

Prognostic significance of high-risk HPV persistence after laser CO₂ conization for high-grade CIN: a prospective clinical study

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Summary

Purpose of investigation: To estimate the persistence rate of high-risk HPV DNA (HR-HPV DNA) in a population treated totally by laser CO₂ conization for high-grade cervical intraepithelial neoplasia (HG-CIN), and to examine if this persistence might be considered an independent risk factor for relapsing disease. **Methods.** All women with a histological diagnosis of HG-CIN and planned for laser CO₂ conization from January 2003 to December 2004 were prospectively submitted to a HR-HPV test prior to surgery and at three and six months of follow-up. Women providing written informed consent with 24 months of follow-up were enrolled in the study group. A positive HPV test, involvement of resection margins, age at first intercourse, smoking habits, parity and age at conization > 50 years old were considered as risk factors for relapsing HG-CIN during follow-up, and were univariately and multivariately analyzed to discover any independent influencing factors. **Results:** Of HG-CIN 15.4% resulted not to be HPV related nor relapsing. The HPV clearance rate after treatment was 78.8%. Involvement of resection margins and HR-HPV DNA persistence post-treatment resulted as the only two statistically significant risk factors for HG-CIN recurrence (rate 3.8%). HR-HPV DNA persistence in follow-up resulted to be independent from other risk factors at multivariate analysis. **Conclusions:** Although able to reach a low recurrence rate of HG-CIN, laser CO₂ conization does not remove HPV infection completely from the cervix with a case of persistence in every five treated patients. In our experience this persistence in itself represents an independent risk factor for developing relapsing disease and constitutes the basis to introduce HPV testing even in the follow-up of patients treated for HG-CIN by laser CO₂ conization.

Key words: Laser CO₂ conization; HPV persistence; Recurrent CIN.

Introduction

There is general agreement in treating high-grade cervical intraepithelial neoplasia (HG-CIN) by conservative outpatient ablative or excisional procedures.

The main purpose of conservative treatment of CIN is the prevention of invasive cervical cancer along with preservation of the integrity of the genital system among a population of women who have a strong desire for subsequent pregnancies [1].

The use of conservative treatments in women with a diagnosis of CIN produces a reduction in the risk of invasive cancer by 95% during the first eight years after the procedure [2].

However women conservatively treated for HG-CIN represent a subgroup of the population in which the incidence of invasive cervical cancer still remains about five times greater than in the general population requiring a careful, long-term, post-treatment follow-up.

Due to this incremented risk compared with the screened population, the main intention of post-treatment follow-up protocols is maximizing test sensitivity in the early detection of persistent or recurrent diseases, suggesting the introduction of high-risk human papilloma virus (HR-HPV) DNA detection by Hybrid Capture II (HCII) or polymerase chain reaction (PCR) as a matching surveillance tool together with the current recommended cervical cytological checks [3].

The rationale of this association is based on former prospective studies about the natural history of HPV infection conducted following-up untreated lesions that reported a regression rate of 25%, a persistence rate of 61% and a progression rate of 14%, revealing a direct correlation between HPV infection and HPV-related CIN behavior [4].

Clinical trials comparing different conservative treatments have generally failed to show significant differences in outcome [5] and to our knowledge almost all the studies evaluating the risk of post-treatment CIN related to HR-HPV persistence have been conducted on women treated by the loop electrosurgical excision procedure (LEEP) or cold knife conization, with only one published report in which a subgroup of the population was treated by laser CO₂ conization [6-8].

The aim of this prospective cohort study was to evaluate the persistence rate of HR-HPV infection after laser CO₂ conization for HG-CIN and to examine if virus persistence might be considered an independent risk factor for residual/recurrent disease during follow-up.

Materials and Methods

All women submitted to laser CO₂ conization from January 2003 to December 2004 at our institution for a biopsy-proven HG-CIN were submitted to a HPV test before the surgical procedure and during follow-up examinations.

The personal history of established risk factors for cervical cancer (age, smoking habits, age at first intercourse, parity) and

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of comorbidities (other sexually transmitted infections, diabetes, chronic diseases and subsequent therapies) were collected for each patient before treatment.

All procedures were performed in an outpatient setting by two skilled laser surgeons using a SHARPLAN 733A CO₂ laser (ESC Sharplan, Yokneam, Israel) with a maximum power output of 50 Watt, used in continuous mode, connected to a Zeiss OPMI colposcope (Carl Zeiss, Oberkochen, Germany).

A preoperative colposcopic evaluation was first performed to identify the external limit of the lesion and to determine the extent of the excision. A 30-gauge needle was used to inject 5-8 ml of a 2% lidocaine solution with epinephrine at the 3, 6, 9 and 12 o'clock positions of the cervix.

The first step of the laser conization procedure was to direct the laser beam perpendicularly to the cervical surface achieving an initial 0.5-1 cm deep circular section and then to guide it obliquely by manipulating the on-going excised specimen using a small steel hook. To assure complete clearance of the lesion after excision, the crater base and the walls were vaporized with a defocused laser beam. Defocalized laser beam action was sufficient in every case to reach hemostasis without need for sutures.

Histological analysis reports of excised specimens were collected recording the status of margins.

Patient follow-up consisted of six close cytological and colposcopic examinations taking place at 3, 6, 9, 12, 18 and 24 months after conization and once per year up to five years before returning to the screening program.

A punch biopsy was performed in all cases of colposcopic abnormality observed during follow-up and only histologically-proven HG-CIN was considered as a persistent/recurrent lesion.

HPV DNA test was introduced at the 3-month and 6-month examinations in patients providing informed consent for HPV testing and was performed as follows, independent of the cyto-histological examination. After speculum insertion cervical cells were collected with a swab and suspended in 3 ml of saline solution prior to colposcopy. After centrifugation the pellet was incubated with proteinase K and digesting buffer at 56° C for 2-5 h followed by automated DNA purification (BioRobot EZ1, QIAGEN, Germany). PCR amplification of HPV-DNA sequences, using commercial multiplex PCR kits (Nanogen), was carried out first by L1 consensus primers for screening (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 66 and 68) and by E6-E7 primers for typing (6,11 for low risk; 16, 18, 31, 33, 35, 45, 52 and 58 for high risk). The amplified products were identified by agarose gel electrophoresis. The quality of the sample DNA was validated by detection of the housekeeping gene beta-globin as an internal control.

Only women with both 3-month and 6-month positive HR-HPV tests were considered as having persistent post-treatment infection.

A retrospective evaluation of the prospectively collected data was performed enrolling in the study group only women providing written informed consent to HPV testing with 24 months of follow-up without having missed more than one of the scheduled examinations. Diabetes, HIV and chronic steroidal therapy were considered as exclusion criteria commonly related to potential immunodepression.

Data about risk factors for relapsing disease were firstly analyzed in an univariate fashion applying Pearson's chi-square test for categorical variables, with $p < 0.05$ considered as significant. The same variables were then included in a multiple logistic regression analysis to assess if they were likely influencing HR-HPV persistence at follow-up or if, otherwise, this charac-

teristic might have an independent role in predicting relapsing CIN. Effect estimates are expressed as odds ratio (OR) with a profile likelihood-based on 95% confidence limits.

Results

Ninety-two women underwent laser CO₂ conization for HG-CIN during the study period. Among these, 78 patients matched the inclusion criteria and constituted the study group.

The mean age of the studied population was 38.3 years (22-62); the mean age at first intercourse was 17.8 years (range 12-25); at the time of laser conization 35/78 (44.9%) were nulliparous and 50/78 (64.1%) were smokers.

Histological analysis of cone-specimens resulted as HG-CIN in all cases with a margin-involvement rate of 12.8% (10/78).

Twelve women of 78 (15.4%) had a negative HPV test before the procedure. In this subgroup resection margins were negative in all cases, no patient presented a positive HPV test at follow-up and no relapsing CIN was detected after conization.

Among the remaining 66 women (84.6%), who presented a positive HPV test before treatment, 52 (78.8%) became negative at HPV testing in the follow-up, while 14 (21.2%) continued to be HPV-positive at the three- and six-month evaluations.

Among HPV-negative women in the follow-up, 48 of the 52 (92.3%) were still negative at the test performed within three months of the treatment, while four (7.7%), resulting positive at three months, were cleared of HPV infection at the six-month evaluation.

No negative test at three months became positive afterwards.

Three women of 66 (3.8%) developed a relapsing HG-CIN during follow-up and were submitted to a second laser conization; one case (1.9%) was observed six months after conization in the subgroup of 52 negative women at follow-up HPV testing and two (at 9- and 12-month evaluations) in the subgroup of 14 persistent positive patients (14.3%).

Involvement of margins and HPV infection persisting at follow-up (Table 1), resulted to be the only two statistically significant risk factors for relapsing HG-CIN on univariate analysis ($p < 0.01$ and $p < 0.05$, respectively).

Multivariate analysis revealed that none of the evaluated risk factors enhanced the likelihood of the HPV test to

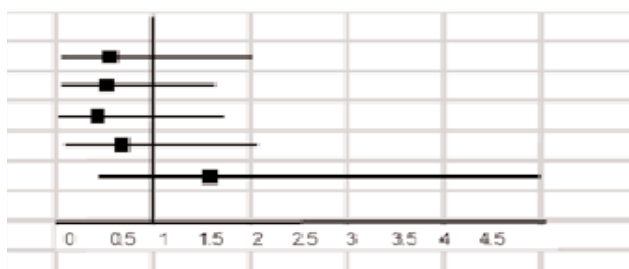
Table 1. — Pearson's chi-square analysis on residual/recurrent CIN2 + risk factors.

	Recurrence (n: 3)	No recurrence (n: 63)	p
Positive margins	2	8	$p < 0.01$
HPV DNA at follow-up	2	12	$p < 0.05$
Smoking	2	48	ns
Parity	2	41	ns
Age > 50 at conization	1	8	ns
> 17 yrs old at first intercourse	1	20	ns

Table 2. — Multivariate logistic regression on factors influencing HR-HPV DNA persistence at follow-up.

	OR	C.I. (95%)
Positive margins	0.45	0.10 - 2.01
Smoking	0.42	0.10 - 1.67
> 50 yrs old at conization	0.37	0.08 - 1.74
< 17 yrs old at first intercourse	0.60	0.17 - 2.05
Parity	1.53	0.47 - 4.99

OR = odds ratio.



remain positive in the follow-up so that HPV DNA persistence after laser conization for HG-CIN appeared to be an independent risk factor for relapsing disease (Table 2).

Discussion

Persistence of HR-HPV infection after conservative treatment of CIN has already been widely pointed out to be strongly correlated with relapse of the disease. This is probably due to conservative treatment which, by excision of infected epithelium, is able to reduce HPV DNA presence – as demonstrated by the declining HPV antibody levels – but not to completely remove it [9].

The persistence rate of HPV infection after conservative treatment of CIN varies in the literature from 6% [10] to 50% [11]. This large variability probably derives from differences in patient selection criteria and treatment methods. For example Nagai *et al.* [8] evaluated HPV DNA persistence in a group of patients consistent in the indications for treatment (histologically proven CIN III in all cases), but not in the method of treatment since they were differentially submitted to cold knife or laser CO₂ conization. Conversely Kucera *et al.* [10] evaluated the rate of HPV persistent infection in a group of patients treated totally by electrosurgical excision of the transformation zone (LLETZ) for CIN, without distinguishing between cases of low- and high-grade disease. In the same way Bollen *et al.* [12] evaluated a group of patients treated by LEEP for cervical dysplasia without classifying them into grades of disease severity.

Moreover some of the studies reporting high rates of HPV DNA persistence include patients treated by destructive techniques with higher rates of relapsing CIN compared with patients treated by excisional methods. Distefano *et al.* [11] found that only 75% of their patients treated by electrosurgical or diathermic ablation were negative for CIN at follow-up and furthermore that 50% of them remained HPV DNA positive.

To our knowledge with the exception of Nagai *et al.* [8], who included in their series some patients treated by laser CO₂ conization, all the previous studies evaluating the role of persisting HR-HPV infection after conservative treatment for CIN were conducted in patients treated by LEEP/LLETZ, cold knife conization or destructive techniques.

For the first time our study has provided an evaluation of persisting HR-HPV infection in an homogeneous

group of patients treated totally by laser CO₂ conization for HG-CIN.

We observed that in approximately 21% of HPV infected patients, HPV DNA was persistently detected in the cervix after therapeutic conization and that it can be considered an independent risk factor for residual/recurrent disease.

The recurrence rate observed in our experience by laser CO₂ conization (3.8%) is one of the lowest reported in literature where rates range from 0.3% to 23% of women with uninvolved excisional margins up to 84.8% of women with positive margins [13, 14]. This is probably due to the fact that our study population can be considered consistent from a methodological view point, in that conization was technically standardized and performed by only two skilled laser surgeons. The low rate of recurrence may be correlated even to the advantage brought about by the conization using the laser technique, which guarantees a sufficient depth of resection and vaporization of the crater walls, combining the advantages of excisional and destructive procedures, as previously observed [15].

The close selection of patients and the standardization of the operative technique overcome methodological bias in analyzing risk factors for persistence of HPV DNA, thus reducing to a minimum the impact of differences in treatment modalities and selection criteria observed in previous experiences.

Moreover an HPV test before conization was introduced into the study to exclude a population subgroup from the analysis whose cervical lesions were not HPV-related and which presumably had different biology in response to the treatment and persistence/recurrence rate. This hypothesis was derived from Alonso *et al.*'s results where a linear relationship between pretreatment HR-HPV load and risk of residual/recurrent disease was described [6].

In the present study 15.4% of patients with HG-CIN resulted to be HPV negative, in agreement with some previous studies such as the one by Kucera *et al.* [10] who observed that HPV tests resulted negative before treatment in approximately 16% of their case series.

In our experience among pretreatment negative HPV patients, no CIN recurrence or HPV DNA persistence developed, confirming Alonso *et al.*'s hypothesis that absence of HPV DNA prior to treatment might be considered a positive prognostic factor.

In our study HPV testing was performed at the third and sixth month evaluations to obtain data comparable with previous studies reporting conflicting outcomes on time of clearance of HPV DNA after treatment for CIN [7, 9, 16].

In our series 92.3% of the clearance rate was reached within three months of the treatment and only 7.7% of patients achieved absence of HPV DNA afterwards. Moreover none of these ultimate patients was complicated by relapsing lesions which occurred only in the case of persisting HPV DNA up to six months from the conization. This suggests, in agreement with Costa *et al.* [7], that HPV DNA tests might be introduced at least singularly at six-month evaluations to improve follow-up sensitivity in distinguishing high-risk patients for relapsing CIN.

Together with some established risk factors for cervical cancer we decided to include age at the time of treatment as part of the analyzed risk factors for relapsing disease on the basis of a previous study by Verguts *et al.* [17] which described women older than 50 at conization having a higher recurrence rate than younger ones with a statistically significant difference.

The present study did not confirm Vergut *et al.*'s result and none of the chosen risk factors for cervical cancer (age at first intercourse, smoking and parity) resulted to statistically influence residual/recurrent HG-CIN after laser CO₂ conization.

In our experience HPV persistence was not the only significant risk factor for CIN at follow-up because involvement of the resection margins, in agreement with others [18-22], also appeared to be a predictor of disease recurrence.

However, multivariate analysis cleared up that HPV DNA persistence at follow-up was not influenced by any of the other analyzed risk factors, including involvement of margins. This confirms that women with persistent HPV infection after laser CO₂ conization for HG-CIN have a higher risk of residual/recurrent disease – aside from the fact that this risk could be enhanced by other variables such as involvement of the resection margins – and deserve closer follow-up than women with negative HPV tests after treatment.

Conversely to Gok *et al.* [23] and Verguts *et al.* [17] who found a 100% negative predictive value (NPV) (absence of recurrent disease among the population with negative HPV tests at follow-up), our study revealed that approximately 2% of patients with negative HPV tests after treatment will have relapsing HG-CIN at a subsequent follow-up. This result is in agreement with the meta-analysis of Zielinsky *et al.* [19] who described a NPV of HPV testing alone in the follow-up of treated patients of 98%.

Even if our study is biased by a small case series, 2% of false negatives is, in our opinion, too large a risk to run in promoting HPV testing as only a single initial follow-up evaluation and to skip subsequent controls for patients resulting negative. We could agree with Paraskevaidis *et al.* [24] who favor the use of HPV tests only in conjunc-

tion with cytology to primarily select a precise population subgroup at major risk for relapsing disease, and eventually skip visits only for those resulting negative to both HPV tests and cytology.

In conclusion, laser CO₂ conization, although able to reach a low recurrence rate of HG-CIN – taking advantage of being a mixed excisional and destructive technique – does not remove HPV infection completely from the cervix with a case persistence of every five treated patients. In our experience this persistence in itself represents an independent risk factor for developing recurrent disease and constitutes the basis to introduce HPV tests at six-month evaluations even in the follow-up of patients treated for HG-CIN by laser CO₂ conization.

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