Cervical Cancer Prevention: New Frontiers of Diagnostic Strategies

Guest Editors: Massimo Origoni, Walter Prendiville, and Evangelos Paraskevaidis
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Editorial

Cervical Cancer Prevention: New Frontiers of Diagnostic Strategies

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Invasive cervical cancer still represents one of the major issues of preventive oncology either in developed countries or in developing countries, accounting for the fourth leading cause of cancer related deaths in women worldwide and the second leading cause of deaths in women in developing countries [1]. It is the greatest cause of cancer death in women in sub saharan Africa, even outstripping breast cancer. Since the introduction of population-based organized cytological screening programs, a dramatic decrease of incidence of cervical cancer has been obtained in many western countries; despite this reassuring result, the overall performance of cervical cytological screening, the Pap test, is still far from being optimal [2]. Because Human Papillomavirus (HPV) is the recognized necessary cause for the development of cervical cancer, the present research is aimed at the investigation of new approaches towards its prevention and is particularly focused upon several biomolecular patterns and detection tools of the virological contamination of the lower female genital tract. The identification of high-risk viral strains DNA (HPV 16 and 18) is now worldwide recognized as more effective than cervical cytology in several settings: primary screening, triage of atypical cytology, and follow-up after treatment [3]. In this model, the traditional approach of looking for the early stages of the cervical neoplastic disease has nowadays shifted to the biological interpretation of the effects of HPV persistent infection on cervical epithelia and thus to the identification of “at-risk” individuals or groups rather than affected patients. The concept of risk stratification for cervical cancer is the final result of a cultural revolution in the field of cervical cancer natural history knowledge and isolation of progression risk factors. Molecular techniques are better than cervical cytology with respect to diagnostic sensitivity and reproducibility to detect cervical intraepithelial neoplasia grade 2 (CIN2) or cervical intraepithelial neoplasia grade 3 (CIN3)—the high-grade lesion precursors of invasive cervical cancer. According to data from four European randomized trials comparing HPV-based cervical cancer screening with cytology-based cervical cancer screening, HPV-based screening resulted in a 60–70% reduction in invasive cervical cancer incidence compared with cytology-based screening. The decrease in incidence of invasive cervical cancer with HPV testing was not significant within 2.5 years of enrolment, but the effect became decisive with longer follow-up [4]. Besides these results that have significantly contributed to the shift to a molecular screening strategy, a large amount of research works has been performed and is still ongoing in the field of identifying additional and novel aspects of the HPV causal effect of determining the neoplastic transformation of cervical tissues: genotyping, E6/E7 oncoproteins overexpression, high-risk HPV mRNA determination, novel progression biomarkers such as p16INK4a and Ki67, DNA methylation markers, and, last but not least, the future role of colposcopy in the biomolecular era. In this special issue, readers will find articles that focus on these promising aspects of investigation, providing interesting results that could potentially open new
windows of knowledge and new options of interventions in the still unfinished battle towards cervical cancer that started with Papanicolaou.

Massimo Origoni  
Walter Prendiville  
Evangelos Paraskevaidis

References


Research Article

HPV Testing from Dried Urine Spots as a Tool for Cervical Cancer Screening in Low-Income Countries

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Nowadays, several screening strategies are available to prevent cervical cancer, but inadequate resources, sociocultural barriers, and sampling issues impede their success in low-income countries. To overcome these issues, this study aimed to evaluate the performance of human papillomavirus (HPV) testing from dried urine spots (DUS). Eighty-eight urine samples (including 56 HPV DNA positive specimens) were spotted on filter paper, dried, and stored in paper-bags. HPV DNA was detected from the DUS after 1 week and 4 weeks of storage using a polymerase chain reaction (PCR) assay. The sensitivity, specificity, and concordance of the DUS-based HPV test were evaluated by comparing the results with those of HPV testing on fresh urine samples as the gold standard.

The sensitivity of the test was 98.21% (95% CI: 90.56–99.68) for DUS stored for 1 week and 96.42% (95% CI: 87.88–99.01) for DUS stored for 4 weeks. The specificity was 100% (95% CI: 89.28–100) at both time points. The concordance between DUS and fresh urine HPV testing was “almost perfect” using the \( \kappa \) statistic. These preliminary data suggest that a DUS-based assay could bypass sociocultural barriers and sampling issues and therefore could be a suitable, effective tool for epidemiological surveillance and screening programs, especially in low-income countries.

1. Introduction

Cervical cancer is a relevant public health problem for women worldwide, being the third most frequent cancer and the fourth most common cause of death from cancer in women [1, 2]. Overall, the World Health Organization (WHO) estimates that there are 530,000 new cases of cervical cancer each year and more than 270,000 deaths, with 85% of deaths occurring in low- and middle-income countries [3]. All cervical cancers can be attributable to a sexually transmitted infection (STI) that is caused by the human papillomavirus (HPV). HPV infections usually clear up without any intervention within a few months after acquisition, and approximately 90% of infections clear up within two years. A small proportion of infections with certain types of HPV can persist and progress to cancer [3].

HPVs are DNA viruses that are grouped into cutaneous and mucosal types according to their infection site and further subdivided into high-risk (HR) and low-risk (LR) types, depending on their association with disease malignancy. The International Agency for Research on Cancer (IARC) has included 25 types of HPV in the high-risk clade (HR-clade) by subdividing them into three groups [4]. HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 are classified in group 1 as “carcinogenic to humans,” while groups 2A and 2B include the “probably carcinogenic to humans” and “possibly carcinogenic to humans” genotypes, respectively. HPV16 and HPV18 are the two most common HR types in cervical cancer, causing approximately 70% of all cases worldwide [4].

Different types of screening for cervical cancer are now available, such as the conventional cytology test (Pap test) and liquid based cytology (LBC), visual inspection with acetic acid (VIA), and HPV testing for HR-HPV from cervical brush. These types of screening require adequate financial resources, a developed infrastructure, trained labour, and surveillance mechanisms for screening, investigating, treating, and following up on targeted women [5, 6]. Moreover,
women's educational levels, misconceptions, and prejudices are barriers to access and to the success of cervical cancer prevention programs in low-resource countries. Due to these difficulties, the disease is often identified in the late stage, resulting in higher rates of cervical cancer incidence and mortality [7–9].

Alternative tools that can overcome these problems could improve screening coverage and reach the female population at risk in developing countries.

The use of HPV testing on urine, a noninvasive and easy-to-collect sample, could be more attractive to women because it bypasses medical examination, as well as sociocultural and religious implications. The correlation between the detection of HPV in urine and cervical samples has been reported in several studies in the literature [10–12]. In addition, HPV DNA testing is more sensitive than cytology for detecting high-grade cervical intraepithelial neoplasia (CIN), and it would provide an automated and objective assay, which would improve quality control [13].

However, urine samples require restrictive conditions for storage and transportation, especially when samples must be transported over long distances in a warm climate. This factor is particularly relevant in low-income countries where analysis laboratories can be located far away from the rural areas where women live. An alternative method of sample collection is the use of dried urine spots (DUS), that is, a urine sample spotted on blotting paper, which allows for stabilization by drying. This approach has logistical benefits because DUS are small and easily transported and the specimens can be stored at room temperature.

DUS sample collection solves several problems associated with the sampling and storage of fresh urine samples. Therefore, the DUS approach is particularly interesting, especially in developing countries, even considering that dried spots on filter paper can be successfully used for the detection of various infectious agents, as well as metabolic and genetic diseases [14–16].

Few studies have evaluated the use of dried samples on filter paper for the detection of HPV infection, and these investigations examined exclusively cervical brush samples [17–19]. No data are presently available about the use of urine samples on this type of medium.

Thus, the aim of this study was to evaluate the performance of HPV testing from DUS and to compare the results obtained with those from paired fresh urine samples.

2. Materials and Methods

2.1. Sample Collection. Urine samples were obtained from 88 immigrant women (median age: 34 years; interquartile range (IQR): 28–43 years) who attended NAGA Onlus in Milan, Italy, between June 2012 and December 2013 and were included in a large epidemiological study on HPV and Chlamydia trachomatis infections [20]. All of the women provided informed consent for further anonymous research testing on the residual samples. Ethics approval was obtained from the Ethics Committee of the University of Milan, Italy.

Of the urine samples, 56/88 (63.6%) were HPV DNA positive and 32 (36.3%) were HPV DNA negative. Of the HPV DNA positive samples, 40/56 (71.4%) were sustained by single infections (24 belonging to HR-clade genotypes and 16 to LR genotypes) and 16/56 (28.6%) were caused by multiple infections (4 were caused by LR genotypes and 12 by at least 1 genotype of the HR-clade).

2.2. Sample Preparation. Each 400 μL urine sample was subdivided into eight 50 μL aliquots that were each spotted on preprinted circles on a piece of filter paper (Mascia Brunelli, Italy). The DUS filter papers were dried for 3 h and then stored in paper bags in a dry location at room temperature (RT; 25–30°C) for either 1 week or 4 weeks until the analyses took place. The analyses were carried out at the Laboratories of the Department of Biomedical Sciences for Health, University of Milan, Italy.

2.3. Nucleic Acids Extraction. Four preprinted circles were punched or cut out from each piece of DUS filter paper using a sterile single-cut paper-punching machine or a new sterile scalpel blade. The circles were transferred into a 1.5 mL tube containing 1 mL of NucliSENS Lysis Buffer (bioMérieux, Lyon, France) and incubated on a roller mixer for 30 minutes at RT. Then the tube was centrifuged for 15 s at 1500 × g. The lysate, with a volume of approximately 750 μL, was extracted using the commercial NucliSENS EasyMAG method (bioMérieux, Lyon, France), according to the manufacturer’s instructions. The nucleic acids were eluted in 100 μL of the NucliSENS elution buffer.

The concentration of the extracted DNA was evaluated using a spectrophotometer (Nanodrop 2000c, Thermo Fisher Scientific Inc., Wilmington, DE, USA). The quality of DNA was validated by detection of a 268 bp fragment of the housekeeping beta-globin gene using an in-house polymerase chain reaction (PCR) assay [21].

2.4. HPV DNA Detection. HPV DNA was detected using an in-house nested-PCR assay based on the amplification of a 150 bp open reading frame late gene 1 (ORF L1) fragment. The nested-PCR assay was performed using a two-step amplification to either increase sensitivity or mitigate the inhibitory effect of substances potentially present in the sample. Every PCR reaction included positive (HPV-16 positive cells, Caski) and negative (water) controls. Strict laboratory precautions and quality assurance/quality control measures were followed to avoid cross contamination and carry over PCR. ELSI_F/ELSI_R primers were used for the first cycle of amplification and GP5+/GP6+ primers were used for the nested reaction, as previously described [11, 22].

The amplification products were visualized by means of electrophoresis analysis on 2% agarose gels containing ethidium bromide (0.5 mg/mL). The amplified products were compared with molecular weight standards (DNA Molecular Weight, Marker 100, Sigma-Aldrich, St. Louis, MO, USA).

2.5. HPV Genotyping. HPV DNA positive DUS isolates were genotyped using INNO-LiPA HPV Genotyping Extra
(Fujirebio Italia, Rome, Italy), a line probe assay based on the principle of reverse hybridization, according to the manufacturer's instructions. The resulting line patterns allowed for identification of 28 different HPV genotypes: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 69, 70, 71, 73, 74, and 82.

2.6. Statistical Analysis. The sensitivity and specificity of HPV testing from DUS were evaluated in comparison to HPV testing from fresh urine samples as the gold standard. These results were presented as percentages with 95% confidence intervals (95% CIs). Proportions (95% CI) were calculated using the Wilson score model by the OpenEpi statistical program (version 3.01), which is available online [23].

To determine the proportion of agreement between DUS and fresh urine testing, Cohen's unweighted kappa (κ) statistic was calculated by dividing the difference between the observed proportion of agreement and the expected proportion of agreement by 1 minus the expected proportion of agreement. The concordance was defined as "poor" (κ = 0), "slight" (0.01 ≤ κ ≤ 0.20), "fair" (0.21 ≤ κ ≤ 0.40), "moderate" (0.41 ≤ κ ≤ 0.60), "substantial" (0.61 ≤ κ ≤ 0.80), "almost perfect" (0.81 ≤ κ < 1.00), or "perfect" (κ = 1.00).

3. Results

DNA was successfully extracted from all of the DUS samples stored at RT for either 1 week or 4 weeks. The evaluated DNA concentrations ranged from 6.4 to 50.2 ng/μL for 1 week of DUS storage and from 7 to 30.8 ng/μL for 4 weeks of DUS storage. The housekeeping beta-globin gene was amplified from all DUS samples, confirming the suitability of the DNA extraction method.

3.1. Detection of HPV DNA from DUS after 1 Week or 4 Weeks of Storage. Of the 56 DUS samples prepared from HPV DNA positive fresh urine, 55/56 (98.21%, 95% CI: 90.56–99.68) tested HPV DNA positive after 1 week. In contrast, HPV DNA was detected in 54/56 (96.42%, 95% CI: 87.88–99.01) of the DUS samples after storage for 4 weeks.

The DUS sample that tested HPV DNA negative after both 1 and 4 weeks of storage was prepared from an HPV-72 (LR HPV) infected urine sample. The other DUS that tested HPV DNA negative only after 4 weeks of storage was prepared from an HPV-56 (HR HPV) infected urine sample.

All DUS prepared using HPV DNA negative urine samples tested negative for HPV DNA.

The sensitivity of the HPV DNA test in DUS at 1 and 4 weeks was 98.21% (95% CI: 90.56–99.68) and 96.42% (95% CI: 87.88–99.01), respectively. The specificity for the test was 100% (95% CI: 89.28–100) after both 1 week and 4 weeks of storage. The proportion of agreement between the DUS and fresh urine tests was "almost perfect" (κ statistic ≥ 0.81) (Table 1).

3.2. HPV Genotyping. Of the 40 DUS prepared from fresh urine samples of women infected by a single HPV genotype, 38 (95%) tested HPV DNA positive after four weeks of storage. All amplified fragments from the DUS were properly genotyped, and the distribution of HPV genotypes matched across the two sample types (Figure 1). The distribution of HR-clade and LR HPV genotypes was similar across the paired samples. In particular, 23 HPV DNA positive DUS were sustained by HR-clade genotypes and 15 were caused by LR genotypes.

4. Discussion

To our knowledge, this is the first report of HPV detection from urine samples stored on filter paper. The quantity and the quality of DNA extracted from DUS were comparable to those obtained by standard collection from fresh urine, as estimated by the spectrophotometric readings and the detection of the housekeeping beta-globin gene using an in-house PCR assay. Accordingly, the high molecular weight DNA extracted from DUS samples is sufficient to perform molecular assays, either traditional or high throughput.

The data obtained showed an elevated concordance between HPV DNA detection in DUS and fresh urine samples. Cohen's unweighted kappa values were very high, indicating an "almost perfect" agreement.
These preliminary data support the use of DUS as simple, rapid, and safe sampling for HPV DNA detection and genotyping by using molecular tests, such as PCR and line probe assay based on the principle of reverse hybridization. Furthermore, the use of DUS strengthens the already recognized advantages of urine samples. Collection of these noninvasive specimens is more acceptable and can bypass the ethical, social, and religious barriers of speculum exams for the collection of conventional cervical brush [7, 8, 11].

Moreover, drying urine samples on filter paper allows the DNA to be protected from degradation for a long period, as shown by the high percentage (96.4%) of HPV DNA positive samples detected after four weeks of storage at room temperature.

Due to the introduction and increasing availability of novel and powerful high-throughput molecular technologies, the optimization of methods for biological samples collection and storage has become a critical issue. These results highlight the potential use of DUS samples as an alternative means of biobanking, avoiding the high costs and logistical problems associated with the storage and transportation, especially where a cold chain is absent [19, 24]. Finally, DUS have a small size and can be easily mailed to a reference laboratory.

5. Conclusions

HPV testing from DUS showed an elevated sensitivity and specificity and a high concordance rate compared to HPV testing from fresh urine samples. These preliminary data suggest that a DUS-based assay could bypass sociocultural barriers and sampling issues. This approach could be a suitable and effective tool for epidemiological surveillance and screening programs, especially in low-income countries.

Ethical Approval

Ethics approval was obtained from the Ethics Committee of the University of Milan in Milan, Italy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References


Research Article

Underscreened Women Remain Overrepresented in the Pool of Cervical Cancer Cases in Spain: A Need to Rethink the Screening Interventions

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Objective. Audit of women with invasive cervical cancer (CC) is critical for quality control within screening activities. We analysed the screening history in the 10 years preceding the study entry in women with and without CC during 2000–2011. Methods. 323 women with CC from six pathology departments in Catalonia (Spain) and 23,782 women with negative cytology were compared. Age, previous history of cytologies, and histological type and FIGO stage were collected from the pathology registries. Logistic regression analysis was used to estimate odds ratios (OR) and 95% confidence intervals (CI95%). Results. History of cytology was registered in 26.2% of CC cases and in 78% of the control women (P < 0.0001) and its frequency decreased with increasing age. Compared to women with squamous cell carcinoma, adenocarcinoma cases were significantly more likely to have a cytology within the 3-year interval preceding cancer diagnosis (OR = 2.6 CI 95%; 1.2–5.6) and to have normal cytology results in previous screenings (OR = 2.4 CI 95%; 1.2–4.5). FIGO II–IV cases were more common among older women (older than 60 years). Conclusions. Absence of prior screening history was extremely common among CC cases compared to controls. Organized actions to reduce underscreened women and use of highly sensitive HPV-based tests could be important to reduce CC burden.

1. Introduction

The major cause of cervical cancer (CC) is the persistent infection with oncogenic types of human papillomavirus (HPV) [1]. CC is preceded by visible morphological cervical intraepithelial lesions (CIN) that can be detected through regular exam of exfoliated cells of the cervix. Although vaccines to prevent infection with specific oncogenic HPVs are now available, it will take at least 2-3 decades for their effects on CC burden to be seen [2].

Meanwhile, adult unvaccinated women remain target for CC screening. However, to guarantee an adequate population impact of screening, large population coverage and adequate follow-up have been fundamental in decreasing incidence and mortality of CC [3, 4].

In the autonomic region of Catalonia, CC screening is opportunistic. Efforts to increase CC screening coverage were initiated in 2006 within the public sector with the introduction of new screening protocol [5]. Routine screening with cervical cytology is recommended in the region to women
aged 25–65 with a 3-year interval. Cervical cytology coverage was estimated for the period 2008–2011 to be around 70% if public as well as private coverage is considered [6, 7]. Every year, there will be around 378 new cancer cases of CC in the region implying a lifetime risk of one out of 106 women [8].

As part of quality assessment of the screening activities in the region, we aimed to monitor screening uptake among women who have developed invasive CC. For this purpose, we analysed the screening history in the 10 years preceding the study entry among women with and without CC who attended within the Public Health System from a predefined study area for the period 2000–2011.

2. Patients and Methods

2.1. Data Collection. The study includes all women with an incident invasive CC diagnosed in six pathology departments of Catalonia during January 2000 to December 2011 (Hospital General de L’Hospitalet, Corporació Sanitaria Parc Taulí, Hospital Mutua de Terrassa, Consorci Hospitalari de Vic, Hospital Althaia, and Consorci Hospitalari de Terrassa). The aforementioned hospitals encompass 2 of the 7 health counties that compose Catalonia’s Health System. The female population over the age of 24 in the area was 360,008 women [9].

A total of 323 newly diagnosed CC cases were identified during the study period. Information collected from clinical records during the 10 years prior to CC diagnosis included history of previous cytologies, time since the last cytology to cancer diagnosis, age of the patient at time of cancer diagnosis, and type and stage of CC. We assumed that cytologies taken within the previous 6 months to the cancer diagnosis were obtained as part of the diagnostic process and excluded them for the analysis. Women were categorised as never screened if there was no record on cervical cytology.

A comparison group consisted of 23,782 women with a normal cytology retrieved from one of the six pathology departments and resident in the study area in 2007. Thereafter, these women were referred to as control group. Information about the presence or absence of prior cytologies during 10 years before negative cytology and ages of the women were collected from the same source as the cases.

The overall project was approved by the ethical committee of the Catalan Institute of Oncology. Any information regarding the identification of patients was anonymized before analysis.

2.2. Screening Tests and Stage of Cervical Cancers. In all centres, conventional cytology was used. All the cytological results were classified or adapted if needed, according to the 2001 Bethesda system [10].

CC cases were staged according to the International Federation of Gynaecology and Obstetrics (FIGO) classification system [11].

2.3. Statistical Analysis. For the women with CC, information consisted of histological type, FIGO stage (unknown, I, II, III, and IV), and registration to previous screening, which included result of prior cytology (no previous cytology, normal or abnormal), time since the last previous cytology (<3 years and >3 years before cancer diagnostic according to established 3-year screening interval), and numbers of previous cytologies (without, 1, or >1 prior cytology). The large majority of cases were squamous cell carcinoma (SCC) or adenocarcinoma (ADC). The remaining cases (N = 15) were reclassified as follows: three clear cell adenocarcinoma and one adenoid cystic carcinoma cases were included in the ADC group while one small cell carcinoma and ten adenosquamous carcinoma were included in the SCC group. Data were analysed with and without these rare histological types and the result was similar in both situations, so they were included in the analysis. Women with nonevaluable previous cytology (N = 2) or missing age were excluded (N = 2).

Logistic regression was performed to estimate the odds ratio (OR) with the corresponding 95% confidential intervals (CI 95%) of developing ADC or SCC. Adjustment was done by geographical area and women’s age.

Differences in the presence of cervical cytology in the previous ten years between the CC cases and the control group were estimated taking into account the different age structure of both groups. Proportional differences were compared using chi-square test. Statistical significance was defined as P < 0.05.

3. Results

Table 1 shows the age distribution and the percentage of women with prior cytology during the previous 10 years among 323 CC cases and 23,782 control women. Women with CC were on average 12.6 years older than control women (54.4 versus 41.9). History of previous cytology was identified in 78.8% of the control women and in 26.2% of CC cases (P < 0.0001). After adjustment for differences in the age structure, the global use of prior cytology among CC was 70% lower compared to that in controls.

Table 2 describes the age distribution, the period, and the FIGO stages at diagnosis of the 323 CC cases by histological type. Overall, 248 (76.8%) of the CC were SCC and 75 (23.2%) were ADC. The average age was 54.4 years with a range of 23–96 years. The majority of the CC were diagnosed in the age range of 40 to 49 years (26.2%). In 40.9% of CC cases, the cancer stage was unknown. No statistically significant differences were observed between histological types and the general characteristics explored.

Women with ADC were significantly more likely to have had a prior cytology (OR = 2.1 CI 95%: 1.2–3.8), more than 1 previous cytology (OR = 3.2 CI 95%: 1.5–6.5), a cytology 3 years before cancer diagnosis (OR = 2.6 CI 95%: 1.2–5.6), and a normal cytology (OR = 2.4 CI 95%: 1.2–4.5) as compared to women with SCC (Table 3).

Age was strongly associated with FIGO stages (P < 0.05) (Table 4). Women aged less than 40 years were more likely to have a stage I CC while stages II–IV were more common among women aged 60 or more. Older women were less likely to have a prior cytology (82.9% and 79.2% in age groups of
Table 1: Age and history of previous screening cytology in women with cervical cancer and women with a normal cytology.

<table>
<thead>
<tr>
<th></th>
<th>Control women</th>
<th>Women with cervical cancer*</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>N = 23,782</td>
<td>N = 313</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
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<tr>
<td>&lt;30 years</td>
<td>5.224</td>
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<td>30–39 years</td>
<td>5.879</td>
<td>56</td>
<td>18.0%</td>
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<td>40–49 years</td>
<td>5.426</td>
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<td>50–59 years</td>
<td>4.277</td>
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<td>15.1%</td>
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<td>60–69 years</td>
<td>2.378</td>
<td>41</td>
<td>13.2%</td>
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<tr>
<td>≥70 years</td>
<td>598</td>
<td>71</td>
<td>22.8%</td>
</tr>
<tr>
<td>With at least one cytology in the last 10 years**</td>
<td></td>
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<td></td>
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<tr>
<td>With cytology</td>
<td>18.733</td>
<td>82</td>
<td>26.2%</td>
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<tr>
<td>Without cytology</td>
<td>5.049</td>
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<td>73.8%</td>
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<tr>
<td>Age-standardized coverage ratio</td>
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</tbody>
</table>

*There are 2 ages missing.

**10 women with cervical cancer had a prior cytology performed over 10 years ago and they were excluded for the analysis.

Table 2: General characteristics of the study population by histological types.

<table>
<thead>
<tr>
<th>General characteristics</th>
<th>Total N = 323</th>
<th>%</th>
<th>Squamous carcinoma N = 248</th>
<th>%</th>
<th>Adenocarcinoma N = 75</th>
<th>%</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;30 years</td>
<td>12</td>
<td>3.7%</td>
<td>8</td>
<td>3.3%</td>
<td>4</td>
<td>5.3%</td>
<td>0.62</td>
</tr>
<tr>
<td>30–39 years</td>
<td>56</td>
<td>17.4%</td>
<td>43</td>
<td>17.5%</td>
<td>13</td>
<td>17.3%</td>
<td>0.54</td>
</tr>
<tr>
<td>40–49 years</td>
<td>84</td>
<td>26.2%</td>
<td>65</td>
<td>26.4%</td>
<td>19</td>
<td>25.3%</td>
<td>0.54</td>
</tr>
<tr>
<td>50–59 years</td>
<td>51</td>
<td>15.9%</td>
<td>43</td>
<td>17.5%</td>
<td>8</td>
<td>10.7%</td>
<td>0.11</td>
</tr>
<tr>
<td>60–69 years</td>
<td>41</td>
<td>12.8%</td>
<td>31</td>
<td>12.6%</td>
<td>10</td>
<td>13.3%</td>
<td>0.75</td>
</tr>
<tr>
<td>≥70 years</td>
<td>77</td>
<td>24.0%</td>
<td>56</td>
<td>22.8%</td>
<td>21</td>
<td>28.0%</td>
<td>0.64</td>
</tr>
<tr>
<td>Year of diagnosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000–2003</td>
<td>119</td>
<td>36.8%</td>
<td>92</td>
<td>37.1%</td>
<td>27</td>
<td>36.0%</td>
<td>0.44</td>
</tr>
<tr>
<td>2004–2007</td>
<td>73</td>
<td>22.6%</td>
<td>60</td>
<td>24.2%</td>
<td>13</td>
<td>17.3%</td>
<td>0.17</td>
</tr>
<tr>
<td>2008–2011</td>
<td>131</td>
<td>40.6%</td>
<td>96</td>
<td>38.7%</td>
<td>35</td>
<td>46.7%</td>
<td>0.37</td>
</tr>
<tr>
<td>FIGO stages</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown stage</td>
<td>132</td>
<td>40.9%</td>
<td>101</td>
<td>40.7%</td>
<td>31</td>
<td>41.3%</td>
<td>0.70</td>
</tr>
<tr>
<td>I</td>
<td>85</td>
<td>26.3%</td>
<td>63</td>
<td>25.4%</td>
<td>22</td>
<td>29.3%</td>
<td>0.78</td>
</tr>
<tr>
<td>II</td>
<td>40</td>
<td>12.4%</td>
<td>31</td>
<td>12.5%</td>
<td>9</td>
<td>12.0%</td>
<td>0.49</td>
</tr>
<tr>
<td>III</td>
<td>48</td>
<td>14.9%</td>
<td>37</td>
<td>14.9%</td>
<td>11</td>
<td>14.7%</td>
<td>0.56</td>
</tr>
<tr>
<td>IV</td>
<td>18</td>
<td>5.6%</td>
<td>16</td>
<td>6.5%</td>
<td>2</td>
<td>2.7%</td>
<td>0.17</td>
</tr>
</tbody>
</table>

*There are 2 ages missing.

All the variables are adjusted by area and groups of age.

FIGO: International Federation of Gynecology and Obstetrics.

60–69 and ≥70 years, resp.) or to have had a cervical cytology within an interval longer than 3 years. In the presence of a previous screening history, women younger than 40 years old were more likely to have an abnormal cytological result compared to older women (P = 0.05). Women with normal cytology were, on average, older than women with an abnormal cytology (54.6 versus 43.8 years, resp., P = 0.003). Most of the atypical squamous cell of undetermined significance (ASC-US), atypical squamous cells cannot exclude a high grade squamous intraepithelial lesion (ASC-H) and atypical glandular cells of undetermined significance (AGC) results were diagnosed in the group of women aged 40–49 years (33.3%) while the low grade squamous intraepithelial lesions (LSIL) results were mostly diagnosed in women younger than 40 years (85.7%). About half of the negative cytologies (56.7%) and 80% of the positive cytologies were performed within 3 years prior to CC diagnosis (P = 0.029) (data not shown). Among all cases, 12 were in women younger than
### Table 3: Screening history of the study population by histological type.

<table>
<thead>
<tr>
<th>Screening history</th>
<th>Total N = 323</th>
<th>Squamous carcinoma N = 248</th>
<th>Adenocarcinoma N = 75</th>
<th>Histological type</th>
<th>P value</th>
<th>OR</th>
<th>CI 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Previous cytologies to cancer diagnosis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without prior cytology</td>
<td>231</td>
<td>71,5%</td>
<td>187</td>
<td>75,4%</td>
<td>44</td>
<td>58,4%</td>
<td>0,01</td>
</tr>
<tr>
<td>With prior cytology</td>
<td>92</td>
<td>28,5%</td>
<td>61</td>
<td>24,6%</td>
<td>31</td>
<td>41,3%</td>
<td>0,01</td>
</tr>
<tr>
<td><strong>Number of previous cytologies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without prior cytology</td>
<td>231</td>
<td>71,5%</td>
<td>187</td>
<td>75,4%</td>
<td>44</td>
<td>58,7%</td>
<td>0,01</td>
</tr>
<tr>
<td>1 previous cytology</td>
<td>50</td>
<td>15,5%</td>
<td>37</td>
<td>14,9%</td>
<td>13</td>
<td>17,3%</td>
<td>0,31</td>
</tr>
<tr>
<td>&gt;1 previous cytologies</td>
<td>42</td>
<td>13,0%</td>
<td>24</td>
<td>9,7%</td>
<td>18</td>
<td>24,0%</td>
<td>0,00</td>
</tr>
<tr>
<td><strong>Time between previous cytology and cancer diagnosis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without prior cytology</td>
<td>231</td>
<td>71,5%</td>
<td>187</td>
<td>75,4%</td>
<td>44</td>
<td>58,7%</td>
<td>0,03</td>
</tr>
<tr>
<td>Prior cytology ≤3 years before cancer diagnostic</td>
<td>58</td>
<td>18,0%</td>
<td>40</td>
<td>16,1%</td>
<td>18</td>
<td>24,0%</td>
<td>0,07</td>
</tr>
<tr>
<td>Prior cytology &gt;3 years before cancer diagnostic</td>
<td>34</td>
<td>10,5%</td>
<td>21</td>
<td>8,5%</td>
<td>13</td>
<td>17,3%</td>
<td>0,02</td>
</tr>
<tr>
<td><strong>General results of previous cytologies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Without prior cytology</td>
<td>231</td>
<td>72,0%</td>
<td>187</td>
<td>76,0%</td>
<td>44</td>
<td>58,7%</td>
<td>0,02</td>
</tr>
<tr>
<td>Normal</td>
<td>60</td>
<td>18,7%</td>
<td>38</td>
<td>15,4%</td>
<td>22</td>
<td>29,3%</td>
<td>0,01</td>
</tr>
<tr>
<td>Abnormal</td>
<td>30</td>
<td>9,3%</td>
<td>21</td>
<td>8,5%</td>
<td>9</td>
<td>12,0%</td>
<td>0,18</td>
</tr>
<tr>
<td>ASC-US-AGC-H</td>
<td>18</td>
<td>60,0%</td>
<td>11</td>
<td>52,4%</td>
<td>7</td>
<td>77,8%</td>
<td></td>
</tr>
<tr>
<td>LSIL</td>
<td>7</td>
<td>23,3%</td>
<td>6</td>
<td>28,6%</td>
<td>1</td>
<td>11,1%</td>
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</tr>
<tr>
<td>HSIL</td>
<td>5</td>
<td>16,7%</td>
<td>4</td>
<td>19,0%</td>
<td>1</td>
<td>11,1%</td>
<td></td>
</tr>
</tbody>
</table>

*Cytopologies taken within the previous 6 months to the cancer diagnosis were excluded for the analysis because they were considered as part of the diagnostic process. There are 2 women with a nonevaluable previous cytology. These cases were excluded for the analysis in which result of previous cytology is involved.

*Percentages of specific cytological abnormalities are among the abnormal cytologies.

All the variables are adjusted by area and groups of age.

ASC-US: atypical squamous cell of undetermined signifcancy, ASC-H: atypical squamous cells which cannot exclude a high grade squamous intraepithelial lesion, AGC: atypical glandular cells of undetermined signifcancy, LSIL: low grade squamous intraepithelial lesion, and HSIL: high grade squamous intraepithelial lesion.
Table 4: Screening history of the study population by groups of age.

<table>
<thead>
<tr>
<th>Screening history</th>
<th>Age*</th>
<th>&lt;30 years</th>
<th>30–39 years</th>
<th>40–49 years</th>
<th>50–59 years</th>
<th>60–69 years</th>
<th>&gt;70 years</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N = 12</td>
<td>N = 56</td>
<td>N = 84</td>
<td>N = 51</td>
<td>N = 41</td>
<td>N = 77</td>
<td></td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td>% column</td>
<td>% column</td>
<td>% column</td>
<td>% column</td>
<td>% column</td>
<td>% column</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td>4 33.3%</td>
<td>20 35.7%</td>
<td>39 46.4%</td>
<td>19 37.3%</td>
<td>19 46.3%</td>
<td>30 39.0%</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td></td>
<td>7 58.3%</td>
<td>24 42.9%</td>
<td>27 32.1%</td>
<td>10 19.6%</td>
<td>7 17.1%</td>
<td>10 13.0%</td>
<td>0.02</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td>0 0.0%</td>
<td>7 12.5%</td>
<td>9 10.7%</td>
<td>5 9.8%</td>
<td>3 7.3%</td>
<td>5 6.5%</td>
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</tr>
<tr>
<td>III</td>
<td></td>
<td>2 4.3%</td>
<td>7 12.5%</td>
<td>8 10.3%</td>
<td>3 5.1%</td>
<td>6 8.4%</td>
<td>2 2.5%</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td>0 0.0%</td>
<td>4 6.8%</td>
<td>8 9.8%</td>
<td>3 6.0%</td>
<td>3 4.2%</td>
<td>2 2.5%</td>
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<tr>
<td>Cancer diagnosis</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Squamous carcinoma</td>
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<td>8 66.7%</td>
<td>43 76.8%</td>
<td>65 77.4%</td>
<td>43 84.3%</td>
<td>31 75.6%</td>
<td>56 72.7%</td>
<td>0.69</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td></td>
<td>4 33.3%</td>
<td>13 23.2%</td>
<td>19 22.6%</td>
<td>8 15.7%</td>
<td>10 24.4%</td>
<td>21 27.3%</td>
<td></td>
</tr>
<tr>
<td>Previous cytologies to cancer diagnosis</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without prior cytology</td>
<td>8 66.7%</td>
<td>36 64.3%</td>
<td>59 70.2%</td>
<td>31 60.8%</td>
<td>34 82.9%</td>
<td>61 79.2%</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>With prior cytology</td>
<td>4 33.3%</td>
<td>20 35.7%</td>
<td>25 29.8%</td>
<td>20 39.2%</td>
<td>7 17.1%</td>
<td>16 20.8%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within those with previous cytology**</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 previous cytology</td>
<td></td>
<td>1 25.0%</td>
<td>10 50.0%</td>
<td>14 56.0%</td>
<td>12 66.7%</td>
<td>2 28.6%</td>
<td>9 56.3%</td>
<td>0.48</td>
</tr>
<tr>
<td>&gt;1 previous cytologies</td>
<td></td>
<td>3 75.0%</td>
<td>10 50.0%</td>
<td>11 44.0%</td>
<td>6 33.3%</td>
<td>5 71.4%</td>
<td>7 43.8%</td>
<td></td>
</tr>
<tr>
<td>Time between previous cytology and cancer diagnosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior cytology ≤3 years before cancer diagnostic</td>
<td>3 75.0%</td>
<td>17 85.0%</td>
<td>18 72.0%</td>
<td>12 66.7%</td>
<td>4 57.1%</td>
<td>4 25.0%</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Prior cytology &gt;3 years before cancer diagnostic</td>
<td>1 25.0%</td>
<td>3 15.0%</td>
<td>7 28.0%</td>
<td>6 33.3%</td>
<td>3 42.9%</td>
<td>12 75.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Results of previous cytologies</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td>1 25.0%</td>
<td>10 50.0%</td>
<td>17 68.0%</td>
<td>12 66.7%</td>
<td>5 71.4%</td>
<td>15 93.8%</td>
<td>0.05</td>
</tr>
<tr>
<td>Abnormal</td>
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<td>3 75.0%</td>
<td>10 50.0%</td>
<td>8 32.0%</td>
<td>6 33.3%</td>
<td>2 28.6%</td>
<td>1 63.3%</td>
<td></td>
</tr>
<tr>
<td>ASC-US-AGC-Ha*</td>
<td></td>
<td>1 33.3%</td>
<td>4 40.0%</td>
<td>6 75.0%</td>
<td>4 66.7%</td>
<td>2 100.0%</td>
<td>1 100.0%</td>
<td></td>
</tr>
<tr>
<td>LSIL*</td>
<td></td>
<td>2 66.7%</td>
<td>4 40.0%</td>
<td>0 0.0%</td>
<td>1 16.7%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td></td>
</tr>
<tr>
<td>HSIL*</td>
<td></td>
<td>0 0.0%</td>
<td>2 20.0%</td>
<td>2 25.0%</td>
<td>1 16.7%</td>
<td>0 0.0%</td>
<td>0 0.0%</td>
<td></td>
</tr>
</tbody>
</table>

*There are 2 ages missing.

* Cytologies taken within the previous 6 months to the cancer diagnosis were excluded for the analysis because they were considered as part of the diagnostic process. There are 2 women with a nonevaluable previous cytology. These cases were excluded for the analysis in which result of previous cytology is involved.

**Percentages of specific cytological abnormalities are among the abnormal cytologies.

30 years of age. Of them, 66.7% did not have any previous cytology, nearly half of the cases (41.6%) were diagnosed in stage I, and four were ADC as histological type. Figure 1 shows the distribution of the time since previous cytology to cancer diagnosis by FIGO stages. Although the differences were not statistically significant, women without previous cytology were more likely to be diagnosed at more advanced stages.

4. Discussion

Our study confirms that lack of CC screening history was substantially common among women with CC. While 73.8% of women with CC did not have a previous cervical cytology record within the Public Health System, less than a quarter of women attending screening with a normal cytological result had no previous cytology data in the previous ten years. The natural history of invasive CC, a disease with long preneoplastic changes, more than 10 years in the majority of the cases, generally allows its early detection. Our comparison with the women attending CC screening having a negative cytology shows clearly a different behaviour towards screening. Although our study was not designed to be a case-control study, it shows, in our opinion, that lack of screening is an outstanding feature among women with CC.

The proportion of unscreened women among CC cases in our study was significantly higher than the 23%–68% range reported by others [12–24]. Organized screening programs consistently have lower percentages when compared to opportunistic screening situations [25–27]. van der Aa et al. [26] from the Netherlands reported a twofold increased risk of death comparing women with CC screened through an opportunistic approach with those detected by organized screening programs. In most studies, advanced age was an additional factor that contributed to absence of screening [16, 17, 19, 21, 22]. In our study, women with no prior history of cytology were significantly older than women with a history of screening (55.8 versus 51.1 years) and over a third of CC cases were diagnosed in women aged over 60. It was in this age group that over 80% of the women had no previous screening history. A recent study about screening coverage in Catalonia [7] confirmed the poor screening history of women aged between 66 and 69 years with only 16% of them reporting a prior cytology in the 3 years prior to the evaluation [7].

Advanced age has consistently been associated with increased disease stages in consistence with our observations where the cases in stages III-IV were double among women aged over 65 years compared to women younger than 40 years old. Most likely, the absence of an adequate screening history plays a major impact in these observations although we cannot rule out that a certain proportion of cases could be newly developed after the end of the screening recommendations [15, 21, 26]. Efforts to reduce this group of underscreened women have been recommended [28]. We have now an ongoing program to actively identify this population [5]. A recent evaluation of this strategy [29] showed that underscreened women had a high burden of cervical disease. Attempts to extend these initiatives into an organized activity are undergoing [30] a randomized trial inviting these women to participate in the screening program.

In contrast with the above data, the utility of CC screening in women younger than 30 years old is questionable given the probability of regression of precancer lesions and the potential harm of the interventions [31]. In our study, 3.7% (n = 12) of the women were younger than 30 years of age. According to population-based cancer registries, the specific rate of CC in women aged 20–24 and 25–29 years for the year 2007 was 2.37/100,000 and 5.09/100,000 women, respectively, with a total specific rate of 1.70/100,000 among those younger than 30 years old [8]. One study carried out in Canada [31] among women aged 15 to 29 concluded that CC in adolescents (15–19 years old) was rare and does not justify a population-based screening. Castañón et al. [32] reported, in a study carried out in 1,800 women diagnosed with CC at ages 20–29 from England, that most cases were detected with microinvasive cancer (stage IA) with excellent prognosis and although cancers diagnosed between 20 and 24 years were more likely to be diagnosed at more advanced stages, their frequency was rarer (2% of all the cancers diagnosed in
England in 2010) as the majority of the cancers fell between
the range of 26–29 years (63.2%). Prophylactic vaccination
will likely play an important role in these age groups as it is
expected to reduce the incidence of CC substantially [33].

In the present study, among women with prior cytol-
yogy, 37% had a previous normal cytology in the 3-year
screening interval prior to CC diagnosis. In Andràe et al.
[15], 24% of all cases had developed CC despite having a
normal cytology within the recommended interval but the
percentage increased to 40% for women aged older than 65
years. We could only review a small fraction of previous
cytologies, but, in a second reading, three out of 30 could be
considered to be false negatives, two of them being among
cases with a diagnosis of ADC group and one being with
a SCC, confirming the poor sensitivity of a single cervical
cytology [22, 25, 34, 35]. The use of HPV testing is now being
proposed in many settings for its better prediction of CIN2+
cases [22, 35, 36].

It is well recognized that women with ADC have higher
risk of having a previous negative cytology [25, 26, 37–39]. In
our study, women with ADC were twice more likely to have a
previous negative cytology than women with a SCC. Besides,
of all ADC, 17.8% had a prior cytology within a period not
exceeding 3 years before cancer diagnosis while this pro-
portion was 9.9% in SCC. Glandular lesions can be missed,
especially when they do not involve the transformation zone
but are located higher in the endocervical canal. Despite
the wide use of cervical brushes that have improved the capture
of endocervical cells, the risk of underdetection is likely to
remain. HPV testing as primary screening seems to be highly
recommended to improve overall sensitivity of screening
and in particular for the optimization of adenocarcinoma
diagnosis [20].

In a much lower proportion, CC cases could be
attributable to poor follow-up [25]. In our study, 40% of the
cancers with previous cytology had a result of LSIL/HSIL and
most of them (80% of LSIL and 60% of HSIL) were diagnosed
in a period of 3 years or lower at cancer diagnosis. The
reasons why these women did not have an adequate follow-
up are unknown. In a certain proportion, women refrained
from follow-up suggesting that the adequacy of the message
is probably not optimal [40]. Others have reported that a
potential cause of loss of follow-up is a repeated negative
cytology or negative colposcopy [15, 35], suggesting that a
single negative test at follow-up is not enough to send back
women to regular screening when there is a positive test.

The proportion of ADC and SCC found in our study
did not differ from other studies [12–24]. Cytological results
of ASC-US, AGC and ASC-H were found in 4.4% of SCC
while this percentage was more frequent in ADC cases (9.3%).
Despite small numbers (3/75), AGC was only seen in the ADC
group and all of them had been diagnosed within the 3-year
screening interval. Cytologies classified as AGC, although
relatively uncommon, are likely to be a reliable marker of
cancer varying the incidence of cases from 0.05% to 2.1 [41]
suggesting that immediate colposcopy referral is probably the
best option for these women [39, 41].

There is much controversy about what is the appropriate
age of stopping screening. A case-control study carried out
by Castañón et al. [42] suggested that women with adequate
negative screening at the age of 50–64 years substantially
reduced their risk of CC at the age of 65 years and older
compared with women who were not screened. However,
the magnitude of that protection decreased with time since
the last screen, recommending exiting the screening only if
the last three tests were all negatives. In our data, 81% of
women aged 65 years or more did not have any previous
cytology registered. Among the 18 women with a previous
cytology, 13 had a negative cytology performed over 3 years
of CC diagnosis and only 4 women had a negative cytology
preceding CC diagnosis within the 3-year screening interval.
Interestingly, 3 of these 4 women were diagnosed with ADC,
in agreement with the poorer sensitivity of cytology in the
diagnosis of glandular lesions as compared to squamous
ones. Our data suggest that the number of CC cases that
occurred in women over the age of 65 when exiting the
screening following the recommendations is likely to be very
small.

4.1. Study Limitations and Strengths. We have been able to
explore screening history among women attending the Public
Health System. It is unknown if women attended within the
private gynaecology sector have a different behaviour and,
thus, we cannot extrapolate our results to them. Our control
group consisted of a large sample of women without CC
diagnosis and normal cytology for the purpose to contrast the
absence of screening history by age group in women with and
without CC. We cannot exclude that additional explanations
due to factors other than age could partially explain the huge
difference in screening uptake.

Unfortunately, our data was limited in relation to poor
knowledge on stage of disease. This is explained by the fact
that we used pathology registries and not clinical records
where stage is likely to be more complete.

We could only review the negative previous cytologies for
the period 2000–2007 due to logistic limitations but no major
changes have taken place in cervical cytology guidelines in
the region for the recent years. Thus, we think that the percent
of false negative results should be similar across the years
evaluated.

Strengths of this study are that information was reported
by six hospitals covering a predeterminate geographical area.
No differences in the data collected were observed between
them. The control population, despite not being matched
to the cases by year of diagnosis, was composed of 23,782
women, providing a robust indication of the screening uptake
when age adjusted analysis is presented.

5. Conclusions

In summary, the results of this study indicate that lack of
screening is a major limitation in CC prevention. Efforts
to increase population coverage of screening, especially in
older women, in which a high number of nonscreened and
higher stages of cancer were observed, have to be paired
with improving the sensitivity of the principal screening
test for a better CC diagnosis. Use of HPV-based screening
tests may significantly improve the efficiency of screening interventions.

Conflict of Interests

Silvia de Sanjosé received occasional travel funds to conferences/symposia/meetings by either GlaxoSmithKline, Sanofi Pasteur MSD, Merck & Co., or Qiagen. She is consultant for Merck & Co. Francesc Xavier Bosch is member of the advisory board of GlaxoSmithKline, Merck Sharp & Dohme, and Sanofi Pasteur MSD and of the speakers’ bureau of GlaxoSmithKline. He received occasional travel fund to conferences/symposia/meetings by either GlaxoSmithKline, Sanofi Pasteur MSD, Merck & Co., or Qiagen. The rest of the authors declared no conflict of interests.

Acknowledgments

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References


Review Article

Current Advances in the Application of Raman Spectroscopy for Molecular Diagnosis of Cervical Cancer

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Raman spectroscopy provides a unique biochemical fingerprint capable of identifying and characterizing the structure of molecules, cells, and tissues. In cervical cancer, it is acknowledged as a promising biochemical tool due to its ability to detect premalignancy and early malignancy stages. This review summarizes the key research in the area and the evidence compiled is very encouraging for ongoing and further research. In addition to the diagnostic potential, promising results for HPV detection and monitoring treatment response suggest more than just a diagnosis prospective. A greater body of evidence is however necessary before Raman spectroscopy is fully validated for clinical use and larger comprehensive studies are required to fully establish the role of Raman spectroscopy in the molecular diagnostics of cervical cancer.

1. Raman Spectroscopy—What Is It and How Does It Work?

The physical phenomenon of Raman scattering, also known as the Raman effect, has been extensively studied since it was first discovered in 1928 by the Indian physicist C. V. Raman. It works on the principle that a small fraction (approximately 1 in 10 million) of the radiation scattered by certain molecules differs from that of the incident beam, and that the shift in wavelength depends upon the chemical structure of the molecules responsible for the scattering [1]. Raman spectra are acquired by irradiating a sample with a powerful laser source of usually visible or near-infrared monochromatic radiation and measuring the scattered radiation with a suitable spectrometer [1, 2]. Figure 1 shows the process involved in collection of Raman spectra.

Knowing the frequency of the incident light and measuring the frequency of the Raman scattered light, it is possible to calculate the vibrational energy difference. This energy is known as the Raman shift and is usually expressed in wavenumbers (cm\(^{-1}\)) in a plot known as the Raman spectrum. Raman spectral features can be used as identification markers of particular substances because complex molecules have several specific vibrational energy modes allowing the Raman spectrum of each substance to be highly specific and distinctive [3]. Figure 2 shows an example of a Raman spectrum recorded from a cervical cancer cell line, CaSki. The full spectral range is shown from 400 to 3500 cm\(^{-1}\), including the fingerprint region, 400 to 1800 cm\(^{-1}\), and the high wavenumber (HW) region, 2800 to 3500 cm\(^{-1}\). Figure 3 shows the fingerprint region in more detail with the major assignments related to glycogen, proteins, lipids, and nucleic acids highlighted.

Raman spectroscopy has been applied in numerous scientific fields, from chemistry and biochemistry to arts and archaeology, as a powerful spectroscopic technique which allows a spectral fingerprint capable of identifying the structure and function of molecules, cells, tissues, or materials [4, 5]. In particular, its application to medical diagnostics has been of increasing interest in the past few decades [6].
Figure 1: Schematic showing the process involved in Raman spectra collection. When the sample is illuminated by an incident monochromatic light, the majority of the scattered light is of the same wavelength—elastically scattered (green arrow). A notch filter is therefore used to block the elastically scattered light which would otherwise overwhelm the weak signal of the Raman or inelastically scattered light (orange arrow). The Raman scattered light may be dispersed according to wavelength through a grating and detected by a CCD (charge-coupled device) detector. A Raman spectrum is finally shown upon software analysis.

Figure 2: Raman spectrum of cervical cancer CaSki cell line. The variation of Raman shift wavelength is expressed in wavenumbers (cm$^{-1}$) and can be observed along the $X$-axis whilst the intensity is represented along the $Y$-axis. The fingerprint and the high wavenumber (HW) regions of the spectrum are indicated by the arrows.

Raman spectroscopy has been reported for the detection of different types of pathologies, including cancer [4, 6–11]. A large number of studies concerning the investigation of cervical cancer with this particular vibrational spectroscopic technique have demonstrated its usefulness in understanding the disease progression at the molecular level. This review aims to compile the most significant achievements in this emerging research area. Methodologically, PubMed, Web of Science, and publicly available websites were searched for original data and literature in English using the following keyword combination: cervical cancer and Raman spectroscopy.

2. Cervical Cancer

Cervical cancer refers to any malignant neoplasm arising from the uteri cervix. As the fourth most common cancer in women worldwide and the fourth leading cause of female cancer deaths, cervical cancer is a key research area [12]. Its most common onset site is the cellular junction or transformation zone, where the stratified squamous epithelium of the ectocervix meets the columnar mucus-secreting epithelium of the endocervix. The most frequent types of cervical cancer are thus squamous cell carcinoma (SCC) and adenocarcinoma (ADC) [13, 14].

Persistent Human Papilloma Virus (HPV) infection is accepted as the leading aetiological agent for cervical cancer [15]. HPV is a circular double-strand DNA virus of almost 8000 bp belonging to the Papillomaviridae family. From more than 150 different genotypes, only 40 are reported to infect the anogenital tract, typically classified as high- or low-risk according to their ability to cause a recurrent infection [15, 16]. After HPV infection, dysplasia usually develops in the transformation zone. Low grade dysplasia can spontaneously regress without leading to cervical cancer [13, 17]. However some lesions progress to moderate and subsequently severe dysplasia, finally progressing to invasive cancer. For this reason, cervical cancer is postulated as a progressive disease [13, 17].

The implementation of coordinated and organized cytology screening programmes in developed countries has resulted in a marked decrease of the disease over the past decades; however cervical cancer is still a major problem in developing countries where approximately 80% of the cases occur [18]. The existing screening programmes are based on the microscopic evaluation of liquid based cytology and despite a high specificity of 95 to 98%, sensitivity varies from 74 to 96% [19, 20]. For this reason, other methods such as automated cytology and HPV testing have been studied in an attempt to reduce false negative rates.
An abnormal Pap smear is usually followed by colposcopy, biopsy, and histological confirmation of the diagnosis. Despite its slowness the major concerns about this procedure are the subjectivity of the grading characteristics and the fact that premalignancy or early malignancy stages could be missed due to their low morphologic perceptibility. Alongside other spectroscopy techniques such as FTIR (Fourier Transform Infrared) [21–23] and fluorescence spectroscopy [24–26], Raman spectroscopy has, in recent years, been acknowledged as a promising biomedical tool.

3. Raman Spectroscopy for Cervical Cancer

Table 1 compiles all the Raman spectroscopy studies concerning cervical cancer reported in the literature until September 2014 and discussed in this review. For clarity purposes it is important to explain a few terms that will be recurrent throughout the review. In vivo measurements relate to those acquired directly from the cervix of patients, ex vivo refers to the measurements acquired from the surface of biopsies and other surgical material extracted from the patients’ cervix, and in vitro refers to spectra obtained from cell lines. Formalin fixed paraffin preserved (FFPP) histological sections and cytology samples are referred to separately.

3.1. In Vivo Spectra Recorded from the Patient. Mahadevan-Jansen et al. in 1998 were the first to show the potential of Near Infrared (NIR) Raman spectroscopy to detect cervical precancers amongst other pathologies. They developed a compact fibre-optic probe which they used to record ex vivo and in vivo spectra [27, 28].

The overall ex vivo conclusions stated that, in the Raman spectrum of squamous intraepithelial lesions, peaks attributed to collagen (1656, 1070 cm⁻¹) consistently decreased in intensity while peaks assigned to phospholipids, DNA, and glucose 1-phosphate (1454, 1330, and 978 cm⁻¹) increased in intensity. These findings were attributed with tumour progression, as the number of cells in the epithelium increases with lesion development. Furthermore, multivariate statistical analysis allowed the differentiation of precancers from all other tissues with sensitivity and specificity rates of 82% and 92%, respectively [28]. Their exploratory in vivo results showed broadly similar Raman spectra at the fingerprint region [27]. The main differences were (1) a band at 936 cm⁻¹ only observed in vivo, (2) a peak at 978 cm⁻¹ that was not consistently observed in ex vivo spectra, and (3) an amide band at 1252 cm⁻¹ that was more prominent in the in vivo spectra. The authors highlighted the need to increase patient numbers so the in vivo technology could be clinically relevant [28].

Advances in fiber-optic technology led Utzinger et al. to further assess the viability of Raman spectroscopy to detect and classify cervical precancer lesions [29]. In a small clinical trial it was concluded that Raman spectra acquired from in vivo sampling were comparable with histopathology reports. The results showed increased Raman intensity of phospholipids and DNA assignments, ~1330, 1454, and 1650 cm⁻¹, respectively, as the lesions progressed to high-grade dysplasia [29]. Despite these encouraging results, the authors noted the heterogeneity of the tissue and thus the possible contribution of normal epithelial cells to the spectral data. It was also suggested that further technological advances were once again needed to assess performance in large scale clinical trials [29].

The same group have investigated the influence of hormonal changes, particularly menstrual cycle and menopausal state, and by introducing these into the in vivo diagnosis algorithm, Kanter et al. improved the overall accuracy of Raman spectroscopy to 94% [30] reaching 97% for low-grade dysplasia detection [31]. Postmenopausal, perimenopausal, and premenopausal normal cervix before and after ovulation showed subtle but consistent differences at 1250 cm⁻¹ and 1300–1320 cm⁻¹, assigned to collagen and other cellular features like lipids, Amide III, and nucleotides [31]. Similarly, previous disease history and the proximity to malignant lesions were also shown to influence Raman spectral profiles. The principal qualitative differences between “true” normal and “previous disease” normal spectra were found in the 1200–1400 cm⁻¹ range where assignments to proteins and collagen type I were higher in “true” normal spectra whilst the DNA and glycogen assignments (~1330 cm⁻¹) were higher in “previous disease” normal Raman spectra [32]. The same range was also found to comprise the most significant differences between Raman spectra of “true” normal, “adjacent to disease” normal, and low- and high-grade dysplasia. Collagen assignment was again higher in both “true” and “adjacent to disease” normal whereas DNA was higher in low and high-grade dysplasia [32].

In an attempt to further establish the greatest sources of intraclass variation among normal Raman spectra, Vargis et al. investigated race and ethnicity, body mass index (BMI), parity, and socioeconomic status in their in vivo study. The results showed that only BMI and parity were significant sources of variation within normal spectra. Their influence on dysplasia and disease remains to be assessed [33].

3.2. Ex Vivo Spectra Recorded from Excised Patient Tissue. Krishna et al. reported Raman spectral differences between normal and malignant biopsy samples. Amides I and III and structural proteins such as collagen seemed to be characteristic of normal tissue whilst DNA, lipids, and noncollagenous proteins dominated the abnormal spectral features [34]. Keller et al. showed that Raman spectral profiles from the stroma below epithelium with HPV associated histological changes had differences in DNA (1316 and 1334 cm⁻¹) and glycogen (1048, 1083, 1256, and 1333 cm⁻¹) assignments [35]. Further differences at 1260 and 1304 cm⁻¹ Amide III band were proposed to be related with the angiogenesis process or to the fact that disease may have extended without visible histologically effects [35]. While increased DNA levels and decreased glycogen levels as dysplasia progresses had been described before, this was the first report of alterations of the histologically normal stroma below diseased epithelium. Further study is therefore warranted as disease classification depends on stromal invasion.
Table 1: Raman spectroscopy studies concerning cervical cancer reported in the literature until September 2014 sorted by diagnosis (D), treatment response (R), and further conditions analysed. Sampling numbers and data analysis methodology are also indicated as maximum representation and discrimination feature (MRDF), sparse multinomial logistic regression (SMLR), principal component analysis (PCA), linear discriminant analysis (LDA), genetic algorithm-partial least squares-discriminant analysis (GA-PLS-DA), partial least squares-discriminant analysis (PLS-DA), Fisher’s discriminant analysis (FDA), principal component analysis logistic regression (PCA-LR), and spectral analysis when no multivariate statistical method was reported.

<table>
<thead>
<tr>
<th>Sampling type</th>
<th>Sampling numbers</th>
<th>Year</th>
<th>Authors (research group)</th>
<th>Raman spectroscopy [spectral region; laser used]</th>
<th>Sort category</th>
<th>Data analysis methodology</th>
<th>Other considerations</th>
</tr>
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<tbody>
<tr>
<td><strong>In vivo</strong> n = 11</td>
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<td></td>
<td></td>
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<tr>
<td>Not disclosed</td>
<td>1998</td>
<td>Mahadevan-Jansen et al. [28]</td>
<td>Fingerprint region; 789 nm</td>
<td>1000–1800 cm⁻¹; 789 nm</td>
<td>D</td>
<td>Spectral Analysis</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2001</td>
<td>Utzinger et al. [29] (Mahadevan-Jansen group)</td>
<td>Fingerprint region; 785 nm</td>
<td></td>
<td>D</td>
<td>Spectral analysis</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2009</td>
<td>Kanter et al. [30] (Mahadevan-Jansen group)</td>
<td>Fingerprint region; 785 nm</td>
<td></td>
<td>D</td>
<td>MRDF and SMLR</td>
<td>Multiclass development</td>
</tr>
<tr>
<td></td>
<td>2009</td>
<td>Kanter et al. [31] (Mahadevan-Jansen group)</td>
<td>Fingerprint region; 785 nm</td>
<td>HW (2800–3700 cm⁻¹) region; 785 nm</td>
<td>D</td>
<td>MRDF and SMLR</td>
<td>Hormonal variation influence</td>
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<td>785 nm</td>
<td>D</td>
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<td>D</td>
<td>SMLR</td>
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<td>Fingerprint region; 785 nm</td>
<td></td>
<td>D</td>
<td>GA-PLS-DA</td>
<td>Additional genetic algorithm techniques</td>
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<td>Fingerprint region; 785 nm</td>
<td></td>
<td>D</td>
<td>MRDF and SMLR</td>
<td>Investigation of normal patient variability</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>D</td>
<td>SMLR</td>
<td>—</td>
</tr>
<tr>
<td></td>
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<td>Fingerprint &amp; HW (2800–3700 cm⁻¹) region; 785 nm</td>
<td></td>
<td>D</td>
<td>PCA-LDA</td>
<td>—</td>
</tr>
<tr>
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<td>D</td>
<td>FDA and PCA</td>
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<td>D</td>
<td>PCA</td>
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<td>D</td>
<td>PCA</td>
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</tr>
<tr>
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<td></td>
<td>D</td>
<td>MRDF and SMLR</td>
<td>Investigation of temporal and spatial effects</td>
</tr>
<tr>
<td></td>
<td>2008</td>
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<td>D</td>
<td>PCA-LR</td>
<td>Cervicitis influence</td>
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<td>Kamamoto et al. [37]</td>
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<td>D</td>
<td>Spectral analysis</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>Rubina et al. [47] (Krishna group)</td>
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<td></td>
<td>D</td>
<td>PCA-LDA</td>
<td>Chemoradiotherapy</td>
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<tr>
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<td>D</td>
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<td>—</td>
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<td>2007</td>
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<td>D</td>
<td>PCA</td>
<td>—</td>
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<tr>
<td></td>
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<td>Ostrowska et al. [42] (Lyng group)</td>
<td>Fingerprint region; 532 nm</td>
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<td>D</td>
<td>PCA</td>
<td>HPV influence</td>
</tr>
<tr>
<td></td>
<td>2010</td>
<td>Kim et al. [43] (Goodacre group)</td>
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<td></td>
<td>D</td>
<td>Spectral analysis</td>
<td>HPV16 influence (E6 protein)</td>
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<td></td>
<td>D</td>
<td>SMLR</td>
<td>HPV detection</td>
</tr>
<tr>
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<td>D</td>
<td>PCA-LDA</td>
<td>—</td>
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<tr>
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<td>Fingerprint region; 785 nm</td>
<td></td>
<td>D</td>
<td>PCA-LDA</td>
<td>—</td>
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<td>Blood n = 2</td>
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<td>Lyng et al. [39]</td>
<td>Fingerprint region; 785 nm</td>
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<td>D</td>
<td>PCA-LDA</td>
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<td>Fingerprint region; 830 nm</td>
<td></td>
<td>D</td>
<td>PCA</td>
<td>—</td>
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</tbody>
</table>
The role of cervicitis in Raman spectroscopy diagnosis of low-grade dysplasia was investigated by da Silva Martinho et al. Despite an overall sensitivity and specificity of 93% and 85%, the results showed that spectral changes observed at 857, 925, ~1247, 1370, and 1525 cm\(^{-1}\) vibrational bands resulted in the cervicitis group falling mid-way between the normal and low-grade dysplasia groups. The data showed that a severe inflammatory condition such as cervicitis makes the identification and correct diagnosis of early malignancy stages such as low-grade dysplasia difficult and must therefore be taken into account when developing data analysis algorithms [36].

Finally, NIR micro-Raman spectroscopy study by Kamemoto et al. showed that Raman spectra from collagen bands at the low frequency 775–975 cm\(^{-1}\) region distinguish normal from cervical cancer cells, and that this is concordant with the analysis of C–H stretching in high wavenumber region (2800–3700 cm\(^{-1}\)) [37].

### 3.3. FFPP Sections Spectra Recorded from Histological Sections.

Archival FFPP material is extremely valuable as it allows retrospective studies to be undertaken after diagnosis and outcome is known. Raman spectroscopy studies have been undertaken on histological FFPP sections further confirming Raman spectroscopy as a powerful informative tool in cervical cancer research.

Krishna et al. studied formalin fixed cervical tissues by both Raman and FTIR spectroscopy, reporting the discrimination of malignant tissues through both techniques. In Raman spectra, differences in protein, lipids, and nucleic acid peaks were observed along with stronger Amide III assignments which is supportive of disordered, helical secondary structure of protein components in malignant conditions [38].

Further confirmation of the potential of Raman spectroscopy for cervical cancer was reported by Lyng et al. who demonstrated the viability of using FFPP samples and investigated the underlying biochemical changes associated with cervical precancer and cancer [39]. Results showed a reduction in glycoprotein bands at 482, 849, and 938 cm\(^{-1}\) and an increase in nucleic acid bands at 724, 779, 829, 852, 1002, 1098, 1240, and 1578 cm\(^{-1}\) in cervical precancer and cancer. An increase intensity of Amide I was also reported [39].

### 3.4. In Vitro Spectra Recorded from Cell Lines.

Yazdi et al. described the use of UV resonance Raman spectroscopy at 257 nm to distinguish between normal and malignant breast [MCF-10A, MCF-7 McGuire, and MDA-MB435] and cervical [CrEc-Ec 4665 (primary culture from normal cervix epithelium), SiHa, and HeLa] cultured cells. They reported an increase in DNA/protein ratio and a change in the purine scattering in malignant cells, suggesting the application of resonance Raman spectroscopy in cytology screening by monitoring DNA and RNA differences between normal and abnormal cells [40].

Despite being the main aetiological factor in cervical cancer, HPV was only investigated by Raman spectroscopy towards the end of the last decade with a cell culture study by Jess et al. [41]. Raman microspectroscopy was used to discriminate PHK (primary human keratinocytes), PHK E7 and CaSki cells, where PHK E7 cells express the E7 gene of HPV16 and CaSki expresses HPV16. The mean Raman spectra showed variations at DNA and protein level, consistent with HPV gene expression and malignancy in both live and fixed cells. Together with principal component analysis (PCA) results, Raman spectroscopy was shown to be a valuable tool in identifying and characterizing the different stages of HPV-associated malignancies [41].

Ostrowska et al. applied both FTIR and Raman spectroscopy to the study of cervical cancer cell lines. Their data suggest that HPV negative (C33a) and low HPV copy number (SiHa with 1-2 copies) cell lines are biochemically very similar but significantly different from mid (HeLa) and high (CaSki) HPV copy number cell lines. The main variations were observed for protein, nucleic acid, and lipid levels and were confirmed by both mean spectra and PCA analysis [42]. Discrimination of the cell lines based on HPV integration shows the potential of Raman spectroscopy to identify HPV induced biochemical changes [42].

Worthy of highlight is also a comparative study by Kim et al. [43] of the distribution of intracellular components in cells expressing HPV16 E6 oncoprotein. The key finding of this Raman mapping study is that E6 oncoprotein expression induces major phenotypic changes in the cells which are also targeted by an HIV antiviral drug—Indinavir [43].

Vargis et al. [44] also showed Raman microspectroscopy to successfully detect HPV and differentiate specific virus strains, in a complementary cell line and in vitro study with cellular pellets from cytology samples. Normal HPV negative cell line NHK was used alongside three cervical carcinoma cell lines: HPV positive (HeLa and SiHa) and HPV negative C33a. Specificity values of 89–97% for cell lines and 98.5% for cytology samples are extremely encouraging and confirm the enormous potential of Raman spectroscopy to provide an accurate differential diagnosis [44].

### 3.5. Cytology Spectra Recorded from Exfoliated Cells.

Rubina et al. used Raman spectroscopy to distinguish between 49 cervical cancer and 45 negative control cytology samples. Cellular pellets were generated from ThinPrep material and subjected to Raman analysis. Amide I (1660 cm\(^{-1}\)), Amide II (1538 cm\(^{-1}\)), and Amide III (1247 cm\(^{-1}\)) were the main features dominating the control Raman spectra whereas the spectra of cervical cancer samples were dominated by blood features such as fibrin (1570 cm\(^{-1}\)) and heme (1620 cm\(^{-1}\)). PCA-LDA (linear discriminant analysis) showed a classification efficiency of ~90% but the loadings were suggestive of blood as the major discriminative factor between the two groups. As bleeding is a common occurrence in cervical infections, uterine cancer, and menstrual cycle, 57 samples (28 controls and 29 cancers) were further treated with red blood cell lysis buffer prior to Raman acquisition. The absence of heme and fibrin bands confirmed the effective removal of blood from the samples and an increase in protein content (at 1006, 1450, and 1660 cm\(^{-1}\)) and change in their secondary structure due to positive Amide III bands was observed. In this case the PCA-LDA analysis showed a classification...
efficiency of ~80%. Sample heterogeneity and the fact that the distribution of the abnormal cells in the cervical cancer specimens can vary from 1-2% to 20–40% were suggested as the major causes of misclassification. The authors suggested further studies on pure cancerous and precancerous specimens as a means to build standard and validation models that could then be applied to blinded specimens [45].

3.6. Treatment Response. In their dual Raman and FTIR study, already mentioned in the FFPP section, Krishna et al. also presented data concerning Raman spectra after radiotherapy cycles, showing small changes, especially in antioxidant levels [38]. In a further ex vivo pilot study to detect radiotherapy response [46], tissues were collected after a second fraction of radiotherapy and classified based on clinical evaluation into complete, partial, and no response. Raman spectra were acquired and PCA provided a clear separation between responding and nonresponding samples as well as between complete and partial radiotherapy response.

In a more recent ex vivo study, Rubina et al. explored the feasibility of fibre-optic-based Raman spectroscopy in predicting tumour response to concurrent chemoradiotherapy. Their PCA classification pattern also showed encouraging results despite the need of a greater body of evidence [47].

A study by Duraipandian et al. used HW Raman spectroscopy to noninvasively assess, in vivo, the effect of Vagifem (oestrogen therapy) treatment in women [48]. A bimolecular Raman spectroscopy model could not only successfully identify hormone menopausal related changes in cervical epithelium, but also assess the effect of Vagifem treatment during colposcopic inspections as the protein and lipid Raman signals increase after treatment and start to resemble premenopausal values [48].

3.7. Improving Data Analysis and Recording. Improving overall sensitivity and specificity of Raman spectroscopy for in vivo diagnosis of cervical cancer has also led researchers to add better algorithms and methods for statistical analysis. A study by Kanter et al. explored binary and multiclass discrimination algorithms to analyse Raman spectroscopy data: maximum representation and discrimination feature (MRDF) and sparse multinomial logistic regression (SMLR). Although both algorithms provided an improvement over the current method of diagnosis, colposcopy-guided biopsy (with sensitivity of 87% and specificity of 72%), the use of a multiclass algorithm improved the overall Raman spectroscopy sensitivity from 92% to 98% and the specificity from 81% to 96% [49].

Similarly, Duraipandian et al. investigated the application of genetic algorithm-partial least squares-discriminant analysis (GA-PLS-DA) with double cross-validation (dCV). By employing a GA-PLS-DA algorithm which used significant Raman bands selected from 925–935, 979–999, 1080–1090, 1240–1460, 1320–1340, 1400–1420, and 1625–1645 cm$^{-1}$, a 72.5% specificity and 89.2% sensitivity for precancer detection were achieved and could therefore be further investigated as a feasible alternative to current PCA methods [50].

Still in the in vivo context, modifications in the recording process have also been considered and reported in the literature. HW Raman spectroscopy, 2800–3700 cm$^{-1}$, was successfully described by Mo et al. with 93.5% and 97.8% diagnostic sensitivity and specificity, respectively [51]. The results showed that the intensity of the Raman signal within the 2800–3035 cm$^{-1}$ range, which comprises proteins and lipids, from dysplastic tissue, was significantly lower than that observed for normal tissue. An increase in the vibrational signal of water from the dysplastic tissue was also observed and in line with that reported by FTIR spectroscopy [10, 11]. The authors further supported these observations with literature concerning the increase of aquaporins at the dysplastic cell membrane and the fact that higher DNA levels or hydration of DNA due to the unfolding step in cell division could also account for this observation [51].

Simultaneous fingerprint and HW Raman spectroscopy have also been described by Duraipandian et al. who showed their complementary potential and ability to improve early disease detection. The sensitivity and specificity values of 85% and 81.7%, respectively, for integrated fingerprint and HW Raman spectroscopy were shown to be higher than those of fingerprint or HW Raman spectroscopy alone [52].

3.8. Future Perspectives. An exploratory work on surface-enhanced Raman spectroscopy (SERS) for cervical cancer diagnosis through blood plasma analysis was recently reported by Feng et al. Comparing blood plasma samples from clinically and histopathologically confirmed healthy volunteers and cervical cancer patients, results showed that PCA-LDA algorithms yielded better sensitivity (96.7%) and specificity (92%) than empirical algorithms based only on the integration area of SERS spectral bands of 1310–1430 and 1560–1700 cm$^{-1}$ [53].

Along the same lines, Gonzalez-Solis et al. published a study on cervical cancer detection based on Raman spectroscopy of serum samples. The study reported higher levels of carotenoids and protein components in control samples whereas assignments to glutathione and tryptophan were more intense in the spectra of abnormal samples. Despite a small number of patient samples (3 CIN I and 19 SCC), PCA analysis yielded a sensitivity of 100% and a specificity of 97.1% [54].

4. Summary

All pathologies are marked by fundamental biochemical changes at the molecular, cellular, and tissue level. The identification and further understanding of these changes would allow improved diagnosis and treatments, as well as overall management and disease survival. The potential of Raman spectroscopy in molecular diagnostics relies on its ability to determine and characterize the unique fingerprint of a sample at the biochemical level.

The high incidence rate of cervical cancer as well as its aggressiveness has led researchers to continually examine and pursue better diagnosis, prognosis, and treatment techniques to decrease mortality rates and comorbidity from the disease. As highlighted, not only does Raman spectroscopy have the potential to identify cancerous and precancerous tissue, it also has the ability to probe deeper into the
disease fingerprint to elucidate its underlying mechanisms. By implementing variations of the technique to study a wide range of samples such as commercially available cell lines, FFPP sections, and in vivo and ex vivo tissue, the potential of Raman spectroscopy as a viable option for a future diagnostic technology of cervical cancer and other disease states has been shown. In cervical cancer, a number of different factors including HPV infection, hormonal imbalances, and inflammatory infection have already been reported to influence Raman spectra. Whilst these could be seen as limitations, they actually prove the sensitivity of the technique and support additional evidence generated by other approaches such as proteomics and virology.

However, lack of information regarding which data was considered for the sensitivity and specificity values reported, as well as the lack of positive and negative predictive values, calls for the standardization in the reporting of these important performance measurements. Likewise, sample handling and processing ought to be reported as it can influence Raman spectroscopy profiles. Finally, whilst acknowledging the exploratory nature of most studies and the difficulty in obtaining patient samples, a frank criticism is the small sample size of most reported studies. Although spectroscopically significant due to the high number of spectroscopic measurements, more samples are required to assess the biological and pathological relevance and reproducibility.

A far greater body of evidence is still required before this technology can make head way in a clinical setting. For instance, the engagement of the clinical community in supporting more comprehensive studies both in vivo and ex vivo, cognisant of all variables and considering a wide range of controls, gathered from a representative spectrum of the population would be vital to take the technique a step closer to cervical cancer diagnosis. If such studies could be undertaken and the reliability of the technique proven, Raman spectroscopy could have a real future in clinical diagnostics of cervical cancer and similar pathologies.

Conflict of Interests

The authors declare no conflict of interests.

Authors’ Contribution

Inês Raquel Martins Ramos performed the literature review and drafted the paper with input on design from Alison Malkin and Fiona Mary Lyng. Fiona Mary Lyng and Alison Malkin revised and approved the paper for submission.

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References


Clinical Study

Long-Term Clinical Outcome after Treatment for High-Grade Cervical Lesions: A Retrospective Monoinstitutional Cohort Study

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Background. The aim of this retrospective observational study of women treated for cervical intraepithelial neoplasia grade 2 or worse (CIN2+) was to assess the long-term risk of residual/recurrent high-grade CIN. Materials and Methods. We evaluated 760 women treated by loop electrosurgical excision procedure (LEEP) or conization (76) between 2000 and 2009, and followed up to June 30, 2014 (median follow-up 6.7 years, range 4–14). Visits every 6 months for the first year after treatment and yearly for up to the following 10 years included cytology, colposcopy when indicated, and HPV testing (search and typing). Results. CIN2+ or vaginal intraepithelial neoplasia grade 2 or worse (VAIN2+) was detected in 67 cases (8.8%), 39 at first follow-up and 28 after one/more negative visits. The risk of CIN2+ was higher in case of positive margins (odds ratio (OR) 8.04, 95% CI 4.31–15.0), type 3 transformation zone (OR for CIN3 27.7, 95% CI 2.07–36.9), CIN3+ excision (OR 6.02, 95% CI 1.73–20.9), and positive high-risk HPV test at first follow-up (OR for HPV16: 20.6, 95% CI 6.8–62.6; OR for other hrHPV types: 18.3, 95% CI 5.9–57.0). Conclusion. Residual/recurrent high-grade CIN occurred in <9% cases, and the risk was associated with transformation zone type, lesion grade, margins status, and hrHPV test result at 6–12 months of follow-up.

1. Introduction

Detection and treatment of preneoplastic high-grade lesions (cervical intraepithelial neoplasia grades 2 and 3 or worse (CIN2+)) can prevent the development of invasive cervical cancer and are the aim of the cervical cancer screening programmes. Different guidelines recommend treatment of all high-grade lesions since their risk of progression is approximately 30% [1, 2]. A proportion (estimated to be about 20–30% for CIN3 and 50–60% for CIN2 and even higher in young women) would undergo spontaneous regression if left untreated, but unfortunately no reliable biomarkers for discriminating regressive from progressive lesions have been identified to date [3].

The treatment should be effective in eradicating the high-grade lesion and have minimum morbidity and adverse effects on future fertility and pregnancy outcomes, particularly in young women. Conservative excisional methods are therefore the treatment of choice. Loop electrosurgical excision procedure (LEEP) and needle conization are the most widely used methods (especially in the last 10 years); the major advantages are specific tailoring of the treatment (which minimizes adverse effects) and histological evaluation of the treated lesion. These modalities show high efficacy in eradicating intraepithelial lesions, although failure rates of 5–30% are reported [4]. As a consequence of treatment failure, women treated for high-grade lesions have a risk of progression to invasive cancer 4–5 times higher than
the general population [5–7]. This implies that follow-up procedures must be put in place and need to be effective in detecting residual/recurrent disease, while containing the number of visits for successfully treated women. Follow-up has been traditionally carried out by regular Pap smears, with or without colposcopy, for some years. In the last years, several studies have demonstrated that human papillomavirus (HPV) testing performed 6–12 months after treatment is a valuable tool: it is more sensitive than cytology in identifying women with residual/recurrent disease and has a very high negative predictive value (NPV) [8–12]. Search for high-risk HPV (hrHPV) types as a pool is the modality used in most studies, and specific typing is not recommended, although an increased risk of recurrence has been demonstrated for women treated for HPV16-associated high-grade lesions [13, 14]. Implementation of hrHPV testing in clinical practice depends on several factors and can take time before reaching a regular and consistent application.

The aim of the present study was to analyze the clinical outcome of 760 women treated for high-grade lesions and followed up for a minimum of 2 to more than 14 years, in order to assess the risk of residual/recurrent high-grade CIN and identify the best predictive indicators for recurrence and their role in the diagnostic strategy.

### 2. Patients, Materials, and Methods

#### 2.1. Patients and Clinical Procedures

Subjects eligible for this retrospective monoinstitutional (Azienda Ospedaliera di Padova, Department of Woman and Child Health, Gynecology Service) analysis were women diagnosed with and treated for CIN2+ by loop electrosurgical excision procedure (LEEP) or conization by electric needle, having a follow-up of minimum 2 years.

All involved colposcopists performed regularly more than 500 procedures per year and had a total experience ranging from 20 to 40 years. Colposcopic features were recorded and described according to Barcelona nomenclature of the International Federation for Cervical Pathology and Colposcopy (IFCPC) [15]. Biopsies were performed on abnormal areas under coloscopic guide (directed biopsies); endocervical brushing was done in cases of not fully visible squamocolumnar junction (SCJ) or presence of atypical glandular cells (AGC) in the Pap smear, with curettage in cases of high-grade lesion and/or type 3 SCJ. Data on cytology, colposcopy, and diagnostic biopsies done before treatment were recorded.

The diagnostic work-up leading to lesion's treatment is depicted in Figure 1.

LEEP and conization were performed under coloscopic guide in local anesthesia in an outpatient facility by experienced personnel [16]; loops of different sizes were used according to lesion's characteristics and cervix conformation; in all cases, care was taken to personalize the loop size. Excision margins were kept 2-3 mm out of the lesion, and completeness of lesion's removal was colposcopically verified.

At the time of treatment, the following data were collected: conformation and size of the cervix; transformation zone (TZ) type; size, grade, and number of the lesion(s) and their relation with endocervical canal and vagina; treatment modality; histology of the excised lesion (including margin section status), and patient's age.

Follow-up procedures included the following:

(i) cytology and colposcopy 6 and 12 months after treatment (an additional visit 3 months after treatment was recommended in cases with positive section margins or microinvasive carcinoma),

(ii) HPV testing (search and typing) at 6 and 12 months after treatment which was added from 2005 and gradually implemented,

(iii) cytology at yearly intervals for the following 10 years, with colposcopy in case of abnormal Pap.
Patients showing a cervical (CIN) or vaginal (VAIN) high-grade lesion at first follow-up visit after treatment were considered as having residual disease; patients showing a cervical or vaginal high-grade lesion after one or more follow-up visits with negative cytology and colposcopy were considered as having recurrent disease [17]. Patients with invasive lesions were considered as having progressive disease. Patients with residual or recurrent CIN2+ or VAIN2+ lesion were referred for a second treatment.

2.2. HPV Analyses. Search and typing of HPV DNA sequences was performed, as described in [18], by polymerase chain reaction (PCR) with consensus MY09/MY11 primers (which detect most high- and low-risk types); type identification was accomplished by restriction fragment length polymorphism (RFLP) analysis of MY amplimers, as well as PCR with HPV 16 type-specific 16H1/16R3 primers. DNA amplificability of all samples was verified by PCR with primers GH20/PC04 for the β-globin gene. For analysis of the HPV results, samples were classified as negative (no HPV DNA detected); positive for hrHPV types [19], with or without other types, differentiating HPV16 from the other hrHPV types; positive for low-risk (lrHPV) types (presence of any other HPV type).

2.3. Statistical Analyses. Cumulative incidence rates of residual and recurrent CIN2 and CIN3+ were computed by histological diagnosis at baseline (CIN2 versus CIN3+), overall (panel A: all 760 women treated for CIN2+ included in the study), and among women without residual high-grade disease at first follow-up visit, who had a hrHPV test (panel B: 506 women without residual high-grade disease at first follow-up visit and with HPV test). For statistical analyses, VAIN2 and VAIN3 cases were cumulated with CIN2 and CIN3+ cases, respectively.

The predictors of residual disease and/or recurrence risk (age at excision, pretreatment (initial) cytology, extension and number of lesions, vaginal involvement, and endocervical canal involvement at colposcopy) were assessed computing odds ratio (OR), with 95% confidence intervals (95% CI). Multivariate logistic regression models were used for each panel to predict the probability of a CIN2 or a CIN3+ event. All the parameters of interest were considered as covariates in the models, using a forward-stepwise selection to determine which variables could be considered significant at $P = 0.05$.

3. Results

During the period between January 1, 2000, and December 31, 2009, a total of 810 women were treated for CIN2+ in our institution; 520 (64.2%) were attending the organized screening programme (target population: women 25–64 years old); 290 had been referred by other centres (women of any age). Among them, 20 moved in a distant area, 15 had less than 2 years of follow-up, and 15 did not attend any follow-up. Therefore, 760 women were included in our retrospective analysis; cases were classified according to the worst diagnosis, made either on the biopsy or the excised lesion. Of these, 415 had a diagnosis of CIN2, 330 of CIN3/in situ carcinoma, and 15 of microinvasive carcinoma (Table 1). Mean age at time of excision was 39 years (median 37.5; range 19–71); in particular, 287 women had <35 years (38%), 370 were in the 35–50 years age range (49%), and 103 were older than 50 years (13%).

LEEP was performed in 684 and conization in 76 cases, respectively; all but 15 (who requested general anesthesia for the presence of comorbidities) were treated in local anesthesia in an outpatient facility.

Histological evaluation of the excised specimens disclosed no lesion of any grade in 5 (6.6%) and CIN1 only in 116 (16%) cases. In all other cases, a high-grade lesion was confirmed. Margins status of the excised tissues were disease-free in 506 cases, positive in 126, not assessable in 20 (in 15 because of artefacts, and in 5 because no lesion was present in the excised specimen), while the data was unavailable in 108 (14%).

Median follow-up was 6.7 years (range 4–14; mean 7 years). Overall, during follow-up, 67 women (8.8%) were diagnosed with histologically confirmed CIN2+ or VAIN2+. The large majority of the lesions developed within the first two years of follow-up, at a higher rate after treatment of CIN3+ compared to CIN2 and with a plateau after two years only for cases treated for CIN3+.

3.1. Residual High-Grade Disease. The first posttreatment follow-up was performed after 3 months in women treated for microinvasive carcinoma and in most of the cases with positive margins and after 6 months in the remaining cases. Residual or progressive disease was detected at first follow-up visit in 39 women; 17 cases were diagnosed as CIN2, 15 cases were diagnosed as CIN3, 3 cases were diagnosed as microinvasive carcinomas, 1 case was diagnosed as invasive carcinoma (this was a FIGO stage IA2 and occurred in an immunodepressed woman treated for CIN3, with positive endocervical margins, and subsequently treated by hysterectomy), 2 cases were diagnosed as VAIN2, and 1 case was diagnosed as VAIN3. In 6 cases, a diagnostic biopsy or a new excision was performed directly; in all other cases, the cytology was positive and guided the diagnostic work-up. The colposcopic impression was normal in 14/39 (36%) cases; 5 of these women had a type 3 TZ, and in 2 cases a VAIN2 was detected.

3.2. Recurrent High-Grade Disease. Among the 721 remaining cases, recurrent high-grade disease was found during subsequent follow-up in other 28 women (17 CIN2, 5 CIN3, 5 VAIN2, and 1 VAIN3; no invasive lesions occurred in this group). The colposcopic impression at the time of recurrence was normal in 9/28 (32%) cases; 5 of these women had a type 3 TZ, and in 3 cases a VAIN2 was detected. After 7 years of FU, the cumulative incidence rate for CIN3 was 6.7% among women treated for CIN3+ and 4.3% among women treated for CIN2; the cumulative incidence rate for CIN2 was 0.7% and 6.3%, respectively.

3.3. Predictors of Disease. Among the parameters analyzed as predictors of overall residual or recurrence risk, pretreatment
Table 1: Principal characteristics of the 760 cases included in the study.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histological diagnosis at baseline</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN2</td>
<td>415</td>
<td>54.6</td>
</tr>
<tr>
<td>CIN3/in situ carcinoma</td>
<td>330</td>
<td>43.4</td>
</tr>
<tr>
<td>Microinvasive carcinoma</td>
<td>15</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>Colposcopic diagnosis at baseline</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>87</td>
<td>11.4</td>
</tr>
<tr>
<td>G1 (abnormal grade 1)</td>
<td>378</td>
<td>49.7</td>
</tr>
<tr>
<td>G2 (abnormal grade 2)</td>
<td>279</td>
<td>36.7</td>
</tr>
<tr>
<td>Suspected invasive carcinoma</td>
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<td>0.1</td>
</tr>
<tr>
<td>Missing</td>
<td>15</td>
<td>2.0</td>
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<tr>
<td><strong>Squamocolumnar junction location</strong></td>
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<td></td>
</tr>
<tr>
<td>Type 1 (visible, ectocervical)</td>
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<td>70.0</td>
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<tr>
<td>Type 2 (visible, endocervical)</td>
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</tr>
<tr>
<td>Type 3 (nonvisible, in endocervix)</td>
<td>52</td>
<td>6.8</td>
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<tr>
<td>Missing</td>
<td>18</td>
<td>2.4</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
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<td></td>
</tr>
<tr>
<td>LEEP</td>
<td>684</td>
<td>90.0</td>
</tr>
<tr>
<td>Conization</td>
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<td><strong>Margins status of the excised specimens</strong></td>
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<tr>
<td>Negative</td>
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</tr>
<tr>
<td>Positive</td>
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<tr>
<td>Missing</td>
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</tr>
<tr>
<td><strong>HPV test at first follow-up</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>350</td>
<td>48.5</td>
</tr>
<tr>
<td>HPV 16</td>
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<tr>
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<tr>
<td>Low-risk HPV</td>
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<tr>
<td>Not performed</td>
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<td>29.8</td>
</tr>
<tr>
<td><strong>Residual</strong> lesions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN2</td>
<td>17</td>
<td>2.2</td>
</tr>
<tr>
<td>CIN3</td>
<td>15</td>
<td>2.0</td>
</tr>
<tr>
<td>Microinvasive carcinoma</td>
<td>3</td>
<td>0.4</td>
</tr>
<tr>
<td>Invasive carcinoma</td>
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<td>0.1</td>
</tr>
<tr>
<td>VAIN2</td>
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<td>0.3</td>
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<tr>
<td>VAIN3</td>
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<tr>
<td><strong>Recurrent</strong> lesions</td>
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<td></td>
</tr>
<tr>
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<td>2.2</td>
</tr>
<tr>
<td>CIN3</td>
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<tr>
<td>VAIN2</td>
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<td>0.7</td>
</tr>
<tr>
<td>VAIN3</td>
<td>1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*Residual: lesions detected at first follow-up.
**Recurrent: lesions detected after one or more negative follow-up visits.

A higher CIN2+ risk in women with increasing age and with not fully visible junction at the time of treatment was observed, but the associations are statistically not significant.

Instead, the risk of posttreatment CIN2+ occurrence was higher in women with an original G2 colposcopic diagnosis (OR versus G1 2.55, 95% CI 1.28–5.08) and in cases with positive margins (OR 8.04, 95% CI 4.31–15.0). Moreover, the risk of residual or recurrent CIN3+ was higher in women whose original lesion was CIN3+ (OR 6.02, 95% CI 1.73–20.9) (Table 2).

HPV testing at 6–12 months after treatment was added from 2005 and gradually implemented; it was available for a minority of the 39 cases with residual disease (4/4 positive for hrHPV) and for 506 out of the 721 cases (70%) without residual high-grade lesions. No HPV DNA sequences were detected in 350 cases (69%), while HPV typing of the 156 HPV-positive cases disclosed a high-risk type in 67 (13%; HPV16 in 34, and other hrHPV types in 33 cases) and a low-risk type in 89 (18%) cases. Recurrent lesions (17 CIN2, 5 CIN3, 5 VAIN2, and 1 VAIN3) developed almost exclusively among women who had a positive hrHPV test at 6–12-month follow-up (Figure 2, panel B). The cumulative incidence rate of recurrent CIN2+ was steeper in HPV16+ cases, reaching a plateau 18 months after treatment, compared to 36 months in those with other hrHPV types. The cumulative CIN2+ incidence rates at the end of the follow-up were 26.5% in HPV16+ cases and 24.2% in other hrHPV+ cases; the corresponding figures for CIN3 were 8.8% and 3.0%, respectively. Among women positive for a low-risk HPV type at 6–12-month follow-up, a recurrent CIN2 lesion occurred in 4 cases, with a cumulative incidence rate of 5.9%.

Cytology was regularly performed throughout follow-up. It was repeatedly negative in more than half of the women, among whom 1 single CIN2 lesion occurred (0.25%). It was abnormal (ASC-US or worse) at least once in the others; this prompted additional interventions, and the frequency of detection of high-grade lesions was proportional to the (initial) cytology, extension and number of lesions, vaginal involvement, and endocervical canal involvement at colposcopy did not show any statistically significant correlation.

![Figure 2: Cumulative incidence rates of recurrent CIN2+, by result of HPV typing at first FU after treatment and time since excision. Only women without residual high-grade disease at first follow-up visit, who had an HPV test result (panel B) are included.](image-url)
Table 2: Multivariate logistic regression analyses to identify predictive factors of the risk of residual or recurrent high-grade lesions in 760 women treated for CIN2+ (upper part) and among 506 women without residual high-grade disease at first follow-up visit who had a HPV test (lower part). Only variables that resulted significant after a forward-stepwise selection are reported.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Women treated for CIN2+ (n = 760)</th>
<th></th>
<th></th>
<th>Women without residual high-grade disease at first follow-up visit with HPV test (n = 506)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Risk of residual or recurrent CIN2</td>
<td>Odds ratio(^\wedge) (95% CI) &amp; P value</td>
<td>Risk of residual or recurrent CIN3+</td>
<td>Odds ratio(^\wedge) (95% CI) &amp; P value</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histological diagnosis at baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN2(\wedge)</td>
<td></td>
<td>1.00 (1.00–1.00) &amp; 1.00 (1.00–1.00) &amp; —</td>
<td>1.00 — §</td>
<td>1.00 — §</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN3+</td>
<td></td>
<td>1.00 — —</td>
<td></td>
<td>1.00 — —</td>
<td>1.00 — —</td>
<td></td>
</tr>
<tr>
<td>Margins status of the excised lesions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative(\wedge)</td>
<td></td>
<td>1.00 — —</td>
<td></td>
<td>1.00 — —</td>
<td>1.00 — —</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td>5.11 (2.42–10.8) &amp; &lt;0.001</td>
<td>13.8 (4.98–38.5) &amp; &lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamocolumnar junction location</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 1(\wedge)</td>
<td></td>
<td>1.00 — —</td>
<td></td>
<td>1.00 — —</td>
<td>1.00 — —</td>
<td></td>
</tr>
<tr>
<td>Type 2</td>
<td></td>
<td>$</td>
<td></td>
<td>1.68 (1.04–20.5) &amp; 0.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 3</td>
<td></td>
<td>27.7 (2.07–369) &amp; 0.012</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colposcopic diagnosis at baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1(\wedge)</td>
<td></td>
<td>1.00 — —</td>
<td></td>
<td>1.00 — —</td>
<td>1.00 — —</td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td></td>
<td>4.17 (1.28–13.6) &amp; 0.018</td>
<td>$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td>1.67 (0.37–7.61) &amp; 0.503</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV test at first follow-up</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative(\wedge)</td>
<td></td>
<td>1.00 — —</td>
<td></td>
<td>1.00 — —</td>
<td>— (\wedge)</td>
<td></td>
</tr>
<tr>
<td>HPV 16</td>
<td></td>
<td>13.3 (3.48–50.5) &amp; &lt;0.001</td>
<td>63.6 (4.45–909) &amp; 0.002</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other high-risk HPV</td>
<td></td>
<td>22.3 (5.69–87.3) &amp; &lt;0.001</td>
<td>7.76 (0.42–142) &amp; 0.168</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-risk HPV</td>
<td></td>
<td>2.94 (0.67–12.8) &amp; 0.152</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(\wedge\) Reference.
\(\wedge\) Adjusted for all the variables in the table.
\(\wedge\) Nonsignificant, excluded from the model.
\(\wedge\) No CIN3+ events in the Low-risk group, excluded from the model.

Degree of the cytological abnormality, ranging from <5% for ASC-US and LSIL (low-grade squamous intraepithelial lesion) to almost 80% for HGSIL (high-grade squamous intraepithelial lesion).

Among the 721 women without residual disease (panel B), the overall risk of recurrent CIN2 or CIN3 was correlated only with infection with HPV16 (OR 20.6, 95% CI 6.8–62.6) or other hrHPV types (OR 18.3, 95% CI 5.9–57.0). Table 2 shows the parameters associated with the recurrence of CIN2 and CIN3 separately. Besides HPV status at 6–12-month follow-up, recurrence of CIN2 was higher in women with a G2 colposcopic diagnosis (OR 4.17, 95% CI 1.28–13.6) and in those with an original histological diagnosis of CIN2 (statistically not significant). The risk of recurrent CIN3 was associated with HPV status at 6–12-month follow-up (OR 63.6, 95% CI 4.45–900, for HPV16 positivity) and type 3 squamocolumnar junction at baseline (OR 27.7, 95% CI 2.07–369).

4. Discussion

This is a retrospective observational study on 760 women treated for histologically confirmed CIN2+ lesions in a single institution and followed-up for a median of 6.7 years (range 4–14). Aim of the study was to assess their long-term risk of developing (new) high-grade lesions. Overall, the residual/recurrence rate was 8.8%; 5.1% were detected at first follow-up (residual disease) and 3.7% after a negative follow-up (recurrent disease). Progressive disease was recorded in 4 cases (0.5%), all of which were after CIN3(+) and within the first year after treatment: 1 invasive carcinoma (hysterectomy performed 10 months after conization) and
3 microinvasive carcinomas. No cases of invasive squamous or adenocarcinoma were recorded during the long-term follow-up. The most used treatment modality was loop electrosurgical excision procedure (LEEP), personalized and performed by highly experienced personnel; the 8.8% rate of residual/recurrent high-grade lesions was at the lower end of the 5–30% published rates [4, 20]. The risk to develop invasive cervical disease in women treated for a high-grade lesion has been reported to persist for many years [5, 6, 21]. In a recent cohort study covering the whole Swedish population for half a century [7], the risk of developing or dying from cervical or vaginal cancer in women treated for CIN3 was two to three times higher than that in the general female population, and the risk increased with increasing age at treatment and with ageing of treated women. In our study group, no invasive disease was detected after the first year after treatment, and only a (statistically not significant) trend for higher risk of CIN2/CIN3 recurrence in older women was observed. Our study is completely different for design, number of women evaluated, and length of follow-up and no direct comparison of the two studies can be made.

The identification of biomarkers predictive of precancer and cancer development after excisional treatment is important to modulate the follow-up in order to guarantee high sensitivity for detecting recurring lesions and to avoid excessive controls of cured women. Follow-up protocols show some differences among different countries and are subject to additional changes as a result of the technological innovations and the new cervical cancer preventive strategies (i.e., HPV-based screening, HPV vaccination, and new biomarkers) [22, 23].

Positive margins of the excised lesion were predictive for both residual and recurrent CIN2+ lesions, with higher OR for CIN3+ compared to that for CIN2, a result comparable to what is reported in another study of women treated for high-grade lesions [17] and in a study of women treated for stage Ia1 squamous cervical cancer [24].

Among the baseline anatomoclinical characteristics analyzed as long-term predictors of recurrent disease, statistically significant ORs were found for type 3 SCJ, G2 colposcopic diagnosis, and CIN3+ histological diagnosis; interestingly, they partly differed for CIN2 and CIN3 recurrence.

G2 as original colposcopic diagnosis was a significant predictor for recurrent CIN2 but not for CIN3; this parameter was dropped by our model as nonsignificant, probably because of the existence of stronger predictors for CIN3. CIN3+ as original diagnosis was predictive of CIN3+ recurrence.

Colposcopy represents a crucial step in the management of women with abnormal screening tests, since it is the method used to identify the type and features of the transformation zone and determines the reliability of the diagnostic biopsies, but conflicting results have been reported on its accuracy [25]. In our study, a type 3 TZ was associated with a quite high odds ratio for CIN3 posttreatment development. This is a rare occurrence, more often observed among older women; in our study, it was recorded in 7% of the 760 treated women, but it was present in 15% (10/67) of the cases with residual/recurrent lesions. Both experience and ability of the colposcopist influence the capacity to detect and sample a lesion, particularly when it is small in size; increasing the number of biopsies has been suggested as a potential way to improve colposcopy sensitivity [26], but this could eventually represent an overdiagnosis [27]. Indeed, HPV-based screening and HPV prophylactic vaccination will likely modify the frequency and extent of cervical abnormalities in the future; this might adversely affect colposcopic performance, and efforts to improve its quality assurance are of utmost importance [28].

A positive hrHPV test at first follow-up (6–12 months after treatment) was found in 13% of the cases with available data without residual disease and was positively associated with recurrent disease. Moreover, positivity for HPV 16 was associated with a very high OR for CIN3. Posttreatment hrHPV testing at 6-month follow-up has been clearly demonstrated to have higher sensitivity than cytology and comparable specificity [8, 11, 12], while a potential value of genotyping has been suggested [13]. Indeed, HPV 16 is known to have a higher oncogenic capacity than the other known high-risk types [29], with implications for natural history (faster development and higher persistence rate) and management [30].

The distribution over time of the residual/recurrent high-grade lesions showed that most of them developed within 2 years, as already reported in the literature [4], but disclosed some differences between CIN2 and CIN3. In particular, while a plateau was always observed for CIN3 lesions (irrespective of the grade of the initial lesion) and CIN2 after CIN3+, CIN2 following CIN2 showed a constant distribution over time. Indeed, a debate is ongoing on whether CIN2 is a definite entity and a truly intermediary step or the result of misclassification of CIN1 and CIN3 [3]; its diagnostic reproducibility is very low and its regression rate is rather high, especially in young women. Moreover, differences in risk factors, showing that CIN3 is more similar to cancer than CIN2, have been recently highlighted [31]. Our data show that CIN2 recurred mostly as CIN2, with a specific temporal behaviour, and biomarkers for CIN2 recurrence partly differed from those for CIN3. It is known that clinical outcome of an HPV infection is the result of a complex balance between immune system responses and viral immune evasion mechanisms; our data suggest that CIN2 (particularly when associated with a non-HPV16 type) may represent a persistent poorly controlled HPV infection rather than a true preneoplastic lesion.

The strengths of our study are the large number of patients followed up for much more than two years and treated in the same institution by experienced personnel, in a routine setting. Long-term evaluation is particularly important to understand the real risk of progressive disease in women treated for CIN2+, and homogeneity in treatment modality minimizes the differences present when analyzing multicentre cohorts.

The limitations of our study are mainly represented by the retrospective nature of data analysis, the late use of HPV testing and the missing results for some of the analyzed parameters; nonetheless, these weaknesses reflect what occurs in the every-day routine clinical setting.
Although our study is not powered to give information that may immediately translate into a modification of the follow-up protocols, the long-term clinical outcome and the results on the analyzed biomarkers allow some considerations and practical applications. Accurate definition of the type of transformation zone, the histological grade, and the margins status of the excised lesion appear to be very important; closer follow-up is necessary in case of type 3 TZ, CIN3+ lesion, and positive margins. Accuracy and experience of the personnel performing the excision also play a major role, stressing the need for training and quality assurance. Testing for hrHPV (with eventually partial typing for HPV16) 6–12 months after treatment is very effective in discriminating the women at higher and lower risk of recurrence.

The role and frequency of colposcopy after treatment are a matter of debate also in Italy [32]. While it has been performed in all women of our study group (treated between 2000 and 2009) at 6- and 12-month posttreatment follow-up visits, most recent guidelines (i.e., United Kingdom [33]) are not recommending colposcopy as routine practice in posttreatment follow-up. Indeed, while the colposcopy at 12 months after excision is deemed not necessary [34] (no longer performed in our institution), the colposcopy at first follow-up visit could be useful to localize the new SC, and as autoverification on the quality of the excision procedure.

5. Conclusions

Our retrospective analysis to assess the risk of high-grade CIN in women treated for CIN2+ lesions in a single institution on a routine basis and followed up for up to 14 years shows a favourable long-term clinical outcome in the great majority. Progressive disease was detected only in the first year after treatment. The type of transformation zone, the lesion grade, the status of the margins, and the result of hrHPV test at 6–12-month follow-up are the most useful parameters to predict long-term treatment outcomes.

Disclosure

The data have been partly presented at the Italian Workshop of Eurogin 2013 (Florence, Italy; November 3–6, 2013). Lorena Baboci was a recipient of a Ph.D. fellowship granted by IOV-IRCCS, Padua, Italy.

Conflict of Interests

All the authors of the present paper state under their responsibility that no conflict of interests exists with third parties.

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References


Research Article

Accuracy of Colposcopically Directed Biopsy: Results from an Online Quality Assurance Programme for Colposcopy in a Population-Based Cervical Screening Setting in Italy

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6 Department of Health, Regione Emilia-Romagna, Viale Aldo Moro 21, 40127 Bologna, Italy
7 Romagna Cancer Registry, Romagna Cancer Institute (IRST) IRCCS, Via Piero Maroncelli 40, Meldola, 47014 Forli, Italy

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Purpose. To report the accuracy of colposcopically directed biopsy in an internet-based colposcopy quality assurance programme in northern Italy. Methods. A web application was made accessible on the website of the regional Administration. Fifty-nine colposcopists out of the registered 65 logged in, viewed a posted set of 50 digital colpophotographs, classified them for colposcopic impression and need for biopsy, and indicated the most appropriate site for biopsy with a left-button mouse click on the image. Results. Total biopsy failure rate, comprising both nonbiopsy and incorrect selection of biopsy site, was 0.20 in CIN1, 0.11 in CIN2, 0.09 in CIN3, and 0.02 in carcinoma. Errors in the selection of biopsy site were stable between 0.08 and 0.09 in the three grades of CIN while decreasing to 0.01 in carcinoma. In multivariate analysis, the risk of incorrect selection of biopsy site was 1.97 for CIN2, 2.52 for CIN3, and 0.29 for carcinoma versus CIN1. Conclusions. Although total biopsy failure rate decreased regularly with increasing severity of histological diagnosis, the rate of incorrect selection of biopsy site was stable up to CIN3. In multivariate analysis, CIN2 and CIN3 had an independently increased risk of incorrect selection of biopsy site.

1. Introduction

Colposcopy aims at detecting macroscopic changes in colour and morphology of cervical mucosa. Comparison of these features with established patterns of disease allows classifying the observed lesions and identifying abnormal areas that warrant biopsy.

The colposcopic impression of any abnormality, however, is prone to observer variation. This is potentially associated with a low inter- and intraobserver agreement in interpretation of colposcopic abnormalities and with a low accuracy of colposcopically directed biopsy in defining extent and severity of lesions. Several cross-sectional and prospective studies published between the 1990s [1, 2] and the last decade [3–6] have cast doubt on the effectiveness of biopsy in detecting the presence of high-grade cervical intraepithelial neoplasia (CIN).

Low sensitivity for detection of high-grade disease may have serious clinical consequences. In particular, it may cause early invasive lesions to be inadvertently treated by an ablative technique [2, 7]. Disease relapse, which has been described in these patients [8], may erode the clinicians’ confidence about conservative treatments. Nondiagnosis of carcinoma flaws quality control procedures for cytology [9] and invalidates the clinical studies of preinvasive disease that use biopsy as a gold standard [1].
These problems are complicated by the insufficient diffusion of quality assurance (QA) programmes for colposcopy. These programmes should be based on interactive retraining sessions and large agreement and accuracy studies would allow identifying specific areas of improvement, selecting a set of well-defined and highly reproducible colposcopic features of cervical abnormalities, and increasing the colposcopists’ competence as well as the appropriateness of their clinical decisions.

In the first session of a permanent online colposcopy QA programme that is being conducted in Italy, the participants evaluated a test set of digital colpophotographs. The current article reports an analysis of the correctness of their decisions for biopsy.

2. Materials and Methods

2.1. Setting. The population-based, triennial Pap smear screening service that covers women aged 25–64 years living in the Emilia-Romagna Region of northern Italy is described elsewhere [10]. Colposcopy assessment for women with abnormal screening results is carried out by specially appointed gynaecologists and gynaecologist oncologists. Over the past decade, the colposcopists working in the screening centres have been targeted by several on-site colposcopy QA initiatives. In 2009-2010, an internet-based QA programme was developed.

2.2. Design. A detailed protocol of the programme can be found and free-accessed elsewhere [11]. In brief, a log-in web application was created and made accessible on the website of the regional Administration. Between December 2010 and February 2011, the 65 screening colposcopists were invited to participate on a voluntary basis. Fifty-nine registered, logged-in, viewed a posted set of 50 colpophotographs selected by an expert committee, and classified them according to colposcopic impression, visibility of the squamocolumnar junction, and need for biopsy. The images were accompanied by a caption with information about patient age, last Pap smear result, and human papillomavirus test result (if any). The participants indicated the single most appropriate site for biopsy with a left-button mouse click on the image. This site was automatically checked against an area identified by the committee as the most appropriate one from which to take a sample. The size and shape of the area varied according to its colposcopic appearance. Its coordinates were mapped inside the source code of the software. The site selected by the colposcopists was automatically classified into correct and incorrect. After completing the test, they received online a set of personal results. The programme had no administrative functions (ranking, accreditation, etc.).

The committee classified the colposcopic impression and identified the single most appropriate biopsy site with a joint discussion. Original histological information, including normal histology and biopsy not performed, was known to the selectors but was not assumed to represent a gold standard for the colposcopic impression and the need of a biopsy [11].

2.3. Colpophotographs. Technical details of acquisition of the test set of images can be found elsewhere [11]. In brief, 250 high-definition digital colpophotographs were obtained from women with abnormal Pap smear results consecutively attending two screening centres randomly selected out of the total 11 centres. From this basic set, 50 images were selected based on the following criteria: they were well-representative of major normal and abnormal colposcopic findings; the cervix was entirely visible; there were no light reflections, colour artifacts, shaded areas, or mucus accumulation; and the patient had not been treated previously. The rationale for these criteria is discussed elsewhere [11].

2.4. Classification of Colposcopic Impression. Colposcopic impression was classified as negative; abnormal, grade 1 (G1); abnormal, grade 2 (G2); and suspected invasive cancer (Cancer). These categories were equivalent to the colposcopic patterns that the International Federation for Cervical Pathology and Colposcopy classification of 2002 [12] designated as normal colposcopic findings; abnormal colposcopic findings, minor changes; abnormal colposcopic findings, major changes; and colposcopic features suggestive of invasive cancer.

2.5. Rationale and Objectives of the Current Study. In May 2011, a plenary seminar was organized to discuss the overall results and to perform an interactive review of the test set of images. An article reporting agreement data on colposcopic impression has recently been published [13].

The rationale of the study that is presented here has been described in detail elsewhere [11]. In brief, although the committee did not consider the original histological diagnosis as a gold standard, comparing the interpretation of colposcopic findings and the decision for biopsy with the histological diagnosis of the underlying lesion was nevertheless important in that it provided an approximate measure of the probability for women with abnormal Pap smear results to receive a false-negative or false-positive colposcopic assessment.

In particular, the current study was undertaken to determine the probability of a patient with abnormal colposcopic findings and a histologically confirmed cervical lesion not having biopsy or having biopsy in an incorrect cervical site. We evaluated (1) the non-biopsy rate and the rate of incorrect selection of biopsy site according to colposcopic impression formulated by the committee; (2) the non-biopsy rate and the rate of incorrect selection of biopsy site according to original histological diagnosis; and (3) the association of patient characteristics and colposcopist characteristics with the probability of biopsy failure of both types.

2.6. Data Analysis. Data analysis was based on a total of 2950 paired colposcopist-committee observations resulting from the product of 59 colposcopists and 50 images. Ninety-five percent confidence intervals (CI) around rates were calculated according to standard methods [14].

In the analysis of factors associated with biopsy failures, all variables were treated as categorical. The patient age and colposcopist age were dichotomized by the median values. The chi-square test for heterogeneity and trend was used to
Table 1: Nonbiopsy and incorrect selection of biopsy site according to the colposcopic impression formulated by the committee.

<table>
<thead>
<tr>
<th>Colposcopist decision on biopsy</th>
<th>Biopsy: no (negative)</th>
<th>Biopsy: yes</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1</td>
<td>G2</td>
<td>Cancer</td>
</tr>
<tr>
<td>No</td>
<td>803</td>
<td>64</td>
<td>12</td>
</tr>
<tr>
<td>Yes, incorrect site</td>
<td>NA</td>
<td>58</td>
<td>85</td>
</tr>
<tr>
<td>Yes, correct site</td>
<td>NA</td>
<td>468</td>
<td>1024</td>
</tr>
<tr>
<td>Yes, subtotal</td>
<td>200</td>
<td>526</td>
<td>1109</td>
</tr>
<tr>
<td>Total</td>
<td>1003</td>
<td>590</td>
<td>1121</td>
</tr>
</tbody>
</table>

|                                | Nonbiopsy rate        | Incorrect biopsy site rate | Total biopsy failure rate |
|                                | 0.11 (0.08–0.13)      | 0.01 (0.01–0.02)           | 0.04 (0.03–0.05)         |
| No                             | 0.10 (0.08–0.12)      | 0.08 (0.06–0.09)           | 0.07 (0.06–0.09)         |
| Total                         | 0.21 (0.17–0.24)      | 0.09 (0.07–0.11)           | 0.11 (0.10–0.13)         |

G1: abnormal, grade 1; G2: abnormal, grade 2; Cancer: suspected invasive cancer.

Table 2: Nonbiopsy and incorrect selection of biopsy site according to original histological diagnosis.

<table>
<thead>
<tr>
<th>CIN1a</th>
<th>CIN2</th>
<th>CIN3/AIS</th>
<th>Carcinoma</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>48</td>
<td>5</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Yes, incorrect site</td>
<td>35</td>
<td>21</td>
<td>75</td>
<td>2</td>
</tr>
<tr>
<td>Yes, correct site</td>
<td>330</td>
<td>210</td>
<td>860</td>
<td>232</td>
</tr>
<tr>
<td>Yes, subtotal</td>
<td>365</td>
<td>231</td>
<td>935</td>
<td>234</td>
</tr>
<tr>
<td>Total</td>
<td>413</td>
<td>236</td>
<td>944</td>
<td>236</td>
</tr>
</tbody>
</table>

|                                | Nonbiopsy rate | Incorrect biopsy site rate | Total biopsy failure rate |
|                                | 0.12 (0.09–0.15) | 0.02 (0.01–0.05) | 0.01 (0.00–0.02) | 0.01 (0.00–0.03) | 0.03 (0.03–0.04) |
| No                             | 0.08 (0.06–0.12) | 0.09 (0.06–0.13) | 0.08 (0.06–0.10) | 0.01 (0.00–0.03) | 0.07 (0.06–0.09) |
| Total                         | 0.20 (0.16–0.24) | 0.11 (0.07–0.15) | 0.09 (0.07–0.11) | 0.02 (0.00–0.04) | 0.11 (0.09–0.12) |

CIN: cervical intraepithelial neoplasia; AIS: adenocarcinoma in situ.

a Fifty-nine CIN1 cases were excluded from evaluation because the committee did not consider them worthy of biopsy based on colposcopic findings and, thus, did not classify the correctness of biopsy site as indicated (if any) by the colposcopists.

estimate the strength of univariate associations. A P value < 0.05 was considered statistically significant. Multivariate analysis was performed using a multiple logistic regression model (backward stepwise selection). The level for removal of variables was set at P = 0.10. An odds ratio with a 95% CI that did not include the unity was considered statistically significant.

3. Results

3.1. Nonbiopsy and Incorrect Selection of Biopsy Site by Colposcopic Impression. Table 1 shows the colposcopists’ performance according to the colposcopic impression formulated by the committee. Overall, the colposcopists considered biopsy to be indicated more often than the committee, that is, in 2071 of 2950 instances versus 1947 (rate, 0.70 versus 0.66; ratio, 1.06; 95% CI, 1.00 to 1.13). This was entirely explained by the fact that they opted for biopsy in 20% of cases interpreted to be negative (and thus unworthy of further investigations) by the committee. Conversely, biopsy was omitted in about 10% of G1 changes, and 1% of G2 changes. No such cases were observed when the committee formulated the impression of Cancer. As far as the biopsy site is concerned, the rate of errors was stable at about 0.10 in both G1 and G2 and decreased to 0.01 in Cancer. Total biopsy failure rate, which peaked at 0.21 in G1, decreased regularly to 0.09 in G2 and 0.01 in Cancer.

3.2. Nonbiopsy and Incorrect Selection of Biopsy Site by Original Histological Diagnosis. Table 2 shows the frequency of nonbiopsy and incorrect selection of biopsy site according to original histological diagnosis. The pattern of results was closely similar to that in Table 1. Total biopsy failure rate was 0.20 in CIN1 and then decreased to approximately 0.10 in CIN2 and CIN3/AIS and 0.02 in carcinoma. However, the decreasing trend was more rapid for nonbiopsy rate, while errors in the selection of biopsy site were stable in all grades of CIN and decreased only in carcinoma. For this reason, they were the majority of total biopsy failures (133/197 or 68%), and this was entirely accounted for by their greater proportion in CIN2 and CIN3.

3.3. Factors Associated with Incorrect Selection of Biopsy Site. On account of the above finding, analysis of factors associated with biopsy failures was restricted to incorrect selection of site. Results are shown in Table 3. In univariate analysis, the probability of biopsy site being incorrectly selected decreased with increasing severity of the colposcopic impression and...
Table 3: Factors associated with the odds ratio for incorrect selection of biopsy site (total number of observations 1829).

<table>
<thead>
<tr>
<th>Factor</th>
<th>Number of observations</th>
<th>Number (%) with incorrect biopsy site</th>
<th>P</th>
<th>Univariate odds ratio (95% CI)</th>
<th>Multivariate odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colposcopic impression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>472</td>
<td>46 (9.7)</td>
<td>0.000</td>
<td>1.00 (referent)</td>
<td>1.00 (referent)</td>
</tr>
<tr>
<td>G2</td>
<td>1121</td>
<td>85 (7.6)</td>
<td></td>
<td>0.76 (0.52–1.11)</td>
<td>0.46 (0.27–0.80)</td>
</tr>
<tr>
<td>Cancer</td>
<td>236</td>
<td>2 (0.8)</td>
<td></td>
<td>0.08 (0.02–0.33)</td>
<td>0.09 (0.02–0.42)</td>
</tr>
<tr>
<td>Squamocolumnar junction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visible</td>
<td>1416</td>
<td>85 (6.0)</td>
<td>0.000</td>
<td>1.00 (referent)</td>
<td>1.00 (referent)</td>
</tr>
<tr>
<td>Not, or not entirely, visible</td>
<td>413</td>
<td>48 (11.6)</td>
<td></td>
<td>2.06 (1.42–2.99)</td>
<td>2.46 (1.62–3.73)</td>
</tr>
<tr>
<td>Original histological diagnosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN1</td>
<td>413</td>
<td>35 (8.5)</td>
<td></td>
<td>1.00 (referent)</td>
<td>1.00 (referent)</td>
</tr>
<tr>
<td>CIN2</td>
<td>236</td>
<td>21 (8.9)</td>
<td>0.001</td>
<td>1.05 (0.60–1.86)</td>
<td>1.97 (1.01–3.85)</td>
</tr>
<tr>
<td>CIN3/AIS</td>
<td>944</td>
<td>75 (7.9)</td>
<td></td>
<td>0.93 (0.61–1.42)</td>
<td>2.52 (1.32–4.80)</td>
</tr>
<tr>
<td>Invasive carcinoma</td>
<td>236</td>
<td>2 (0.8)</td>
<td></td>
<td>0.09 (0.02–0.39)</td>
<td>0.29 (0.06–1.34)</td>
</tr>
</tbody>
</table>

G1: abnormal, grade 1; G2: abnormal, grade 2; Cancer: suspected invasive cancer; CIN: cervical intraepithelial neoplasia; AIS: adenocarcinoma in situ; CI: confidence interval; NA: not applicable.

Patient age, colposcopist age, and participation in previous local quality assurance initiatives were not associated with incorrect selection of biopsy site in univariate analysis (P > 0.05) and were not included in logistic regression analysis.

From a multiple logistic regression model with backward stepwise selection of variables. The level for removal of variables was set at P = 0.10.

Table 3: Factors associated with the odds ratio for incorrect selection of biopsy site (total number of observations 1829).

4. Discussion

4.1. Rationale Issues. Colposcopically directed punch biopsy is affected by well-known biases that arise from colposcopic pattern recognition and from collection, processing, and reporting of biopsy samples. In addition to this, it is increasingly understood that the colposcopic pattern is an independent risk stratifier in the patient management algorithm, which includes many pieces of clinical information. Moreover, biopsy in medicine is typically used to confirm the diagnosis of a suspected condition, whereas assessment of precancerous cervical disease is often done with the excision of the entire lesion.

Despite these changing concepts, however, assessment of cervical disease status still relies in most instances on the histological report of colposcopically directed punch biopsy (or biopsies), which remains a critical step in the management of women with abnormal Pap smear results.

4.2. Test Conditions. We have previously discussed the methodological problems involved in colposcopy QA and, thus, in our own programme [11, 13]. The basic problem is that the test was conducted under artificial conditions. This facilitated the recognition of colposcopic features and the accuracy in indicating the need for biopsy and in selecting the place from which to take the sample. Opposite biases also existed, such as the impossibility of increasing the magnification of tissues. It appears that the overall sensitivity of the diagnostic process cannot be directly inferred from the sensitivity of colposcopically guided biopsy in a QA environment.

In addition, the participants were allowed a single opportunity to choose the biopsy site. This is different from the clinical real-world situation, although it enabled them to receive a direct feedback of the correctness of the chosen site.

4.3. Design. Correlating the colposcopic interpretation and the decision for biopsy with the histological diagnosis provides an approximate estimate of the probability of a false-negative or false-positive colposcopy assessment [11, 13]. The problem with this approach is that there is no absolute historical gold standard on which to rely. This can be established by cone biopsy or loop excision biopsy [2, 6, 7], by biopsy of colposcopically detected abnormalities plus random biopsies from normal-appearing quadrants [4] or by endocervical curettage plus random biopsies from normal-appearing areas [15].

The current study was not comparable with these designs. We used virtual substrates and we made the unproven assumption that the biopsy site chosen by the committee was on the worst-looking area. Moreover, given the relative subjectivity of the colposcopic impression, a single-shot biopsy decreased the chance of selecting the area with higher-grade colposcopic abnormalities and did not exclude the possibility that foci of severe squamous lesions could be found in specimens taken from areas with minor colposcopic changes.

4.4. Histological Diagnosis. The patients whose colpophotographs and data were used in the current study were originally diagnosed in two screening centres that follow certified
QA procedures including those for cytology and histology in cancer screening [16]. These facts notwithstanding our assumption that the original histological diagnosis reflected the actual state of disease are unwarranted. It may have occurred, for example, that quality and amount of biopsied tissue were insufficient and that the pathologist’s reporting was inaccurate.

However, our results must be viewed from the perspective that misclassifications do not create an association between two variables. Rather, they weaken or abolish an association if it exists. Following this line of reasoning, we can safely assume that the observed steep decrease in total biopsy failure rate from CIN1 to carcinoma was not generated by a misclassification bias.

4.5. Interpretation of Results. Due to the above considerations, extrapolation of our results to a field situation as well as external comparisons with other studies should be done with caution. Conversely, internal comparisons are free of biases resulting from test conditions. From this perspective, some findings deserve attention. First, overall biopsy rate was higher among the participating colposcopists compared with the expert committee, reflecting a higher level of diagnostic uncertainties. Specifically, among colposcopists with limited experience, a non-conservative approach to biopsy is positively associated with the probability of disease detection [17–19].

Second, total biopsy failure rate decreased steadily with increasing histological severity of the lesion. This is explained by our previous finding of a strong correlation between colposcopic impression and original histological diagnosis [15]. The correlation between the visual aspects of the cervix and the severity of the underlying epithelial changes is imperfect [15, 18] but not weak.

Third, incorrect selection of biopsy site was the most common type of biopsy failure, and this was entirely due to the fact that it was more frequent than nonperformance of biopsy in CIN2 and CIN3. In multivariate analysis, after adjustment for the colposcopic impression and the visibility of the squamocolumnar junction, the risk of incorrect selection of biopsy site was confirmed to be significantly increased in CIN2 and CIN3 and extremely low in carcinoma.

Problems with biopsy site in high-grade CIN are difficult to interpret. Errors in selecting a biopsy site in a large surface lesion have often been postulated to explain nondiagnosis of CIN3 [20] and microinvasive carcinoma [21]. Complex atypical areas of the transformation zone including central small high-grade foci and an external large low-grade lesion may be incorrectly interpreted by colposcopists. Probably, lesions of this type are most commonly encountered in a regularly repeated screening setting because they are of recent onset and at an early stage of development.

In any case, our finding provides another confirmation that the concerns regarding the sensitivity of biopsy for high-grade CIN are justified [1–6]. Several potential solutions to this problem are under consideration. Some options that are being proposed include increasing the number of biopsy specimens [17], that is, taking multiple biopsies from the worst-looking lesion and other abnormal areas and taking random biopsies from all normal-appearing areas [18]. The latter approach is particularly controversial.

Several factors have been hypothesised to influence the accuracy of biopsy. The level of supporting evidences, however, is low [7]. Two important, although expected, findings of our study were that the risk of incorrect selection of biopsy site decreased steadily with increasing severity of the colposcopic impression and that it was greater when the squamocolumnar junction was not, or not entirely, visible.

4.6. Conclusions. Although the existence of a problem with selection of biopsy site specifically in high-grade CIN was confirmed, total biopsy failure rate decreased with increasing severity of both colposcopic impression and histological diagnosis and was almost nil for invasive carcinoma. Before undertaking aggressive biopsy strategies aimed at increasing the number of specimens, an attempt should be made to improve the detectability of high-grade CIN through large training programmes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References


miR-34a and miR-125b Expression in HPV Infection and Cervical Cancer Development

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1. Introduction

Cervical cancer is the third most common cancer among women with approximately 530,000 new cancer cases and 275,100 deaths each year [1, 2]. Persistent infection by human papillomavirus (HPV) has been considered the etiological cause of squamous intraepithelial lesions of the cervix that may develop into high-grade dysplasia or to invasive carcinoma. The majority of HPV infections are asymptomatic and are efficiently controlled by the host immune system; therefore, the outcome of HPV infection is variable [3]. High-risk HPVs (HR-HPV) are recognized to be a necessary but nonsufficient condition for the development of cervical cancer and clinicians are still demanding for the identification of useful predictive/prognostic biomarkers for HPV infection [4–7].

Recently, microRNAs (miRNAs), noncoding RNAs with approximately 18–25 nucleotides in length, have been proposed as biomarkers of cancer development. miRNAs are responsible for modulating gene expression by binding to
complementary segments present in the untranslated region (UTR) of messenger RNA (mRNA) leading to the suppression of translation and/or degradation of mRNA [8]. miRNAs are thought to potentially target up to one-third of human coding genes managing cellular activity, including proliferation, differentiation, and apoptosis [8, 9]. These molecules have been described as key regulators in cancer, and, in fact, several studies have been addressing the impact of miRNAs in tumor development either by acting as oncogenes or tumor suppressor genes [9, 10].

Several studies have attempted to identify potential biomarkers of HPV infection outcome by studying the events of HPV-related carcinogenesis [11, 12]. Recently, it was suggested that some miRNAs could be biomarkers for the occurrence and development of the HPV-associated cancers, including cervical cancer [13]. Moreover, studies have described several interactions between miRNAs and HPV oncoproteins and specific miRNAs have been located in cancer-related genomic regions, which include fragile sites at or near HPV integration sites. Therefore, the identification of different tumor-specific miRNA signatures might be an important tool to distinguish the different HPV-associated lesions or cancers [14–16].

The aim of this study was to characterize the expression of two miRNAs (miR-34a and miR-125b) in cytological samples from women with different cervical lesions, including invasive cervical cancers, and evaluate its impact as predictive/prognostic biomarkers of cervical cancer and HPV infection.

2. Subjects, Materials, and Methods

2.1. Study Population. The study was performed in exfoliated cervical cells collected from randomly selected women (n = 114, median age 40 ± 12.6 years old) attended at the Gynaecological Service from the Portuguese Institute of Oncology of Porto (IPO Porto) during routine clinical observations. These women are followed up in our institution due to previous history of cancer (not specifically cervical cancer). All samples were submitted to cytological examination and classified according to the Bethesda classification by qualified pathologists from our institution: 49 women with normal cytology, 28 with low-grade intraepithelial squamous lesions (LSIL), 29 with high-grade intraepithelial squamous lesions (HSIL), and 8 with invasive cervical carcinomas (ICC).

2.2. Sample Processing. Samples were collected in ThinPrep tubes (Hologic, USA) and stored at room temperature prior to processing: a 4 mL aliquot was used for digene HC2 High-Risk HPV DNA Test (QIAGEN, Germany); and 1 mL was used for total nucleic acids extraction using High Pure Viral Nucleic Acid Kit (Roche, Germany). DNA/RNA was quantified using the NanoDrop 1000 Spectrophotometer v3.7 (Thermo Scientific, Wilmington, DE, USA).

2.3. HPV Status. HPV was detected at the Virology Service of IPO Porto as part of routine procedures using digene HC2 High-Risk HPV DNA Test (QIAGEN, Germany) (HC2). HC2 test detects 13 high-risk HPV (HR-HPV) types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. HPV infection was detected in 67.3% of all cervical specimens, with a prevalence of 40.8% (20/49) for normal cytology, 75.0% (21/28) for LSIL, 96.6% (28/29) for HSIL, and 87.5% (7/8) for ICC.

2.4. miRNA Analysis. miR-125b, miR-34a, and miR-23a were analysed using two-step real-time PCR protocols with TaqMan MicroRNA Assays: hsa-miR-125b-5p_000449; hsa-miR-34a-5p_000426; hsa-miR-23a-3p_000399 (Applied Biosystems, Foster, CA, USA). Reverse transcriptase reactions were performed using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA) in a 15 μL of total volume reaction mix with 7 μL of master mix containing 1x RT buffer, 1.0 mM of total dNTPs, 50 U MultiScribe Reverse Transcriptase Enzyme, and 0.25 U of RNase inhibitor; 3 μL of RT primers (Applied Biosystems, Foster, CA, USA); and 5 μL of RNA sample. The amplification conditions were as follows: annealing at 16 °C for 30 min, extension at 42 °C for 52 min, and RT inactivation at 85 °C for 10 min. All reverse transcriptase reactions included two nontemplate controls using double distilled water to replace template RNA. qPCRs were performed on Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster, CA, USA) with a 20 μL final volume mixture containing 1x TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, California, USA), 1x MicroRNA Assay (Applied Biosystems, Foster City, California, USA), and 2 μL cDNA (RT product). Thermal cycling conditions were 10 min. at 95 °C followed by 45 cycles of 15 sec. at 95 °C and 1 min. at 60 °C.

2.5. Data Analysis. The endpoint of qPCR data is the threshold cycle (Ct), which represents the fractional cycle number at which the fluorescence reaches the fixed threshold. All qPCR reactions were run in duplicate and all experimental replicates had less than 0.99 Ct difference.

The relative quantification of miRNA expression was analyzed using Livak method (also known as 2^(-ΔΔCt) method). In this method, Cts from the target miRNAs (miR-125b and miR-34a) in both test and reference cases were adjusted in relation to the Ct of a normalizer miRNA (miR-23a) resulting in ΔCt. The reference group in this study was women with normal cytology and without HPV infection. The difference between the ΔCt of cases and ΔCt reference gives the ΔΔCt value, which is incorporated to determine the fold-difference in expression. The range of fold-differences was also calculated for each type of lesion.

2.6. Statistical Analysis. Statistical analysis was performed using the computer software IBM SPSS (Statistical Package for Social Sciences) Statistics version 20.0 for Mac. All groups were compared, whenever appropriate, by Student's t-test, ANOVA, and Chi-square (χ²) test. Results are considered to be significantly different when P < 0.05. Receiver operating characteristic (ROC) curves were used to assess the predictability of cervical disease progression.
3. Results

3.1. qPCR Analysis. qPCR results are shown in Table 1 and Figure 1. Firstly, we noticed that qPCR was able to detect both low and high quantities of the miRNAs (overall Ct values range was 24.53 to 41.35). Additionally, the variation coefficient (VC) for all miRNAs was below 10%, which reveals that the variation of Ct within samples was very low. These data also show that the variation of Ct within samples was very low. These data also show that the High Pure Viral Nucleic Acid Kit (Roche, Germany) was able to efficiently extract miRNA from this type of samples.

3.2. miRNAs Expression. Expression of selected miRNAs was determined for all samples, grouped according to cytological result, using women with normal cytology and without HPV infection as control group. Firstly, we have tested the influence of the presence of HPV in the different cervical lesions in data analysis; nevertheless, the results showed no statistically significant differences (data not shown).

According to the Livak method, the ratio between the $2^{-\Delta \Delta Ct}$ of miR23a Average Ct of controls and the $2^{-\Delta \Delta Ct}$ of miR23a Average Ct of all cases of miR-23a revealed no significant difference on the overall data regarding the miR-23a distribution in all subgroups (ratio = 1.18) [17]. Moreover, the t-test showed no statistically significant differences between the distribution of miR-23a in the two groups ($P = 0.555$). Therefore, we have considered that miR-23a could be used as the normalizer of the analysis of miR-125b and miR-34a expression.

Considering the results of miR-125b expression (Table 2) there are interesting data: firstly, we observed a twofold ($2^{-\Delta \Delta Ct} = 2.11; P = 0.038$) increase in the group of women with normal cytology with HPV infection; then, despite not being statistically significant, we also found a decrease of miR-125b expression for LSIL or HSIL ($2^{-\Delta \Delta Ct} = 0.75$ and $2^{-\Delta \Delta Ct} = 0.55$, resp.); and, finally, the analysis revealed that miR-125b expression was significantly decreased in women with ICC ($2^{-\Delta \Delta Ct} = 0.21; P = 0.004$). The accuracy of miR-125b levels to discriminate between the different cervical lesions and normal cases was evaluated using ROC curves; nevertheless, significant results were only observed for ICC where the cutoff of $-5.8$ (ΔCt value) allows discriminating ICC with 88% sensitivity and 69% specificity (area under the curve = 0.802; $P = 0.010$) (Figure 2).

Similar to miR-125b, miR-34a expression in women with normal cytology and with HPV infection was also significantly different from the control group ($2^{-\Delta \Delta Ct} = 1.69; P = 0.049$) and women with ICC ($2^{-\Delta \Delta Ct} = 2.08; P = 0.042$) (Table 2). Despite these differences and in contrast to miR-125b, there was no trend for miR-34a expression in the different cervical lesions.

<table>
<thead>
<tr>
<th></th>
<th>miR-125b</th>
<th>miR-34a</th>
<th>miR-23a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (HPV−) ($n = 29$)</td>
<td>$28.43 \pm 2.48$</td>
<td>$34.14 \pm 1.82$</td>
<td>$34.94 \pm 1.72$</td>
</tr>
<tr>
<td>Normal (HPV+) ($n = 20$)</td>
<td>$28.06 \pm 2.20$</td>
<td>$34.09 \pm 1.58$</td>
<td>$35.65 \pm 2.58$</td>
</tr>
<tr>
<td>LSIL ($n = 28$)</td>
<td>$29.34 \pm 2.12$</td>
<td>$34.60 \pm 1.71$</td>
<td>$35.42 \pm 2.04$</td>
</tr>
<tr>
<td>HSIL ($n = 29$)</td>
<td>$29.31 \pm 2.13$</td>
<td>$34.02 \pm 1.18$</td>
<td>$34.96 \pm 1.70$</td>
</tr>
<tr>
<td>ICC ($n = 8$)</td>
<td>$29.78 \pm 2.46$</td>
<td>$32.17 \pm 1.90$</td>
<td>$34.02 \pm 0.79$</td>
</tr>
</tbody>
</table>

Table 1: qPCR data analysis.

Figure 1: Cycle threshold of analysed miRNAs. LSIL: low-grade squamous intraepithelial lesions; HSIL: high-grade squamous intraepithelial lesions; ICC: invasive cervical carcinoma.
cervical lesions as the results showed that it seems to be stably expressed in LSIL and HSIL ($2^{-\Delta\Delta Ct} = 1.01$ and $2^{-\Delta\Delta Ct} = 1.10$, resp.).

4. Discussion

Actually, miRNA expression and biological functions are highly influenced by cellular context, probably due to the differential expression of their target mRNAs [9]. In fact, the miRNAs expression varies from tissue to tissue and several studies proved that they are differentially expressed in normal tissues when compared with normal adjacent tissues and therefore have been described as possible biomarkers of cancer development. Moreover, miRNAs are thought to be highly stable when compared with mRNA and, therefore, their detection is relatively easy and reproducible, suggesting they could be used as good candidates for biomarkers and diagnostic tools in oncology [9]. However, it is important to consider that microRNA expression can be affected by several factors such as the type of treatment that the patients are submitted to. Recent studies have been developed to identify proof-valuable biomarkers for HPV infection outcome considering the cellular modifications caused by HPV infection and the cascade of distinct events of HPV carcinogenesis [11]. Literature has shown differential miRNA expression in cervical lesions and cancer tissues and also revealed potential interactions of miRNAs in HPV-associated carcinogenesis [13]. In our study, we investigated the expression of two miRNAs (miR-125b and miR-34a) that have been described as interacting in crucial steps of cervical cancer development.

Our study is the first using qRT-PCR methodology to evaluate the expression of miRNAs in exfoliated cervical cells with high sensitivity and specificity. Nevertheless, we are aware that our study has some limitations such as the following: the possibility of potential selection bias cannot be ruled out since the study population was hospital-based; the limited number of samples, mainly in ICC group, may have some impact on the precision of data and, therefore, it would be useful to develop a further study with increased number of samples; the type of sample used, cervical exfoliated cells, is not the most frequently used in these types of studies, although clinicians frequently request the use of noninvasive techniques for the research of new biomarkers; therefore further studies should be performed, especially in matched cytology-histology samples, to evaluate the correlation between the specificity/sensitivity of the analysis; and, finally, the fact that we have not used a specific kit for miRNA extraction could have some impact on data quality. Although the use of TaqMan assays provides feasibility to the study and considering that the specificity is extremely high, the exclusion of DNA amplification would be an important factor to address since we have not used a specific kit for miRNA extraction. Nevertheless, regarding this last point, in our study, the amplification of reference miRNA has shown very good coefficient of variation (<7%). Moreover, in RT-qPCR methodology, data normalization is crucial to obtain accurate results. According to Shen et al., who analysed the stability of candidate miRs and small RNA reference genes in cervical cancer cell lines and other cervical specimens, miR-23a has proven to be optimal reference microRNA and was suggested to be used for normalization [18]. In our study, the data considering miR-23a also revealed that this microRNA is a good endogenous reference to cervical samples.

miR-125b has been described to be involved in different cellular processes such as inflammation, cell proliferation, and cell cycle regulation and, therefore, it can act as oncogene

<table>
<thead>
<tr>
<th></th>
<th>miR-125b</th>
<th></th>
<th></th>
<th>miR-34a</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔCt ± sd</td>
<td>$2^{\Delta\Delta Ct}$</td>
<td>t-test</td>
<td>ΔCt ± sd</td>
<td>$2^{\Delta\Delta Ct}$</td>
<td>t-test</td>
</tr>
<tr>
<td>Normal (HPV−) (n = 29)</td>
<td>$-6.51 ± 1.73$</td>
<td>Reference</td>
<td></td>
<td>$-0.80 ± 1.16$</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>Normal (HPV+) (n = 20)</td>
<td>$-7.59 ± 1.77$</td>
<td>$2.11 (0.64–7.01)$</td>
<td><strong>0.038</strong></td>
<td>$-1.56 ± 1.47$</td>
<td>$1.69 (0.76–3.78)$</td>
<td><strong>0.049</strong></td>
</tr>
<tr>
<td>LSIL (n = 28)</td>
<td>$-6.09 ± 1.44$</td>
<td>$0.75 (0.23–2.48)$</td>
<td>$0.320$</td>
<td>$-0.82 ± 1.81$</td>
<td>$1.01 (0.45–2.27)$</td>
<td>$0.955$</td>
</tr>
<tr>
<td>HSIL (n = 29)</td>
<td>$-5.65 ± 2.15$</td>
<td>$0.55 (0.17–1.83)$</td>
<td>$0.099$</td>
<td>$-0.94 ± 1.59$</td>
<td>$1.10 (0.49–2.46)$</td>
<td>$0.707$</td>
</tr>
<tr>
<td>ICC (n = 8)</td>
<td>$-4.24 ± 2.10$</td>
<td>$0.21 (0.06–0.69)$</td>
<td><strong>0.004</strong></td>
<td>$-1.86 ± 1.56$</td>
<td>$2.08 (0.93–4.66)$</td>
<td><strong>0.042</strong></td>
</tr>
</tbody>
</table>

Ct: cycle threshold; Sd: standard deviation; LSIL: low-grade squamous intraepithelial lesions; HSIL: high-grade squamous intraepithelial lesions; ICC: invasive cervical carcinoma.

**Figure 2:** Prediction of ICC development using miR-125b levels. AUC: area under the curve.
or tumor suppressor depending on cell type [19–22]. We demonstrated that miR-125b expression is increased in normal cervix infected with HPV, while the relative expression seems to decrease as lesions progress (Table 2). According to literature, an increased expression of miR-125b shortly after HPV infection can be explained by interaction with viral proteins [23, 24]. Based on a recent study that revealed strong homology between miR-125b and the HPV-L2, an increased expression of miR-125b is a quick response to HPV productive infection shortly after viral infection. Nuovo and colleagues showed that after persistent infection, HPV-L2 can induce inactivation of miR-125b and this event seems to be associated with cytological changes of the koilocytes [25]. Although literature refers to the fact that miR-125b is a negative regulator of p53 and its expression would allow a more effective suppression of p53 pathways leading to tumor development [3, 20, 26, 27], it has been shown that overexpression of miR-125b leads to decreased cell proliferation, apoptosis, and suppression of tumor growth by targeting the PI3K/Akt/mTOR signaling pathway [28]. This evidence must be further studied to clarify what is promoting the underexpression of miR-125b in cervical carcinogenesis after HPV infection.

miR-34a is a key regulator of tumor suppression and has been reported to be downregulated in several cancers [29]. This miRNA is implicated in the p53 network and has been described as direct target of p53: when p53 is activated, it induces the transcription of miR-34a, which is able to target several molecules involved in cellular transformation and carcinogenesis [14, 15, 29–32]. Theoretically, in HPV-associated carcinogenesis, the expression of HPV-E6 will downregulate the expression of miR-34a by targeting p53 to degradation through the ubiquitin-proteasome system [26, 29, 33, 34]. Therefore, it is expected that miR-34a deregulation occurs after HPV infection, probably after viral genome integration into host's genome. Li and colleagues reported that inhibition after HPV infection, probably after viral genome integration therefore induce miR-34a expression. Surprisingly, in our study, we did not observe significant differences in relative expression when considering LSIL and HSIL. The increase in miR-34a expression in HPV infected cells is probably explained by the activation of cellular repair mechanisms after viral infection that would activate p53 pathways and therefore induce miR-34a expression. Surprisingly, in our study, we observed a significant increase of miR-34a expression in women with invasive carcinomas, which increased the discussion on this subject since previous studies have shown controversial results regarding miR-34a and invasive cervical carcinoma [29, 33, 36]. Hence, it is important to perform more studies on miR-34a levels in HPV infection and cervical lesions/cancer and to clarify whether there are other pathways besides the p53-associated pathways, which are able to activate miR-34a expression in HPV-associated lesions.

In conclusion, our study revealed that both miR-125b and miR-34a are overexpressed in HPV infected cells and this should be further investigated to establish whether this is a direct consequence of viral infection or a cellular response. Additionally, our study revealed that miR-125b levels decrease as cervical lesions progress to invasive cervical cancer and ROC curve revealed that its levels could be used with good sensitivity and specificity in ICC diagnosis. Thus, being preliminary, these results suggest that miR-125b should be further investigated as a predictive/prognostic noninvasive tool of cervical cancer development.

Conflict of Interests

All the authors declare no competing financial interests for the work described in this paper.

Authors’ Contribution

All authors have contributed to this paper.

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References


Acceptability of Human Papillomavirus Vaccine: A Survey among Master of Business Administration Students in KwaZulu-Natal, South Africa

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Cervical cancer is a preventable public health problem. The two new human papillomavirus (HPV) vaccines are available but not accessible to everyone in South Africa, as they are very expensive. This study aimed to investigate educated peoples acceptability regarding HPV vaccination. This was a cross-sectional survey conducted among 146 master of business administration students by self-administered, anonymous questionnaire. The majority (74%) of the participants ever heard of cervical cancer, but only 26.2% heard about HPV. After reading the fact information regarding cervical cancer and HPV, the intention to vaccinate their daughters increased from 88% to 97.2% (P = 0.003). The majority (75.4%) indicated that HPV vaccination should be given before their daughters are mature enough to understand about sex, and 80.3% reported that they will discuss matters related to sex with their daughters if their daughters want to know about the vaccine. Those who did not want to vaccinate their daughters highlighted that they want more information regarding safety of the vaccine which might change their decision towards HPV vaccination. A health education information method can increase the vaccination acceptance rate in South Africa.

1. Introduction

Cervical cancer is a preventable disease, but it accounts for around 275,100 deaths, and more than half a million women are diagnosed each year in the world [1]. In South Africa, it is also the second common cancer after breast cancer. It is reported that at least 3,000 women die from cervical cancer every year, and by the year 2025, at least 4,000 women will die from cervical cancer in South Africa [2]. Because of unequal access to healthcare, differences in socioeconomic status, and exposure to HPV and HIV infection, black women are disproportionately affected [3, 4]. Cervical cancer screening in South Africa is free for women over 30 years of age as to achieve 70% coverage of this age group. But the screening in this group of women is below 20%. A recent study conducted from January 2007 to December 2010 found that annual screening coverage was between 2.9% and 4.2% [5]. Another study conducted among women who were diagnosed and managed at a Gynecological Oncology unit in South Africa reported that about 40% of women had a gynecological examination at their first visit and 15% were referred appropriately [6].

Thanks to the new development of a HPV vaccine with high efficacy, cervical cancer incidence can significantly be reduced (with 70%) if a comprehensive vaccination program could be implemented [7–9]. Many countries that have low cervical cancer rates have already implemented country wide HPV vaccination programs. But full coverage was not achieved in any country. A survey conducted in the USA in 2010 reported that 60% of parents were not interested or not sure to vaccinate their daughters [10]. School-based vaccination programs have the highest vaccination coverage in many countries like Spain, Scotland, England, and The Netherlands [11–14]. The most notable finding was in Uganda, where a well-planned school-based campaign achieved about 95% coverage in the country [15].

The two vaccines, Cervarix (a bivalent vaccine) and Gardasil (the quadrivalent vaccine), are currently available in
South Africa. But they are very expensive. The majority of the population cannot afford these vaccines for their daughters. The national Department of Health of South Africa is planning to implement HPV vaccination countrywide. Successful vaccine implementation will depend on how different population groups are aware of these vaccines.

This study investigates the acceptability of HPV vaccination among educated people like master of business administration (MBA) students in South Africa. These groups of people are holding managerial positions/owning small to medium businesses in the country. They have the capacity and capability to develop new projects towards reducing cervical cancer incidence as well as they can disseminate the information regarding HPV vaccination and cervical cancer to their colleagues or employees. Moreover, in South Africa, corporate social responsibility (CSR) is a very serious topic for discussion in the business sector. CSR means voluntary involvement, or investment, of companies in social projects that help or uplift the community in which they operate in areas such as health care, housing, education, and basic services. CSR can also be defined as the commitment by business contributing to economic development and at the same time improving the quality of life of the workforce, their families and the local community, and society at large.

2. Methodology

This was a cross-sectional survey study, including a factsheet, conducted among MBA students who are currently registered at the University of KwaZulu-Natal. A total of 245 students were enrolled for the MBA program. All these students were given the possibility to participate in the study.

A self-administered questionnaire with closed and a few open-ended questions was used based on a study conducted among educated parents in India [16]. The questionnaire had three sections. The first part included sociodemographic information and the awareness regarding cervical cancer and HPV. The second section consisted of a factsheet which provided basic information on cervical cancer, how HPV is transmitted, and efficacy and safety of the HPV vaccine. Once participants finished reading the factsheet information they were asked to complete the third section. This section asked questions regarding parental acceptance and perception of HPV vaccination. It took about 15 minutes to complete the questionnaire.

University of KwaZulu-Natal research and ethics committee approved the study prior to data collection. Participation in the study was voluntary. Anonymity and confidentiality were maintained at all times. Prior to data collection, the main researcher communicated with the corresponding lecturers about the data collection procedure. Once the lecturers agreed, the researcher went to the corresponding lecturers' lecture room during the lecture break. The researcher then explained the aims and objectives of the study. Each participant signed a consent document before completing the questionnaire. Students who were present in the class during that day and voluntarily participated were included in the study.

Data were checked for completeness and then entered into a Microsoft Excel 2003 spread sheet and imported into SPSS 21.0, for Windows for analysis. The results were summarized using descriptive statistics: expressed as mean (SD) for continuous variables and percentages for categorical variables. Comparisons of categorical responses were evaluated using the Chi-square or Fisher’s exact test. A P value < 0.05 was considered as statistically significant.

3. Results

A total of 146 students participated in the survey. Only these students were present on the day of data collection. Those who were not present had similar background characteristics because of the same admission criteria. Table 1 summarizes sociodemographic information of the participants. Results
Table 2: Important reasons and concerns regarding vaccination of children (n = 79).

<table>
<thead>
<tr>
<th>Frequency</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Most important reason to vaccinate your child</td>
<td></td>
</tr>
<tr>
<td>You were aware that vaccines prevent certain diseases</td>
<td>68</td>
</tr>
<tr>
<td>Your physician/pediatrician advised you</td>
<td>9</td>
</tr>
<tr>
<td>Your friends and/or relatives advised you</td>
<td>1</td>
</tr>
<tr>
<td>Vaccination necessary for admission to school</td>
<td>1</td>
</tr>
<tr>
<td>Most important concern to vaccinate your child</td>
<td></td>
</tr>
<tr>
<td>The seriousness of the disease that the vaccine prevents</td>
<td>41</td>
</tr>
<tr>
<td>The effectiveness of the vaccine</td>
<td>18</td>
</tr>
<tr>
<td>Side effects of the vaccine</td>
<td>16</td>
</tr>
<tr>
<td>If adequate care was taken to preserve the vaccine</td>
<td>2</td>
</tr>
<tr>
<td>Price of the vaccine</td>
<td>2</td>
</tr>
</tbody>
</table>

indicated that the average age of the respondents was 34.8 years with a standard deviation of 5.9 years. More than half of the participants were males (58.9%) and Africans (55.5%) and were married (60.3%) and have a degree higher than the bachelor degree (59.3%). Regarding profession, more than two-thirds (70%) of the participants were working in a middle or senior managerial position and 77.1% were earning more than R 20,000 per month.

Participants, who had children, were asked if their children were vaccinated. Results showed that almost all the participants (90%) had given regular vaccination to their children. The most important reason to vaccinate was that vaccines prevent certain diseases (86.1%) and the main concern to vaccinate was the seriousness of the disease that the vaccine prevents. Reasons and concerns for vaccinations are shown in Table 2. Nine participants had their children not vaccinated because of not knowing about the vaccine (n = 5) or their physician did not advise them (n = 4) to have their children vaccinated.

Overall, 74% of the participants ever heard of cervical cancer and only 26.2% heard about HPV. Initially, 88.8% of the participants were willing to have their daughters vaccinated against cervical cancer. After reading the fact sheet information regarding cervical cancer and HPV, the intention to have their daughters vaccinated significantly increased to 97.2% (P = 0.003) (Figure 1). Participants were also asked to indicate the best time for HPV vaccination. More than three-quarters (75.4%) indicated that HPV vaccination should be given before their daughters are mature enough to understand about sex. One out of 5 (19.6%) reported that they will wait until their daughter has grown up and can decide for herself and 5.0% suggested to have their daughters vaccinated just before they get married.

Participants’ perception regarding HPV vaccination (%).

<table>
<thead>
<tr>
<th>Statements</th>
<th>SD</th>
<th>D</th>
<th>N</th>
<th>A</th>
<th>SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>It is necessary to explain to your daughter before vaccination that the vaccine protects against a sexually transmitted infection</td>
<td>22.5</td>
<td>7.2</td>
<td>11.6</td>
<td>26.1</td>
<td>32.6</td>
</tr>
<tr>
<td>Vaccination may send a no-objection message from the parents to start sexual relationships</td>
<td>37.7</td>
<td>28.3</td>
<td>13.0</td>
<td>18.1</td>
<td>2.9</td>
</tr>
<tr>
<td>Avoid discussing matters related to sex with your daughter if she wants to know about the vaccine/papilloma virus</td>
<td>45.3</td>
<td>35.0</td>
<td>10.2</td>
<td>5.8</td>
<td>3.6</td>
</tr>
</tbody>
</table>

SD = strongly disagree, D = disagree, N = neutral, A = agree, and SA = strongly agree.

More than half (58.7%) of the participants had indicated that it is necessary to explain to their daughters before vaccination that it protects against sexually transmitted infection, whereas about two-thirds (66.0%) did not think that vaccination will send no-objection message to start sexual relationship (Table 3). The majority (80.3%) reported that they will discuss matters related to sex with their daughters if their daughters want to know about the vaccine. More than a third (35.6%) of the respondents indicated that vaccination should be given by gynecologists, 29.6% had a preference for the GP to vaccinate their daughter, 10.4% had a preference for the pediatrician, and 24.4% mentioned that it does not matter to them who gives the vaccination.

After reading the fact sheet information, four participants still did not want to give HPV vaccination to their daughters. Amongst them, three participants reasoned that the vaccine is new and they are not sure if it will be safe for their children.
and one participant did not give a reason. When asked what can change their mind regarding accepting the vaccine, two participants wanted a detailed report on the safety and effectiveness of the vaccine, one participant was willing to have his daughter vaccinated if it is recommended from the child's school, and the last participant did not indicate anything (data not shown).

4. Discussion

As far as we know, this is the first study to investigate the acceptability of HPV vaccination among educated, urban people in South Africa. Awareness regarding cervical cancer varies. This study found that a majority of the participants ever heard of cervical cancer, but only 26.2% heard about HPV. An Indian study conducted among urban, educated couples found that about a third of the participants heard about cervical cancer [16]. A cross-sectional study conducted among adults in Botswana, reported that 71% respondents heard of cervical cancer and 35% heard of HPV [17]. A Ghanaian study found that 87% of the surveyed women were aware of cervical cancer [18].

It is well known that vaccination prevents serious diseases. Globally as well as in South Africa, general vaccination coverage has increased over the last few decades. So, it was expected that all the educated people like our surveyed population vaccinated their children for their general, regular vaccination. This study found that about 90% vaccinated their daughters for general vaccination. This finding is in line with South African vaccination coverage. In South Africa, full vaccination coverage is 80% or more for one-year-old children in eight of its nine provinces [19]. Considering our educated participants, this result is low but similar to the study conducted among educated parents in India [16]. Researchers have concluded that health care workers not providing accurate information, vaccine unavailability, and lack of access to health care facilities are some of the reasons of low vaccination coverage in South Africa [20].

Globally, vaccination acceptability is high among different population groups. Studies have found high acceptability for HPV vaccination in all the countries that investigated HPV vaccination acceptability among women as well as for their daughters. Our study found that 88.8% of the participants were willing to vaccinate their daughters against cervical cancer. A recent South African study conducted among female undergraduate students found that 77.3% of the participants were willing to take HPV vaccination for themselves [21]. A study from Mali reported that 74.5% of the participants would vaccinate their children against HPV [22]. Other studies conducted from African countries reported higher acceptance rates for HPV vaccination for themselves or for their daughters despite having little knowledge regarding cervical cancer and HPV. For example, 88% of women from Botswana and 94% of Ghanaian women were willing to vaccinate themselves or their daughters [17, 18].

The effectiveness of health education on secondary prevention of cervical cancer is well documented. After reading the fact sheet information regarding cervical cancer and HPV, the respondents' intention to have their daughters vaccinated increased from 88% to 97.2%. This finding is similar to other studies conducted elsewhere. A qualitative study conducted among women aged 18–45 years in Malawi highlighted that before the study none of the participants heard of a HPV vaccine, but after given basic information, all the participants were willing to accept HPV vaccination [23]. Another study conducted in Tanzania among parents, female pupils, teachers, health workers, and religious leaders reported that after providing information about cervical cancer and HPV vaccination, most of the participants agreed that they would give HPV vaccination to their daughters [24]. A systematic review study highlighted that more information and recommendation from health care workers are the most important factors affecting parental decision making towards HPV vaccination for their daughters [25]. It is also important to note that none of the participants were undecided after reading the fact information. This suggests that health information is very important to make the desired decision.

The appropriate age to vaccinate HPV vaccines varies across different population groups. HPV vaccines are effective if they are given before the girl is not yet sexually active. In the present study, a majority mentioned that HPV vaccination should be given to their daughters before children understand about sex. A South African study reported that because of early sexual activity by the adolescents, health care providers and community respondents suggested that HPV vaccination should commence around the age of nine [26]. The present study also found similar finding as a majority of the participants mentioned that HPV vaccination should be administered before their daughters understand about sex. The study from Ghana reported that the acceptable age for HPV vaccination was 13 [18]. It was also reported that health care workers have different opinions regarding the best age for HPV vaccination. For example, one study from America found that 35% of the medical personnel would recommend HPV vaccination to 9-to-12 year-old girls, while another study reported that 77% of the paediatricians would recommend HPV vaccination to 13-to-15-year-old girls [27, 28]. Therefore, before national vaccination program implementation, health care workers need to be on the same page regarding the best age for HPV vaccination and disseminate this information to the general public.

Since HPV vaccines are new, it will face challenges to be accepted by the general population. Researchers have reported that potential side effects, health need, and benefit of the vaccine are the most imported challenges faced by the vaccine to get accepted by the general population [29]. In this study, after providing accurate information about HPV vaccines, some participants are still not happy to vaccinate their daughters. Also when asked what could be done to change their mind regarding accepting the vaccine, half of the participants reported they would be willing to accept the vaccine, but after given basic information, all the participants were willing to accept HPV vaccination [23]. Another study conducted in South Africa reported that some participants were concerned about vaccine safety and effectiveness, but they still supported the vaccine [26]. Among Ghanaian women, a majority were concerned about vaccine safety and side effects [18]. A Tanzanian study reported that 35% of the teachers did not want to vaccinate their daughters because of side effects.
and potential effects of the vaccine on future reproduction [24].

This study only sampled people from one university. As they are educated people, the results cannot be generalized. One important aspect of this study is that this group of people, who can significantly impact the society at large regarding HPV vaccination, has never been investigated before. The findings will be helpful for nationwide vaccination coverage as the business community plays a significant role in the community upliftment.

5. Conclusion
The present study found a high acceptability rate of HPV vaccination for their daughters in an educated group of MBA students. Educational information increased the vaccine acceptability rate. These findings are important, because due to their position in the South African society, this group of educated people can play an important role in disseminating the knowledge and attitude regarding HPV vaccination among a large part of the South African population.

Conflict of Interests
The authors declare that there is no conflict of interests regarding the publication of this paper.

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