Determination of Cervicovaginal Microorganisms in Women with Abnormal Cervical Cytology: The Role of *Ureaplasma urealyticum*

A. LUKIC[1], C. CANZIO[1], A. PATELLA[1], MR. GIOVAGNOLI[2], P. CIPRIANI[3], A. FREGA[1] and M. MOSCARINI[1]

Departments of [1]Gynecologic Sciences, Perinatology and Puericulture, [2]Cytopathology and [3]Public Health, Second Faculty of Medicine and Surgery, "La Sapienza" University of Studies of Rome, Sant'Andrea Hospital, Rome, Italy

Abstract. *Objective: To evaluate the existence of an association between cervicovaginal infections and precancerous lesions of the uterine cervix, through determination of prevalent cervicovaginal micro-organisms, alone and in association with human papillomavirus (HPV), in patients with abnormal and normal vaginal cytology. Patients and Methods: Patients with abnormal vaginal cytology were divided into three study groups according to cytological findings: ASC-US, L-SIL and H-SIL. All patients underwent colposcopic examination and exo-endocervical and vaginal sampling for microbiological and molecular analysis for detection of HPV-DNA, Ureaplasma urealyticum, Chlamydia trachomatis, Trichomonas vaginalis, mycetes and common bacteria. Results were compared with the patient group asymptomatic for cervicovaginal inflammation with negative vaginal cytology and colposcopy. Results: A high association between Ureaplasma urealyticum infection and the grade of cytological cervical lesion (27% for ASC-US, 35% for L-SIL and 45% for H-SIL) was found. Furthermore, 19% of the control group samplings were positive for Ureaplasma urealyticum, significantly less than that observed in the positive cytology groups. An interesting association of HPV with Ureaplasma urealyticum in patients with H-SIL vaginal cytology (83%), much higher than that observed in patients with slightly abnormal or normal vaginal cytology (56% for ASC-US, 49% for L-SIL, 40% for normal cytology) was also identified. In contrast, the association between Papillomavirus and multiple microorganisms seemed to decrease with the level of cellular dysplasia in 30% of controls, 33% of ASC-US, 32% of L-SIL and 17% of H-SIL. Conclusion: The presence of a high Ureaplasma urealyticum level seems to be a cofactor of HPV infection, a necessary cause of precancerous lesions of the uterine cervix. The presence of Ureaplasma urealyticum may play a role both in initiating viral cellular anomalies and in viral persistence. It can be hypothesized that these initial processes are helped by a state of cervical inflammation, also supported by multiple microorganisms. It would, thus, be suggested for all patients who present with an abnormal PAP test to undergo a cervicovaginal microbiological examination to detect potentially pathogenic microbes for correct diagnosis and treatment, as well as a more complete follow-up of cervical cytological lesions.*

The human papillomavirus (HPV) is one of the most common sexually transmitted pathogens and is strongly associated with pre-neoplastic and neoplastic lesions of the uterine cervix.

It has been suggested that HPV infection alone may not be sufficient to promote cervical carcinogenesis and that other cofactors could be involved, such as smoking, oral contraceptives, immunosuppression, vitamin deficiency and other sexually transmitted diseases (1-3).

Many pathogenic agents have been studied both as risk factors *per se* and as cofactors interacting with HPV in the development of precancerous and cancerous lesions of the uterine cervix, with the conclusion that some sexually transmitted agents could be directly or indirectly involved in the development of cervical lesions, through production of metabolites or other carcinogenic substances, or by increasing the susceptibility of the inflamed epithelium (1).

Whether the herpes simplex virus (HSV) is implicated or not in the development of cervical lesions has been the subject of long discussion; the most recent molecular studies did not convincingly demonstrate the association of HSV infection with cervical lesions (4, 5). In one of our previous studies, the presence of HSV-1 and HSV-2 infection had

Corresponence to: A. Lukic, MD, Sant'Andrea Hospital, Via Michele Amari 47, 00179, Rome, Italy. Tel: +390633775479, Fax: +39063350611, e-mail: luki.anki@tin.it

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already been reported in patients positive for HPV DNA, who presented with an abnormal transformation zone grade 1 (ANTZ-G1) in colposcopic examination (6). In contrast, a recent IARC case-controlled multicenter study (7) has shown strong evidence of HSV-2 as a cofactor of HPV in increasing the risk of developing invasive cervical carcinoma: HSV-2 seropositivity was significantly higher among case patients with invasive squamous-cell carcinoma (44.4%) or adeno- or adenosquamous-cell carcinoma (43.8%) than among the control subjects (25.6%), while among the HPV-positive women, HSV-2 seropositivity was associated with an increased risk of such neoplasia. However, anti-HSV antibodies and precancerous lesions may have only a chance association, as both are linked to the woman's sexual habits (8).

The possible association of *Trichomonas vaginalis (Tv)* with cervical lesions has been studied since the 1950s. Zhang and Begg (9) showed a relative risk (RR) of 1.93, indicating a doubled risk of developing intra-epithelial lesions in the presence of Tv. Despite this, the exact mechanism by which Tv exerts its influence is still obscure (8).

The direct role of *Chlamydia trachomatis (Ct)* infection as a risk factor in the development of cervical lesions is rather controversial. Strong epidemiological evidence suggests that combined infection with HPV and *Ct* plays a central role in the etiology of intra-epithelial lesions of the uterine cervix and represents a risk for the subsequent development of invasive cervical neoplasia when associated with other factors, such as smoking and sexual promiscuity (2, 3, 10-12). However, other authors have suggested that infections due to HPV or *Ct* are independently-occurring factors and that there is no association between the two in the pathogenesis of cervical intra-epithelial lesions (13).

The possible relationship between *Bacterial vaginosis (Bv)* and cervical intra-epithelial lesions has been hypothesized since the 1970s. Platz-Christensen *et al.* (14) found the presence of grades 1, 2 and 3 cervical intra-epithelial neoplasia (CIN) in 5% of women who also presented *Bv* and only in 1.4% of women without *Bv*. The percentage was similar for women with CIN grades 2 and 3 only: 2.9% in women with *Bv* and 0.4% in women without *Bv*, respectively. The authors then calculated an RR of CIN 3/Ca *in situ* of 5.0 and an RR of CIN 1, 2, 3 of 8.0 for women with *Bv*. Eltabbakh *et al.* (15) found that 50% of women who presented with a cervical abnormality at the Pap-test had a cervicovaginal infection, 28% of which were *Bv*. In contrast, Peters *et al.* (16) found no association between *Bv* and intra-epithelial lesions.

Some authors (6, 18) found that women with cervical cytological abnormalities presented with higher frequencies of *Mycoplasma hominis* and *Ureaplasma urealyticum (Uu)* infection (high concentration). In light of this evidence, it was suggested that patients who presented with abnormal PAP tests should undergo cervicovaginal microbiological examinations for potential pathogens, especially before any treatment of squamous intra-epithelial lesions (SIL) and even more in persistent or recurrent cases of papillomavirus infections (6).

The purpose of this study was to evaluate the existence of an association between cervicovaginal infections and pre-neoplastic lesions of the uterine cervix through determination of single microorganisms in various cytological groups.

Patients and Methods

From the patients attending the Sant'Andrea Hospital Colposcopy and Cervicovaginal Pathology Unit, II Faculty of Medicine and Surgery, "La Sapienza" University of Rome, Italy, from June 2003 to December 2004, a sample of 239 patients with abnormal vaginal cytology was selected. The patients were sexually active, asymptomatic, aged 18 to 50, pre-menopausal, not pregnant, HIV-negative and had not been undergoing any oral contraceptive, antibiotic, immunosuppressive or topical therapy for at least one month prior. Informed consent was obtained from all women participating in the study. The patients were divided by cytological report into three study groups: 66 (18.6%) presented atypical squamous cells of undetermined significance (ASC-US), 115 (65%) presented low-grade squamous intra-epithelial lesions (L-SIL) and 58 (16.4%) had high-grade squamous intra-epithelial lesions (H-SIL). All patients underwent exo-endocervical and vaginal smears for molecular and microbiological examinations and colposcopic examination. In particular, HPV DNA, *Uu, Ct, Tv*, mycetes and common bacteria in the cervicovaginal secretion were studied. In all cases, colposcopy was carried out by the same operator.

In the control group of 118 pre-menopausal, asymptomatic patients with normal vaginal cytology and colposcopy and with similar characteristics (age, therapy, risk factors) as those of the case group, cervicovaginal microbiological sample examination included the study of HPV DNA, *Uu, Ct, Tv,* mycetes and common bacteria as well. The evaluation of colposcopic signs of inflammation was performed before collecting cervicovaginal smears.

PAP test. Material was collected from the uterine cervix by rotating the Ayre brush 360˚ around the uterine cervix for ectocervix smears and by completely introducing and rotating the cytobrush at least 45˚C in the cervical canal for endocervix smears. The two specimens were streaked onto a single slide and set with a fixative spray. The cytological preparations were sent to the cytopathology laboratory for staining and reading through the optical microscope. The cytological report utilized the 2001 Bethesda System (19) and identified 3 subgroups of patients with abnormal vaginal cytology: 1 – those with atypical squamous cells of undetermined significance (ASC-US); 2 – those with low-grade squamous intraepithelial lesion (L-SIL); 3 – those with high-grade squamous lesion (H-SIL).

HPV DNA study. The Hybrid Capture II microplate method was used for the detection of high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 and low-risk HPV types 6, 11, 42, 43, 44 (17). Specimens were processed according to the manufacturer's instructions, using 5% of the cervical specimen for testing. Triplicate assay controls representing 1 pg/ml of HPV DNA and a negative control of carrier DNA in specimen transport medium were included.

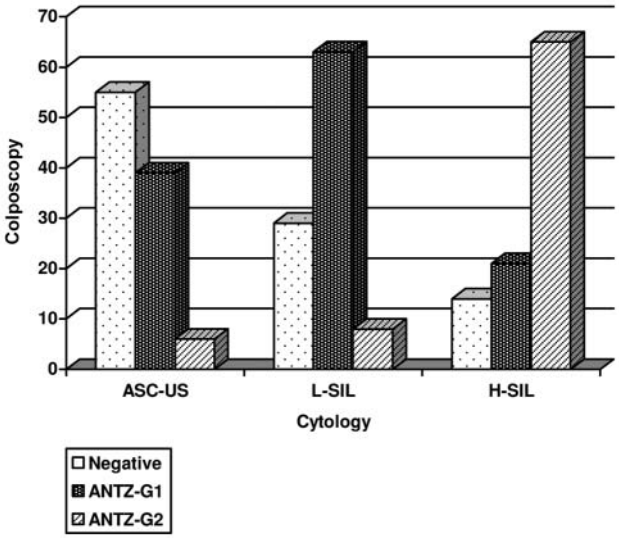

Figure 1. *Relationship between cytological findings and colposcopy. ASC-US: Abnormal cervical cytology of undetermined significance. L-SIL: Low-grade squamous intra-epithelial lesions. H-SIL: High-grade squamous intra-epithelial lesions.*

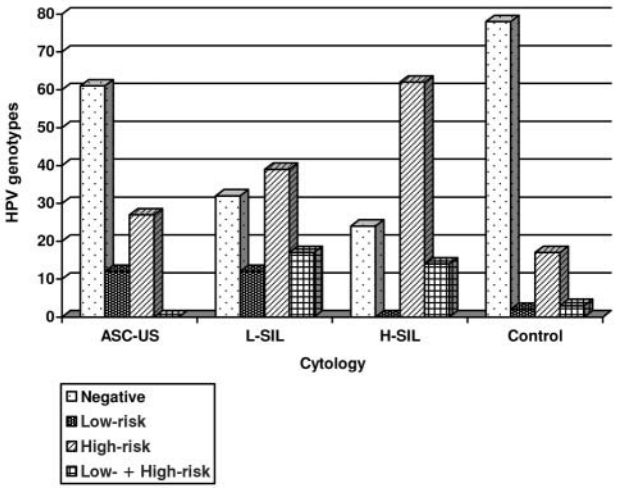

Figure 2. *Relationship between cytological findings and human papillomavirus (HPV) genotypes. ASC-US: Abnormal cervical cytology of undetermined significance. L-SIL: Low-grade squamous intra-epithelial lesions. H-SIL: High-grade squamous intra-epithelial lesions.*

Specimens were considered positive for high-risk and/or for low-risk HPV if their assay chemiluminescence was at least that of the average of three positive assay controls.

Detection of Uu. Detection of *Uu* was conducted through a culture examination of the collected material, using the Mycoplasma IST 2 kit. The sampling was carried out both at the endocervix, inserting a plug approximately 1 cm into it, and at the ectocervix.

Samples were considered as positive for *Uu* for values $>10^4$ UFC.

Detection of Ct. Ct was detected using a special slide for carrying out direct immunofluorescence with monoclonal antibodies marked with fluorescein (IFD). The smear was collected at the endocervix, inserting the plug beyond the squamocolumnar junction.

Detection of mycetes, common microbes, and Tv. Mycetes and common microbes were detected through cultural examination of the material collected by sterile plugs from the vaginal posterior fornix, while *Tv* was detected by fresh bacterioscopic examination.

Colposcopy. Saline solution, 5% acetic acid solution and 5% Lugol's iodo-iodized solution, in that order, were used for Schiller's Test. The colposcopic profiles were classified in conformance to the 1990 International Federation of Cervical Pathology and Colposcopy Classification (IFCPC) (20) and led to the identification of 3 subgroups of patients: 1 – normal colposcopic pattern (native squamous epithelium, ectropion, normal zone of transformation, cervicitis, *etc*.); 2 – abnormal transformation zone grade 1 (ANTZ G1); 3 – abnormal transformation zone grade 2 (ANTZ G2). A cervical biopsy was performed in all cases of high grade transformation zone on recruitment. In all cases, colposcopy was carried out by the same operator who evaluated colposcopic signs of inflammation in the control group patients before collecting cervicovaginal smears.

Statistical analysis. A logistic regression model and the Chi-square test were applied.

Results

In the ASC-US group, 55% of the 66 patients presented with a normal colposcopic pattern, 39% were positive for abnormal transformation zone grade 1 (ANTZ G1), while the remaining 6% presented with an abnormal transformation zone G2 (ANTZ G2) (Figure 1). The HPV DNA study of the exo-endocervical secretion was negative in 61%, positive for low risk oncogenic HPV DNA in 12% and positive for high risk HPV in 27% (Figure 2); microbiological examination of the cervicovaginal secretion was positive for *Uu* in 27%, mycetes in 9%, *Ct* in 3%, Streptococcus agalactiae (Sa) in 12%, enterococci in 6% of cases, multiple agents (*Ct* + *Sa*; *Uu* + myces) in 9% and was negative in 34%, with the PAP test showing evidence of ASC-US (Figure 3).

Of the 115 L-SIL patients, 29% presented with a normal profile on colposcopy, 63% ANTZ-G1 and 8% ANTZ-G2 (Figure 1). The viral DNA study was negative for HPV DNA in 32%, positive for low risk HPV DNA in 12%, positive for high risk HPV DNA in 39% and positive for low and high risk HPV DNA in 17% (Figure 2). The cervicovaginal secretion was positive for *Uu* in 35%, mycetes in 4%, *Ct* in 4%, *Sa* in 4%, enterococci in 4%, *Tv* in 1%, multiple agents (*Uu* + *Ct*; *Uu* + myces; *Uu* + Sa; *Uu* + *Ct* + Tv; *Uu* + *E. coli* + Enterococci; *Uu* + Gardnerella; *Ct* + myces, *Sa* + myces) in 23% and was negative in 25% (Figure 3).

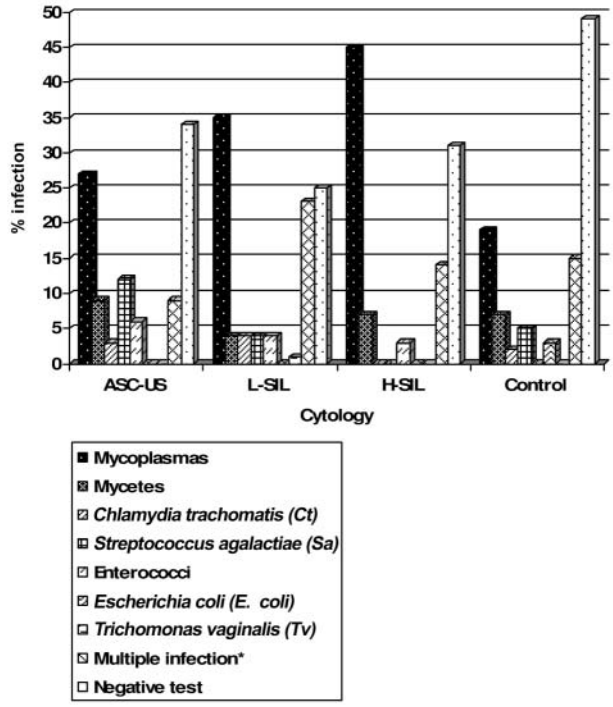

Figure 3. *Microbiological tests in various cytological groups and control group. ASC-US: Abnormal cervical cytology of undetermined significance. L-SIL: Low-grade squamous intra-epithelial lesions. H-SIL: High-grade squamous intra-epithelial lesions. *Multiple infection: ASC-US: 4 Ureaplasma urealyticym (Uu) + myces; 2 Ct + Sa. L-SIL: 4 Uu + Ct; 5 Uu + myces; 6 Uu + Sa; 2 Uu + Ct + Tv; 3 Uu + E. coli + Enterococci; 3 Uu + Gardnerella; 2 Ct + myces, 2 Sa + myces. H-SIL: 4 Uu + E. coli; 4 Uu + Sa. Control: 5 Uu + myces ; 4 Uu + Ct ; 9 Uu + Staphylococcus.*

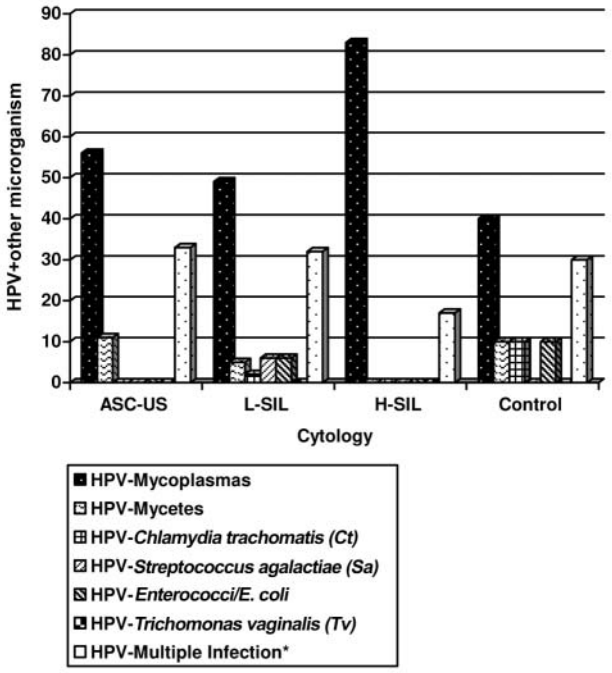

Figure 4. *Relationship between cytological findings and Human Papillomavirus (HPV) + other microorganism co-infections. ASC-US: Abnormal cervical cytology of undetermined significance. L-SIL: Low-grade squamous intra-epithelial lesions. H-SIL: High-grade squamous intra-epithelial lesions. *Multiple infection: ASC-US: 4 Ureaplasma urealyticum (Uu)+ Myces; 2 Ct + Sa. L-SIL: 3 Uu + Ct; 4 Uu + Myces; 4 Uu + Sa; 1 Uu + Ct + Tv; 2 Uu + E. Coli + Enterococci; 1 Uu + Gardnerella; 1 Ct + Myces, 1 Sa + Myces. H-SIL: 2 Uu + E. coli; 2 Uu + Sa. Control: 2 Uu + Myces ; 2 Uu + Ct ; 2 Uu + Staphylococcus.*

In the H-SIL group, 14% of the 58 patients presented a normal or unsatisfactory colposcopy, 21% showed ANTZ-G1 and 65% presented ANTZ-G2 (Figure 1). The viral DNA study was negative for HPV DNA in 24%, positive for high-risk HPV DNA in 62% and positive for low- and high-risk HPV DNA in 14% (Figure 2). The cervicovaginal secretion was positive for *Uu* in 45%, mycetes in 7%, enterococci in 3%, multiple agents (*Uu* + *Sa*; *Uu* + *E. coli*) in 14% and was negative in 31% (Figure 3).

The simultaneous presence of HPV and other microorganisms was observed in 18 (27%) cases of ASC-US, in 53 (46%) cases of L-SIL and in 24 (41%) cases of H-SIL patients and in only 20 (17%) cases of controls; in these co-infection groups an association of HPV and *Uu* was observed (56% in ASC-US patients, 49% of L-SIL patients and 83% of H-SIL patients with simultaneous presence of HPV and other microrganisms) (Figure 4). In addition, the association between HPV and multiple microorganisms compared with the level of cellular dysplasia was observed in 30% of controls, in 33% of ASC-US, in 32% of L-SIL and in 17% of the H-SIL cases.

On recruitment, a biopsy was performed for 39% of women with ASC-US, 37% of those with L-SIL and all H-SIL during colposcopic examination. The 8 patients with cytological H-SIL and negative colposcopy underwent LEEP (negative histology in 2 cases only).

The correlation between cytology and histology is illustrated in Figure 5: 30% of ASC-US and 44% of L-SIL cases were associated with the histological pattern of koilocytosis, while 97% of the severe atypical cytology cases were associated with the histological patterns of CIN 2-3.

Of the 118 control patients of the control group, 49% were negative on microbiological study, 19% were positive for *Uu*, 8% for mycetes, 2% for *Ct*, 5% for *Sa*, 3% for *Escherichia coli (Ec)* and 15% for multiple infection (*Uu* + myces ; *Uu* + *Ct* ; *Uu* + Staphylococcus) (Figure 3). In no case were *Gardnerella vaginalis* (*Gv*) or *Tv* found in the cultural examination.

HPV-DNA was found in 22% of patients with normal cytology (Figure 2). In cases with HPV in association with other microorganisms in patients with negative cytology, the

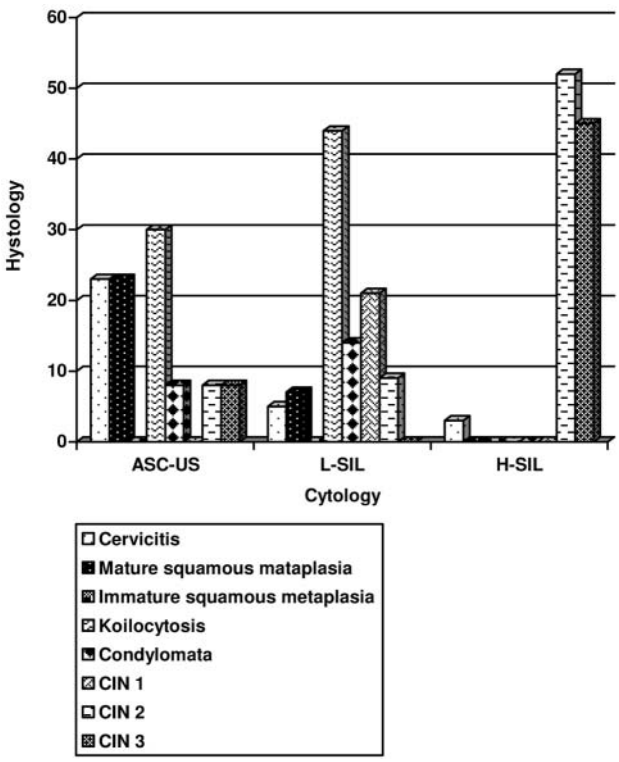

Figure 5. *Relationship between cytological reports and histology. ASC-US: Abnormal cervical cytology of undetermined significance. L-SIL: Low-grade squamous intra-epithelial lesions. H-SIL: High-grade squamous intra-epithelial lesions.*

virus was associated more frequently with *Uu* (40%) and multiple infection (30%) (Figure 4).

Discussion

Sexually transmitted diseases (STD) other than HPV have been considered as possible cofactors in the pathogenesis of carcinoma of the uterine cervix, even if no single agent has been identified as particularly significant (21). In the absence of specific pathogens such as HSV, Chlamydia and other oncogenic risk factors, such as smoking and estroprogestinic agents, bacterial vaginosis has been considered as an important cofactor in the persistence and/or progression of high-grade lesions in women infected with HPV (21, 22). Some authors studied SIL prevalence in the population with cervicovaginal infection, a risk factor for the development of cervical pre-neoplastic lesions.

In a microbiological study of STD Eckert and colleagues reported 17% SIL in the STD population compared to 8% SIL in the control population and a high percentage of Pap tests not assessable for SIL due to strong inflammation (23). In a cohort study of more than 10,000 women, Castle *et al.* (21) found a statistically significant risk – two-fold increase – of high-grade lesions for women who presented cervicitis at the Pap test compared to those who were negative.

In our study the prevalence of the infections in a population with cellular atypia was examined.

In a study on the importance of the microbial flora in CIN pathogenesis in 106 cases (including CIN 1-3) and 74 negative controls, Guijon *et al.* (18) have reported respectively 41.9% and 24% smear positivity for *Mycoplasma hominis* infection ($p=0.006$). It has, thus, been hypothesized that *Mycoplasmas* can induce clinical progression from a latent to a subclinical stage of HPV infection by direct interaction with other microorganisms, squamous epithelial cells and the immune system, even without demonstration of a clear causality between *Mycoplasmas* and CIN (6, 18).

The microbiological study in our group of patients showed a low positivity for *Ct*, *Tv*, mycetes, Enterococci and Streptococci, due to which we cannot speculate on the role of such microorganisms as possible cofactors of HPV infection in low- and high-grade lesions.

The presence of infection by Enterococchi, Mycetes, *Sa* and *Tv* were found not to substantially change the odd ratios in any comparisons.

In contrast, our study revealed a high association between *Uu* infection and the grade of cytological cervix lesion (27% for ASC-US, 35% for L-SIL and 45% for H-SIL). Furthermore, only 19% of our control group was positive for *Uu*, clearly less than that observed in the positive cytology groups.

Compared to our observations in the control group, mycoplasma infection frequency was higher in the H-SIL ($p<0.002$) and L-SIL groups ($p<0.004$), while the rate was similar in the ASC-US group ($p<0.12$). These data are comparable to those reported for mycoplasma infection (18).

In addition, a high percentage of association of HPV with *Uu* was found in patients with H-SIL cytology (83%), much higher than that observed in patients with slightly abnormal or normal vaginal cytology (56% for ASC-US, 49% for L-SIL, 40% for normal cytology). The HPV-*Uu* association was stronger in the HSIL group than in the control group ($p<0.004$) or in the LSIL group ($p<0.005$) and it was less strong in the ASC-US group ($p<0.05$). HPV detection increases the risk of developing HSIL compared to the controls in patients with *Uu* infection (OR=10; CI 1,2-82,7).

According to the literature, on a given day, approximately 5% to 27% of normal women will test positive for high-risk HPV DNA (24-28), in contrast to 90% to 100% of women with a high-grade lesion (28-32). Our data show a 20% prevalence of HR-HPV infection in the control group.

Even the presence of high-risk (or high-risk + low-risk) HPV types increased in different subgroups of cytology: 20%, 27%, 56% and 76%, respectively, in normal, ASC-US, LSIL and HSIL cases (Figure 2).

The association between the virus and multiple organisms seems to decrease with the degree of cellular dysplasia (in 30% of controls, 33% of ASC-US and 32% of L-SIL *versus* 17% of H-SIL) without reaching statistical significance. *Uu* was quite always present in cases of HPV infection in association with multiple infection (Figure 4).

The presence of *Uu* may play a role both in initiating viral cellular anomalies and in viral persistence. It can be hypothesized that these initial processes are helped by a state of cervical inflammation, also supported by multiple microorganisms (33-35).

Of the common microorganisms, this study considered only those grown by culture examination. However, there may be others and if so, what is their role? How can we overcome this limit to culture examination and recognize the presence of these microorganisms in the cervicovaginal secretion? When does a saprophytic microorganism take on a pathogenic role?

As colony counting is not applicable to culture examination of the cervicovaginal secretion as it is for urine culture or a pharyngeal swab, diagnosis of common microorganism infection is often clinical. The biological information provided by the cultural examination must in any case be suitably correlated within the clinical picture. Cervicovaginal microbial flora characterization is necessary, including the use of molecular biological technology enabling the identification of the characteristic DNA fragment for each microbial species found. Diagnosis can then be based on this determination, independently of or in association with the microbiological culture.

Microbiological testing of women who present with abnormal Pap tests is clearly extremely important in identifying and treating any inflammations, even if asymptomatic, which might represent a cofactor of HPV infection in the pathogenesis of pre-neoplastic cervical lesions, as well as being a confounding factor in the study of cases with uncertain cytology.

In addition, the treatment of mildly symptomatic or asymptomatic infections, such as those caused by *Mycoplasmas*, also takes on a prognostic significance in terms of the regression of cellular abnormalities and low-grade lesions: eliminating the presence of a pathogen or excessive population of a commensal/saprophytic microorganism which compromises the local immune system also enables the latter to keep better control over HPV, prevent its replication and eliminate infected cells, *i.e.,* the lesion.

It would, thus, be opportune for all patients who present with an abnormal Pap test to undergo a cervicovaginal microbiological examination to detect potentially pathogenic microbes for the correct diagnosis, study, treatment and more complete follow-up of cervical cytological lesions.

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