 months for 3 years; at the end of this period, the results of Pap test, HPV- DNA test and HPV-mRNA test were analyzed. The samples for both molecular tests were obtained from the ectocervix and the endocervical canal. The DNA genotyping was performed with the LiPA (Innogenetics, Gent, Belgium, EU) using biotinylated SPF10 PCR primers for the amplification of a 65-bp region of the L1 gene of a broad spectrum of HPV types (AmpliTaq Gold DNA polymerase; Applied Biosystems, Foster City, CA, USA). LiPA is capable of detecting 26 HPV types: high-risk HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and 70; low-risk HPV genotypes 6, 11, 34, 40, 42, 43, 44, 53, 54 and 74. The assay was performed according to the manufacture's protocol. For the detection of E6/E7 mRNA of HPV types 16, 18, 31, 33 and 45 the PreTect HPV-Proofer Kit (referred to as the mRNA test) (NorChip, Klokkaarstua, Norway) was used, according to the manufacturer's instructions. The mRNA was extracted using Rneasy Mini Kit (QIAGEN, Gaithersburg, MD, USA).

The diagnosis of BV was based on the presence of at least three of four Amsel's criteria: 1) thin, gray/white discharge; 2) malodorous "fishy" discharge upon adding 10 % potassium hydroxide; 3) high vaginal pH (>4.5), and 4) identification of vaginal epithelial cells heavily coated with bacteria, "clue cells".

Statistical Analysis

Patient age, number of follow-up months, number of follow-up check-ups with a pathologic finding (presence of BV, HPV-m RNA positivity, relapse of the lesion) per patient, as mean ± standard deviation and median with range were described. We used patient stratified, time-dependent Cox regression models to evaluate the effect of the age of the patient, the presence of BV and HPV-m RNA positivity during each follow-up on the occurrence of relapse at the check-ups. Time-dependent Cox regression models allow to study the effect of time-varying risk factors on the incidence of a recurrent event during multiple observations of one patient, while the standard Cox model evaluates only the risk that such event can happen just once per patient.

We first analyzed the univariate relationship between the observed variables and the occurrence of relapse of the lesion; in this analysis, age was grouped into four categories (27-35, 36-42, 43-49, 50-58 years) and the younger age group was used as reference. Then, we built a multivariable model which evaluate also how the interaction between presence of BV and HPV-m RNA positivity influences such risk. Finally, we evaluated the effect of this interaction separately with a multivariable model for each age group, in order to evaluate the influence of age on such relationship. We report incident rate ratios (IRR), which represent the multiplicative effect on the risk of a relapse at each check-up, together with the 95% Confidence Intervals (95% CI), the statistic of the relationship

Table I. Number of women who have had at least one relapse, a diagnosis of BV, a positive HPV mRNA test to the controls, mean ± SD number and range of these women's events.

	No. of patients	Average ± DS women's events	Max – min women's events
Relapse	23 (22.1%)	0.683 ± 1.4	0 – 5
Presence of BV	51 (49.0%)	1.9 ± 1.99	0 – 5
HPV mRNA pos.	36 (34.6%)	0.865 ± 1.34	0 – 5

Table II. Statistical relationship between the variables and the risk of relapse. The table shows the results of three separate models, independent of each other.

Age group	aHR	IC 95%	Statistics	*p*-value
36-42 years	0.906	[0.238, 3.44]	-0.145	0.884 ns
43-49 years	0.957	[0.298, 3.08]	-0.0732	0.942 ns
50-58 years	1.36	[0.41, 4.49]	0.5	0.617 ns
Presence of BV	1.74	[0.73, 4.15]	1.25	0.211 ns
HPV mRNA pos.	10.1	[4.83, 21]	6.17	<0.001 ***

and the p-value. When describing multivariable models, we reported the adjusted IRR (aIRR). Data analysis and plotting were performed using the R statistical language v. 3.2.0.

Results

The average age of patients was 42.5 ± 8.92 years (median: 42.5, range from 27 to 58 years). The minimum follow-up was 6 months and the maximum 36 months (average: 22.8 ± 4.53, median: 24).

In Table I are shown the data relating to the CIN lesion's relapse, the presence of BV and positive of HPV-mRNA test. In Table II are described the variables: patients' age, presence of BV, positivity to HPV-mRNA test. Figure 1 shows financial results of the Cox models for each of the 2 table variables.

We divided the age of the patients into quartiles (27-35, 36-42, 43-49, 50-58 years) (Figure 1): in the first quartile, 23% of patients relapsed, in the second one 19% relapsed, in the third one

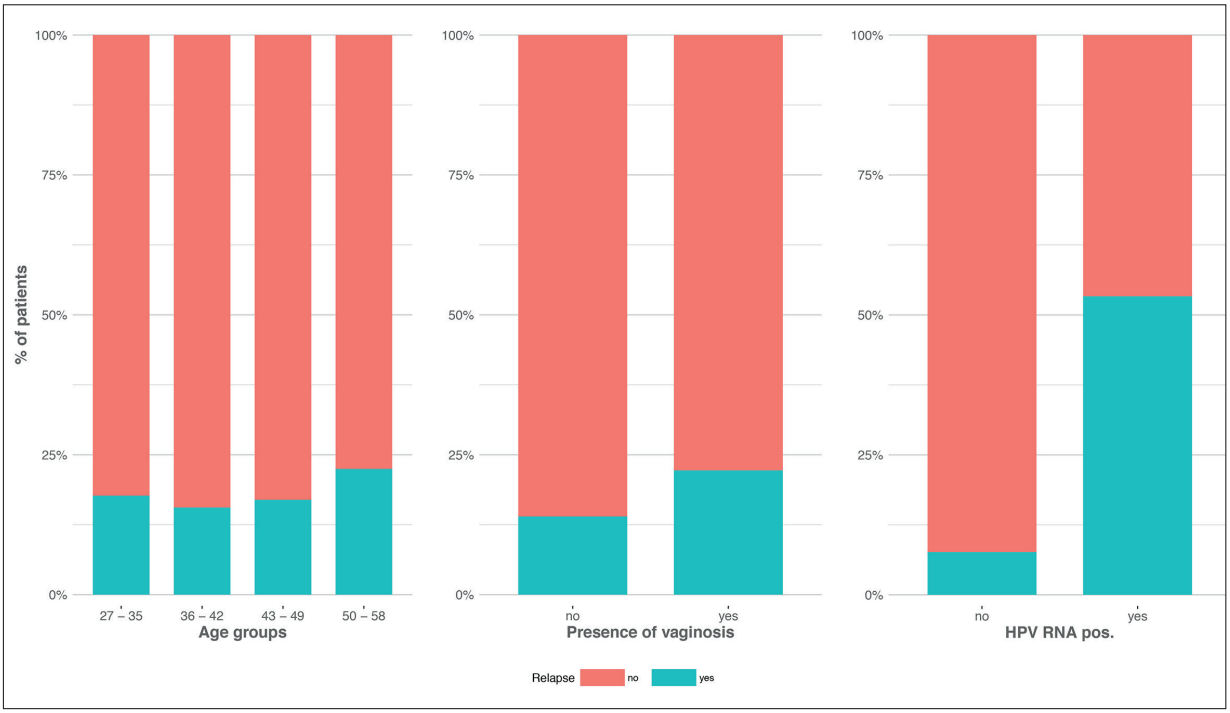

Figure 1. Relationship between risk of relapse and the age group, presence of BV and positive to mRNA test.

Table III. Model with independent effect and interations between the presence of BV, positive mRNA test and risk of relapse.

Age group	aHR	IC 95%	Statistics	*p*-value
HPV mRNA pos.	18.5	[6.17, 55.2]	5.22	<0.001 ***
Presence of BV	2.9	[0.767, 11]	1.57	0.116 ns
BV X mRNA pos.	0.369	[0.0886, 1.54]	-1.37	0.171 ns

23% relapsed, in the last one 18% relapsed. Figure 1 shows the relapse based on the presence of BV. Among the 53 patients without BV, the 20% had relapsed compared with 23% of 51 patients with diagnosis of BV.

Finally, Figure 1 shows the relapse based on positivity of mRNA test. The 10% of the patients with HPV-mRNA test negative had relapsed compared to 45% of patients with HPV-mRNA test positive. Table III shows the independent contribution of the presence of BV and HPV-mRNA on the risk of relapse and the interaction between these two factors.

In Table IV is shown as the relationship between BV, HPV-mRNA, relapse and age, changes according to age group. In Figure 2, the graphic rendering of mentioned relationship is summarized.

Discussion

Human papillomavirus (HPV) is one of the most common sexually transmitted infection[8].

The natural history of HPV demonstrates that the virus can disappear naturally[9] and the lesions may undergo a spontaneous regression[10]. Persistent high-risk HPV infection is associated with neoplastic lesions of the uterine cervix; therefore, many studies suggest that HPV is a necessary, but not sufficient, condition for cervical carcinogenesis[8].

Risk factors for HPV disease can be young age at first intercourse, multiple sexual partners, cigarette smoking, race, high parity, oral contraceptive use and low socio-economic status[11,12].

Since 1970s several trials report a possible association between vaginal microenvironment, BV, and cervical carcinogenesis that it is actually debated.

Hudson et al[13] found that in phase of squamous metaplasia, basic pH typical of BV could make the transformation zone more vulnerable to agents promoting dysplasia such as HPV. According to the study of Bauer et al[14] lactobacilli could have a protective effect through oxygen species interaction: producing lactobacilli could be a natural anti-cancer in vaginal microbioma of healthy women. In the presence of bacterial vaginosis, the depletion of lactobacilli and a relative absence of hydrogen peroxide (H_2O_2) alters this microbicidal vaginal environment reducing anti-tumor effect. Furthermore, biochemical changes in vaginal secretions of women with BV include the presence of nitrosamine produced by anaerobic bacteria: harmful substance, capable of forming DNA adducts and consequently mutagenic events and

Table IV. Variable model that influence relapse, divided by age groups.

	aHR	IC 95%	Statistics	*p*-value
27-35 years				
Presence of BV	32.2	[5.75, 181]	3.95	<0.001 ***
HPV mRNA pos.	1.13	[0.0634, 20.1]	0.0821	0.935 ns
BV X mRNA pos.	0.748	[0.0546, 10.2]	-0.218	0.828 ns
36-42 years				
Presence of BV	4.16e+09	[1.61e+09, 1.07e+10]	45.9	<0.001 ***
HPV mRNA pos.	3.9e+08	[4.51e+07, 3.37e+09]	18	<0.001 ***
BV X mRNA pos.	7.9e-10	[1.5e-10, 4.16e-09]	-24.8	<0.001 ***
43-49 years				
Presence of BV	30	[4.46, 202]	3.5	<0.001 ***
HPV mRNA pos.	9.48	[0.935, 96]	1.9	0.057 .
BV X mRNA pos.	0.277	[0.0283, 2.71]	-1.1	0.270 ns
50-58 years				
Presence of BV	6.06e-08	[5.63e-09, 6.51e-07]	-13.7	<0.001 ***
HPV mRNA pos.	1	[0.141, 7.16]	0.00347	0.997 ns
BV X mRNA pos.	1.03e+08	[5.97e+06, 1.77e+09]	12.7	<0.001 ***

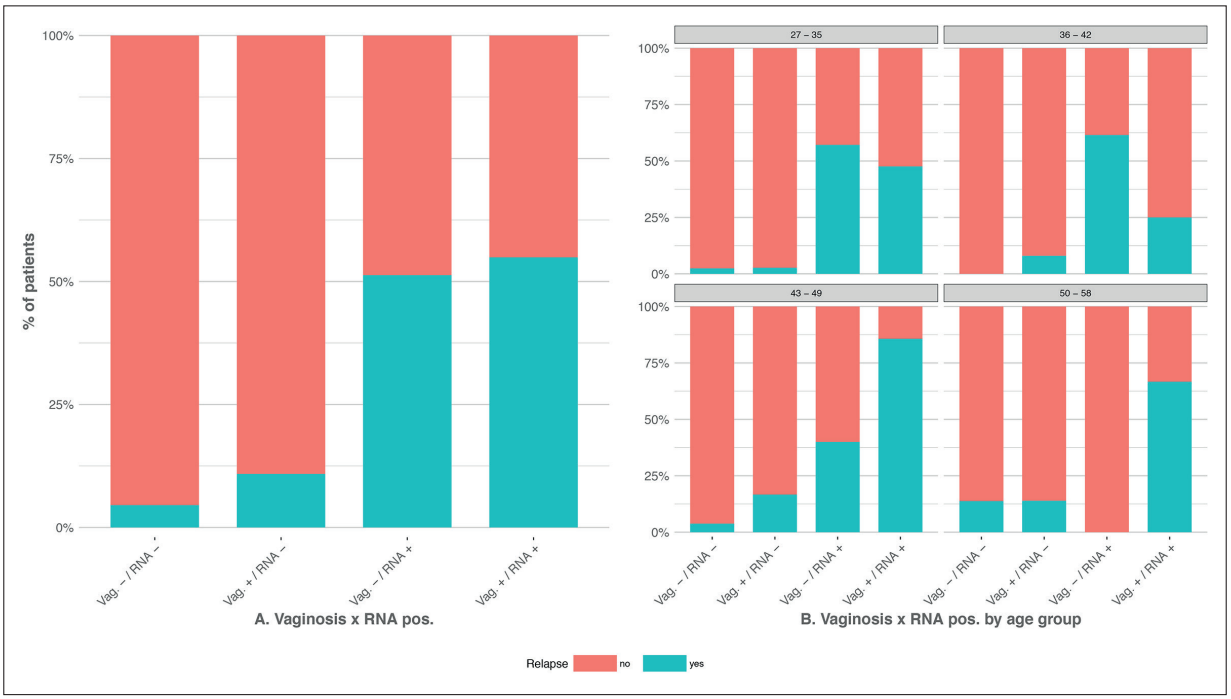

Figure 2. Relationship between relapse and age group, presence of BV, positive HPV mRNA test.

metabolic by-products, like propionate and butyrate, capable of damaging epithelial cells[15,16] and interfere with the TLR-mediated inflammatory response[17,18]. Briselden et al[19] reported a positivity for mucin-degrading enzymes, in particularly sialidases, in 84% of BV-positive women. These enzymes destroy the gel layer coating the cervical epithelium, promoting susceptibility to cervical HPV infection[19,20].

Gardnerella vaginalis, anaerobic bacteria frequently involved in BV, through sialidases, degrades host defence molecules such as Immunoglobulin-A (IgA) against G vaginalis hemolysin[21]. This enzyme removes sialic acid from secretory IgA and consequently reduces local immune response[22].

Furthermore, studies investigating the BV role in vaginal immunity disorders and, in the decreased clearance of high-risk papilloma virus infection, it assumed a favorable factor of vaginosis in the persistence of HPV infection[23-24].

BV and HPV infection occur in sexually-active women, but their temporal relationship is still controversial. Mao et al[26] reported that HPV infection generally occurs first or at the same time as BV, while Guo et al[24] showed that BV is previous to HPV infection and that a vaginal microenvironment is a protective factor.

Indeed the composition of vaginal flora and commensal vaginal Lactobacillus spp play an important role against urogenital diseases, such as bacterial vaginosis, sexually transmitted infections or urinary tract infections. Ravel et al[27] reported in their study five major community state types present in vaginal microbiota: the vaginal microbioma characterized by low numbers of *Lactobacillus* and higher diversity of anaerobic bacteria (typical of BV) has been associated with slow regression of HPV[28]. According to Mitra et al[29], this vaginal microenvironment can be associated with higher incidence of HPV infection and CIN; through the use of Next Generation Sequencing (NGS) they identified 5 major community – state types (CST): CST I, II, III, V characterized by *Lactobacillus crispatus, L. gasseri, L. iners* and *L. jensenii* respectively and CST IV characterized by low numbers of *Lactobacillus* spp. and higher of presence of anaerobic bacteria. The results of their study showed that CST IV microbiome is associated with greater risk of progression of HPV infection and of CIN severity. Indeed the differences in the composition of the vaginal microenvironment may be correlated with increasing severity of HPV lesion. In the presence of LSIL there is a significant concentration of Lactobacilli (*lactobacillus: jensenii* p-value < 0.01, *coleohominis* p-value < 0.05) while in the presence of HSIL, high levels of enterobacteriaceae were found as *Peptostrep-*

tococcus anaerobium (*p*-value applies < 0.05) and *Anaerococcus tetradius p*-value <0.05, *Fusobacteria* to *Sneathia sanguinegens p*-value < 0.01. However, the relationship between BV and cervical intraepithelial neoplasia is still debated.

A clinical trial[30] proved that cervical cytological abnormalities are more frequently in women with a disrupted vaginal flora and suggested a possible link between BV and the development of cervical cancer. Another retrospective cohort-study[32] described in follow-up smear of women with BV higher rate of LSIL and HSIL (OR 1.89, 95% CI 1.41-2.52).

Lehtovirta et al[31] found an increasing risk of CIN in patients with BV in an univariate analysis (Hazard ratio 1.85, 95% CI 1.04-3.28) and in 2012, Gillet et al[33] evaluated the association between BV and CIN in a meta-analytic survey.

Even if there was substantial heterogeneity among the various studies, (X^2 = 164.7) a positive correlation between BV and CIN (odd ratio 1.51; 95% CI, 1.24-1.83) has been found, in patients showing positive Pap test or testing positive for vaginal smear and for HPV-DNA test. However, this correlation could be affected by the presence of other risk factors, therefore we enrolled in our study patients, without others risk factors[34] for the CIN. In literature, the relationship between BV and onset of CIN has been widely analyzed compared to the relationship between BV and relapse of CIN subsequently LEEP surgery. Therefore we focused on the recurrence in patients previously surgically treated for CIN2+ after LEEP. Our data showed that the BV has a role in the risk of relapse (1.74 aHR), but the risk assessment has been made difficult by the presence of not considered confounding and interaction factors and the small number of patients (*p*-value = 0.211 ns). We divided the patients into quartiles to evaluate the role of age on the risk of relapse but the results obtained were not statistically significant (age 27-35 *p*-value =, age 36-42 *p*-value = 0.884, age 50-58 *p*-value = 0.617). We also decided to analyze the role use of viral biomarkers as HPV DNA test or HPV mRNA test in the presence of cervical HPV infection because they can improve a correct clinical approach.

A positive HPV-DNA test for 12-24 months represents a risk index of the onset of CIN. Our previous study[35] analyzed the relationship BV and CIN and whether this correlation was increased in the presence of HPV-DNA positive test. In the current study, has been employed not only the HPV-DNA test, but also the HPV-mRNA test that is based on the lesser expression of viral oncoproteins in transient infections than transformative. This molecular test was used to evaluate the follow-up and relapse of CIN2+ after LEEP. We observed no important differences about the presence of HPV in the two groups as the patients were tested positive for HPV DNA test. Furthermore, the results of our study showed the relationship between the positivity of HPV- mRNA test and the occurrence of recurrences: it was greater than ten times of controls with mRNA negative test. In Table III and Figure 2a, we analyzed the interaction between VB and the positivity of HPV- mRNA test. The patients were divided into four groups (VB / mRNA-; VB+ / HPV-mRNA-; VB- / HPV-mRNA+; VB+ / HPV-mRNA+). In the first and second group (HPV- mRNA negative) of Figure 2, the presence of the VB increased the risk of recurrence, but not significantly (*p*-value = 0.116 ns), while in the third and fourth group (HPV mRNA positive) the recurrence appeared in a higher rate of cases. Analyzing the data of the fourth group the simultaneous presence of VB and HPV-mRNA positive test increased the risk of recurrence, but less than the sum of the effects of the two variables (*p*-value: 0.171 ns). Referring to Figure 2b and Table IV, the positivity to BV in presence of HPV-mRNA did not modify the risk of relapse in age groups between 27-35 years (*p*-value = 0.935 ns) and between 50-58 years (*p*-value = 0.997), and conversely the positivity to BV in presence of HPV- mRNA modified the risk of recurrence of CIN in age groups between 36-42 years (*p*-value < 0.001) and 43-49 years (*p*-value < 0.059). Our results show that the presence of VB and of mRNA decreases the risk of recurrence in first, second and third age group, especially in second age group (36-42 years). Instead, HPV-mRNA positivity seemed to have a protective role on relapse in older patients, but the interaction between the BV and HPV-mRNA in this group seemed to promote the relapse.

The division into age groups decreases the size of the sample becoming a limiting factor for the clinical results.

Conclusions

No significant statistical differences can be demonstrated comparing the results between the positive BV group and the negative BV group in terms of cervical intraepithelial lesions' relapse.

Further studies would be needed to define the role of BV and its correlation with CIN occurrence in a larger cohort of patients.

Conflict of interest

The authors declare no conflicts of interest.

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