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Prevalence and Distribution of High-Risk Human Papillomavirus Genotypes in Invasive Carcinoma of the Uterine Cervix in Uruguay. An update on clinical outcome.

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ABSTRACT

Although the causal role of Human papillomavirus (HPV) in cervical cancer CC is accepted, controversial reports have been published about the prognostic significance of different genotypes. Our aim was to evaluate the HPV-status and its relationship with clinical-pathological variables and clinical outcome in invasive cervical cancer of Uruguayan women. Main prevalent genotypes were HPV16 (63.6%), HPV18 and HPV45 (8.3% each genotype), while other genotypes (HPV31, 33, 35, 39, 51, 52, 58, 66, 73 and one undetermined genotype) account for 12.4%, and in 8.3% HPV DNA could not be detected. With a mean follow-up of ten years, we found a significant correlation of overall survival with International Federation of Gynecology and Obstetrics (FIGO) staging and lymph node metastasis. Relating to HPV, three prognostic groups were observed. The better clinical outcome was related to genotypes other than HPV16/18/45, while HPV16/18 genotypes belong to an intermediate risk group and the worse prognosis was related to HPV negative and HPV45. HPV independent tumors have been suggested as a different entity compared with virally driven ones. Improvement in knowledge of molecular pathogenesis could impact in CC patients care. In conclusion, worse prognosis was related to HPV negative and HPV45 related tumors. More research is warranted for better understanding molecular basis of virally driven or independent cervical cancer oncogenesis.

Keywords: Human Papillomavirus, cervical cancer, clinical outcome

1. INTRODUCTION

Despite diagnosis progress and preventive actions, cervical cancer (CC) is still a public health concern, especially in developing countries. According to data from the International Agency for Research on Cancer (IARC), of the World Health Organization, it is the fourth most frequent cancer worldwide, and the fourth cause of cancer-related death in women (GLOBOCAN 2012). CC is one of the main examples of health inequity as 85% of cases, as well as 9/10 related death occur in developing countries (Ferlay, 2015). In Uruguay, it is the third most frequent cancer in women, after breast and colorectal cancer, with an average of 330 new cases per year, which represent an incidence rate of 15.69 and a mortality rate of 5.33 death per 100,000 population (approximately 133 deaths per year) (Barrios, 2014a; Barrios, 2014b).

The carcinogenic implication of HPV infection in CC development is largely accepted, although progression to CC only occur in a small percentage of women, and most of them clear the infection (Schiffman, 2007). Understanding the genetic basis of HPV oncogenicity is highly complex, but innovative analytic methods are improving our knowledge about susceptibilities to HPV type-specific infection and cervical progression (Zou, 2016). Among the accepted HPV oncogenic genotypes (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59), prevalence has shown to be highly variable by geographical region, sexual behavior and age. It is widely accepted that genotypes 16 and 18 are the main contributors to HPV-related cervical carcinogenesis. These genotypes are also responsible for a subset of tumors in other locations such as oropharyngeal, and anogenital cancers in both sexes, and seem to play a role in other digestive cancers like esophagus and colorectal cancer although evidences are still inconclusive for these localizations (Zumsteg, 2016; Bucchi. 2016). Vaccination represents a good strategy to reduce the burden of HPV-related cancers. Two first approaches (bivalent and tetravalent) include main oncogenic genotypes (HPV16 and HPV18), covering upto 70% of HPV-related CC, and a second generation vaccine (nonavalent) covers upto 90%. Vaccine implementation programs led to controversial discussions and a conclusion on its actual efficacy require a 20 years follow-up period. After the first ten years it can be concluded that vaccines are safety and efficient, but the superiority of vaccination in preventing CC compared to HPV screening is not proven yet (Angioli, 2016).

Numerous methods are available for HPV detection and genotyping, although not all of them have the same performance, which could explain the variation in reported prevalence in the literature. Although the PCR-based methods seem to be one of the most sensitive, several cases of CC remain HPV negative after re-analysis, and it has been suggested that this subset of tumors could represent a more aggressive group (Rodríguez-Carunchio, 2015). Similar results were found for head and neck (H&N) tumors (Liu, 2017) and anal cancer (Mai, 2015). For invasive CC, the main prognostic factors are stage at diagnosis, tumor size, parametrial spread, regional lymph node status, perivascular invasion and deep stromal invasion (Pecorelli, 2009). Although etiology of CC is related to HPV infection, the prognostic role of different genotypes, if any, is not clear. Several authors did not find any correlation between HPV genotypes and survival (Tong, 2007; Cuschieri, 2014; Lau, 2015), while others found intermediate risk HPV correlating with better outcome than high risk genotypes (de Cremoux, 2009; Huang, 2004). Indeed, controversial results involve also the two most frequent high risk genotypes. While some authors found HPV16 as an independent prognostic factor for overall survival (Pilch, 2001), other reports state that HPV16 is more frequent in patients surviving more than 5 years (Dahlgren, 2006). Otherwise, greater consensus seems to hold HPV18 as a worse prognosis factor, especially for low stages (Schwartz, 2001; Lai, 2007; Yang, 2014).

We previously reported the only study of prevalence and distribution of HPV genotypes in invasive CC carried out in Uruguayan women, which showed HPV16, 18 and 45 as main prevalent genotypes (Berois, 2013). In view of contradictory data concerning the influence of HPV genotypes on the clinical outcome of CC patients, at the present work our aim was to review clinical records of same patients in order to evaluate the prognosis significance of HPV-status and its relationship with clinical-pathological variables.

2. MATERIALS AND METHODS

2.1 Patients and clinical-pathological data

The present study is an update of our previous report about the prevalence and genotype distribution of HPV infection in 176 patients with invasive CC treated at the *Centro Hospitalario Pereira Rossell* in Montevideo, Uruguay (Berois, 2013). Medical records were reviewed in order to collect information and patients were selected based on the availability of data about their stage at diagnosis, histopathological features, treatments and outcome. A total of 121 patients met these inclusion criteria. The study was reviewed and approved by the ethical review boards of the School of Medicine (*Universidad de la República*) and the Ministry of Public Health of Uruguay.

2.2 Treatments

Patients were treated according to accepted protocols and following the FIGO staging system (FIGO Committee on Gynecologic Oncology, 2014). Treatment plans were decided in Gynecologic Tumor Board meetings formed by gynecologists, medical and radiation oncologists as well as pathologists. To sum up, surgery (cone, simple or radical trachelectomy, radical hysterectomy, and pelvic lymphadenectomy), radiotherapy (RT) or concurrent chemoradiotherapy (CCRT) was chosen depending on different factors, such as the FIGO stage, the prognostic factors and the reproductive desires of the patient. Although surgery and RT show similar results in early stages, surgical is the treatment of choice. Patients with locally advanced disease received RT (combination of external beam RT and intracavitary brachytherapy) or CCRT with radical purpose. The treatment standardized according to the lesion extension based on clinical examination and computed tomography consisted of 48.8 Gy delivered to the whole pelvis, followed by a parametrial reinforcement of 14 Gy. Brachytherapy is individually adjusted according to the tumor size, and the dosage as well, depending on the tolerance of the tissues. As far as CCRT is concerned, the regimens used were based on cisplatin at doses of 40 mg / m2.

2.3 Specimen preparation and HPV genotyping

HPV genotyping was performed by Polymerase chain reaction (PCR), as previously described (Berois, 2013). In short, formalin-fixed paraffin-embedded tissue blocks having more than 10% of tumor cells were used for DNA extraction by a commercial kit (QIA ampDNA mini kit, Qiagen, Valencia, CA). PCR amplification was performed using GAPDH gene sequence primers, in order to evaluate DNA quality, and HPV detection was assessed by generic primers GP5+/6+ and specific primers for HPV16, 18, 33 and 45 genotypes. Positive GP5+/GP6+ samples -but negative for all HPV-specific types screened- were further analyzed by sequencing and aligned with reference sequences for genotype identification.

2.4 Statistical Methods

Contingency tables involving qualitative variables were tested using Chi square test or Fisher exact test, when needed. Overall survival distribution time was estimated using the Kaplan-Meier method. Survival curves were compared using logrank test. Cox Proportional Hazard Model was used for multivariate analysis, but sample size limitations were critic in order to reach statistical significance. A value of $P<0.05$ was considered statistically

significant. Statistical analysis was performed using STATA version 14.2 software.

3. RESULTS

The population included in this analysis was 121 patients. Although it is fewer than the population of our first report (n = 176), the characteristics of the patients are similar. Mean age was 44 years (range 23-79). Following FIGO staging guidelines (FIGO, 2014), 9 patients (7.4 %) had stage IA1, 3 patients (2.5 %) had stage IA2, 49 patients (40.5 %) had stage IB1, 8 patients (6.6 %) had stage IB2, 14 patients (11.6 %) had stage IIA and IIB, 2 patients (1.6 %) had stage IIIA, 18 patients (14.9 %) had stage IIIB, and 2 patients (1.6 %) had stage IIIC, and IVA. Staging patients with prognostic criteria in early and advanced stages account for 76 cases (62.8%) in the former group and 45 cases (37.2%) in the later.

The histological type was predominantly squamous cell carcinoma (SCC) (104/121; 86%), followed by adeno-squamous carcinoma (ASC) (11/121; 9%), and adenocarcinoma (ADC) (6/121; 5%). Primary treatment, according to FIGO stage, was surgery in 83 cases (simple hysterectomy in 9 cases or hysterectomy plus pelvic lymph node dissection in 74 cases) and RT/CCRT in 38 cases.

HPV DNA was found in 111/121 (91.7%), and the most prevalent genotypes were HPV16 (63.6%), HPV18 and HPV45 (8.3% each genotype). One patient showed co-infection HPV16 and HPV45. Other genotypes (HPV31, 33, 35, 39, 51, 52, 58, 66, 73 and one undetermined genotype) account for 12.4% of cases, while in 10/121 (8.3%), HPV DNA could not be detected. Correlation between clinical features and HPV genotype is shown in Table I. Similar to our previous report, HPV16, 18 and 45 are predominant in younger women (below 60 years) while intermediate risk genotypes are more frequent in older women. Concerning histological type, HPV 16 was present in 66.3% of SCC, 50.0% of ADC and 45.4% of ASC, while HPV18 was found in 5.6% of SCC, 50.0% of ADC and 9.1% of ASC. HPV45 was positive in 8.4% of SCC, no ADC and 27.3% of ASC. Other genotypes were present only in SCC and no ADC was HPV negative.

Table I: Correlation between HPV genotype and clinical-pathologic parameters (n = 121)

Characteristics	Nº of patients	HPV16 (n=77)	HPV18 (n=10)	HPV45 (n=10)	Other HPV genotypes (n=15)	HPV negative (n=10)	*p*
Age (years)							
< 35	**21**	**12**	**2**	**4**	**2**	**1**	**0.487**
35 - 60	**84***	**55**	**8**	**5**	**9**	**8**	
> 60	**16**	**10**	**0**	**1**	**4**	**1**	
FIGO stage							
I	**69***	**46**	**5**	**6**	**7**	**6**	**0.308**
II	**28**	**19**	**4**	**0**	**4**	**1**	
III-IV	**24**	**12**	**1**	**4**	**4**	**3**	
Histological type							
Squamous cell carcinoma	**104***	**69**	**6**	**7**	**15**	**8**	**0.014**
Adenocarcinoma	**6**	**3**	**3**	**0**	**0**	**0**	
Adeno-squamous carcinoma	**11**	**5**	**1**	**3**	**0**	**2**	
Primary treatment							
Simple hysterectomy	**9**	**8**	**0**	**0**	**1**	**0**	**0.953**
Radical hysterectomy	**74***	**47**	**7**	**6**	**8**	**7**	
RT/CCRT	**38**	**22**	**3**	**4**	**6**	**3**	

HPV: Human Papillomavirus; FIGO: International Federation of Gynecology and Obstetrics; RT: radiotherapy; CCRT: concurrent chemoradiotherapy

* One patient coinfected HPV16/45

Mean follow-up was 135 months (95% CI: 123.9-146.1). There were 33 deaths at the time of study analysis, 28 of which were related to cervical cancer and the remaining 5 to other causes. Overall survival (88/121, 72.7%), related to clinical-pathological features and HPV genotype is shown in Table II. As expected, significant correlation was found between FIGO stage and survival rate, which was 82.6% for stage I, 75.0% for stage II and 41.7% for stage III-IV (p = 0.001) (Figure 1). Table III shows survival analysis of patients with CC who underwent radical hysterectomy. Twenty out of 74 patients had lymph node metastasis and survival rate was 55% for node-positive patients and 88.9% for node-negative ones (p = 0.001) (Table III). Kaplan–Meier curves for overall survival stratified by lymph node status was statistically significant (p = 0.0018). Extrauterine invasion was seen in 30/64 patients (46.9%), but survival rate compared with non-disseminated disease was not significant. Survival rate by HPV genotype was 76.6% for HPV16, 70.0% for HPV18, 50.0% for HPV45, 86.6% for other genotypes, and 50.0% for HPV negative patients. Kaplan–Meier curves allow to identify a three group modal trend, where genotypes others than HPV16/18/45 exhibit better survival, an intermediate group represented by HPV16/18 genotypes, and a worse prognostic group related to HPV45 and HPV negative CC (Figure 3).

Table II: Survival analysis of 121 patients with CC

Characteristics	Nº of patients	Survival patients n (%)	*p*
Age (years)			
< 35	**21**	**13 (61.9%)**	**0.391**
35 – 60	**84**	**64 (76.2%)**	
> 60	**16**	**11 (68.7%)**	
FIGO stage			
I	**69**	**57 (82.6 %)**	**0.001**
II	**28**	**21 (75.0 %)**	
III-IV	**24**	**10 (41.7 %)**	
Histological type			
Squamous cell carcinoma	**104**	**76 (71.1 %)**	**0.665**
Adenocarcinoma	**6**	**5 (83.3 %)**	
Adeno-squamous carcinoma	**11**	**7 (63.3 %)**	
HPV status*			
HPV16	**77**	**59 (76.6 %)**	**0.121**
HPV18	**10**	**7 (70.0 %)**	
HPV45	**10**	**5 (50.0 %)**	
Other HPV genotype	**15**	**13 (86.6 %)**	
HPV negative	**10**	**5 (50.0 %)**	
Primary treatment			
Surgery	**83**	**64 (77.1 %)**	**0.003**
RT/CCRT	**38**	**19 (50.0 %)**	

CC: cervical cancer; FIGO: International Federation of Gynecology and Obstetrics; HPV: Human Papillomavirus; RT: radiotherapy; CCRT: concurrent chemoradiotherapy
* One patient coinfected HPV16/45

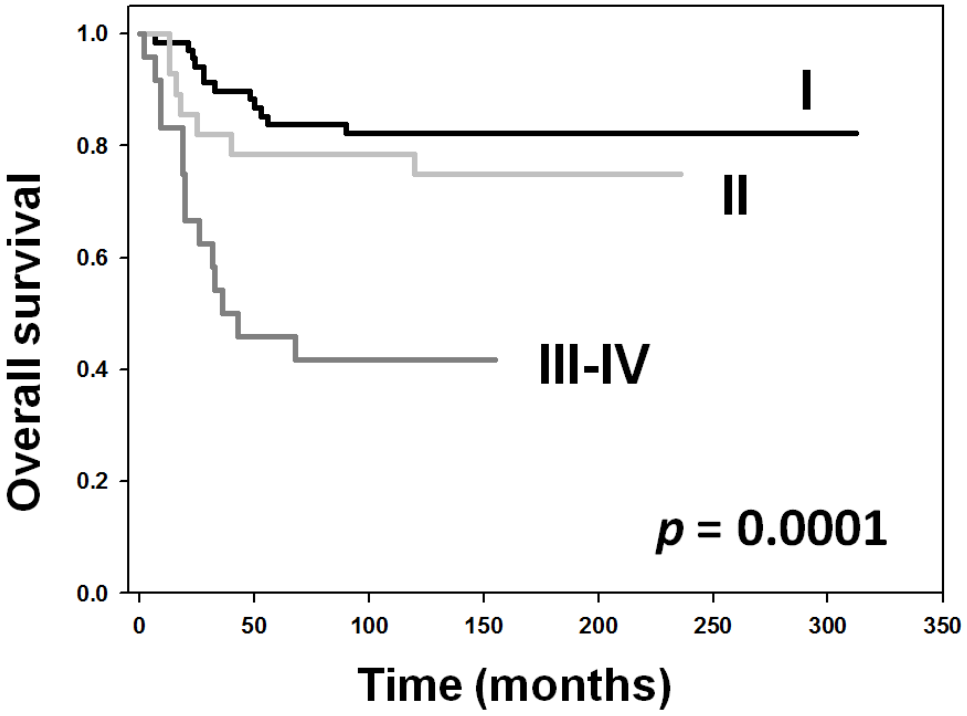

Figure 1: Kaplan–Meier curves for overall survival stratified by FIGO stage.

Table III: Survival analysis of patients with CC who underwent radical hysterectomy

Characteristics	Nº of patients	Survival patients n (%)	*p*
Lymph node status (n = 74)			
Positive	**20**	**11 (55.0 %)**	**0.001**
Negative	**54**	**48 (88.9 %)**	
Extrauterine invasion (n = 64)			
Positive	**30**	**23 (76.6 %)**	**0.558**
Negative	**34**	**28 (82.3 %)**	

CC: cervical cancer

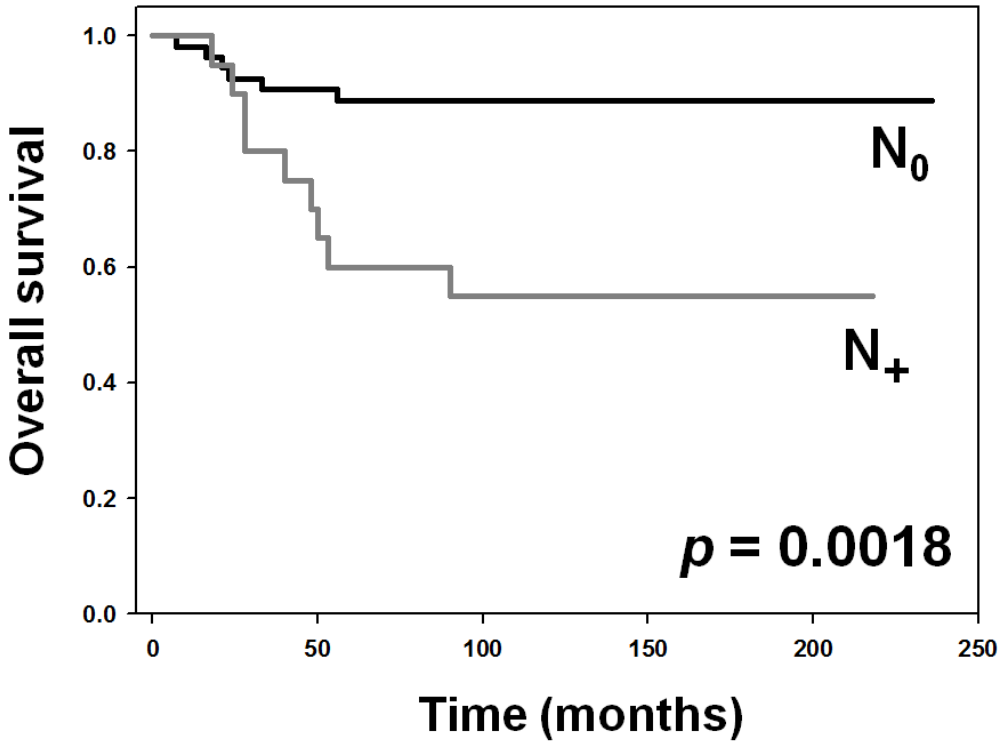

Figure 2: Kaplan–Meier curves for overall survival stratified by lymph node status.

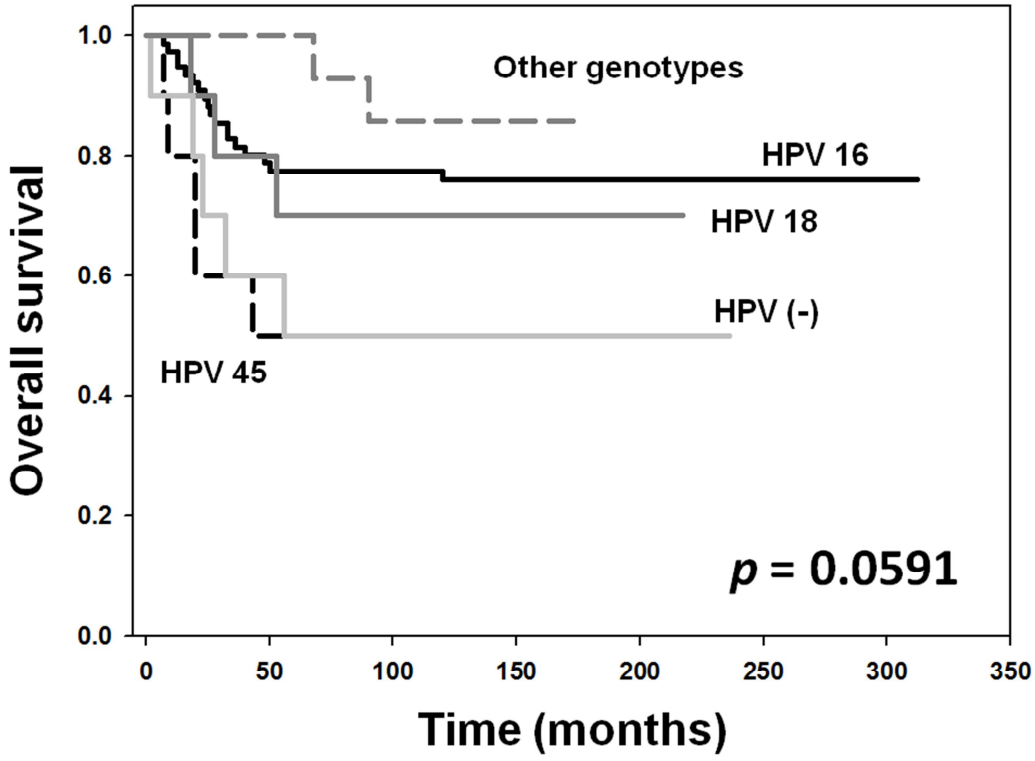

Figure 3: Kaplan–Meier curves for overall survival stratified by HPV status, all stages included.

1. DISCUSSION

The most important prognostic variables of CC are FIGO stage, lymph node status and clinical-pathological features of primary tumor such as size, depth of stromal invasion, parametrial or vaginal involvement, lymph-vascular space involvement and histological type (Berek 2010). Although histological types have been suggested as independent prognostic factors (Mabuchi, 2012; Zhou, 2017), no significant differences in OS were found in our population. In agreement with Bradbury *et al*. we found significant correlation of OS with lymph node metastasis but not extrauterine invasion (Table III) (Bradbury, 2015), although other authors reported significant correlation of parametrial involvement with disease-free and overall survival (Jiamset, 2016). FIGO stage is crucial for treatment choice. For early stages IA, IB1 and IIA1 (disease limited to the cervix or with involvement of up to the upper two thirds of the vagina) there is no treatment of choice in terms of survival because both, surgery and RT, offer similar results (Landoni, 2017). However, surgical treatment offers advantages such as preservation of ovarian function, maintains a more functional vagina and facilitates the knowledge of pathological prognostic factors. It is the treatment performed for early stage patients at our Hospital (extrafascial hysterectomy in cases of stages IA1, or radical hysterectomy and pelvic lymphadenectomy in the rest). As extensive lymphadenectomy is a main

cause of postoperative morbidity, controversial opinions have been arguing about the number of lymph nodes removed in their role as an independent prognostic factor for OS. Mao *et al.* suggest that histology and depth of invasion, instead the increased number of removed nodes, are associated with survival for early stage CC (Mao, 2016). Current literature suggests that patients with low-risk early-stage CC may be candidates for more conservative approaches, preserving reproductive function (Baiocchi, 2017; Willows, 2016). In contrast, CCRT is the standard of care in locally advanced stages of CC, which exceed the limit of the cervix towards parametria, annexes or pelvic organs (FIGO stages IB2, IIA2-IV) (Meng, 2016). Neoadjuvant chemotherapy followed by radical hysterectomy is a promising therapeutic option for stage IB2-IIB disease (Gadducci, 2017). Although in our OS analysis (Table II) it seems that surgery treatment is a better option compared with RT/CCR, certainly this is because most non operated patients belong to advanced stages. Only 2 patients with early stage were treated by RT without surgery, and both are alive (data not shown).

Recently, in the era of precision medicine, increasing evidence in databases suggest the potential usefulness of deregulated genes as biomarkers able to predict both, response to treatment and survival, as well as molecular targets in CC patients (Niu, 2017). Among several examples, MVP (major vault protein), IGF-1R (insulin-like growth factor 1 receptor), and BCL2 (B cell lymphoma 2) expression in tumor tissues have been suggested as useful biomarkers for an optimal planning of therapeutic strategy (Valenciano, 2014). Furthermore Foxp3 (forkhead/winged helix transcription factor p3) and TLR4 (toll-like receptor 4) could play a role in promoting the immune escape of CC suggesting a potential rational for new immunotherapeutic strategies (Zhang, 2017). However, lacking of phase III trial conclusions, no tissue biological variables can still be used nowadays in the clinical practice to better define the prognosis or to tailor treatment strategies of patients with CC (Liu, 2016).

The etiopathogenic association between HPV infection and CC is well established (Bosch, 2002). However, although increasing evidence supports the link between HPV status and disease prognosis, a large number of controversial reports do not allow conclusive remarks. Some authors suggest that HPV genotype has significant value to predict OS and disease free survival (Lai, 2007; Wang, 2010), while other reports state otherwise (Lau, 2015; Tong, 2007). In our series a three group modal trend was observed. Whereas genotype others than HPV16/18/45 exhibit better survival, an intermediate risk-group was represented by HPV16/18 genotypes, and a worse prognostic group was related to HPV45 and HPV negative. Several reports showed different clinical outcome for high risk HPV genotypes compared with intermediate risk. For example, de Cremoux *et al*. reported that high-risk HPV types 16/18/45 were associated with reduced disease-free survival as compared to intermediate-risk genotypes (HPV 31, 33, 35, 39, 52, 53, 58, 59, 73) although OS did not reach statistical significance (de Cremoux, 2009). In Asian population, in which HPV58 and related genotypes (52 and 33) are more prevalent, similar results were found. Ferdousi *et al*, in agreement with Lai *et al*, reported better outcome related to HPV58 (Ferdousi, 2010; Lai, 2007), while in the series of Huang *et al*, HPV31 was a significant predictor of good prognosis, independent of clinical stage (Huang, 2004).

Concerning most prevalent genotypes HPV16/18, several studies have shown that patients with HPV18-containing tumors were at increased risk of death and disease recurrence, especially in early stage disease (Schwartz, 2001; Kang, 2011; Yang, 2014), while for Pilch *et al*. HPV16 genotype had an independent negative impact on overall survival in 204 patients with CC (Pilch, 2001). In our work the survival analysis placed HPV16/18 genotypes in an intermediate risk group, while patients whose

tumors were HPV negative or related to HPV45 exhibited higher mortality. These results are in agreement with Wang *et al*., who compared patients infected with alpha-7 species, alpha-9 species and HPV negative and concluded that patients without HPV infection or the ones infected with alpha-7 species only, make up a high risk group with poor prognostic (Wang, 2010). Baalbergen *et al*. studied the HPV impact on survival only for adenocarcinoma tumors and although their results did not reach statistical significance, a trend was seen for worse 5-year survival of patients with HPV45 related tumors (Baalbergen, 2013). Concerning HPV negative CC patients, other authors have reported a worse survival rate, in agreement with our results (Riou, 1990; Lai, 2013; Rodríguez-Carunchio, 2015; Okuma, 2016). However, contradictory results have been reported by Pilch *et al*. who found higher survival rate for HPV negative compared with HPV16 (Pilch, 2001). On the other hand, as we previously state, some authors found no association of HPV DNA and prognosis of CC. Tong *et al*. divided their studied population in four groups (HPV16-related, HPV18-related, intermediate risk type-related and HPV negative) and conclude that neither the presence nor type of HPV DNA bears any prognostic significance in CC (Tong, 2007). Cuschieri *et al*. also analyzed impact on survival rate with particular reference to HPV16/18 and conclude that those genotypes do not confer worse survival compared to cancers associated with other types (Cuschieri, 2014). In the same way, two studies conducted in Chinese women, where most prevalent HPV genotypes are HPV16, 18, 52 and 58, did not find significant association between OS and infection with a particular HPV type, except for a slightly trend to better survival in HPV58 single-infected patients found by Shah *et al*. (Lau, 2015; Shah, 2009). Another study, aiming to evaluate the possible impact of HPV on the survival of patients with adenocarcinoma CC, conclude that HPV infection do not predict patient prognosis, and only clinical stage and architectural grade are significant predictors for survival in such tumors (Dabic, 2008). Although comparison of results from different trials is difficult because diverse techniques are used, besides the lack of standardized protocols, small patient series, heterogeneous tumor stage and histological type distribution, different treatment protocols, and various statistical analyses, we highlight that HPV negative tumors seem to have worse prognosis in CC and also in extra genital tumors. H&N HPV-related tumors showed significantly less p53 mutations and p16 expression than HPV-negative ones and exhibit different clinical behavior, with favorable overall or disease-specific survival (Liu, 2017). It has been suggested that an underlying mechanism dependent on these two proteins could explain higher sensitivity to RT/CCRT in H&N-HPV positive tumors (Perri, 2015), as well as in genital tumors (Wakeham, 2017; Wang, 2010). Moreover, immune response to HPV infection increases tumor-infiltrating lymphocytes (TILs), which have been related to favorable prognosis in many solid tumors. In H&N tumors a distinct B-cell signature between virally driven tumors compared with virus independent ones have been demonstrated (Wood, 2016), which let to argue about biological variation in adaptive immune responses explaining different clinical outcome. In CC a large polyclonal repertoire of T cells HPV-specific have been demonstrated within the total population of TILs as well as in tumor-draining lymph nodes (de Vos van Steenwijk, 2010). In a therapeutic approach, objective regression of metastasis in CC patients by HPV-TILs enables to discuss about their immunological role, which could explain more aggressive behavior in HPV-negative tumors and encourages research for immunotherapy strategies in CC (Stevanović, 2015).

In conclusion, saving limitations due to the retrospective condition of this study, in which treatment protocols could evolve over time, we observed a significant correlation of FIGO staging and lymph node metastasis with overall survival, in

agreement with most reports in the literature. Our findings support the worse prognosis of HPV negative tumors, and among HPV genotypes we distinguish a trend for three prognostic groups, whereas the better OS was seen for genotypes others than HPV16/18/45, an intermediate OS rate for HPV16/18 and the worse OS for HPV45 and negative tumors. Improvement in knowledge of molecular pathogenesis of negative tumors which let to confirm if it is a different entity, as well as in immunological basis of HPV infection, could impact in CC patients care.

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