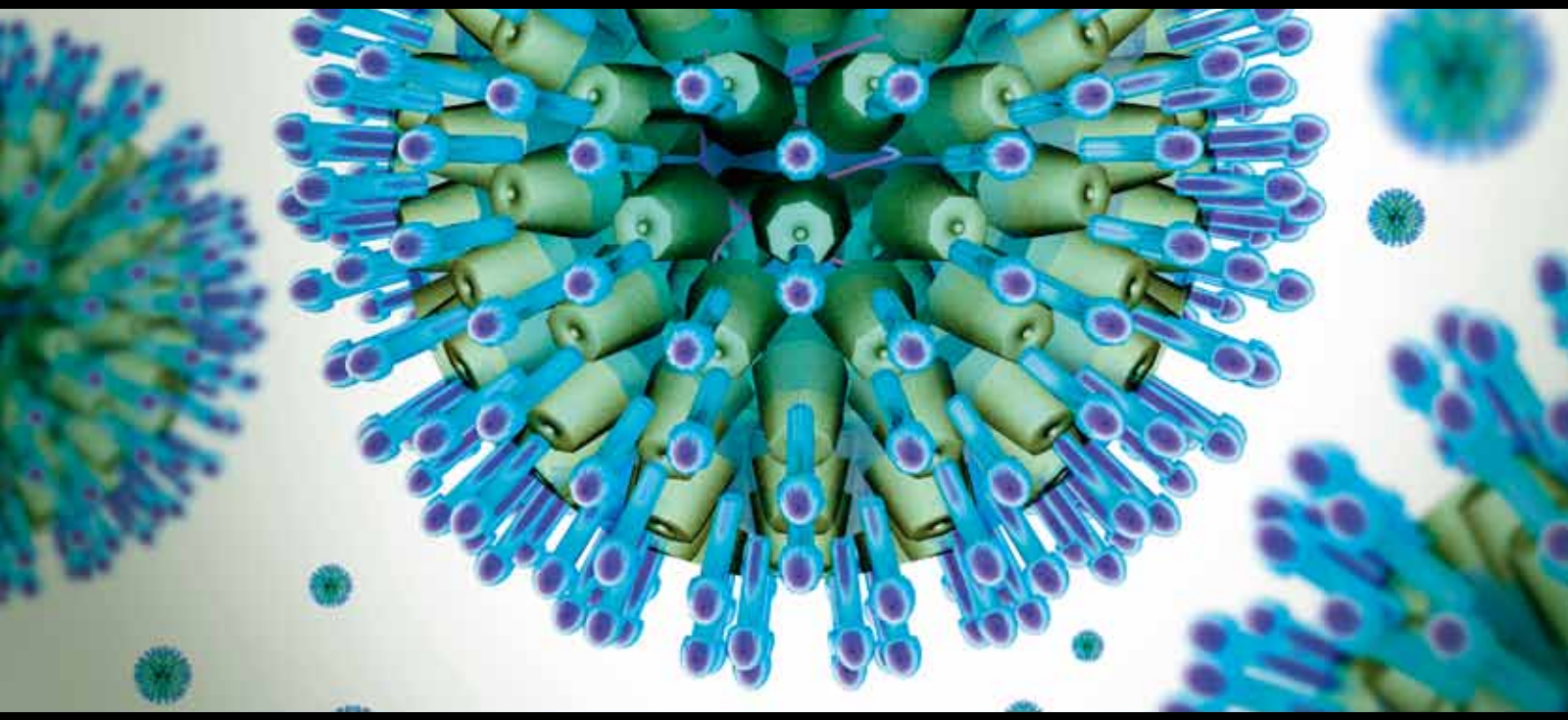
Infectious Diseases in Obstetrics and Gynecology

Prevention of Human Papillomavirus-Related Malignancies of the Female Genital Tract

Guest Editors: George Koliopoulos, Pierre Martin-Hirsch, and Marc Arbyn



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Editorial Board

Contents

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Editorial

Prevention of Human Papillomavirus-Related Malignancies of the Female Genital Tract

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Persistent infection from high-risk HPV types is necessary for the development of cervical cancer and constitutes a main cause for a significant proportion of cancers in other sites of the female anogenital tract such as the vulva, the vagina, and the anus [1]. Cervical cancer in particular being the third most common cancer in women (with approximately 530,000 cases and 275,000 deaths per year) [2] is an important global health issue warranting intensive preventive efforts [3]. Secondary prevention through cervical cytology screening has been effective in reducing the incidence and the mortality of the disease [4]. However, cervical cytology has certain limitations one of which is poor reproducibility. In the study by A. C. Barcelos et al. titled "*Atypical squamous cells of undetermined significance: bethesda classification and association with human papillomavirus*" which appears in this special issue, observers would agree in the diagnosis of ASCUS only in two out of three cases.

Secondary screening efforts focus on the detection and subsequent treatment of cervical intraepithelial neoplasia (CIN). However, the sensitivity of cytology in the detection of CIN has been questioned [5]. Obviously failure of this mechanism might result in the development of cervical cancer. HPV DNA testing emerged as a way to improve the deficiencies of cytology [6]. The study by Barcelos et al. [7] confirmed that HPV DNA testing with Hybrid Capture II (HCII) can be applied in the triage of ASCUS cytology. Interestingly the HCII positivity rate in this cohort of ASCUS smears (22%) was lower than in the ALTS (55%) [8].

HCII, however, does not allow HPV typing. Certain PCR-based tests allow the identification of the specific genotypes responsible for the infection, which could have future clinical applications. One of the first steps in PCR is DNA extraction. The study by Chranioti et al. [9] compared the accuracy of the method after manual and after automated DNA extraction and found better results with the former.

Cell cycle biomarkers such as E6&7 mRNA testing and P16INK4a are further tools that could be used in the prevention of cervical cancer [10]. However, biomarkers could also have prognostic value and influence management of cervical (pre-)cancer. In the study by F. M. Abu Backer et al. titled "*Clinicopathological comparison of adenocarcinoma of cervix and endometrium using cell cycle markers: P16ink4a, P21waf1, and p27Kip1 on 132 Cancers*" the expression of two less studied proteins, p21WAF1 and p27Kip1 was significantly associated with lymph node invasion in cervical adenocarcinoma.

Most HPV infections are transient without clinical significance. However, in a minority of women HPV will persist and will initiate the neoplastic process. Epigenetic event such as gene methylation might play a role in the selection of the women who will progress to cancer. The study by A. Spathis et al. titled "*Promoter methylation of* $p16^{INK4A}$*, hMLH1, and MGMT in liquid-based cervical cytology samples compared with clinicopathological findings and HPV presence*" examined the methylation-dependent inactivation of tumor suppressor genes connected with cell cycle regulation as p16INK4A and DNA repair mechanisms as human MutL Homolog 1 (hMLH1) and O6-methylguanine DNA methyl transferase (MGMT). Even though the accuracy of methylation was found lower than traditional tests, epigenetic effects are areas of promising future research.

The deficiencies of secondary prevention, such as the need for sufficient infrastructure, which is absent in developing countries, the false negative results, the adverse effects of overtreatment on subsequent pregnancy outcome [11, 12], as well as the psychological morbidity resulting from HPV infection [13] have left plenty of room for improvement. With the introduction of HPV vaccines a combination of secondary with primary prevention is now possible [14, 15]. The article by K. Natunen et al. titled "*Aspects of prophylactic vaccination against cervical cancer and other human papillomavirus-related cancers in developing countries*" outlines certain issues that arise with respect to application of the vaccine in the developing countries where the need for cervical cancer prevention is the greatest. They suggest that given the low coverage rates, vaccination of the males might have to be considered in order to produce herd immunity. They also argue that strategies for vaccine implementation would have to be school based with community outreach activities in regions where school attendance is low.

Progress in the elucidation of the mechanisms of carcinogenesis has led to the development of exciting technologies that can be used for lower anogenital tract cancer prevention. The main challenge we face now is to find the proper way to use all these discoveries for the benefit of women's health.

George Koliopoulos
Pierre Martin-Hirsch
Marc Arbyn

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Research Article

Clinicopathological Comparison of Adenocarcinoma of Cervix and Endometrium Using Cell Cycle Markers: P16ink4a, P21waf1, and p27Kip1 on 132 Cancers

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Objective. We studied the clinicopathological parameters of adenocarcinoma arising from endocervix (ECA) and from endometrium (EMA) based on the expression of P16ink4a, P21waf1, and p27Kip1 proteins. *Study Design*. Immunohistochemistry was done on sections of confirmed ECA and EMA from hysterectomy specimens which have had no prior chemotherapy/radiotherapy. *Results*. There were 40 ECAs and 92 EMAs. The mean age of ECA was 49.82 (SD 10.29); the youngest was 30 years old and the oldest 75 years old. The mean age of EMA was 54.45 (SD 10.92); the youngest was 30 years old and the oldest was 82 years old. For ECA, the size of the tumour is significantly associated with age and with depth of infiltration. FIGO stage is associated with histological grade. p21WAF1 expression is significantly associated with infiltration of the corpus and lymph node metastasis. p27Kip1 expression is significantly associated with lymph node invasion. The presence of lymph node metastasis is strongly associated when p16INK4a and p27Kip1 expressions are analyzed in combination. For EMA, p16INK4a expression is associated with histologic grade. *Conclusion*. Our study shows that we could use these cell cycle markers as predictors for more aggressive subsets of adenocarcinoma of the cervix and endometrium.

1. Introduction

The trend of adenocarcinoma of the cervix (ECA) is increasing [1–3] particularly among younger women [4]. Adenocarcinoma of the endometrium on the other hand affect older women, in the fifth to six decade [5]. In routine clinical practice, the presentation of these two cancers often overlap [6] and each requires different management [7]. ECA has more risk of recurrence [8] than endometrial adenocarcinoma. In Malaysia, adenocarcinoma of the cervix accounts for 5–15% of the total cervical cancers and the trend is also increasing [9]. The histological appearance of these two cancers under routine H/E staining is almost similar in many instances. In a study on fractional curettage specimens from fifteen women who had tumours in the endocervical and endometrial specimens, only 34.1% were diagnosed as endometrial carcinomas [10]. Carcinoembryonic antigen (CEA), estrogen receptor (ER), vimentin, and a panel of histochemical stains [11] are routinely used to differentiate these two cancers [12, 13]. Routine IHC studies may not be helpful for synchronous endometrial and endocervical tumors [14]. In such, clonality studies using robust molecular techniques may help diagnose cases in which conventional imunohistochemical studies are not helpful [14].

p16INK4A is a molecular biomarker that consistently discriminates uterine cervix adenocarcinoma from endometrial adenocarcinoma [15]. It is a cell cycle regulatory tumour suppressor gene that has intimate interplay with the retinoblastoma gene [16]. p16INK4A could differentiate similar skin conditions: actinic keratosis from Bowens disease of which differentiating them before definitive treatment is important as each has different management [16]. p21WAF1 is a cyclin-dependent kinase inhibitor which acts as both a sensor and an effector of multiple antiproliferative signals

[17]. p27Kip1 is a negative regulator of the G1 phase of the cell cycle [18]. Attempts at differentiating the clinicopathological parameters of adenocarcinoma of the cervix from endometrium have been done by a number of researchers using immunohistochemical stains [13, 14] and molecular techniques such as Western blotting [19] and PCR [20, 21]. Among the parameters studied was presence of estrogen receptor status [19, 22].

We studied the clinical parameters of adenocarcinomas arising from endocervix based on the expression of P16ink4a, P21waf1, and p27Kip1 proteins and whether the presence/absence of such protein expression correlate with clinical presentations/behaviour of these cancers.

2. Methodology

This is a retrospective study on archived tissue blocks of histologically confirmed adenocarcinoma of the cervix and endometrium diagnosed from 2005 to 2008. The samples were hysterectomy specimens with or without removal of ovaries and fallopian tubes. Biopsies (either colposcopic or by curettage) were excluded. Specimens from patients who have had chemotherapy or radiotherapy before hysterectomy were excluded. These samples were taken from two public hospitals located in two different localities: Hospital Universiti Sains Malaysia (HUSM) (north-eastern region) and Hospital Sultanah Bahiyah (HSB) (north-western region of Malaysia).

The demography and the clinical details were obtained from the case folders. The histological diagnoses of ECA and EMA were categorized according to the main histological feature as endocervical NOS, adenosquamous, endometrioid, serous, and clear cell. The cancers are called "mixed" when the histological findings show more than one histological patterns. Immunohistochemistry staining was done on 4 micron thickness sections from paraffin blocks using the standard immunohistochemical staining technique. p16INK4a, P21WAF1, and P27Kip1 primary antibodies were at 1 : 100 dilutions. Positive controls were squamous cell carcinoma for p16INK4a, colonic adenocarcinoma for p21KIP1, and normal prostatic tissue for p27Kip1 tissue as per suggestion by manufacturers of these antibodies. The association was determined using multiple logistic regression tests. The results were analysed using SPSS version 16, and the level of confidence (P value) was set at 0.05.

The study was approved by the Research Ethics Committee of Universiti Sains Malaysia.

3. Results

There were 40 adenocarcinomas (ECAs) of the endocervix and 92 adenocarcinomas of the endometrium (EMAs). The median age for adenocarcinoma of the cervix was 48.5 years, and the mean age was 49.82 (SD 10.3). The youngest patient was 30 years old and the oldest 75 year old. The median age for adenocarcinoma of the endometrium was 55.0 years and the mean age was 54.45 (SD 10.9). The youngest patient was 30 years old and the oldest 82 years old. For adenocarcinoma of the cervix, there were 74% Malays, 18% Chinese, 8% Siamese, and no Indian patients while for endometrial carcinoma, there were 79% Malays, 11% Chinese, 9% Indian, and 1% Siamese ethnic group.

The most common histological type of adenocarcinoma of the cervix was mucinous type: 62.5% (25/40), and the commonest histological subtype of endometrial

Table 1: Summary of the clinicopathological parameters in adenocarcinoma of the cervix (ECA) and adenocarcinoma of the endometrium (EMA).

Variables of ECA	Frequency (%) of ECA	Variables of EMA	Frequency (%) of EMA
Age (years)		Age (years)	
<35	3 (7.5)	<50	28 (30.4)
35–54	22 (55.0)	50–60	35 (38.0)
≥55	15 (37.5)	>60	29 (31.5)
FIGO stage		FIGO stage	
I	27 (57.5)	I	50 (54.3)
ll	7 (17.5)	II	13 (14.1)
lll	5 ((12.5)	lll	13 (14.1)
IV	5 (12.5)	lV	16 (17.4)
Histology tumor differentiation		Myometrial invasion	
Well/moderate	25 (62.5)	< 1/2	45 (48.9)
Poor and Special type	15 (37.5)	> 1/2	47 (51.1)
Infiltration depth		Histologic grade	
Inner 2/3	13 (32.5)	G1	39 (42.4)
Outer 1/3 or through	27 (67.5)	G2	23 (25.0)
		G3	30 (32.6)
Tumor length		Postmenopausal status	
<20 mm	11 (32.5)	Yes	54 (58.7)
>20 mm	29 (67.5)	No	38 (41.3)
Tumor thickness			
<5 mm	4 (10.0)		
>5 mm	36 (90.0)		
Infiltration to corpus			
Yes	17 (42.5)		
No	23 (57.5)		
Vascular invasion			
Yes	20 (50.0)		
No	20 (50.0)		
Lymph node invasion			
Yes	12 (30.0)		
No	28 (70.0)		
Histologic type:		Histologic type:	
Endocervical NOS	25 (62.5)	Endometrioid	75 (81.5)
Adenosquamous	6 (15.0)	Adenosquamous	4 (4.4)
Endometrioid	5 (12.5)	Clear cell	5 (5.4)
Serous	1 (2.5)	Serous	5 (5.4)
"Mixed"	2 (5.0)	"Mixed"	3 (3.3)

Table 2: Interrelationship* between clinicopathologic parameters in patients with adenocarcinoma of the cervix (ECA).

	Age group (*P* value)	Ethnic (*P* value)	Diagnosis (*P* value)	Histo grade (*P* value)	FIGO stage (*P* value)	Infiltration depth (*P* value)	Tumour size (diameter) (*P* value)	Tumor thickness (*P* value)	Corpus infiltration (*P* value)	Vascular invasion (*P* value)	LN invasion (*P* value)
Age group	—	0.140	0.377	0.372	0.582	0.392	**0.006**	0.364	0.301	0.165	0.056
Ethnic		—	0.090	0.850	0.206	0.559	0.838	0.224	0.858	0.140	**0.017**
Histologic type			—	**<0.0001**	0.572	0.191	0.411	0.586	0.804	0.744	0.722
Histologic grade				—	0.090	0.542	0.120	0.586	0.680	0.102	**0.012**
FIGO stage					—	0.330	0.152	1.000	**0.042**	**0.003**	**<0.0001**
Infiltration depth						—	**0.010**	0.056	0.085	**0.018**	0.507
Tumor length							—	**0.025**	0.230	**0.013**	0.076
Tumor thickness								—	0.749	0.292	0.818
Corpus infiltration									—	0.337	**0.043**
Vascular invasion										—	**0.038**
LN invasion											—

* Calculated using Chi-square.

Table 3: Interrelationship* between clinicopathological parameters in patients with adenocarcinoma of endometrium (EMA).

	Age group (*P* value)	Menopausal status (*P* value)	Ethnic (*P* value)	Diagnosis (*P* value)	Histology grade (*P* value)	Myometrial invasion (*P* value)	FIGO stage (*P* value)
Age group	—	**<0.0001**	0.139	0.178	0.175	0.101	0.343
Meno status	—	—	0.194	0.270	0.641	0.062	0.260
Ethnic	—	—	—	0.825	0.703	0.248	0.026
Histologic type	—	—	—	—	**<0.0001**	0.213	0.081
Histologic grade	—	—	—	—	—	0.060	**<0.0001**
Myometrial invasion	—	—	—	—	—	—	**<0.0001**
FIGO stage	—	—	—	—	—	—	—

* Calculated using Chi-square.

adenocarcinoma was endometrioid type (75/92). Majority of the patients were in FIGO stage I (ECA 57.5%, EMA 54.3%). The summary of the clinicopathological findings of the 132 cancers is in Table 1.

The interrelationship between the various clinicopathological correlations is as shown in Table 2 for ECA and Table 3 for EMA. For adenocarcinoma of the cervix, the grade was significantly associated with histologic type and with lymph node. The age of the patients was significantly associated with the size of the tumor. The tumor size is also associated with the depth of infiltration. FIGO stage is strongly associated with vascular and lymph node invasion (Table 2). For adenocarcinoma of the endometrium, FIGO stage is associated with histological grade and expectedly with myometrial invasion (Table 3).

For adenocarcinoma of the cervix, none of the clinicopathological parameters is significantly associated with p16INK4a expression (Table 4) including the histologic subtypes. p21WAF1 expression (Table 5) is significantly associated with infiltration of the corpus ($P = 0.043$) and lymph node metastasis ($P = 0.071$), and p27Kip1 expression (Table 6) is significantly associated with lymph node invasion ($P = 0.030$). The presence of lymph node metastasis is strongly associated ($P = 0.013$) when p16INK4a and p27Kip1 expressions are analyzed in combination.

For adenocarcinoma of the endometrium, p16INK4a expression (Table 7) is associated with histologic grade ($P = 0.014$) but not the histologic type ($P = 0.888$). p21WAF1 and p27Kip1 did not show significant associations with clinicopathologic parameters ($P > 0.05$).

4. Discussion

Adenocarcinoma arising from the cervix and the endometrium in our series shows overlapping clinicopathological presentations. The youngest patients for both cancers were 30 years of age though the mean age of endocervical adenocarcinoma is about five years younger. There was a number of adenocarcinomas of the cervix which was in the mean age of adenocarcinoma of endometrium. These two cancers also have similar ethnic distribution. The ethnic distribution of our cases is comparable with the background population implying fair representation of the sampling population. They are more seen among the ethnic Malay than other ethnic groups in this series depicting the ethnic distributions of the community served by the two hospitals.

Table 4: Associations between p16INK4a expression and clinicopathologic parameter for endocervical adenocarcinoma.

Characteristics	No. of patients	Positive, n (%) ($n = 32$)	Negative, n (%) ($n = 8$)	P value
Age (years)				
<35	3	2 (66.7)	1 (33.3)	0.256
35–54	22	16 (72.7)	6 (27.3)	
≥55	15	14 (93.3)	1 (6.7)	
FIGO stage				
I-II	30	24 (80.0)	6 (20.0)	1.000
III-IV	10	8 (80.0)	2 (20.0)	
Histology tumor differentiation				
Well/moderate	25	18 (72.0)	7 (28.0)	0.102
Poor	15	14 (93.3)	1 (6.7)	
Infiltration depth				
Inner 2/3	13	9 (69.2)	4 (30.8)	0.237
Outer 1/3 or through	27	23 (85.2)	4 (14.8)	
Tumor size ([diameter)				
<20 mm	11	8 (72.7)	3 (27.3)	0.479
>20 mm	29	24 (82.8)	5 (17.2)	
Tumor thickness				
<5 mm	4	3 (75.0)	1 (25.0)	0.792
>5 mm	36	29 (80.6)	7 (19.4)	
Infiltration to corpus				
Yes	17	13 (76.5)	4 (23.5)	0.631
No	23	19 (82.6)	4 (17.4)	
Vascular invasion				
Yes	20	17 (85.0)	3 (15.0)	0.429
No	20	15 (75.0)	5 (25.0)	
Lymph node invasion				
Yes	12	8 (66.7)	4 (33.3)	0.168
No	28	24 (85.7)	4 (14.3)	
Histologic type				
Endocervical	25	20 (80.0)	5 (20.0)	1.000
Non endocervical	15	12 (80.0)	3 (20.0)	

Table 5: Associations between P21waf1 expression and clinicopathologic parameter for adenocarcinoma of the cervix.

Characteristics	No. of patients	Positive, n (%) ($n = 28$)	Negative, n (%) ($n = 12$)	P value
Age (years)				
<35	3	2 (66.7)	1(33.3)	0.937
35–54	22	15 (68.1)	7 (31.8)	
≥55	15	11 (73.3)	4 (33.7)	
FIGO stage				
I-II	30	23 (76.7)	7 (23.3)	0.111
III-IV	10	5 (50.0)	5 (50.0)	
Histology tumor differentiation				
Well/moderate	25	16 (64.0)	9 (36.0)	0.285
Poor	15	12 (80.0)	3 (20.0)	
Infiltration depth				
Inner 2/3	13	10 (76.9)	3 (23.1)	0.507
Outer 1/3 or through	27	18 (66.7)	9 (33.3)	
Tumor length				
<20 mm	11	7 (63.6)	4 (36.4)	0.589
>20 mm	29	21 (72.4)	8 (27.6)	
Tumor thickness				
<5 mm	4	1 (25.0)	3 (75.0)	0.101
>5 mm	36	27 (75.0)	9 (25.0)	
Infiltration to corpus				
Yes	17	9 (52.9)	8 (47.1)	**0.043**
No	23	19 (82.6)	4 (17.4)	
Vascular invasion				
Yes	20	15 (75.0)	5 (25.0)	0.490
No	20	13 (65.0)	7 (35.0)	
Lymph node invasion				
Yes	12	6 (50.0)	6 (50.0)	**0.071**
No	28	22 (78.6)	6 (21.4)	
Histologic type				
Endocervical	25	17 (68.0)	8 (32.0)	0.722
Non endocervical	15	11 (73.3)	4 (26.7)	

We noted that certain clinicopathological parameters were significantly associated with other parameters within the same cancer. For adenocarcinoma of the cervix, the age of the patients was significantly associated with tumor size and with depth of infiltration. The histologic grade was strongly associated with presence of lymph node metastasis. FIGO staging was strongly associated with presence of corpus infiltration, vascular invasion, and lymph node metastasis. For adenocarcinoma of endometrium, the age of the patients was associated with menopausal status, FIGO staging with histological grading of the cancer, and presence/absence of myometrial invasion.

When these clinicopathological parameters were tested against the expression of the three cell cycle markers, none of the clinicopathological parameters of adenocarcinoma of the cervix including the histologic subtypes had significant association with p16INK4a expression alone. However, when p16INK4a expression and p27Kip1 expressions were analyzed in combination, there were strong associations with presence/absence of lymph node metastasis. p21WAF1 expression alone is significantly associated with infiltration of the corpus and lymph node metastasis. Lymph node involvement was also significantly associated with p27Kip1 expression. For adenocarcinoma of endometrium, only P16INK4a expression had strong association with the histologic grade. p21WAF1 and p27Kip1 expressions were not significantly associated with any clinicopathological parameters. Our study shows that we could use these cell cycle markers as predictors for more aggressive subsets of adenocarcinomas of the cervix and endometrium.

Table 6: Associations between p27Kip1 expression and clinicopathologic parameter for endocervical adenocarcinoma.

Characteristics	No. of patients	Positive, n (%) ($n = 17$)	Negative, n (%) ($n = 23$)	P value
Age (years)				
<35	3	2 (66.7)	1 (33.3)	0.292
35–54	22	7 (31.8)	15 (68.2)	
≥55	15	8 (53.3)	7 (46.7)	
FIGO stage				
I-II	30	15 (50.0)	15 (50.0)	0.097
III-IV	10	2 (20.0)	8 (80.0)	
Histology tumor differentiation				
Well/moderate	25	11 (44.0)	14 (56.0)	0.804
Poor	15	6 (40.0)	9 (60.0)	
Infiltration depth				
Inner 2/3	13	5 (38.5)	8 (61.5)	0.720
Outer 1/3 or through	27	12 (44.4)	15 (55.6)	
Tumor length				
<20 mm	11	5 (45.5)	6 (54.5)	0.818
>20 mm	29	12 (41.4)	17 (58.6)	
Tumor thickness				
<5 mm	4	1 (25.0)	3 (75.0)	0.395
>5 mm	36	16 (44.4)	20 (55.6)	
Infiltration to corpus				
Yes	17	6 (35.3)	11 (64.7)	0.428
No	23	11 (47.8)	12 (52.2)	
Vascular invasion				
Yes	20	7 (35.0)	13 (65.0)	0.337
No	20	10 (50.0)	10 (50.0)	
Lymph node invasion				
Yes	12	2 (16.7)	10 (83.3)	**0.030**
No	28	15 (53.6)	13 (46.4)	
Histologic type				
Endocervical	25	9 (36.0)	16 (64.0)	0.283
Non endocervical	15	8 (53.3)	7 (46.7)	

Table 7: Associations between P16INK4a expression and clinicopathologic parameter for endometrial adenocarcinoma.

Characteristics	No. patients	Positive, n (%) ($n = 23$)	Negative, n (%) ($n = 69$)	P value
Age (years)				
<50	28	5 (17.9)	23 (82.1)	0.148
50–60	35	7 (20.0)	28 (80.0)	
>60	29	11 (38.0)	18 (62.0)	
FIGO stage				
I	50	12 (24.0)	38 (76.0)	0.809
II–IV	42	11 (26.2)	31 (73.8)	
Myometrial invasion				
< 1/2	45	9 (20.0)	36 (80.0)	0.278
> 1/2	47	14 (29.8)	33 (70.2)	
Histologic grade				
G1-G2	62	11 (17.7)	51 (82.2)	**0.014**
G3	30	12 (40.0)	18 (60.0)	
Postmenopausal status				
Yes	54	16 (29.6)	38 (70.4)	0.222
No	38	7 (18.4)	31 (81.6)	
Histologic type				
Endometrioid	75	16 (21.3)	59 (78.7)	0.888
Non endometrioid	17	7 (41.2)	10 (58.8)	

There are a number of studies which have utilized the use of cell cycle regulatory genes such as P16INK4a, p21WAF1, and p27Kip1 in predicting the behavior of certain cancers such as malignant astrocytomas [23], oral squamous cell carcinomas [24], vulval carcinomas [25], and primary large-cell neuroendocrine carcinoma of the parotid gland [26]. In the latter, markedly reduced expressions of p21Waf1 and p27Kip1 were noted in the salivary gland cancer cells indicating highly aggressive biologic behavior [26]. Methylation of p16INK4a was seen to be correlated with gender and tumor size ($P = 0.005$ and $P = 0.035$, resp.) in colorectal carcinoma (CRC) and could be used as a marker of poor prognosis in CRC [27]. In 224 vulvar squamous cell carcinomas stained with p16, p21, and p27, Knopp et al., noted a high expression of p16 as indicator of a better prognosis in the multivariate analysis (RR = 0.5, 95% CI = 0.2–1.0) and less risk of developing lymph node metastasis (OR = 0.3, 95% CI = 0.2–0.7) [25]. They also noted that a high level of p21 was significantly associated with shorter survival in patients staged FIGO I and II (RR = 3.4, 95% CI = 1.3–9.3). We could not find any published literature on utilizing the use of these markers on adenocarcinoma of the cervix and endometrium

In conclusion, for adenocarcinoma of the cervix, p21WAF1 expression is significantly associated with infiltration of the corpus and lymph node metastasis. p27Kip1 expression is significantly associated with lymph node invasion. The presence of lymph node metastasis is strongly associated when p16INK4a and p27Kip1 expressions are analyzed in combination. For adenocarcinoma of the endometrium, p16INK4a expression is associated with histologic grade but not histologic type. Our study shows that we could use these cell cycle markers as predictors for more aggressive subsets of adenocarcinoma of the cervix and endometrium.

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Review Article

Aspects of Prophylactic Vaccination against Cervical Cancer and Other Human Papillomavirus-Related Cancers in Developing Countries

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Cervical cancer and other human papillomavirus- (HPV-) related cancers are preventable, but preventive measures implemented in developing countries and especially in low-income rural regions have not been effective. Cervical cancer burden derived from sexually transmitted HPV infections is the heaviest in developing countries, and a dramatic increase in the number of cervical cancer cases is predicted, if no intervention is implemented in the near future. HPV vaccines offer an efficient way to prevent related cancers. Recently implemented school-based HPV vaccination demonstration programmes can help tackle the challenges linked with vaccine coverage, and access to vaccination and health services, but prevention strategies need to be modified according to regional characteristics. In urban regions WHO-recommended vaccination strategies might be enough to significantly reduce HPV-related disease burden, but in the rural regions additional vaccination strategies, vaccinating both sexes rather than only females when school attendance is the highest and applying a two-dose regime, need to be considered. From the point of view of both public health and ethics identification of the most effective prevention strategies is pivotal, especially when access to health services is limited. Considering cost-effectiveness versus justice further research on optional vaccination strategies is warranted.

1. Background

1.1. Human Papillomavirus and Cervical Cancer. Human papillomavirus (HPV) is the main cause of cervical cancer [1], the third most common cancer in women [2]. There is, however, growing evidence linking genital HPV infection to other anogenital cancers (anus, vulva, vagina, and penis) and head and neck cancers in both men and women [3–11]. HPV is carried by both females and males and can spread with high (up to 0,6 per act for HPV16) transmission probability [12], and most (up to 70–80%) people will get infected during their lifetime [13]. Thus, HPV can be characterised as a ubiquitous, sexually transmitted infection (STI) causing significant disease burden in both sexes, but especially in women with up to 6-7% lifetime risk of developing cervical cancer in Latin America [14].

Of the estimated 530000 annual cases of cervical cancer, 86% occur in developing countries [2]. Its standardised incidence ratio (world, 100) is substantially higher in the developing countries (116) than in the developed countries (60). It is important to note that cervical cancer is the most common cancer in women in most parts of Africa, Central America, Southern Asia, and Melanesia. [3] It is also the most important cause of years of life lost in Latin America and the Caribbean, and among cancers in the populous regions of Sub-Saharan Africa and South-Central Asia [14]. Furthermore, largely due to changes in sexual risk taking behaviour and (in some countries) dynamic state of epidemics by high-risk (hr) HPV types [15, 16], the number of cervical cancer cases has been predicted to rapidly increase (up to 90%) by 2020 in developing countries if no intervention is implemented [17, 18].

1.2. Occurrence of Other HPV-Related Cancers. It has been established that the HPV attributable proportion in cancers of the anus, vagina, penis, vulva, oropharynx, and oral cavity

is 25% or higher [8, 11, 19–21]. There are clear signs that the incidence of most HPV-related cancers is increasing. The incidence rates of anal cancer in Scotland and England have nearly doubled in both women and men from 1986 to 2003 [22]. Increase in the incidence of anal cancer is also reported in Australia [23]. In the developed countries, also the incidence of HPV-related head and neck cancer is rapidly increasing especially in males and in the younger birth cohorts [5, 6, 9–11, 24]. In Australia, Netherlands, Sweden, and USA, the incidence of HPV-related tonsillar cancer has rapidly increased during recent years [7, 23, 25, 26]. Similar trends have not yet been reported for the developing countries.

1.3. Occurrence of Infections with High-Risk HPV Types. Most of the genital infections with hrHPV type(s) are asymptomatic and heal without treatment. The risk of cervical cancer increases as the hrHPV infection persists. Over 70% of cervical cancers are attributed to HPV types 16 and 18 and approximately 20% to HPV types 31, 33, 35, 45, 52, and 58 [3, 27]. Precancerous cervical lesions usually appear within 5 years in individuals with an established persistent infection with HPV types 16 and 18. A majority of other HPV-related cancers are also attributable to HPV types 16 and 18. For example, it is estimated that 24% of cancers in the mouth are associated with HPV and 95% of these cancers are attributable to HPV types 16 and 18. Over 80% of anal cancers are associated with HPV and 92% of these cancers are attributable to HPV types 16 and 18. Thus, it can be assumed that targeting HPV types 16 and 18 and a remarkable proportion of phylogenetically related hrHPV types (31, 33, 45) by prophylactic vaccination [28–30] would play a significant role in preventing all HPV-related cancers [27].

Prevention of hrHPV infections could decrease the incidence of numerous cancers in both sexes [5, 31]. In healthy men, HPV infection in the genital tract is very common (from 35% to 73%) [32]. Alike other STIs, HPV transmits more easily from men to women than from women to men [3]. Male circumcision and use of condoms prevent the spreading of hrHPV infections, which probably explains the low incidence of cervical cancer in countries such as Israel where circumcision is widespread [3, 33]. Racial differences have been reported showing that African-Americans are less likely to have HPV-positive head and neck (oropharyngeal) cancers [34, 35]. Reasons for the recent increase of oropharyngeal HPV-related cancers, especially in younger male birth cohorts in the developed countries, are not clear, but changes in sexual behaviour, efficiency of HPV transmission through oral sex, and lack of protective immunity from hrHPV infections of the genital mucosae have been suggested [6, 36].

Various studies indicate that a high number of lifetime sexual partners, tobacco smoking, parity, oral contraceptive use, and coinfections with *Chlamydia trachomatis* and HIV increase the risk of acquisition of hrHPV infection [37–42]. The impact of cofactors on the acquisition of infections with multiple hrHPV types has not been studied largely [43, 44].

STIs are a serious health problem in developing countries, and several studies indicate that conventional STIs increase the likelihood of HIV transmission [45]. HPV infections in both females and males are also risk factors for HIV acquisition [46–48]. Prevention of HPV infections could play a part in preventing other STIs as well [49]. It is also highly likely that HPV-related neoplasia progresses faster in HIV-positive people [50].

2. Prevention of HPV-Related Cancers

2.1. HPV-Related Cancers and Cancer Mortality Are Preventable. Cervical cancer is a disease which can be prevented. Similar to liver cancer that is secondary to hepatitis B infection, cervical cancer has a recognized, single necessary cause, HPV. Application of cervical cytology in population-based screening programmes has significantly lowered the incidence of cervical cancer in developed countries [3]. Cervical cancer incidence and mortality decreased markedly in the Nordic countries, Europe, Canada, and USA due to the implementation of cervical cytology in health care, most notably in population-based screening programmes [51–54]. Mortality from cervical cancer has also substantially declined since the 1960s in Europe, but there are still large country-specific differences [55]. Cervical cancer mortality is substantially higher in Eastern Europe than in other parts of Europe. Increases in the incidence of and mortality from cervical cancer have been reported in the last 15 years resulting from HPV epidemics and a drop in the number of women participating in the screening programmes [55].

Mortality rates of cervical cancer are lower than incidence rates with a ratio of mortality to incidence of 55% [3]. Survival rates are, however, lower in the developing countries [56–58], and the differences in the ratios of mortality to incidence between developing and developed countries are significant. The ratios range from 20% in Switzerland to 80% in most African countries. Latin America and South Asia have ratios of 40–55% [3]. The differences can be explained by the stage at which cancer is detected, access to health services, and adequacy of treatment.

2.2. New Means for Primary Prevention of HPV-Related Cancer. Currently there are two licensed prophylactic HPV vaccines, a bivalent vaccine (Cervarix) against HPV types 16 and 18 and a quadrivalent (Gardasil) vaccine against HPV types 6, 11, 16, and 18 available (FDA 2006; EMEA 2007). In order to be prophylactic, both vaccines need to be administered before the individual is exposed to HPV types covered by the vaccine. According to reports from the major phase III trials, the vaccines prevent from 97 to 98% of infections caused by HPV types 16 and 18 [59, 60]. Both vaccines have shown a significant cross-protection also against HPV types 31 and 45 [28, 29]. The bivalent vaccine has also shown cross-protection against HPV types 33 and 51 [30]. As indicated above, over 70% of cervical cancers are attributed to HPV types 16 and 18, and approximately 20% to HPV types 31, 33, 35, 45, 52, and 58 [3, 27] which fits the cross-protection efficacy and reported 87% overall

vaccine efficacy against CIN3+ [29]. It is assumed that both HPV vaccines, in preventing hrHPV infections, prevent other HPV-related cancers besides cervical cancer with high to moderate efficacy [61].

It is known that the existing vaccines are most efficient for antibody production when administered to early adolescents. Both males and females had higher antibody responses at the age of 9–15 compared to the age of 16–26 [62, 63]. There is, however, no concrete information available on long-term (>10 years) efficacy of the vaccines and necessity for a booster. One model has predicted an over 20-year protection but at the moment the predictions rely only on assumptions [64]. It is not known if HIV infection will affect the efficacy of HPV vaccines [65], but smoking does not seem to affect HPV vaccine-induced antibody response [42]. Booster doses work very well and produce higher antibody levels when measured one month later [62]. HIV positivity as such does not hamper development of HPV antibodies following natural infection [66], whereas smoking does [42]. Type replacement, that is, how nontargeted hrHPV types that may have competitive advantage [67] will behave following mass vaccination, is an open question, but the likelihood of the kind of type replacement seen following bacterial vaccination is small due to the different biology of viral and bacterial infections [68, 69].

3. Primary Prevention Strategies in Developed Countries

According to WHO, 22 countries in low-resource regions have included an HPV vaccine in their vaccine programmes [70]. Adopted vaccination strategies include offering the vaccine only to 12–15-year-old girls or only to a certain proportion of the female population of the same age. The vaccination strategies are in line with the results of numerous cost-effectiveness studies which suggest that with high vaccine coverage (≥75%) vaccination of males would not be cost effective [71–82]. Vaccinating males becomes cost effective by assuming a low to moderate (30%–50%) coverage in females [74].

The assumptions behind these recommendations and the superior cost-effectiveness of female vaccination strategy are that the health service system covers all the regions equitably and that adolescent girls have both access to the health services and are willing to use it. However, the probability of preventing other HPV-related cancers both in females and males has not been taken into account. Emerging information on HPV-related cancers in men may change the conclusions of cost-effectiveness modelling. In Australia, it is estimated that one quarter of the preventable cancers are in men [23], but this most likely varies between countries.

There is not enough information on how vaccinating males would change the transmission of HPV, but it is possible that vaccinating only females could result in an increase in HPV transmission like in the case of Rubella vaccination and Rubella acquisition by young adult females in the UK [83]. British data also suggests that due to sexual behaviour characteristics British men are at greater risk of being exposed to, contracting, and transmitting HPV infection than females. Each vaccinated male would therefore reduce the infection risk more than a vaccinated female [84]. It is important to estimate if this assumption is true, and the extent of the possible difference. Overall, the highly infectious nature (high transmission probability) of HPV supports the idea of vaccinating both sexes. It is also possible that vaccinating the same number of males and females as in the female-only vaccination strategy would decrease the prevalence of hrHPV infections slightly in a steady state of hrHPV epidemics [85] and in dynamic state of the hrHPV epidemics (as is the case in many countries), and it is likely that the impact of male vaccination would be higher.

The questions concerning HPV vaccine efficacy on males and the possible effect of vaccination on HPV transmission are valid as it is known that the vaccine against herpes simplex virus type 2 is not effective on males [86] but it could still have a profound effect on HSV-2 occurrence through herd immunity provided viral shedding is significantly reduced [87]. Prevention of infectious diseases comparable to HPV with vaccines is based on producing herd immunity through a sufficient coverage in susceptible individuals to reduce transmission [87]. The quadrivalent HPV vaccine is proven efficacious in males, likely prevents HPV transmission [88], and has been shown to reduce HPV 6/11-associated disease burden. Vaccinating males is currently not recommended by the WHO, but the impact of herd immunity on female cancers and other HPV-related cancers may need to be reconsidered [89, 90].

High vaccine coverage is needed to produce herd immunity, and the key question is whether sufficient coverage can be achieved by vaccinating only females. Sufficient coverage has been achieved only in the UK (75%) and Australia (70%). In the Netherlands (50%), Germany (40%), and USA (25%), the coverage is neither enough to protect significant proportions of females nor to produce herd immunity. Protection is effective on an individual level, but in the absence of herd immunity the unvaccinated remain unprotected. At present, the questions concerning coverage in both sexes and the magnitude of herd immunity remain open [65, 87, 90]. Mathematical models suggest that vaccinating both males and females could produce herd immunity and an impact both on hrHPV prevalence and occurrence of cervical cancer with considerably lower coverage than vaccinating females only [12, 89, 90]. This could be a decisive factor in the low-income areas where there are problems with access to health services. Other reports indicate better results (reduction of HPV prevalence in unvaccinated females by 86–96% versus 7–31%) with a vaccine coverage of 80% in females and males versus 80% in females only [91], but there is no evidence-based data available yet.

4. Prevention Strategies of HPV-Related Cancers in the Developing Countries

4.1. A Different Point of View. The need for cervical cancer prevention is the greatest in developing countries where the burden of cervical cancer and other HPV-associated

cancers is the heaviest, and preventive measures have not been/cannot be implemented consistently. From the point of view of global justice, the prevention of cervical cancer should be a priority in countries where its burden is the heaviest. The Global Alliance for Vaccines and Immunisation (GAVI) considers HPV vaccines among the vaccines that would have the biggest impact on the disease burden in developing countries. Within the developing countries (and in some developed or middle-income countries), the situation in rural regions with major problems of access to health services poses various questions concerning equity and justice [92]. In the reality of overall scarce resources, rural regions tend to suffer the most [93]. This is exemplified in how screening has failed to make an impact in developing countries and especially in rural regions with problems of access to all cervical cancer prevention health services, including screening, diagnosis, treatment, and followup [53, 94]. The poor (<50%) acceptance of cervical screening by a significant proportion of females in the younger birth cohorts has resulted in a comparable loss of impact with consequent increase in cervical cancer incidence in the developed countries. In the developing countries the accessibility of health services is far too low to guarantee desired impact overall. HPV vaccines offer qualitatively different (primary/complete versus secondary/incomplete prevention) possibilities for preventing cervical cancer in women and other HPV-related cancers in both women and men.

Due to the assortative nature of common sexually transmitted infections like HPV, the sufficient coverage to produce herd immunity effect is relatively low [95]. In the case of HPV, with a narrow window of applicability (before sexual debut), it is advisable to decide early enough if the vaccine is offered only to females or to both sexes, that is, all potential carriers of HPV infection. The question is pertinent in all regions where achieving (access to or acceptability of an HPV vaccine) high vaccine coverage is challenging. The challenges are numerous varying from financial restraints of access to cultural or religious acceptability of the vaccine.

In addition to access to health services (in this case vaccination or screening), it should be noted that the success of a vaccination or a screening programme is partly dependent on decision making by the objects of the intervention or other parties like parents, spouses, other family members, influential persons in the community, and politicians [96]. In the case of HPV, attitudes towards vaccination are more positive in persons who have more information on the vaccine, HPV, and the causality between HPV and cancer [97]. It is probable that those who have the least information are less likely to participate than those with solid/improved information. The average, complete nonacceptability does not exceed 10–15% [96–106] but can be further reduced with health education. According to numerous studies, women and men in general are unaware of the causality between HPV and cervical cancer, and it can be assumed that the connection between HPV and HPV-related cancers is even less generally known. Attitudes towards HPV vaccination are, however, generally positive [106] which is probably due to positive attitudes towards vaccinations in general [107].

HPV is such a common infection that a risk-group intervention alone is not likely to produce good results and have an impact. A high-risk group strategy based on behavioural characteristics would also be problematic to implement for a variety of reasons, most notably the inability to identify people at risk. Moreover, HPV does not have a tight core group as many classical STIs [108]. A useful strategy here might be to implement "add-ons" for risk groups in addition to a general vaccination programme. In the case of HPV, there may be two different risk groups: those who are at risk of infection because of risk-taking behaviour and those who are at risk of nondetection of sequelae because of geographical differences in access, that is, rural versus urban residence. Due to the ubiquitous nature of HPV infection it is reasonable that the WHO recommendation takes only the latter risk group into account by recommending starting a phased introduction in populations who do not have access to screening [109].

Cervical cancer is associated with poverty on both the global [3] and regional scalen [110–112]. The same association can be seen in cancer incidence and mortality in general [113, 114]. Poverty is the most common source of inequity along with gender, ethnicity, religion, geography, age, education, and social status [115–118]. It has been argued that health inequity in the developing countries is likely to increase if HPV vaccination programmes are not implemented [92, 119]. Cervical cancer mortality rate, which can indicate the effectiveness of screening, diagnosis, treatment, and followup, is proportionally higher (up to 18 times) in rural than in urban areas [110–112, 120]. People with low social status living in the developing (or middle-income) countries and in the rural regions are the least well off in the case of HPV-related diseases. This again highly favours HPV vaccination as a prevention strategy in low-income areas with special emphasis on regional characteristics.

This regional aspect has its implications in terms of judging different methods and optional preventive strategies that can guarantee access to and coverage of primary prevention. In the urban areas the recommended prevention strategies might be efficient to significantly reduce HPV disease burden because there are fewer problems connected to access and coverage. The challenges of implementing HPV vaccination programmes in practice are similar to some elements of implementing screening programmes, that is, financial constraints, competing health needs, and limited human resources. The infrastructure and logistical capacity needed are much more limited in rural regions and will require investments.

4.2. Optional Strategies for Rural and Urban Regions. The question is whether we can opt for a single strategy for a country or a group of countries or we should look at regional characteristics linked to access in deciding which strategy to adopt case by case. Malmqvist et al. [121] have stated that strategies aiming at herd immunity (providing the vaccine to males and females) might be the best way to prevent cervical cancer from the point of view of justice. Strategies aiming at

herd immunity in the developed countries would also protect those who do not have access to the vaccine (or screening) or who do not accept vaccination or screening. Strategies that are doable in developed countries may not be practical in low-resource regions. Thus, it is appealing to contemplate different strategies for rural and urban regions taking into account access/distance to health services and opting for regional and/or population subgroup-specific strategies in order to achieve the highest possible impact.

In case mass vaccination would be offered, several strategies exist. A few relevant regional or even community-level strategies from the point of view of developing countries and/or regions are described as follows:

(1) vaccinating females at the age of 12–15:

  (i) unrealistic targeting a coverage of 70%,
  (ii) questionable herd immunity effect,
  (iii) marginalized females excluded;

(2) vaccinating females when school attendance is at the highest (10–12 years of age):

  (i) unrealistic targeting a coverage of >80%,
  (ii) prioritizing vaccinating younger girls before school attendance drops—need for boosters?
  (iii) questionable herd immunity effect,
  (iv) marginalized females excluded;

(3) vaccinating females and males at the age of 12–15:

  (i) targeting a coverage of >40% in all regions,
  (ii) cost effectiveness,
  (iii) acceptance of the vaccine for boys?
  (iv) marginalization tackled by the herd immunity effect?

(4) vaccinating females and males when school attendance is at the highest (10–12 years of age):

  (I) targeting a coverage of >50% in all regions,
  (ii) acceptance of the vaccine for boys?
  (iii) marginalization tackled by the herd-immunity effect.

All strategies would be school based with community outreach activities in regions where school attendance is low and equally there would be a need for information campaigns to adolescents, service providers, decision makers, schools, and parents [122]. Information campaigns for both sexes with a notion to all HPV-related cancers might further increase the acceptance of HPV vaccine. It would also be important to address local or cultural issues which are linked to vaccines [93].

In terms of HPV vaccine coverage in developed countries, the highest rates have been achieved by school-based vaccination programmes [123]. School-based demonstration projects have shown promise in terms of coverage and compliance. In a universal school-based vaccination programm the adolescents who go to school may bring also nonattendees to the vaccination site [124]. School attendance of girls has increased in developing countries from 78% in 1990 to 85% in 2005 [125, 126]. Rates of primary school completion follow closely the enrolment figures [127]. The gender gap between boys and girls has disappeared in East Asia, Latin America, and Eastern and Southern Africa and is diminishing both in urban and rural regions and within economic quintiles [125, 127].

The challenges of a school-based programme are many. School-based programmes may not be feasible if sufficient resources are not allocated to providers [128]. School attendance in girls during adolescence may be lower than is needed for effective coverage, and certain high-risk groups might not be reached at all [124, 129].

The project funded by PATH, an American NGO, in Peru, Uganda, India, and Vietnam reached coverage of 80–95% in 9–14-year-old girls in selected schools demonstrating a high acceptability of the HPV vaccine [130]. The PATH project is also producing vital practical information on introducing HPV vaccines in developing countries (e.g., in certain cultures parents do not have documentation concerning date of birth, there may be undue concerns for fertility, emphasis on cancer prevention, etc.). Even though HPV vaccination shares many barriers with cervical screening [131], it seems that barriers linked to acceptation of HPV vaccine might be easier to deal with. For those females who agreed to be vaccinated, completion of the three-dose regimen was over 90% in Peru [130]. It is not clear whether vaccination rates such as these would be achievable in a nonresearch setting.

Optional preventive strategies, regional strategies, or mixed strategies, offering vaccination only to girls in some wealthier regions, and to both sexes in certain low-income regions could tackle the problems linked with coverage and access. In some areas, it might be more feasible to have boys vaccinated because of higher school attendance, and this could lessen problems of coverage even though fewer girls would be reached. Other preventive acts such as male circumcision [132] and use of condoms [133] make this a problem which has a solution that includes actions by both males and females. Tackling the problem as gender-free problem might promote vaccine acceptance and create political will [128]. Hence, the proposed "add-ons" would include vaccinating both sexes to achieve maximal coverage and acceptance. Targeting the early adolescents when school attendance is at the highest but before sexual debut might be a problem. In a study conducted in South Africa, some parents expressed their fear that vaccination at 11 years or older would already be too late [128].

The questions concerning dosage are closely linked with logistics, vaccine storage, vaccine acceptability, and cost effectiveness. The present vaccines are administered in three doses, but it is possible that a two-dose regimen could be enough to provide protection. This would have a significant effect mainly on costs but equally to vaccine acceptability and accessibility. In a study on Kenyan women, the acceptability of a three-dose vaccine regimen was only 31% compared to

86% for a one-dose regimen [105]. It is known that present vaccines do not provide effective protection in a one-dose regimen but a two-dose regimen remains possible.

Further cost-effectiveness studies are needed taking into account other HPV-related cancers besides cervical cancer. Regional characteristics and problems linked with access and coverage should be addressed as well. Vaccination strategies including catch-up vaccination in older females or addressing early adolescents after the sexual debut should be linked with the most accessible screening and treatment methods such as the single visit approach (VIA and cryotherapy) to ensure that the means of prevention would protect those who may be already HPV infected and for whom the prophylactic vaccine cannot be effective.

Information about HPV vaccination and HPV-related cancers continues to emerge, but more research is needed especially on the long-term impact of vaccination, duration of protection, male vaccination, and reduction of HPV transmission. The GAVI Alliance subsidises the provision of vaccines to the poorest countries and is currently reviewing HPV vaccine as a candidate for sustainable financing. Even with secured financing, there is no simple answer concerning which strategy should be adopted in the developing countries.

5. Conclusion

HPV vaccination is the most promising way to prevent cervical and other HPV-related cancers in developing countries. The vaccine is effective, safe, and widely accepted. Vaccination strategies have an important effect on the success of any vaccination programme. In the case of HPV, it is crucial to reach at least 70% of females or 40–50% of both sexes before sexual debut. Currently, only the option of vaccinating females is recommended by WHO.

Optional preventive strategies, regional strategies, or mixed strategies in rural low-income regions could solve the problems linked with HPV vaccination coverage, access, and acceptability. Regional characteristics affect fundamentally the feasibility of HPV vaccination strategies as it has already been proven with screening. Differences between regions in terms of access to health services increase the need to adopt region-specific HPV vaccination strategies that are not currently deemed as cost effective. Emerging information on HPV-related cancers in both women and men and the feasibility of achieving high vaccine coverage in rural regions might produce different results in terms of cost effectiveness, and this might result as a change in strategic aims and recommendations.

Targeting rural low-resource regions with specific vaccination strategies should be a priority from the point of view of ethics and public health. The number of cervical cancer cases is estimated to increase dramatically in developing countries if no intervention is implemented, and the trend is probably even stronger in regions where access to health services is limited. Whatever option is chosen, it is vital to merge any vaccination strategy with appropriate screening methods and sexual education.

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Research Article

Performance Evaluation of Manual and Automated (MagNA Pure) Nucleic Acid Isolation in HPV Detection and Genotyping Using Roche Linear Array HPV Test

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Nucleic acids of human papillomavirus (HPV) isolated by manual extraction method (AmpliLute) and automated MagNA pure system were compared and evaluated with cytohistological findings in 253 women. The concordance level between AmpliLute and MagNA was very good 93.3% ($\kappa = 0.864$, $P < .0001$). Overall HPVpositivity detected by AmpliLute was 57.3% (30.4% as single and 27% as multiple infections) in contrast to MagNA 54.5% (32% and 23%, resp.). Discrepant results observed in 25 cases: 11 MagNA(−)/AmpliLute(+), 10 of which had positive histology; 5 MagNA(+)/AmpliLute(−) with negative histology; 8 MagNA(+)/AmpliLute(+): in 7 of which AmpliLute detected extra HPV genotypes and 1 MagNA(invalid)/AmpliLute(+) with positive histology. Both methods performed well when compared against cytological (area under curve (AUC) of AmpliLute 0.712 versus 0.672 of MagNA) and histological diagnoses (AUC of AmpliLute 0.935 versus 0.877 of MagNA), with AmpliLute showing a slightly predominance over MagNA. However, higher sensitivities, specificities, and positive/negative predictive values were obtained by AmpliLute.

1. Introduction

It is now well established and widely accepted that virtually all cervical cancer and its immediate precancerous lesions arise from persisting cervical infection by some highly oncogenic HPV genotypes [1, 2]. The most important of these HPV genotypes are HPV16 and HPV18 which account for ~70% of all invasive cervical cancers with minor variations in this percentage between continents [3].

Fifteen HPV genotypes have been to date classified as high-risk (HR) types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), 3 as probably HR (26, 53, 66) and 12 as low-risk (LR) (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108) [1, 4].

The majority of HPV infections are transient, but persistence of an HR HPV is a significant risk factor for the development of cervical cancer. This occurs only in a minority of infections and is an unpredicted event. It could be a genetic predisposition with an inadequate immune response and/or possible uncontrolled reaction with tumor suppressor genes [5, 6].

Type-specific detection of HPV is increasingly important for monitoring the impact of HPV vaccine implementation and as a tool for cervical cancer screening. As a consequence, standardization of laboratory methods for HPV detection and typing is important. The commercial HPV detection kits: Hybrid Capture II (Digene Corporation, Gaithersburg, Md, USA), Cervista HPV HR (Third Wave Technologies, Inc., Madison, USA) and Cervista 16/18 tests are approved by the FDA for use in routine screening of HPV. However, the above assays are unable to discriminate specific genotypes or to identify infections involving multiple genotypes and the Cervista assay detects only two HPV types (types 16 and 18).

Various molecular assays for HPV detection and typing have been used in epidemiological studies, and they are based on two different technologies: (1) hybridization-based assays (e.g., HC II) and (2) PCR-based tests (e.g., GP5+/GP6+, PGMY09/11, INNO-LiPA HPV Genotyping (Innogenetics, Belgium), Linear Arrays HPV test (Roche Molecular Systems, Inc. Branchburg, NJ, USA), CLART HPV2 (Genomica, Madrid, Spain). The advantages and disadvantages of these two basically different methodologies have been extensively discussed [7–19].

The qualitative Linear Array HPV (LA-HPV) HPV genotyping test, developed by Roche Molecular Systems offers a reliable, sensitive, and standardized approach for HPV typing in cervical specimens. It is distributed as a research use only but it has been submitted for FDA review. This test utilizes amplification of target DNA by PCR and nucleic acid hybridization for the detection of 37 types in cervical cells collected into an LBC media. This test includes four steps: specimen preparation—DNA extraction, PCR amplification, hybridization of the amplified products with specific probes and colourimetric detection on the hybrids on strip [13, 17, 20–22]. Current specimen processing protocols recommend the use of manual extraction of DNA using the AmpliLute liquid media extraction kit, based on the QIAamp method (QIAGEN, Inc., Valencia, Calif, USA). An alternative method for DNA extraction is the automated MagNA Pure LC extraction system, developed by the same company.

The objective of this study was to evaluate and compare the automated MagNA pure DNA extraction method with the AmpliLute DNA extraction method in detecting HPV DNA form ThinPrep Pap tests using the linear array (LA) HPV genotyping and detection assays and also to correlate these results to cytological and histological diagnosis.

2. Methods

2.1. Clinical Specimens. In the present study, cervical brush specimens were obtained from women aged from 17 to 70 years who attended the gynecologic outpatient clinic of "Attikon" University Hospital, Athens, Greece, for opportunistic examination, between July 2009 and May 2010. Women considered eligible for the study if they fulfilled the following criteria: (a) they agreed to undergo colposcopy and if necessary cervical biopsy and (b) there was enough residual biological material, after cytological examination, for the two molecular assays to be completed. A total of 253 women met these criteria and were enrolled in the study. This patient population does not represent the general population of women attending public screening programs. Approval from the ethics committee was obtained before inclusion.

2.2. Cytological Diagnosis. Samples of ThinPrep Pap tests were collected by means of a Brun's-like brush. The PreservCyt vials (Corporate Headquarters: Hologic, Inc., Ltd., UK), containing the cell samples were addressed to the Department of Cytopathology of the aforementioned hospital for preparation of thin-layer slides using the ThinPrep 2000 Automated Slide Processor (Corporate Headquarters: Hologic, Inc., Ltd., UK) according to the manufacturer's instructions. Cytological findings were interpreted according to the Bethesda classification system and were classified as follows: (a) within Normal Limits (WNL), (b) atypical squamous cells of undetermined significance (ASC-US), (c) low-grade squamous intraepithelial lesion (LSIL), and (d) high-grade squamous intraepithelial lesion (HSIL). The cytopathologists and the biologist conducting HPV testing were all blinded to the clinical profile to ensure unbiased reporting.

2.3. Histological Diagnosis. A cervical biopsy was performed if lesions were present upon colposcopy. All histological assessments were made blinded to the HPV DNA status of the participants. The histological evaluation revealed the following categories: negative HPV, CIN 1, CIN 2, and CIN 3. In case histology showed a CIN 2 or CIN 3, the patient was referred for appropriate treatment.

2.4. DNA Extraction Methods. After slide preparation for cytological examination, the remaining PreservCyt samples were vortexed vigorously for 15 sec to maximize homogeneity and two aliquots of 250 μL and 1 mL were generated from each clinical specimen.

DNA was isolated using two different procedures (i) a 250 μL aliquot was extracted by AmpliLute liquid media kit (Roche Molecular Systems) in conjunction with a QIAvac 24 plus vacuum system, according to the manufacturer's instructions in the product insert and (ii) a 1 mL aliquot was extracted by MagNA Pure LC extraction system using the DNA-I kit (blood cells high-performance protocol) (Roche Molecular Systems). Briefly, for the manual extraction, samples and HPV positive/negative controls were processed in parallel. Clinical samples were mixed by vortexing to form a homologous state, and 250 μL were removed and lysed with proteinase K solution and buffer ATL at 56°C for 30 min. The samples underwent a second incubation at 70°C for 15 min in the presence of buffer AL containing a carrier RNA. The lysate was then transferred to vacuum columns where isolation and purification of DNA was completed via washing of different solutions to bind DNA and remove other cellular materials. Extracted DNA was eluted into 120 μL of buffer AVE. Specimens and controls were immediately stored at 2°C–8°C for up to 7 days or frozen at −20°C for up to 8 weeks.

For automated extraction, the samples were prepared using a modified procedure involving the centrifugation of 1 mL aliquots of the PreservCyt samples at 13000 × g for 20 min prior to discarding of the supernatant. The resulted cell pellets were resuspended into 200 μL of sterile phosphate-buffered saline, and the procedure of automated extraction was followed according to the manufacturer's instruction, using the DNA-I kit. The method is based on magnetic-bead technology with a special buffer containing chaotropic salts and proteinase K. Nucleic acids are bound to the surface of the magnetic glass particles. Cellular debris was removed by several washing steps, and the purified nucleic acids were eluted. 100 μL in volume of extracted

genomic DNA product was obtained, after the magnetic beads were separated from the solution. Specimens and controls were immediately stored at 2°C–8°C for up to 7 days or frozen at −20°C for up to 8 weeks. After nucleic acid purification all samples were analyzed by LA HPV assay for HPV genotyping.

2.5. LA-HPV Amplification (PCR). The LA genotyping test use a pool of biotinylated primers designed to amplify an approximately 450 bp sequence within the polymorphic L1 region of the genome of the 37 HPV genotypes. An additional 268 bp primer pair which targets the human β-globin gene is included in the assay to provide a control for cell adequacy, extraction, and amplification. PCR was carried out on each of the samples and controls, using the Linear Array HPV genotyping mastermix which contains: Tris buffer, potassium chloride, AmpliTaq, gold DNA polymerase (microbial), AmpErase, (uracil-N-glycosylate) enzyme (microbial), dATP, dCTP, dUTP, dGTP, dTTP, each of upstream and downstream primers (biotinylated) and β-globin primers, sodium azide, magnesium chloride, and amaranth dye. The reaction mixture contained 50 μL of HPV mastermix and 50 μL of eluted DNA. The amplification was performed on the Applied Biosystems Gold-plated 96-well GeneAmp PCR System 9700 (Applied Biosystems, Foster City, Calif, USA) using the following thermal profile: 2 min at 50°C, 9 min at 95°C; 30 sec at 95°C, 1 min at 55°C, 1 min at 72°C (40 cycles) with a ramp rate set at 50% followed by 5 min at 72°C and a final hold at 72°C indefinitely. PCR amplicons were immediately denatured by the addition of 100 μL of (DN) denaturation reagent and stored at 4°C for further analysis within 7 days.

2.6. LA-HPV Detection. The detection of the HPV genotypes was carried out using the LA HPV detection kit. Once the amplification was completed, 75 μL of denatured amplicon were added to the linear array strips that contain multiple copies of HPV genotype-specific probes in a defined area for all 37 genotypes and the β-globin reference lines. The HPV types detected are HPV6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51–56, 58, 59, 61, 62, 64, 66–73, 81–84, IS39, and CP6108. The biotin-labeled amplicon was bound to the strips using a hybridization buffer in a shaking waterbath at 53°C. Once bound, the strips were washed at high stringency to remove nonbound material and streptavidin-horseradish peroxidase conjugate was then added and bound to the biotin-labeled amplicon hybridized to the oligonucleotide probes. The strips were then washed with a substrate solution containing hydrogen peroxide and 3,3′,5,5′-tetramethylbenzidine (TMB). In the presence of hydrogen peroxide, the bound streptavidin-horseradish peroxidase catalyzed the oxidation of TMB to form a blue-colored complex, which precipitated at the probe positions where hybridization had occurred (colourimetric determination). This color precipitation allowed for manual reading of the strips and genotype detection by comparison with the HPV reference guide provided. LA test does not directly detect HPV52 but combines a set of probes that detects HPV33, −35 and −58 (HPV mix). Specimens that test negative for HPV33, −35 and −58 individually but are positive for HPV mix are considered to be HPV52 positive. The specimens that test positive for HPV mix and for HPV33, −35 and/or −58 have an uncertain HPV52 status, for this analysis, these specimens were considered to be HPV52 negative, since coinfection with HPV52 cannot be ruled out by this test.

The procedure performed into two physically separated areas (pre-PCR and post-PCR) in order to avoid contamination of samples with previously amplified products. All washes and hybridization steps were undertaken in a 24-well tray with lid. The reading of the strips, produced by the two methods, was made by one well-experienced biomedical scientist. Discrepant interpretations were resolved by a second biomedical scientist and consensus review performed without knowledge of prior results. The LA-HPV test does not cross-react with a variety of viruses, bacteria, protozoa and yeast that could be present in cervical specimens.

2.7. Statistical Analysis. Pairwise comparison of AmpliLute method and MagNA pure method was performed by using kappa (κ) statistics. A κ value of 0 indicates no agreement better than chance, and κ value of 1 indicates perfect agreement, κ values from 0 to 0.20, 0.21 to 0.40, 0.41 to 0.60, 0.61 to 0.80 and 0.81 to 1.00 indicate poor, fair, moderate, good, and very good strengths of agreement, respectively. All P values <.001 are considered statistically significant. Receiver operating characteristics (ROC) curve analysis was applied to calculate and compare AmpliLute method and MagNA pure method with cytological findings. In addition ROC analysis was applied to calculate and compare AmpliLute method and MagNA pure method with histological findings. All the statistical analyses were obtained with the statistical package for the social sciences (SPSS) computer software.

3. Results

A total of 253 women were analyzed in the present study by means of screening for the presence of HPV DNA by using two different DNA extraction methods. Out of the 253 cervical smears, 253 nucleic acid extracts were produced using the AmpliLute liquid media extraction method and 253 DNA extracts were generated using the MagNA pure automated extraction system. All women referred to colposcopy and if visible lesions were found, they were sampled. Patients with severe cervical diseases were further assessed.

The DNA extracts were evaluated by the LA-HPV genotyping test and compared against the reported cytological and histological diagnoses. The levels of sample adequacy for cytological examination and for nucleic acid extraction and amplification efficiency among the specimens, based on β-globin positivity, did not differ dramatically between the tests (Table 1). Sample adequacy was higher with AmpliLute extracts (100%) than with the MagNA pure method (99.6%) and cytology examination (97.6%). Only one nucleic acid extract generated by the MagNA pure LC was invalid after LA

Table 1: Adequacy of 253 samples extracted by the two methods tested by HPV LA test.

Adequate sample	No. of samples (%)		
	Cytology	AmpliLute	MagNA pure*
Yes	247 (97,6%)	253 (100%)	252 (99,6%)
No	6 (2,4%)	0 (0,0%)	1 (0,4%)
Total	253	253	253

* DNA-I modified protocol using 1 mL PreservCyt sample.

genotyping test due to the absence of high and low β-globin result.

The comparison of HPV LA test results using AmpliLute extracts with equivalent MagNA pure extracts showed an overall concordance of 93.3% ($\kappa = 0.864$, $P < .001$) (Table 2). HPV genotype profiles were identical in 228/253 (90.1%) of specimens, including of 103 HPV negative samples and 125 positive samples with identical HPV status detected by both methods. Out of 125 cases, the same HPV profile was identified in 73 single infections and in 52 multiple infections. Discrepant results observed in 25 samples including 11 cases which were AmpliLute(+)/MagNA(−), 5 cases AmpliLute(−)/MagNA(+), 5 cases identified as single infections in MagNA but as multiple type infections in AmpliLute, 3 cases in which multiple infections were detected with different HPV profile between the two methods, and in one case, MagNA generated an invalid result as opposed to AmpliLute (Table 3).

Overall, 94/253 (37%) women were diagnosed with normal cytology, 50/253 (19.8%) were exhibited ASCUS, 91/253 (36%) were diagnosed with LSIL, 5/253 (2%) with LSIL but having some positions with HSIL, and 7/253 (3%) with HSIL. In 6 cases (2.4%) the cytological diagnosis was difficult due to inadequacy of the clinical samples (Tables 4 and 5).

HPV positivity detected by AmpliLute was slightly higher compared with MagNA Pure, 57.3% and 54.9%, respectively. The largest percentage of samples negative for HPV DNA was found in WNL category. Analytically, for the cytological category WNL, HPV positivity observed by AmpliLute method was 29/94 (31%), for ASCUS was 46%, for LSIL 77%, for LSIL/HSIL 100% and for HSIL 100%. HPV positivity detected by the MagNA pure LC method for the aforementioned categories was: 33%, 44%, 78%, 80%, and 100%, respectively, (Tables 4 and 5).

Within the studied population, single and multiple type infections were present in every cytological diagnosis. In total, single HPV infections detected by the AmpliLute was 28.5% and by MagNA Pure 30.4% whereas multiple type of infection was observed in 26.5% and 23%, respectively. In this study, multiple infections composed of up to five HPV genotypes in a plethora of combinations. The HPV distribution of single infections (divided into two categories according to the HPV genotype oncogenicity: LR and HR) as well as multiple infections (divided into three categories: LR (when only low-risk HPV types were present), HR-LR (when low- and high-risk HPV types were present) and HR (when only high-risk HPV types were present) in all cytological categories studied are given in Figure 1

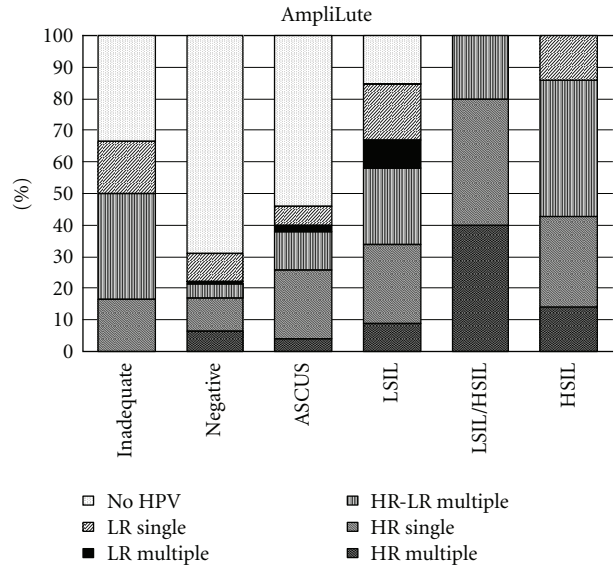

Figure 1: Prevalence of HR-HPV and LR-HPV types according to cytological diagnosis as detected by the AmpliLute extraction method.

for the AmpliLute method and in Figure 2 for the MagNA pure. More multiple infections were detected by AmpliLute method in all cytological categories compared with the MagNA pure (P not statistical significant). Analytically, for the cytological category WNL, the composition of multiple infections observed by AmpliLute method was at 6.4% with HR, 4.2% with HR-LR, and 1% with LR, for ASCUS at 4% with HR, 12% with HR-LR, and 2% with LR, for LSIL at 9% with HR, 24% with HR-LR, and 9% with LR, for LSIL/HSIL at 40% with HR and 20% with HR-LR and for HSIL at 40% with HR, 20% with HR-LR. Multiple infections detected by the MagNA Pure LC method for the aforementioned categories was WNL at 4.2% with HR, 4.2% with HR-LR, and 2% with LR, for ASCUS at 4% with HR, 8% with HR-LR and 2% with LR, for LSIL at 4% with HR, 23% with HR-LR and 9% with LR, for LSIL/HSIL at 20% with HR and 20% with HR-LR and for HSIL at 14% with HR, 43% with HR-LR. The HPV type prevalence according to the two extraction methods is given below with HPV16 being the most frequent type detected, in both types of infections and by the two methods, followed by HPV31, HPV53, HPV6, HPV33, HPV45, HPV42, and HPV51 as detected by the AmpliLute method and by HPV31, HPV53, HPV18, HPV51, HPV18, HPV6, and HPV33 as detected by the MagNA method (data not showed).

The two extraction methods were compared against the cytological findings (inadequate cytological samples were excluded). In terms of processing evaluation, AmpliLute method obtained better results than MagNA pure method (AmpliLute: sensitivity (SE) = 73.2%, specificity (SP) = 69.15%, positive predictive value (PPV) = 79.43%, negative predictive value (NPV) = 61.32%; MagNA Pure: SE = 67.32%, SP = 67.02%, PPV = 76.87%, NPV = 55.75%). Both methods performed well when compared against the cytological diagnosis; nevertheless, the AmpliLute method

Table 2: Concordance between AmpliLute and MagNA Pure (DNA-I, 1 mL)* DNA extracts, in single and multiple HPV infections, identified by LA assay.

MagNA	No. of samples with the following LA results AmpliLute				
	Single	Multiple (same)	Multiple (different)	Negative	Total
Single HPV type	73	5		2	80
Multiple HPV type (same)		52		3	55
Multiple HPV type (different)			3		3
Negative	4	7		103	114
Invalid	1				1
Total	78	64	3	108	253

Table 3: Analysis of cases with discrepant results.

Cytological findings	Histological findings	AmpliLute	MagNA Pure
#1 WNL	HPV	33, 67, 70	67, 70
#2 WNL	HPV	33, 45	(−)
#3 WNL	No biopsy	16, 31, 33, 45	(−)
#4 WNL	No biopsy	(−)	18
#5 WNL	Negative	33, 45	45
#6 WNL	No biopsy	(−)	6, 58
#7 WNL	No biopsy	(−)	16, 33, 35
#8 WNL	No biopsy	(−)	16, 62
#9 WNL	Negative	31, 33, 42	45
#10 ASCUS	HPV	35, 53	35, 53, 54
#11 ASCUS	HPV	39, 42, 52, 84	52
#12 ASCUS	HPV	6, 16, 52	52
#13 ASCUS	HPV	16	(−)
#14 LSIL	HPV	16	(−)
#15 LSIL	CIN 1	16, 31, 33, 45	16, 31
#16 LSIL	CIN 1	16, 31, 33, 45	(−)
#17 LSIL	CIN 1	42	Invalid
#18 LSIL	CIN 1	31, 16	(−)
#19 LSIL	Negative	(−)	59
#20 LSIL	HPV	16	(−)
#21 LSIL	CIN 1	6, 16, 33, 45	(−)
#22 LSIL	CIN 1	52, 82	52
#23 LSIL	CIN 1	16, 31	(−)
#24 LSIL	HPV	16	(−)
#25 LSIL/HSIL	CIN 2	16, 33, 51	(−)

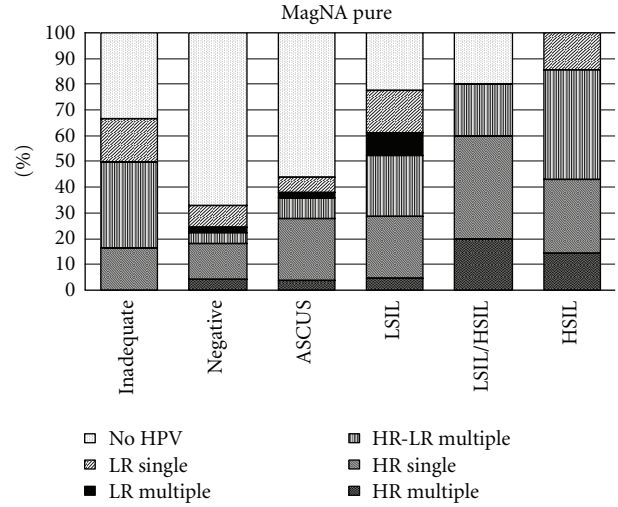

Figure 2: Prevalence of HR-HPV and LR-HPV types according to cytological diagnosis as detected by the MagNA pure extraction method.

demonstrated a slightest higher area under curve (AUC) 0.712 (Std. Error 0.035, 95% CI: 0.644–0.779, $P < .001$) compared to AUC of MagNA pure method 0.672 (std. error 0.036, 95% CI: 0.602–0.742, $P < .001$).

The comparison of ThinPrep diagnosis and histological results is presented at Table 6. Out of 253 colposcopic examinations, 145 women underwent biopsy. In 108 women, no visible lesions were found, and therefore, those women were not sampled. Positive histological result was found in 129 cases. From patients with normal cytology, only 13 out of 94 (13.8%) in biopsy had a HPV lesion or CIN 1 diagnosis. From patients with ASCUS, 14 out of 50 (28%) had HPV in biopsy, 8/50 (16%) had CIN 1, whereas only 1 (2%) patient had CIN 2. In LSIL, 29/91 (31.8%) had a biopsy diagnosis of HPV, 39/91 (42.8%) had CIN 1, 7/91 (7.7%) had CIN 2, and only 2/91 (2.2%) had CIN 3. All cases classified as HSIL with ThinPrep cytology had a biopsy diagnosis of either CIN2 (4/7, 57.2%) or CIN3 (3/7, 42.8%). In LSIL/HSIL category, 1 patient (20%) had CIN1, 3/5 (60%) had CIN2 and 1/5 (20%) had CIN3. Four patients with no cytological diagnosis, due to inadequate of sampling, biopsy revealed lesions with HPV.

Results of biopsy reading and HPV genotyping by the two extraction methods are demonstrated in Tables 7 and 8. Once more, AmpliLute method showed greater performance over MagNA pure (AmpliLute: SE = 100%, SP = 87.5%, PPV = 98.47%, NPV = 100%, FPR = 12.5%, FNR = 0.00%, OA = 98.62%, MagNA pure: SE = 91.47%, SP = 81.25%, PPV = 97.52%, NPV = 54.17%, FPR = 18.75%, FNR = 8.53%, OA = 90.34%). Comparison of AUC for AmpliLute method related to histological diagnosis was 0.935 (std. error 0.018, 95% CI (0.900–0.971), $P < .001$) and it was higher compared to

Table 4: Distribution of HPV types (in single infections) detected by AmpliLute method against cytological diagnosis.

Cytology	AmpliLute																					
	HPV Negative	6	16	18	31	42	51	52	53	54	58	59	61	62	66	73	81	83	84	Multiple	Total
Negative	65	2	7		1				1			2	3			1		1		11	94
ASCUS	27		6	1	2			2	2					1						9	50
LSIL	14	4	14	1	3	4	1	1	2	1	2				1	1	1		2	39	91
LSIL/HSIL			2																	3	5
HSIL			1		1											1				4	7
Inadequate	2			1										1						2	6
Total	108	6	30	3	7	4	1	3	5	1	2	2	3	2	1	3	1	1	2	68	253

Table 5: Distribution of HPV types (in single infections) detected by MagNA pure method against cytological diagnosis.

Cytology	MagNA Pure																						
	HPV Negative	6	16	18	31	42	45	51	52	53	54	58	59	61	62	66	73	81	83	84	Multiple	Inadequate	Total
Negative	63	2	7	1	1		2			1			2	3			1		1		10		94
ASCUS	28		5	1	2				4	2					1						7		50
LSIL	20	4	11	2	3	3		1	2	2	1	2	1			1	1	1		2	33	1	91
LSIL/HSIL	1		2																		2		5
HSIL			1		1												1				4		7
Inadequate	2			1											1						2		6
Total	114	6	26	5	7	3	2	1	6	5	1	2	3	3	2	1	3	1	1	2	58	1	253

AUC of MagNA pure method 0.877 (std. error 0.024, 95% CI (0.830–0.924), $P < .001$).

4. Discussion

Various research assays for HPV detection and typing have been used in epidemiological studies. The LA-HPV genotyping test provides a standardized, consistent and rapid means for HPV detection and genotyping. This test provides the capacity to identify 37 individual HPV genotypes within a given specimen and ascertain whether recurrent HPV positivity is, in fact, due to the persistence of a specific HR HPV genotype, meaning a substantially increased risk of disease progression [23–25]. Current specimen processing protocols recommend the use of manual extraction of DNA using the AmpliLute liquid media extraction kit, based on the QIAamp method (QIAGEN, Inc., Valencia, Calif, USA). This method of DNA preparation is time consuming and labor intensive and is prone to potential specimen cross-contamination, particularly when large numbers of specimens are being processed. An alternative method for DNA extraction is the automated MagNA pure LC extraction system, developed by the same company, which could facilitate the assay by minimizing the potential sample-contamination, hands-on time as well as increase labor efficiency and sample accuracy.

In the present study, we assessed DNA extracts form PreservCyt cervical samples, generated by the automated MagNA pure extraction system and by the manual AmpliLute method (both recommended by Roche) for HPV testing using the LA-HPV genotyping and detection assays. In addition, we correlated those results with the cytological and histological findings of the enrolled participants.

Among the 253 ThinPrep Pap tests analyzed in our study, only one extract from the MagNA pure modified DNA-I extraction protocol was found to be invalid due to the absence of low and high β-globin. In contrast, all nucleic acids generated from the AmpliLute protocol were valid for HPV DNA genotyping. This marginal difference in sample adequacy could be either due to the high AmpliLute protocol efficiency or to the variations in aliquoting the specific sample resulting in an inadequacy of cellular material for the automated procedure. Comparison of the HPV genotyping results, obtained with the AmpliLute DNA to those from the MagNA demonstrated a substantial level of agreement (93.3%), with κ value of 0.864. Both extraction methods, in terms of qualitative results performed equally well when compared against the cytological diagnosis, with AmpliLute method demonstrating a small predominance (AUC of AmpliLute: 0.712 versus AUC of MagNA: 0.672). Nevertheless, the AmpliLute method exhibited higher sensitivity, specificity, positive and negative predictive values as opposed to MagNA method. The same outcome, AmpliLute method being more efficient than the MagNA, was noticed when we compared the two methods with the histological diagnosis. AUC of AmpliLute was 0.935 in contrast with the AUC of MagNA which was 0.877.

HPV types identified in individual samples by each method are largely in agreement 90.1% (228/253). In all studied cases, AmpliLute showed a slightly higher detection rate of HPV compared with MagNA. For the former, HPV

Table 6: Correlation of cytological findings to histological diagnosis.

Cytology	Histology						
	No biopsy	Negative	HPV	CIN 1	CIN 2	CIN 3	Total
WNL	79	2	4	9			94
ASCUS	27		14	8	1		50
LGIL		14	29	39	7	2	91
HGIL					4	3	7
LSIL/HSIL				1	3	1	5
Inadequate	2		4				6
Total	108	16	51	57	15	6	253

Table 7: Distribution of HPV types (in single infections) detected by AmpliLute method against Histological Diagnosis.

Histology	AmpliLute																				
	HPV6	16	18	31	42	51	52	53	54	58	59	61	62	66	73	81	83	84	Multiple	Negative	Total
Negative																			2	14	16
HPV	4	12	1		3	1	1	4	1	2			2	1	1	1		2	15		51
CIN 1		11	2	6	1		2												35		57
CIN 2		4		1											1				9		15
CIN 3		3																	3		6
No biopsy*	2							1			2	3			1		1		4	94	108
Total	6	30	3	7	4	1	3	5	1	2	2	3	2	1	3	1	1	2	68	108	253

*Women without colposcopic findings.

overall positivity was calculated at 57.3% comprising 30.4% of HPV detected as single infection and 27% as multiple. And for the latter, the respective positivity was 54.5%, 32% as single and 23% as multiple type of infection. The prevalence of HPV-infected samples increased, in both methods, with the severity of cytological diagnoses: 30.8% of AmpliLute versus 33% of MagNA in the WNL, 46% versus 44% of ASCUS, 84.6% versus 77% of LSIL, 100% versus 80% of LSIL/HSIL and 100% by both of HSIL. Since, there are limited Greek epidemiological data available, studies that yielded similar findings in healthy women to our results, were the report by Papachristou et al. [26] who found that the corresponding prevalence was 31.5% and Agorastos et al. [27] at 36.3%. Other studies in our country demonstrated that HPV DNA presence in WNL varied from 24% [28, 29] to 18% [30, 31] with the lowest prevalence reported at 2.5% [32]. This variability is also observed widely in the literature and is mainly due to the different criteria used for selecting the study population and also due to different molecular test applied. The biological meaning out of this is that latent HPV infections with no apparent underlying disease, which would otherwise not be diagnosed on cytological evaluation, are detectable with highly sensitive PCR-based methods.

AmpliLute correctly identified 87.5% of the negative histological cases as HPV negative samples compared to 81.2% of MagNA. In addition, in cases with histological evaluation from HPV up to CIN2, MagNA missed 10 cases counting for 8% (10/123) of the population with these specific histological abnormalities, whereas all those cases were accurately detected as HPV positive samples by AmpliLute. In 144 cases with cytological findings of WNL and ASCUS, HPV was detected approximately in 52 cases (more than 50% of which were HR HPV types) and from which only 33 participants exhibited histological lesions of HPV up to CIN2. The remaining cases need to be followed up closely due to their elevated risk for developing a high-grade cervical lesion in the future.

The small number of cases investigated in this present study limits our ability to conclude correct and representative epidemiological data on HPV prevalence in Greek women. Nevertheless, data of this report on HPV distribution add to a rich body on literature demonstrating that HPV 16 was the most frequent type detected in both types of infections followed by HR HPV 31, 53, 33, 45, 18, and 51. The observation of HPV 53 being among the three most prevalent HR HPV types detected is consistent with findings of previous Greek studies [28, 31]. However, the prevalence and clinical role of HPV 51 needs to be clarified through further studies. Critical points on multiple infections are succinctly presented, since the detailed analysis of multiple infections identified in the clinical specimens was beyond the scope of this work and they will be discussed analytically on other report. Nevertheless, they were highly detected among the HPV positive participants: 47% (68/145) by the AmpliLute and 42% (58/138) by the MagNA. Multiple type HPV infections were identified in approximately 50% of the HPV-infected individuals in WNL category, at 34% in ASCUS, at 50% in LSIL and in LSIL/HSIL and finally at 60% in HSIL category. The elevated incidence rate of multiple infections in our results are in line with the results described by Sandri et al. [10] who found multiple infections in 43% of the studied population and by Gargiulo et al. in 49.7% [33].

Table 8: Distribution of HPV types (in single infections) detected by MagNA Pure method against Histological Diagnosis.

Histology	MagNA pure																							
	HPV6	16	18	31	42	45	51	52	53	54	58	59	61	62	66	73	81	83	84	Inadequate	Multiple	Negative	Total
Negative						2						1										13	16
HPV	4	8	1		3		1	3	4	1	2			2	1	1	1		2		12	5	51
CIN 1		11	3	6				3												1	29	4	57
CIN 2		4		1												1					8	1	15
CIN 3		3																			3		6
No biopsy*	2		1						1			2	3			1		1			6	91	108
Total	6	26	5	7	3	2	1	6	5	1	2	3	3	2	1	3	1	1	2	1	58	114	253

*Women without colposcopic findings.

Regarding the discordant results observed between the two extraction methods, as showed in Table 3, in 44% (11/25) of the cases, MagNA failed in detecting HPV as opposed to the AmpliLute. Ten of these eleven cases were histological confirmed as ≥HPV and correctly identified by the AmpliLute. In 20% (5/25) of the cases, which were positive by the MagNA but negative by the AmpliLute method, there were either negative in histology or there were with normal cytology and without visible lesion upon colposcopy (thus for those women, cervical biopsy was not taken). In this regard, AmpliLute method gave a correct negative call and those cases could be considered as MagNA false positive results. In 32% (8/25) of cases, which were positive by the two methods but differing in the number of HPV genotypes detected, AmpliLute demonstrated higher level of detecting additional HPV genotypes in seven cases, apart from the common shared types, as opposed to MagNA. Those extra genotypes detected carry an increased clinical significance, since there were HR genotypes and could alter the clinical outcome of the patient. Only in one case, 4% (1/25) the HPV genotypes were completely different by the two methods and also in one case MagNA detected one extra genotype than the AmpliLute. The invalid result generated by the MagNA was HPV42 with CIN1 histology. Even though the patient population studied does not represent the general population attending our hospital, but only women who agreed to undergo further examination if necessary, the clinical samples tested covered a range of pathologies, from samples that were cytologically normal to samples that had HSIL. Therefore, the results (as well as the discordant result rate) for HPV detection generated by the two extraction methods demonstrated in the present study can be representative of the HPV infection in a screening population.

It is important to mention that the decision of utilizing the modified DNA protocol for the automated MagNA pure extraction system, was made based on a recent report. It compares DNA extraction efficiencies using the same extraction system with the incorporation of three different working DNA extraction kits: (i) blood cells high-performance protol (DNA-I kit), (ii) total nucleic acid (TNA) kit, and (iii) a modified DNA-I kit with the manual AmpliLute protocol for both AMPLICOR and LA HPV tests in 150 specimens [34]. Although the women enrolled in the above study had histological confirmed cervical abnormalities, no comparison was made between the DNA extracts and the HPV genotyping test with their cytohistological findings. We used the modified DNA-I kit (blood cells high-performance protol) using 1 mL of PreservCyt sample as reported by Stevens et al. [34], since it performed better than the other two protocols and it was recommended by the author.

At this point, it is important to emphasise that even though for the manual AmpliLute method we used one fourth of biological material (250 μL) as opposed to the automated MagNA DNA-I modified protocol (1000 μL) and equal amount of DNA extract inputs were used for PCR amplification (50 μL) and subsequently HPV detection, more HPV-positive cases were detected by the manual method. Someone would assume that increased HPV genotype detection would occur when a bigger amount of clinical sample is incorporated in the DNA extraction procedure, since more representative epithelial cells would be present in the sample tested, and thus increasing the possibility of HPV genotypes been detected by the assay used. The findings of our report, which are in contrast with previous reported one [34], declared the opposite, indicating that the current manual AmpliLute protocol for DNA preparation, provides adequate DNA quality, and consequently, it is capable of detecting HPV infections with high sensitivity. Having in mind that both methods gave comparative results when tested against cytology and histology, our data provide an additional advantage to AmpliLute, since reliable results can be obtained even when small volumes of biological material are available for molecular use.

In the literature, there is also a report that utilizing the same MagNA pure automated extraction system, compares the AMPLICOR HPV test to the INNO-LiPA HPV genotyping test, using only the TNA extraction kit for DNA isolation for AMPLICOR test [35], making, thus, difficult the direct comparison with this work. Several studies have undertaken assessment of the utility of various automated DNA extraction platforms in conjunction with the LA HPV test without comparing them with manual extraction methods [34, 36, 37]. Moreover, in the literature, there are limited studies that address the variability in HPV genotyping introduced by small changes in front-end DNA extraction procedures prior to use in the LA HPV genotyping test [38, 39]. From those reports, it was interesting found that minor changes to equally valid DNA extraction methods appeared to vary the assay's performance. For example, varying the volume of PreservCyt for DNA extraction or varying the centrifuge speed

during DNA extraction or varying the amount of template DNA used for amplification can impact assay results. It is well documented and widely accepted that it is difficult to achieve reproducible and accurate HPV genotyping results using PCR-based methods, particularly when individual specimens may contain multiple concurrent infections and/or low viral copy numbers. Each of the many steps of testing, from collection of the cervical sample to the final recording of the result, can introduce important variability. Large-scale data comparing different methods of DNA isolation are needed to reach an optimal protocol for the HPV presence detection and accurate genotyping in order to monitor viral clearance, and most importantly HPV persistence, which is considered as a key factor in cervical cancer development. Moreover, accurate and sensitive methods for detection of HPV should be determined, since their performance can strongly affect the results of epidemiological studies and the clinical treatment strategy selected. Therefore, the MagNA extraction method should be tested against other automated and manual nucleic acids isolation techniques and in large population studies before being implemented and routinely used in laboratories. If would be proven accurate in detecting HPV infection, laboratories particularly those involved in large-scale HPV genotyping studies or handling a large amount of clinical specimens or can afford the cost of the automated procedure (more than two and a half times most pricey than the manual procedure) could profit from this automated nucleic acid isolation technique.

5. Conclusion

Accurate laboratory assays for the diagnosis of HPV infection are being recognized increasingly as essential for clinical management of women with cervical precancerous lesions. The first and most important step in molecular diagnosis of HPV infection is the nucleic acid isolation. An alternative approach to manual extraction procedures, which are time consuming and labor intense, is the automated processing of clinical specimens for HPV detection and genotyping which minimizes the potential sample contamination and the hands-on time. From our data, it was concluded that both DNA extraction methods demonstrated similar clinical performance, with no significant difference for any of the outcomes assessed even if for some outcomes the AmpliLute method exhibited higher sensitivity, specificity positive and negative predictive values as opposed to MagNA methods. Based on the results of this study, the automated nucleic acid isolation method should be tested versus other automated and manual techniques before it is routinely implemented. In addition, additional studies with larger populations are required to be carried out using the automated extraction system in order for its potential value to accurate HPV detection been determined.

Authors Contribution

A. Chranioti and P. Karakitsos conceived and coordinated the study; E. Age and C. Kottaridi performed the molecular analysis; N. Margari performed the statistical analysis; A. Pappas collected thin prep samples and performed colposcopy/biopsy; I. Panayiotides performed the pathological evaluation; P. Karakitsos performed the thin prep evaluation; A. Chranioti wrote the paper.

Conflict of Interests

The authors declare there is no conflict of interests.

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Clinical Study

Atypical Squamous Cells of Undetermined Significance: Bethesda Classification and Association with Human Papillomavirus

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Introduction. To analyze patients with atypical squamous cells of undetermined significance (ASCUS) through a cytology review and the presence of microbiological agents, with consideration of colposcopy and semiannual tracking. *Methods*. 103 women with ASCUS were reviewed and reclassified: normal/inflammatory, ASCUS, low-grade squamous intraepithelial lesion (LSIL), or high-grade squamous intraepithelial lesion (HSIL). If ASCUS confirmed, it was subclassified in reactive or neoplastic ASCUS, ASC-US, or ASC-H; and Regione Emilia Romagna Screening Protocol. Patients underwent a colposcopic examination, and test for *Candida* sp., bacterial vaginosis, *Trichomonas vaginalis*, and human papillomavirus (HPV) were performed. *Results*. Upon review, ASCUS was diagnosis in 70/103 (67.9%), being 38 (54.2%) reactive ASCUS and 32 (45.71%) neoplastic ASCUS; 62 (88.5%) ASC-US and 8 (11.41%) ASC-H. ASCUS (Regione Protocol), respectively 1-5: 15 (21.4%), 19 (27.1%), 3 (27.1%), 16 (22.8%), and 1 (1.4%). A higher number of cases of cervical intraepithelial neoplasia (CIN) II/III in the biopsies of patients with ASC-H compared to ASC-US ($P = .0021$). High-risk HPV test and presence of CIN II/III are more frequent in ASC-H than ASC-US ($P = .031$). *Conclusions*. ASC-H is associated with clinically significant disease. High-risk HPV-positive status in the triage for colposcopy of patients with ASC-US is associated with increased of CIN.

1. Introduction

Since Papanicolaou's introduction of the Pap smear (1943), a variety of terms have been used to describe accompanying cytological diagnoses [1]. The Bethesda classification proposed in 1988 (revised in 1991) reflects the biological behavior of squamous intraepithelial lesions (SILS) of the cervix [2]. This classification scheme subdivides abnormal squamous epithelial cells into four groups: (1) atypical squamous cells of undermined significance (ASCUS); (2) low-grade squamous intraepithelial lesions (LSILS), encompassing light dysplasia/cervical intraepithelial neoplasia (CIN) I as well as cell changes associated with the human papillomavirus (HPV); (3) high-grade squamous intraepithelial lesions (HSIL), including moderate dysplasia/CIN II, severe dysplasia, and carcinoma *in situ*/CIN III; (4) squamous cell carcinoma.

The ASCUS diagnosis has caused confusion and controversy with respect to its significance and appropriate use [3–5]. More than 2 million women in the United States receive a diagnosis of ASCUS in cervical-vaginal cytology each year [4–6]. The ideal clinical tracking of these women has been highly controversial, and doubts remain regarding which control and treatment strategies are best [3, 7].

Comparisons between laboratories have shown that the diagnostic frequency of ASCUS varies from 1.6% to 9% [8, 9]. Gerber et al. detected ASCUS in 5% (1,387) out of a total of 29,827 patients in their study [9]. Lee et al. reported 1,035 ASCUS diagnoses out of 49,882 oncological cytologies performed, a 2% frequency rate [10]. According to some

authors, the frequency of ASCUS should not exceed 2- to -3 times the frequency of LSIL [2–11].

Guerrini et al. have attempted to better define the ASCUS diagnosis by using the morphological criteria recommended by the Regione Emilia Romagna Screening Protocol (1997) [12]. This subdivision improved treatment courses, as cases of ASCUS at levels 1 to 3 corresponded to more substantial colposcopic findings which are related to the presence of CIN in biopsies [13].

The new Bethesda reclassification released in 2001 included a major change with respect to ASCUS. The category was subdivided into two categories: ASC-US (atypical squamous cells of undetermined significance), which reflects the difficulties in distinguishing between reactive changes and LSIL, and ASC-H (atypical squamous cells, cannot rule out a high-grade lesion), which reflects a differential diagnosis between immature reactive metaplasia and HSIL [10, 14]. Morin et al. showed the presence of CIN in 22.2% of the biopsies of 360 women with ASCUS, with 16.1% having CIN I and 5.3% having CIN II/III [1].

Research on HPV has begun to shape part of the evaluation of patients with ASCUS and other cytological changes [15]. The addition of a biomolecular test for HPV increases the sensitivity of detection for CIN in women with ASCUS relative to repeated cytology [16]. On the other hand, a negative test for HPV in women with ASCUS can reduce the number of times colposcopy is needed and reduce unnecessary biopsies [1, 17]. The HART (*HPV Testing in Addition to Routine Testing Study*), HPV testing with reflex results, was found to be highly sensitive for the diagnosis of subjacent CIN in women between the ages of 30 and 60, compared to a repetition of cytology [18].

The correct interpretation of the intensity of ASCUS morphological changes with respect to patient prognosis, the significance of the findings, and the appropriate clinical course to be followed have yet to be clarified. Therefore, the objectives of this study were (1) to evaluate the variation in the ASCUS diagnosis in routine Papanicolaou exams and other morphological classifications of ASCUS, (2) to analyze the microbiological agents that cause inflammation, and (3) to evaluate the use of HPV testing in the triage of patients with ASCUS based on colposcopy.

2. Materials and Methods

2.1. Study Design. A prospective study was performed at the walk-in clinic of the Gynecology and Obstetrics Division of the Federal University of the Triângulo Mineiro between January 2003 and December 2007. In the first part of the study, women with a diagnosis of ASCUS were evaluated by cervical-vaginal cytology in a routine exam performed by four medical cytopathologists. Cytology findings were used in triage, and the order of patient entry followed the series sequence of the Pap smear registrations of the institution's cytopathology service.

Patients with a diagnosis of ASCUS, who were taken to the colposcopy service, were informed about the study and its purpose. Those who agreed to participate signed terms of informed consent approved by the ethics committee of the Federal University of the Triângulo Mineiro. Women became part of the study when they met the inclusion criteria: a diagnosis of ASCUS by cytology; not pregnant; no bleeding during the exam; no use of oral antibiotics, fungicides, or vaginal creams in the last 30 days; sexual abstinence for at least two days; no previous history of SIL or cervical procedures.

2.2. Methods

2.2.1. Clinical Methods. Information about the age, habits, and lifestyles (parity, number of partners, age of sexarche, age of first pregnancy, and smoking status), contraceptive methods used, and history of sexually transmitted diseases was initially collected.

2.2.2. Microbiological and Biochemical Methods. After providing the above data, the patients underwent a gynecological examination, colposcopy, and collection of cervical-vaginal material for the study of microbiological agents and HPV by the hybrid capture technique. A variety of procedures were used to collect the samples as described hereafter. To search for *Trichomonas vaginalis*, material was collected from the base of the vaginal fornix with a swab, and the secretion was placed on a glass slide. After adding drops of saline solution and placing a coverslip on the specimen, a search for moving and flagellated microorganisms was performed by optical microscopy (fresh examination). To search for *Candida sp.*, vaginal material was collected with a swab and seeding performed in a Petri dish, containing the Sabouraud Agar culture medium, where the growth of fungus was verified. To search for bacterial vaginosis, the widely accepted clinical criteria originally proposed by Amsel et al. were used [19]: (1) homogenous vaginal secretion that adheres to the vaginal walls, (2) vaginal pH above 4.5, (3) the presence of a characteristic smell after adding a solution of 10% potassium hydroxide to the vaginal secretion, and (4) the presence of "clue cells" in the Gram-colored smear. The presence of 3 out of 4 of the above criteria was considered sufficient for establishing a diagnosis. Measurement of pH was performed by collecting vaginal material using a swab and then introducing it into a sterile test tube, containing 1 mL of distilled, deionized water. The material was taken to the laboratory at the end of the consult, and vaginal pH was gauged using a designated digital Sentron brand pH meter, which uses a 0 to 14 pH scale [20]. For this determination, the contents of the test tube were used. After homogenization in a vortex for about 10 seconds, the swab was removed and introduced into the flask of the pH measuring machine, with an electrode located at its end. Quantification of pH was done digitally on the spot. This procedure took place no longer than 1 hour after each sample had been collected. To search for human HPV, endocervical and ectocervical material was initially collected with a special brush, a component of the Digene hybrid capture kit, and placed in its own tube containing material to preserve it, and then maintained frozen at a temperature of -20°C.

At the end of the collection period, once a sufficient number of samples had been obtained, the tubes were defrosted and analyzed according to hybrid capture techniques.

Hybrid Capture. The Hybrid Capture II *System* DML 2000 brand microplate system machine with signal amplification was used for chemiluminescence. The information and methodology described below were derived from the instruction manual provided by the product vendor (Digene of Brazil) and are consistent with previously described techniques [20].

The *kit* used to detect HPV had 18 viral types grouped into two pools of probes. The probes for low-risk virus included types 6, 11, 42, 43, and 44, representing approximately 70% of this viral group. With respect to the high-risk virus, the system had probes for types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68, representing approximately 99% of this viral group. According to the vendor, the microplating sensitivity for HPV is 1 pg/mL, equivalent to 0.1 copies of the virus.

Colposcopic Exam. After the materials had been collected, the patients underwent video-colposcopy with image capture. The Barcelona classification scheme [21], proposed in 2002, was used to describe the findings. Briefly, samples were divided into two classes: normal colposcopic findings (original squamous epithelium, columnar epithelium, and normal transformation zone) and abnormal colposcopic findings (acetowhite epithelium, spotted, mosaic, leucoplasia, iodine-negative zone, and atypical vessels), with the latter category subdivided into minor or major changes depending on the intensity of the changes observed. The following reagents were used to perform the colposcopy: aqueous solution of 3% glacial acetic acid, lugol's solution, and sodium bisulfite. When changes were observed, the patients subsequently underwent a directed biopsy using Gaylor-Medina forceps.

Cytological Evaluation and Histopathology. In a second step, the smears of patients with an initial diagnosis of ASCUS by the cytopathologists during the routine exam were reviewed and reclassified by the same cytopathologists, in collaboration with the author of this study, as normal/inflammatory cytology, ASCUS, LSIL, or HSIL. When a diagnosis of ASCUS was confirmed upon review, the case was subclassified as either probably reactive or probably neoplastic (Bethesda 1988–1991) and, also according to the Bethesda 2001 norms, as ASC-US or ASC-H. Cases of ASCUS were newly subclassified according to their morphological changes following the recommendations of the Regione Emilia Romagna Screening Protocol [12] into the following groups: atypical squamous cells with mature-intermediate type cytoplasm (ASCUS 1), metaplastic atypical squamous cells (ASCUS 2), atypical squamous cells with parakeratosis (ASCUS 3), reactive atypical cells (ASCUS 4), and atypical squamous cells with atrophy (ASCUS 5).

The cytology smear was colored using the Papanicolaou technique and evaluated according to the morphological criteria of amphophilia, perinuclear halo, dyskeratosis, nuclear criteria (binucleation, multinucleation), increase in the nucleus/cytoplasm relation, anisokaryosis, hyperchromasia, nuclear atypias, and karyorrhexis. The biopsies were also reviewed and reclassified by the same pathologist, in conjunction with the author of this study. The patients underwent new cytological and colposcopic evaluations after 6 months. The results were analyzed by comparing cytological and colposcopic criteria, the presence of microbiological agents that cause infection and HPV in patients with ASCUS of a probably reactive character and ASCUS of a probably dysplastic character, ASC-US, and ASC-H.

2.2.3. Statistical Analysis. The GraphPad InStat program, version 3.0, was used for statistical analysis. The results were compared using Fisher's exact test with a significance level of less than 5% ($P < .05$).

3. Results

Between January 1, 2003, and December 31, 2007, 46,362 Pap smears were performed by the cytopathology service of the Gynecology and Obstetrics Division. Of these, 41,349 (89.18%) had a negative cytology for oncological changes, 2,309 (4.98%) had a diagnosis of ASCUS, 265 (0.57%) had a diagnosis of AGUS (atypical glandular changes of an undermined significance), 1,760 (3.79%) had a diagnosis of LSIL, 551 (1.18%) of CIN II/III, and 128 (0.27%) of invasive carcinoma of the cervix. The screening is opportunistic and covers some areas of Uberaba (Minas Gerais, Brazil) and some neighboring towns. The population served is of low socioeconomic status.

A total of 103 women with an initial diagnosis of ASCUS were randomly selected and evaluated. Their average age was 35.76 (range, 18–50 years old). Of the 103 participants, 32 (31%) were smokers. In terms of contraceptive use, 40 (38.83%) had received tubal ligation, 30 (29.12%) used hormonal methods, 3 (2.91%) used only condoms, 1 (0.97%) used an intrauterine device, and 29 (28.15%) did not use any form of contraceptive. The women's average number of sexual partners was 2.15 (range, 1–10). The average age of their first sexual relationships was 17.62 years old (range, 12–27 years old) and the average age of their first pregnancy was 19.67 years old (range, 12–31 years old). Sixteen women (15.53%) were nulliparous, 60 (58.25%) had between one and three children, and 27 (26.21%) had more than three children.

The slides of all 103 of these initial cases of ASCUS were reviewed by the same examiner in conjunction with the author of this study, evaluating a variety of changes according to the protocol. Of the 103 cases, 70 (67.96%) were confirmed as ASCUS at the second reading; 30 (29.12%) were reclassified as normal/inflammatory smears, 2 (1.94%) as LSIL, and 1 (0.97%) as HSIL. Of the 70 cases of ASCUS, 38 (54.28%) were reclassified as ASCUS of a probably reactive nature and 32 (45.71%) as ASCUS of a probable neoplastic nature (Bethesda, 1991); 62 (88.57%) were reclassified as ASC-US and 8 (11.41%) as ASC-H (Bethesda, 2001). Patients

Table 1: Distribution of patients with an initial diagnosis of ASCUS which, upon review, were reclassified as normal/inflammatory, ASCUS of a probably reactive nature, and ASCUS of a probably neoplastic nature, in relation to infection.

Agent	Cytology				
	Normal/inflammatory *N* (%)	ASCUS			
		Probably reactive *N* (%)	Probably neoplastic *N* (%)	ASC-US *N* (%)	ASC-H *N* (%)
Candida sp.	4 (13.4)	9 (23.6)	4 (12.5)	13 (21)	0
Bacterial vaginosis	2 (6.6)	6 (15.8)	6 (18.7)	12 (19.3)	0
Trichomonas vaginalis	0	2 (5.2)	0	2 (3.2)	0
No infection	24 (80.0)	21 (55.2)	22 (68.7)	35 (56.4)	8 (100)
Total cases by group	30	38	32	62*	8

* $P = .0196$ versus ASC-H, Fisher's exact test.

Table 2: Principal colposcopic findings in patients with an initial diagnosis of ASCUS who, upon review, were reclassified as normal/inflammatory cytology, probably reactive ASCUS, probably neoplastic ASCUS, ASC-US, and ASC-H.

Colposcopy	Cytology				
	Normal/inflammatory *N* (%)	ASCUS			
		Probably reactive *N* (%)	Probably neoplastic *N* (%)	ASC-US *N* (%)	ASC-H *N* (%)
Normal findings	17 (56.6)	23 (60.5)	9 (28.1)	32 (51.6)	0
Abnormal findings Major*	0	0	6 (18.7)*	1 (1.6)	5 (62.5)**
Minor	8 (26.6)	9 (23.6)	15 (46.8)	21 (33.9)	3 (37.5)
Unsatisfactory	5 (16.6)	6 (15.8)	2 (6.2)	8 (12.9)	0
Total cases by group	30	38	32	62	8

* $P = .0020$ and $P = .0018$ for the presence of abnormal and major abnormal colposcopic findings, respectively, versus the probably reactive ASCUS group, Fisher's exact test.
** $P = .0017$ and $P < .0001$ for the presence of abnormal and major abnormal colposcopic findings, respectively, versus the ASC-US group, Fisher's exact test.

with a diagnosis of SIL at the review were excluded from the reporting of the results.

The results of the microbiological examinations for *Candida sp.*, bacterial vaginosis. and *T. vaginalis* of 100 of the patients reevaluated in this study are shown in Table 1. No statistically significant difference was found in the comparison of the presence of infection (bacterial vaginosis, *Candida sp.* and *T. vaginalis*) between the group with probable reactive ASCUS and probable neoplastic ASCUS. A statistically significant difference was observed, however, with respect to the presence of infection between the ASCU-US and ASC-H groups.

The colposcopic findings are shown in Table 2. The colposcopy was considered unsatisfactory when it was not possible to see the squamocolumnar junction. When comparing the presence of abnormal colposcopic findings in the probably reactive and probably neoplastic groups, a statistically significant difference was found, with more abnormal findings being observed in the neoplastic group. When the same groups were compared, but only major colposcopic findings were evaluated, the difference remained significant, being greater in the group with changes that were probably neoplastic. Analysis of the colposcopic findings in the patients with cytology reviewed according to the Bethesda 2001 classification revealed significantly more abnormal colposcopic findings in the ASC-H group than in the ASC-US group. A similar comparison for major colposcopic findings also revealed significantly more incidences in the ASC-H group relative to the ASC-US group.

Among the 70 patients with ASCUS after cytology review, 30 (42.8%) underwent biopsy. Five of these (16.6%) were infected with HPV, 9 (30%) had CIN (3 CIN I, 3 CIN II and 3 CIN III), and 16 (53.3%) had normal biopsies. HPV infection was present in 4 patients with probable neoplastic ASCUS and in 1 patient with probable reactive ASCUS; in the Bethesda 2001 classification, the 5 HPV cases all involved patients with ASC-US that were subjected to biopsies. Three cases of CIN I were present in biopsies of patients with probable reactive ASCUS (Bethesda 1991) and ASC-US (Bethesda 2001). CIN II was present in 1 patient with reactive ASCUS and 2 with probable neoplastic ASCUS; and in the 2001 classification, all cases of CIN II were present in the biopsies of patients with ASC-H. All three CIN III diagnoses were made in patients in the neoplastic ASCUS and ASC-H groups. In the group of 30 patients with normal/inflammatory cytology, 8 underwent biopsy, revealing CIN I in 1 case and HPV infection in 1 case.

The anatomopathological results of biopsies performed in patients with changes found during the colposcopic examination are shown in Table 3. No statistically significant differences were found when the presence of changes was compared between the normal/inflammatory, probably

Table 3: Results of biopsies performed on patients with an initial diagnosis of ASCUS who, upon review, were reclassified as having normal/inflammatory cytology, probably reactive ASCUS, and probably neoplastic ASCUS, and who, in accordance with the Regione Emilia Romagna (ASCUS 1 to 5), showed evidence of changes in the colposcopic exam.

Biopsy	Cytology							
	Normal/inflammatory *N* (%)	ASCUS						
		Probably reactive *N* (%)	Probably neoplastic *N* (%)	1 *N* (%)	2 *N* (%)	3 *N* (%)	4 *N* (%)	5 *N* (%)
Normal	6 (20.0)	7 (18.4)	9 (28.1)	1 (6.6)	5 (26.3)	5 (26.3)	4 (25.0)	1 (100.0)
HPV/CINI	2 (6.6)	1 (2.6)	7 (21.8)	2 (13.3)	4 (21.0)	2 (10.5)	0	0
CIN II/III	0	1 (2.6)	5 (15.6)	3 (20.0)	2 (10.5)	0	1 (6.25)	0
Not performed	22 (73.3)	29 (76.3)	11 (34.3)	9 (60.0)	8 (42.1)	12 (63.1)	11 (68.7)	0
Total cases by group	30	38	32	15	19	19	16	1

All *P*s > .05; Fisher's exact test.

Table 4: Results of the biopsies performed on the total group of patients with an initial diagnosis of ASCUS who, after review, were reclassified as having cytologies that were normal/inflammatory, ASC-US, and ASC-H, which showed changes in the colposcopic examination.

Biopsy	Cytology		
	Normal/inflammatory *N* (%)	ASCUS	
		ASC-US *N* (%)	ASC-H *N* (%)
Normal	6 (20.0)	13 (21.0)	3 (37.5)
HPV/CIN I	2 (6.6)	8 (13.0)	0
CIN II/III	0	1 (1.6)	5 (62.5)*
Not performed	22 (73.3)	40 (64.5)	0
Total cases by group	30	62	8

* $P = .0256$ and $P = .0021$, relative to ASC-US group, versus ASC-H group with normal/inflammatory cytology and cases of CIN II/III in biopsies of patients with ASC-H, respectively, Fisher's exact test.

reactive ASCUS, and probably neoplastic ASCUS groups. The reconfirmed cases of ASCUS ($n = 70$) were then subclassified according to the recommendations of the Regione Emilia Romagna Screening Protocol as follows: 15 cases of ASCUS 1 (21.4%), 19 of ASCUS 2 (27.1%), 19 of ASCUS 3 (27.1%), 16 of ASCUS 4 (22.8%), and 1 case of ASCUS 5 (1.42%). The results of the biopsies showed 3 cases of CIN II/III among patients with ASCUS 1, 2 cases among patients with ASCUS 2, and 1 case among patients with ASCUS 4. There were no cases of CIN II/III among the patients with ASCUS 3 and 5. When the results of the colposcopic findings as classified according to the Regione Emilia Romagna were analyzed, there were no statistically significant group differences.

When the results of the biopsies were reevaluated against the cytologies according to Bethesda 2001 (Table 4), we found a greater frequency of CIN II/III in the group of patients with ASC-H, compared to the group with normal/inflammatory cytology. There were a greater number of cases of CIN II/III in the biopsies of patients with ASC-H relative to the group with ASC-US. When CIN II/III findings were compared between the morphological ASCUS groups, we found a significantly greater frequency among patients with ASC-H than those with ASCUS 1 and those with probable neoplastic ASCUS. The distribution of cases of CIN II/III (absent/present – *n*, %) at biopsy in relation to the cytological diagnoses of probably neoplastic ASCUS, ASC-H, and ASCUS 1 (Regione Emilia Romagna Classification) showed 27/5 (84.3%/15.6), 12/5 (80/20), and 3/5 (37.5/62.5), respectively, Bethesda 1991, probably neoplastic ASCUS, Regione Emilia Romagna, ASCUS 1 and Bethesda 2001, ASC-H, being $P = .0713$ and $P = .0145$, respectively, Bethesda 2001, ASC-H versus ASCUS 1 and probably neoplastic ASCUS (Fisher's exact test).

With regard to the hybrid capture test in the 100 cases with an initial diagnosis of ASCUS, the presence of high-risk HPV DNA was detected in 19 (27.1%) women with reconfirmed ASCUS and in 8 (26.6%) with normal cytology on review. There was a greater frequency of high-risk HPV infection in the ASC-H group compared to the ASC-US group. The results of DNA/high-risk HPV experiment (negative/high-risk HPV – *n*, %) using the hybrid capture technique in patients with an initial diagnosis of ASCUS who, upon review, were reclassified as having cytologies that were normal/inflammatory, ASC-US and ASC-H showed 22/8 (73.3/26.6), 48/14 (77.4/22.6), 3/5 (37.5/62.5), respectively, normal, ASC-US, and ASC-H cytologies, being $P = .0296$, ASC-H versus ASC-US for high-risk HPV (Fisher's exact test). Of the 6 cases of high-grade CIN, after a biopsy guided by colposcopy (out of the 100 cases evaluated in the study), 5 (83.3%) tested positive for high-risk HPV by the hybrid capture technique. Table 5 shows the correlations between ASC-US, ASC-H, and normal/inflammatory biopsies and the presence of high-risk HPV and the biopsy of high-grade

Table 5: Presence of CIN II/III in biopsies, in relation to positivity for high-risk HPV through hybrid capture, and reclassification of ASCUS cytology according to Bethesda 2001.

Cytology	High-risk HPV positive			High-risk HPV negative		
	Biopsy with CIN II/III *N* (%)	Biopsy without CIN II/III *N* (%)	Biopsy not performed *N* (%)	Biopsy with CIN II/III *N* (%)	Biopsy without CIN II/III *N* (%)	Biopsy not performed *N* (%)
ASC-US	1 (20.0)	7 (70.0)	6 (50.0)	0	14 (63.6)	34 (68.0)
ASC-H	4 (80.0)*	1 (10.0)	0	1 (100.0)	2 (9.0)	0
Normal	0	2 (20.0)	6 (50.0)	0	6 (27.3)	16 (32.0)
Total	5	10	12	1	22	50

* $P = .0319$, presence of CIN II/III at biopsy versus ASC-US among patients who tested positive for high-risk HPV, Fisher's exact test.

lesions. We found a greater proportion of CIN II/III in the biopsies of patients testing positive for high-risk HPV in the ASC-H group than in the ASC-US group.

Out of a total of 70 patients with a diagnosis of ASCUS, after review, 6 were transferred to the oncology service for treatment because of biopsies showing CIN II or III. Of the 64 remaining cases, 7 did not come for followups. Of the 57 patients diagnosed with ASCUS, 43 had normal/inflammatory cytology and normal colposcopy at their semi-annual followup, 7 had ASC-US and normal colposcopy, and 7 had LSIL (4 CIN I and 3 HPV; with all cases of CIN I being proven by directed biopsy). Of these, all cases of CIN I and II were diagnosed in the first biopsy. Of the 4 cases of CIN I, 3 came from the initial group with probable neoplastic ASCUS and 1 came from the probably reactive ASCUS group. Based on the 2001 classification, all CIN I cases belonged to the ASC-US group. Of the 3 patients with HPV, 1 came from the initial probably neoplastic ASCUS group and 2 came from the probably reactive group; all 3 belonged to the ASC-US group.

Of the 30 patients for whom the diagnosis of ASCUS was not confirmed upon cytological reclassification, no tracking was performed. At their semi-annual followups, 25 (83.3%) had normal colposcopic exams and normal/inflammatory cytology and 3 had ASC-US. There were no cases of CIN in semesterly tracking in patients who had a normal/inflammatory cytology diagnosis on reclassification.

4. Discussion

A diagnosis of ASCUS not only depends on well-defined cytological patterns, but also on many subjective criteria [6, 12]. The reproduction of the interpretation of ASCUS is lower than 50% [6]. We observed that the number of cytological diagnoses of ASCUS in our service was equivalent to 1.3× the frequency of LSIS, or 4.98% of all cytologies performed. ASCUS frequency rates in the literature vary from 2% to 7% [10, 22, 23]. The present results from our service demonstrate an ASCUS frequency compatible with the Bethesda expectations.

In the women with a confirmed diagnosis of ASCUS, altered biopsies were found in 20% of the cases, with 11.4% of these being HPV/CIN I and 8.6% being CIN II/III. Some studies have reported a concomitance of CIN II/III in 5–10% of patients with ASCUS [1], results similar to those from our study. Roche and Spicer, who tracked patients with ASCUS for 2 years, reported 18% of cases with HPV/CIN I and 15% with CIN II/III [24]. Eltabbakh et al. found a 15.9% frequency of neoplasia in patients with ASCUS [25]. A prior study performed by our group evaluating 1,244 women with ASCUS revealed CIN I in 60.3%, CIN II/III in 17.46%, and invasive neoplasia in 6.3% of the cases [26]. Thus it was concluded that CIN or invasive lesions can occur in women with ASCUS, and therefore new cytology or colposcopy and rigorous tracking should be considered for these patients.

All patients in our study with a diagnosis of ASCUS, upon review, were reclassified as having lesions of a probable neoplastic nature (45.7%) or of a probable reactive nature (54.2%), a result quite similar to previous work by our group (45.4% probable neoplastic ASCUS and 54.5% probable reactive ASCUS after reviewing the ASCUS cytologies) [13]. Other studies in the literature have also made this subdivision. Guerrini et al. reclassified 107 patients with a diagnosis of ASCUS and found 78.5% of them to be probably reactive and 21.5% to be probably neoplastic [12]. The absence of well-defined criteria, as well as the subjectivity of the diagnosis, may account for this variation. After reclassification according to the Regione Emilia Romagna, our findings showed the presence of CIN II/III in 20% of the ASCUS 1 cases, 10.5% of the ASCUS 2 cases, 1% of the ASCUS 4 cases, and none of the ASCUS 3 and 5 cases. We observed a higher frequency of CIN II/III in the biopsies of patients with ASCUS 1, a finding similar to that of Guerrini et al. [12].

Women with a cytological diagnosis of ASC-H have been shown to have a higher association with CIN II/III than those with ASC-US [14]. Barreth et al. studied 517 women with a cytology of ASC-H, and found a 2.9% presence of cervical cancer, 1.7% with *in situ* adenocarcinoma, and 65.6% with CIN II/III [27]. In another study, 85 women with ASC-H underwent colposcopies and histological analysis of biopsies of the areas of change, with CIN II and III being found in 52 (61.2%) of the cases and invasive cancer being found in 7 (8.2%) of the cases [28]. Similar results were found in our study, which verified levels of 62.6% of CIN II/III for patients classified as ASC-H. The diagnosis of ASC-H in the Papanicolaou exam is associated with the risk of clinically significant

disease, and a biopsy directed by colposcopy should be considered the proper course of action in these cases.

We performed a comparison of the Bethesda 1991, Bethesda 2001, and Regione Emilia Romagna classifications to better diagnose CIN II/III. We did not find any prior studies in the literature that had compared these three classification systems. When we performed the analysis, we observed a higher frequency of CIN II/III in cases of ASC-H than in cases of probable neoplastic ASCUS and ASCUS 1. Nevertheless, a limitation of our study is a low number of CIN II/III cases.

The detection of high-risk HPV DNA is thought to be useful in supplementing an abnormal cytological result [29, 30] and that in women with ASCUS, the presence of an HPV-positive group can substantially increase the chances of finding CIN II/III and cervical cancer, even though in the majority of these women significant lesions are not found [6]. Therefore, some authors recommend that testing for HPV DNA be performed on women with ASCUS [15, 31], while a biopsy guided by colposcopy is only recommended if high-risk HPV is present, while other women are treated more conservatively [5].

Various studies have shown a difference in the presence of HPV DNA when the cytologies of ASC-US and ASC-H are compared. Srodon et al. studied patients with ASCUS classified according to the Bethesda 2001 system and found HPV to be present in 67% of women with ASC-H and 45% of women with ASC-US [32]. In evaluating the presence of high-risk HPV by hybrid capture, Kurman and Solomon found it to be present in 14.2% of ASC-US cases and 66.6% of ASC-H cases [2]. In our study, we found high-risk HPV in 22.5% of patients with ASC-US and 62.5% of patients with ASC-H. When the cytologies of ASC-US and ASC-H were compared, the presence of high-risk HPV DNA was statistically greater in the latter, highlighting the importance of this division in the detection of clinically significant disease.

Srodon et al. evaluated the presence of HPV and high-grade CIN in patients with ASC-US and ASC-H and found CIN II/III in 10.2% of HPV-positive patients with ASC-US and in 5.9% of HPV-negative patients with ASC-US [32]. We found that 7.14% of the cases of high-grade CIN II/III were in HPV-positive women with ASC-US. Furthermore, we found higher rates of CIN II/III in HPV-positive patients with ASC-US than in HPV-negative ASC-US group (no cases of high-grade CIN). The presence of CIN II/III in 7.14% of the HPV-positive women with ASC-US suggests that the HPV test could be used in the triage of patients with ASC-US for a colposcopy and that HPV positivity may be associated with an increased probability of CIN. The diagnosis of ASC-H appears to be associated with an increased risk of clinically significant lesions, especially when associated with oncogenic HPV. Excision of the lesion may be indicated because the risk for histologic CIN II found was high for women with HPV positive tests, HSIL cytology, and a high-grade impression at colposcopy [33]. Other methods as liquid-based cervical cytology do not show more efficacies in diagnosis HSIL compared to Pap smears [34]. Actually, the frontiers of cervical cancer prevention is the preventing persistent HPV infections using vaccination or HPV testing utilizing opportunistic or organized screening [35].

We believe that the data obtained in this study provide an important confirmation of the usefulness of both the ASCUS subdivision according to the Bethesda 2001 protocol and the hybrid capture test. We propose that an initial cytology of ASC-US be reviewed for diagnostic confirmation and that, if it stands, two strategies should be followed: (1) an HPV test should be performed, and (2) if there is a positive high-risk HPV finding, there should be a colposcopy or semi-annual followup with cytology. In cases classified as normal after a review of the ASC-US diagnosis, we suggest a new cytology be performed after 6 months. This finding is in agreement with the data obtained by Chen et al. [36], whose findings also lead to the conclusion that a cytology of ASC-US, especially without a prior Pap smear, is quite likely to develop into cervical cancer and that of the most aggressive kind. Another study about ASC-H showed that HPV DNA testing has an extremely high negative predictive value for histologic CIN II/III, reaching 100% in women 40 years and older [37]. Based on 2006 consensus guidelines, a program of DNA testing for high-risk types of HPV, repeat cervical cytologic testing, or colposcopy are all acceptable methods for managing women over the age of 20 years with ASC-US [38].

This study allows us to conclude that the level of agreement between observers in diagnosing ASCUS upon review was 67.96%. The frequency of infection was higher in patients with ASC-US than in patients in the ASC-H group, and the frequency of abnormal colposcopic findings was greater in the neoplastic ASCUS and ASC-H groups than in the reactive ASCUS and ASC-US groups, respectively. A positive HPV test, used in the triage of patients with ASC-US for colposcopy indication, appears to be associated with an increased chance of detecting CIN.

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Research Article

Promoter Methylation of $p16^{INK4A}$, hMLH1, and MGMT in Liquid-Based Cervical Cytology Samples Compared with Clinicopathological Findings and HPV Presence

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Cervical cancer is a common cancer inflicting women worldwide. Even though, persistent infection with oncogenic Human Papillomavirus (HPV) types is considered the most important risk factor for cervical cancer development, less than 5% of women with HPV will eventually develop cervical cancer supporting that other molecular events, like methylation-dependent inactivation of tumor suppressor genes, may cocontribute in cervical carcinogenesis. We analyzed promoter methylation of three candidate genes (p16, MGMT, and hMLH1) in 403 liquid-based cytology samples. Methylation was commonly identified in both benign and pathologic samples and correlated with higher lesion grade determined by cytological, colposcopical, or histological findings, with HPV DNA and mRNA positivity of specific HPV types and $p16^{INK4A}$ protein expression. Overall accuracy of methylation is much lower than traditional diagnostic tests ranking it as an ancillary technique with more data needed to identify the exact value of methylation status in cervical carcinogenesis.

1. Introduction

Cervical cancer still remains the third most commonly diagnosed cancer type in women worldwide, particularly in developing countries, with over 500,000 estimated new cases and over 250,000 estimated deaths [1]. The main cause of cervical cancer development is infection with Human Papilloma Viruses (HPVs) [2], that are small DNA viruses with oncogenic properties [3–5]. There are over 100 different HPV types, but only around 40 have been found in cervical epithelium and about 20 have been considered as high-risk factors for cancer development [6, 7]. Even though, persistent infection with oncogenic Human Papillomavirus (HPV) types is considered the most important risk factor for cervical cancer development, less than 5% of women with HPV will eventually develop cervical cancer [8], supporting the notion that other molecular events cocontribute in cervical carcinogenesis.

Inactivation of tumor suppressor genes has been shown to be a critical step in tumor development [9]. Apart from well-monitored suppression mechanisms as mutational inactivation, chromosome deletions, and loss of heterozygosity, epigenetic inactivation of tumor suppressor genes is a more recent discovery, where promoter methylation of a tumor suppressor gene abolishes its expression [10]. A significant amount of studies have provided evidence that promoter methylation of tumor suppressor genes is linked with cervical carcinogenesis [11–13] and even with specific severity of lesions [14].

Methylation-specific PCR (MSP) is a sensitive technique widely used to identify promoter methylation, mainly due to its low cost [15]. With MSP, promoter methylation has been discovered in various tumor suppressor genes connected with cell cycle regulation as $p16^{INK4A}$ and DNA repair mechanisms as human MutL Homolog 1 (hMLH1) and O6-Methylguanine DNA Methyl Transferase (MGMT) [11, 13, 16, 17]. $p16^{INK4A}$ is a protein shown to be overexpressed in high-grade lesions as a result of HPV oncoprotein over-expression, while inhibition of DNA repair mechanisms has been shown to occur in many types of carcinomas [4, 5, 9, 13].

In this study we used MSP to identify promoter methylation of the three above referred tumor suppressor genes in normal and pathological cervical liquid-based cytology samples, in order to evaluate their use in identifying lesions. Next we assessed the relation of promoter methylation to HPV presence, mRNA expression, $p16^{INK4A}$ protein expression, and clinicopathological features, in order to clarify whether methylation is correlated with HPV presence and lesion progression.

2. Materials and Methods

2.1. Specimens. Samples were part of a larger pool of samples from primary screening for cervical cancer in Greece. A total of 403 liquid-based cytological (LBC) smears from women that underwent colposcopy were included in the present study. These consisted of 340 histologically confirmed samples and 63 samples with normal cytology that were added in order to increase the number of cytologically negative samples and have a better baseline of promoter methylation in "normal" samples. The study population consisted of women with a mean of 36.8 years of age (min–max: 18–81), a start of sexual intercourse at 18.9 years of age (13–30), and with a mean of 3.9 sexual partners (1–16). Cytology smears were collected in liquid-based media (ThinPrep, Hologic, Marlborough, USA), a single-layer smear was prepared by automated means (TP2000 processor), stained according to Papanikolaou, and diagnosis was set according to the Bethesda system by a skilled cytopathologist [18]. All molecular tests were performed on residual LBC specimens. Histology diagnosis was set by a skilled histopathologist and for statistical purposes CIN-I were classified as LSIL, while CINII and CINIII were classified as HSILs.

2.2. Bisulfite Conversion MSP. A commercially available kit for bisulfate conversion and PCR amplification was used (Amplicolon, Bird Srl, Siena, Italy) to identify promoter methylation of $p16^{INK4A}$, hMLH1, and MGMT. PCR products were analyzed in a 2% agarose gel, stained with ethidium bromide and visualized under UV light. If a PCR product was detected only in the unmethylated reaction, sample was characterized as unmethylated, while presence of a PCR product in the methylated reaction characterized the sample as methylated, regardless of the result of the unmethylated reaction. Absence of a product from both reactions characterized the sample as invalid. An unmethylated DNA control is included in the kit, while a methylated DNA control was created after treatment of the unmethylated control with SssI methyltransferase (NEB, Massachusetts, USA) (see Figure 1).

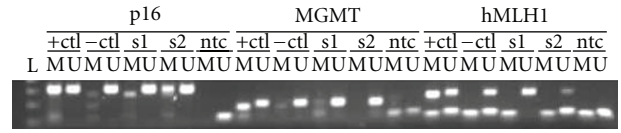

Figure 1: Agarose gel electrophoresis of PCR products of gene promoter methylation of (a) MGMT, (b) hMLH1, and (c) $p16^{INK4A}$. L: DNA ladder 50 bp, +ctl: DNA treated with SssI, −ctl: unmethylated DNA control, s1,s2: clinical samples negative for methylation for MGMT and hMLH1. s2 is positive for p16 methylation.

2.3. HPV Typing and E6/E7 mRNA Expression. DNA HPV typing was performed using a commercially available kit (CLART HPV2, Genomica, Madrid, Spain), according to manufacturer's instructions. The kit can identify 35 different HPV types, that are categorized as low risk (HPVs 6, 11, 40, 42, 43, 44, 54, 61, 62, 71, 72, 81, 83, 84, and 89) and high risk (HPVs 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 82, and 85) based on their epidemiology in specific grade of lesions [6]. E6/E7 mRNA expression was identified using the commercially available Nuclisens EasyQ HPV kit (Biomerieux SA, Marcy l'Etoile, France), that is, able to detect mRNA of five high-risk HPV types (HPV16, HPV18, HPV31, HPV33, and HPV45). Positive and no template controls were included in all experiments.

2.4. $p16^{INK4A}$ Protein Expression. Protein expression of $p16^{INK4A}$ was identified by immunocytochemistry using a commercial kit according to the manufacturer's instructions (CINtec Cytology, mtm Laboratories AG, Heidelberg, Germany). Evaluation of positive staining was performed by an experienced cytopathologist.

2.5. Statistical Analysis. All statistic tests were performed using IBM Statistics SPSS 19 (IBM Corporation, NY, USA) using χ^2 analysis, two-paired t-test and bivariate correlation analysis. All analysis tests were two tailed with significance at 95%. Overall accuracy was estimated by ROC analysis using histology (HSIL+) as the golden standard.

3. Results and Discussion

Promoter methylation status was successfully analyzed for 403 samples for MGMT, 372 samples for hMLH1 and 290 samples for $p16^{INK4A}$. $p16^{INK4A}$ protein expression was available for 358 samples, HPV DNA typing for 398 samples and mRNA expression for 355 samples. Cytological, colposcopical, and histological findings were well correlated as indicated by the area under the curve (AUC) values of both cytology (0.863) and colposcopy (0.861) compared to histology.

3.1. Promoter Methylation and Clinicopathological Findings. Results of promoter methylation status are summarized in

Table 1: Promoter methylation results.

		MGMT				hMLH1				$p16^{INK4A}$				Any gene			
		M	(%)	*N*	*P*	*M*	(%)	*N*	*P*	*M*	(%)	*N*	*p*	*M*	(%)	*N*	*P*
Cytology	WNL	22	22.7	97		5	5.7	88		7	13.5	52		31	32.0	97	
	ASCUS	26	40.7	64		4	7.0	57		9	18.4	49		33	51.6	64	
	LgSIL	51	38.6	132		22	17.9	123		18	17.5	103		75	56.8	132	
	ASC-H	3	37.5	8	***	1	14.3	7	*	1	33.3	3		4	50.0	8	***
	HgSIL	43	47.3	91		13	14.8	88		15	19.7	76		54	59.3	91	
	SCC	5	80.0	6		0	0	5		1	33.3	3		5	83.3	6	
	AdenoCa	5	100	5		2	50.0	4		3	75.0	4		5	100	5	
Colposcopy	NSF	3	42.9	7		0	0	7		0	0	5		3	42.9	7	
	Negative	25	27.2	93		6	7.3	82		11	21.2	52		35	38.0	93	
	LGSIL	74	38.7	191	***	24	13.6	177	*	25	17.6	142		100	52.4	191	***
	HGSIL	40	40.8	98		15	16.1	93		15	18.5	81		56	57.1	98	
	SCC	9	81.8	11		1	10.0	10		1	14.3	7		9	81.8	11	
	AdenoCa	4	100	4		1	33.3	3		2	66.7	3		4	100	4	
Histology	Negative	13	22.8	57		8	15.1	53		5	12.2	41		21	36.8	57	
	LSIL	70	42.7	164		21	13.5	155		25	20.7	121		95	57.9	164	
	HSIL	43	42.6	101	***	14	14.6	96		15	17.6	85		57	56.4	101	**
	SCC	8	66.7	12		1	9.1	11		1	12.5	8		8	66.7	12	
	AdenoCa	6	100	6		2	40.0	5		3	75.0	4		6	100	6	

M: Methylated, *N*: Number of cases, NSF: Nonsatisfactory, χ^2 for trend *P*: $^*P < .05$, $^{**}P < .005$, $^{***}P < .001$.

Table 1. MGMT methylation was the most aberrant methylated gene, followed by $p16^{INK4A}$ and finally by hMLH1. MGMT methylation increased statistically significant with lesion severity as determined by either cytological ($P < .0001$), colposcopical ($P < .0001$), or histological ($P < .0001$) findings. hMLH1 methylation, on the other hand, displayed a significant increase with lesion severity only with cytological ($P = .0173$) and colposcopical ($P = .0489$) findings, while $p16^{INK4A}$ methylation showed no significant difference. Any of the three genes was statistically more often methylated in more severe lesions, as determined by either cytological ($P < .0001$), colposcopical ($P = .0002$), or histological ($P = .0031$) findings.

3.2. Promoter Methylation and Molecular/Immunocytochemical Findings. Positivity of $p16^{INK4A}$ protein expression increased with MGMT and $p16^{INK4A}$ methylation ($P = .001$ and $P = .013$). Methylation of MGMT increased with HPV DNA positivity ($P = .021$), overall mRNA positivity ($P = .017$), expression of HPV16 mRNA ($P = .001$), and DNA positivity for HPV16, HPV18, and HPV68 ($P < .01$). hMLH1 methylation increased with positivity for low-risk HPV DNA ($P = .001$), HPV16 mRNA ($P = .035$) and DNA positivity for HPV40, HPV51, and HPV61 ($P < 0.01$). Methylation of $p16^{INK4A}$ increased with overall mRNA positivity ($P = .046$), expression of HPV16 mRNA ($P = .05$), HPV33 mRNA ($P = .036$) HPV16, HPV43, and HPV85 DNA positivity. Presence of either HPV16, HPV45, HPV53, HPV61, HPV68 DNA positivity or HPV16 and HPV45 mRNA positivity was correlated with an increase of the number of methylated genes ($P < .01$).

3.3. HPV Status, $p16^{INK4A}$ Protein Expression and Clinicopathological Findings. Severity of the lesion, whether determined by cytology, colposcopy, or histology, was statistically higher with $p16^{INK4A}$ protein expression, mRNA expression, and presence of HPV DNA of high-risk types ($P < .001$). HPV DNA positivity could either be only from high-risk types or mixed with low-risk types. Presence of low-risk HPV DNA was only correlated with low grade lesions ($P < .01$). HPV DNA positivity was more common in younger women (35.5 versus 39.3, $P = .003$) with more sex partners (4.3 versus 2.8, $P = .039$), while $p16^{INK4A}$ protein expression was more common in older women (39.6 versus 36.8, $P = .04$) with earlier sex life initiation (17.8 versus 19.5, $P = .019$).

3.4. Discussion. Promoter methylation has been proposed to be a significant cofactor in carcinogenesis, especially in nonhereditary carcinomas. Its role in epigenetic inactivation of tumor suppressor genes has been shown to be common in many types of carcinomas, while recent evidence supports its contribution in cancer development in the cervix [10–14].

Findings of this study, as far as promoter methylation increase during lesion progression is concerned, are partially consistent with previous studies [11, 12, 14, 19]. The main difference with the present study is the significantly larger amount of samples that are included in our study and the significantly higher positivity rate of methylation in our study. As far as diagnostic or screening utility of promoter methylation, none of the genes displayed an AUC of over 0.6 when plotted against histology, with HSIL as the cutoff, ranking it as an ancillary technique with more data needed

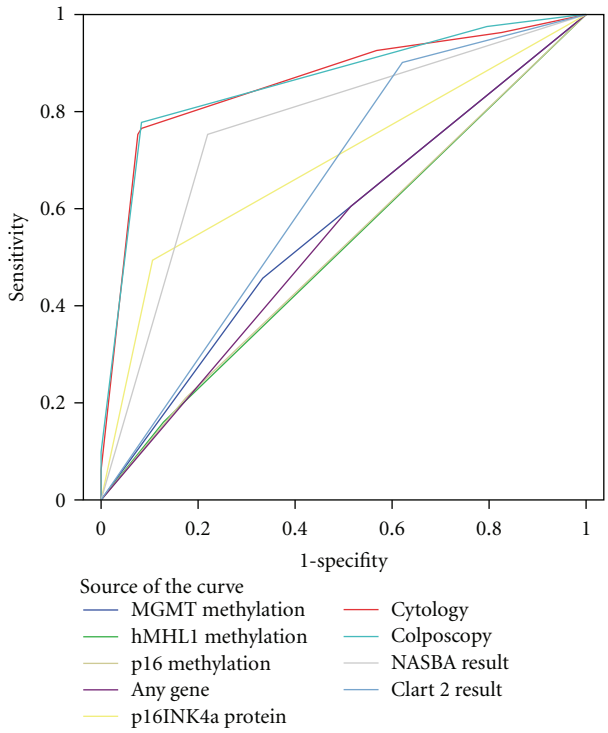

Figure 2: ROC curve analysis. Diagonal segments are produced by ties.

to identify the exact value of methylation status in cervical carcinogenesis.

As expected HPV DNA testing displayed high sensitivity with low specificity depicted in the ROC curve (Figure 2, AUC 0.641), while mRNA higher specificity with lower sensitivity (AUC 0.767). $p16^{INK4A}$ protein expression showed displayed better specificity than mRNA testing, but worse sensitivity (AUC 0.694). The above findings are consistent with previous studies, that have shown the use of these techniques in triaging women [6, 7, 20–23].

Presence of both $p16^{INK4A}$ protein expression and promoter methylation was identified in both HSILs and carcinomas. Due to the heterogeneity of cytological samples this could reflect different pathways that are activated in specific types of cells or could be due to the partial methylation that can be identified with MSP but is not enough to abolish protein expression [24]. MGMT methylation was correlated with previously reported risk factors for severe lesions [7, 20, 21, 25], while interestingly enough hMLH1 methylation was correlated with low-risk HPV types especially when co-infection of a low-risk with a high-risk was identified.

4. Conclusion

Aberrant DNA promoter methylation of MGMT, hMLH1, and $p16^{INK4A}$ is a common finding in liquid-based cytology samples of the cervix. Even though, there is a statistically significant increase of DNA methylation as the severity of the lesion increases, either for a single gene, or for the total number of methylated genes, the accuracy of promoter DNA methylation in identifying severe lesions is low. As a result wide use in screening programs is not recommended, since more studies with larger methylation panels should be performed before the exact significance of methylation in cervical carcinogenesis is elucidated.

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