

Polymorphism of clinical manifestation of HPV infection in the genital mucosa – 3-year retrospective study

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Abstract

Background: Human papillomaviruses (HPVs) are associated with a wide variety of cutaneous and mucosal infections and with malignancies in humans. More than 100 HPV types have been identified, some of which have affinity for skin and others for mucosal sites. **Aim:** The purpose of this study came from our desire to support the "Stop Cervical Cancer" campaign, adopted as "Cervical Cancer Removed as a Public Health Problem", following the *World Health Organization* (WHO) initiative in 2019–2020. At European level, cervical cancer mortality has fallen by more than 30% over the past 30 years, through coherent, consistent and comprehensive prevention programs based on accurate and consistent public information. Romania is in an unfavorable situation with regard to incidence (32.8 new cases/100 000 women) and cervical cancer mortality (10.9 deaths/100 000 women). Free Babeș-Papanicolaou (Pap) testing for early detection of cervical cancer was valid this year until March. **Patients, Materials and Methods:** The study runs over a period of three years, between 2016–2018, in the "Elena Doamna" Hospital of Obstetrics and Gynecology, Iași, Romania. Of the 8500 patients hospitalized for various diseases (ovarian cysts, uterine fibroids, endometriosis, abnormal vaginal bleeding, dysmenorrhea, ectopic pregnancies, *placenta praevia*, spontaneous abortions and on demand, benign, malignant tumors), only 382 were present in the ambulatory for Pap test. For the other conditions, Pap test denied because the patients did not want this but to solve the condition for which they presented themselves. **Results:** We retrospectively review 382 Pap tests of patients who presented themselves in the Ambulatory Service of the Hospital both at the advice of the gynecologist and due to the program initiated by the WHO and supported by the Department of Public Health, Iași: "Cervical cancer can be eliminated as a problem of public health". Lesions equal to or worse than high-grade squamous intraepithelial lesion (HSIL) equvalate with high-grade lesions including HSIL (cervical intraepithelial neoplasia – CIN1, CIN2). Endometrial lesions were excluded from the study. As seen of this campaign, the number of patients has increased in 2018 (242 cases), compared to 46 cases in 2017 and 94 cases in 2016. **Conclusions:** Our study demonstrated that 35% (7/20) of HSIL-confirmed biopsies previously had negative HPV assays. Despite the previous negative HPV tests, a wide variety of HPV genotypes has been detected in most biopsies. In our case, we frequently identified the HPV 59 and 45 strains, 51 cases with HSIL lesions presented a first positive HPV test, 13 cases with low-grade squamous intraepithelial lesion (LSIL) showed three negative HPV tests. Prevention plays an important role in reducing the incidence of cervical cancer cases. The Pap test is now considered the primary prevention method, but consecutive vaccination significantly increases protection against high-risk HPV strains. Education plays an important role in the prophylaxis of HPV infection and cancer. It should be instituted in schools, from puberty age through partnerships or government programs with public health directorates and university hospitals or using European funds.

Keywords: human papillomavirus test, Papanicolaou test, HPV-cytology co-testing high-grade cervical lesion, cervical cancer.

Introduction

Human papillomaviruses (HPVs) are associated with a wide variety of cutaneous and mucosal infections and with malignancies in humans. More than 100 HPV types have been identified, some of which have affinity for skin and others for mucosal sites [1]. Different HPV types can cause common warts, anogenital warts, respiratory papillomatosis, low- or high-grade squamous intraepithelial cervical lesions and cervical, anogenital or oropharyngeal malignancies. HPV genotypes designated as low risk (LR) are usually associated with the development of skin warts and non-carcinogenic lesions and high-risk (HR) genotypes are associated with cancers [2].

The nomenclature of HPV is based on recommendation derived from the *Study Group of Papillomavirus* and established comprises of a diverse group of more than 150 related viruses than have slowly evolved with their

host. HPV is a small double-stranded, non-enveloped deoxyribonucleic acid (DNA) virus, around 60 of these HPV types are detected in the mucosa epithelium and are restricted to the basal cell located in the stratified epithelium [3, 4]. HPV has the ability to exploit their host's enzymatic mechanism, thus guaranteeing a low mutation rate and a high degree of proof reading [5].

HPVs can be divided into the following classes: *alpha*, *beta*, *gamma*, *mu* and *nu*; viruses from the *beta* group can infect cutaneous epithelia, whereas the remaining types are responsible for the transformation of papilloma's that are typically not subject to neoplastic transformation [3, 4]. HPV 6 and 11 subtypes of the *alpha* 10 species are mainly responsible for genital warts and respiratory papilloma's, which are both LR types, as they tend to cause warts but not cancer, the HPV 39, 56, 59, 66 and 68 subtypes are considered LR. Despite individuals with condyloma acuminatum with LR-HPV subtypes infection,

they have an amplified risk of acquiring anal cancer since they are more likely to acquire a HR-HPV subtype, such as 16, 18, 31, 33 and 45. These subtypes are liable for over 80% of cervical cancers, HPV 16 is the most likely to cause anal cancer [4, 6].

Genital infection is considered mainly a sexually transmitted disease. Its incidence increases after the first sexual intercourse and female adolescents who are sexually active [7]. Recently, the focus has shifted to measures to prevent HPV infections as well as cervical cancer screening for early diagnosis and treatment of cervical cancer. Despite progress made to date on prevention of HPV infection in both females and males, there are continuing controversies and debates regarding the long-term efficacy or HPV vaccines, also whether condom use and male circumcision offer protective effects [8].

Due to the association between squamous cell oropharyngeal cancer and HPV 16 and 18 genotypes, Gardasil® and Cervarix® vaccines were approved in 2016, which was shown to prevent the transmission of these viruses in the 90–95% proposal [5].

Cervical cancer is the fourth most common cancer among women worldwide: 527 624 new cases and 265 672 deaths each year. In Romania, 3300 new cases of cervical cancer are database recorded each year and 1700 deaths from this disease are database reported. 7.5% of the cervical cancers diagnosed annually in Europe come from Romania [9, 10]. The incidence is three times higher than the European Union (EU) average.

Romania ranks first in the EU countries in terms of cervical cancer mortality (14.2 per 100 000 women) [11]. This means a mortality rate 20 times higher than that of Iceland and about four times higher than the EU rate [12].

Only 86% of Romanian women have heard of cervical cancer but more than half of them do not associate this disease with persistent HPV infection, and one in 10 women finds false information that cervical cancer is caused by infection. A 68% of Romanian women have not heard of HPV infection. Half of the women who have heard of the HPV virus do not know or believe that both women and men can be carriers of the virus. Only one in five Romanian women find out about the affection from your family doctor or gynecologist. Seven out of 10 Romanian women have not had a test for precancerous lesions or HPV detection [neither the Babeş–Papanicolaou (Pap) test nor the HPV–DNA test] during the past three years. Only 23% of the Romanian women have done Pap test in the last three years, and 5% have both tested [13].

Aim

The aim of our study is to raise awareness in the rural/urban population regarding the need to perform the Pap test once a year and for consecutive testing for HPV strain detection. This analysis should become a compulsory analysis for any woman because any early-onset cancer is treatable as compared to late-stage cancer.

Patients, Materials and Methods

The study was conducted over a period of three years between 2016–2018 in the “Elena Doamna” Hospital of Obstetrics and Gynecology, Iaşi, Romania. Of the 8500 patients hospitalized for various diseases (ovarian cysts,

uterine fibroids, endometriosis, abnormal vaginal bleeding, dysmenorrhea, ectopic pregnancies, *placenta praevia*, spontaneous abortions and on demand, benign, malignant tumors), only 382 were present in the ambulatory for Pap test. For the other conditions, Pap test denied because the patients did not want this but to solve the condition for which they presented themselves.

We retrospectively review 382 Pap tests of patients who presented themselves in the Ambulatory Service of the Hospital both at the advice of the gynecologist and due to the program initiated by the *World Health Organization* (WHO) and supported by the Department of Public Health, Iaşi: “Cervical cancer can be eliminated as a problem of public health”. Lesions equal to or worse than high-grade squamous intraepithelial lesion (HSIL) were considered high-grade lesions including HSIL (cervical intraepithelial neoplasia – CIN1, CIN2). Endometrial lesions equvalate with from the study. As seen of this campaign, the number of patients has increased in 2018 (242 cases), compared to 46 cases in 2017 and 94 cases in 2016 (Table 1).

Table 1 – Cytology diagnosis of LSIL

Age [years]	No. of cases	No. of tests	Negative HPV tests	HPV	Biopsy diagnosis	DNA microarray / (No. of cases)
19–30	16	3	3			
	3	3	2	HPV		56/59/66 / (2) 35/58/91 / (1)
	13	3	3			
31–40	4	3	2	HPV		6/35/39 / (1) 40/52/82 / (1) 44/33/58 / (1)
	2	2	1	HPV		56/59/66 / (1) 43/68/45 / (1)
	21	3	3			
41–50	2	3	2	HPV		33/66 / (1) 54/31 / (1) 43/33/58 / (1) 56/59/39 / (1) 55 / (1)
	9	2	1	HPV		43/73 / (1) 44/33/73 / (1) 43/35/39 / (1) 59/59/66 / (1) 31/73/26 / (1) 44/35 / (1)
	1	1	0	HPV	Trichom.	33/58 / (1)
51–60	17	3	3			
	2	3	2	HPV		39/43/66 / (1) 45 / (1)
	2	2	1	HPV		43/33/39/68 / (1) 44/35/39/68 / (1)
61–70	4	3	3			
	2	2	1	HPV	Trichom. (1)	45/33 / (1) 59/59/66 / (1)

LSIL: Low-grade squamous intraepithelial lesion; HPV: Human papillomavirus; DNA: Deoxyribonucleic acid; *Trichom.*: *Trichomonas*.

The Pap tests were collected and analyzed in the Ambulatory Service of “Elena Doamna” Hospital of Obstetrics and Gynecology, Iaşi; the patients were trained to show up between 10–14 days of the menstrual cycle, do not perform vaginal care, do not use ova, vaginal creams with 48 hours before harvesting and avoid sexual intercourse. The HPV genotyping take place in a private lab.

According to the recommendations of experts from the *International Agency for Research on Cancer* (IARC), the HPV virus was divided into four groups: HR, possibly HR, LR, not classifiable. The HR-HPV category includes the (a) strains: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 68A/68B (carcinogen 1A), (b) probably/possible carcinogen: 26, 53, 66, 67, 70, 73, 82 (groups 2A and 2B). Of the LR-HPV category are the (a) strains: 6, 11, 40, 43, 44, 54, 70, 61, 81, (b) the unclassified strains category are still: 69, 71 and 74 [12].

Results

We examined the biopsy and Pap tests for 382 women between the ages of 22 and 80. Of these, we found with atypical glandular cells (AGC; $n=5$, 1.3%) between the ages of 23 and 63 years (Table 2). With atypical endocervical, endometrial glandular cells not otherwise specified (AGC-NOS; $n=28$, 7.32%) aged between 25 and 77 years (Table 3). With AGC-NOS and atypical squamous cells cannot exclude HSIL (ASC-H) ($n=6$, 1.57%) aged between 23 and 40 years (Tables 1 and 4).

Table 2 – Cytology diagnosis of AGC

Age [years]	No. of cases	No. of tests	Negative HPV tests	HPV	Biopsy diagnosis	DNA microarray / (No. of cases)
19–30	2	1	1			
41–50	2	1	1			
51–60	1	1	1			

AGC: Atypical glandular cells; HPV: Human papillomavirus; DNA: Deoxyribonucleic acid.

Table 3 – Cytology diagnosis of AGC-NOS

Age [years]	No. of cases	No. of tests	Negative HPV tests	HPV	Biopsy diagnosis	DNA microarray / (No. of cases)
19–30	9	1	1			
31–40	12	1	1			
41–50	4	1	1			
61–70	2	1	1			
71–80	1	1	1			

AGC-NOS: Atypical glandular cells not otherwise specified; HPV: Human papillomavirus; DNA: Deoxyribonucleic acid.

Table 4 – Cytology diagnosis of AGC-NOS and ASC-H

Age [years]	No. of cases	No. of tests	Negative HPV tests	HPV	Biopsy diagnosis	DNA microarray / (No. of cases)
19–30	3	1	1			
31–40	3	1	1			

AGC-NOS: Atypical glandular cells not otherwise specified; ASC-H: Atypical squamous cells cannot exclude high-grade squamous intraepithelial lesion (HSIL); HPV: Human papillomavirus; DNA: Deoxyribonucleic acid.

With ASC-H, we found 57 (14.92%) cases between the ages of 25 and 77 years (Table 5). Of these, only one case showed low-grade squamous intraepithelial lesion (LSIL) type biopsy for which HPV genotyping is envisionable. The patient performed three tests (at that time 0, three months and six months, respectively) and the results of the first two tests were negative but the six months testing confirmed the presence of HPV 70/39/68 strains.

With atypical squamous cells of undetermined significance (ASCUS), we found 128 cases (33.5%) between the ages of 23 and 70 (Figures 1 and 2) (Table 6).

With ASCUS and AGC-NOS, we found three (0.78%) cases between the ages of 22 and 72 years (Table 7).

With ASCUS and AGC, we found a single case (0.26%) aged 39 years (Table 8).

With atypical squamous cells not otherwise specified (ASC-NOS), we found a single case (0.26%), aged 35, with negative HPV test (Table 9).

With LSIL type, we found 96 (25.13%) cases between the ages of 19 and 24. Of these, one case was LSIL and AGC-NOS in a 19-year-old patient who had negative all three HPV genotyping tests (Table 10).

Table 5 – Cytology diagnosis of ASC-H

Age [years]	No. of cases	No. of tests	Negative HPV tests	HPV	Biopsy diagnosis	DNA microarray / (No. of cases)
19–30	11	1	1			
31–40	12	1	1			
	1	3	2			70/39/68 / (1)
41–50	18	1	1			
51–60	9	1	1			
61–70	5	1	1			
71–80	2	1	1			

ASC-H: Atypical squamous cells cannot exclude high-grade squamous intraepithelial lesion (HSIL); HPV: Human papillomavirus; DNA: Deoxyribonucleic acid.

Table 6 – Cytology diagnosis of ASCUS

Age [years]	No. of cases	No. of tests	Negative HPV tests	HPV	Biopsy diagnosis	DNA microarray / (No. of cases)
19–30	20	1	1			
31–40	31	1	1			
41–50	48	1	1			
51–60	10	1	1			
61–70	18	1	1			
71–80	1	1	1			
81–90	1	1	1			

ASCUS: Atypical squamous cells of undetermined significance; HPV: Human papillomavirus; DNA: Deoxyribonucleic acid.

Table 7 – Cytology diagnosis of ASCUS and AGC-NOS

Age [years]	No. of cases	No. of tests	Negative HPV tests	HPV	Biopsy diagnosis	DNA microarray / (No. of cases)
19–30	1	1	1			
61–70	1	1	1			
71–80	1	1	1			

ASCUS: Atypical squamous cells of undetermined significance; AGC-NOS: Atypical glandular cells not otherwise specified; HPV: Human papillomavirus; DNA: Deoxyribonucleic acid.

Table 8 – Cytology diagnosis of ASCUS and AGC

Age [years]	No. of cases	No. of tests	Negative HPV tests	HPV	Biopsy diagnosis	DNA microarray / (No. of cases)
31–40	1	1	1			

ASCUS: Atypical squamous cells of undetermined significance; AGC: Atypical glandular cells; HPV: Human papillomavirus; DNA: Deoxyribonucleic acid.

Table 9 – Cytology diagnosis of ASC-NOS

Age [years]	No. of cases	No. of tests	Negative HPV tests	HPV	Biopsy diagnosis	DNA microarray / (No. of cases)
31–40	1	1	1			

ASC-NOS: Atypical squamous cells not otherwise specified; HPV: Human papillomavirus; DNA: Deoxyribonucleic acid.

Table 10 – Cytology diagnosis of LSIL and AGC-NOS

Age [years]	No. of cases	No. of tests	Negative HPV tests	HPV	Biopsy diagnosis	DNA microarray / (No. of cases)
19–30	1	3	3			

LSIL: Low-grade squamous intraepithelial lesion; ASC-NOS: Atypical squamous cells not otherwise specified; HPV: Human papillomavirus; DNA: Deoxyribonucleic acid.

A case with LSIL type and *Trichomonas* infection discovered in a 41-year-old patient who popped up with 33.58 spikes at the first HPV genotyping.

Thirteen cases with LSIL type lesions between the ages of 20 and 54 popped up with two HPV negative genotyping tests for three.

Seventy-one cases of LSIL type injuries and between the ages of 34 and 65 popped up with three HPV negative genotyping tests (Figures 3 and 4).

With HSIL type injuries, we found 51 (13.35%) cases between the ages of 26 and 80 years. Of these, in 31 (60.78%) cases, the first HPV genotyping was found for 56, 59, 66, 51, 54, 91, 69, 16, 52, 33, 68, 61, 18, 83, 66, 58, 83, 67, 11, 45, 53, 59, 35, 45, 22, 11, 82, 44, 39, 40, 31, 39, 73, 31 strains (Figures 5 and 6) with ages between 26 and 65 (Tables 1 and 11). Of the remaining 20 (39.21%) cases with HSIL type injuries, we found three (15%) cases that had two HPV negative genotypes and the third positive. In the first case, it is a 71-year-old patient who received the positive result in the third HPV test for 70, 73, 80 strains. The second patient is 40 years old and received the positive result in the third HPV test for 43, 39, 68 strains. The third patient is 45 years of age and received the positive result for the third HPV genotyping test for 16, 35, 45 strains (Figures 7 and 8).

Of the 17 (8.5%) cases remaining with HSIL type injuries, we found one case of CIN1 ($n=1$, 5.88%) with 80 years old and CIN2 patients ($n=12$, 70.58%) aged between 28 and 56 years. HPV strains identified for CIN1 case were 16, 59, 90, and for CIN2 patients were 69, 66, 58, 59, 18, 67, 83, 53, 59, 90 (Figures 9 and 10).

In the last four (23.52%) cases with HSIL type injuries, HPV genotyping was identified for 54, 45, 52, 33, 68 strains, genotyping that was negative in the first two tests, then to the third test HPV strains to be identified. Patient ages were between 47 and 65 years.

Most precursor and premalignant HPV infections are asymptomatic. It a proved fact that HPV affects basal keratinocytes.

The most important proof in that HPV infection requires a microabrasion of the genital epithelium that results in epithelial denudation but retention of the epithelial basement membrane. The thinner more fragile metaplastic epithelium may be more susceptible to the micro-wounding process and thus HPV infection.

Diagnosis of squamous intraepithelial lesions was studied on variation in nuclear size and shape, hyperchromia and coarse chromatin granules, nuclear/cytoplasmic ratio.

Low-grade dysplasia (koilocytosis) takes in on koilocytic changes: histologically, the changes involve only the lower 1/3 of the epithelium or there are koilocytic changes in the upper epithelium (maturation seen). Koilo-

cytes are superficial or intermediate squamous cells with large and irregular well-defined perinuclear halo, with a cookie cutter border and cytoplasmic thickening. Bi- or multi-nucleation is often identified, nuclei are enlarged (2–3 times) normal size.

High-grade dysplasia (CIN2 and CIN3) striking nuclear atypia involving all layers of the epithelium, lack of or minimal maturation, nuclear changes include enlargement, membrane irregularities, variable shapes and abnormal chromatin, the nuclear/cytoplasmic ratio was high.

Table 11 – Cytology diagnosis of HSIL

Age [years]	No. of cases	No. of tests	Negative HPV tests	HPV	Biopsy diagnosis	DNA microarray / (No. of cases)
19–30	3	1	0	HPV	CIN2 (1)	18/67/54 / (1) 16/51 / (1) 18/67/83 / (1)
31–40	9	1	0	HPV		16/58 / (1) 16/59 / (1) 56/59/66 / (1) 44/59/61 / (1) 52/73/66 / (1) 16/56/58 / (1) 18/11 / (1) 40/16 / (1) 16/58/91 / (1)
						43/39/68 / (1)
						16/35/45 / (1)
						52/33/58 / (1) 45/54/52 / (1) 45/52/33 / (1)
						18/58/33 / (1) 18/68/59 / (1) 31/59 / (1) 59/69 / (2) 18/69 / (1) 18/66/53 / (1) 18/66 / (3)
41–50	14	1	0	HPV	CIN2 (6)	56/68/91 / (1) 68/82/18 / (1) 16/35/45 / (1) 56/59/66/91 / (1) 52/33/58 / (1) 66/39/73 / (1) 45/54/52 / (1) 45/52/33 / (1) 58/59/66 / (1) 53/59/66 / (1) 26/66/52 / (1) 18/67 / (1) 18/61 / (1) 18/90 / (1) 18/67/83 / (1) 16/66 / (1) 18/58/31 / (1) 31/68/54 / (1) 51/54 / (1)
51–60	11	1	0	HPV	CIN2 (5)	11/45/59 / (1) 68/66/73 / (1) 56/59/91 / (1) 39/45/91 / (1) 39/51 / (1) 33/58 / (1)
						45/33 / (1)
						70/73/82 / (1)
61–70	6	1	0	HPV		16/59/90 / (1)
71–80	1	3	2	HPV		
	1	1	0	HPV	CIN1 (1)	

HSIL: High-grade squamous intraepithelial lesion; HPV: Human papillomavirus; CIN: Cervical intraepithelial neoplasia; DNA: Deoxyribonucleic acid.

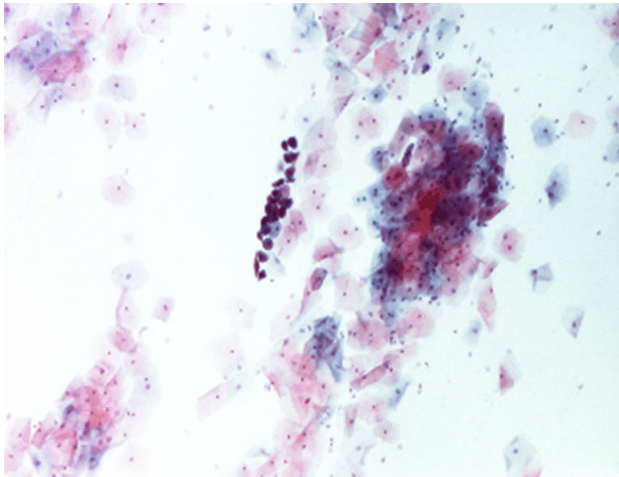


Figure 1 – ASCUS: parabasal cells with perinuclear cytoplasmic clearing and enlarged hyperchromatic nuclei. Papanicolaou (Pap) staining, $\times 40$. ASCUS: Atypical squamous cells of undetermined significance.

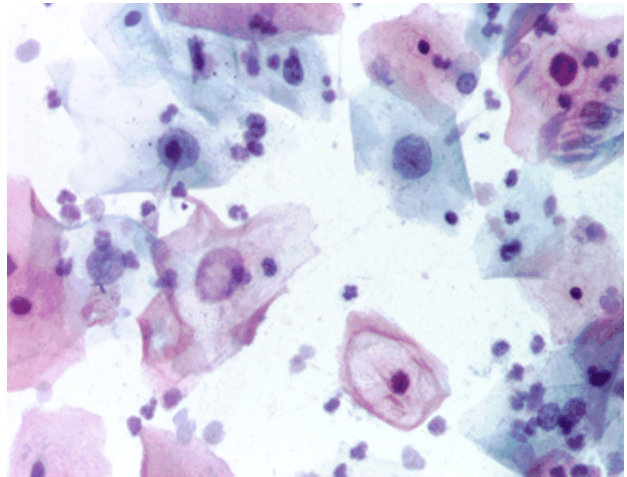


Figure 2 – ASCUS: squamous cells with enlarged and hyperchromatic nuclei. Pap staining, $\times 200$.

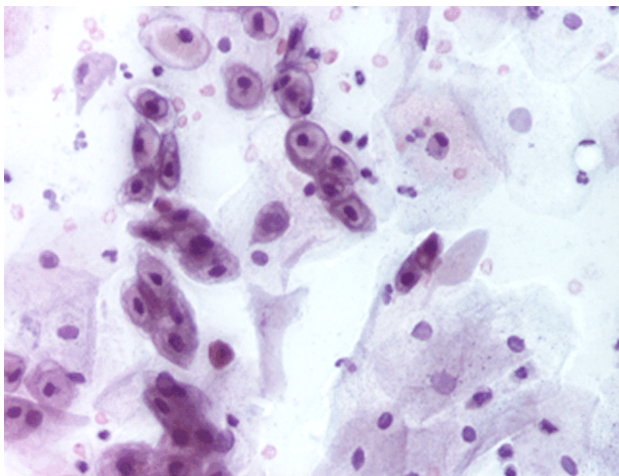


Figure 3 – LSIL: squamous cells with perinuclear empty spaces surrounded by cytoplasmic thickening associated with moderate nuclear enlargement. Pap staining, $\times 200$. LSIL: Low-grade squamous intraepithelial lesion.

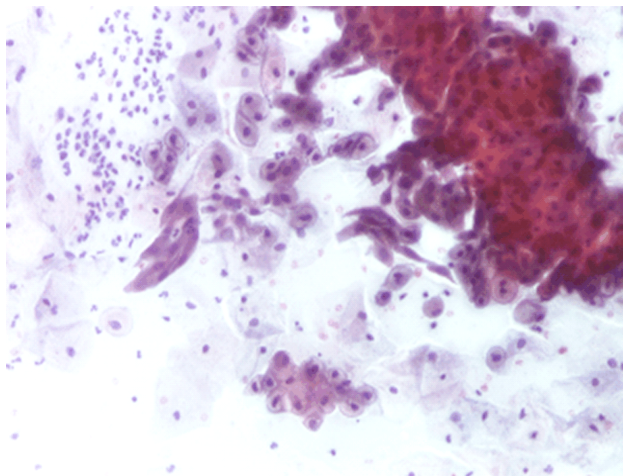


Figure 4 – LSIL: eosinophilic and basophilic koilocytes associated with parakeratotic features. Pap staining, $\times 100$.

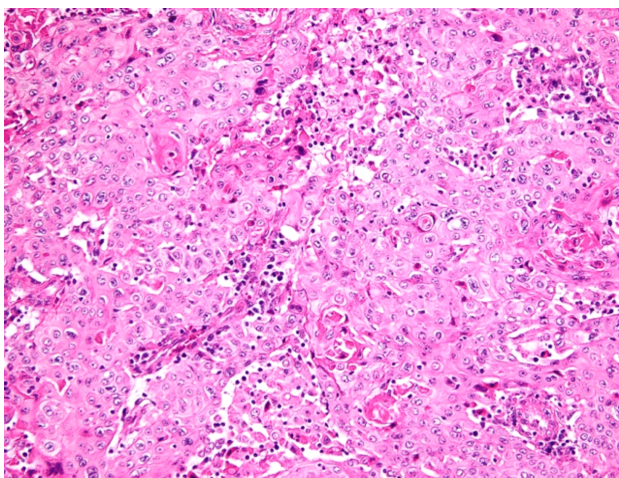


Figure 5 – Moderately differentiated cervical squamous cell carcinoma with inflammation. Hematoxylin-Eosin (HE) staining, $\times 40$.

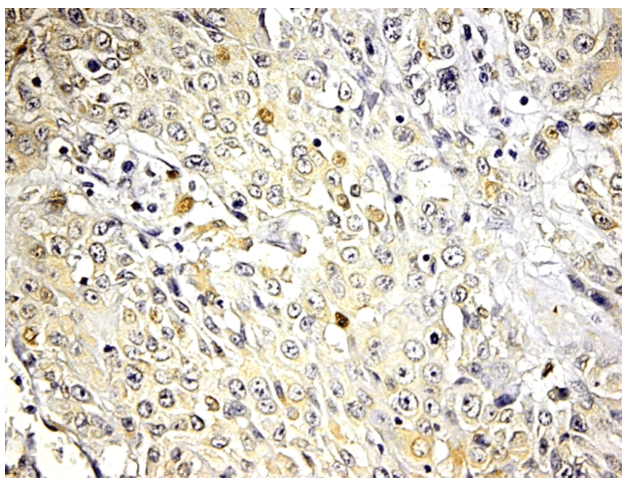


Figure 6 – Few tumor cell nuclei are positive for HPV. Anti-HPV 16/18 antibodies immunomarking, $\times 100$. HPV: Human papillomavirus.

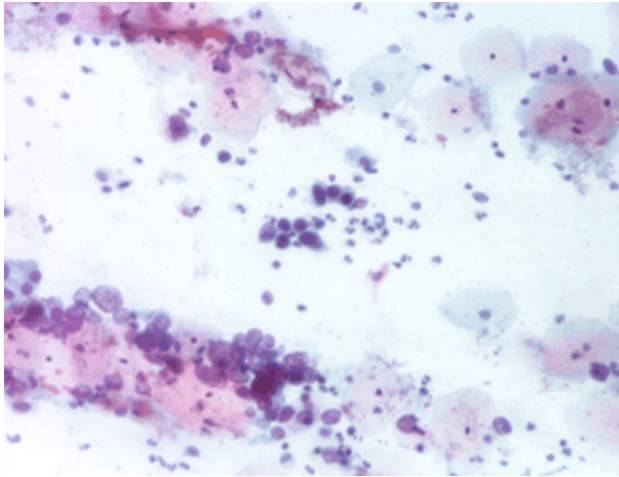


Figure 7 – HSIL: parabasal cells with enlarged, irregular nuclei with coarse chromatin. Pap staining, $\times 100$. HSIL: High-grade squamous intraepithelial lesion.

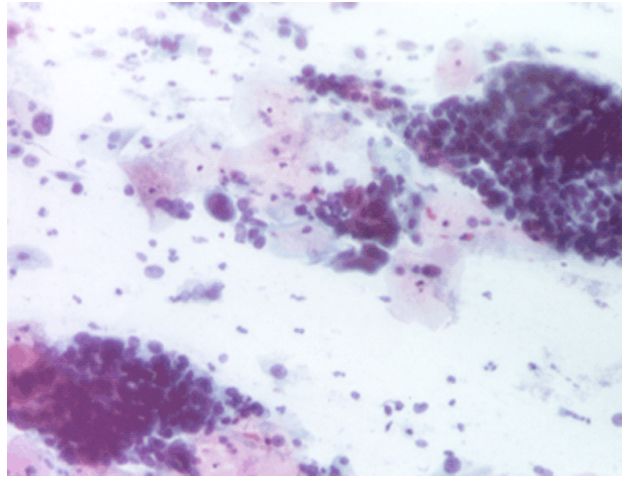


Figure 8 – HSIL: isolated/clustered parabasal or basal cells with single or multiple enlarged, irregular nuclei, with coarse chromatin. Pap staining, $\times 100$.

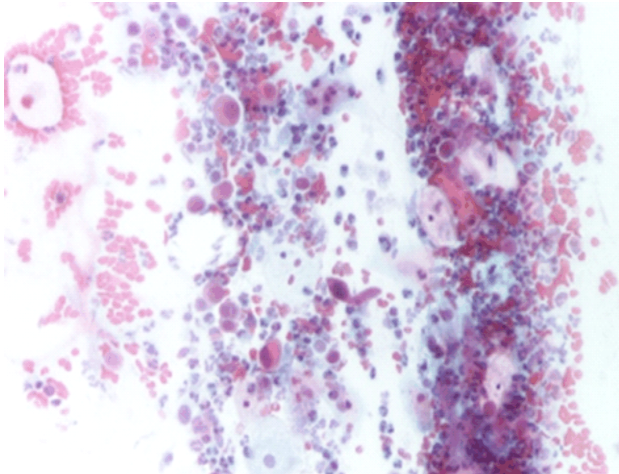


Figure 9 – Carcinoma: pleomorphic malignant cells, inflammation, blood and necrosis. Pap staining, $\times 100$.

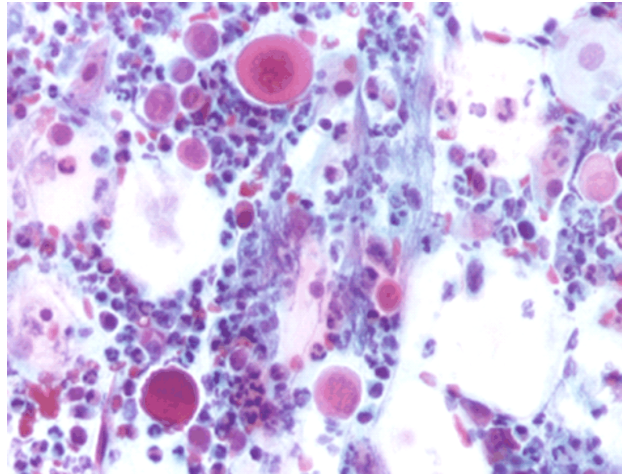


Figure 10 – Carcinoma: pleomorphic malignant cells – details. Pap staining, $\times 200$.

✉ Discussions

The risk of contracting an HPV infection is more common in people who have numerous sexual partners and have a promiscuous behavior.

HPV infection can be through direct contact with the skin of the infected person and during sexual intercourse through vaginal, anal or oral sex penetration and not all of these are needed at the same time [5].

HPV infection can spread from a wide infected area, e.g., genital origin infection spreads to the anus. The virus spreads through the direct contact of the hands with the genital region and in this way both symptomatic and asymptomatic persons can become infected. As with other sexually transmitted diseases, such as human immunodeficiency virus (HIV), hepatitis B virus, *Herpes simplex virus type 2* (HSV-2), *Neisseria gonorrhoeae*, HPV genitalia can be transmitted from mother to child during pregnancy and labor. This is evidence that HPV in girls or even virgin women is possible [5, 14].

It is a known fact that HPV testing has a high sensitivity when compared to cytomorphology in the detection of cervico-vaginal dysplasia, as evidenced by a multitude

of studies [15, 16]. In this study, we determined that 15% ($n=3/20$) of biopsies that confirmed HSIL-type lesions showed three previous HPV negative and 20% ($n=4/20$) biopsy tests confirmed HSIL-type injuries had two previous HPV negative trials. For example, in a study involving 256 649 Pap assays, HPV tests and co-tests demonstrated that 19% of women with cervical cancer had a history of HPV-negative tests [17]. HPV-negative tests counts in 25% of patients with cervical cancer [18].

We further analyzed the seven biopsies of patients who had HSIL-type injuries with three HPV-negative tests in the past or two HPV-negative assays. HPV genotyping indicated different HPV strains despite previous HPV-negative trials. In these cases, it is about HR-HPV 73, 68, 16, 35, 45, 33 strains.

In the case of a single LSIL-type lesion biopsy and subsequent *Trichomonas* infection, with a history of HPV-negative testing, we identified the following increased risk strains: 33 and 58.

In the other 13 biopsies with LSIL-type lesions and 2 previous HPV-negative tests, we identified the following increased risk strains: 35, 31, 56, 59, 45, 39, 52, 82.

These findings demonstrate that HPV infection was associated with some of the LSIL 13.54% lesion biopsies ($n=13/96$) but also those with HSIL 13.72% ($n=7/51$), the percentages being significantly different.

Another reason that led us to the HPV infection in both LSIL and HSIL patients was the presence of koilocyte cells. According to specialized studies, the viral assembly is verified in most differentiated epithelial layers. The release of the virus occurs through differential degeneration of the cells that lead to the formation of koilocytes. The term koilocytes comes from Greek “*kilos*” (cavity). Kors & Durfee, introduced the term in 1956, with reference to the cytopathic alteration characterized by a prominent halo and pyknotic nucleus [19].

Koilocytosis is in theory discussed as a pathognomonic marker of HPV infection. The koilocyte formation is due to E5 and E6 oncoprotein, although the vacuolization mechanism remains unclear up to date [20–22]. Some studies suggest that the cytoplasmic vacuolization contributes to keratinocytes fragility, facilitating the virion release [20].

Therefore, koilocytes are cells destined to apoptosis, which is a consequence of macromolecules synthesis inhibition [23].

Koilocytes were present in the biopsy of the LSIL-type lesion and the lympho-plasmopathic polyp whose previous HPV test was negative. Subsequent to the second HPV genotype, at 3-month difference was positive for 45 high-oncogenic strains.

In our study, in the 13 cases with CIN1 and CIN2, the HR-HPV strains identified by the first genotyping were 18, 16, 59, 58. The underlying reasons for biopsy discrepancies and previously HPV-negative tests are unknown. Several factors may contribute to HPV-negative biopsy tests including low viremia, inappropriate samples, technical errors or interference with different materials. We have found 20 cases with HSIL- and LSIL-type lesions that had HPV-negative trials. In addition to possible inappropriate sampling, high-grade cervical lesions frequently occur in women with persistent HPV infection characterized by overexpression of *E6/E7* oncogenes after integration of viral DNA into the host genome [24]. Compared to the early phase of productive infection, virion production and *L1* gene expression may be significantly lower in women with HPV-persistent infection when the incidence of high-grade lesions increases. In support of this, recent studies have found that HPV *E6/E7* messenger ribonucleic acid (mRNA) testing reach improvements for CIN2 cervical lesions compared to HPV DNA tests [25–27]. By using immunohistochemistry, Grapsa *et al.* [27] demonstrated that the p16-positive/*L1*-negative pattern was significant by more common in HSIL than in LSIL. Given that a sufficient amount of the HPV *L1* gene is required for most of the HPV DNA assays, including Cobas[®] test reduced *L1* expression could contribute to false negative HPV tests in a number of women with high-grade cervical lesions [28].

It is noteworthy that HPV 16 and 18 strains were less detectable in HSIL- and LSIL-type lesions than other HPV non-16/18 strains ($n=24/147$, 16.32%). This pattern of genomic prevalence differs significantly from that observed in women in the same study population in

confirmed HSIL-type biopsies and previous positive tests in which the prevalence for HPV 16/18 and HPV non-16/18 was approximately equal [27].

More, HPV 59 and 45 were the two identified genotypes in the proportion of 80% in the case of HPV-negative non-16/18 (Table 1). Zhan *et al.* (2014) showed in a study that the pattern of genomic prevalence we identified in our study is incapable with their own, which showed that HPV 45 and 59 strains ranked 7th and 14th, respectively, in a study on cervical dysplasia [29]. This suggests that HPV 59 and 45 strains may have lower levels of *L1* gene expression compared to other HPV genotypes, especially for high-grade lesions with HPV DNA integration. Another possibility is that Cobas[®] tests may have a relatively low sensitivity in detecting HPV non-16/18 genotypes, especially for 59 and 45 genotypes [26, 28].

HR-HPV non-16/18 strains identified in HSIL-type biopsies with three HPV-negative trials ($n=3/20$, 15%) are 73, 39, 68, 35, 45. In the case of HSIL-type biopsies with two HPV-negative tests ($n=4/20$, 20%), the strains found are 45, 52, 33, 68. In the case of biopsies with LSIL-type lesions, HR-HPV non-16/18 strains ($n=13/96$, 13.54%) that have been identified are 35, 58, 31, 56, 59, 45, 39, 52, 82.

Abnormal cytology was detected in previous Pap tests in 15 cases including ASCUS ($n=6$), AGC-NOS ($n=1$), ASC-H and AGC-NOS ($n=1$), and LSIL ($n=4$). Follow-up biopsies showed CIN1 ($n=1$) and CIN2 ($n=12$) in 13 cases.

Based on the IARC Classification, these non-HPV genotypes belong to the three groups and, defined as non-HPV due to insufficient data on oncogenic potential. It can be argued that these genotypes have no benign potential as they think they are responsible for cervical dysplasia or even cancer, particularly for women with HPV-negative tests in a past [27]. Quiroga-Garza *et al.* [29] reported that the HPV 90 genotype, one of the non-HPV strains, was associated with HPV cervical dysplasia including HSIL, in the North American population. In our study, we identified the association of LR non-HPV genotypes (61, 81, 83) in patients with HSIL-type lesions. These unconventional HPV genotypes under overlook because they are undetectable in most HPV assays commonly used in practice. Moreover, these genotypes are generally under perception as LR or even non-oncogenic strains. For the 13 CIN1 and CIN2 cases identified in our study, the HPV 90 strain was present.

Our study demonstrated a high rate of multi-genotypic HPV infection in women with HSIL confirmed biopsies and prior HPV-negative trials. The rate was 13.72% ($n=7/51$), close to the 20–40% percentage reported by other previous studies, with variations that depended on the age and severity of cervical lesions [28, 30, 31].

Several studies including ours have indicated that HPV genotypes can compete with other genotypic infections. However, is yet to be demonstrating whether such interactions of HPV genotypes are sufficient to interfere with HPV detoxification? Several studies are needed in this respect [26, 32, 33].

In our study, the three cases with HSIL-type lesions and three previous HPV-negative tests, along with the other four cases with two previous HPV tests, have posed the question whether HPV-negative tests were HSIL or

HPV-negative tests. This is yet to be uncovered. However, persistent infection with HPV is to be taken in consideration as the primary cause of most cervical cancers. HPV is rarely detected at the cervical diagnosis.

Besides these cases with HSIL-type injuries in our study, we also found 13 cases with LSIL-type lesions with two HPV-negative tests in the past. In any case, several factors are to be taken in consideration before considering a negative HSIL–HPV case or cancer [34]. As discussed earlier, false negative trials is to be caused by low viremia, low lesion size, material interference, technical errors, limited test panel, or insufficient testing sensitivity.

In our case, several factors could affect the interpretation of the results. First, we could talk about our study being a retrospective and not a controlled one. Although the selection of patients takes place, the study reflected clinical practice without a controlled trial. Secondly, the study is relatively small, with few cases of glandular lesions and carcinomas. Certainly, future prospective studies should take place.

✉ Conclusions

Our study demonstrated that 35% ($n=7/20$) of HSIL-type confirmed biopsies previously had HPV-negative assays. Despite the previously HPV-negative tests, a wide variety of HPV genotypes appears in most biopsies. In our case, we frequently identified HPV 59 and 45 strains, 51 cases with HSIL-type lesions presented a first HPV-positive test, 13 cases with LSIL-type lesions showed three HPV-negative tests. Prevention plays an important role in reducing the incidence of cervical cancer cases. The Pap test is a golden standard in the primary prevention method, but consecutive vaccination significantly increases protection against HR-HPV strains. Education plays an important role in the prophylaxis of HPV infection and cancer. It is required taking place in schools (voluntarily), from puberty age through partnerships or government programs with public health directorates and university hospitals or using European funds.

Conflict of interests

The authors declare that they have no conflict of interests.

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