Universitat de Girona
Facultat de Medicina

Intraoperative post-conization human papillomavirus testing: an earlier predictive factor of recurrences in patients treated for cervical intraepithelial neoplasia

FINAL DEGREE PROJECT

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1. ABBREVIATIONS

HPV	Human Papillomavirus
HR-HPV	High-Risk Human Papillomavirus
CIN	Cervical Intraepithelial Neoplasia
LSIL	Low-grade Squamous Intraepithelial Lesion
HSIL	High-grade Squamous Intraepithelial Lesion
LEEP	Loop Electrosurgical Excision Procedure
VLPs	Virus-Like Particles
IOP-HPV	Intraoperative Post-conization Human Papillomavirus

2. ABSTRACT

Background: Human papillomavirus (HPV) testing is now the better predictive factor of recurrent disease during follow-up in women operated for cervical intraepithelial neoplasia grades 2-3. The time between the surgery and the first HPV test depends on the surgical margins: 6 months (if margins are negative) and 4 months (if margins are positive). So, we have to wait a considerable time to know if the virus is still present after the treatment.

Purpose: The aim of the present work is to determine if the effectiveness of human papillomavirus testing performed in the same surgical procedure (immediately after the conization), is the same in comparison to human papillomavirus testing performed after 4-6 months of the operation. In this way, cases with recurrent disease during follow-up would be earlier predicted.

Design: Prospective, observational and multicentric study.

Population: Women diagnosed with cervical intraepithelial neoplasia grades 2-3 undergoing cervical conization in a public hospital located in the province of Girona, between 2017 and 2020.

Methods: An endocervical sample will be obtained intraoperatively with a cytobrush from the cervix remaining after the conization. The material will be kept in Specimen Transport Medium and processed using the commercially available Hybrid Capture System. Patients will be followed-up for 3 years. The intraoperative HPV test will be compared with HPV test at 4 and 6 months. It will also be compared with the conventional cone margins indicator of recurrences.

Keywords: Cervical intraepithelial neoplasia, conization, human papillomavirus testing, post-treatment follow-up.

3. INTRODUCTION

3.1. Epidemiology of cervical cancer

Cervical cancer is the third most common neoplasia among women worldwide. It is estimated that in the world are diagnosed approximately 500.000 new cases of cervical cancer each year, of which about 80% occur in developing countries (1).

In Spain the incidence of cervical cancer is low, with a rate population mean of 7.6 per 100.000 woman-years. Incidence rates have been, on the whole, stable in recent years, although there has been a clear and sustained increase in women under 45 years old. This is reflected in mortality caused by cervical cancer in our country: 3.6 per 100.000 woman-years, being the thirteenth cause of death for cancer in women at any age, but the sixth for women between 15 and 44 years (2).

At a local level, in the sanitary region of Girona, according to the last CanGir report submitted by la Unitat d'Epidemiologia i Registre de Càncer de Girona, between 2007 and 2009 the incidence of cervical cancer was approximately 33 (2,4%), 24 (1,7%) and 26 (1,8%) respectively. In addition, incidence projections for 2014 were about 26 cases.

The natural history of the cancer is known. The slow progression and processing of premalignant cancer lesions confers a prolonged latency period. Those, bring the possibility to establish screening measures against this cancer. It is possible not only to recognize these premalignant phases but also to treat them simply and effectively.

Furthermore, recognition of a necessary infectious cause has opened the possibility of vaccinating against this disease (2).

Once the invasive cancer has been established, the prognosis is directly related to the stage at time of diagnosis. In developed countries, thanks to the knowledge of the prognostic factors and multidisciplinary treatments availability, cervical cancer mortality has decreased a 75% over the last 50 years. However, in developing countries it remains a cancer with a mortality rate close to 50% (2).

3.2. The association between human papillomavirus and cervical cancer

Human papillomavirus (HPV) persistent infection of the uterine cervix is a necessary risk factor for development of cervical intraepithelial neoplasia and cervical cancer. Also after treatment for cervical dysplasia, HPV can be present and promote the recurrence of cervical disease (3).

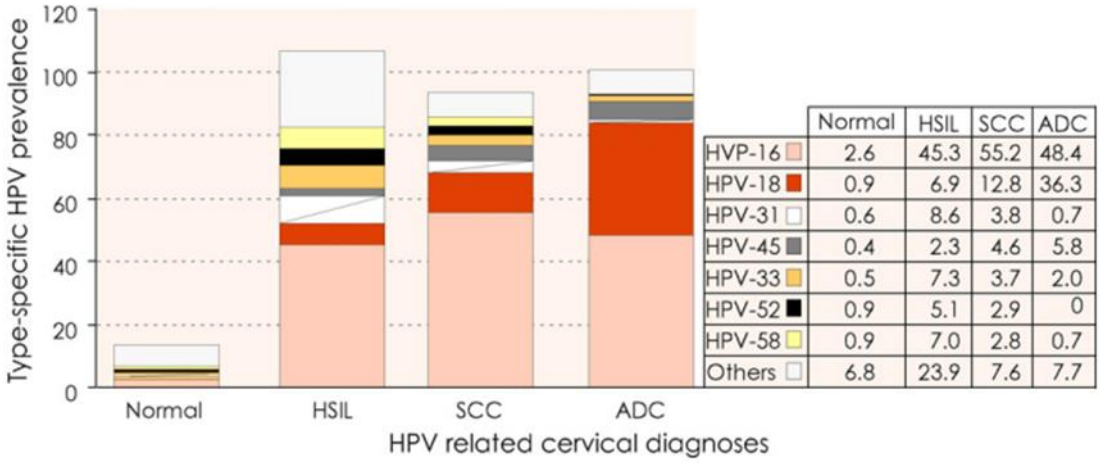
While the involvement of HPV in causing benign warts was already known, the first evidence of association between human cancer and certain HPV types was proposed more than thirty years ago by H. Hausen and colleagues. Subsequent epidemiological and biological studies have confirmed the direct role of several mucosal HPV types in the development of cervical cancer and other epithelial tumors (4).

In 2007, a meta-analysis was published giving data on the worldwide HPV prevalence in women with normal cytology (5). According to its results, at a given point in time, 10.4% of the women worldwide are positive for HPV DNA in the cervix, indicating that HPV is one of the most common sexually transmitted infections. In Spain, it is estimated that of all sexually active women, 14.3% have a detectable HPV infection (1).

Natural history studies have contemplated the HPV DNA incidence by age groups. Rates of exposure in young women are high and often include multiple types. More than 90% of infections in this group of women are transient and irrelevant from the oncogenic point of view. However, women over 30 years have lower rates of HPV infection, but with higher percentage of persistence, leading to increased risk and incidence of precursor lesions from this age (6).

Risk factors for progression of an HPV infection identified in case-control studies include cigarette smoking, long-duration oral contraceptive use and multiple live births (7). It is among women with HPV persistence that cervical cancer may develop (3).

As has been said before, it is important to underline not all types of HPVs are associated with cervical cancer. Of the more than 100 different varieties of HPV existing, only serotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 are consistently classified as high risk (HR) HPVs. On the other hand, low risk HPVs (e.g. types 6 and 11), are mainly linked to benign genital warts (4).



	Normal	HSIL	SCC	ADC
HVP-16	2.6	45.3	55.2	48.4
HPV-18	0.9	6.9	12.8	36.3
HPV-31	0.6	8.6	3.8	0.7
HPV-45	0.4	2.3	4.6	5.8
HPV-33	0.5	7.3	3.7	2.0
HPV-52	0.9	5.1	2.9	0
HPV-58	0.9	7.0	2.8	0.7
Others	6.8	23.9	7.6	7.7

Figure 1. Type-specific HPV prevalence across the spectrum of HPV related cervical diagnoses. Multiple infections counted several times. ADC: Adenocarcinoma; HSIL: High squamous intraepithelial lesions; SCC: Squamous cervical carcinoma (5).

Infections with HPV 16 and HPV 18 are the most carcinogenic. These two virus types are known to be responsible for 70% of all invasive cervical carcinomas (50% and 20% respectively), followed by HPV 45, 31, and 33 (4,8).

3.3. The effects of HPV in cervical cells: physiopathology of the infection

The genome of all HPV types consists of a small double stranded DNA filament and encodes approximately eight genes. According to protein expression during the viral cycle two functional genome regions have been identified: one coding region containing the early genes, E1, E2, E4, E5, E6, and E7 and another region containing two late genes, the major (L1) and minor (L2) capsid proteins. In addition, the HPV genome has a non-coding region, named long control region (LCR), which includes most of the regulatory elements involved in viral DNA replication and transcription.

Women acquire HPV through sexual intercourse with an infected partner. In the female genital tract the area adjacent to the border of the endocervix and ectocervix, known as the transformation zone or squamocolumnar junction, appears to be the preferential site for infection. Once there, HR-HPVs get into epithelial cells of the cervix and have the capacity to pass from episomal to host genome integrated form.

Biological studies have been mainly focused on HPV16 and HPV18, since they are the most frequent types detected in cervical cancer worldwide. These studies have clearly demonstrated that E6 and E7 play a key role in carcinogenesis because they are directly

involved in promoting cellular transformation and altering pathways related to the immune response, as well as cellular transformation by targeting several cellular proteins.

The most characterized example of viral/cellular protein interaction is the ability of HPV16 E6 to degrade the tumour suppressor protein p53 via the proteasome pathway. The p53 is a transcription factor that is activated in response to stress or DNA damage and positively regulates the expression of genes involved in the control of cell cycle arrest or apoptosis. Since the major role of p53 is to safeguard the integrity of the genome, cells expressing HPV16 E6 show chromosomal instability, which greatly increases the probability that HPV-infected cells will evolve towards malignancy (4,9).

3.4. Precancerous lesions: types and management

Over the years, there have been many different classifications of precursor lesions of cervical cancer that have been changing as progress in knowledge has been made.

In the early 70s, Ralph M. Richart introduced a histological classification for the precancerous lesions. He introduced the term 'Cervical Intraepithelial Neoplasia', known as CIN, and established 3 different levels. Laboratory-based studies showed that the differences between the different grades of dysplasia are quantitative as well as qualitative:

- CIN 1: Corresponds to mild dysplasia and abnormal squamous cells are located in the lower one-third of the epithelium.
- CIN 2: Corresponds to moderate dysplasia and abnormal squamous cells are located in the basal two-thirds of the epithelium.
- CIN 3: Corresponds to severe dysplasia and abnormal squamous cells are located throughout almost or all the thickness of the epithelium.

Years later, the Bethesda first classification of cervical cytology was introduced. It combined similar intraepithelial diagnoses into 2 broad categories:

- LSIL (Low-grade Squamous Intraepithelial Lesions): represents the changes of CIN 1.
- HSIL (High-grade Squamous Intraepithelial Lesions): represents the changes of CIN 2 and 3.

Both classifications are still indistinctly used in diagnosis of cervical precancerous lesions. While CIN1/LSIL is relatively common and represents the usually benign cytopathological signs of HPV infection, CIN 2-3/HSIL is rare and represents a truly premalignant condition (10,11).

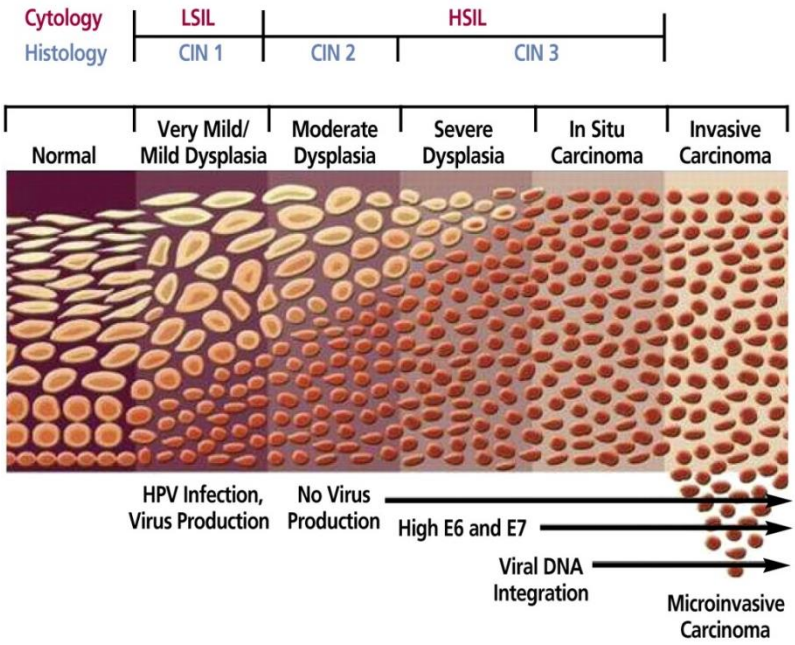


Figure 2: Progression of persistent human papillomavirus (HPV) infection in the cells of the cervix (21).

So, early detection and treatment of HSIL are important for the prevention of cervical cancer (12). These, if left untreated, bears a significant risk of developing into invasive carcinoma (13). In fact, it is known that more than one-third of CIN 3 lesions will progress to invasive cervical cancer within 10–20 years (7).

Incidence and mortality resulting from cervical cancer has dramatically decrease thanks to the implementation of conventional Papanicolaou test cytological screening for detection cervical precancerous lesions since 1940s (14). In countries where it has been appropriately apliccated with a coverage of over 70% of the population, both the incidence and mortality has decreased an 80% (6).

Lately, some studies have found that using HPV DNA testing alone in primary screening has performance superior to Papanicolaou test (7). In addition, the high predictive value of a negative HPV DNA test allows for safe extension of screening intervals to at least once every 5 years, increasing the efficiency of cervical cancer screening with minimal impact on cancer risk (15). Despite knowing this, exclusive cervical cytology continuous current in the primary prevention of cervical cancer. The transition to HPV screening test is still a target that should be achieved within 3-5 years. In regard of co-test (HPV test + cytology), it doesn't add higher performance and effectiveness to HPV DNA test as a unique method

and involves a greater expenditure of resources. Hence, the choice of co-test must have a temporary purpose while technology for the detection of HPV DNA is incorporated and implemented (6).

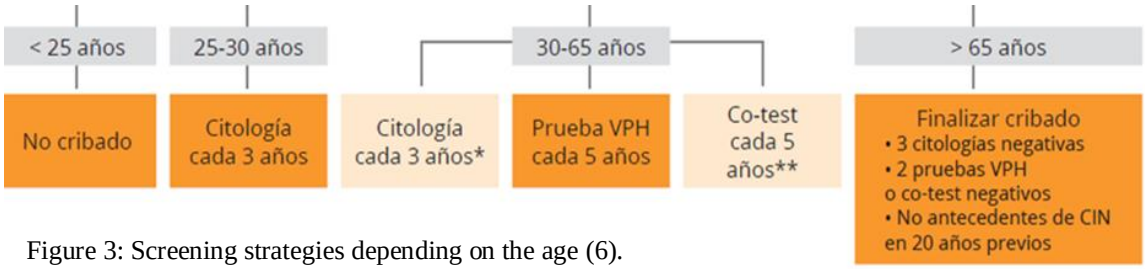


Figure 3: Screening strategies depending on the age (6).

After finding atypical squamous cervical cells with the cytology, biopsy of the lesion guided by colposcopy and subsequent histological examination, are indispensable to reveal the changes that classify the pathology.

LSIL, initially, doesn't need a specific treatment due to the high probability of spontaneous regression (16). Conversely, since the early 1990s the loop electrosurgical excision procedure (LEEP) has been a popular modality for local treatment of HSIL (called conization) because of its many advantages over other resection techniques, as cryosurgery and laser vaporization (17). Conservative treatment with LEEP is both a diagnostic and a therapeutic procedure that can effectively eradicate CIN 2-3 at every stage of a woman's life (18).

3.5. HPV vaccination in patients treated due to HSIL

Since 2006 two HPV vaccines, Gardasil and Cervarix, are commercially available. Both vaccines contain virus-like particles (VLPs) from HPV16 and HPV18, which, as described above, are responsible for approximately 70% of the cervical cancers worldwide. In addition, Gardasil includes VLPs from the low-risk HPV 6 and 11, which are associated with approximately 90% of the genital warts (4).

Women treated for cervical lesions are a particularly susceptible group to develop new lesions or even cervical cancer. The risk of cancer 10-20 years after HSIL/CIN 2-3 treatment is 5-10 times higher than in general population (6).

Several clinical studies has clearly demonstrated that so far both products showed high efficacy in preventing the development of recurrence in women who have undergone

treatment for cervical lesions, inducing a robust response in immunized patients. HPV vaccines administered in women after conization for HSIL/CIN 2-3 can reduce the recurrence of ≥ HSIL/ CIN2 lesions in 60-80% of cases compared with patients who have not been vaccinated (6).

Another advantage of the vaccine is that relapse prevention has repercussions on future pregnancies, thus a second conization is associated with a risk 2 times higher of preterm birth (6).

An important drawback is the high cost of these vaccines. The vaccine consists in three doses and each one costs about 120 €. Unfortunately, this prevents their widespread application in the vast majority of the treated women.

3.6. Current management guidelines to detect recurrent disease

The incidence of recurrent disease after LEEP conization varies between 5% and 30% (13). Therefore, close follow-up of patients after treatment is recommended to identify failures requiring re-excision.

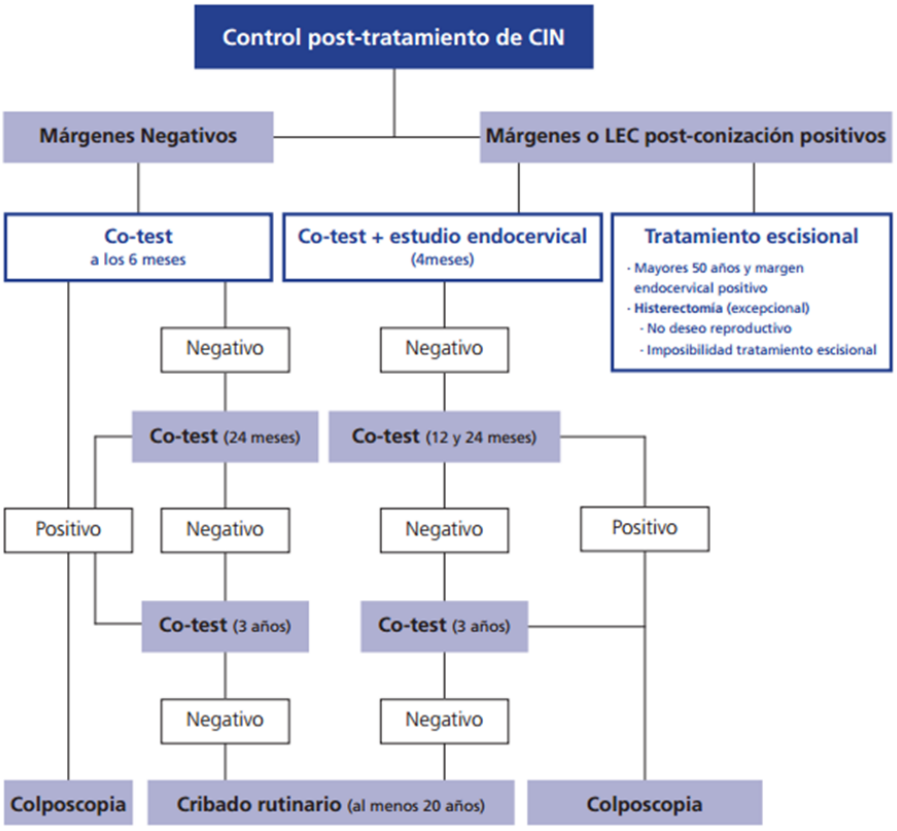


Figure 4: Current management protocol after CIN 2-3 treatment (20).

Current follow-up protocol of patients treated by conization is mainly based on the combination of cytological analysis + HR-HPV testing (co-test) (18).

Several studies have shown the association between persistent HPV infection after HSIL treatment and its recurrence on time (12). In fact, HR-HPV testing is more sensitive than cytology at the expense of specificity (19).

Depending on surgical margins, co-test is done 6 months after the operation (if margins are negative) or 4 months after the operation (if margins are positive) (20).

The time between the surgery and the first HPV test is considerable, because during this period neither the physician nor the patient has information about the truly risk of recurrent disease during follow-up.

The margin status of the conization material has been found suboptimal to predict relapse of cervical disease (19). Although pathological margin status is generally considered as a risk factor for the development of recurrent CIN, a free margin does not always indicate complete excision because of the possibility of multifocal lesions or inadequate specimen tissue caused by ablative conization. On the other hand, most women with involved margins will not develop recurrent CIN (13).

At present, patients are quoted after 1 month of the LEEP with two finalities: to verify that the wound has healed properly, and to know the histological results of the surgical piece. This last includes: corroborating the type of lesion and its location, and knowing the degree of margins affectation. But, as it has been said before, free margins don’t allow the doctor to guarantee the disease is not going to reappear.

Taking into account de high rates of relapse, it is not easy for the patient to stay calm. The HPV caused the lesion. It will be necessary four or six months more to know if the virus is still present.

4. JUSTIFICATION

If the HPV test was performed in the same surgical procedure, immediately after the conization, in the first visit (1 month after the operation) doctors would have information about the truly risk of recurrence.

After excision of the lesion, a high percentage of women clear not only the lesion but also the infection, and become negative for HPV. So, HPV testing performed intraoperatively should be negative when the lesion and the infection has completely been removed, or positive in the case of persistence of the infection.

The purpose of this longitudinal study is to evaluate the effectiveness of intraoperative post-conization human papillomavirus (IOP-HPV) testing to predict recurrent CIN during follow-up. We want to compare it with the current strategy of performing HPV test 4 or 6 months after conization. If the diagnostic efficacy of both strategies to identify treatment failure (i.e. recurrent disease during follow-up) is the same, IOP-HPV testing would earlier identify the patients with high probability of relapse. Hence, an earlier identification of patients with treatment failure might contribute to avoiding delays in the re-treatment of these patients.

Another advantage of IOP-HPV testing is that it will always reflect a persistence of the previous HPV infection. Instead, conventional follow-up doesn't differentiate between persistent or new post-treatment HPV infection. This is important because most of the new ones will clear spontaneously (according to the natural history of HPV infection) and they will not produce another CIN.

Moreover, thanks to the high negative predictive value of the test, it would be possible to alleviate patients' anxiety about treatment failure months earlier than nowadays.

Finally, although several studies have found that it isn't a good tool to predict treatment failure, the follow-up protocol is actually mainly guided by the margins of the surgical specimen. This study is a chance to corroborate this suboptimal utility of the cone margins proving that IOP-HPV testing detects risk of recurrence more accurately. Thus, this study would give a good opportunity to raise an update of the current management protocol after CIN 2-3 treatment.

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6. HYPOTHESES

6.1. Main hypothesis

This study will assess the following null hypothesis: To predict recurrent disease during follow-up in women operated for cervical intraepithelial neoplasia grades 2-3, the effectiveness of intraoperative post-conization human papillomavirus (IOP-HPV) testing is the same in comparison to human papillomavirus testing performed after 4-6 months of the operation. So, IOP-HPV testing can earlier predict cases with recurrent disease during follow-up.

6.2. Secondary hypothesis

Intraoperative post-conization human papillomavirus testing is a better predictive factor of recurrent disease during follow-up than the conventional cone margins indicator in women operated for cervical intraepithelial neoplasia grades 2-3.

7. OBJECTIVES

7.1. Main objective

The aim of the present work is to evaluate the effectiveness of intraoperative post-conization human papillomavirus testing. We want to compare it with the current approach of performing the human papillomavirus test 4-6 months after the conization. The finality is to predict recurrent disease during follow-up earlier than nowadays in women operated for cervical intraepithelial neoplasia grades 2-3.

7.2. Secondary objective

To compare the effectiveness of intraoperative post-conization human papillomavirus testing versus the margins of the surgical specimen to predict recurrent disease during follow-up in women operated for cervical intraepithelial neoplasia grades 2-3.

8. METHODS

8.1. Study design

It's a prospective and observational study.

8.2. Study population

The study population will be women diagnosed with CIN 2-3 undergoing cervical conization in a public hospital located in the province of Girona, between 2017 and 2020. Therefore, it will be a multicentric study involving the following 9 hospitals: Hospital Universitari Dr. Josep Trueta de Girona, Hospital Santa Caterina de Salt, Hospital de Blanes, Hospital de Palamós, Hospital de Campdevànol, Hospital de Figueres, Hospital de Puigcerdà, Hospital de Calella and Hospital Sant Jaume d'Olot.

A biopsy of the lesion guided by colposcopy will be used for preoperative diagnosis. The operation will be done using the LEEP technique.

8.2.1. *Inclusion criteria*

- Women diagnosed with CIN 2-3.
- Women undergoing cervical conization by the LEEP technique.
- Women who have given informed written consent.

8.2.2. *Exclusion criteria*

- Women under 18 years old.
- Women not able or not willing to attend to the follow-up visits.
- Re-treatment of a CIN relapse.

8.3. Sample selection

A consecutive non-probabilistic sampling will be used in this study. The recruitment will take place in each hospital involved in the study throughout three years.

A cervical biopsy is usually done after an abnormality has been found during a routine pelvic exam or Papanicolau test. Women who have undergone cervical biopsy will attend to the doctor appointment to search for the diagnostic results. If pathologists confirm a CIN 2-3, the necessity of a conization to remove the lesion will be explained. Women accepting the operation will be our candidates. After signing the informed consent for the conization, they will be given an information sheet describing the study (annex I). If a woman is interested, doctor will obtain her informed consent (annex II) and she will become a member of our study.

8.4. Sample size

The sample size has been obtained with the program GRANMO Calculator. Accepting an alpha risk of 0.05 and a beta risk of 0.2 in a two-sided test, 414 subjects are necessary to recognize as statistically significant a difference greater than or equal to 7%. A dropout rate of 10% has been anticipated. It is estimated that in the province of Girona approximately 140 conizations might take place within a year. This means that we will need 3 years in order to collect all the amount of patients needed for the study.

If the results of the study find no differences in the efficacy between the two approaches (IOP-HPV test and HPV test after 4-6 months of the conization) to predict recurrences, we will be able to support the null hypothesis, taking into account that minimum differences can exist (less than 7%).

8.5. Interventions

8.5.1. *Cases selection*

To provide the most accuracy and validity of the preoperative diagnostic cervical biopsy, the extraction and the following specimen evaluation will be performed as it is explained below:

The patient will lie down on an exam table with her feet in stirrups. Then, an experienced gynecologist will insert a speculum into the vagina to keep the canal open during the procedure. After preparing the cervix with 5% acetic acid, a colposcope will be used to magnify the view. Once located the transformation zone, the gynecologist will focus on identifying some lesion. Colposcopic findings will be described following the criteria of

the International Federation for Cervical Pathology and Colposcopy (annex III). If a lesion is identified, a punch biopsy will be obtained. The instrument used for these biopsies is called biopsy forceps. Finally, gynecologist may put silver nitrate to the biopsy site to reduce the amount of bleeding. In addition, the patient will be advised to avoid having sexual intercourse at least 24 hours.

The small pieces of tissue taken from the cervix will be put in a container with formalin. The container will be labeled with the patient's identifying information. It will be then sent to the pathology lab. There, the tissue will be put in a small box called cassette to be processed by a machine. After processing, which may take a few hours, the tissue sample will be put into a mold with hot paraffin wax. The wax cools to form a solid block that protects the tissue. This paraffin wax block with the embedded tissue will be then cut into very thin slices by a microtome. These thin slices of the specimen will be placed on glass slides and dipped into a specific dye to change the color of the tissue. The color makes cells easier to see under a microscope. At this point, the pathologist will look at the tissue under a microscope. Histological findings will be described following the Ralph M. Richart histological classification for the precancerous lesions explained above in the *introduction* section.

8.5.2. *LEEP conization and IOP-HPV testing*

The LEEP conization is an ambulatory surgery. Therefore, patients will usually come to the hospital the same day of the operation. The procedure will be as it follows:

The entire operation takes less than an hour. It will be done with general or regional anesthesia. Firstly, the cervix will be exposed using a speculum. Then, the area of abnormality will be delineated with acetic acid and lugol iodine. The loop size will be selected according to the size of the area to be excised. In all cases the excision will be performed under colposcopic guidance. After the excision, careful inspection of the cervical bed will be performed with selective coagulation of the bleeding areas.

Subsequently, an endocervical sample will be obtained with a cytobrush from the cervix remaining after the conization and kept in a vial with 1ml of Specimen Transport Medium (STM).

After the operation, the patient will be usually authorized to leave the hospital (with an accompanying person) after a short resting period. She will be given some instructions such as avoiding carrying huge weights or sexual intercourse at least 1 month.

8.5.3. *Conization specimen and IOP-HPV test processing*

LEEP conization specimen will be put in a container with formalin. The container will be labeled with the patient's identifying information. It will be then sent to the pathology lab. There, the specimen margins will be painted with Indian ink. Then, the specimen will be cut and put in different cassettes. After being processed, cut and placed on glass slides like cervical biopsies, the excisional sample will be thoroughly examined under the microscope. In this way, margins affectation will be known.

As for the detection of HPV, it will be performed using the commercially available Hybrid Capture System on the material collected in the liquid-based media. All the samples will be analyzed for the presence of the following high-risk HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. The test will provide relative quantification of the viral load present in each individual sample. A relative light unit (RLU) of 1 will be used as the cut-off to classify a cervical sample as positive or negative for HPV.

8.5.4. Follow-up routine

Post-treatment follow-up visits will be scheduled as is depicted in *Figure 5*.

The aim of these appointments will be to find recurrences of the disease. For the study, the absolute number and the percentage of recurrent CIN (1, 2 or 3) cases will be recorded over a 3-year follow-up period, when most of the recurrences take place. This is not to say that patients won't be followed after this period, but they will reenter the routine screening of the general population.

One key point in the follow-up period is the first appointment (after 1 month of the operation): On the one hand, it will be when the doctor and the patient will know the IOP-HPV testing result and if cone margins are affected (positive) or not (negative). The cone margins status will guide the time frequency and the tests of the following visits. On the other hand, the doctor should be in charge of transmitting to the women the importance of using protection methods if they have sexual intercourse. This prevents the patients from acquiring a new post-treatment HPV infection. A part from the relevance in respect of the

patients' health, it guarantees that positive HPV tests after 4-6 months of the operation will only reflect persistence cases of HPV infection.

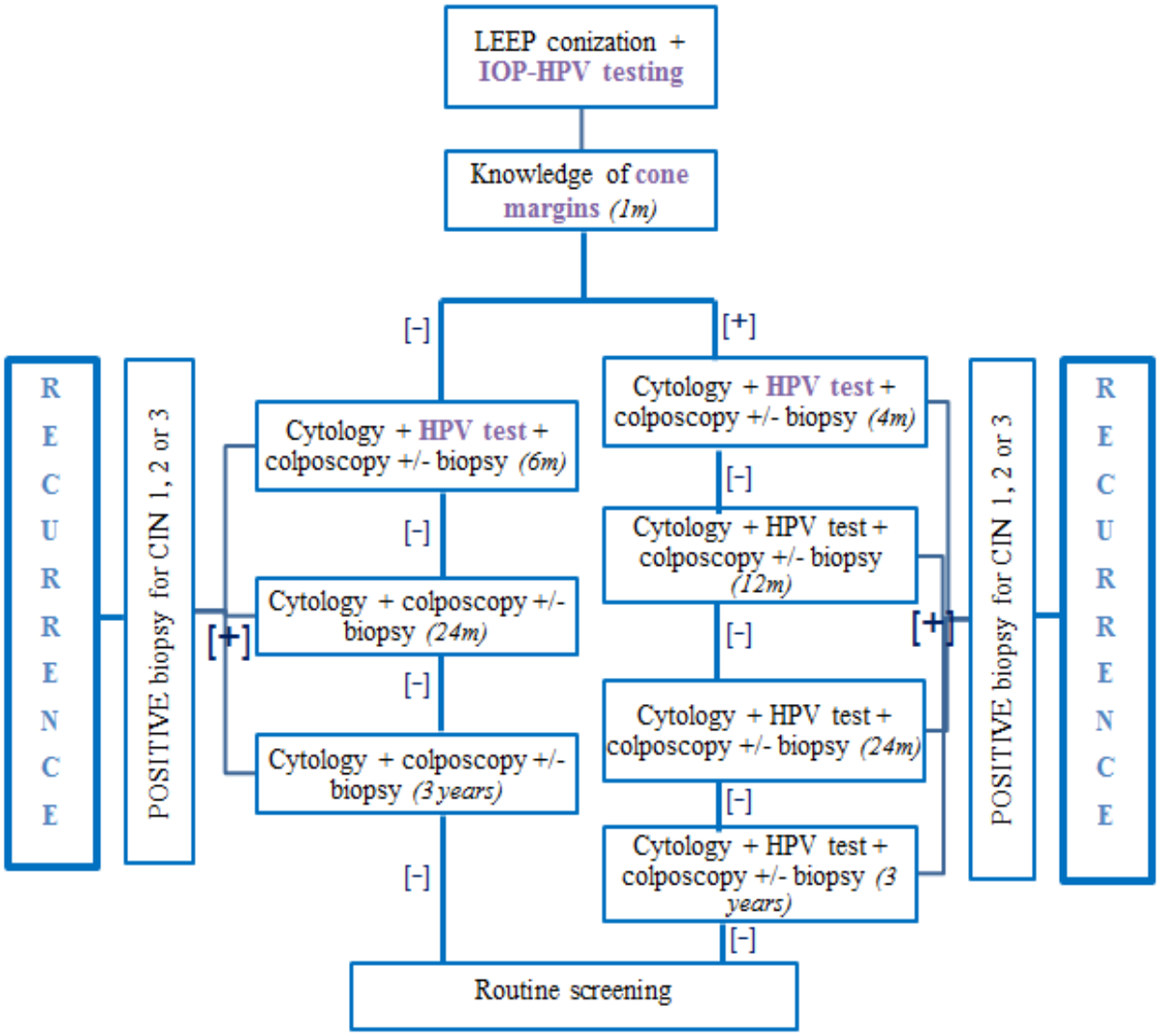


Figure 5. ( ): Time after LEEP conization; IOP-HPV: Intraoperative post-conization human papillomavirus

Regarding the cervical cytologies, they will be performed in each visit after the first appointment. The conventional following technique will be used to collect and process the specimens:

The patient will be in the dorsolithotomy position. A speculum of appropriate size will be inserted into the vagina without lubrication. First of all, a sample of the exocervix will be taken using a wooden spatula. Then, the endocervix sample will be obtained with a brush.

The sample on the spatula will be spread lengthwise down one half of the slide surface, using a single uniform motion. The endocervical brush will be then rolled along the remaining half of the slide surface. Finally, the entire slide will be labeled with the patient's identifying information. It will be then sent to the pathology lab. There, the sample will be stained using the Papanicolau method and will be evaluated by a skilled cytopathologist. Cytological findings will be described following the 2001 Bethesda classification (annex IV).

Regarding the HPV tests, they will be performed and processed exactly like intraoperative ones (IOP-HPV testing). In order to detect persistent HPV infections, they will be done after 4 months of the operation (if cone margins are positive) or 6 months after the operation (if cone margins are negative). Thereafter, HPV testing will be performed in all the following visits in cases with margins affected. Instead, HPV detection will be interrupted in patients with free margins and also a negative result in the 6-month after treatment HPV test.

Finally, a colposcopy will be performed in each visit after the first appointment. If a lesion is identified, a biopsy will be obtained. The technique will be exactly the one explained above in the *cases selection* section.

Treatment failure will be considered in cases diagnosed with recurrent CIN (1, 2 or 3) over the 3-year follow-up period.

8.6. Variables

8.6.1. *Independent variable*

The independent variables are represented by the two compared approaches used for detecting treatment failure. Both are based on the same technique: the HPV test. What differentiates them is the interval between LEEP conization and the realization of the test. IOP-HPV testing will be performed in the same surgery procedure, immediately after removing the surgical specimen. This approach will be compared with the currently one of doing HPV test 4-6 months after the operation. The results will be expressed by the absolute number and percentages of positive and negative cases.

8.6.2. *Dependent variable*

The dependent variable in this study is the treatment failure. It will be estimated by measuring the absolute number and the percentage of recurrent CIN (1, 2 or 3) over a 3-year follow-up period.

8.6.4. *Covariables*

These variables are included in the study to get further information about our participants. All of which will be relevant to perform a multivariate analysis to evaluate its individual interaction effects with regard to recurrent CIN.

1) Age: in years.
2) Tobacco: number of cigarettes.
3) VIH: viral load < or ≥ 100.000 copies/ml.
4) Preoperative histological grade: CIN 2 o 3, based on the biopsy findings. Histological classification is explained above in the introduction section.
5) Viral load of IOP-HPV testing: Measured in relative light units (RLUs).
6) Cone margins status: Positive (in some point the lesion is in contact with the margins) or negative (there is a space between the lesion and the margin throughout all the edge of the surgical piece). This information will also allow us to analyze if it is a worse predictive factor of recurrence disease than IOP-HPV. This way, we will fulfill the secondary objective of the study.
7) HPV vaccination: yes (all 3 doses) or not.
8) Follow-up cytologies: Some abnormal cytology or not. Cytological findings are described in annex IV.
9) Follow-up colposcopies: Some abnormal colposcopy or not. Colposcopic findings are described in annex III.

8.7. Data collection

For data collection, investigators and collaborators will attend to a coordination meeting prior to the start of the study. Homogeneity in data collection must be ensured. So, all members involved in the study will have to follow the established guidelines during its execution.

All the data from participating women will be recorded in their medical history and also collected in a study database.

The following table summarizes the data collection process:

Appointment	Collected data	Data resources	Study member
Visit when women accept to be part of the study group	▪ Age ▪ Tobacco ▪ VIH	▪ Medical history and patient survey	Gynecologist Pathologist
	▪ Preoperative histological grade	▪ Biopsy guided by colposcope	
1st follow-up visit after 1 month of the operation	▪ Cone margins status	▪ Excised specimen	Gynecologist Pathologist
	▪ IOP-HPV test result ▪ Viral load of IOP-HPV testing	▪ Sample for IOP-HPV testing	
Consecutive follow-up visits up to 3 years after the operation	▪ HPV vaccination	▪ Patient survey	Gynecologist Pathologist
	▪ HPV test result after 4-6 months of the operation	▪ Sample for HPV test	
	▪ Relapse of the disease	▪ Biopsy guided by colposcope	
	▪ Abnormal follow-up cytologies	▪ Sample for cytology	
	▪ Abnormal follow-up colposcopies	▪ Colposcopy findings	

9. STATISTICAL ANALYSIS

UNIVARIATE ANALYSIS

First, we will define variables as categorical or quantitative:

- Categorical variables are the two compared approaches used for detecting treatment failure, recurrences, VIH, preoperative histological grade of CIN, cone margins status, HPV vaccination, abnormal follow-up cytologies and abnormal follow-up colposcopies.
- Quantitative variables age, tobacco and viral load of IOP-HPV testing.

Categorical variables will be expressed as percentages and proportions. Quantitative variables will be described by mean +/- standard deviation (when assuming a normal distribution) or median and the quartiles (if not possible to assume a normal distribution).

BIVARIATE ANALYSIS

In regard of the main objective of the study, we will follow the steps below:

First, we will analyse the association between recurrent disease (dependent variable) and the following tests (independent variables):

a. IOP-HPV testing performed in women with negative surgical margins.
b. HPV testing performed after 6 months of the operation (will be in women with negative surgical margins).
c. IOP-HPV testing performed in women with positive margins.
d. HPV testing performed after 4 months of the operation (will be in women with positive surgical margins).

Chi-square or Fisher's exact tests will be used as both analysed variables are categorical. The results will be presented as absolute numbers and percentages.

Secondly, we aim to determine if the efficacy of HPV test to identify recurrent disease changes if it is performed at different times. So, the diagnostic efficacy of these tests will be evaluated as sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). The results will be presented as means and SD.

If the efficacy of the tests a and b and the efficacy of the tests c and d are the same, we will be able to accept as correct our main hypothesis.

In regard of the secondary objective of the study, we will follow the steps below:

First, we will analyse the association between recurrent disease (dependent variable) and the following predictive factors (independent variables):

e. IOP-HPV testing
f. Cone margins status

Chi-square or Fisher's exact tests will be used as both analysed variables are categorical. The results will be presented as absolute numbers and percentages.

Secondly, we aim to compare the efficacy of these predictive factors to identify recurrent disease. So, its diagnostic efficacy will be evaluated as sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). The results will be presented as means and SD.

If the efficacy of the IOP-HPV testing is better than the efficacy of cone margins status, we will be able to accept as correct our secondary hypothesis.

MULTIVARIATE ANALYSIS

A multivariate Cox regression model will be applied in order to evaluate interaction effects of the covariables with regard to recurrent CIN.

We will considerate all variables statistically significant if p value is <0.05.

Statistical analysis will be performed using the IBM Statistical Package for Social Sciences (SPSS) for Windows program. To manage computed data, Microsoft Excel tool will be used.

10. ETHICAL CONSIDERATIONS

We will submit the study for an ethics review to the Clinical Research Ethics Committee (CEIC) of the Hospital Universitari de Girona Dr. Josep Trueta. If the study fulfills the required criteria, it will assess its approval. In addition, all recommendations given by the committee will be taken into account.

We will respect the confidentiality of the database used during the procedure according to the Spanish Organic Law *15/1999, de 13 de diciembre, de Regulación del Tratamiento Automatizado de los Datos de Carácter Personal*. All women's data will be managed anonymously in order to protect their privacy. Moreover, the right of accessing to any kind of information concerning the patient will be guaranteed as well as the right of modifying or erasing their personal data.

Study participants will be given the information sheet (annex I) and they will be asked to sign the informed consent (annex II) in order to be included in the study.

The study will be carried out following the principles of the WMA Declaration of Helsinki and in accordance with the Medical Research Involving Human Subjects Act (last revised in 64th General Assembly, Fortaleza, Brazil, in October 2013).

11. STUDY LIMITATIONS

- Relatively short follow-up period will be reported in this study. Long-term follow-up would be necessary to confirm the accuracy of the results and to determine the significance of persistent long-term infections. Nevertheless, all the patients will be followed for at least 3 years, a period of time in which most of the recurrences are expected to develop.

- The study does not provide information on the type of HPV causing the lesion. Although women will be advice on the importance of using protection methods if they have sexual intercourse between the first and the second appointments, we can't be sure that all women will follow the recommendations. Consequently, the knowledge of the type of HPV that have caused the lesion would be necessary. In this way, we would obtain stronger evidence on persistent disease or newly acquired infection.

- There are 3 moments in the study where we assume that we will have patients lost: women who do not come to the operation, women being directly re-operated after the conization (if endocervical resection margin is affected) and women who don't attend the follow-up visits.

- In this study, the obtaining and processing of cytologies, HPV tests, biopsies guided by colposcope and the surgical pieces are operator-dependent techniques. Hence, we need skilled gynecologists with close collaboration with experienced pathologists. Both of them should be familiar with the methods used.

- The present study will be carried out in a small portion of territory, so additional research would be required to confirm our findings.

12. WORK PLAN

Investigators: Sílvia Feliu, Ester Vila, Alejandra Azkargorta, Eduard Sala.

Collaborators: Biologist of Hospital Trueta, statistical consultant, gynecologists and pathologists of each hospital involved in the study.

The study has been designed in 5 phases that are described below:

1. **Coordination phase** (2 months).

   It will involve all the investigators and collaborators.

   At the beginning of the study, all investigators and collaborators will attend to a coordination meeting. There, the timeline of the study will be planned and the methods of data collection will be set up. In addition, problems identification, suggestions and final elaboration of the research protocol will be carried out.

2. **Field work** (6 years and a half).

   It will involve all the investigators, biologist of hospital Trueta and the gynecologists and pathologists of each hospital involved in the study.

   In order to recruit the 414 women needed for our study, a detailed field sampling will be executed according to the inclusion and exclusion criteria during a period of 3 years. With each patient: (A) They will be asked to join the study voluntarily. Once they agree to participate, they will have to read the information sheet and sign de informed consent. (B) In the moment of the operation and right after the LEEP conization, an endocervical sample of the remaining cervix will be collected for HPV testing. Then, the surgical piece and the sample for HPV testing will be sent to pathology service in order to be analyzed. (C) After the treatment, women will attend to the follow-up visits during a 3-year period. (D) All relevant information (study variables and covariables) will be recorded in the database. (E) We will communicate our gratitude to every patient for their cooperation with the study.

3. **Data extraction and processing database** (7 years).

   It will involve all the investigators and collaborators.

   Data will be entered in the database simultaneously with the study development. Regularly, an analysis of data will be performed by the statistical consultant in order to control its evolution.

4. **Data analysis** (3 months).

   It will involve all the investigators and the statistical consultant.

   After processing the database, all data collected will be analyzed using the appropriate statistical test.

5. **Interpretation, publication and dissemination of the results** (5 months).

   It will involve all the investigators and collaborators.

   An interpretation of the outcomes will be performed and the results will be discussed among all the investigators and collaborators.

   After that, we will try to spread the obtained knowledge by publishing articles in prestigious scientific journals.

   Finally, the dissemination strategy will include conferences, meetings and training sessions, among others.

The time schedule of the study is shown below:

		TIME								
PHASE	**PERSONNEL**	**Jan 2017 – Feb 2017**	**Mar 2017 – Ago 2018**	**Sept 2018 – Feb 2020**	**Mar 2020 – Ago 2021**	**Sept 2021 – Feb 2023**	**Mar 2023 – Ago 2023**	**Sept 2023 – Feb 2024**	**Mar 2024 – May 2024**	**Jun 2024 – Jul 2024**
Coordination phase	All the investigators and collaborators									
Field work	All the investigators, biologist of hospital Trueta and the gynecologists and pathologists of each hospital									
Data extraction and processing database	All the investigators and collaborators									
Data analysis	All the investigators and the statistical consultant									
Interpretation, publication and dissemination of the results	All the investigators and collaborators									

13. AVAILABLE MEANS TO CARRY OUT THE PROJECT

The project will take place in public hospitals located in the province of Girona. All the hospitals have the informatics equipment suitable for accessing and filling the database for the study development.

In order to get the results of HPV tests, only the Hospital Universitari de Girona Dr. Josep Trueta has the resources to analyze the samples. So, all the samples from the other hospitals will be sent to the pathology service of Hospital Trueta. There, the biologist and her assistants will use the Hybrid Capture System to analyze all the samples.

In regards to the analysis of cytologies, cervical biopsies and the specimen from the operation, each hospital will sent the samples to their own pathology service or, in cases where it is not available, to the Hospital Trueta.

The statistical consultant, the biologist service and the reagent kit needed for obtaining de results of IOP-HPV tests will be paid by the project.

14. BUDGET

All the clinical techniques, except the IOP-HPV tests, are normally made following the current protocol of the hospitals. So, extra money will be needed just for the extra reagent kits that will be used for obtaining the IOP-HPV results with the Hybrid Capture System. Each kit costs 1500€ and contains reagents to analyze 88 samples. So, we will need 5 kits to analyze the 414 samples. Then, the estimated cost is 7.500€. Cytobrushes and vials with Specimen Transport Medium (to obtain and transport the HPV samples) are distributed for free in hospitals of the province of Girona by the trading house from where the Hybrid Capture machine of hospital Trueta is. So, no extra money will be needed.

The biologist of hospital Trueta will have to validate and inform the results of the IOP-HPV tests. The estimated salary for this extra work is 800€. In addition, we will hire a statistical specialist in order to perform a regular analysis of the database during the data extraction period. Then, he will also analyze all data collected using the appropriate statistical test. The estimated salary is 35€ per hour and approximately 30 hours of statistical support will be needed. Then, the estimated cost is 1050€.

Finally, we will need money for the coordination meeting at the beginning of the study, and for the publication and dissemination of the findings.

Material resources	
• Cytobrushes and vials with Specimen Transport Medium for HPV testing	0€
• 5 reagent kits	7.500€
Human resources	
• Biologist	800€
• Statistician	1.050€
Travel expenses	
• Coordination meeting	500€
• Conferences, meetings and training sessions to disseminate the findings	2.500€
Publication costs	
• Articles publication	1.500€
TOTAL	**13.850€**

15. ANNEXES

15.1. Annex I: Information sheet for participants

FULL D'INFORMACIÓ PER A LA PACIENT

Títol de l'estudi: El test del virus del papil·loma humà intraoperatori després de la conització: un factor predictiu de recurrències més precoç en pacients tractades per una neoplàsia epitelial intracervical.

Vostè ha estat convidada a participar en un estudi d'investigació sobre els controls post-quirúrgics en pacients conitzades per una neoplàsia intraepitelial cervical grau 2 o 3. Abans de decidir si vol formar-ne part o no, és important que entengui perquè s'està realitzant la recerca i què inclou. Siusplau, prenguis el temps necessari per llegir aquest formulari que inclou un resum informatiu sobre l'estudi. L'equip que en forma part la convidem a preguntar-nos tot allò que no li hagi quedat clar o si li agradaria rebre més informació.

Per què es realitza aquest estudi?

Aquest estudi té com a principal objectiu avaluar l'eficàcia del test del HPV (virus del papil·loma humà) realitzat intraoperatòriament. Es compararà amb el test del HPV dut a terme, actualment, al cap de 4 o 6 mesos després de la intervenció. L'objectiu final és comprovar si el mateix procediment realitzat en diferents moments del temps prediu amb la mateixa eficàcia les pacients que patiran una recidiva de la malaltia. Si és així, no caldria esperar mesos per saber si la pacient continua o no amb el virus un cop tractada amb la conització.

A més a més, l'estudi també donarà la oportunitat de comparar el test del HPV intraoperatori amb un altre factor predictor de recidives utilitzat actualment: els marges de la peça quirúrgica. Ja que aquest és el que s'utilitza per guiar les conseqüents visites de seguiment, si es demostrés que el test del HPV intraoperatori és més eficaç per predir les futures recidives, es podrien replantejar els protocols actuals de seguiment de les pacients conitzades.

Quantes dones participaran en aquest estudi?

En aquest estudi participaran aproximadament 414 dones diagnosticades d'una neoplàsia intraepitelial cervical grau 2 o 3 que rebran tractament amb conització en algun hospital de la província de Girona.

Què passarà si hi participo?

Si vostè accepta participar en l'estudi, seguirà el procediment terapèutic i de control post-quirúrgic habitual segons el protocol establert per la *Asociación Española de Patología Cervical y Colposcopia (AEPCC)*. Així doncs, aquest estudi només suposarà la realització d'un test pronòstic addicional en el moment de l'acte quirúrgic: el test del HPV just després de la conització.

A part d'això, se li sol·licitarà que ens faciliti la informació sobre les seves dades personals que sigui rellevant per a l'estudi si aquesta no figura en la seva història clínica.

És obligatori participar-hi?

La participació en aquest estudi és totalment voluntària. Si decideix participar-hi, haurà de firmar el consentiment informat en el qual vostè declara haver entès tot el que fa referència a participar en l'estudi. Pel contrari, si decideix no participar-hi, la seva atenció mèdica no es veurà afectada en cap moment.

Com es mantindrà la confidencialitat de la meva informació personal?

Per tal de garantir en tot moment el compliment de la Llei Orgànica de Protecció de Dades de Caràcter Personal (15/1999), tota la informació recopilada durant aquest estudi es mantindrà confidencial i només serà utilitzada amb finalitat d'investigació. No s'utilitzarà el seu nom en cap informe de l'estudi sinó que la seva identificació personal estarà codificada a través d'una sèrie numèrica aleatoritzada.

A més, tindrà dret a consultar tota la informació recopilada sobre vostè en aquest estudi i a rectificar qualsevol dada errònia.

Què passarà si canvio d'opinió en el decurs de l'estudi?

Pot decidir abandonar l'estudi en qualsevol moment i sense que afecti la seva atenció en el futur.

Per altra banda, el metge de l'estudi també podrà decidir retirar-la per qualsevol de les següents raons: si no segueix les normes de l'estudi, si no assisteix a les visites programades o si existeixen raons mèdiques o personals que ho facin necessari.

Què se'n farà de la informació obtinguda a partir de l'estudi?

Una vegada finalitzat l'estudi, es preveu publicar els resultats en revistes d'interès científic de l'àrea de coneixement de la patologia cervical per tal que altres centres i pacients puguin beneficiar-se de les troballes del nostre estudi.

La publicació dels resultats sempre es durà a terme mantenint la confidencialitat de les seves dades personals.

Qui ha revisat l'estudi?

L'estudi ha estat revisat pel Comitè d'Ètica i Investigació Clínica (CEIC) de l'Hospital Universitari de Girona Dr. Josep Trueta, el qual n'ha donat una opinió favorable.

Amb qui puc contactar si tinc preguntes o problemes?

Si té qualsevol pregunta pot trucar a la coordinadora de l'estudi al 619 030 032

Moltes gràcies per la seva atenció.

15.2. Annex II: Informed consent sheet for participants

FORMULARI DE CONSENTIMENT INFORMAT

Títol de l'estudi: El test del virus del papil·loma humà intraoperatori després de la conització: un factor predictiu de recurrències més precoç en pacients tractades per una neoplàsia epitelial intracervical.

- He llegit i entès el full d'informació per a la pacient i el formulari de consentiment informat.
- He parlat sobre l'estudi amb el meu metge i ha respòs a les meves preguntes de forma satisfactòria.
- Entenc que la meva participació en l'estudi és totalment voluntària, que puc canviar d'opinió més endavant sense haver de donar explicacions i que, independentment de la meva decisió, la meva atenció mèdica i els meus drets legals no es veuran afectats.
- Dono permís al personal de l'estudi perquè pugui consultar la meva història clínica amb la finalitat de recopilar les dades necessàries per a la realització de l'estudi.
- Accepto voluntàriament participar en aquest estudi d'investigació.

Participant:

Nom, Cognoms i DNI: Firma: Data:

Doctor que ha informat al pacient:

Nom, Cognoms i DNI: Firma: Data:

15.3. Annex III: Colposcopic findings

Following the criteria of the International Federation for Cervical Pathology and Colposcopy, colposcopic findings are described as it follows:

I. Normal colposcopic findings:
   a. Original squamous epithelium
   b. Columnar epithelium
   c. Transformation zone

II. Abnormal colposcopic findings:
   a. Flat acetowhite epithelium
   b. Dense acetowhite epithelium*
   c. Fine mosaic
   d. Coarse mosaic*
   e. Fine punctation
   f. Coarse punctation*
   g. Iodine partial positivity
   h. Iodine negativity*
   i. Atypical vessels*

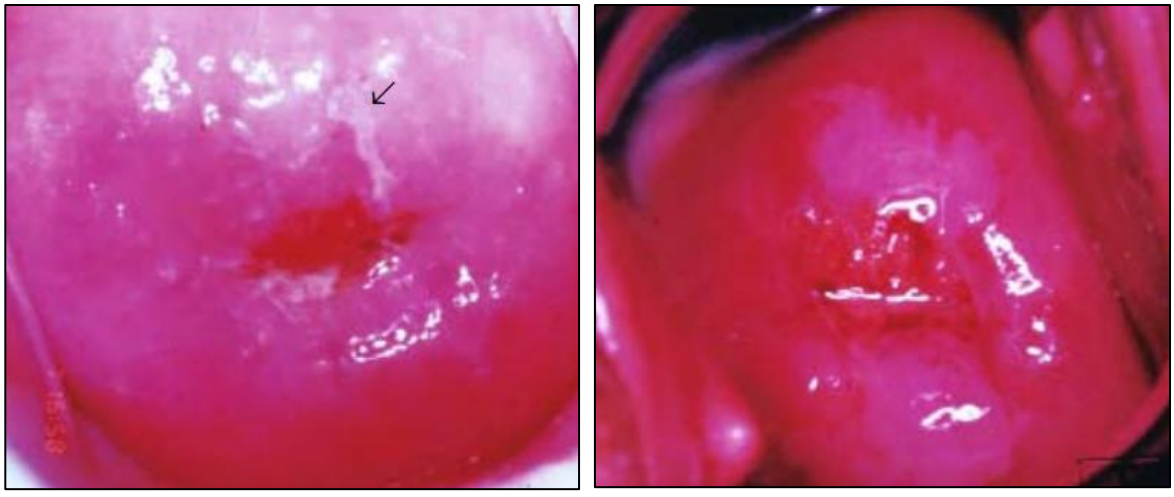
*Major changes.

III. Colposcopic features suggestive of invasive cancer.

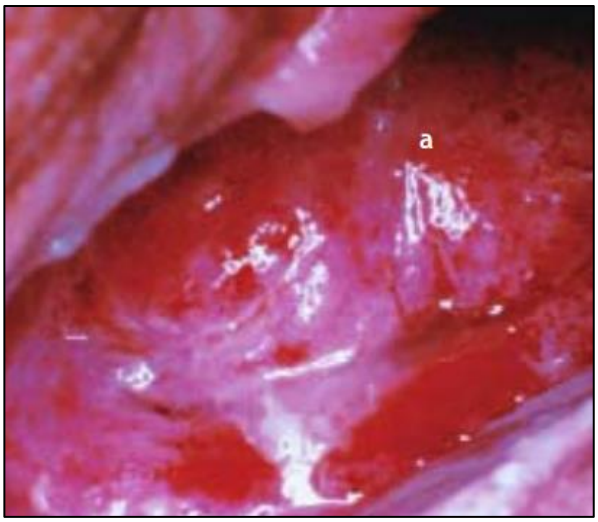
IV. Unsatisfactory colposcopy:
   a. Squamocolumnar junction not visible
   b. Severe inflammation, severe atrophy, trauma
   c. Cervix not visible

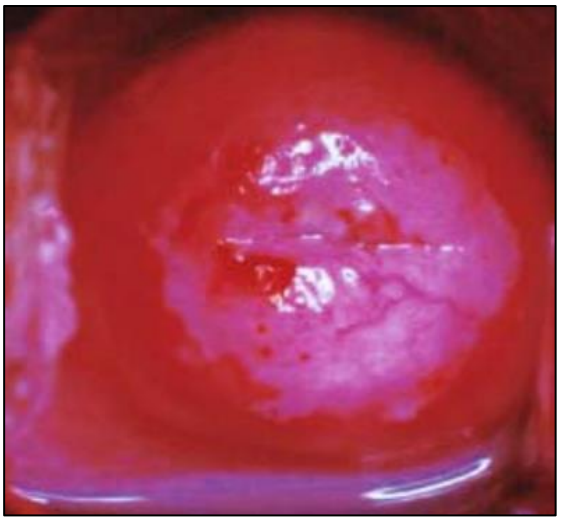
V. Miscellaneous findings:
   a. Condylomata
   b. Keratosis
   c. Erosion
   d. Inflammation
   e. Atrophy
   f. Deciduosis
   g. Polyps

Taken from: Walker P, Dexeus S, De Palo G, Barrasso R, Campion M, Girardi F, et al. International terminology of colposcopy: an updated report from the International Federation for Cervical Pathology and Colposcopy. *Obstet Gynecol* 2003; 101:175-7.

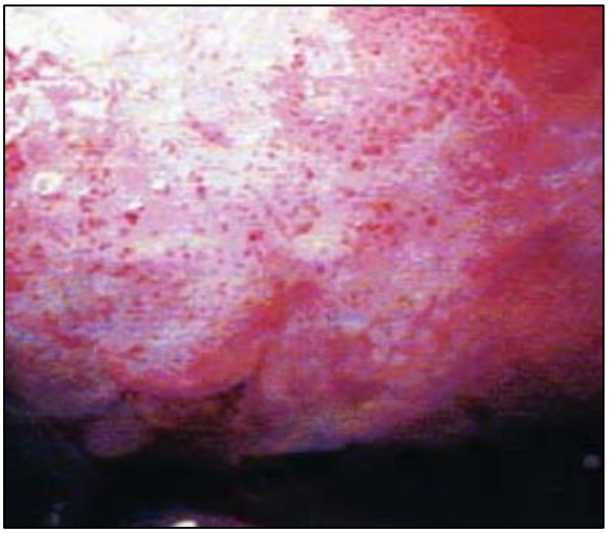


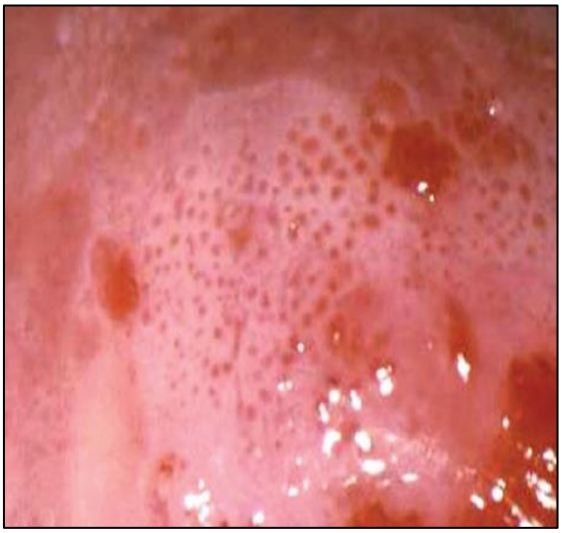
Figures 1 and 2: Acetowhite lesions





Figures 3 and 4: A dense opaque acetowhite areas with coarse mosaics





Figures 5 and 6: Coarse punctation

Figures taken from: International Agency for Research on Cancer. Chapter 7: Colposcopic assessment of cervical intraepithelial neoplasia [Internet]. Available from: http://screening.iarc.fr/colpochap.php?lang=1&chap=7

15.4. Annex IV: Cytological findings

Following the criteria of the 2001 Bethesda System, cytological findings are described as it follows:

Epithelial Cell Abnormalities:

- Squamous cell:
  - Atypical squamous cells (ASC)
    - of undetermined significance (ASC-US)
    - cannot exclude HSIL (ASC-H)
  - Low-grade squamous intraepithelial lesion (LSIL)
  - High-grade squamous intraepithelial lesion (HSIL)
  - Squamous cell carcinoma
- Glandular cell:
  - Atypical glandular cells (AGC) (specify endocervical, endometrial, or not otherwise specified)
  - Endocervical adenocarcinoma in situ (AIS)
  - Adenocarcinoma
- Other

Taken from: Solomon D, Davery D, Kurman R, Moriarty A, O'Connor D, Prey M, et al. The 2001 Bethesda System: terminology for reporting results of cervical cytology. *JAMA* 2002; 287:2114-9.