Università degli Studi di Ferrara

DOCTORAL COURSE IN BIOMEDICAL SCIENCES AND BIOTECHNOLOGY

CYCLE XXXIII

COORDINATOR Prof. Paolo Pinton

NEW BIOMARKERS FOR HUMAN PAPILLOMAVIRUS POSITIVE-HEAD AND NECK SQUAMOUS CELL CARCINOMAS

Scientific/Disciplinary Sector (SDS) BIO/13

Candidate
Oton Gonzalez, Lucia
(signature)

Scientific tutor
Prof. Tognon, Mauro
Mauro Tognon
(signature)

Academic Years 2017-18; 2018-19; 2019-20

INDEX

CHAPTER 1

General introduction

1.1. General introduction

The head and neck anatomical region consists of a series of structures in the oral cavity, oropharynx, and larynx. Squamous Cell Carcinomas of the Head and Neck (HNSCCs) are the sixth most common type of cancers. Up to 2018, approximately 850,000 new patients worldwide were diagnosed per year, including about 151,000 in Europe (Paolo Boscolo-Rizzo et al. 2017; Johnson et al. 2020). The most well-established risk factors for HNSCCs are tobacco and alcohol consumption (Liu et al. 2018). Since the 1980s the incidence of these HNSCCs has decreased over time due to an increased public awareness, and consecutive reduced smoking and alcohol consumption rates (Paolo Boscolo-Rizzo et al. 2017; Rosenberg and Vokes 2021). However, the incidence of oropharyngeal squamous cell carcinoma (OPSCC), including squamous cell carcinomas (SCC) of the tonsils (Figure 1), soft palate, base of tongue and lateral/posterior pharyngeal walls, which include a significantly high proportion of HNSCCs, is rising (Paolo Boscolo-Rizzo et al. 2017; Näsman, Du, and Dalianis 2020; McIlwain et al. 2014).

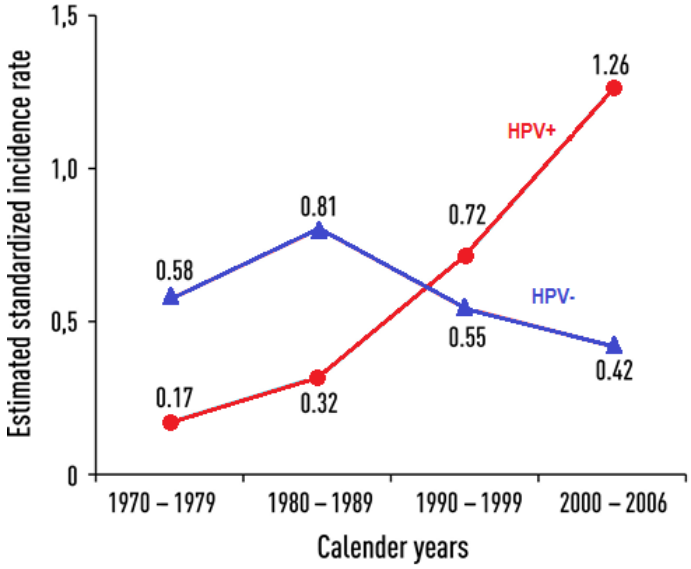


Figure 1. Estimated age-standardized incidence rate with 95% CI of HPV-positive and HPV-negative tonsillar squamous cell carcinoma (SCCs) cases per 100,000 individuals/year in Stockholm between 1970-2006. Adapted from Näsman et al. 2020.

The increased fraction of oropharyngeal HNSCC, registered during the past decade and independent of tobacco and alcohol use, was associated with high-risk human papillomaviruses (HPVs), with a remarkable contribution of HPV16 type (Rosenberg and Vokes 2021). It is worth mentioning that before 2000 HPV16 accounted for up to 40.5%

of OPSCC cases, rising up to 70% of OPSCC cases since 2009 (McIlwain et al. 2014; Ekanayake Weeramange et al. 2020).

High-risk HPV16 is not only responsible for oropharynx cancers, including tonsil, base of tongue and other oropharyngeal sites. Indeed, the last evaluation of the International Agency for Research in Cancer (IARC) concluded that there is enough evidence for HPV16 carcinogenicity in the oral cavity as well. Limited evidence for laryngeal cancer and HPV16 has been established so far (Bruni et al. 2019).

In several Countries prophylactic vaccination programs are now available, whereas they are in progress in other Regions. Notwithstanding, a significant reduction in HPV-OPSCC incidence is not expected until 2060. Altogether these data indicate the need of investigations to improve disease prevention, diagnosis, and treatment (Chaturvedi et al. 2018; Gillison et al. 2015).

1.2. Clinical and pathological features of OPSCC

HPV-related OPSCC constitute a distinct epidemiological, molecular and clinical form as compared to non HPV-related OPSCC (Bruni et al. 2019; Seiwert et al. 2015). HPV-negative OPSCCs tend to affect older patients with a history of heavy tobacco smoking and alcohol consumption, whereas they have an onset similar to other squamous cell carcinomas (SCCs) of the head and neck. HPV-positive OPSCCs affect younger patients who show a reduced history of smoking and alcohol intake (McIlwain et al. 2014). Some studies indicate that the most likely explanation for the origin of this distinct form of head and neck cancers associated with HPV is a sexually acquired oral HPV infection that is not cleared, persists and evolves into a neoplastic lesion (Bruni et al. 2019; Chaturvedi et al. 2015).

HPV-associated OPSCCs present different features compared to HPV-negative tumors. For instance, histopathologically, HPV-positive tumors tend to have a poorly differentiated and frequently basaloid histology (Lewis 2017; P. Boscolo-Rizzo et al. 2013), which in the classic sense of tumor classification and differentiation status, could mislead clinicians since, classically, poorly differentiated looking tumors are more aggressive when HPV-negative (Thompson 2013). Interestingly, HPV-positive tumors tend to have overall a better prognosis (Thariat et al. 2010).

Tumor morphology of HPV-related OPSCC is unique amongst head and neck tumors, partially because of the anatomical region of the oropharynx, but also because of the way HPV infection occurs. Indeed, the tumorigenesis process begins at the tonsillar crypts after HPV infection that is not cleared by the host's immune system (Figure 2). Over time, SCC may spread to the neck lymph nodes, and progress once tumors have metastasized (Lewis 2017).

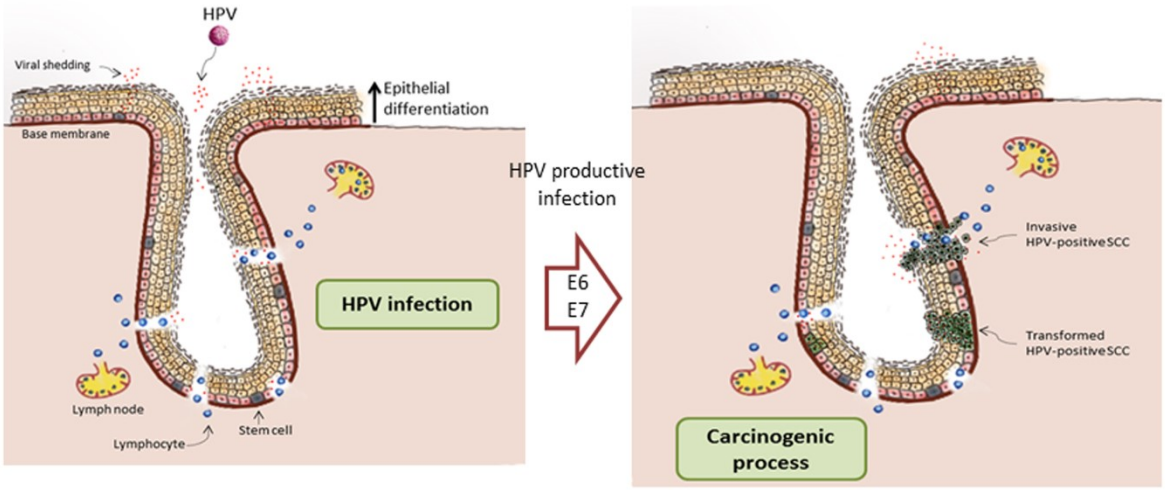


Figure 2. HPV infection of the tonsil crypt and the onset of HPV-positive OPSCC. HPV: Human papillomavirus; SCC: Squamous cell carcinoma. Adapted from Johnson et al. 2020.

1.3. Role of the human papillomavirus in oncogenic transformation

The HPV family consists of approximately 8,000 base pairs, circular, double-stranded, non-enveloped DNA viruses. Viral genome consists of early and late genes and a non-coding sequence named long control region (LCR). Early genes encode for proteins involved in viral replication, such as, E1 and E2/E4, and the accessory proteins; E5, E6 and E7, whereas the late genes transcribe for the capsidic L1 and L2 proteins (Sabatini and Chiocca 2020; Egawa et al. 2015) (Figure 3).

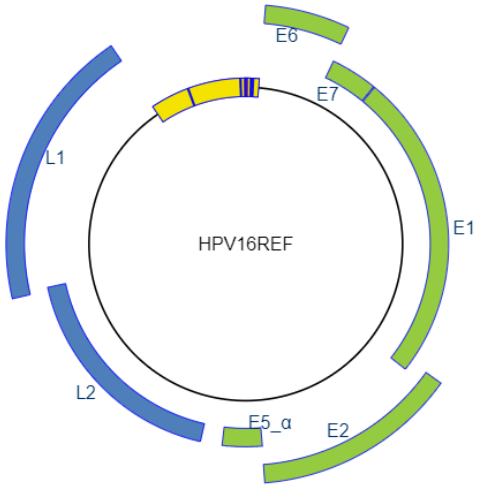


Figure 3. Reference HPV16 genome. From PaVe database.

High-risk HPVs are able to induce carcinogenic transformation of the infected mucosal epithelium by escaping cell-cycle checkpoints (Sabatini and Chiocca 2020). The contribution of HPV to the HNSCC onset occurs commonly within the tonsillar crypt of the oropharynx. Crypt cells are arranged in a discontinuous single-layer epithelium, which is susceptible to carcinogenic transformation (Sabatini and Chiocca 2020).

HPVs normally infect the basal layer of the epithelium, then exploit the epithelial-to-keratinocyte proliferation and differentiation pathways to complete the viral life cycle (Rotondo et al. 2020b). The primary transforming activity of HR HPVs depend on the E5, E6 and E7 oncoproteins. In particular, the two major viral oncoproteins E6 and E7 inhibit apoptosis and promote tumor cell growth (Zhang et al. 2016). E6 binds and disrupts the tumor suppressor protein p53, the major protein involved in the control of cell cycle, while E7 binds to the tumor suppressor pRb protein, which regulates the G1-S phase transition through interaction with the E2F transcription factor.

E7 binding disrupts the Rb-E2F complex, leading to the inactivation of Rb through proteasomal degradation. As a result, genes promoting the entry of cells into S phase are continuously activated, whereas the p16 gene, the inhibitor protein of the cyclin D1 CDK4/CDK6 complex, is continuously overexpressed (C. A. Moody and Laimins 2010a) (Figure 4).

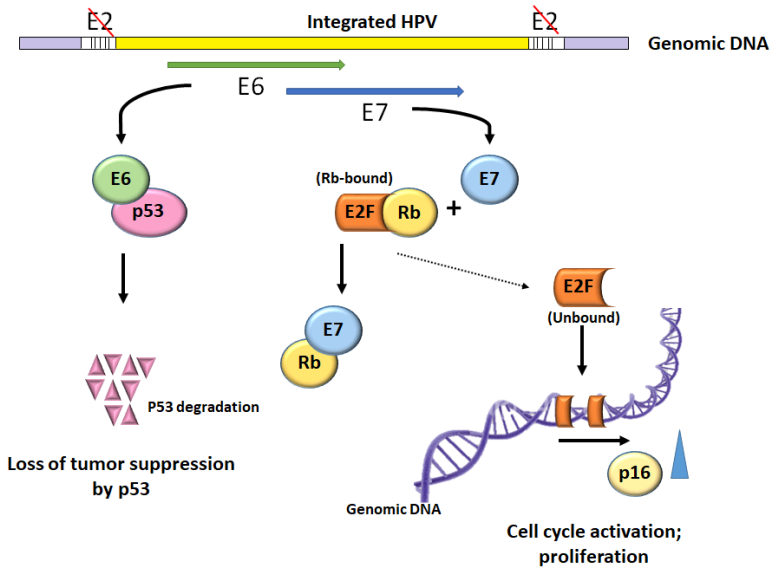


Figure 4. Classical mechanism of action, during the transformation, operated by HPV E6 and E7 oncoproteins.

Overexpression of the p16 protein has been reported in different HPV-associated cancers. In cervical intraepithelial lesions (CIN), overexpression of p16 protein is considered a reliable marker of tumor progression (Agoff et al. 2003), whereas in HPV-positive HNSCC, p16 overexpression has been associated to favorable outcome (Pinatti et al. 2021). On the other hand, in about 20% of HPV-positive HNSCC cases the p16 protein presents normal or downregulated expression (Henley-Smith et al. 2021).

HR-HPV E5, E6 and E7 oncoproteins are key players in tumor development by activating cellular oncogenes and inactivating tumor suppressor genes (R. Yang et al. 2019). Usually, HPVs replicate in epithelial cells, where the viral DNA is maintained in episomal form (Kurita et al. 2019). In this phase, HPV expression is regulated by cellular and viral transcription factors. In particular, HPV E2 protein, regulates viral expression (McBride and Warburton 2017, 6), through the interaction between two E2 proteins with four E2 binding sites (E2BSs) located in the long control region (LCR) of the viral genome (Balderas-Loaeza et al. 2007) (Figure 5).

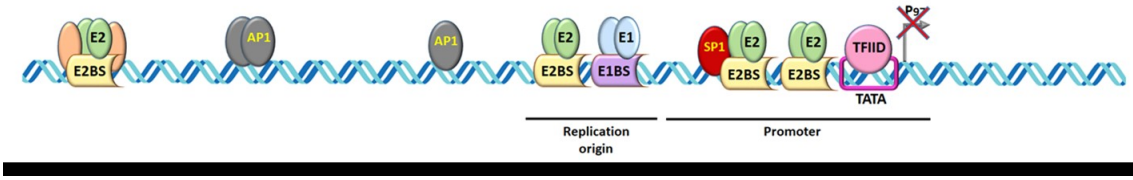


Figure 5. Schematic representation of the four E2 binding sites (E2BSs) in the LCR of HPV16. When E2 binds to the high affinity E2BS1 (left) the early promoter p97 is activated until increased levels of E2 are produced to bind to the low affinity E2BS 3 and 4 (right) and result in inhibition of the p97 promoter. This mechanism leads to a fine-tuned auto-regulatory feed-back inhibition of the HPV16 LCR.

The transcription of HPV early genes leads to inactivation of cellular control functions, and triggers reprogramming of cell proliferation, apoptosis, differentiation, epigenetic reorganization, and genomic instability (Zhang et al. 2016). These changes support the integration of episomal HPV DNAs into the genome of the host cell (McBride and Warburton 2017; Akagi et al. 2014), whereas the loss of E2 protein repression of viral transcription due to loss of E2 regions upon integration (Pinatti et al. 2021), contributing to E6 and E7 oncogenes overexpression and the initiation of the carcinogenesis process (Egawa et al. 2015; Preti et al. 2020) (Figure 4).

Despite data from cervical carcinoma studies (Rotondo et al. 2015), viral integration seems to be unnecessary for carcinogenic transformation in HNSCC, where integration, altered methylation patterns or a combination of both have been observed to play a role in the cancer onset (Wang et al. 2017; Khanal et al. 2018).

1.4. Diagnosis

Many studies have investigated the clinical presentation of HNSCC at different locations with efforts to improve early diagnosis (Kwon et al. 2021). The assignment of a proper clinical and pathological stage is key to facilitate prognosis estimation and to determine the best treatment option upon diagnosis (Lydiatt et al. 2017).

HNSCC patients present low 5-year survival rates associated to failure in early diagnosis. Diagnosis at advanced tumor stages is fairly common due to the lack of early detection/screening tests for HNSCCs detection (McIlwain et al. 2014), which is usually based on clinical examination of the upper aerodigestive tract and histologic analysis of tissues under analysis. This type of analysis may miss hidden tumor sites, such as the tongue base or tonsil crypts (Punyadeera and Slowey 2019).

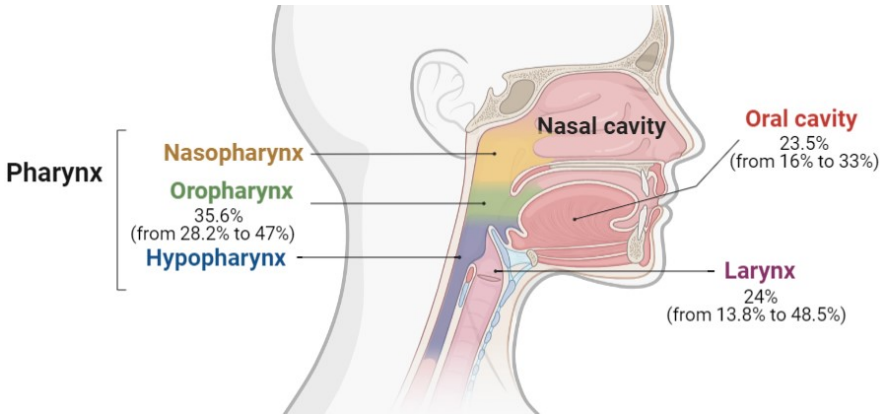


Figure 6. Anatomy of the head and neck region; parts of the head and neck that are susceptible to HPV infection and percentage of HPV-positive tumors per part according to Betiol et al 2013.

Usually, at early disease stage (I or II), tumors of the oral cavity and oropharynx present a small size (less than 2cm) and are often asymptomatic, while patients at late disease stage (III and IV) present symptoms, such as pain, otalgia, dysphagia, sore throat, etc., often accompanied of lymph node involvement (McIlwain et al. 2014). Furthermore, HPV-positive HNSCC, being a distinct form of HNSCC from HPV-negative HNSCC, presents a different clinical feature (Ekanayake Weeramange et al. 2020; Betiol, Villa, and Sichero 2013). For instance, HPV-positive patients commonly present a neck mass as the initial symptom, whereas HPV-negative patients more commonly present symptoms related to the primary tumor site, such as sore throat, dysphagia, and/or odynophagia (McIlwain et al. 2014). Most HPV infections cause no symptoms, whereas due to the anatomical complexity of the affected region, it is difficult to find preneoplastic lesions, as routinely occurs for cervical cancers (Figure 6). Taken the inability to screen for and detect precursor lesions or early-stage cancers (Gillison et al. 2015), most HPV-positive patients are typically diagnosed with late-stage disease (Dahlstrom, Anderson, et al. 2015), usually presenting early tumor stage and advanced nodal stage compared to HPV-negative, in general, TNM stage disease III and IV at presentation (Machczyński et al. 2020).

The absence of defined early warning signs puts in evidence the need to find specific biomarkers for early disease diagnosis, prediction of prognosis, and ultimately, appropriate selection of therapy (Economopoulou, de Bree, et al. 2019; Punyadeera and Slowey 2019).

HPV status has a significant effect on patient prognosis, whereas it may help to determine the appropriate therapy. HPV positivity is the most favorable non-anatomic predictor for HNSCC outcome (Machczyński et al. 2020) and accordingly, HPV DNA status is one of the first markers analyzed upon HNSCC diagnosis (Ekanayake Weeramange et al. 2020). Since current classification methods fail to differentiate between HPV-positive and HPV-driven HNSCC tumors, the recognition of distinctive molecular profiles to allow for correct classification of HPV-positive HNSCCs is still imperative for the choice of best treatment in order to prevent tumor recurrences after primary treatment (Ekanayake Weeramange et al. 2020).

1.5. Treatment and prognosis

HNSCC treatment approach is generally guided by tumor anatomical location, stage, and disease and functional characteristics (Johnson et al. 2020). The use of the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) staging systems are the prevalent choices for patient stratification and treatment selection, although personalized nomograms and molecular analyses for therapy choice (MATCH) approaches are emerging holding the promise of better stratification (Lydiatt et al. 2017).

The head and neck are a complex anatomic area, involved in vital functions, such as breathing, swallowing or speech. Usually, SCC treatment is very aggressive, leading to permanent impairment of speech or swallowing. The treatment selection is difficult, as it should aim for function preservation while maintaining highly curative approaches (Johnson et al. 2020). Currently, the main choices for HNSCC treatment are resection, radiation and chemotherapy.

For early-stage tumors (stages I or II), the primary treatment consists on tumor resection and/or radiation. In the event of primary treatment failure, after single modality radiation or surgery, salvage with radiation or chemotherapy will reduce the risk of recurrence and improve survival. Resection is also used as a rescue method for late stage patients presenting tumor relapse (McIlwain et al. 2014; Johnson et al. 2020).

For patients with advanced disease stage (stages III or IV) with extra-nodal extension, close or involved surgical margins, or perineural invasion; multimodality treatment with combinations of surgery, radiation, and/or chemotherapy is commonly used. The administration of high-dose cisplatin chemotherapy in combination with radiation further

improves disease-free survival, whereas it impacts patient survival in high risk groups (Johnson et al. 2020).

Complexity of HNSCC is suggested by molecular biology/genetic analyses that reveal heterogeneity independently from the tumor location and stage (Ekanayake Weeramange et al. 2020), whereas it evidences the need for a better understanding of the biology of HNSCCs to find biomarkers for personalized treatments. Some altered targets have already been found, but clinical improvements are slow. For instance, the monoclonal antibody targeting EGFR, Cetuximab, has been approved for both HPV-positive and -negative HNSCCs, with limited efficacy (Alsahafi et al. 2019). Recently, the use of immunotherapy has been also approved for HNSCC. In 2016, the anti-programmed cell death protein (PD-1) monoclonal antibodies, nivolumab and pembrolizumab, for the treatment of patients with recurrent/metastatic HNSCC refractory to platinum-based therapy were approved. In 2019, the use of pembrolizumab was approved for the first-line treatment of patients with unresectable recurrent/metastatic HNSCC (Cohen et al. 2019). For patients with recurrent/metastatic HNSCC expressing PD-L1 with a combined positive score (CPS) ≥1, the use of pembrolizumab in combination with platinum and fluorouracil was approved as the frontline therapy (Cohen et al. 2019; Johnson et al. 2020).

Since the use of chemoradiotherapy (CRT) following surgery is associated to increased radiation toxicity, and a wide range of side effects, it is of extreme importance to accurately predict the extent of disease prior to treatment administration (Johnson et al. 2020). In fact, there is over 50% of likelihood for distant metastases in patients with HPV-positive oropharyngeal cancer that are primarily managed with resection surgery, whereas 65% of these patients need to be further treated with CRT (Rosenberg and Vokes 2021). About 10-25% of HPV-positive patients will present recurrence and/or distant metastasis within the first two years of treatment (Cohen et al. 2019; Economopoulou, Koutsodontis, et al. 2019).

Only now current staging systems are beginning to differentiate HPV-related tumors from other HNSCCs (Lewis 2017). HPV-positive tumors are usually highly responsive to therapy, resulting in better overall survival (OS) with increased rates of locoregional control (Machczyński et al. 2020). Therefore, treatment de-escalation for HPV-positive patients has been proposed, but because of the risk of recurrence the decision on patients

that would be good candidates for treatment de-intensification is difficult (Rosenberg and Vokes 2021; Alsahafi et al. 2019).

1.6. Aim and outline of my experimental thesis

Overall, my thesis has been organized in four chapters:

1. Chapter 1 provides a general introduction on Head and neck squamous cell carcinoma (HNSCC); (1.1) The site of onset and etiopathogenesis, (1.2) the main clinical and pathological features; (1.3) the role of the human papillomavirus in the onset of oropharyngeal squamous cell carcinoma (OPSCC), a subset of HNSCCs; (1.4) how HNSCC is diagnosed and (1.5) treated based on classical considerations and molecular characterizations.

2. The second chapter of my thesis discusses the materials and methods used for the study of several markers in HNSCC, including the developed methods that have been useful for the publication of other four studies (Oton-Gonzalez et al. 2021; Rotondo et al. 2020b; Tognon et al. 2020; Malagutti et al. 2020).

3. In the third chapter, the study results are presented in the form of three different subchapters. Each one of these chapters corresponds to a manuscript in preparation; therefore, the main structure of the article has been maintained with an introduction, results and a discussion section. The overall aim of these studies was to find new markers serving as molecular targets in HNSCCs for the planning of targeted therapeutic strategies to improve patient's survival.

3.1. Treatment de-escalation has been proposed on HPV-positive patients based on their improved prognosis compared to HPV-negative, but still a subset of HPV-patients present high relapse rates and metastasis, making difficult to decide which patients are good candidates for treatment de-intensification. The identification of molecular markers for HPV-positive tumors that allow for the stratification of this group of patients is necessary. In this subchapter a prognostic model based on the identification of circulating viral oncoproteins as a marker for patient stratification is proposed.

3.2. HPV plays an important role in the prognosis of HNSCC patients. HPV-positive tumors present an overall improved survival compared to HPV-negative. The main transforming activity of HPV relies on the oncoproteins E6 and E7; for this reason, HPV

mRNA expression, a marker of transcriptional activity of the virus, is considered necessary to initiate the carcinogenesis process. In the second subchapter, the different mechanisms involved in HPV oncogene expression are studied, focusing mainly on the impact that different variants of the HPV long control region (LCR) and its methylation status have on oncogene expression and patient's prognosis.

3.3. Head and neck squamous cell carcinoma (HNSCC) is a heterogeneous disease. Several attempts have been made to classify these tumors based on anatomic location, clinical stage and tumor phenotype; once these characteristics are individualized, the appropriate treatment is chosen, but not always classical characterization correlates to prognosis; therefore, there is a need to find new prognostic in HNSCCs for planning targeted therapeutic strategies to improve patient's survival. In this subchapter, a set of tumor suppressor genes and proto-oncogenes are studied in correlation to HNSCC; finally, a combination of molecular markers in HNSCC are proposed in order to plan personalized treatment strategies.

4. Finally, in chapter four a general discussion of the contents presented in the thesis is presented.

CHAPTER 2

Materials and Methods

2.1 Study population

Head and neck squamous cell carcinoma (HNSCC) specimens (n=84) from patients, with a mean age ± standard deviation [SD] 65.91±11.39 years old, together with control tumor-free samples (n=58) from non-oncological patients (normal controls) who underwent tonsillar surgery; 39±15.17 years old, were collected at the ear, nose and throat (ENT) Clinic, University Hospital of Ferrara, Ferrara, Italy.

Patients were assessed for eligibility of this study in agreement with the Declaration of Helsinki and local rules. All patients gave written informed consent to participate in the studies. Clinical data of patients were available from hospital records. The studies were approved by the Ethics Committee of Ferrara. Inclusion criteria were (i) detection of primary OPSCC in patients 18-95 y old; (ii) exclusion criteria were the radiotherapy and/or chemotherapy treatments before ascertainment and patients' recruitment. Tumor and non-tumor tissue biopsies, together with blood, were collected at the time of surgery.

2.2 Nucleic acid extraction

2.2.1. Fresh tissue

DNA/RNA extractions from HNSCC (n=67) and control biopsies (n=58) were carried out using the AllPrep DNA/RNA/Protein Extraction Kit (Qiagen, Milan, Italy). DNA and RNA were quantified with the NanoDrop 2000 (Thermo Scientific, Milan, Italy) and stored at -80°C until the analyses. DNA suitability was assessed by qualitative PCR for β-Globin gene amplification as done before (Oton-Gonzalez et al. 2021). Due to transport/extraction methods, some RNA samples degraded and were not available for further analyses. Matched RNA/DNA samples were n=41.

2.2.2. FFPE tissue

To increase the sample size of HPV-positive HNSCC samples formalin-fixed and paraffin embedded tissues (FFPE) (n=17) were used. DNA and RNA were extracted from FFPE tissues using the QIAamp DNA FFPE tissue kit and RNeasy FFPE kit, respectively (Qiagen, Milan, Italy) as done before (Rotondo et al. 2016, 6). DNA and RNA were quantified with the NanoDrop 2000 (Thermo Scientific, Milan, Italy) and stored at -80°C

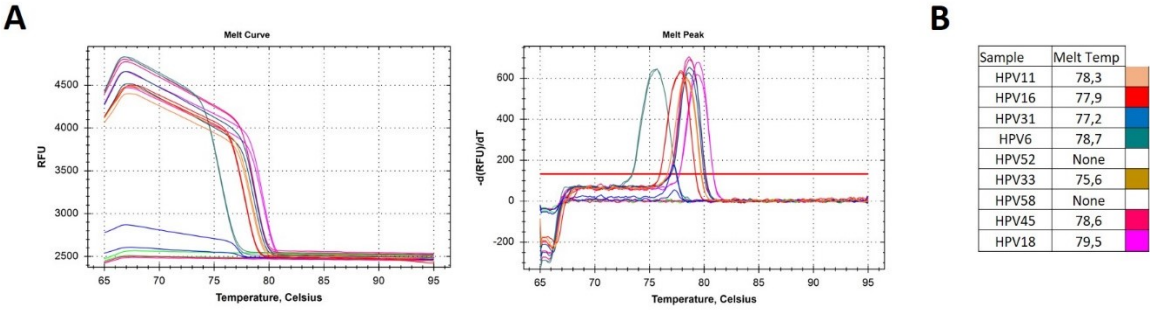
until the analyses. DNA suitability was assessed by qualitative PCR for β-Globin gene amplification as done before (Rotondo et al. 2018a).

2.3 HPV screening

DNAs were tested for HPV DNA sequences by RT-qPCR using the universal primer pair for HPV L1 detection GP5+/GP6+ (Table 1) (Malagutti et al. 2020; Oton-Gonzalez et al. 2021). Each RT-qPCR assay was performed using a recombinant plasmid vector containing the complete HPV16 genome, as positive control and two negative controls; a HPV-negative DNA obtained from human placenta (Rotondo et al. 2020b) and a mock sample consisting of distilled H_2O. Briefly, 50ng of human genomic DNA were used in 10 µl qPCR reactions, including: 2x of the SsoAdvanced Universal SYBR Green Supermix, Bio-Rad (Hercules, CA, USA) and a final concentration of 500nM for each primer. Thermal conditions were: initial step of 95°C for 5 min and 45 cycles of 95°C for 15 s followed by a 60°C for 30s step. Each sample was analyzed in triplicate.

2.4 Genotyping

At the end of the RT-qPCR reaction, a final high resolution melting step was added from 65-95°C, increasing 0.1°C every 0.03 s. Plasmids containing different HPV types were used as positive controls. DNA void of HPV genome was used as negative control. Initial genotyping was done by comparing the HNSCC samples melting peaks to the plasmid controls; similar melting peaks indicated same genotype as the control (Figure 1). Further characterization was performed using the specific genotype primers for HPV amplification.



Sample	Melt Temp
HPV11	78,3
HPV16	77,9
HPV31	77,2
HPV6	78,7
HPV52	None
HPV33	75,6
HPV58	None
HPV45	78,6
HPV18	79,5

Figure 1. Melting curve analysis for HPV detection obtained by qPCR. (A) Representative melting curve analysis of qPCR products employing recombinant

plasmid vectors containing the complete genomes of HPV6, 11, 16, 18, 31, 33, 45, 52 and 58 types, used as positive controls. The melting curve assay showed a differential melting temperature (Tm), i.e., 77.9 ◦C for HPV16 and 79.5 ◦C for HPV18. (B) HPV genotype in correspondence to its Tm. Each color represents one sample from graph A.

2.5 Viral DNA load

After HPV genotype determination on the basis of differential melting temperature (T_m) (de Araujo et al. 2009; Oton-Gonzalez et al. 2021), a standard curve of specific recombinant plasmids was used to calculate the viral DNA load. HPV DNA load values were reported as viral copies per 10^4 human cell equivalents (copy/10^4 cells).

Quantification of the viral DNA load was performed against a plasmid-HPV-specific standard curve. Viral load was expressed as HPV copies per cell. Since at least one HPV genome copy/ cell is expected in transformed cells, 1 copy/ cell was defined as the cutoff for high viral load, samples below the cutoff were determined to have low viral load.

2.6 HPV DNA physical status

HPV DNA physical integration/episomal status was investigated using the qPCR as described (Table 1) (Peitsaro, Johansson, and Syrjanen 2002). Specific primers amplifying the viral regions E2 and E6 were used. Briefly, 50 ng of template DNA were analyzed in triplicate in 10µl multiplex qPCR reactions consisting of 2x TaqMan Universal Master Mix II, no UNG, Thermo Fisher Scientific (Waltham, MA, USA). Primers were used in multiplex at a final concentration of 500nM and 300nM for E6 and E2, respectively. Probes labelled with reporter dyes FAM for E6 and ROX for E2, were used at a final concentration of 100nM each. The thermal conditions were: an initial step of 95ºC for 5 min and 45 cycles of 95ºC for 15 s followed by a 60ºC for 30s step. A standard curve using the HPV16 recombinant plasmid was used to calculate the amount of E2 and E6 amplified. Then, the E2/E6 ratio was assessed to determine the HPV DNA physical integration/episomal status. A ratio E2/E6 of 0, i.e. no E2 presence, indicated integration, while the ratio greater than 1 and lower than 1 indicated episomal form and concomitant integrated and episomal form, respectively. The HPV16-positive cells, employed as controls, SiHa and CaSki cell lines, were used as comparative samples.

2.7 Total mRNA retrotranscription

Total mRNA was retro-transcribed using the Improm II (Promega, Wisconsin, USA) reverse transcription system, as performed before (Torreggiani et al. 2019). Briefly, 1050ng of total mRNA were used for retrotranscription in 21µl of reaction mixture. Each reaction contained: the diluted RNA in DEPC-treated water up to a 10µl volume, 1 µl of random examers; and the RT MIX composed of: 4 µl 5X IMPROM buffer, 4 µl $MgCl_2$ 25Mm, 1 µl dNTPs (10Mm) and 1 µl of RT IMPROM. RT-PCR conditions were: 70°C for 10 min after mixing the diluted RNA with the random primers; and 42°C for 60 min, followed by 15 min incubation at 70°C, after addition of the RT mix. cDNA was finally diluted to 1:2 to a final concentration of 25ng/µl to be used in PCR reactions.

2.8 Gene expression

2.8.1. Viral gene expression

cDNAs were analyzed for the expression of HPV E2, E5, E6 and E7 genes, using the specific ddPCR assay QX200 Droplet Digital PCR System-Bio-Rad (Bio-Rad, Segrate, Italy). DdPCR reactions contained; 11 µL of a 2 ×ddPCR super mix (QX200 EvaGreen ddPCR, Bio-Rad, Segrate, Italy), primers at final concentration of 225nM each, and 10 µL of DNA/ddH2O (~50 ng per reaction). Specific primers for each HPV gene were used (Table 1). The mixture was added to the DG8 cartridge with 20 µL of droplet formation oil, using an automated droplet generator (Bio-Rad, Segrate, Italy). Every sample was partitioned into ~20,000 droplets, which were then transferred into a 96-well PCR plate, covered with pierceable foil, heat-sealed using a PX1 PCR Plate Sealer (Bio-Rad, Segrate, Italy), and placed in a thermal cycler (SimpliAmp, Applied Biosystem, Milan, Italy). Cycling conditions were as follows: heat to 95 °C for 5 min, followed by 94 °C for 30 s, 60 °C and for 1 min for a total of 45 cycles, then 5 min at 90 °C, ending at 4 °C. After PCR, the 96-well PCR plate was placed in the reader and data were analyzed using with the QuantaSoft analysis tool (Bio-Rad, Segrate, Italy). The GAPDH housekeeping gene was employed as control for the gene expression analysis (Rotondo et al. 2020b). Each ddPCR experiment included one DNA extraction negative control, an additional negative control (H_2O) without DNA, and a positive control consisting on HPV16 recombinant plasmid (Oton-Gonzalez et al. 2021).

2.8.2. Host gene expression

cDNAs were analyzed for the expression of cellular genes. Real-time qPCR was performed using 2x SsoAdvanced Universal SYBR Green Supermix, Bio-Rad (Segrate, Italy). A final concentration of 500nM of each primer (Table 1) was used in single assays of 10µl each, samples were run in triplicate, along with no-template controls. Thermal cycling consisted of an initial cycle of 95°C for 5 min, 40 cycles of 95°C for 15 s and 60°C for 30 s. Fold changes of the host mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method, using as control the cellular housekeeping gene, GAPDH. Furthermore, data was normalized against the control group.

2.9 Promoter methylation analysis

2.9.1. Bisulfite conversion

DNA from (i) fresh HNSCC biopsies and control non-tumor specimens, together with (ii) FFPE OPSCC tissues, were subjected to sodium bisulfite treatment using the EpiTect Bisulfite Kit (Qiagen, Milan, Italy). Briefly, 500ng of DNA were converted in 140µl reactions containing; 20µl of DNA dilution in ddH_2O, 85µl of bisulfite mix and 35µl of DNA protect buffer, provided by the kit. Reactions were left to incubate overnight following manufacturer instructions. DNA was extracted following the protocol for fresh tissue samples (i): "*Sodium bisulfite conversion of unmethylated cytosines in DNA*" and for FFPE tissues (ii): "*Sodium bisulfite conversion of unmethylated cytosines in DNA isolated from FFPE tissue samples*", both contained in the EpiTect Bisulfite Handbook (09/2009).

2.9.2. HPV promoter methylation

Since bisulfite treatment is known to introduce nicks in the DNA, HPV16 long control region (LCR) sequence was divided into three segments, to preserve the high efficiency of the PCR reaction as done before (Kalantari et al. 2004). Bisulfite-treated DNA was amplified using three primer copies: 16msp3F/R (positions 7049 to 7560), 16msp4F/7R (positions 7465 to 7703) and 16msp5F/R (positions 7748 to 86), which amplify in a noncontiguous manner the HPV16 5'LCR, the central LCR known as the enhancer region, and the 3'LCR containing ORI and p97 promoter regions, respectively (Kalantari

et al. 2008; Milutin Gašperov et al. 2015). The PCR started at 95°C for 5 min, followed by 45 amplification cycles of 95°C for 30s, annealing at variable temperature according to primer pair for 1 min (Table 1), and extension at 68°C for 1 min as done before (Kalantari et al. 2004). There was a final extension at 68°C for 7 min. A PCR negative control, containing distilled water, and three positive controls, containing the HPV16 plasmid, SiHa and CaSki cell lines DNA, were included per reaction. PCR products were run onto 2% agarose gels.

2.9.3. Host gene promoter methylation

Methylation was studied at the RARB (OMIM: 180220) promoter region of DNA from HNSCC patients and healthy subjects; these promoters contains 9 CpG dinucleotides. Amplification was done by PCR, using specific BSF primers as done before (Rotondo et al. 2018b; 2016) (Table 1). Briefly, The PCR started at 95°C for 5 min, followed by 50 amplification cycles of 95°C for 1 min, annealing at variable temperature according to primer pair for 1 min, and extension at 72°C for 1 min. There was a final extension at 72°C for 10 min. A PCR negative control, containing distilled water and a positive control were included per reaction. PCR products were run onto 2% agarose gels.

2.9.4. Direct DNA sequencing and sequence analysis

All DNA amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Milan, Italy) and then subjected to direct sequencing using the automated ABI-Prism-3130X DNA sequencer (Applied Biosystems, Monza, Italy), as performed before (Mazzoni, Rotondo, et al. 2017; Rotondo et al. 2017). The obtained sequences were manually edited with Chromas Lite 2.1.1. (Technelysium Pty Ltd, South Brisbane, AU) and aligned to that available in the GenBank database using the BiQ analyzer software (Bock et al. 2005).

Table 1. Validated primer sets used in qPCR and/or ddPCR to detect and quantify HPV DNA and both, viral and cellular genes.

	Target	Primers names	Primer sequence (5'→ 3')	Amplicon size (bp)	Annealing temp. (°C)	Reference
DNA						
Viral	HPV L1	GP5+	TTTGTTACTGTGGTAGATACTAC	139-145	60	(Rotondo et al. 2020a)
		GP6+	GAAAAATAAACTGTAAATCATATTC			
	HPV16 E2	E- HPV16 E2 F	AACGAAGTATCCTCTCCTGAAATTATTAG	82	60	(Peitsaro, Johansson, and Syrjanen 2002)
		E- HPV16 E2 R	CCAAGGCGACGGCTTTG			
		E Probe 16E2PRO	[ROX]CACCCCGCCGCGACCCATA[BHQ2]			
	HPV16 E6	I+E- HPV16 E6 F	GAGAACTGCAATGTTTCAGGACC	81	60	
		I+E- HPV16 E6 R	TGTATAGTTGTTTGCAGCTCTGTGC			
		I+E Probe 16E6PRO	[6FAM]CAGGAGCGACCCAGAAAGTTACCACAGTT[BHQ1]			
Host	β-Globin	β-Globin F	TGGGTTTCTGATAGGCACTGACT	152	56	(Martone et al. 2007)
		β-Globin R	AACAGCATCAGGAGTGGACAGAT			
Bisulfite treated DNA						
Viral	HPV16 promoter	E2-M FWD (16msp5F)	TAAGGTTTAAATTTTTAAGGTTAATTAAAT	287	57	(Kalantari et al. 2004)
		E2-M REV (16msp5r)	ATCCTAAAACATTACAATTCTCTTTTAATA			
	HPV16 5′LCR	16msp3F	AAGTAGGATTGAAGGTTAAATTAAAATTTA	543	55	
		16msp3r	AACAAACAATACAAATCAAAAAAACAAAAA			
	HPV16 enhancer	16msp4F	TATGTTTTTTGGTATAAAATGTGTTTTT	278	53	
		16msp7R	TAAATTAATTAAAACAAACCAAAAATATAT			
Host	RARB promoter	RARB_SQ_F	AGAGGTAGGAGGGTTTATTTTTTGT	86	48	(Rotondo et al. 2018b)
		RARB_SQ_R	AATCATTTACCATTTTCCAAACTTACT			
RNA						
Viral	HPV16 E2	HPV16 E2 F	AACGAAGTATCCTCTCCTGAAATTATTAG	82	60	(Peitsaro, Johansson, and Syrjanen 2002)
		HPV16 E2 R	CCAAGGCGACGGCTTTG			
	HPV16 E6	HPV16 E6 F	GAGAACTGCAATGTTTCAGGACC	81	60	
		HPV16 E6 R	TGTATAGTTGTTTGCAGCTCTGTGC			
	HPV16 E5	16-E5 FWD	CGTCCGCTGCTTTTGTCTGTGTCTACATAC	89	60	(Weyn et al. 2011)
		16-E5 REV	CACCTAAACGCAGAGGCTGCTGTTATCCAC			

	HPV16 E7	E7 FWD	AGGAGGATGAAATAGATGGTCCAG	112	60	(Pett et al. 2006)
		E7 REV	CTTTGTACGCACAACCGAAGC			
Host	P16INK4A	p16 ink4a FWD	CCAACGCACCGAATAGTTACG	58	60	(Marcoux et al. 2013)
		p16 ink4a REV	GCGCTGCCCATCATCATG			
	IRF6	IRF6_F	GCTCTCTCCCAATGACTGACCTGGA	239	60	(Zengin et al. 2015)
		IRF6_R	CCATGACGTCCAGCAGCTTGCTA			
	P63	P63_F	AGCAGCAAGTTTCGGACAGT	236	60	(Yalcin-Ozuysal et al. 2010)
		P63_R	TGCTGTTGCCTGTACGTTTC			
	RARB	Rarb_f	TGAAAATCACAGATCTCCGTAGCA	75	60	(Rotondo et al. 2018b)
		Rarb_r	CCAGGAATTTCCATTTTCAAGGT			
	c-Jun	cJun_f	GAGAGGAAGCGCATGAGGAA	76	60	(Kolomeichuk et al. 2008)
		cJun_r	CCAGCCGGGCGATTC			
	EGFR	EGFR fwd	AACTGTGAGGTGGTCCTTGG	116	60	(Ganly et al. 2007)
		EGFR rev	GTTGAGGGCAATGAGGACAT			
	GAPDH	GAPDH F	GAAGGTGAAGGTCGGAGTC	226	60	(Xiao et al. 2011)
		GAPDH R	GAAGATGGTGATGGGATTTC			

2.10 Serological testing

Blood samples were collected for HNSCC patients at the time of diagnosis for all HNSCC patients and during the follow-up of 3, 6, 12, 24 months for HPV-positive HNSCC patients. Upon collection, blood samples were allowed to clot for 30 min at room temperature and then centrifuged at 1,300 g for 15min to separate the serum. Sera were transferred into new tubes and stored at -80°C until the analyses. Serum HPV16L1 IgG antibodies and E7 oncoproteins were evaluated in HNSCC patients, HPV-positive and HPV-negative at the time of diagnosis for all patients, and during a follow-up at 3, 6, 12 and 24 months for HPV-positive patients.

2.10.1. Serum HPV16 L1 Antibodies

HPV16 L1 IgG antibodies were analyzed by ELISA with a commercial kit (HPV16L1, Cusabio, TX, USA). The test was performed according to manufacturer's instructions. Serum samples were diluted 1:1000 and thoroughly mixed before use. The signal intensity was measured as Optical Density (OD) at 450 nm (Thermo Electron Corp., model Multiskan EX, Finland) (Bononi et al. 2018). The cutoff value was calculated according to manufacturer's instructions, i.e., an OD sample/OD negative ratio, equal or greater than 2.1, was considered HPV16 IgG positive.

2.10.2. E7 oncoprotein levels

HPV16 E7 oncoprotein concentration in sera was assessed using the "HPV16 E7 Oncoprotein ELISA Kit" (Cell Biolabs, California, US), according to manufacturer's instructions. The ELISA plates were read spectrophotometrically at 450 nm (OD) as done previously (Mazzoni et al. 2018). HPV16 E7 protein concentration was calculated using standard curves of recombinant E7 proteins provided by the kit. Briefly, according to manufacturer's instructions, seven serial dilutions (1:3) from; 135 ng/ml were prepared and used for sample interpolation. Presence or absence E7 HPV16 oncoprotein was determined by comparing the absorbance of the sample to the cutoff value, calculated as done before (Pietrobon et al. 2017). The cutoff for HPV16 E7 oncoprotein was 0.75ng/ml. HPV16 E7 oncoprotein variation during the follow-up was assessed by the ratio between protein amount the time of relapse/the previous time point; ratios >1 indicated increment of protein prior to relapse, while ratios <1 indicated decrement.

2.11. Statistical analysis

Statistical analyses were carried out using the GraphPad Prism for Windows (version 8.0, GraphPad). Student's t-test was used to compare the mean between groups for gene expression analyses and viral load (Mazzoni, Di Stefano, et al. 2017). Pearson/Spearman correlation tests were used to analyze correlation between viral gene expression and HPV DNA load; between E7 oncogene and p16 expression in HPV-positive HNSCCs; to estimate the correlation of viral DNA with viral mRNA expression; establish the associations between HPV LCR epigenotypes and viral gene expression; to check for correlation between nucleotide variation/methylation within transcription binding sequences and viral gene expression; and to assess the univariate differences of clinicopathological features according to E7 oncoprotein presence in serum.

For methylation analyses; sequences showing more than 50% of methylated CpGs were considered hypermethylated. Fisher's exact test was used to test the hypotheses that the methylation frequencies correlated to HPV physical status. All HPV16 LCR sequences were compared with the prototype clone (K02718.1), isolated from a human invasive cervical carcinoma and regularly used for this type of studies (Yao et al. 2019; Escobar-Escamilla et al. 2019). The PROMO-ALGGEN database (Messeguer et al. 2002; Farré et al. 2003) was used to predict the cellular transcription factor binding sites (TFBS) affected by nucleotide variations in HPV16 LCR. A dissimilarity rate of 5% was permitted.

Finally, all parameters were correlated to patient's progression-free survival (PFS) and overall survival (OS) using the Kaplan-Meier model; tick marks indicated censored subjects and drop-offs. Kaplan-Meier statistical significance was estimated using the log-rank test. P values less than 0.05 were considered statistically significant ($p<0.05$) for all analyses.

CHAPTER 3

RESULTS

CHAPTER 3.1

Serum circulating E7 HPV oncoprotein as a recurrence marker of Head and neck Squamous Cell carcinomas

3.1.1. Introduction

Despite the great improvements in treatment options for HNSCC, and in particular, OPSCC, preventing relapses and treating patients remains a challenge. Optimization of therapy is the primary purpose, aiming to increase survival rates through more targeted options and personalized treatments for all patients (Wittekindt et al. 2019). However, treatment protocols for OPSCC management do not take into account the biological differences between HPV-positive and HPV-negative tumors, and therefore standard treatments for both subtypes are currently employed (Hargreaves et al. 2019; Qian et al. 2019).

Several clinical trials, such as PATHOS (NCT02215265) or OPTIMA (NCT02258659), are now in progress (Hargreaves et al. 2019; Mirghani and Blanchard 2018) aiming to determine whether treatment de-intensification confers improved quality of life for HPV-positive OPSCC patients whilst maintaining high rates of cure. In fact, while many HPV-positive OPSCC patients respond well to treatment de-escalation, some others present with complex cases, marked by high relapse rates and worse prognosis (Hargreaves et al. 2019; Roberts et al. 2019), indicating heterogeneity within this subgroup of patients. About 10-25% of HPV-positive patients will present recurrence and/or distant metastasis within the first two years of treatment (Cohen et al. 2019; Economopoulou, Koutsodontis, et al. 2019).

Hence, a correct stratification of HPV-positive patients is necessary to select patients that will benefit from treatment de-intensification (Mehanna et al. 2019). In the effort to improve stratification, many studies investigate HPV status, p16 overexpression, the surrogate marker of HPV transformation (Lydiatt et al. 2017; Cohen et al. 2019), and HPV E6/E7 mRNA expression (Reuschenbach et al. 2019). However, the current stratification system leads to several pitfalls: first, HPV might be present as a transient infection, but not active in the tumors (Reuschenbach et al. 2019); second, p16 expression is not always observed in HPV-positive tumors (Lechner et al. 2018); third, HPV mRNA levels could be too low to be detected (Kitamura et al. 2020); last, many HNSCC tumors are anatomically difficult to access and could eventually be missed, especially during the patient's follow-up (Van Abel and Moore 2012).

Serological testing has gained interest in the past few years for HPV-positive OPSCC prognostic studies. The immune response of the host has been studied in association to

both HPV-positive tumors and patient's prognosis (Wittekindt et al. 2019; Holzinger et al. 2017). HPV16 L1 capsid antibodies have been found several years before OPSCC presentation, but are also cumulative markers of viral exposure (Dahlstrom, Anderson, et al. 2015; Piontek et al. 2019). Antibodies to HPV16 E6 and E7 oncoproteins at the time of diagnosis may be useful to predict disease-free survival in HPV-positive OPSCC patients (Spector et al. 2017; Lang Kuhs et al. 2017). However, routine testing for antibodies against HPV oncoproteins are difficult to perform due to the lack of available commercial kits.

Studies on cervical cancer have shown that detection of HPV E6 and E7 oncoprotein in cervical scrapings may constitute valuable markers for disease progression (Y.-S. Yang et al. 2012; Kong et al. 2020). Moreover, the presence of HPV16 E6 and E7 oncoproteins has been demonstrated by direct ELISA in culture supernatant of cervical cancer cell lines SiHa and CaSki, indicating release of viral oncoproteins from tumor cells (Lee et al. 2001). On this ground, we hypothesized that HPV E6 and E7 oncoproteins could be present in serum of HPV HNSCC patients, whereas their serum detection could be useful for prognostic purpose. The presence of HPV E6 and E7 oncoproteins in serum of HPV-associated cancer patients has not been investigated yet. Lack of studies on this field may be due to unavailable commercial kits for serum HPV E6/E7 oncoprotein detection, although new kits for testing serum HPV E7 protein are starting to be commercialized in the past few years.

The aim of this study was to identify markers for a better stratification of HPV-positive HNSCC patients. To this purpose, classical tumor markers, such as HPV DNA, p16 expression and HPV E7 mRNA were studied in different HNSCC subtypes, including OPSCC. Serum from HNSCC patients was analyzed for HPV16 L1 antibody titers and, for the first time, for HPV16 E7 oncoprotein levels at time of diagnosis and during the follow-up at 3, 6, 12 and 24 months. Finally, results were correlated to patient's relapse-free survival (RFS) and overall survival (OS).

3.1.2. Results

3.1.2.1. HPV DNA analysis

Head and Neck squamous cell carcinomas (HNSCCs) and control samples were analyzed for HPV DNA sequences and genotype. HPV DNA was found in 20/67 (29.85%) HNSCC

samples. HPV-genotype was determined by high resolution melting (HRM) to be HPV16 in 19/20 (95%) of the HNSCC HPV-positive cases and HPV33 in 1/20 (5%) of the cases. Control DNAs were found to be HPV11-positive in 1/58 (1.7%) of the cases. Our further studies were hereafter focused on HPV type 16 due to the high prevalence in HNSCC. Viral DNA load in cancer specimens ranged from 2.52×10-4 to 426.7 copies of HPV DNA per cell (Figure 1A).

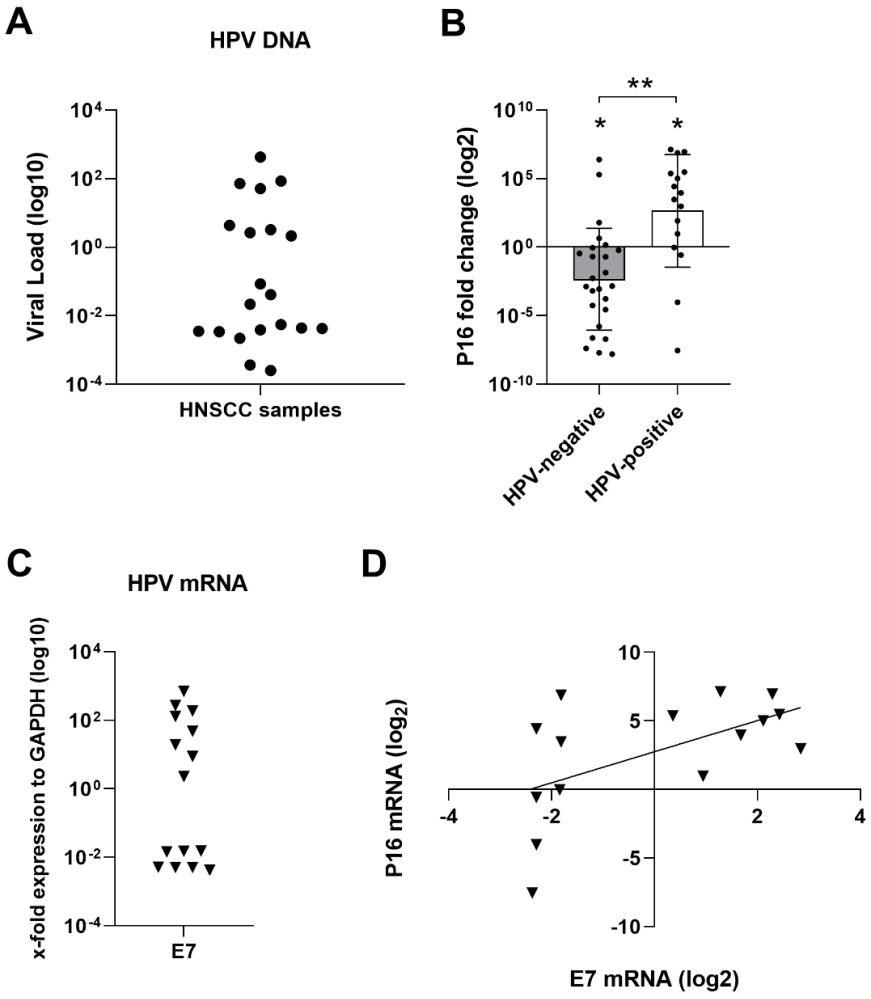


Figure 1. Analysis of classical markers for stratification of HNSCC samples. Statistical significance was indicated as * for $p<0.05$ and ** for $p<0.0001$; (**A**) viral load quantification of HPV-positive HNSCC samples by qPCR; (**B**) differential p16 mRNA expression in HNSCC samples analyzed by qPCR; (**C**) Viral E7 mRNA expression in HPV-positive HNSCC samples; p16 analysis in HNSCC samples; (**D**) Spearman correlation analyses between the expression of E7 (log_2) oncogene and p16 (log_2) showed correlation ($r=0.59$; $p<0.05$) in HPV-positive HNSCC tumor samples.

3.1.2.2. P16 gene expression

Due to transport/extraction methods, some RNA samples degraded, and therefore 41/67 HNSCC matching DNA/RNA samples were available for further analyses. P16 mRNA expression was investigated in HNSCC samples, showing upregulation in 12/16 (75%) of the HPV16-positive HNSCC and in 5/25 (24%) of the HPV-negative HNSCC samples compared to controls ($p<0.001$). HPV-positive patients presented overall p16 gene upregulation compared to controls (Mean ± [SD], 2.60 ± 3.98 $\log_2$ fold, $p<0.05$) with the exception of two samples that harboured p16 downregulation. HPV-negative samples were downregulated compared to control samples (Mean ± [SD], -2.34 ± 3.71 $\log_2$ fold, $p<0.05$). Differences in p16 expression between HPV-positive and negative were also significant ($p<0.0001$) (Figure 1B).

3.1.2.3. HPV mRNA expression

HPV-positive HNSCC samples were analyzed for HPV16 E7 gene expression by qPCR. Specifically, HPV16 E7 gene expression was analyzed in 16 HPV-positive HNSCC samples. mRNA expression of E7 was detected in 15/16 (93.75%) (Figure 1C). Pearson correlation test showed no correlation between the expression levels ($\log_{10}$) of E7 and HPV DNA load ($r=0.42$, $p>0.05$).

Furthermore, Pearson correlation analyses showed correlation between the expression of E7 and the up-regulation of p16 ($r=0.46$; $p>0.05$) (Figure 1D). But, HPV E7 mRNA expression did not correlate to p16 upregulation for all samples, since two samples presented E7 expression with p16 downregulation, and one sample presented p16 upregulation but no E7 expression; therefore p16 is not always a good marker indicating HPV active infection.

3.1.2.4. Serological studies

3.1.2.4.1. HPV16 L1 antibody titer

HPV16 L1 antibodies were found with a similar proportion in 18/20 (90%) HPV–positive HNSCC and 7/8 (87.5%) HPV-negative HNSCC patients ($p>0.05$). HPV DNA-positive HNSCC patients presented higher Optical Density (OD) readings for antibodies anti-HPV16 L1 compared to -negative (Mean±[SD], 4.001±2.11 vs. 2.29±0.32; $p<0.05$)

(Figure 2A). The antibody response was further compared during the follow-up at 3, 6, 12 and 24 months. Results indicated that HPV16 L1 antibody titers did not vary significantly during follow-up ($p>0.05$) (Figure 2B).

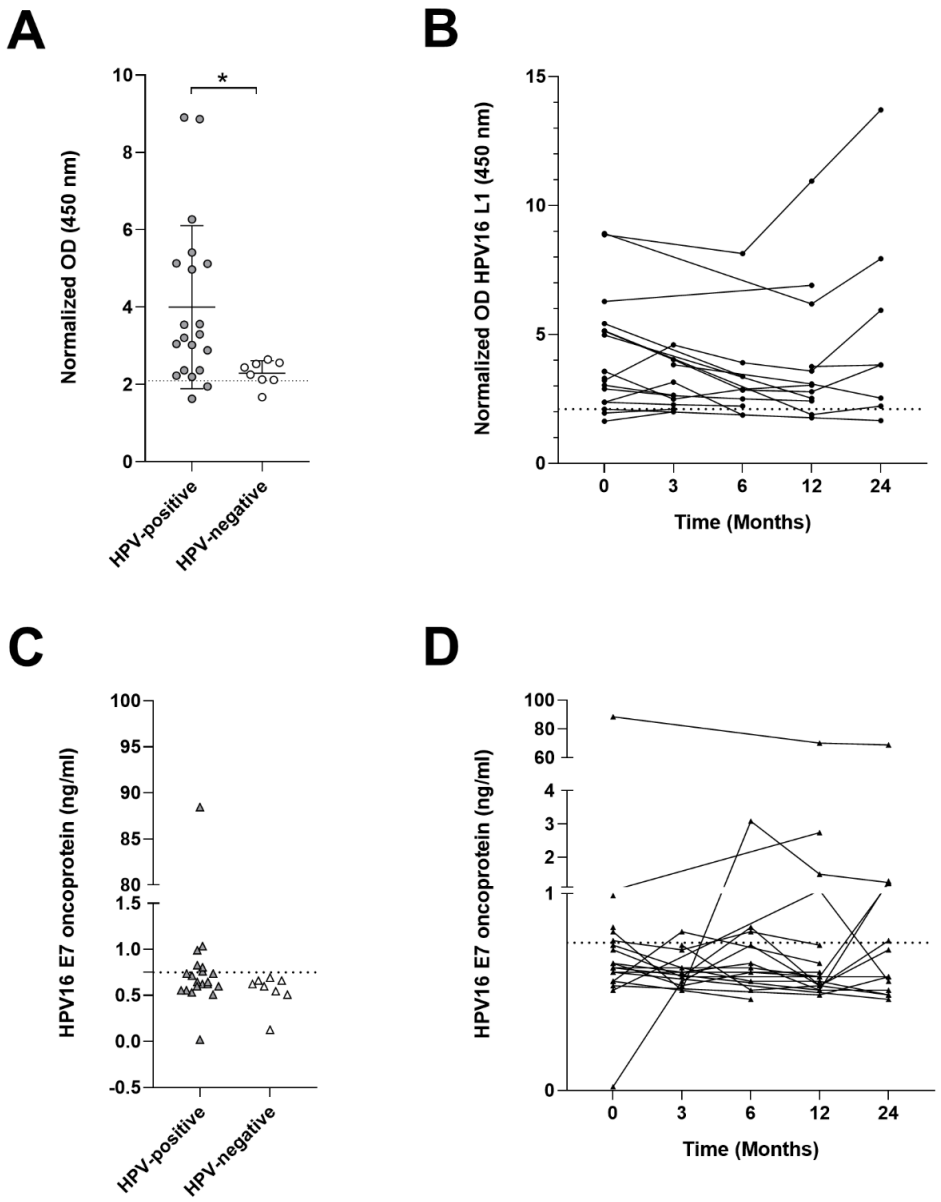


Figure 2. ELISA tests on HNSCC serum samples. Statistical significance was indicated as * for $p<0.05$; (**A**) Serum antibody levels against HPV16 L1 in HNSCC patients. Differential OD between HPV-positive and HPV-negative patients ($p<0.05$); (**B**) HPV16 L1 antibody variation during the follow-up of HPV-positive patients; (**C**) HPV16 E7 oncoprotein quantification in serum shows no difference between HPV-positive and HPV-negative patients ($p>0.05$); (**D**) HPV16 E7 oncoprotein variation during the follow-up of HPV-positive patients.

3.1.2.4.2. HPV16 E7 oncoproteins in sera

HPV16 E7 oncoproteins (ng/ml) amount was evaluated at the time of diagnosis and during a follow-up of 3, 6, 12 and 24 months. At T0; HPV16 E7 oncoprotein was detected in 6/20 (30%) HPV-positive patients' serum and none of HPV-negative ($p>0.05$) (Figure 2C). Variation in the amount of E7 oncoprotein during the follow-up was studied; for instance 9/20 (45%) samples remained negative for HPV E7 oncoprotein during the follow-up, 9/20 (45%) presented increment in the amount of oncoprotein; 4/9 (44.44%) of those, were positive at the time of diagnosis; while 5/9 (55.55%) became positive during the follow-up. Two samples out of 20 (10%), positive at the time of diagnosis, presented HPV E7 decrement over-time, one became negative. HPV E7 variation among samples, during the follow-up, resulted not statistically significant ($p>0.05$) (Figure 2D).

Finally, HPV16 E7 protein level in serum was studied in correlation to the viral mRNA expression in the tumor samples. Results showed correlation between the amount of HPV16 E7 mRNA expressed in the tumors and E7 oncoprotein in serum ($r=0.79$, $p<0.01$), suggesting that circulating E7 protein may be due to release from tumor site.

3.1.2.5. Survival analysis

3.1.2.5.1. RFS and OS in correlation to HPV DNA, p16 expression and HPV mRNA

The median follow-up time for this study was 24 months. The relapse free survival (RFS) and overall survival (OS) were assessed in HPV-positive HNSCC patients (n=20) compared to HPV-negative (n=47); different RFS rates were observed for both groups 72.11% and 48.77% for HPV-positive and –negative, respectively ($p>0.05$) (Figure 3A); also OS was improved for HPV-positive patients; 88.89% compared to 52.08% in HPV-negative OPSCCs ($p<0.01$) (Figure 3B).

To study the effect of p16 expression on survival, all HNSCC samples were subdivided into p16-over or –underexpression in the tumor samples. High and low expression were considered when $\log_2$ of the fold change was greater than 1 (n=13) or lower than -1 (n=17), respectively. Samples with a $\log_2$ of the fold change between 1 and -1 (n=11) were considered to have normal expression, therefore were not represented. RFS was 73.84%, in patients carrying p16 upregulation, compared to p16 downregulation, 48.12% ($p>0.05$) (Figure 3C). OS was 100% in patients with higher expression of p16 compared to 52.94% of patients with p16 downregulation ($p<0.01$) (Figure 3D).

RFS and OS were also assessed for HPV E7 mRNA expression in HNSCC samples. All HPV DNA-negative samples with matching mRNA, were considered as HPV mRNA-negative. Samples were divided into expressing E7 oncogene (n=15) and not expressing (n=26). Survival proportions retrieved from the analysis indicated that RFS was 64.61% in patients positive for E7 mRNA, while 48.77% in patients HPV mRNA-negative ($p>0.05$) (Figure 3E). OS was higher in patients carrying HPV E7 mRNA, 92.85%, compared to those HPV mRNA-negative, 52.08% ($p<0.05$) (Figure 3F).

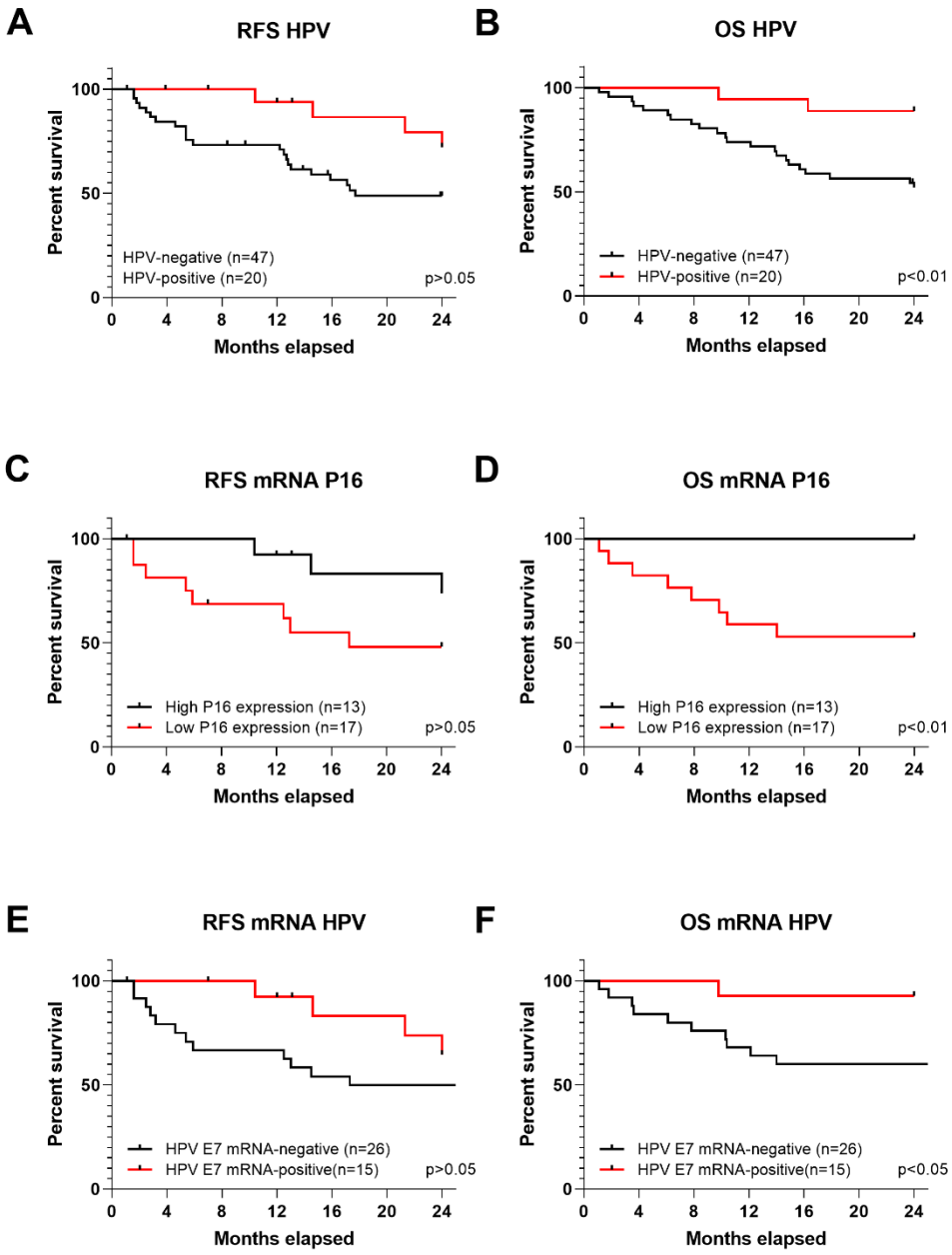


Figure 3. Kaplan-Meier (KM) curves for RFS and OS in HNSCC; (**A, B**) KM curves of RFS (a) and OS (b) for HPV DNA presence in HNSCC tumor samples;

(**C, D**) KM curves of RFS (e) and OS (f) for p16 over- or under-expression in HNSCC samples; (**E, F**) KM curves of RFS (c) and OS (d) for HPV E7 mRNA expression in HNSCC tumor samples. Statistical significance was indicated as $p<0.01$ or $p<0.05$.

3.1.2.5.2. RFS and OS in relation to serum HPV16 L1 antibodies

The next step was to study the association of serological markers of HPV infection, such as HPV16 L1 antibody and E7 oncoprotein presence, with patient's survival. We did not observe significant differences of HPV16 L1 antibodies in RFS or OS for HPV-positive patients (n=20) at the time of diagnosis and during the follow up. RFS was 51.28% and 100% for HPV16 L1 antibodies-positive patients (n=18), and 100% for HPV16 L1 antibodies-negative (n=2) ($p>0.05$) (Figure 4A). OS was also similar between HPV-positive patients and HPV16 L1 antibody positivity or negativity, being 63.64% and 100%, respectively ($p>0.05$) (Figure 4B). Overall these results indicate that HPV16 L1 is not a good indicator of prognosis and since it is a cumulative marker of exposure, it may be only used for epidemiological purposes.

3.1.2.5.3. RFS and OS in relation to serum HPV16 E7 oncoprotein

HPV16 E7 oncoprotein presence in serum was correlated to patients' clinicopathological characteristics (Table 1). Interestingly, E7 oncoprotein in serum was strongly associated to recurrence in HNSCC patients ($p<0.0001$) and in the OPSCC subgroup ($p<0.001$). Statistical analyses on the other HNSCC subtypes were not possible due to the small sample size (Table 1). RFS was 90.9% for patients testing negative for E7 protein (n=14) compared to 0% for HNSCC patients presenting E7 positivity (n=6) ($p<0.0001$) (Figure 4C). OS was higher in patients negative for E7 oncoprotein (n=14) compared to those positive for E7 (n=6), 100% and 50%, respectively ($p<0.01$) (Figure 4D).

The variation of serum E7 oncoprotein was also studied in correlation to patients' survival. RFS was 42.85% in HNSCC patients who increased the amount of E7 oncoprotein during the follow-up or remained positive (n=9), compared to 79.55% of those who experienced E7 decrement or remained negative during the follow-up (n=11)

(p>0.05) (Figure 4E). OS proportion was 85.71% for patients who increased E7 oncoprotein or remained positive (n=9), and 90.9% for those who decremented E7 oncoprotein or remained negative (n=11) (p>0.05) (Figure 4F). These results put in evidence the necessity of patients' monitoring for recurrence after circulating HPV E7 oncoprotein finding at the time of diagnosis, and eventually, its increasing levels during the follow-up.

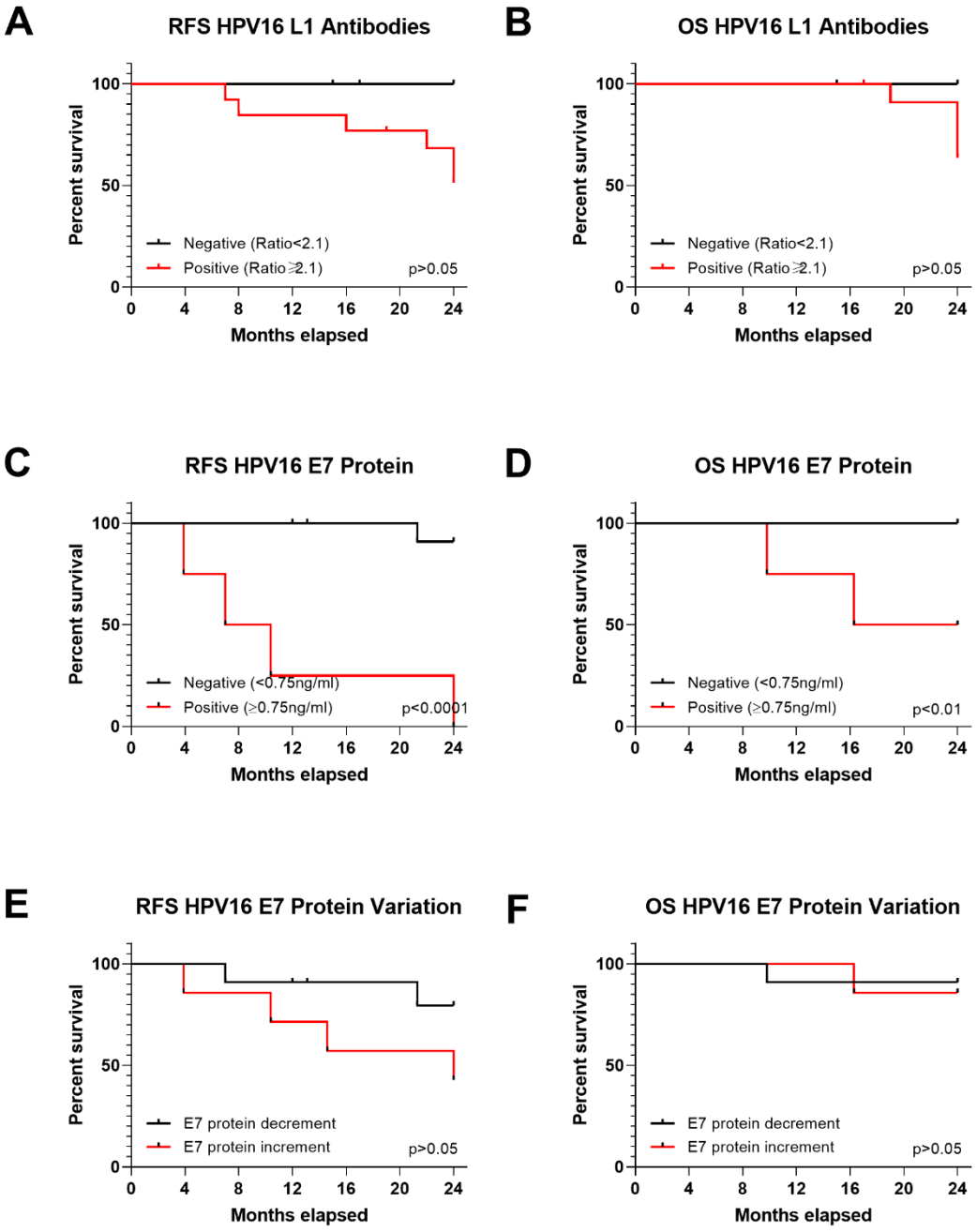


Figure 4. Kaplan-Meier (KM) curves for serological tests representing RFS and OS in HNSCC patients for HPV16 L1 and OPSCC for E7 oncoprotein; (**A, B**) KM of

RFS and OS for HPV16 L1 presence in HNSCC patients; (**C, D**) KM of RFS and OS for HPV E7 oncoprotein presence in serum of HPV-positive OPSCC patients; (**E, F**) KM of RFS and OS for increment or decrement of E7 oncoprotein in serum of OPSCC patients during the follow-up. Drop-out patients, where no follow-up was available, were not represented in the graphs. Statistical significance was indicated as $p<0.05$.

3.1.2.6. TNM, stage in correlation to OPSCC patient prognosis and E7 oncoprotein in serum

Survival analyses showed that patients with smaller tumor size had similar RFS and OS rates to those with greater size. RFS was 72.9% and 57.14% for patients with T (1-2) and T (3-4), respectively ($p>0.05$) (Figure 5A), while OS 87.5% and 90%, respectively ($p>0.05$) (Figure 5B). Similarly, no statistically significant differences were observed for RFS nor OS survival rates when studied in correlation to lymph node involvement; RFS was 100% vs 58.18% for patients without and with lymph node involvement ($p>0.05$) (Figure 5C), while OS was similar; 100% and 86.67%, respectively ($p>0.05$) (Figure 5D).

Patients in stages III/IV are more likely to recur. Indeed, RFS for patients in stage III/IV was 42.86% compared to 100% for patients in stage I/II ($p<0.05$) (Figure 5E), while OS was similar for both groups 83.33% and 100%, respectively ($p>0.05$) (Figure 5F).

Last, we studied the correlation between serum E7 presence and tumor size, lymph node involvement and disease stage. E7 presence in serum correlated to tumor size ($p<0.05$), but not to lymph node involvement in OPSCC ($p>0.05$) (Table 1). Out of 6 HPV-positive patients with stage I/II, none presented the E7 oncoprotein in serum at T0, while 5/12 (41.66%) of the patients in stage III/IV presented E7 oncoprotein in serum ($p>0.05$).

Table 1. Clinicopathological features of HNSCC patients

Clinicopathological variables	HPV-negative	HPV-positive	p-value	Tumor Site (HPV-Positive)							
Tumor Site				Oral	p-value*	Oropharynx	p-value	Hypopharynx	p-value*	Larynx	p-value*
Oral	25/47 (53,20%)	2/20 (10%)									
Oropharynx	13/47 (27,66%)	15/20 (75%)									
Hypopharynx	1/47 (2,13%)	2/20 (10%)									
Larynx	6/47 (12,77%)	1/20 (5%)									
Hidden	2/47 (4,25%)	-									
Tumor Size											
T1	7/47 (14,89%)	3/20 (15%)	0,225	-	NA	2/15 (13.33%)	0,031	1/2 (50%)	NA	-	NA
T2	14/47 (29,79%)	5/20 (25%)		-		4/15 (26.67%)		1/2 (50%)		-	
T3	9/47 (19,15%)	3/20 (15%)		-		3/15 (20.00%)		-		-	
T4	17/47 (36,17%)	9/20 (45%)		2/2 (100%)		6/15 (40.00%)		-		1/1 (100%)	
Node Status											
N0	9/47 (19,15%)	3/20 (15%)	0,108	-	NA	2/15 (13.33%)	0,096	-	NA	1/1 (100%)	NA
N+	38/47 (80,85%)	17/20 (85%)		2/2 (100%)		13/15 (86.67%)		2/2 (100%)		-	
Clinical Stage											
I	1/47 (2,13%)	1/20 (5%)	0,467	-		1/15 (6.67%)	0,336	-	NA	-	NA
II	7/47 (14,89%)	5/20 (25%)		-		5/15 (33.33%)		-		-	
III	7/47 (14,89%)	5/20 (25%)		-		4/15 (26.67%)		1/2 (50%)		-	
IVa	25/47 (53,19%)	9/20 (45%)		2/2 (100%)		5/15 (33.33%)		1/2 (50%)		1/1 (100%)	
Ivb/c	7/47 (14,89%)	-		-		-		-		-	
Recurrence	16/47 (42,55%)	6/20 (30%)	0,0001	1/2 (50%)	NA	4/15 (26.66%)	0,001	1/2 (50%)	NA	0/1 (0%)	NA
Persistance	4/47 (8.51%)	2/20 (10%)				2/15 (13.33%)					
N/A	2/47 (4,25%)	2/20 (10%)		-		2/15 (13.33%)		-		-	
Tobacco consumption											
No	5/47 (10,64%)	2/20 (10%)	0,481	-	NA	2/15 (13.33%)	0,582	-	NA	-	NA
Ex Smoker	13/47 (27,66%)	7/20 (35%)		-		4/15 (26.67%)		2/2 (100%)		1/1 (100%)	
Smoker	25/47 (53,19%)	7/20 (35%)		2/2 (100%)		5/15 (33.33%)		-		-	
N/A	4/47 (8,51%)	4/20 (20%)		-		4/15 (26.67%)		-		-	
Alcohol consumption											
No	10/47 (21,28%)	5/20 (25%)	0,962	-	NA	4/15 (26.67%)	0.725	-	NA	1/1 (100%)	NA
Ex consumer	3/47 (6,38%)	2/20 (10%)		1/2 (50%)		0/15 (0%)		1/2 (50%)		-	
Consumer	27/47 (57,45%)	9/20 (45%)		1/2 (50%)		7/15 (46.67%)		1/2 (50%)		-	
N/A	7/47 (14,89%)	4/20 (20%)		-		4/15 (26.67%)		-		-	
Age	64,04±11,55	67,05±9,05	0,709	63±1.41	NA	65.73±9.15	0.409	73±1.41	NA	83±NA	NA
Gender											
Male	33/47 (68,09%)	17/20 (85%)	0,244	2/2 (100%)	NA	12/15 (80%)	0.289	2/2 (100%)	NA	1/1 (100%)	NA
Female	15/47 (31,91%)	3/20 (15%)		-		3/15 (20%)		-		-	

[1] Clinicopathological variables in HNSCC patients both HPV-negative and HPV-positive. P-values are referred to correlation between E7 oncoprotein expression in serum and the different variables in HPV-positive HNSCC patients. P values < 0.05 were considered statistically significant. *Too few pairs were available for correlation analysis.

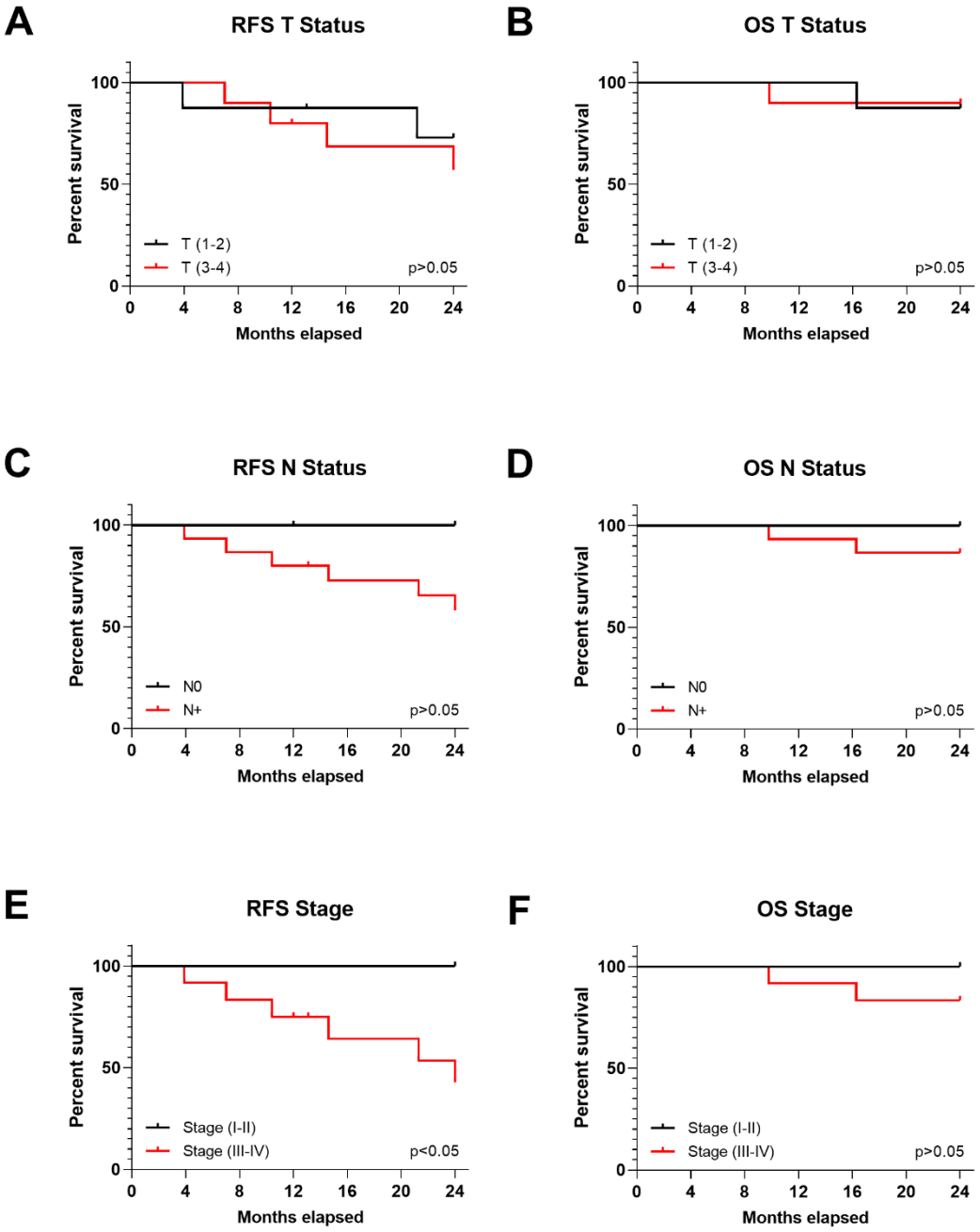


Figure 5. Kaplan-Meier (KM) curves for tumor size (T), node status (N) and stage in HPV-positive HNSCC patients representing RFS and OS; (**A, B**) KM representing RFS and OS for patients divided into tumor size: T (1-2) and T (3-4); (**C, D**) KM representing RFS and OS for patients divided into node status: N0 and N+; (**E, F**) KM representing RFS and OS for patients divided into stages I/II and III/IV; OS for patients divided into stages I/II and III/IV. Statistical significance was indicated as $p<0.05$.

3.1.3. Discussion

The current study aims to find markers for recurrence in HPV-positive patients. For patient stratification we studied classical HPV markers; HPV DNA, P16 mRNA and viral mRNA expression. Once stratified, we studied the presence of potential serological markers; HPV16 L1 antibodies and, for the first time, HPV E7 oncoproteins presence. Serological markers were then correlated to patient's prognosis.

In a first screening, we found that 29.85% of the HNSCC tumor samples, including 75% OPSCC, harbored HPV-DNA, and 95% of them presented HPV16. These findings are in agreement with other studies indicating that HPV is found in 25% of all HNSCC, and up to 70% of OPSCC tumors (International Agency for Research on Cancer 2007; Castellsagué et al. 2016; Betiol, Villa, and Sichero 2013; McIlwain et al. 2014), whereas 90% of all HPV-positive tumors carried HPV type 16 (Pytynia, Dahlstrom, and Sturgis 2014). P16 mRNA expression was found overexpressed in 75% of the HPV-positive HNSCC samples. P16 is an established surrogate marker for tumors with transcriptionally active HPV, which is known to be associated with less genetically altered and less complex tumors that respond better to therapy and have improved outcomes (Lin et al. 2014; Schlecht et al. 2015). Yet, not all HPV-positive tumors present p16 gene upregulation, as shown in ours and previous studies (Reuschenbach et al. 2019). In order to match clinical stratification, based on HPV DNA presence (Reuschenbach et al. 2019; Amin, American Joint Committee on Cancer, and American Cancer Society 2017; Slebos 2006), transcription of E7 viral oncogene was assessed in HPV-DNA positive patients only, showing 93.75% of the HPV16 DNA positive samples expressed HPV16 E7 oncogene. Nevertheless, HPV mRNA has been previously found in HPV-DNA negative tumors (Dwedar et al. 2020). This discrepancy could rely on the sensitivity of the method of detection used, which may vary from one study to another (Forslund et al. 2019).

Since HPV status has a great impact on patient prognosis and may soon guide therapy (Ekanayake Weeramange et al. 2020), the correct stratification of patients is imperative. In our study, we found that 30% of HPV-positive patients presented recurrence within the first two years from diagnosis, similarly to other studies (Hargreaves et al. 2019; Roberts et al. 2019; Cohen et al. 2019; Economopoulou, Koutsodontis, et al. 2019). The study of classical markers of HPV infection, i.e., HPV DNA presence, p16 and HPV mRNA showed improved patient OS, in our cohort of study, although it was not correlated to recurrence.

Antibody response against L1 was studied for the prevalence of viral infection among HNSCC patients. HPV L1 antibodies are cumulative markers of past and present infections and its presence does not imply HPV-driven tumorigenesis (Pierce Campbell et al. 2016). In fact, in our study both HPV-positive and –negative HNSCC patients presented antibodies against HPV16 L1 with a similar prevalence of 90% and 87.5%, respectively. Interestingly, the antibody title in HPV-positive patients was higher compared to HPV-negative, which could be indicative of active infection. We also studied the antibody response during the follow-up to monitor disease status, as proposed by Routman et al. (Routman et al. 2019), but we did not observe a significant change for antibody titers during the follow up, indicating that antibody levels against HPV L1 should not be useful to diagnose or monitor disease.

In the past few years, the study of antibody response against HPV E6/E7 oncoproteins in OPSCC, the major subtype of HNSCC, has been proposed as a marker for disease progression, and in spite of the good perspective for both diagnosis and prognosis (Holzinger et al. 2017; Dahlstrom, Anderson, et al. 2015; Spector et al. 2017; Lang Kuhs et al. 2017; Kreimer et al. 2019), results are still under debate due to the lack of seroconversion of many patients, as evidenced for other diseases related to viral infections as well (Rizzo et al. 2016). In a study conducted by Kreimer et al., 57.6% of OPSCC patients remained HPV E6 seronegative during follow-up (Kreimer et al. 2019). To our knowledge, no previous research has been conducted to detect HPV E7 oncoproteins in HNSCC patient's serum, while the availability of ELISA kits for oncoprotein detection could rapidly facilitate the study translation into the clinic. We specifically found E7 oncoprotein in serum with a frequency of 30% in HPV-positive samples, and detection of the circulating protein at the time of diagnosis strongly correlated to relapse. Our data is in accordance with studies finding higher levels of antibodies against HPV oncoproteins at time of diagnosis in association to significantly increased risk of recurrence (Spector et al. 2017; Fakhry et al. 2016). Similar to previous studies for antibody titer variation during the follow-up we could not associate the variation, increment or decrement, of E7 oncoprotein in OPSCC serum during the two-year follow-up with patient's outcome (Piontek et al. 2019; Schroeder et al. 2018; Dahlstrom, Anderson, et al. 2015). Nevertheless, serum E7 increase or decrease during the follow-up was observed in patients experiencing recurrence or not, respectively, thus, lack of

significant correlation between serum E7 level and relapse may be due to limited sample size.

Of note, circulating E7 protein showed correlation with high E7 mRNA expression in the tumor, suggesting that tumor site may provide circulating oncoprotein. In this view, circulating E7 protein may be considered a marker of tumorigenesis, representing at serological level what occurring at tumor site. Sources of viral oncoproteins in serum are currently not established, but some hypotheses could be proposed. First, the transcriptionally active circulating tumor cells may account for serum viral oncoproteins presence (Economopoulou, Koutsodontis, et al. 2019). In fact, HPV spreading through blood circulation has been previously reported (Vergara et al. 2019), and HPV E6/E7 transcription in circulating tumor cells (CTCs) has been correlated to patient's prognosis (Economopoulou, Koutsodontis, et al. 2019). Second, invasion and the associated development of a tumor vascular bed may result in the release of E6/E7 proteins from the tumor mass, probably as a consequence of necrosis (Stanley 2003). Third, HPV-positive tumor cells may secrete exosomes containing viral oncoproteins, as reported for other DNA viruses (Rajagopal and Harikumar 2018). Whatever the mechanism, we successfully found HPV16 E7 oncoproteins in HNSCC patients' serum and correlated their presence to patient's prognosis. The detection of the E7 oncoprotein in serum at the time of diagnosis displayed strong diagnostic and prognostic reliability in predicting relapses and overall survival of HPV-positive HNSCC patients, especially HPV-positive OPSCC patients.

Moreover, E7 oncoprotein also correlated to tumor size, but not lymph node involvement or disease stage. Overall, 41.66% HNSCC patients with high disease stage III/IV presented E7 oncoprotein in serum, while none of those with low stage I/II, in agreement with previous serologic studies (Dahlstrom, Anderson, et al. 2015; Dahlstrom, Li, et al. 2015), making the detection of E7 oncoprotein in serum an excellent discriminator for HNSCC patients that may relapse.

This study demonstrates for the first time, the presence of circulating E7 oncoproteins in serum of HNSCC patients by using a direct ELISA assay. Our results indicate that presence of E7 oncoprotein in OPSCC patients' serum at the time of diagnosis are indicative of a higher risk for recurrence. Liquid biopsy for the detection of prognostic markers in HPV-positive OPSCC patients provides valuable information on disease progression, and may help stratify and monitor patients over-time; this, can result

extremely useful for patients presenting persistent or occult tumors. For future studies, in order to increase the statistical power of the study, a larger sample size for all HNSCC subtypes will be considered. This study takes one step closer to correct patient stratification for therapy de-intensification. The combination of classical markers with serological markers, may be used to plan personalized treatment strategies for HPV-positive patients.

CHAPTER 3.2

Implication of HPV16 Long Control Region variants and methylation status on HNSCC prognosis

3.2.1. Introduction

Besides HNSCC, HPV16 has been associated to other cancers, such as, vulvar, anogenital, penile, vaginal and the most studied; cervical cancer (Rotondo et al. 2020a; Preti et al. 2020). The main transforming activity of the virus is carried out by the E6 and E7 oncoproteins, which expression is required for immortalization of squamous epithelial cells and that are involved in cell proliferation, promoting tumor cell growth and inhibiting apoptosis (Kurvinen et al. 2000). E6 and E7 gene expression are directly regulated by the p97 promoter situated at the E6-proximal end of the LCR (Kurvinen et al. 2000). The activity of the promoter is controlled by both viral and cellular proteins though the interaction with specific binding sites situated within the LCR that can stimulate or suppress viral transcription, that are: (i) four E2 binding sites (E2BSs), and (ii) several binding sites for cellular factors, such as activator protein-1 (AP1), nuclear factor-1 (NF1), nuclear factor of activated T-cells (NF-AT), yin-yang-1 (YY1), Sex-determining Region Y (SRY), etc. (Ribeiro, Caodaglio, and Sichero 2018b; C. Moody 2017).

The viral protein E1 interacts with the origin of replication (ORI) (Durzynska, Lesniewicz, and Poreba 2017), while the E2 protein can bind as a dimer to the E2BSs and together with the cellular factors regulate the expression of E6 and E7 genes (McBride and Warburton 2017, 6). Depending on the binding position of E2 protein it may act by activating or repressing viral transcription; in particular, binding to the high-affinity E2BS1 promotes the activation of the p97 promoter, inducing expression of E6 and E7 oncogenes at low levels. E2 starts binding the low-affinity E2BS3 and E2BS4 as the levels of E2 increases in the cell. The binding to these sites repress the E6 and E7 transcription (Durzynska, Lesniewicz, and Poreba 2017), therefore, E2 transcription activation/repression works as a fine-tuned mechanism for the regulation of the viral transcription.

Interestingly, methylation of E2BS1 and E2BS2 results in promoter hyper activation, while methylation of the E2BS3 and E2BS4 prevent binding of E2, thereby blocking the transcriptional repressor of E6 and E7, and resulting in increased transcription of these viral oncoproteins, necessary for transformation (Durzynska, Lesniewicz, and Poreba 2017; von Knebel Doeberitz and Prigge 2019).

Transcription of these early genes leads to inactivation of cellular control functions, and triggers reprogramming of cell functions such as proliferation, apoptosis, differentiation, epigenetic reorganization, and genomic instability (C. A. Moody and Laimins 2010b). These changes support the integration of episomal HPV genomes into the genome of the host cell (McBride 2017). Integration is thought to be a critical step in malignant progression, and it has been observed to occur in about 80% of the cervical cancer cases (McBride and Warburton 2017; Rotondo et al. 2020b), and in 60% of HNSCCs (Paolo Boscolo-Rizzo et al. 2017), where tumorigenesis seems to be independent of HPV physical status (Olthof et al. 2014). In HNSCC the deregulation of viral gene expression is, thus, caused by a combination of factors including viral genome integration with E2 disruption, HPV DNA methylation by host cell methyltransferases and cellular factors involvement (Egawa et al. 2015; Preti et al. 2020; Ekanayake Weeramange et al. 2020).

Many cellular transcription factors participate in the regulation of viral gene transcription together with E2, binding to their specific regulatory sequences of the LCR (Durzynska, Lesniewicz, and Poreba 2017). Previous analyses of the regulatory regions of HPV16 in cervical cancer have revealed the presence of nucleotide variations and deletions within transcription factor binding site sequences (Kurvinen et al. 2000). In fact, the LCR has been described as the most variable region of the HPV16 genome, and may exert an essential role for persistent viral infection and cancer progression (Fang et al. 2020; Zhe et al. 2019). Variations of LCR have been previously linked to squamous epithelial differentiation, immune escape and tumor progression in cervical cancer (Dai et al. 2020; Zhe et al. 2019; Ribeiro, Caodaglio, and Sichero 2018a). Different HPV LCR variants affect viral persistence and progression differently, although the results from these studies are still inconclusive (Fang et al. 2020). HPV variants could have an effect on response to therapy, but so far there is no indication that HPV variants research in HNSCC could be clinically relevant (Combes and Franceschi 2018). Only a few studies report HPV16 variants in HNSCC within the E6 sequence (Combes and Franceschi 2018), leaving aside the LCR. Since HPV16 LCR variation can strongly influence cervical cancer risk, there is increasing need to understand if a similar effect occurs in HNSCC (Clifford et al. 2019).

The main aim of the present study was to determine whether different variants of HPV16 LCR may contribute to HNSCC prognosis. Viral gene expression was studied in correlation to HPV16 LCR region variants, and since constitutively E6 and E7 oncoproteins expression allows for cell transformation, mechanisms for deregulated viral

transcription, such as HPV16 LCR methylation and viral DNA integration and DNA load were assessed in correlation to viral gene expression. Finally, these parameters combined, will be studied in correlation to tumor recurrence and patient's survival.

3.2.2. Results

3.2.2.1. HPV DNA characterization in HNSCC

HPV DNA status was analyzed and characterized in 35 HPV-positive HNSCC tumor samples (Table 1). Characterization by high resolution melting (HRM) showed that all samples carried the genotype 16, one of which presented a co-infection with HPV18. Physical status analyses determined that HPV16 DNA was in episomal form in 12/35 (34.28%) of HNSCC samples, integrated in 4/35 (11.43%) and mixed, i.e., coexistence of both episomal and integrated genomes, in 19/35 (54.28%) of the samples. Episomal samples showed higher viral load compared to integrated samples ($p<0.05$) (Figure 1A). Pearson correlation studies showed that, overall, the physical status of the viral DNA correlated to the viral DNA load: $r=0.52$, $P<0.001$. Viral load for episomal, integrated and mixed samples was Mean±SD: 53.95±121.5, 1.87±3.48 and 0.023±0.041 copy/cell, respectively (Table 1, Figure 1A).

Table 1. Clinical characteristic of HNSCC patients with HPV-infection

Patient ID#	Sex	Age	Histological diagnosis	Location	Sub-location	TNM	Stage	HPV type	Viral load*	E2/E6 rate
4	M	57	SCC	Oropharynx	Epiglottic vallecula	T4N2M0	III	HPV16	84,9254	0,99
5	M	86	SCC	Oropharynx	Tonsil	T4N2M0	IV A	HPV16	426,7421	1,32
6	M	64	SCC	Oral	Oral floor	T4N1M0	IV A	HPV16	0,0039	0,72
8	M	65	SCC	Oropharynx	Tongue base	T4N2M0	III	HPV16	0,0055	0,26
9	F	73	SCC	Oropharynx	Tonsil	T2N2Mx	II	HPV16	72,3162	0,97
10	M	68	SCC	Oropharynx	Tonsil	T4N2M0	III	HPV16	2,6571	0,86
11	M	74	SCC	Hypopharynx	Aryepiglottic fold and pyriform sinus	T2N2M0	IV A	HPV16	0,0411	1,05
12	M	83	SCC	Larynx	Supraglottic	T4N0M0	IV A	HPV16	0,0043	0,14
13	M	77	SCC	Oropharynx	Tonsil	T4N0Mx	IV A	HPV16	0,0036	0,60
15	M	72	SCC	Hypopharynx	Pyriform sinus	T1N1M0	III	HPV16	0,0219	0,24
16	F	68	SCC	Oropharynx	Uvula	T2N2M0	IV A	HPV16	0,0003	1,07
18	M	75	SCC	Oropharynx	Tongue base	T2N2M0	IV A	HPV16	0,0035	0,05
26	M	62	SCC	Oropharynx	Tonsil	T3N2Mx	II	HPV16	2,1293	1,04
29	M	50	SCC	Oropharynx	Tonsil	T1N0M0	I	HPV16	0,0849	0,03
36	M	63	SCC	Oropharynx	Tonsil	T4N3M0	III	HPV16	50,9591	1,19
37	M	62	SCC	Oral	Cheek	T4N2M0	IV A	HPV16	0,0004	0,07
54	F	63	SCC	Oropharynx	Tonsil	T3N2M0	II	HPV16	4,3340	1,12
62	M	56	SCC	Oropharynx	Tonsil	T2N1Mx	II	HPV16	3,2576	1,26
67	M	61	SCC	Oropharynx	Tonsil	T1N1Mx	II	HPV16	0,0044	0,00
P01	M	NA	SCC	Oropharynx	NA	NA	NA	HPV16	0,0027	0,12
P02	F	55	SCC	Oropharynx	Tonsil	T4N3M0	III	HPV16	0,1089	0,83
P03	M	81	SCC	Oropharynx	Tongue base	T4N1M0	NA	HPV16	0,0005	0,81
P04	F	48	SCC	Oropharynx	Tonsil	T4N1M0	NA	HPV16	0,2090	0,85
P05	M	83	SCC	Oropharynx	Tonsil	T4N1M0	NA	HPV16	0,0027	0,72
P06	M	67	SCC	Oropharynx	Tonsil	T4N1M0	NA	HPV16	0,0445	0,75
P07	F	73	SCC	Oropharynx	Tonsil	T3N1M0	NA	HPV16	3,4034	0,87
P08	M	88	SCC	Oropharynx	Oropharynx	T4N1Mx	NA	HPV16	0,0253	1,00
P09	M	70	SCC	Oropharynx	Tonsil	T2N2M0	NA	HPV16	0,0273	0,93
P10	M	80	SCC	Oropharynx	Tonsil	T1N0Mx	NA	HPV16	4,7913	0,80
P11	M	NA	SCC	Oropharynx	NA	NA	NA	HPV16	0,0712	0,92
P12	F	54	SCC	Oropharynx	Tonsil	T1N1M0	III	HPV16	7,3274	0,35
P13	M	64	SCC	Oropharynx	Triangle + Tongue base	NA	IV	HPV16, 18	0,0163	1,04
P15	M	79	SCC	Oropharynx	Tonsil	T4N2M0	NA	HPV16	8,2517	0,87
P16	M	81	SCC	Oropharynx	Tonsil	T4N2M0	NA	HPV16	11,3683	0,87
P17	M	65	SCC	Oropharynx	Tonsil	T1N1M0	NA	HPV16	0,0088	0,86

SCC, Squamous cell carcinoma

*HPV16 DNA copy/cell

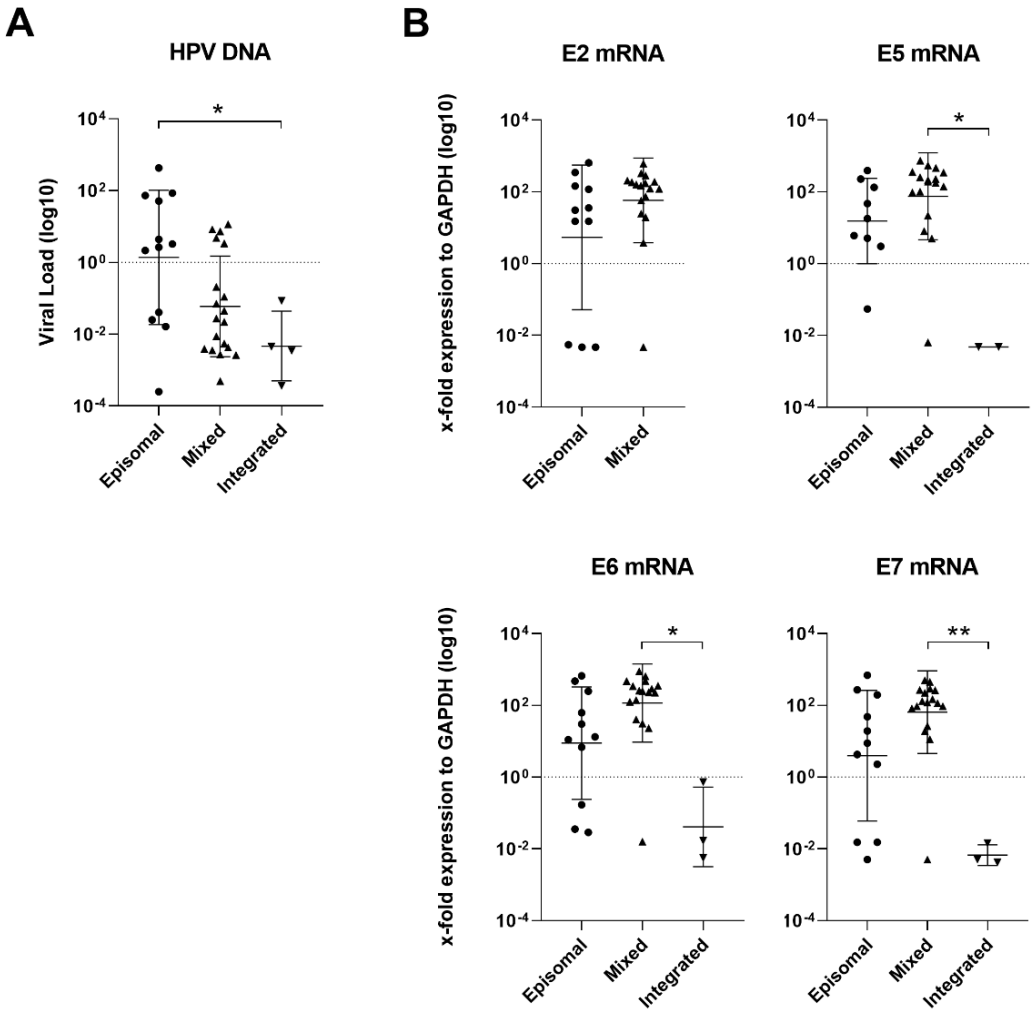


Figure 1. (A) Representation of the viral load (log10) copies/cell according to HPV physical status, viral load was normalized against β-globin; (B) Viral mRNA expression according to HPV DNA status, gene expression was normalized against GAPDH. Statistical significance was indicated as * for $p<0.05$ and ** for $p<0.01$.

3.2.2.2. Expression analysis of HPV16

HPV16 E2, E5, E6 and E7 mRNA expression was analyzed in HPV-positive HNSCC samples by ddPCR. Samples, n=32/35, were available for these analyses. mRNA expression of E2 was detected in 28/32 (87.5%) of the samples, E5 in 28/32 (87.5%), E6 in 31/32 (96.8%) and E7 in 31/32 (96.8%). Gene expression heterogeneity was observed among the samples, depending on the viral physical status and DNA load (Figure 1B). In particular, higher viral gene expression was observed for mixed compared to integrated samples for E5, E6 and E7 ($p<0.05$) (Figure 1B). Pearson correlation test showed no

correlation between E2/E6 ratios and HPV mRNA levels (r=-0.19 for E2; r=0.27 for E5; r=0.21 for E6 and r=0.23 for E7, p>0.05). It also failed to show correlation between the mean log_{10} expression levels of the viral genes E2, E5, E6 or E7 and the viral load; indicating that viral mRNA expression is not dependent on viral load (r=0.36 for E2; r=0.32 for E5; r=0.28 for E6 and r=0.26 for E7, p>0.05), or the physical status of the viral DNA within the cell.

3.2.2.3. Sequence variation analysis of HPV16 LCR

A total of 35 DNA samples were amplified by Endpoint PCR after bisulfite conversion. HPV LCR sequences were analyzed for nucleotide variation. Nucleotide variations were detected in the LCR of 27/35 (77.14%) of the samples analyzed; 19/35 (54.29%) of the sequences presented more than one nucleotide variation (Table 2). A total of 30 variation sites were verified in HNSCC HPV sample sequences compared to the reference sequence for the region analyzed. Sequence variations were overlapped with the correspondent transcription factor binding sites (Table 2). Specifically, The PROMO-ALGGEN database was used to predict the cellular transcription factor binding sites (TFBS) within the LCR sequence analyzed. The consensus binding sequence was: 5'-TGACTCA-3' for C-Jun/c-Fos; 5'-CCGCCATNTT-3' for YY1; 5'-TTGGC$(N)_6$CC-3' for NF1; 5'-RTTGCGYAAY-3' for C/EBP, where R = A or G, and Y = C or T; 5'-CCTTTGA-3' and 5'-CCATTGT-3' for SRY and 5'-[AC]A[AT]T[AG]TT[GT][AG][CT]T[CT]-3' for HNF3-β.

In particular, variation G7521A was frequently detected in 19/35 (54.29%) of the samples. It is located at a YY1 binding site, a repressor for viral gene expression (Mosmann et al. 2015). In-silico prediction showed that sequence variation G>A leads to a loss of recognition site for YY1. Deletions were also detected frequently found in this region, for instance: 7528-7529delAC (3/35, 8.57%), 7529delC (2/35, 5.71%) and 7537delT (7/35, 20%).

Other variants were less common, for instance: A7507T (1/35, 2.86%) creates a binding site for SRY, a repressor for HPV transcription; G7552C, G7552T and G7552A (1/35, 2.86% respectively) were found as well, these variations are located flanking a NF1 TF binding region, that promotes transcription. The C7886G change in 3/35 (8.57%) of the samples affected the signal transducer and activator of transcription 3 (STAT3) binding

Patient ID#	Reference	4	5	6	8	9	10	11	12	13	15	16	18	26	29	36	37	54	62	67	P02	P03	P04	P05	P06	P07	P08	P09	P10	P11	P12	P15	P16	P17	P13	P01	Transcription factor
7504	T																				G							mCpC								
7505	C																					mCpT			mCpT			mCpT	mCpT					mCpT			
7507	A															T																					
7510	T			mCpC																																	
7511	C		mCpA																									mCpA	mCpG								C-Jun / C-Fos
7512	A																												G				G				
7513	G			mCpC																																	
7514	C																					mCpA				mCpA								mCpA			
7516	A																										mCpC										
7517	C							mCpT																					mCpT								
7518	T																												mCpA								
7519	A																										mCpT										
7521	G		A				A				A					A		A	A	A	A	A	A		A	A			A	A	A	A	A	A		A	YY1
7525	T			A												A		A																			
7526	A																																	mCpA			
7528 7529	AC			DelAC												DelAC			DelAC																		
7529	C					DelC					DelC																											
7529 7531	CTT																			mCpAA																	
7531	T																																	mCpG			
7537	T	DelT		DelT		DelT		DelT								DelT			DelT	DelT																	NF1
7538	T																	mCpT																			
7539	T			mCpC																																	
7540	C	mCpC		mCpC				mCpC																													C/EBP-a/b
7544	C								mCpT											mCpT																	
7545	T																			mCpT																	
7549	C																																			mCpA	
7551	T			mCpG																																	NF1
7552	G			T			A	T	T											mCpC																	
7557	C																			mCpC		mCpC					mCpC										
7558	C																				mCpA						mCpA										
7563	C																								mCpC												
7564	C						A															mCpC													mCpC		
7834	G																	T																			SRY
7840	C																										mCpT									mCpT	
7843	C	mCpA	mCpA	mCpA					mCpA							mCpA		mCpA							mCpA					mCpA						mCpA	
7845	C																										mCpA										
7874	C	mCpA					mCpA		mCpA									mCpA																			HNF3-β
7876	C	mCpA					mCpA		mCpA									mCpA																		mCpA	
7886	C																		G									mCpA								G	
7889	C																													mCpT					mCpT		SRY
7900	A							G																													
5	C																	mCpA																			
64	G																			T																	TATA

Table 2. Nucleotide variation and mehylation sites in the HPV16 LCR region.

Table 3. Nucleotide variations and de-novo methylation of HPV-16 LCR

Nucleotide mutation site	Nucleotide change	n. (%)	Transcription factor
7504	T>G	1 (2,86)	C-Jun/C-Fos
7505	T>mC (CpA)	3 (8,57)	
7507	A>T	1 (2,86)	
7510	T>mC (CpC)	1 (2,86)	
7512	A>G	2 (5,71)	
7513	G>mC (CpC)	1 (2,86)	
7516	A>mC (CpC)	1 (2,86)	YY1
7518	T>mC (CpA)	1 (2,86)	
7519	A>mC (CpT)	1 (2,86)	
7521	G>A	19 (54,29)	
7525	T>A	3 (8,57)	
7526	A>mC (CpA)	1 (2,86)	
7528-7529	Del AC	3 (8,57)	
7529	Del C	2 (5,71)	
7529-7531	CTT>mCAA	2 (5,71)	
7531	T>mC (CpG)	1 (2,86)	
7537	Del T	7 (20)	NF1
7538	T>mC (CpT)	1 (2,86)	
7539	T>mC (CpC)	2 (5,71)	
7545	T>mC (CpT)	2 (5,71)	C/EBP
7551	T>mC (CpG)	1 (2,86)	NF1
7552	G>mC (CpC)	1 (2,86)	
	G>T	1 (2,86)	
	G>A	1 (2,86)	
7564	C>A	3 (8,57)	
7834	G>T	1 (2,86)	SRY
7840	T>mC (CpG)	2 (5,71)	
7886	T>G	3 (8,57)	SRY
7900	A>G	1 (2,86)	
64	G>T	1 (2,86)	TATA box

Nucleotide methylation site	De-novo methylation	n. (%)	Transcription factor
7504	C>mC (CpT)	1 (2,86)	C-Jun/C-Fos
7511	C>mC (CpA)	3 (8,57)	
7514	C>mC (CpA)	2 (5,71)	
7517	C>mC (CpT)	1 (2,86)	YY1
7540	C>mC (CpC)	4 (11,43)	C/EBP
7544	C>mC (CpT)	3 (8,57)	
7549	C>mC (CpA)	1 (2,86)	
7557	C>mC (CpC)	2 (5,71)	NF1
7558	C>mC (CpC)	2 (5,71)	
7563	C>mC (CpC)	1 (2,86)	
7843	C>mC (CpA)	9 (25,71)	SRY
7845	C>mC (CpA)	1 (2,86)	
7874	C>mC (CpA)	4 (11,43)	HNF3-β
7876	C>mC (CpA)	5 (14,29)	
7889	C>mC (CpT)	2 (5,71)	SRY
5	C>mC (CpA)	2 (5,71)	

site, located as well at a SRY binding site. All variants found and related transcription factors were reported in Tables 2 and 3.

3.2.2.4. HPV16 LCR promoter was differentially methylated in HNSCC samples.

Collectively, it has been shown that non-CpG methylation is attributable to de-novo activity of the DNMT3A/B-DNMT3L complex (Kalantari et al. 2004; Patil, Ward, and Hesson 2014). New methylation sites, n=16, reported as CpA, CpT and CpC, as described before (Kalantari et al. 2004), were verified in 20/35 (57.14%) samples. Interestingly, upon nucleotide variation research in bisulfite converted sequences, we observed that often, nucleotide changes within the HPV16 LCR region generated new sites for methylation. Specifically, nucleotide variation generated 15 extra methylation sites, not reported before in literature (Table 3). In particular, one de-novo methylated CpA at position 7843 and located in a binding site for SRY, was reported in 9/35 (25.71%) of the samples. De-novo methylation was also verified in 4/35 (11.43%) and 5/35 (14.29%) at positions 7874 and 7876, located at a HNF3-β binding site. The variation verified at 7529-7531 CTT>meCAA created a new binding site for SRY (Table 3).

Despite control by cellular transcription factors, it is widely known that HPV LCR is mainly controlled by E2 interaction with the E2BSs and E1 with the ORI. Cytosine methylation within E2BS1-4 modulates viral gene expression. In particular, E2BS1 alone was methylated in 8/35 (22.85%) of the samples and E2BS2, located at the origin of replication was methylated in 9/35 (25.71%) of the cases. Hypermethylation (half or more of the CpGs methylated) was observed between positions 37-58 in the p97 promoter region containing the E2BS3 and 4, in 13/35 (37.14%) of the samples, that compared to the methylation at the E2BS1, resulted statistically significant ($p<0.01$) (Figure 2). While methylation of E2BS3 and 4 is necessary to repress E2 binding to promote viral gene expression, E2BS1 methylation is not necessary, and methylation at this site and E2BS2, will hyperactivate as well the HPV promoter for viral gene expression.

Other interesting positions at the HPV LCR region, usually studied, are; 7535, methylated in 6/35 (17.14%) of the cases, and position 7553, that was methylated in 7/35 (20%) of the samples. These two sites are known binding sites for the transcription factor NF-1 to promote viral gene transcription. Finally, at position 31, a binding site for SP-1 is present, and was found methylated in 11/35 (31.42%) of the samples (Figure 2).

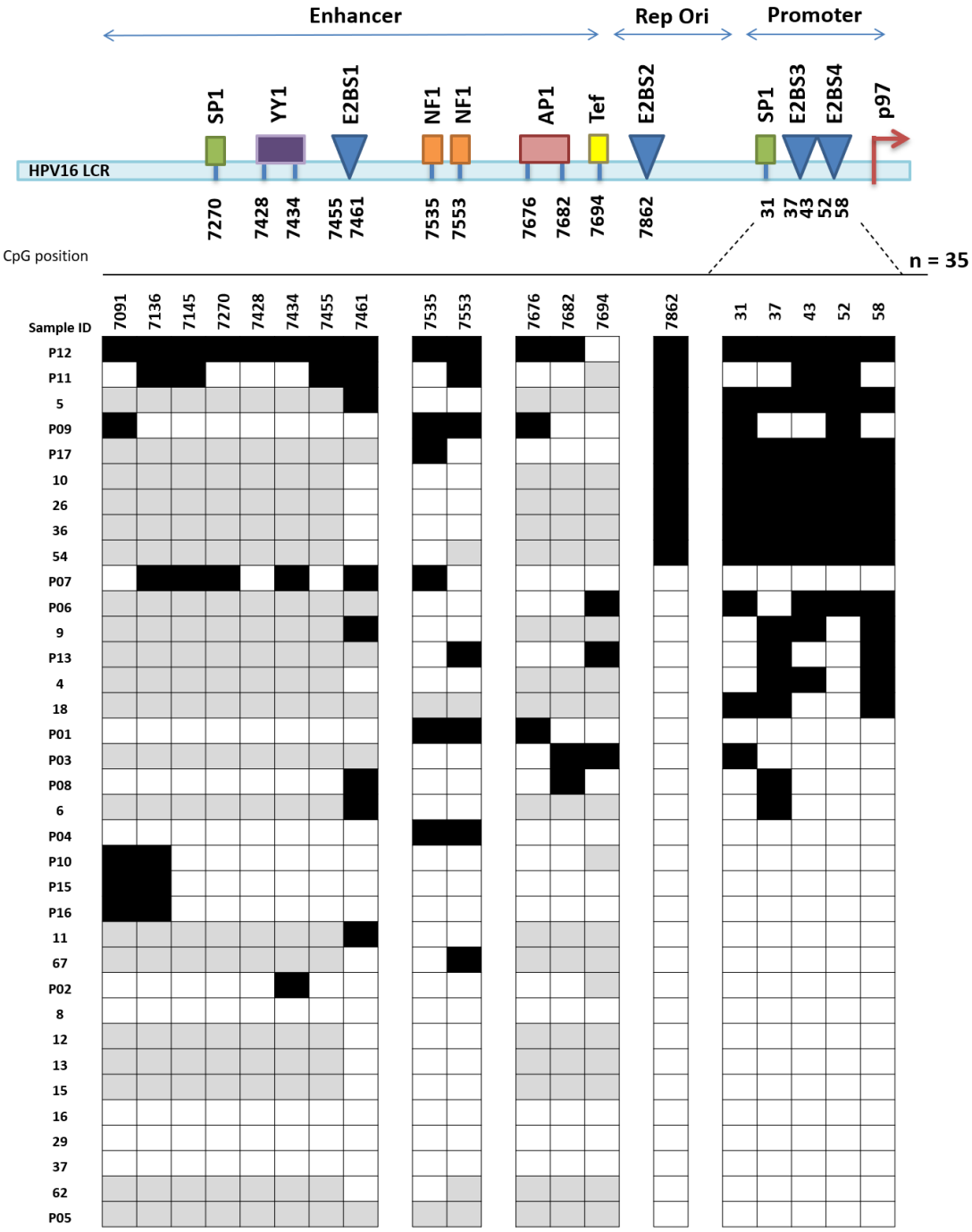


Figure 2. Structure of HPV16 LCR and location of the CpG sites. Transcription factor binding sites are also shown aligned to the corresponding CpG site. Dinucleotide methylation is represented as a colored square (black), while demethylated CpGs are represented as non-colored squares (white). Missing data is presented in grey squares.

3.2.2.5. Influence of nucleotide variation and methylation within HPV16 LCR transcription factor binding sites on viral gene expression.

Since nucleotide variation or methylation can potentially influence transcription factors binding to the HPV16 LCR, resulting in enhanced or reduced viral gene expression, we studied viral gene expression in correlation to HPV LCR sequence variation or methylation on specific transcription factor binding sites.

Nucleotide variation within the LCR affected viral gene expression, in particular for NF1 at position 7535-7539 and C/EBP at position 7540-7549 binding sites. HPV16 variants within NF1 TFBS had a significant reduced expression of viral oncogenes ($p<0.001$), while variations within the C/EBP TFBS result in reduced expression of E6 ($p<0.05$). Sequence variation on the remaining TFBSs also affected viral gene expression, although it was not statistically significant (Figures 3A and B, Table 4).

Gene expression variation in correlation to the variation/methylation within a transcription factor binding site in the HPV16 LCR can be seen in Table 4. Out of the 8 binding sites studied, NF-1 at position 7535-7539, C/EBP situated at position 7540-7549 and HNF3-β at position 7874-7876, showed a negative correlation to the amount of HPV16 gene expression. In particular, samples presenting sequence variation within the NF-1 TFBS, at position 7535-7539, significantly reduced the amount of viral mRNA expressed ($p<0.01$), while samples with variations within the C/EBP binding region expressed less E6 oncoproteins compared to samples with unvaried sequence ($p<0.01$).

Interestingly, Pearson correlation test showed that methylation within the HPV LCR did not correlate to viral gene expression, nor when analyzed by E2BS or cumulatively. For instance, r=0.01; -0.006; 0.04 and 0.04, for E2; E5; E6 and E7 ($P>0.05$) for E2BS1; r=0.27; 0.20; 0.29 and 0.33, for E2, E5, E6 and E7 ($P>0.05$) at the E2BS2; r= -0,09; -0,12; -0,06 and -0,15 for E2; E5; E6 and E7 ($P>0.05$) for E2BS3, and r= 0,16; 0,07; 0,19; 0,12 for E2; E5; E6 and E7 ($P>0.05$) for E2BS4. Cumulative methylation over the HPV16 LCR did not correlate either to viral gene expression; r=046; 0.69; 0.25 and 0.28, for E2; E5; E6 and E7, $P>0.05$).

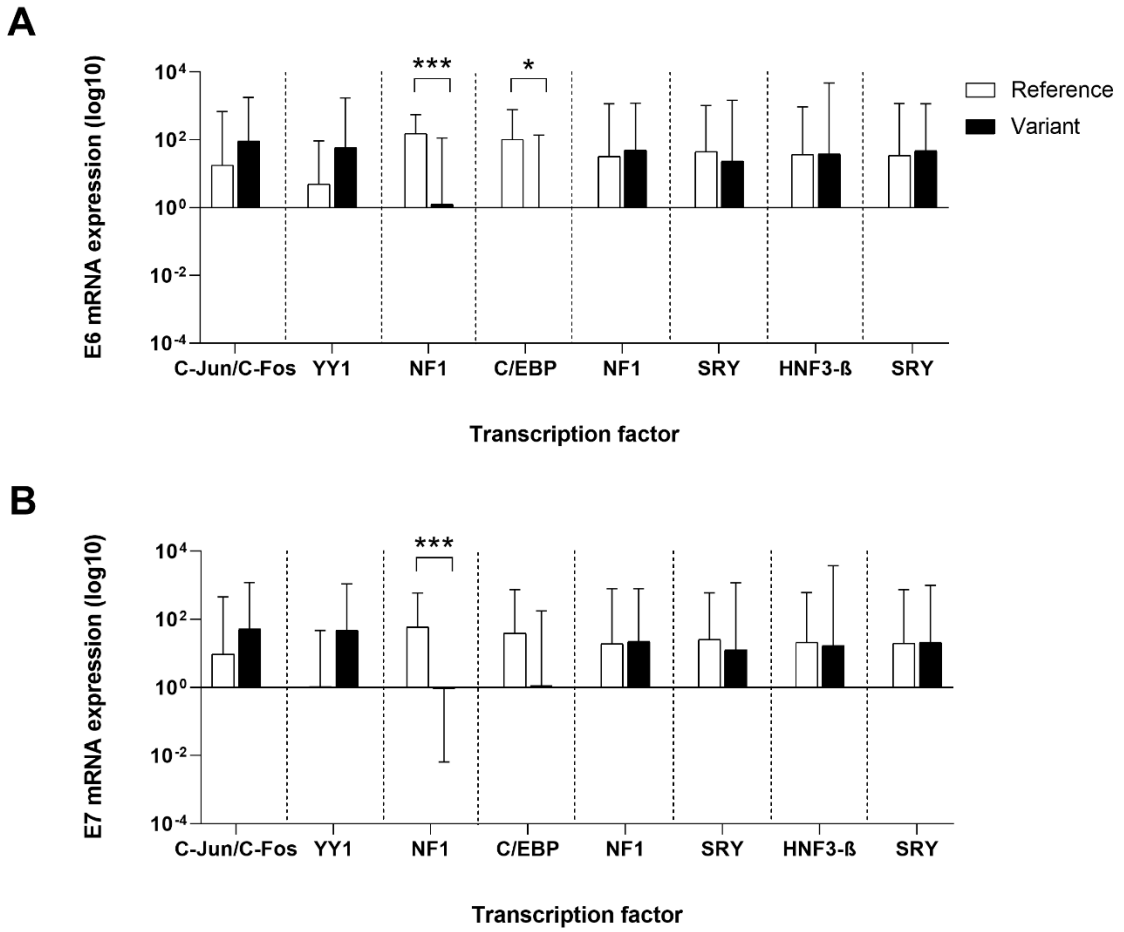


Figure 3. (A) HPV16 E6 mRNA expression correlated to TFBS reference sequence or variation. (B) HPV16 E7 mRNA expression correlated to TFBS reference sequence or variation. Each section represents a position where a TF binds within the HPV16 LCR. Expression in correlation to unvaried sequences are represented with white bars (Reference), while gene expression in correlation to sequence variation is represented with black bars (Variant). Statistical significance was indicated as * for $p<0.05$ and *** for $p<0.001$.

Viral load showed significant association with the methylation frequency of the LCR ($r=0.52$, $p<0.001$). When assessed according to the physical status of the samples; methylation frequency also correlated to E2/E6 ratio ($r= 0.41$, $p<0.05$).

Table 4. Pearson correlation test results: r for every transcription binding site variation/methylation in correlation to viral gene expression.

HPV16 gene	C-Jun / C-Fos	P-value	YY1	P-value	NF1	P-value	C/EBP	P-value	NF1	P-value	SRY	P-value	HNF3-β	P-value	SRY	P-value
E2	0,109	0,595	0,085	0,680	-0,657	2,66E-04*	-0,319	0,112	0,061	0,768	-0,051	0,806	-0,140	0,494	-0,068	0,740
E5	0,143	0,486	0,156	0,448	-0,517	0,007*	-0,060	0,770	0,261	0,197	0,127	0,536	0,234	0,250	0,020	0,924
E6	0,144	0,473	-0,007	0,972	-0,708	3,63E-05*	-0,509	0,007*	0,110	0,584	0,011	0,956	-0,043	0,830	0,039	0,845
E7	0,136	0,499	0,187	0,351	-0,589	0,001*	-0,249	0,210	0,077	0,703	0,010	0,962	-0,067	0,739	0,005	0,979

*Statistically significant values

3.2.2.6. Influence on prognosis of nucleotide variation/methylation within transcription factor binding sites.

Patients (n=35) were followed-up for a 3-year time period where relapse and death events were registered, progression free survival (PFS) and overall survival (OS) were assessed in correlation to variation/methylation for all the transcription factor binding sites studied. Nucleotide variation/methylation at single nucleotides did not yield any significance for PFS nor OS. When analyzed according to TFBS interesting results emerged. While no significant results were observed for PFS and OS when nucleotide variation/methylation was verified within the LCR for viral E2BS1-4, nor cellular C-Jun/C-Fos (at position 7504-7516), NF-1 (at position 7535-7539), C/EBP (at position 7540-7549) and NF-1 (at position 7551-7564), SRY (at position 7834-7843), HNF3-β (at position 7874-7876) or SRY (at position 7886-7900), important results emerged for cellular YY-1 (at position 7517-7531).

For instance, variation/methylation within the YY-1 sequence, resulted to be a strong marker of patient's relapse; PFS was 80.25% for HNSCC patients with nucleotide variation/methylation (n=20) vs. 17.85% for patients carrying the unvaried sequence (n=7) ($p<0.05$), but not OS; 68.55% vs. 66.67% for patients with variation/methylation compared to those with unvaried sequence for YY-1 ($p>0.05$) (Figure 4A).

Variation/methylation within the C/EBP sequence had no effect on patient survival. PFS was 83.33% for patients with variation within the C/EBP sequence (n=7) vs. 47.85% for patients with the wild type sequence (n=20) ($p>0.05$), while OS was 85.71% for patients presenting nucleotide variation/methylation vs. 54.76% for those with unvaried sequence ($p>0.05$) (Figure 4B).

But since nucleotide variants greatly influenced C/EBP binding, and it is known to interact with YY-1 (Y. Shi, Lee, and Galvin 1997), the combination of YY1 and C/EBP for survival studies depending on varied or reference sequence, were performed: PFS was 100% for patients with the variant sequence for the YY1 and C/EBP TFs (n=5), while 0% for patients carrying the HPV with the same sequence as the reference genome (n=5) ($p<0.01$); OS was 80% and 50% for patients carrying the variants, compared to those without them ($p>0.05$) (Figure 4C).

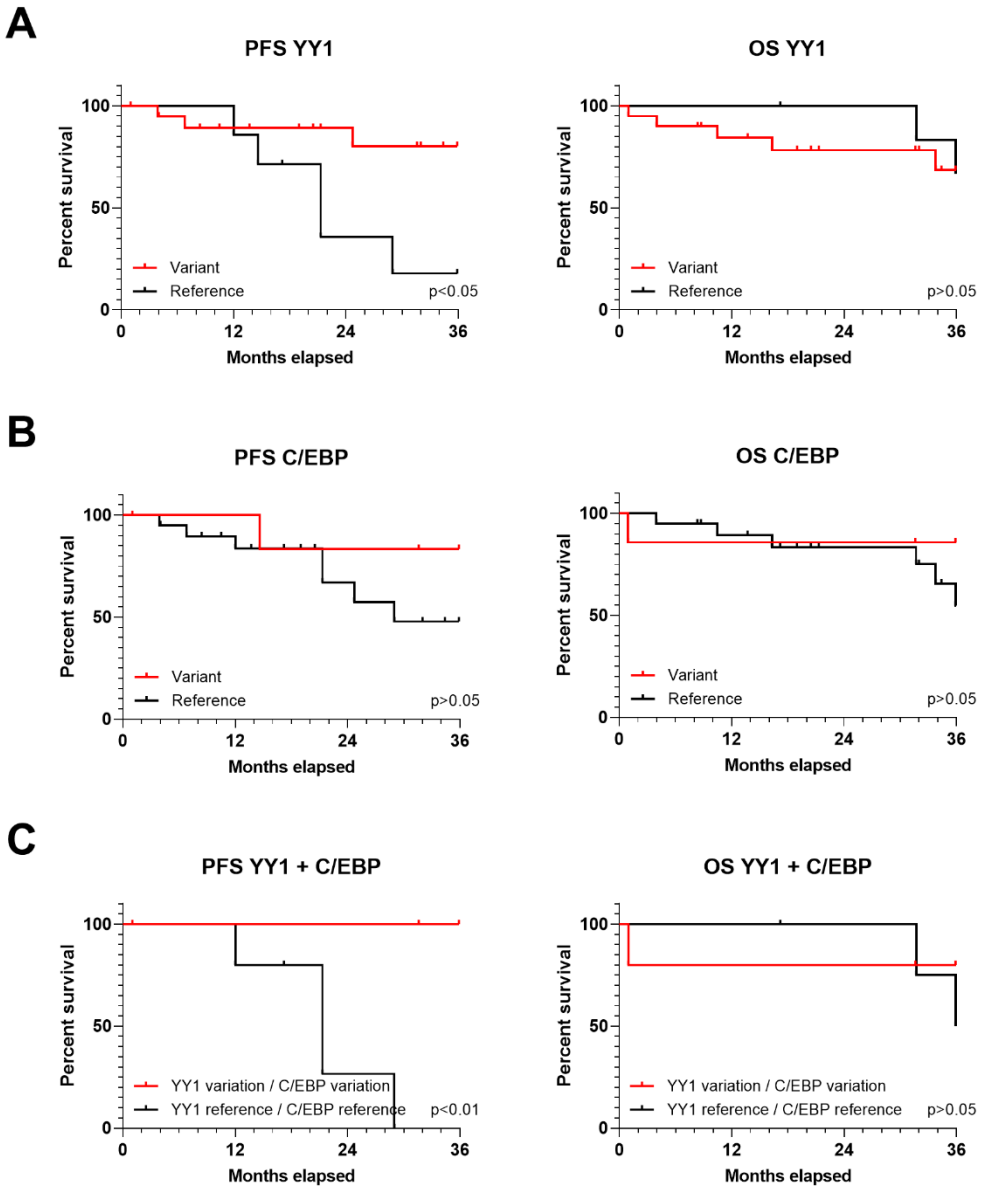


Figure 4. Kapan-Meier curves representing progression free survival (PFS) and overall survival (OS) for HNSCC according to the presence or absence of nucleotide variations and/or methylation within the transcription binding sites sequences present in the LCR of HPV16; (**A**) Kapan-Meier curve for HNSCC patients with or without nucleotide variations/methylation within the YY-1 TFBS situated at position 7517-7531 of the LCR; (**B**) Kapan-Meier curve for HNSCC patients with or without nucleotide variations/methylation within C/EBP TFBS situated at position 7540-7549 of the LCR; (**C**) Kapan-Meier curve for HNSCC patients combining YY-1 and C/EBP TFBS, with or without nucleotide variations/methylation. Statistically significant values are represented as $p<0.05$.

3.2.2.7. HPV classical markers in correlation to patient's survival.

HPV markers such as viral load, the amount of viral mRNA expressed, the physical status of HPV DNA within the sample and methylation within the E2BS1-4 were assessed in correlation to patient's prognosis. None of these parameters were significant when correlated to patient's survival. For instance, PFS was 63.49% vs. 52.39% for patients with high HPV DNA loads compared to those presenting low HPV DNA loads (p>0.05); OS was 35.06% and 64.37%, respectively (p>0.05) (Figure 5A).

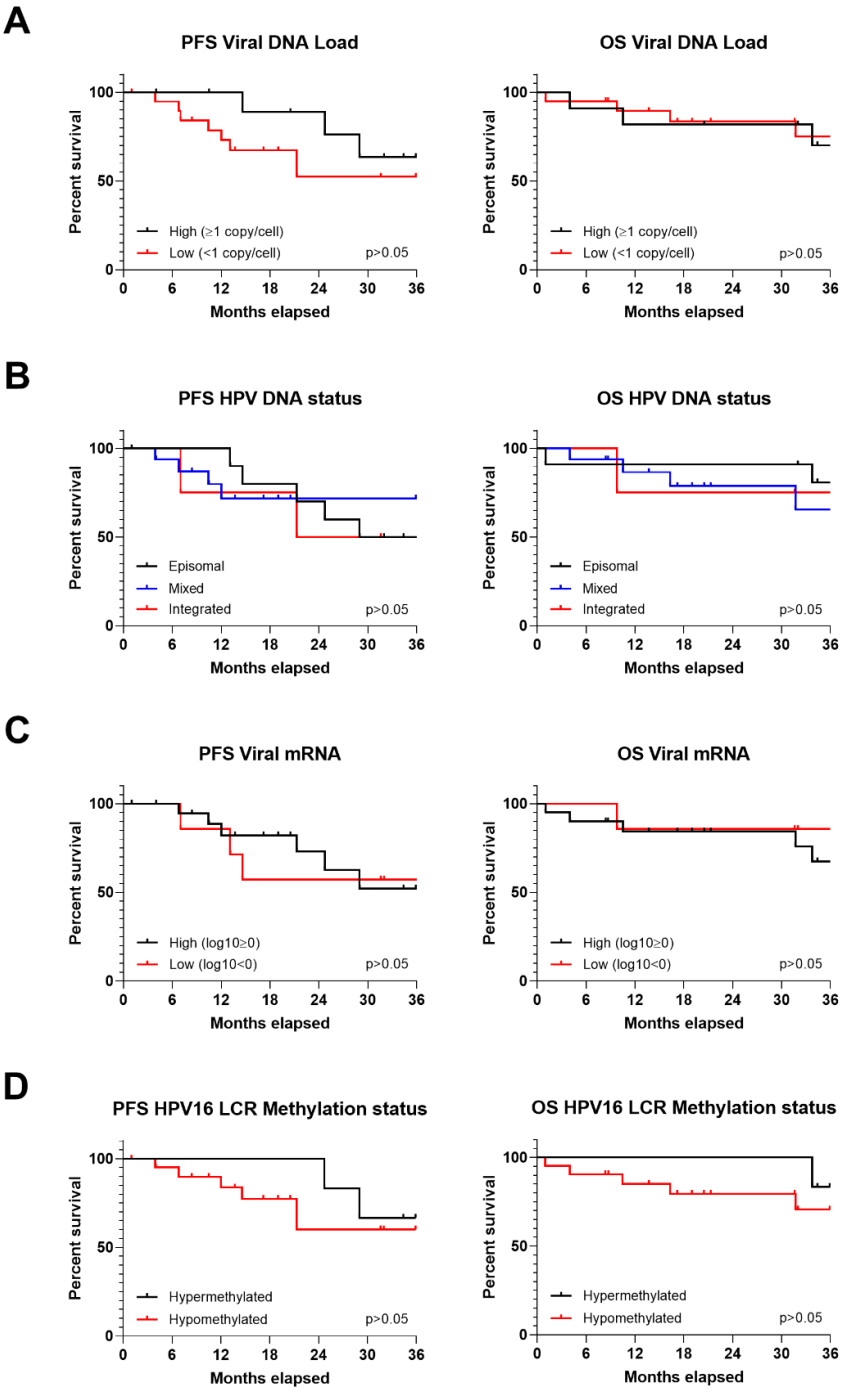


Figure 5. Kapan-Meier curves representing progression free survival (PFS) and overall survival (OS) for HNSCC patients according to HPV16 markers (**A**) Kapan-Meier curves for PFS and OS for HNSCC patients with high or low HPV DNA load; (**B**) Kapan-Meier curve for PFS and OS for HNSCC patients according to the HPV DNA status within the host cell; (**C**) Kapan-Meier curve for PFS and OS for HNSCC patients presenting high or low HPV16 mRNA expression; (**D**) Kapan-Meier curve for PFS and OS for HNSCC patients according to the methylation status of the HPV16 LCR. Statistical significance was indicated as $p<0.05$.

The physical status of the HPV DNA within the host cell did not play a role in patient's prognosis: PFS was 50%, 71.81% and 50% for patients presenting episomal, mixed and integrated HPV DNA ($p>0.05$); OS was 40.40%, 65.55% and 75%, respectively ($p>0.05$) (Figure 5B).

The amount of HPV mRNA expressed was not correlated to survival either; PFS was 52.20% vs. 57.14% for patients with high HPV mRNA expression compared to those with low expression ($p>0.05$); OS was 40.50% and 64.28%, respectively ($p>0.05$) (Figure 5C). Finally, the methylation status of HPV16 LCR was studied in correlation to PFS and OS; results showed that PFS was 60% and 50% for patients presenting HPV16 LCR methylated, compared to those with no methylation ($p>0.05$); similarly, OS was 40% and 54.68%, respectively ($p>0.05$) (Figure 5D).

3.2.3. Discussion

Cellular transcription factors play an important role in HPV LCR transcription regulation, and nucleotide variation could introduce or eliminate transcription factor binding sites from the LCR sequence (Chong et al. 1991, 199), affecting transcription factor binding for their recognition sites, therefore regulating E6/E7 levels and tumorigenesis (Silva et al. 2020). In fact, during a normal infection the p97 promoter is transcriptionally repressed by E2 in order to keep E6 and E7 expression levels low to not activate tumor progression (Graham 2010), while higher oncoprotein expression was associated to tumor progression and aggressiveness (Das et al. 2015). Cellular transcription factors can indeed stimulate or repress HPV16 promoter transcription. In particular, NF-1, AP-1, SP-1 or TEF-1 have been correlated to HPV promoter stimulation and viral gene transcription, contrary to

YY-1 or SRY (Kurvinen et al. 2000). In this study, nucleotide variation in HPV LCR sequences was detected in 77.14% of HNSCC samples. In-silico analysis indicated that these variations overlapped with several TFBSs, influencing TF binding and modulating of viral gene expression.

In particular, the G7521A variation was detected in 54.29% HNSCC and has been often observed in preneoplastic lesions and cervical cancer (Escobar-Escamilla et al. 2019; Mosmann et al. 2015). G7521A overlaps with TFBS for Ying-yang 1 (YY-1), a repressor of LCR, and mutation of any site for YY-1 binding was correlated to an increase in transcriptional activity (O'Connor et al. 1996). Accordingly, HNSCC samples containing this variation experience an increment of viral gene expression compared to those with unvaried site, although this increment resulted not statistically significant. Survival studies showed not significance for the study of single nucleotides, but when analyzed according to the TBFS, results showed that patients carrying variations within the YY-1 sequence were less likely to recur, compared to patient's carrying the unvaried sequence. This finding is very interesting, because variations within this TFBS have been correlated to persistent infection and, in cervical cancer, many HPV16 variants are carriers of YY1-binding sites changes, although no clear association between this marker and carcinogenesis has been made yet (Escobar-Escamilla et al. 2019). In fact, a study carried by Mosmann et al. found that G7521A mutation was mostly present in low-grade squamous intraepithelial lesions (LSIL) (Mosmann et al. 2015), while another study on cervical cancer found no association between this mutation and disease progression (Hildesheim et al. 2001). Furthermore, the reference HPV16 genome that is widely used was initially isolated from a human invasive cervical carcinoma (Yao et al. 2019; Escobar-Escamilla et al. 2019), so it is possible that the lack of variation within YY1-binding site, and in particular, the lack of G7521A variation, could be indicative of a more aggressive HPV16 variant, oppositely to what originally thought (Escobar-Escamilla et al. 2019; Mosmann et al. 2015). YY-1 also can be altered by other cellular transcription factors that are located adjacently in the same promoter; in the HPV18 promoter, interaction between the CCAAT/enhancer-binding protein (C/EBP) and YY-1 was found to be likely responsible for the abrogation of the repressor activity of YY-1 (Y. Shi, Lee, and Galvin 1997). Interestingly, variations within the C/EBP TFBS sequence did not seem to correlate to patient prognosis, although tumors carrying HPV16 with these variations, expressed significantly less E6 mRNA, compared to those with the reference sequence.

Based on this, survival was studied combining YY-1 and C/EBP TFBSs. Patients presenting YY-1 TFBS variations are more likely to recur, and this was reinforced when C/EBP TFBS variations were present, giving a strong indicator for risk of relapse in these patients. Nucleotide variations upon the NF-1 region significantly reduced the amount of all viral mRNAs, but it did not correlate to OS or RFS. It is possible that samples containing variants that hamper viral gene expression could benefit for improved prognosis, although more studies with a greater sample size are necessary to clarify this matter.

Together with cellular factors, viral E2 protein participates in HPV early gene transcription regulation (Verma et al. 2017). E2 gene products may activate or repress transcription depending on the E2BS it binds. While binding to the E2BS1 promotes basal HPV transcription, binding to E2BS3 and 4 represses it (Durzynska, Lesniewicz, and Poreba 2017). Methylation within E2BSs hamper E2 binding and de-repress HPV LCR, allowing for constitutive viral gene expression (von Knebel Doeberitz and Prigge 2019). This mechanism has been well studied in episomal samples that carry intact E2 gene, while viral genome integration with E2 gene disruption often leads to deregulated expression of viral early genes (Sabatini and Chiocca 2020). Our results indicate that HPV DNA was detected in episomal form in 34.28% of HNSCC samples, similar to previous studies (Paolo Boscolo-Rizzo et al. 2017), while integrated and mixed in 11.43% and 54.28% of the samples, respectively, indicating that integration appears to be unnecessary event for viral transformation in HNSCC.

Methylation of the E2BSs in the LCR, often results in overexpression of the E6/E7 early viral proteins (Paolo Boscolo-Rizzo et al. 2017; Ekanayake Weeramange et al. 2020; von Knebel Doeberitz and Prigge 2019), although we could not find this direct correlation in our study, suggesting that methylation of the E2BSs may be not the only events implicated in viral mRNA transcription, but rather, a combination of E2BS and TFBS control of the gene expression is required for viral transcription. It is also possible that due to missing sequences within the LCR of HPV, we were not able to see these correlations, further analyses are ongoing to clarify this matter. Furthermore, methylation level of the HPV LCR correlated to E2/E6 ratios, so epigenetic control of the virus may occur through methylation, in order to promote oncogene expression. In fact, samples carrying episomal or mixed HPV DNA presented overall higher viral gene expression. Interestingly, viral load showed a significant association with the methylation frequency of the LCR as well,

suggesting that methylation occurs upon presence of multiple HPV DNA copies, as seen before (Marongiu et al. 2014). The viral load of cases with higher E2/E6 rates was higher compared to those where no E2 was detected, as seen previously (Ghosh et al. 2012; Lillsunde Larsson et al. 2014), probably because the presence of E2 protein enhances viral DNA replication by interacting and recruiting the viral replication factor E1 to the origin of replication. Also, E2 may facilitate viral genome segregation by tethering the viral DNA to host mitotic chromosomes (Ghosh et al. 2012). Nevertheless, nor methylation within E2BS1-4 nor other HPV markers were positively correlated to patient's prognosis.

Viral gene transcription, therefore, seems to happen under a coordinated series of events, including sequence variation and methylation of certain regions in the LCR. This, to our knowledge, is the first study that demonstrates variation of HPV gene expression upon LCR nucleotide variation/deletion in HNSCC and correlates these variations to patient's prognosis. Previous studies have highlighted the importance of nucleotide variations in the sequence of the viral genes such as E6, E7 or L1, and although no clear establishment to patient's prognosis was made (Combes and Franceschi 2018), this study shows for the first time that, variations in the viral LCR affect the expression of viral genes and influence patient's prognosis.

This study reveals the importance of viral gene expression regulation during carcinogenesis, new markers for patient relapse emerge, improving the understanding of functionally significant epigenetic alterations in HPV-infected cells, and highlighting the importance of variant research to be incorporated to diagnostic strategies and for the planning of treatment strategies for HPV-positive cancers. Knowing the important role that methylation plays in viral gene expression and this, in tumorigenesis, clinical approaches aimed to reduce the amount of oncoprotein expressed should be of particular interest for the treatment of HPV-related HNSCCs. These patients could benefit from treatments with demethylation agents such as like 5-aza-2'-deoxycytidine. In fact, only one clinical trial is currently studying the effects of these treatment in HPV-positive HNSCC patients (NCT02178072). Preliminary results for the use of Vidaza seem promising (Biktasova et al. 2017), and could lead to new treatment strategies for HPV-positive HNSCC; more targeted and less aggressive compared to current protocols.

CHAPTER 3.3

Differential expression profiles in HPV-positive vs HPV negative HNSCCs

3.3.1. Introduction

Head and neck tumors are very heterogeneous and several classifications have been made according to the anatomic region where it arises, etiology, and molecular markers (Klussmann 2017). According to etiology, HNSCC can be associated to tobacco and alcohol consumption, generally for tumors of the oral cavity and larynx, while the human papillomavirus (HPV) has been associated to tumors in the pharynx/oropharynx (Johnson et al. 2020). Generally, the 5-year survival rates for oral, pharyngeal and laryngeal cancers have been estimated to range between 51% and 62%, for all stages combined (Reyes-Gibby et al. 2014; Klussmann 2017), although HPV presence is a good prognosis factor for HNSCC with 5-year survival rates ranging 75–80% (Sabatini and Chiocca 2020). Therefore, HNSCC tumors can be further classified into HPV-negative and HPV-positive.

Despite improved prognosis for HPV-positive HNSCCs, still a subset of patients present high relapse rates (Economopoulou, Koutsodontis, et al. 2019). Thus, the optimal way to predict clinical outcome for all HNSCCs types is to evaluate several biomarkers (Sabatini and Chiocca 2020). In fact, treatment selection criteria based on the presence of biomarkers is now the focused of personalized medicine (J. J. Chen et al. 2015).

Surgery and primary chemoradiotherapy (CRT) treatment are the first line treatments for HNSCC. When comorbidities prevent the use of cytotoxic chemotherapy, the EGFR monoclonal antibody cetuximab is used in combination with radiation (Johnson et al. 2020). EGFR is implicated in HNSCC pathogenesis and its expression is independent of HPV, and therefore treatment with EGFR blockers is recommended in all HNSCC types (Verma et al. 2017). Over 90% of tumors present EGFR upregulation, entailing one of the most prominent oncogenes in HNSCC, and its expression correlates with poorer survival (Cassell and Grandis 2010; Kriegs et al. 2019).

Downstream of EGFR is the PI3K/AKT signaling pathway which is considered one of the most important pathways in HNSCC. PIK3CA oncogene is frequently mutated in HNSCC and plays a role in both HPV-negative and HPV-positive tumors (Psyrri, Seiwert, and Jimeno 2013). The PI3K/AKT signaling pathway plays a key role in the tumor self-renewal process. A recent study on breast cancer demonstrated that suppression of IRF6 enhanced the PI3K/AKT signaling activation PIK3R2, and suggested that IRF6 could be a viable therapeutic target (Xu et al. 2019). IRF6 exhibits tumor suppressor activity in

squamous cell carcinomas and was previously found downregulated due to CpG methylation in its promoter region (Botti et al. 2011; Rotondo et al. 2016). IRF6 is involved in a feedback loop with TP63, a member of the TP53 gene family, to regulate epidermal differentiation (Moretti et al. 2010). The TP63 gene (also known as the p63) encodes for two isoforms. Isoform ΔNp63 has been implicated in HNSCC tumor growth by suppression of apoptosis and p16INK4A expression (Johnson et al. 2020). Treatment with Imatinib has previously demonstrated to downregulate TP63 mRNA expression in HNSCC (Ongkeko et al. 2006; Vanbokhoven et al. 2011).

Several PI3K inhibitors are under clinical testing for HNSCC, such as BYL719, that in combination with SP600125, an inhibitor of c-Jun N-terminal kinase (JNK), has shown in vitro antiproliferative effects in HNSCC cell lines (Badarni et al. 2019). C-Jun expression in HNSCC has been correlated to worse prognosis. Human squamous cell carcinoma gene knockout models have previously shown that c-Jun has tumor promoter effect and its dowregulation was associated with better survival (Verma et al. 2017). C-Jun participates in the formation of the AP-1 complex to promote gene expression, in particular regulates the expression of genes implicated in treatment resistance, such as AXL (Badarni et al. 2019). JNK blocking using the SP600125 compound led to arrest of tumor growth in several cellular models (Badarni et al. 2019; Z. Chen et al. 2008).

In addition to genetic alterations, epigenetic changes also have a role in HNSCC oncogenesis. In particular, RARB promoter has been frequently found hypermethylated with a consequent gene downregulation (Johnson et al. 2020; Rotondo et al. 2018b). RARB is a tumor suppressor protein that antagonizes AP-1 complex (Lubecka et al. 2018). RARB reduction may in turn upregulate c-jun and c-fos genes that contain AP-1 domains in their promoter region (Burris and McCabe 2001). HNSCC patients carrying hypermethylated RARB promoter could benefit from treatments with demethylation agents 5-azacitidine (5-aza-CR; azacitidine) and 5-aza-2′-deoxycytidine (5-aza-CdR; decitabine) (Bais 2019). However, the methylation status of RARB promoter in HNSSCs has not been investigated in depth yet.

The aim of the study was to investigate the expression profile of the EGFR, IRF6, RARB, p16, p63 and c-jun genes in both HPV-negative and HPV-positive HNSSCs, in order to verify if different molecular profiles emerging between groups could be used as molecular markers for the planning of targeted therapeutic strategies aimed to improve patient's survival.

3.3.2. Results

3.3.2.1. Study population and patient samples

Head and neck squamous cell carcinoma (HNSCC) patients (n=84), with a mean age ± standard deviation [SD] 68.15±10.84 years (y) old, and non-oncological patients as controls (n=58) undergoing tonsillar surgery, with a mean age ± [SD] 39±15.17 (y), were enrolled at the ear, nose and throat (ENT) Clinic, University Hospital of Ferrara, Ferrara, Italy.

3.3.2.2. HPV analyses

HNSCC were screened for HPV DNA presence. HPV DNA was found in 37/84 (44.04%) of the samples analyzed. Genotyping with high resolution melting (HRM) showed that 34/37 (91.89%) of HNSCCs carried HPV type 16, 1/37 (2.70%) HPV type 33, 1/37 (2.70%) HPV type 31, and 1/37 (2.70%) presented a coinfection with HPV types 16 and 18. Control DNAs were found to be HPV11-positive in 1/58 (1.7%) of the cases. For further analyses, samples with matching DNA/RNA availability (n=59) were used in this study, for HPV-negative (n=25) and HPV-positive (n=34) samples. Clinicopathological characteristics of these patients, according to HPV status are presented in table 1.

3.3.2.3. Gene expression analysis shows differential gene expression among HNSCC samples

HNSCC samples were analyzed for gene expression. Differences can be observed for HPV-positive and- negative HNSCC groups (Figure 1). EGFR presented higher mRNA expression in HPV-positive tumors compared to control samples and to negative tumors ($p<0.05$). Mean fold change±SD for EGFR expression was 1.32±2.12 and 0.06±1.59 ($p<0.05$) in HPV-positive HNSCCs and HPV-negative, respectively (Figure 1A).

Table 2. Clinicopathological characteristics of HNSCC patients

Clinicopathological variables	HPV-negative	HPV-positive	P value
Tumor Site			
Oral	18/25 (72%)	2/34 (5,88%)	0.0001
Oropharynx	5/25 (20%)	32/34 (94,11%)	0.0001
Hypopharynx	1/25 (4%)	-	-
Larynx	1/25 (4%)	-	-
Tumor Size			
T1	1/25 (4%)	5/34 (14,70%)	0.2283
T2	5/25 (20%)	5/34 (14,70%)	0.7292
T3	6/25 (24%)	4/34 (11,76%)	0.2970
T4	13/25 (52%)	17/34 (50%)	1,0000
NA	-	3/34 (8,82%)	-
Node Status			
N0	5/25 (20%)	3/34 (8,82%)	0.2649
N+	20/25 (80%)	28/34 (82,35%)	1,0000
NA	-	3/34 (8,82%)	-
Clinical Stage			
I	-	1/34 (2,94%)	-
II	4/25 (16%)	5/34 (14,70%)	1,0000
III	3/25 (12%)	6/34 (17,64%)	0.7199
Iva	14/25 (56%)	8/34 (23,52%)	0.0150
Ivb/c	4/25 (16%)	-	-
NA	-	13/34 (38,23%)	-
Recurrence	13/25 (52%)	10/34 (29,41%)	0.1071
N/A	1/25 (4%)	5/34 (14,70%)	
Tobacco consumption			
No	3/25 (12%)	2/34 (5,88%)	0.6414
Ex Smoker	8/25 (32%)	8/34 (23,52%)	0.5585
Smoker	12/25 (48%)	7/34 (20,58%)	0.0950
N/A	2/25 (8%)	20/34 (58,82%)	
Alcohol consumption			
No	6/25 (24%)	5/34 (14,70%)	0.5015
Ex consumer	-	2/34 (5,88%)	-
Consumer	15/25 (60%)	10/34 (29,41%)	0.0322
N/A	4/25 (16%)	20/34 (58,82%)	
Age	68,4±12,67	67,96±10,77	0,8832
Gender			
Male	14/25 (56%)	27/34 (79,41%)	0.0853
Female	7/25 (28%)	7/34 (20,58%)	0.5487

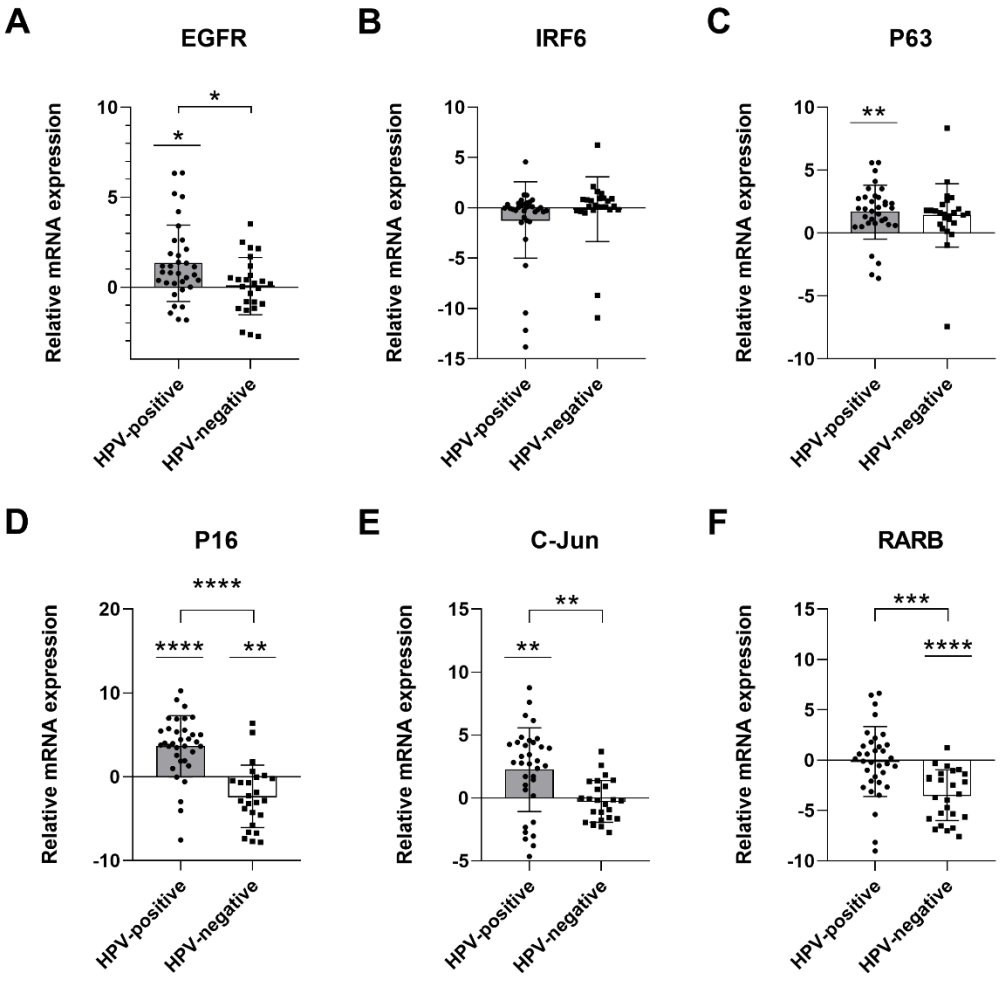


Figure 1. Differential gene expression for samples according to HPV status. Statistical significance is represented as * for p<0.05; ** for p<0.01; *** for p<0.001 and **** for p<0.0001.

For IRF6 no statistical significances were observed between groups and compared to the control group; mean fold change±SD was -1.18±3.79 and -0.12±3.21 ($p>0.05$), in HPV-positive and HPV-negative, respectively (Figure 1B).

P63 gene, was observed upregulated in most HNSCC cases, and in particular, the upregulation in HPV-positive samples was significant compared to normal ($p<0.01$). No significant differences were observed between HPV-negative samples and normal or HPV-positive and HPV-negative samples: mean fold change±SD, 1.67±2.14 and 1.41±2.52, respectively ($p>0.05$) (Figure 1C).

P16, which is by definition the surrogate biomarker of HPV infection was overexpressed in HPV-positive samples ($p<0.0001$) and downregulated in HPV-negative HNSCCs ($p<0.01$) compared to normal. Differences between HPV-positive and negative groups were statistically significant: mean fold change±SD, 3.63±3.65 and -2.34±3.72, respectively, ($p<0.0001$) (Figure 1D).

c-Jun gene showed higher mRNA expression in HPV-positive samples compared to control and to HPV-negative samples; for instance, mean fold change±SD was 2.24±3.32 and -0.26 ±1.66 ($p<0.01$) (Figure 1E).

Lastly, RARB was mostly downregulated in HPV-negative HNSCCs compared to normal ($p<0.0001$). Differences between the HPV-positive HNSCC group and HPV-negative for RARB mRNA expression were also significant: mean fold change± SD, -0.11±3.46 and -3.48±2.52, respectively ($p<0.001$) (Figure 1F).

3.3.2.4. RARB promoter methylation analysis shows inverse correlation to gene expression

The hypermethylation of the tumor suppressor gene promoter RARB, may favor the onset of cancer (Rotondo et al. 2018b). Upon mRNA downregulation observation, the methylation status of the RARB gene promoter was studied in both HNSCC and control samples. Significant hypermethylation was observed in HNSCC samples, for both HPV-positive and negative samples, compared to control (Figure 2).

Furthermore, correlation studies showed a negative correlation between RARB mRNA expression and promoter methylation ($r=-0.37$, $p<0.01$) and in particular associated to HPV-negative ($r=-0.47$, $p<0.01$), while this correlation was not evident when analyzed

HPV-positive samples alone (r=-0.15, p>0.05). Thus, RARB expression may be in part controlled by an independent mechanism rather than promoter methylation; it is possible that, at least in part, HPV mediated.

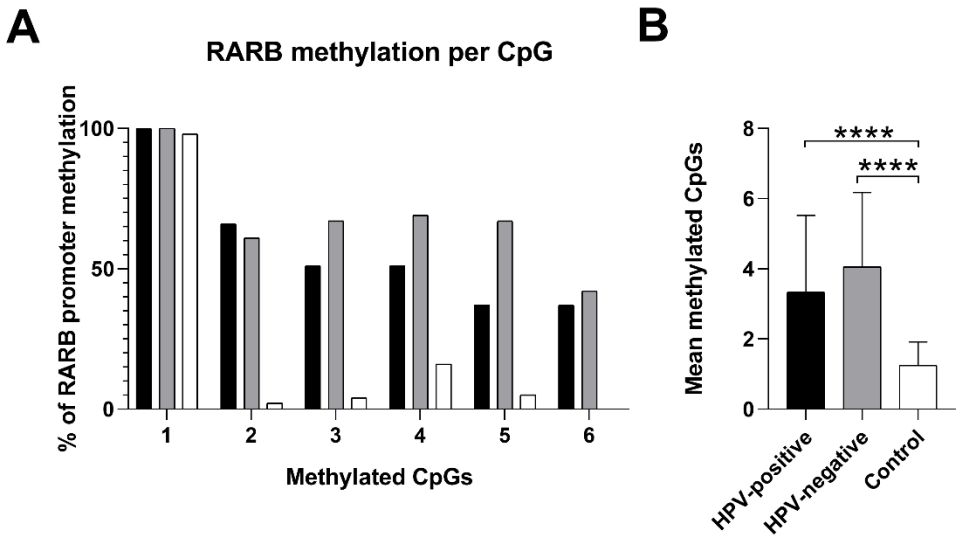


Figure 2. (**A**) Histogram representing methylated CpGs in the RARB promoter. Black columns correspond to HPV-positive HNSCC samples; grey columns represent HPV-negative HNSCC samples; and white columns represent non tumoral samples; (**B**) Percentage of methylated CpGs in the RARB promoter region analyzed. Black columns represent HPV-positive HNSCC samples; grey columns correspond to HPV-negative HNSCC samples; and white columns represent non tumoral samples. Statistical significance is represented as **** for p<0.0001.

3.3.2.5. Survival analyses

Overall survival (OS) and progression free survival (PFS) were studied on HNSCC patients in correlation to each one of the tumor suppressor genes or proto-oncogenes over a two year-follow up. Patients that presented upregulation of RARB had an OS rate of 83.33% compared to 50.65% of those with low expression (p>0.05). PFS was 46.17% for patients with high RARB expression and 47.66% for those with low expression (p>0.05) (Figure 3A).

The related gene c-Jun presented overall survival rates of 68.81% and 60% for patients presenting high and low expression, respectively (p>0.05). PFS was 58.3% and 50% for patients with high and low c-Jun expression (p>0.05) (Figure 3B).

P16 expression has been previously correlated to improved overall survival (Hashmi et al. 2020). OS was 65.72% for patients with P16 overexpression compared to 50% for patients with low gene expression ($p<0.05$). Also, PFS was better, 65.02%, in patients with high P16 mRNA levels compared to 38.82% for patients with gene downregulation ($p<0.05$) (Figure 3C).

Although high EGFR expression has been previously correlated to poor survival in HNSCC (Kriegs et al. 2019), in our study OS was 66.55% when EGFR was upregulated compared to 66.66% when it was downregulated ($p>0.05$). PFS was 40.85% for HNSCC patients with high EGFR and 55.55% for patients with low EGFR mRNA levels ($p>0.05$) (Figure 3D).

IRF6 downregulation has been classically correlated to worse prognosis (Li et al. 2019); we studied the prognosis effect of IRF6 expression in HNSCC and found that OS was 64.28% for patients with high expression, while 57.14% for patients with low expression ($p>0.05$); the PFS was 46.15% for patients with high expression and 42.85% for those with low expression ($p>0.05$) (Figure 3E).

P63 expression is essential for survival of HNSCC cells, due to its suppression of a proapoptotic transcriptional program (Rocco et al. 2006). OS in HNSCC patients with P63 upregulation was 58.4% compared to 80% for patients with low expression ($p>0.05$). PFS was 49.9% vs. 26.66% for patients' samples presenting high vs. low P63 mRNA expression, respectively ($p>0.05$) (Figure 3F).

Survival according to HPV status was also studied. In particular, survival analysis for IRF6 expression in HPV-positive and -negative gave interesting results. HPV-positive patients presented an OS of 100% for those patients with high expression, while 80% for those with low expression ($p>0.05$). PFS was 40% for patients with high expression and 60% for those with low IRF6 expression ($p>0.05$) (Figure 4A).

On the other hand, for HPV-negative patients; OS was 44.44% for patients with high IRF6 expression and 0% for those with low expression ($p>0.05$). PFS was 50% for patients with high expression and 0% for patients with low expression ($p<0.05$) (Figure 4B). These results put in evidence the role of IRF6 in cancer progression, and when focused on HPV-negative samples, it is a strong indicator of relapse. No significant differences were observed for survival when studied the HPV-positive and HPV-negative HNSCC groups separately for the rest of the genes studied.

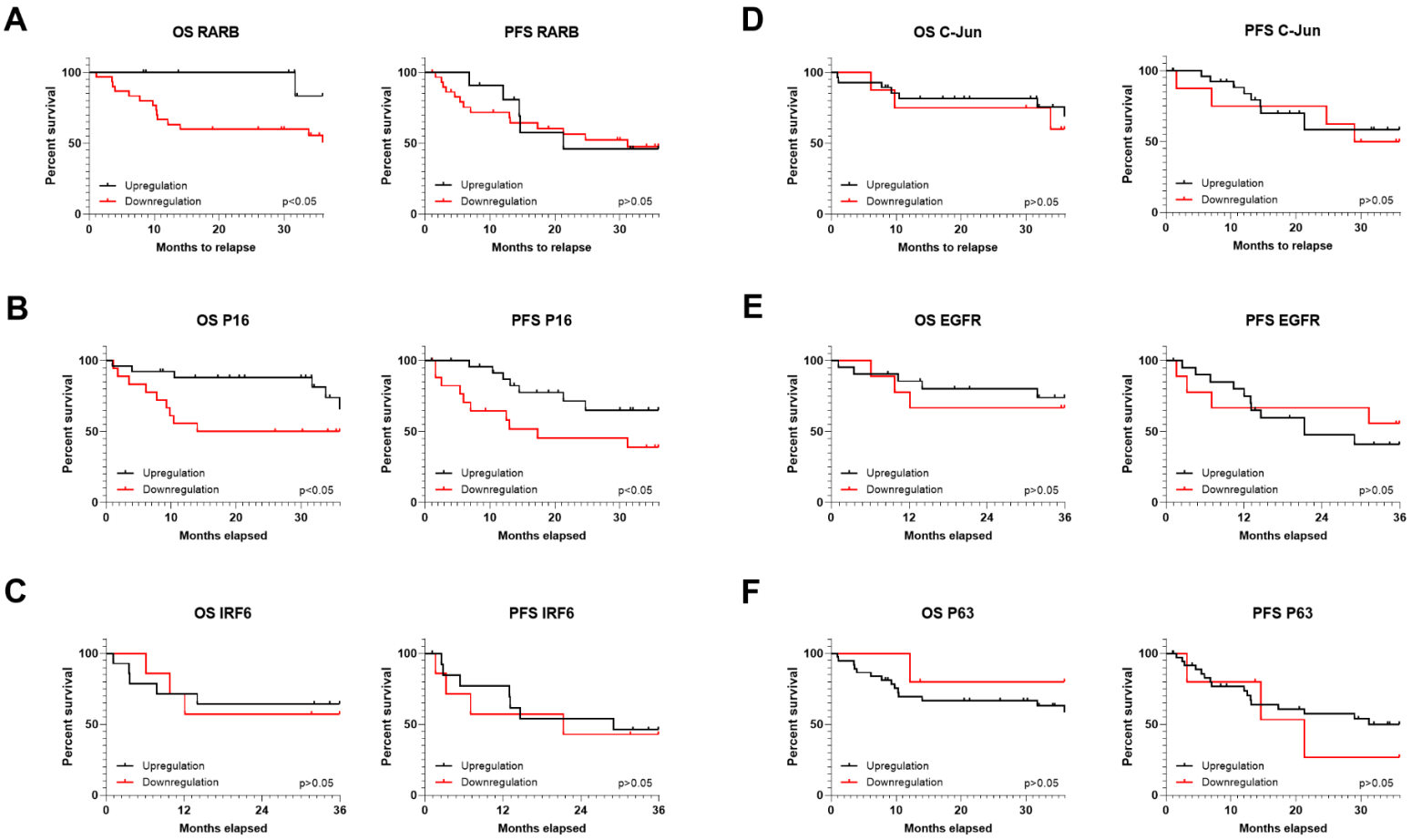


Figure 3. Kaplan-Meier curves representing OS and PFS for HNSCC patients (n=59); (**A**) Represent OS and PFS for RARB gene expression up- or downregulation; (**B**) Represent OS and PFS for c-Jun gene expression up- or downregulation; (**C**) Represent OS and PFS for P16 gene expression up- or downregulation; (**D**) Represent OS and PFS for EGFR gene expression up- or downregulation; (**E**) Represent OS and PFS for IRF6 gene expression up- or downregulation; (**F**) Represent OS and PFS for P63 gene expression up- or downregulation. Statistically significant values are represented as $p<0.05$.

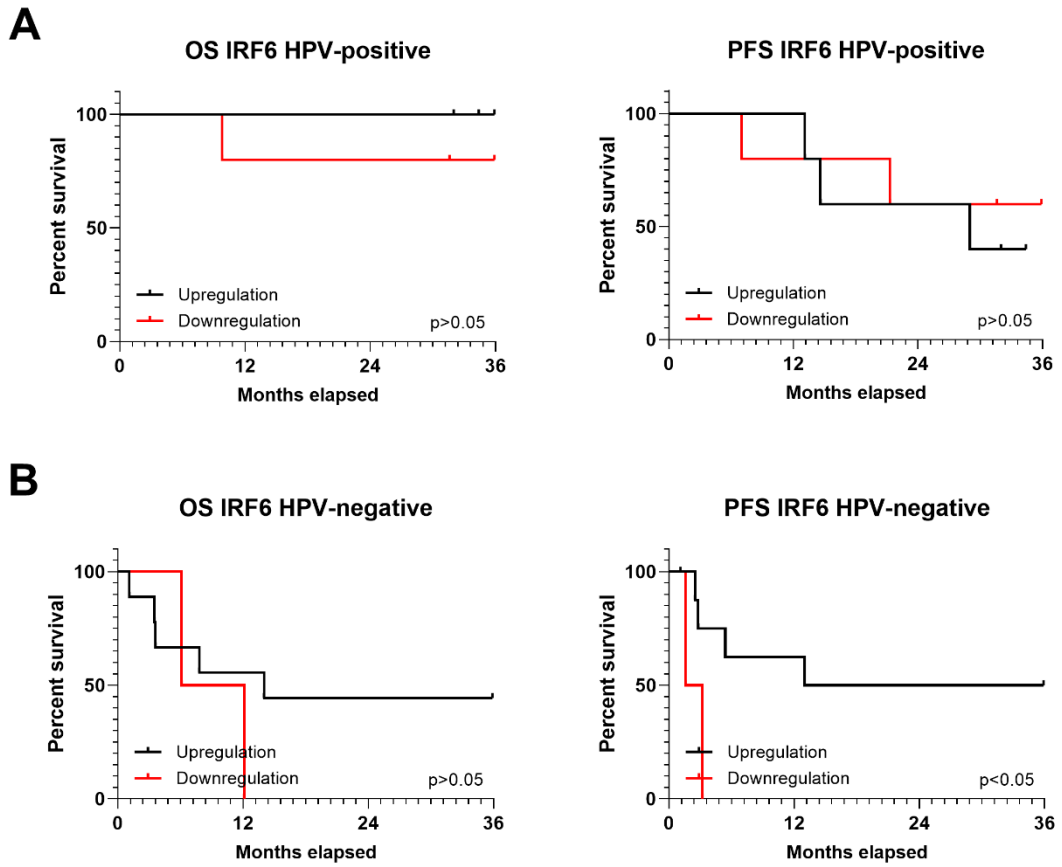


Figure 4. Kaplan-Meier curves (KM) for OS and PFS according to IRF6 expression; (**A**) KM in HPV-positive HNSCC samples; (**B**) KM in HPV-negative HNSCC samples. Statistically significant values are represented as $p<0.05$.

3.3.3. Discussion

Several attempts have been made to classify HNSCC tumors, and based on distinctive characteristics the appropriate treatment is chosen (Canning et al. 2019). While classical characterization based on tumor location and stage is usually used to assess the best treatment option, recent data show that it does not always correlate to patient's response to therapy and prognosis (Leemans, Snijders, and Brakenhoff 2018). Thus, there is a need to find other methods to classify these tumors, such as molecular markers (Canning et al. 2019; Johnson et al. 2020). Different gene expression patterns were found in our cohort of study between the HNSCC subtypes. In particular EGFR, p16, RARB and c-Jun, harbored differential gene expression between HPV-positive and negative samples.

When studied according to survival, p16 resulted to be a good prognostic marker for both HNSCC subtypes, i.e., HPV-positive and –negative. The p16 tumor suppressor gene plays a key role in cell cycle regulation (H. Shi et al. 2015) and has been studied as an independent prognostic value in HNSCC. P16 overexpression has been previously associated to lower T- and N-stage and better survival (Hashmi et al. 2020). In our study, both OS and PFS were associated to p16 expression. In particular, patients showed better prognosis when p16 expression was high, independently of HPV status.

We also studied the gene expression in correlation to survival for RARβ, a tumor suppressor protein that inhibits HNSCC growth by modulating cell proliferation and differentiation, cell cycle progression, and apoptosis (Lubecka et al. 2018). Suppression of RARB has been associated with tumor progression (Lai et al. 2014). In fact, in our study, patients with RARB downregulation had a lower OS compared to those with gene upregulation, and since, RARB gene expression is inhibited by promoter methylation (Johnson et al. 2020; Rotondo et al. 2018b), we studied the methylation status of RARB in our cohort of patients. RARB downregulation following promoter methylation was observed. Based on this, many HNSCC patients could benefit from a combination with demethylation agents such as; 5-azacitidine (5-aza-CR; azacitidine) and 5-aza-2′-deoxycytidine (5-aza-CdR; decitabine) (Bais 2019) added to their current treatment regimens. In fact, treatment combination has demonstrated to have a favorable effect when applied according to the molecular profile of the tumors (Lubecka et al. 2018), and should be considered in the future taking into account the patient's molecular profile.

For instance, current treatment strategies with monoclonal antibodies, such as Cetuximab, targeting EGFR, are effective to inhibit EGFR signaling in HNSCC (Verma et al. 2017; Johnson et al. 2020). Interestingly, EGFR expression seems to be higher in HPV-positive patients in our study, so the patients could especially benefit from this type of treatment. Although EGFR gene overexpression had been previously associated with poor overall survival (OS) and progression-free survival (PFS) (Johnson et al. 2020; Kriegs et al. 2019), in our study population we did not observe great differences in OS and PFS, probably because most of the samples with EGFR upregulation belonged to the HPV-positive subgroup, which overall have an improved OS.

Furthermore, the expression and prognostic value of c-Jun was studied. Although certain studies support a pro-oncogenic function of c-Jun N-terminal kinases (JNK), in particular, c-Jun upregulation has been previously associated to poor survival in HNSCCs (Verma et al. 2017) and was associated to increased resistance to chemotherapy (Badarni et al. 2019). Other studies provide evidence that JNKs act as tumor suppressors in HNSCC (Gkouveris et al. 2016). In our study cohort c-Jun expression did not correlate to OS nor PFS for HNSCC patients. Interestingly, its expression was higher in HPV-positive samples. Further studies are necessary to elucidate the role of c-Jun in these patients.

The P63 gene was also studied in correlation to prognosis. P63 encodes two major isoforms, ΔNp63 and TAp63, and is overexpressed in a majority of HNSCC tumors (Johnson et al. 2020). ΔNp63 promotes HNSCC tumor growth by promoting cell proliferation (Walter et al. 2013). This could be possible for HPV-positive samples in our cohort of study were P63 was found mainly overexpressed, although we did not observe differences for OS and PFS for patients depending on the P63 expression levels.

On the other hand, low IRF6 mRNA expression, correlated to prognosis of HPV-negative HNSCCs. IRF6 is involved in the regulation of squamous differentiation (Stransky et al. 2011); diminished levels of IRF6 have been previously associated with poor prognosis (Xu et al. 2019; Li et al. 2019) and correlated to promoter methylation in several tumors (Rotondo et al. 2016; Nobeyama and Nakagawa 2017; Botti et al. 2011).

In this study, deregulated expression of a combination of candidate genes were studied. A profile for better and worse prognosis based on molecular differences has emerged. These are preliminary results and further studies taking into account the current uncertainties of this work are ongoing. Overall, a better understanding on the expression profile of HNSCC tumors would help plan personalized strategies aimed to target the main deregulated pathways in this pathology. Finally, the development of relevant preclinical models for HNSCC representing the disease at the genetic, histological, and functional levels will provide a method for future studies on the molecular modifiers of response to therapies that are currently in use or now being tested in clinical trials.

CHAPTER 4

Conclusion

Over the past few years there has been a global attempt to classify head and neck squamous cell carcinomas (HNSCCs), in order to improve its prognosis and treatment outcome. For instance, HPV-positive HNSCCs have been defined as a different entity from HPV-negative based on its onset and treatment response. HPV-positive tumors usually respond better to standard treatment choices, while overall survival of these patients is improved. Still, a subset of these patients will present tumor recurrence and/or metastasis within the first two years from initial treatment. The overall survival for this subset of patients is reduced compared to treatment responsive HPV-HNSCCs.

Several studies are ongoing, in order to understand the molecular bases underneath this differential response in the subset of HPV-positive patients. Yet, up to date, no common ground has been reached, and no specific markers to determine outcome for these patients has been established. The main current problem is that there are no standard guidelines to classify HPV-positive patients, and while many clinicians and/or pathologists determine positivity based on HPV-DNA or RNA in situ hybridization (ISH) or HPV-DNA presence, this technique may be misleading and may induce to wrong classification of non-HPV driven tumors into HPV-positive. Another relevant issue is that ISH is not a reliable technique, since small quantities of HPV DNA or RNA may be missed. Recent studies have stated that to be an HPV-driven tumorigenesis, HPV gene expression is fundamental, in order to interact with the host machinery and initiate the carcinogenic process, and although it may indicate which HNSCCs are HPV- driven, it is not able to differentiate patients for improved survival.

Regarding to HPV, classical markers alone, such as HPV DNA, HPV mRNA and p16 expression, were not sufficient to stratify HPV-positive HNSCC patients for disease recurrence in our study, but the combination of classical markers with serological markers, resulted to be strong indicators of prognosis; particularly for oropharyngeal squamous cell carcinomas (OPSCC). HPV16 E7 oncoprotein presence in serum at the time of diagnosis, correlated with disease recurrence and two-year overall survival. The research for E7 oncoproteins in serum may turn useful as a non-invasive procedure for HPV-positive patients' stratification and follow-up, helping identify patients at risk at the time of diagnosis for tumor recurrence during the follow-up, giving a tool for clinicians to determine which patients would be good candidates for treatment de-escalation or should be kept under close surveillance.

Furthermore, differences in the HPV sequence itself may impact patient prognosis and may suppose a molecular tool for the identification of HPV-positive patients at risk of relapse. While methylation of the LCR plays a fundamental role in the control of HPV gene expression, the importance of regulation by other cellular transcription factors emerges, and, interestingly, for the first time, sequence variations within the YY-1 transcription factor binding site, mainly G7521A, were correlated to improved patient survival, contradicting what hypothesized in literature, although never demonstrated before in a cohort of HNSCC patients. The study on the second subchapter shows the importance HPV16 sequence variations have for transcription factor binding and how changes in this sequence can greatly affect viral gene expression, which is, after all, necessary to promote carcinogenesis.

Finally, the content of my thesis describes in detail several differences at molecular level between HPV-positive and negative tumors; differences were significant between EGFR, p16, c-Jun and RARB, and these markers may help create an expression profile in order to stratify HNSCC patients at diagnosis, and to select the best treatment choice according to it. Furthermore, IRF6 emerged as a recurrence marker for HPV-negative patients, since diminished levels of IRF6 correlated to recurrence in HPV-negative patients.

The findings presented in my thesis show molecular differences between HPV-positive and negative tumors, and shed light on fundamental aspects of the HPV biology, that had been poorly studied in HNSCCs so far. The first one being the presence of circulating viral oncoproteins in HPV-positive patients in serum, whose study was difficult due to the lack of commercial kits until a few years ago; and the second one, the importance that HPV16 sequence has on the behavior of the virus and its pathogenic potential. Both aspects turned to be equally important related to patient's progression free survival and should be studied in more depth as candidate markers for patient stratification in the future.

CHAPTER 5

References

Agoff, S. Nicholas, Patricia Lin, Janice Morihara, Constance Mao, Nancy B. Kiviat, and Laura A. Koutsky. 2003. "P16(INK4a) Expression Correlates with Degree of Cervical Neoplasia: A Comparison with Ki-67 Expression and Detection of High-Risk HPV Types." *Modern Pathology: An Official Journal of the United States and Canadian Academy of Pathology, Inc* 16 (7): 665–73. https://doi.org/10.1097/01.MP.0000077518.78046.0C.

Akagi, Keiko, Jingfeng Li, Tatevik R. Broutian, Hesed Padilla-Nash, Weihong Xiao, Bo Jiang, James W. Rocco, et al. 2014. "Genome-Wide Analysis of HPV Integration in Human Cancers Reveals Recurrent, Focal Genomic Instability." *Genome Research* 24 (2): 185–99. https://doi.org/10.1101/gr.164806.113.

Alsahafi, Elham, Katheryn Begg, Ivano Amelio, Nina Raulf, Philippe Lucarelli, Thomas Sauter, and Mahvash Tavassoli. 2019. "Clinical Update on Head and Neck Cancer: Molecular Biology and Ongoing Challenges." *Cell Death & Disease* 10 (8): 540. https://doi.org/10.1038/s41419-019-1769-9.

Amin, Mahul B., American Joint Committee on Cancer, and American Cancer Society, eds. 2017. *AJCC Cancer Staging Manual*. Eight edition / editor-In-Chief, Mahul B. Amin, MD, FCAP ; Editors, Stephen B. Edge, MD, FACS [and 16 others] ; Donna M. Gress, RHIT, CTR-Technical editor ; Laura R. Meyer, CAPM-Managing editor. Chicago IL: American Joint Committee on Cancer, Springer.

Araujo, Melissa Rodrigues de, Laura De Marco, Carlos F. Santos, Izabel Regina Fisher Rubira-Bullen, Guglielmo Ronco, Ilenia Pennini, Loredana Vizzini, Franco Merletti, and Anna Gillio-Tos. 2009. "GP5+/6+ SYBR Green Methodology for Simultaneous Screening and Quantification of Human Papillomavirus." *Journal of Clinical Virology: The Official Publication of the Pan American Society for Clinical Virology* 45 (2): 90–95. https://doi.org/10.1016/j.jcv.2009.03.020.

Badarni, Mai, Manu Prasad, Noa Balaban, Jonathan Zorea, Ksenia M. Yegodayev, Ben-Zion Joshua, Anat Bahat Dinur, et al. 2019. "Repression of AXL Expression by AP-1/JNK Blockage Overcomes Resistance to PI3Ka Therapy." *JCI Insight* 5 (March). https://doi.org/10.1172/jci.insight.125341.

Bais, M.V. 2019. "Impact of Epigenetic Regulation on Head and Neck Squamous Cell Carcinoma." *Journal of Dental Research* 98 (3): 268–76. https://doi.org/10.1177/0022034518816947.

Balderas-Loaeza, Adriana, Gabriela Anaya-Saavedra, Velia A. Ramirez-Amador, Miriam C. Guido-Jimenez, Mina Kalantari, Itzel E. Calleja-Macias, Hans-Ulrich Bernard, and Alejandro Garcia-Carranca. 2007. "Human Papillomavirus-16 DNA Methylation Patterns Support a Causal Association of the Virus with Oral Squamous Cell Carcinomas." *International Journal of Cancer* 120 (10): 2165–69. https://doi.org/10.1002/ijc.22563.

Betiol, J., L.L. Villa, and L. Sichero. 2013. “Impact of HPV Infection on the Development of Head and Neck Cancer.” *Brazilian Journal of Medical and Biological Research* 46 (3): 217–26. https://doi.org/10.1590/1414-431X20132703.

Biktasova, Asel, Michael Hajek, Andrew Sewell, Cyril Gary, Gary Bellinger, Hari A. Deshpande, Aarti Bhatia, et al. 2017. “Demethylation Therapy as a Targeted Treatment for Human Papillomavirus-Associated Head and Neck Cancer.” *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 23 (23): 7276–87. https://doi.org/10.1158/1078-0432.CCR-17-1438.

Bock, C., S. Reither, T. Mikeska, M. Paulsen, J. Walter, and T. Lengauer. 2005. “BiQ Analyzer: Visualization and Quality Control for DNA Methylation Data from Bisulfite Sequencing.” *Bioinformatics* 21 (21): 4067–68. https://doi.org/10.1093/bioinformatics/bti652.

Bononi, Ilaria, Elisa Mazzoni, Silvia Pietrobon, Marco Manfrini, Elena Torreggiani, Marika Rossini, Francesca Lotito, et al. 2018. “Serum IgG Antibodies from Healthy Subjects up to 100 Years Old React to JC Polyomavirus.” *Journal of Cellular Physiology* 233 (8): 5513–22. https://doi.org/10.1002/jcp.26457.

Boscolo-Rizzo, P., A. Del Mistro, F. Bussu, Valentina Lupato, Lorena Baboci, G. ALMADORI, M.C. DA MOSTO, and G. PALUDETTI. 2013. “New Insights into Human Papillomavirus-Associated Head and Neck Squamous Cell Carcinoma.” *Acta Otorhinolaryngologica Italica* 33 (2): 77–87.

Boscolo-Rizzo, Paolo, Carlo Furlan, Valentina Lupato, Jerry Polesel, and Elisabetta Fratta. 2017. “Novel Insights into Epigenetic Drivers of Oropharyngeal Squamous Cell Carcinoma: Role of HPV and Lifestyle Factors.” *Clinical Epigenetics* 9: 124. https://doi.org/10.1186/s13148-017-0424-5.

Botti, Elisabetta, Giulia Spallone, Francesca Moretti, Barbara Marinari, Valentina Pinetti, Sergio Galanti, Paolo D’Onorio De Meo, et al. 2011. “Developmental Factor IRF6 Exhibits Tumor Suppressor Activity in Squamous Cell Carcinomas.” *Proceedings of the National Academy of Sciences of the United States of America* 108 (33): 13710–15. https://doi.org/10.1073/pnas.1110931108.

Bruni, L, G Albero, B Serrano, M Mena, D Gómez, J Muñoz, F. Xavier Bosch, and Silvia de Sanjose. 2019. “Human Papillomavirus and Related Diseases in Europe. Summary Report 17 June 2019.” *ICO/IARC Information Centre on HPV and Cancer (HPV Information Centre).*, June.

Burris, Thomas Patrick, and Edward R. B McCabe. 2001. *Nuclear Receptors and Genetic Disease*. Vol. Retinoid Receptors. San Diego, Calif.: Academic Press. http://catalog.hathitrust.org/api/volumes/oclc/44852083.html.

Canning, Madison, Gang Guo, Miao Yu, Calvin Myint, Michael W. Groves, James Kenneth Byrd, and Yan Cui. 2019. “Heterogeneity of the Head and Neck Squamous Cell

Carcinoma Immune Landscape and Its Impact on Immunotherapy." *Frontiers in Cell and Developmental Biology* 7. https://doi.org/10.3389/fcell.2019.00052.

Cassell, Andre, and Jennifer R. Grandis. 2010. "Investigational EGFR-Targeted Therapies in HNSCC." *Expert Opinion on Investigational Drugs* 19 (6): 709–22. https://doi.org/10.1517/13543781003769844.

Castellsagué, Xavier, Laia Alemany, Miquel Quer, Gordana Halec, Beatriz Quirós, Sara Tous, Omar Clavero, et al. 2016. "HPV Involvement in Head and Neck Cancers: Comprehensive Assessment of Biomarkers in 3680 Patients." *Journal of the National Cancer Institute* 108 (6): djv403. https://doi.org/10.1093/jnci/djv403.

Chaturvedi, Anil K., Barry I. Graubard, Tatevik Broutian, Robert K. L. Pickard, Zhen-Yue Tong, Weihong Xiao, Lisa Kahle, and Maura L. Gillison. 2015. "NHANES 2009-2012 Findings: Association of Sexual Behaviors with Higher Prevalence of Oral Oncogenic Human Papillomavirus Infections in U.S. Men." *Cancer Research* 75 (12): 2468–77. https://doi.org/10.1158/0008-5472.CAN-14-2843.

———. 2018. "Effect of Prophylactic Human Papillomavirus (HPV) Vaccination on Oral HPV Infections Among Young Adults in the United States." *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 36 (3): 262–67. https://doi.org/10.1200/JCO.2017.75.0141.

Chen, James J, Tzu-Pin Lu, Yu-Chuan Chen, and Wei-Jiun Lin. 2015. "Predictive Biomarkers for Treatment Selection: Statistical Considerations." *Biomarkers in Medicine* 9 (11): 1121–35. https://doi.org/10.2217/bmm.15.84.

Chen, Zhong, Justin L. Ricker, Pramit S. Malhotra, Liesl Nottingham, Lorena Bagain, Tin Lap Lee, Ning T Yeh, and Carter Van Waes. 2008. "Differential Bortezomib Sensitivity in Head and Neck Cancer Lines Corresponds to Proteasome, NF-KB and AP-1 Related Mechanisms." *Molecular Cancer Therapeutics* 7 (7): 1949–60. https://doi.org/10.1158/1535-7163.MCT-07-2046.

Chong, T, D Apt, B Gloss, M Isa, and H U Bernard. 1991. "The Enhancer of Human Papillomavirus Type 16: Binding Sites for the Ubiquitous Transcription Factors Oct-1, NFA, TEF-2, NF1, and AP-1 Participate in Epithelial Cell-Specific Transcription." *Journal of Virology* 65 (11): 5933–43. https://doi.org/10.1128/JVI.65.11.5933-5943.1991.

Clifford, Gary M., Vanessa Tenet, Damien Georges, Laia Alemany, Miquel Angel Pavón, Zigui Chen, Meredith Yeager, et al. 2019. "Human Papillomavirus 16 Sub-Lineage Dispersal and Cervical Cancer Risk Worldwide: Whole Viral Genome Sequences from 7116 HPV16-Positive Women." *Papillomavirus Research* 7 (June): 67–74. https://doi.org/10.1016/j.pvr.2019.02.001.

Cohen, Ezra E. W., R. Bryan Bell, Carlo B. Bifulco, Barbara Burtness, Maura L. Gillison, Kevin J. Harrington, Quynh-Thu Le, et al. 2019. "The Society for Immunotherapy of

Cancer Consensus Statement on Immunotherapy for the Treatment of Squamous Cell Carcinoma of the Head and Neck (HNSCC)." *Journal for Immunotherapy of Cancer* 7 (1): 184. https://doi.org/10.1186/s40425-019-0662-5.

Combes, Jean-Damien, and Silvia Franceschi. 2018. "Human Papillomavirus Genome Variants and Head and Neck Cancers: A Perspective." *Infectious Agents and Cancer* 13: 13. https://doi.org/10.1186/s13027-018-0185-6.

Dahlstrom, Kristina R., Karen S. Anderson, Julia N. Cheng, Diego Chowell, Guojun Li, Marshall Posner, and Erich M. Sturgis. 2015. "HPV Serum Antibodies as Predictors of Survival and Disease Progression in Patients with HPV-Positive Squamous Cell Carcinoma of the Oropharynx." *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 21 (12): 2861–69. https://doi.org/10.1158/1078-0432.CCR-14-3323.

Dahlstrom, Kristina R., Guojun Li, Caroline S. Hussey, Jenny T. Vo, Qingyi Wei, Chong Zhao, and Erich M. Sturgis. 2015. "Circulating Human Papillomavirus DNA as a Marker for Disease Extent and Recurrence among Patients with Oropharyngeal Cancer." *Cancer* 121 (19): 3455–64. https://doi.org/10.1002/cncr.29538.

Dai, Shuying, Chuanyin Li, Zhiling Yan, Ziyun Zhou, Xia Wang, Jun Wang, Le Sun, Li Shi, and Yufeng Yao. 2020. "Association of Human Papillomavirus Type 16 Long Control Region Variations with Cervical Cancer in a Han Chinese Population." *International Journal of Medical Sciences* 17 (7): 931–38. https://doi.org/10.7150/ijms.43030.

Das, Poulami, Asha Thomas, Sadhana Kannan, Kedar Deodhar, Shyam K. Shrivastava, Umesh Mahantshetty, and Rita Mulherkar. 2015. "Human Papillomavirus (HPV) Genome Status & Cervical Cancer Outcome--A Retrospective Study." *The Indian Journal of Medical Research* 142 (5): 525–32. https://doi.org/10.4103/0971-5916.171276.

Durzynska, Julia, Krzysztof Lesniewicz, and Elzbieta Poreba. 2017. "Human Papillomaviruses in Epigenetic Regulations." *Mutation Research. Reviews in Mutation Research* 772 (June): 36–50. https://doi.org/10.1016/j.mrrev.2016.09.006.

Dwedar, Reham Ali, Noha Mohamed Omar, Somaia Abdel-Latif Eissa, Abdelrahman Younes Aly Badawy, Dalia Abd El-Kareem, and Lamiaa Abd El-Fattah Ahmed Madkour. 2020. "Diagnostic and Prognostic Impact of E6/E7 MRNA Compared to HPV DNA and P16 Expression in Head and Neck Cancers: An Egyptian Study." *Alexandria Journal of Medicine* 56 (1): 155–65. https://doi.org/10.1080/20905068.2020.1827944.

Economopoulou, Panagiota, Remco de Bree, Ioannis Kotsantis, and Amanda Psyrri. 2019. "Diagnostic Tumor Markers in Head and Neck Squamous Cell Carcinoma (HNSCC) in the Clinical Setting." *Frontiers in Oncology* 9: 827. https://doi.org/10.3389/fonc.2019.00827.

Economopoulou, Panagiota, George Koutsodontis, Margaritis Avgeris, Areti Strati, Christos Kroupis, Ioannis Pateras, Euthymios Kirodimos, et al. 2019. “HPV16 E6/E7 Expression in Circulating Tumor Cells in Oropharyngeal Squamous Cell Cancers: A Pilot Study.” *PloS One* 14 (5): e0215984. https://doi.org/10.1371/journal.pone.0215984.

Egawa, Nagayasu, Kiyofumi Egawa, Heather Griffin, and John Doorbar. 2015. “Human Papillomaviruses; Epithelial Tropisms, and the Development of Neoplasia.” *Viruses* 7 (7): 3863–90. https://doi.org/10.3390/v7072802.

Ekanayake Weeramange, Chameera, Kai Dun Tang, Sarju Vasani, Julian Langton-Lockton, Liz Kenny, and Chamindie Punyadeera. 2020. “DNA Methylation Changes in Human Papillomavirus-Driven Head and Neck Cancers.” *Cells* 9 (6). https://doi.org/10.3390/cells9061359.

Escobar-Escamilla, Noé, Blanca Estela González-Martínez, Adnan Araiza-Rodríguez, David Esaú Fragoso-Fonseca, Abraham Pedroza-Torres, Magaly Guadalupe Landa-Flores, Fabiola Garcés-Ayala, et al. 2019. “Mutational Landscape and Intra-Host Diversity of Human Papillomavirus Type 16 Long Control Region and E6 Variants in Cervical Samples.” *Archives of Virology* 164 (12): 2953–61. https://doi.org/10.1007/s00705-019-04407-6.

Fakhry, Carole, Jesse R. Qualliotine, Zhe Zhang, Nishant Agrawal, Daria A. Gaykalova, Justin A. Bishop, Rathan M. Subramaniam, et al. 2016. “Serum Antibodies to HPV16 Early Proteins Warrant Investigation as Potential Biomarkers for Risk Stratification and Recurrence of HPV-Associated Oropharyngeal Cancer.” *Cancer Prevention Research (Philadelphia, Pa.)* 9 (2): 135–41. https://doi.org/10.1158/1940-6207.CAPR-15-0299.

Fang, Liyuan, Xiaoli Lin, Yasi Yang, Zhilin Song, Xianping Ding, Liping Tan, and Peng Gao. 2020. “Genetic Variability, Phylogeny and Functional Implication of the Long Control Region in Human Papillomavirus Type 16, 18 and 58 in Chengdu, China.” *Virology Journal* 17 (1): 106. https://doi.org/10.1186/s12985-020-01349-3.

Farré, Domènec, Romà Roset, Mario Huerta, José E. Adsuara, Llorenç Roselló, M. Mar Albà, and Xavier Messeguer. 2003. “Identification of Patterns in Biological Sequences at the ALGGEN Server: PROMO and MALGEN.” *Nucleic Acids Research* 31 (13): 3651–53. https://doi.org/10.1093/nar/gkg605.

Forslund, Ola, K. Miriam Elfström, Helena Lamin, and Joakim Dillner. 2019. “HPV-MRNA and HPV-DNA Detection in Samples Taken up to Seven Years before Severe Dysplasia of Cervix Uteri.” *International Journal of Cancer* 144 (5): 1073–81. https://doi.org/10.1002/ijc.31819.

Ganly, Ian, Simon Talbot, Diane Carlson, Agnes Viale, Ellie Maghami, Iman Osman, Eric Sherman, et al. 2007. “Identification of Angiogenesis/Metastases Genes Predicting Chemoradiotherapy Response in Patients With Laryngopharyngeal Carcinoma.” *Journal of Clinical Oncology* 25 (11): 1369–76. https://doi.org/10.1200/JCO.2005.05.3397.

Ghosh, Damayanti Das, Bornali Bhattacharjee, Shrinka Sen, Laikangbam Premi, Indranil Mukhopadhyay, Rahul Roy Chowdhury, Sudipta Roy, and Sharmila Sengupta. 2012. "Some Novel Insights on HPV16 Related Cervical Cancer Pathogenesis Based on Analyses of LCR Methylation, Viral Load, E7 and E2/E4 Expressions." *PLOS ONE* 7 (9): e44678. https://doi.org/10.1371/journal.pone.0044678.

Gillison, Maura L., Anil K. Chaturvedi, William F. Anderson, and Carole Fakhry. 2015. "Epidemiology of Human Papillomavirus-Positive Head and Neck Squamous Cell Carcinoma." *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 33 (29): 3235–42. https://doi.org/10.1200/JCO.2015.61.6995.

Gkouveris, Ioannis, Nikolaos Nikitakis, Maria Karanikou, George Rassidakis, and Alexandra Sklavounou. 2016. "JNK1/2 Expression and Modulation of STAT3 Signaling in Oral Cancer." *Oncology Letters* 12 (1): 699–706. https://doi.org/10.3892/ol.2016.4614.

Graham, Sheila V. 2010. "Human Papillomavirus: Gene Expression, Regulation and Prospects for Novel Diagnostic Methods and Antiviral Therapies." *Future Microbiology* 5 (10): 1493–1506. https://doi.org/10.2217/fmb.10.107.

Hargreaves, Sarah, Matthew Beasley, Chris Hurt, Terry M. Jones, and Mererid Evans. 2019. "Deintensification of Adjuvant Treatment After Transoral Surgery in Patients With Human Papillomavirus-Positive Oropharyngeal Cancer: The Conception of the PATHOS Study and Its Development." *Frontiers in Oncology* 9 (October): 936. https://doi.org/10.3389/fonc.2019.00936.

Hashmi, Atif A., Naila Younus, Samreen Naz, Muhammad Irfan, Zubaida Hussain, Sara T. Shaikh, Javaria Ali, et al. 2020. "P16 Immunohistochemical Expression in Head and Neck Squamous Cell Carcinoma: Association With Prognostic Parameters." *Cureus* 12 (6): e8601. https://doi.org/10.7759/cureus.8601.

Henley-Smith, Rhonda, Alice Santambrogio, Cynthia L. Andoniadou, Edward Odell, and Selvam Thavaraj. 2021. "RNA in Situ Hybridization for Human Papillomavirus Testing in Oropharyngeal Squamous Cell Carcinoma on a Routine Clinical Diagnostic Platform." *Journal of Oral Pathology & Medicine: Official Publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology* 50 (1): 68–75. https://doi.org/10.1111/jop.13103.

Hildesheim, A., M. Schiffman, C. Bromley, S. Wacholder, R. Herrero, A. C. Rodriguez, M. C. Bratti, et al. 2001. "Human Papillomavirus Type 16 Variants and Risk of Cervical Cancer." *JNCI Journal of the National Cancer Institute* 93 (4): 315–18. https://doi.org/10.1093/jnci/93.4.315.

Holzinger, Dana, Gunnar Wichmann, Lorena Baboci, Angelika Michel, Daniela Höfler, Manuel Wiesenfarth, Lea Schroeder, et al. 2017. "Sensitivity and Specificity of Antibodies against HPV16 E6 and Other Early Proteins for the Detection of HPV16-driven Oropharyngeal Squamous Cell Carcinoma." *International Journal of Cancer* 140 (12): 2748–57. https://doi.org/10.1002/ijc.30697.

International Agency for Research on Cancer, ed. 2007. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Volume 90, Human Papillomaviruses: This Publication Represents the Views and Expert Opinions of an IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, Which Met in Lyon, 15 - 22 February 2005*. Lyon: IARC.

Johnson, Daniel E., Barbara Burtness, C. René Leemans, Vivian Wai Yan Lui, Julie E. Bauman, and Jennifer R. Grandis. 2020. "Head and Neck Squamous Cell Carcinoma." *Nature Reviews Disease Primers* 6 (1): 1–22. https://doi.org/10.1038/s41572-020-00224-3.

Kalantari, Mina, Itzel E. Calleja-Macias, Devansu Tewari, Bjørn Hagmar, Kathrine Lie, Hugo A. Barrera-Saldana, Dorothy J. Wiley, and Hans-Ulrich Bernard. 2004. "Conserved Methylation Patterns of Human Papillomavirus Type 16 DNA in Asymptomatic Infection and Cervical Neoplasia." *Journal of Virology* 78 (23): 12762–72. https://doi.org/10.1128/JVI.78.23.12762-12772.2004.

Kalantari, Mina, Denis Lee, Itzel E. Calleja-Macias, Paul F. Lambert, and Hans-Ulrich Bernard. 2008. "Effects of Cellular Differentiation, Chromosomal Integration and 5-Aza-2′-Deoxycytidine Treatment on Human Papillomavirus-16 DNA Methylation in Cultured Cell Lines." *Virology* 374 (2): 292–303. https://doi.org/10.1016/j.virol.2007.12.016.

Khanal, Sujita, Brian S. Shumway, Maryam Zahin, Rebecca A. Redman, John D. Strickley, Patrick J. Trainor, Shesh N. Rai, Shin-Je Ghim, Alfred Bennett Jenson, and Joongho Joh. 2018. "Viral DNA Integration and Methylation of Human Papillomavirus Type 16 in High-Grade Oral Epithelial Dysplasia and Head and Neck Squamous Cell Carcinoma." *Oncotarget* 9 (54): 30419–33. https://doi.org/10.18632/oncotarget.25754.

Kitamura, Koji, Keisuke Nimura, Rie Ito, Kotaro Saga, Hidenori Inohara, and Yasufumi Kaneda. 2020. "Evaluation of HPV16 E7 Expression in Head and Neck Carcinoma Cell Lines and Clinical Specimens." *Scientific Reports* 10 (1): 22138. https://doi.org/10.1038/s41598-020-78345-8.

Klussmann, Jens Peter. 2017. "Head and Neck Cancer - New Insights into a Heterogeneous Disease." *Oncology Research and Treatment* 40 (6): 318–19. https://doi.org/10.1159/000477255.

Knebel Doeberitz, Magnus von, and Elena-Sophie Prigge. 2019. "Role of DNA Methylation in HPV Associated Lesions." *Papillomavirus Research (Amsterdam, Netherlands)* 7 (June): 180–83. https://doi.org/10.1016/j.pvr.2019.03.005.

Kolomeichuk, Sergey N., David T. Terrano, Christopher S. Lyle, Kanaga Sabapathy, and Timothy C. Chambers. 2008. "Distinct Signaling Pathways of Microtubule Inhibitors - Vinblastine and Taxol Induce JNK-Dependent Cell Death but through AP-1-Dependent and AP-1-Independent Mechanisms, Respectively: Taxol and Vinblastine Signal Different Death Pathways." *FEBS Journal* 275 (8): 1889–99. https://doi.org/10.1111/j.1742-4658.2008.06349.x.

Kong, Linghua, Xiaoping Xiao, Huiping Lou, Pengfei Liu, Shuhui Song, Moudu Liu, Tao Xu, et al. 2020. “Analysis of the Role of the Human Papillomavirus 16/18 E7 Protein Assay in Screening for Cervical Intraepithelial Neoplasia: A Case Control Study.” *BMC Cancer* 20 (1): 999. https://doi.org/10.1186/s12885-020-07483-z.

Kreimer, A.R., A. Ferreiro-Iglesias, M. Nygard, N. Bender, L. Schroeder, A. Hildesheim, H.A. Robbins, et al. 2019. “Timing of HPV16-E6 Antibody Seroconversion before OPSCC: Findings from the HPVC3 Consortium.” *Annals of Oncology* 30 (8): 1335–43. https://doi.org/10.1093/annonc/mdz138.

Kriegs, Malte, Till Sebastian Clauditz, Konstantin Hoffer, Joanna Bartels, Sophia Buhs, Helwe Gerull, Henrike Barbara Zech, et al. 2019. “Analyzing Expression and Phosphorylation of the EGF Receptor in HNSCC.” *Scientific Reports* 9 (1): 13564. https://doi.org/10.1038/s41598-019-49885-5.

Kurita, Takashi, Shun-Ichi Chitose, Kiminori Sato, Tomo Sakazaki, Mioko Fukahori, Shintaro Sueyoshi, and Hirohito Umeno. 2019. “Pathological Mechanisms of Laryngeal Papillomatosis Based on Laryngeal Epithelial Characteristics.” *Laryngoscope Investigative Otolaryngology* 4 (1): 89–94. https://doi.org/10.1002/lio2.242.

Kurvinen, K., M. Yliskoski, S. Saarikoski, K. Syrjänen, and S. Syrjänen. 2000. “Variants of the Long Control Region of Human Papillomavirus Type 16.” *European Journal of Cancer* 36 (11): 1402–10. https://doi.org/10.1016/S0959-8049(00)00121-0.

Kwon, Eun Jung, Mihyang Ha, Jeon Yeob Jang, and Yun Hak Kim. 2021. “Identification and Complete Validation of Prognostic Gene Signatures for Human Papillomavirus-Associated Cancers: Integrated Approach Covering Different Anatomical Locations.” *Journal of Virology* 95 (6). https://doi.org/10.1128/JVI.02354-20.

Lai, Zi-Lun, Yung-An Tsou, Shin-Ru Fan, Ming-Hsui Tsai, Hsiao-Ling Chen, Nai-Wen Chang, Ju-Chien Cheng, and Chuan-Mu Chen. 2014. “Methylation-Associated Gene Silencing of RARB in Areca Carcinogens Induced Mouse Oral Squamous Cell Carcinoma.” Research Article. BioMed Research International. Hindawi. August 17, 2014. https://doi.org/10.1155/2014/378358.

Lang Kuhs, Krystle A., Aimée R. Kreimer, Sumita Trivedi, Dana Holzinger, Michael Pawlita, Ruth M. Pfeiffer, Sandra P. Gibson, et al. 2017. “Human Papillomavirus 16 E6 Antibodies Are Sensitive for Human Papillomavirus-Driven Oropharyngeal Cancer and Are Associated with Recurrence.” *Cancer* 123 (22): 4382–90. https://doi.org/10.1002/cncr.30966.

Lechner, Matt, Ankur R. Chakravarthy, Vonn Walter, Liam Masterson, Andrew Feber, Amrita Jay, Paul M. Weinberger, et al. 2018. “Frequent HPV-Independent P16/INK4A Overexpression in Head and Neck Cancer.” *Oral Oncology* 83: 32–37. https://doi.org/10.1016/j.oraloncology.2018.06.006.

Lee, S. J., Y. S. Cho, M. C. Cho, J. H. Shim, K. A. Lee, K. K. Ko, Y. K. Choe, et al. 2001. "Both E6 and E7 Oncoproteins of Human Papillomavirus 16 Inhibit IL-18-Induced IFN-Gamma Production in Human Peripheral Blood Mononuclear and NK Cells." *Journal of Immunology (Baltimore, Md.: 1950)* 167 (1): 497–504. https://doi.org/10.4049/jimmunol.167.1.497.

Leemans, C. René, Peter J. F. Snijders, and Ruud H. Brakenhoff. 2018. "The Molecular Landscape of Head and Neck Cancer." *Nature Reviews Cancer* 18 (10): 662–662. https://doi.org/10.1038/s41568-018-0057-9.

Lewis, James S. 2017. "Morphologic Diversity in Human Papillomavirus-Related Oropharyngeal Squamous Cell Carcinoma: Catch Me If You Can!" *Modern Pathology* 30 (1): S44–53. https://doi.org/10.1038/modpathol.2016.152.

Li, Dandan, Ping Cheng, Jingjie Wang, Xuemei Qiu, Xudong Zhang, Li Xu, Ying Liu, and Shanshan Qin. 2019. "IRF6 Is Directly Regulated by ZEB1 and ELF3, and Predicts a Favorable Prognosis in Gastric Cancer." *Frontiers in Oncology* 9: 220. https://doi.org/10.3389/fonc.2019.00220.

Lillsunde Larsson, Gabriella, Gisela Helenius, Bengt Sorbe, and Mats G. Karlsson. 2014. "Viral Load, Integration and Methylation of E2BS3 and 4 in Human Papilloma Virus (HPV) 16-Positive Vaginal and Vulvar Carcinomas." *PloS One* 9 (11): e112839. https://doi.org/10.1371/journal.pone.0112839.

Lin, Jiaying, Andreas E. Albers, Jinbao Qin, and Andreas M. Kaufmann. 2014. "Prognostic Significance of Overexpressed P16INK4a in Patients with Cervical Cancer: A Meta-Analysis." Edited by Zhi-Ming Zheng. *PLoS ONE* 9 (9): e106384. https://doi.org/10.1371/journal.pone.0106384.

Liu, Changxing, Daljit Mann, Uttam K. Sinha, and Niels C. Kokot. 2018. "The Molecular Mechanisms of Increased Radiosensitivity of HPV-Positive Oropharyngeal Squamous Cell Carcinoma (OPSCC): An Extensive Review." *Journal of Otolaryngology - Head & Neck Surgery = Le Journal D'oto-Rhino-Laryngologie Et De Chirurgie Cervico-Faciale* 47 (1): 59. https://doi.org/10.1186/s40463-018-0302-y.

Lubecka, Katarzyna, Agnieszka Kaufman-Szymczyk, Barbara Cebula-Obrzut, Piotr Smolewski, Janusz Szemraj, and Krystyna Fabianowska-Majewska. 2018. "Novel Clofarabine-Based Combinations with Polyphenols Epigenetically Reactivate Retinoic Acid Receptor Beta, Inhibit Cell Growth, and Induce Apoptosis of Breast Cancer Cells." *International Journal of Molecular Sciences* 19 (12): 3970. https://doi.org/10.3390/ijms19123970.

Lydiatt, William M., Snehal G. Patel, Brian O'Sullivan, Margaret S. Brandwein, John A. Ridge, Jocelyn C. Migliacci, Ashley M. Loomis, and Jatin P. Shah. 2017. "Head and Neck Cancers-Major Changes in the American Joint Committee on Cancer Eighth Edition Cancer Staging Manual." *CA: A Cancer Journal for Clinicians* 67 (2): 122–37. https://doi.org/10.3322/caac.21389.

Machczyński, Piotr, Ewa Majchrzak, Patryk Niewinski, Joanna Marchlewska, and Wojciech Golusiński. 2020. "A Review of the 8th Edition of the AJCC Staging System for Oropharyngeal Cancer According to HPV Status." *European Archives of Oto-Rhino-Laryngology* 277 (9): 2407–12. https://doi.org/10.1007/s00405-020-05979-9.

Malagutti, Nicola, John Charles Rotondo, Luca Cerritelli, Claudio Melchiorri, Monica De Mattei, Rita Selvatici, Lucia Oton-Gonzalez, et al. 2020. "High Human Papillomavirus DNA Loads in Inflammatory Middle Ear Diseases." *Pathogens (Basel, Switzerland)* 9 (3). https://doi.org/10.3390/pathogens9030224.

Marcoux, Sophie, Oanh NL Le, Chloé Langlois-Pelletier, Caroline Laverdière, Afshin Hatami, Philippe Robaey, and Christian M Beauséjour. 2013. "Expression of the Senescence Marker P16INK4a in Skin Biopsies of Acute Lymphoblastic Leukemia Survivors: A Pilot Study." *Radiation Oncology* 8 (1): 252. https://doi.org/10.1186/1748-717X-8-252.

Marongiu, Luigi, Anna Godi, John V. Parry, and Simon Beddows. 2014. "Human Papillomavirus 16, 18, 31 and 45 Viral Load, Integration and Methylation Status Stratified by Cervical Disease Stage." *BMC Cancer* 14 (May): 384. https://doi.org/10.1186/1471-2407-14-384.

Martone, Tiziana, Anna Gillio-Tos, Laura De Marco, Valentina Fiano, Milena Maule, Andrea Cavalot, Massimiliano Garzaro, Franco Merletti, and Giorgio Cortesina. 2007. "Association Between Hypermethylated Tumor and Paired Surgical Margins in Head and Neck Squamous Cell Carcinomas." *Clinical Cancer Research* 13 (17): 5089–94. https://doi.org/10.1158/1078-0432.CCR-07-0119.

Mazzoni, Elisa, Ilaria Bononi, Maria S. Benassi, Piero Picci, Elena Torreggiani, Marika Rossini, Andrea Simioli, et al. 2018. "Serum Antibodies Against Simian Virus 40 Large T Antigen, the Viral Oncoprotein, in Osteosarcoma Patients." *Frontiers in Cell and Developmental Biology* 6: 64. https://doi.org/10.3389/fcell.2018.00064.

Mazzoni, Elisa, Mariantonietta Di Stefano, Josè R. Fiore, Federica Destro, Marco Manfrini, John Charles Rotondo, Maria V. Casali, et al. 2017. "Serum IgG Antibodies from Pregnant Women Reacting to Mimotopes of Simian Virus 40 Large T Antigen, the Viral Oncoprotein." *Frontiers in Immunology* 8: 411. https://doi.org/10.3389/fimmu.2017.00411.

Mazzoni, Elisa, John C. Rotondo, Luisa Marracino, Rita Selvatici, Ilaria Bononi, Elena Torreggiani, Antoine Touzé, Fernanda Martini, and Mauro G. Tognon. 2017. "Detection of Merkel Cell Polyomavirus DNA in Serum Samples of Healthy Blood Donors." *Frontiers in Oncology* 7: 294. https://doi.org/10.3389/fonc.2017.00294.

McBride, Alison A. 2017. "Playing with Fire: Consequences of Human Papillomavirus DNA Replication Adjacent to Genetically Unstable Regions of Host Chromatin." *Current Opinion in Virology* 26 (October): 63–68. https://doi.org/10.1016/j.coviro.2017.07.015.

McBride, Alison A., and Alix Warburton. 2017. “The Role of Integration in Oncogenic Progression of HPV-Associated Cancers.” Edited by Katherine R. Spindler. *PLOS Pathogens* 13 (4): e1006211. https://doi.org/10.1371/journal.ppat.1006211.

McIlwain, Wesley R., Amit J. Sood, Shaun A. Nguyen, and Terry A. Day. 2014. “Initial Symptoms in Patients With HPV-Positive and HPV-Negative Oropharyngeal Cancer.” *JAMA Otolaryngology–Head & Neck Surgery* 140 (5): 441. https://doi.org/10.1001/jamaoto.2014.141.

Mehanna, Hisham, Max Robinson, Andrew Hartley, Anthony Kong, Bernadette Foran, Tessa Fulton-Lieuw, Matthew Dalby, et al. 2019. “Radiotherapy plus Cisplatin or Cetuximab in Low-Risk Human Papillomavirus-Positive Oropharyngeal Cancer (De-ESCALaTE HPV): An Open-Label Randomised Controlled Phase 3 Trial.” *The Lancet* 393 (10166): 51–60. https://doi.org/10.1016/S0140-6736(18)32752-1.

Messeguer, Xavier, Ruth Escudero, Domènec Farré, Oscar Núñez, Javier Martínez, and M. Mar Albà. 2002. “PROMO: Detection of Known Transcription Regulatory Elements Using Species-Tailored Searches.” *Bioinformatics (Oxford, England)* 18 (2): 333–34. https://doi.org/10.1093/bioinformatics/18.2.333.

Milutin Gašperov, Nina, Ivan Sabol, Pavao Planinić, Goran Grubišić, Ivan Fistonić, Ante Ćorušić, and Magdalena Grce. 2015. “Methylated Host Cell Gene Promoters and Human Papillomavirus Type 16 and 18 Predicting Cervical Lesions and Cancer.” *PloS One* 10 (6): e0129452. https://doi.org/10.1371/journal.pone.0129452.

Mirghani, Haitham, and Pierre Blanchard. 2018. “Treatment De-Escalation for HPV-Driven Oropharyngeal Cancer: Where Do We Stand?” *Clinical and Translational Radiation Oncology* 8 (January): 4–11. https://doi.org/10.1016/j.ctro.2017.10.005.

Moody, Cary. 2017. “Mechanisms by Which HPV Induces a Replication Competent Environment in Differentiating Keratinocytes.” *Viruses* 9 (9): 261. https://doi.org/10.3390/v9090261.

Moody, Cary A., and Laimonis A. Laimins. 2010a. “Human Papillomavirus Oncoproteins: Pathways to Transformation.” *Nature Reviews. Cancer* 10 (8): 550–60. https://doi.org/10.1038/nrc2886.

———. 2010b. “Human Papillomavirus Oncoproteins: Pathways to Transformation.” *Nature Reviews. Cancer* 10 (8): 550–60. https://doi.org/10.1038/nrc2886.

Moretti, Francesca, Barbara Marinari, Nadia Lo Iacono, Elisabetta Botti, Alessandro Giunta, Giulia Spallone, Giulia Garaffo, et al. 2010. “A Regulatory Feedback Loop Involving P63 and IRF6 Links the Pathogenesis of 2 Genetically Different Human Ectodermal Dysplasias.” *The Journal of Clinical Investigation* 120 (5): 1570–77. https://doi.org/10.1172/JCI40267.

Mosmann, Jessica P., Marina S. Monetti, Maria C. Frutos, Ana X. Kiguen, Raul F. Venezuela, and Cecilia G. Cuffini. 2015. “Mutation Detection of E6 and LCR Genes from HPV 16 Associated with Carcinogenesis.” *Asian Pacific Journal of Cancer Prevention: APJCP* 16 (3): 1151–57. https://doi.org/10.7314/apjcp.2015.16.3.1151.

Näsman, A., J. Du, and T. Dalianis. 2020. “A Global Epidemic Increase of an HPV-Induced Tonsil and Tongue Base Cancer – Potential Benefit from a Pan-Gender Use of HPV Vaccine.” *Journal of Internal Medicine* 287 (2): 134–52. https://doi.org/10.1111/joim.13010.

Nobeyama, Yoshimasa, and Hidemi Nakagawa. 2017. “Silencing of Interferon Regulatory Factor Gene 6 in Melanoma.” *PLOS ONE* 12 (9): e0184444. https://doi.org/10.1371/journal.pone.0184444.

O’Connor, M. J., S. H. Tan, C. H. Tan, and H. U. Bernard. 1996. “YY1 Represses Human Papillomavirus Type 16 Transcription by Quenching AP-1 Activity.” *Journal of Virology* 70 (10): 6529–39. https://doi.org/10.1128/JVI.70.10.6529-6539.1996.

Olthof, Nadine C., Ernst-Jan M. Speel, Jutta Kolligs, Annick Haesevoets, Mieke Henfling, Frans C. S. Ramaekers, Simon F. Preuss, et al. 2014. “Comprehensive Analysis of HPV16 Integration in OSCC Reveals No Significant Impact of Physical Status on Viral Oncogene and Virally Disrupted Human Gene Expression.” *PLoS ONE* 9 (2). https://doi.org/10.1371/journal.pone.0088718.

Ongkeko, Weg M., Yi An, Theresa S. Chu, Joseph Aguilera, Christopher L. Dang, and Jessica Wang-Rodriguez. 2006. “Gleevec Suppresses P63 Expression in Head and Neck Squamous Cell Carcinoma Despite P63 Activation by DNA-Damaging Agents:” *The Laryngoscope* 116 (8): 1390–96. https://doi.org/10.1097/01.mlg.0000225941.60901.0f.

Oton-Gonzalez, Lucia, John Charles Rotondo, Luca Cerritelli, Nicola Malagutti, Carmen Lanzillotti, Ilaria Bononi, Andrea Ciorba, et al. 2021. “Association between Oncogenic Human Papillomavirus Type 16 and Killian Polyp.” *Infectious Agents and Cancer* 16 (1): 3. https://doi.org/10.1186/s13027-020-00342-3.

Patil, Vibha, Robyn L Ward, and Luke B Hesson. 2014. “The Evidence for Functional Non-CpG Methylation in Mammalian Cells.” *Epigenetics* 9 (6): 823–28. https://doi.org/10.4161/epi.28741.

Peitsaro, P., B. Johansson, and S. Syrjanen. 2002. “Integrated Human Papillomavirus Type 16 Is Frequently Found in Cervical Cancer Precursors as Demonstrated by a Novel Quantitative Real-Time PCR Technique.” *Journal of Clinical Microbiology* 40 (3): 886–91. https://doi.org/10.1128/JCM.40.3.886-891.2002.

Pett, M. R., M. T. Herdman, R. D. Palmer, G. S. H. Yeo, M. K. Shivji, M. A. Stanley, and N. Coleman. 2006. “Selection of Cervical Keratinocytes Containing Integrated HPV16 Associates with Episome Loss and an Endogenous Antiviral Response.”

Proceedings of the National Academy of Sciences 103 (10): 3822–27. https://doi.org/10.1073/pnas.0600078103.

Pierce Campbell, Christine M., Raphael P. Viscidi, B. Nelson Torres, Hui-Yi Lin, William Fulp, Martha Abrahamsen, Eduardo Lazcano-Ponce, Luisa L. Villa, Aimée R. Kreimer, and Anna R. Giuliano. 2016. "Human Papillomavirus (HPV) L1 Serum Antibodies and the Risk of Subsequent Oral HPV Acquisition in Men: The HIM Study." *Journal of Infectious Diseases* 214 (1): 45–48. https://doi.org/10.1093/infdis/jiw083.

Pietrobon, Silvia, Ilaria Bononi, Francesca Lotito, Paolo Perri, Sara Violanti, Elisa Mazzoni, Fernanda Martini, and Mauro G. Tognon. 2017. "Specific Detection of Serum Antibodies against BKPyV, A Small DNA Tumour Virus, in Patients Affected by Choroidal Nevi." *Frontiers in Microbiology* 8: 2059. https://doi.org/10.3389/fmicb.2017.02059.

Pinatti, Lisa M., Hana N. Sinha, Collin V. Brummel, Christine M. Goudsmit, Timothy J. Geddes, George D. Wilson, Jan A. Akervall, Chad J. Brenner, Heather M. Walline, and Thomas E. Carey. 2021. "Association of Human Papillomavirus Integration with Better Patient Outcomes in Oropharyngeal Squamous Cell Carcinoma." *Head & Neck* 43 (2): 544–57. https://doi.org/10.1002/hed.26501.

Piontek, Till, Christoph Harmel, Michael Pawlita, Katrin Carow, Juliane Schröter, Ingo B. Runnebaum, Matthias Dürst, Frederik Graw, and Tim Waterboer. 2019. "Post-Treatment Human Papillomavirus Antibody Kinetics in Cervical Cancer Patients." *Philosophical Transactions of the Royal Society B: Biological Sciences* 374 (1773): 20180295. https://doi.org/10.1098/rstb.2018.0295.

Preti, Mario, John Charles Rotondo, Dana Holzinger, Leonardo Micheletti, Niccolò Gallio, Sandrine McKay-Chopin, Christine Carreira, et al. 2020. "Role of Human Papillomavirus Infection in the Etiology of Vulvar Cancer in Italian Women." *Infectious Agents and Cancer* 15: 20. https://doi.org/10.1186/s13027-020-00286-8.

Psyrri, Amanda, Tanguy Y. Seiwert, and Antonio Jimeno. 2013. "Molecular Pathways in Head and Neck Cancer: EGFR, PI3K, and More." *American Society of Clinical Oncology Educational Book*, no. 33 (May): 246–55. https://doi.org/10.14694/EdBook_AM.2013.33.246.

Punyadeera, Chamindie, and Paul D. Slowey. 2019. "Saliva as an Emerging Biofluid for Clinical Diagnosis and Applications of MEMS/NEMS in Salivary Diagnostics." In *Nanobiomaterials in Clinical Dentistry*, 543–65. Elsevier. https://doi.org/10.1016/B978-0-12-815886-9.00022-X.

Pytynia, Kristen B., Kristina R. Dahlstrom, and Erich M. Sturgis. 2014. "Epidemiology of HPV-Associated Oropharyngeal Cancer." *Oral Oncology* 50 (5): 380–86. https://doi.org/10.1016/j.oraloncology.2013.12.019.

Qian, Xu, Duc T. Nguyen, Yue Dong, Branko Sinikovic, Andreas M. Kaufmann, Jeffrey N. Myers, Andreas E. Albers, and Edward A. Graviss. 2019. “Prognostic Score Predicts Survival in HPV-Negative Head and Neck Squamous Cell Cancer Patients.” *International Journal of Biological Sciences* 15 (7): 1336–44. https://doi.org/10.7150/ijbs.33329.

Rajagopal, Chitra, and K. B. Harikumar. 2018. “The Origin and Functions of Exosomes in Cancer.” *Frontiers in Oncology* 8: 66. https://doi.org/10.3389/fonc.2018.00066.

Reuschenbach, Miriam, Ingeborg Tinhofer, Claus Wittekindt, Steffen Wagner, and Jens Peter Klussmann. 2019. “A Systematic Review of the HPV-attributable Fraction of Oropharyngeal Squamous Cell Carcinomas in Germany.” *Cancer Medicine* 8 (4): 1908–18. https://doi.org/10.1002/cam4.2039.

Reyes-Gibby, Cielito C., Karen O. Anderson, Kelly W. Merriman, Knox H. Todd, Sanjay S. Shete, and Ehab Y. Hanna. 2014. “Survival Patterns in Squamous Cell Carcinoma of the Head and Neck: Pain as an Independent Prognostic Factor for Survival.” *The Journal of Pain* 15 (10): 1015–22. https://doi.org/10.1016/j.jpain.2014.07.003.

Ribeiro, Aline Lopes, Amanda Schiersner Caodaglio, and Laura Sichero. 2018a. “Regulation of HPV Transcription.” *Clinics* 73 (Suppl 1). https://doi.org/10.6061/clinics/2018/e486s.

———. 2018b. “Regulation of HPV Transcription.” *Clinics (Sao Paulo, Brazil)* 73 (suppl 1): e486s. https://doi.org/10.6061/clinics/2018/e486s.

Rizzo, Roberta, Silvia Pietrobon, Elisa Mazzoni, Daria Bortolotti, Fernanda Martini, Massimiliano Castellazzi, Ilaria Casetta, et al. 2016. “Serum IgG against Simian Virus 40 Antigens Are Hampered by High Levels of SHLA-G in Patients Affected by Inflammatory Neurological Diseases, as Multiple Sclerosis.” *Journal of Translational Medicine* 14 (1): 216. https://doi.org/10.1186/s12967-016-0981-y.

Roberts, Sally, Dhananjay Evans, Hisham Mehanna, and Joanna L. Parish. 2019. “Modelling Human Papillomavirus Biology in Oropharyngeal Keratinocytes.” *Philosophical Transactions of the Royal Society B: Biological Sciences* 374 (1773): 20180289. https://doi.org/10.1098/rstb.2018.0289.

Rocco, James W., Chee-Onn Leong, Nicolas Kuperwasser, Maurice Phillip DeYoung, and Leif W. Ellisen. 2006. “P63 Mediates Survival in Squamous Cell Carcinoma by Suppression of P73-Dependent Apoptosis.” *Cancer Cell* 9 (1): 45–56. https://doi.org/10.1016/j.ccr.2005.12.013.

Rosenberg, Ari J., and Everett E. Vokes. 2021. “Optimizing Treatment De-Escalation in Head and Neck Cancer: Current and Future Perspectives.” *The Oncologist* 26 (1): 40–48. https://doi.org/10.1634/theoncologist.2020-0303.

Rotondo, John Charles, Alessandro Borghi, Rita Selvatici, Eros Magri, Enzo Bianchini, Elena Montinari, Monica Corazza, Annarosa Virgili, Mauro Tognon, and Fernanda Martini. 2016. “Hypermethylation-Induced Inactivation of the IRF6 Gene as a Possible Early Event in Progression of Vulvar Squamous Cell Carcinoma Associated With Lichen Sclerosus.” *JAMA Dermatology* 152 (8): 928–33. https://doi.org/10.1001/jamadermatol.2016.1336.

Rotondo, John Charles, Alessandro Borghi, Rita Selvatici, Elisa Mazzoni, Ilaria Bononi, Monica Corazza, Jacqueline Kussini, et al. 2018a. “Association of Retinoic Acid Receptor β Gene With Onset and Progression of Lichen Sclerosus-Associated Vulvar Squamous Cell Carcinoma.” *JAMA Dermatology* 154 (7): 819–23. https://doi.org/10.1001/jamadermatol.2018.1373.

———. 2018b. “Association of Retinoic Acid Receptor β Gene With Onset and Progression of Lichen Sclerosus–Associated Vulvar Squamous Cell Carcinoma.” *JAMA Dermatology* 154 (7): 819. https://doi.org/10.1001/jamadermatol.2018.1373.

Rotondo, John Charles, Silvia Bosi, Cristian Bassi, Manuela Ferracin, Giovanni Lanza, Roberta Gafà, Eros Magri, et al. 2015. “Gene Expression Changes in Progression of Cervical Neoplasia Revealed by Microarray Analysis of Cervical Neoplastic Keratinocytes.” *Journal of Cellular Physiology* 230 (4): 806–12. https://doi.org/10.1002/jcp.24808.

Rotondo, John Charles, Tommaso Candian, Rita Selvatici, Elisa Mazzoni, Gloria Bonaccorsi, Pantaleo Greco, Mauro Tognon, and Fernanda Martini. 2017. “Tracing Males From Different Continents by Genotyping JC Polyomavirus in DNA From Semen Samples.” *Journal of Cellular Physiology* 232 (5): 982–85. https://doi.org/10.1002/jcp.25686.

Rotondo, John Charles, Lucia Oton-Gonzalez, Chiara Mazziotta, Carmen Lanzillotti, Maria Rosa Iaquinta, Mauro Tognon, and Fernanda Martini. 2020a. “Simultaneous Detection and Viral DNA Load Quantification of Different Human Papillomavirus Types in Clinical Specimens by the High Analytical Droplet Digital PCR Method.” *Frontiers in Microbiology* 11: 591452. https://doi.org/10.3389/fmicb.2020.591452.

———. 2020b. “Simultaneous Detection and Viral DNA Load Quantification of Different Human Papillomavirus Types in Clinical Specimens by the High Analytical Droplet Digital PCR Method.” *Frontiers in Microbiology* 11 (November): 591452. https://doi.org/10.3389/fmicb.2020.591452.

Routman, D.M., K.R. Jethwa, A.E. Garda, T.A. DeWees, L. Joern, R. Hilfrich, M.C. Liu, et al. 2019. “HPV16 L1 Capsid Antibody Titers and Prognosis in HPV Associated Malignancy: Oropharyngeal, Anal, Cervical and Vaginal Cancer.” *International Journal of Radiation Oncology*Biology*Physics* 105 (1): E666. https://doi.org/10.1016/j.ijrobp.2019.06.967.

Sabatini, Maria Elisa, and Susanna Chiocca. 2020. “Human Papillomavirus as a Driver of Head and Neck Cancers.” *British Journal of Cancer* 122 (3): 306–14. https://doi.org/10.1038/s41416-019-0602-7.

Schlecht, Nicolas F., Miriam Ben-Dayan, Nicole Anayannis, Roberto A. Lleras, Carlos Thomas, Yanhua Wang, Richard V. Smith, et al. 2015. “Epigenetic Changes in the *CDKN2A* Locus Are Associated with Differential Expression of P16INK4A and P14ARF in HPV-positive Oropharyngeal Squamous Cell Carcinoma.” *Cancer Medicine* 4 (3): 342–53. https://doi.org/10.1002/cam4.374.

Schroeder, Lea, Gunnar Wichmann, Maria Willner, Angelika Michel, Manuel Wiesenfarth, Christa Flechtenmacher, Tanja Gradistanac, et al. 2018. “Antibodies against Human Papillomaviruses as Diagnostic and Prognostic Biomarker in Patients with Neck Squamous Cell Carcinoma from Unknown Primary Tumor: HPV Antibodies as Biomarker in NSCCUP Patients.” *International Journal of Cancer* 142 (7): 1361–68. https://doi.org/10.1002/ijc.31167.

Seiwert, Tanguy Y., Zhixiang Zuo, Michaela K. Keck, Arun Khattri, Chandra S. Pedamallu, Thomas Stricker, Christopher Brown, et al. 2015. “Integrative and Comparative Genomic Analysis of HPV-Positive and HPV-Negative Head and Neck Squamous Cell Carcinomas.” *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 21 (3): 632–41. https://doi.org/10.1158/1078-0432.CCR-13-3310.

Shi, Hao, Xiong Chen, Cheng Lu, Changmei Gu, Hongwei Jiang, RuiWei Meng, Xun Niu, Yangxin Huang, and Meixia Lu. 2015. “Association between P16INK4a Promoter Methylation and HNSCC: A Meta-Analysis of 21 Published Studies.” *PLoS ONE* 10 (4). https://doi.org/10.1371/journal.pone.0122302.

Shi, Y., J. S. Lee, and K. M. Galvin. 1997. “Everything You Have Ever Wanted to Know about Yin Yang 1.....” *Biochimica Et Biophysica Acta* 1332 (2): F49-66. https://doi.org/10.1016/s0304-419x(96)00044-3.

Silva, Ruany C. O., Rita de Cássia P. Lima, Marcelo N. Cordeiro, Daffany L. dos Santos, Lindomar J. Pena, Ana P. A. D. Gurgel, Marcus V. A. Batista, Bárbara S. Chagas, and Antonio C. Freitas. 2020. “Functional Evaluation of Human Papillomavirus Type 31 Long Control Region Variants.” *Genomics* 112 (6): 5066–71. https://doi.org/10.1016/j.ygeno.2020.09.008.

Slebos, R. J.C. 2006. “Gene Expression Differences Associated with Human Papillomavirus Status in Head and Neck Squamous Cell Carcinoma.” *Clinical Cancer Research* 12 (3): 701–9. https://doi.org/10.1158/1078-0432.CCR-05-2017.

Spector, Matthew E., Assuntina G. Sacco, Emily Bellile, Jeremy M. G. Taylor, Tamara Jones, Kan Sun, William C. Brown, et al. 2017. “E6 and E7 Antibody Levels Are Potential Biomarkers of Recurrence in Patients with Advanced-Stage Human Papillomavirus-Positive Oropharyngeal Squamous Cell Carcinoma.” *Clinical Cancer*

Research: An Official Journal of the American Association for Cancer Research 23 (11): 2723–29. https://doi.org/10.1158/1078-0432.CCR-16-1617.

Stanley, Margaret. 2003. "Antibody Reactivity to HPV E6 and E7 Oncoproteins and Early Diagnosis of Invasive Cervical Cancer." *American Journal of Obstetrics and Gynecology* 188 (1): 3–4. https://doi.org/10.1067/mob.2003.97.

Stransky, Nicolas, Ann Marie Egloff, Aaron D. Tward, Aleksandar D. Kostic, Kristian Cibulskis, Andrey Sivachenko, Gregory V. Kryukov, et al. 2011. "The Mutational Landscape of Head and Neck Squamous Cell Carcinoma." *Science (New York, N.Y.)* 333 (6046): 1157–60. https://doi.org/10.1126/science.1208130.

Thariat, J., C. Badoual, C. Faure, C. Butori, P. Y. Marcy, and C. A. Righini. 2010. "Basaloid Squamous Cell Carcinoma of the Head and Neck: Role of HPV and Implication in Treatment and Prognosis." *Journal of Clinical Pathology* 63 (10): 857–66. https://doi.org/10.1136/jcp.2010.078154.

Thompson, Lester D. R. 2013. *Head and Neck Pathology*. Philadelphia, PA: Elsevier Saunders. http://www.clinicalkey.com/dura/browse/bookChapter/3-s2.0-C20100648383.

Tognon, Mauro, Andrea Tagliapietra, Federica Magagnoli, Chiara Mazziotta, Lucia Oton-Gonzalez, Carmen Lanzillotti, Fortunato Vesce, Carlo Contini, John Charles Rotondo, and Fernanda Martini. 2020. "Investigation on Spontaneous Abortion and Human Papillomavirus Infection." *Vaccines* 8 (3): 473. https://doi.org/10.3390/vaccines8030473.

Torreggiani, Elena, Marika Rossini, Ilaria Bononi, Silvia Pietrobon, Elisa Mazzoni, Maria Rosa Iaquinta, Carlo Feo, et al. 2019. "Protocol for the Long-term Culture of Human Primary Keratinocytes from the Normal Colorectal Mucosa." *Journal of Cellular Physiology* 234 (7): 9895–9905. https://doi.org/10.1002/jcp.28300.

Van Abel, Kathryn M., and Eric J. Moore. 2012. "Focus Issue: Neck Dissection for Oropharyngeal Squamous Cell Carcinoma." *ISRN Surgery* 2012: 547017. https://doi.org/10.5402/2012/547017.

Vanbokhoven, Hans, Gerry Melino, Eleonora Candi, and Wim Declercq. 2011. "P63, a Story of Mice and Men." *Journal of Investigative Dermatology* 131 (6): 1196–1207. https://doi.org/10.1038/jid.2011.84.

Vergara, Nicolás, Monserrat Balanda, Deyanira Vidal, Francisco Roldán, Héctor S. Martín, and Eugenio Ramírez. 2019. "Detection and Quantitation of Human Papillomavirus DNA in Peripheral Blood Mononuclear Cells from Blood Donors." *Journal of Medical Virology* 91 (11): 2009–15. https://doi.org/10.1002/jmv.25551.

Verma, Gaurav, Kanchan Vishnoi, Abhishek Tyagi, Mohit Jadli, Tejveer Singh, Ankit Goel, Ankita Sharma, et al. 2017. "Characterization of Key Transcription Factors as

Molecular Signatures of HPV-positive and HPV-negative Oral Cancers." *Cancer Medicine* 6 (3): 591–604. https://doi.org/10.1002/cam4.983.

Walter, Vonn, Xiaoying Yin, Matthew D. Wilkerson, Christopher R. Cabanski, Ni Zhao, Ying Du, Mei Kim Ang, et al. 2013. "Molecular Subtypes in Head and Neck Cancer Exhibit Distinct Patterns of Chromosomal Gain and Loss of Canonical Cancer Genes." *PLOS ONE* 8 (2): e56823. https://doi.org/10.1371/journal.pone.0056823.

Wang, Wei, Zhengrong Sun, Jianhua Liu, Guili Wang, Zhitao Lu, Weiqiang Zhou, Te Qi, and Qiang Ruan. 2017. "Increased Methylation of Human Papillomavirus Type 16 DNA Is Associated with the Severity of Cervical Lesions in Infected Females from Northeast China." *Oncology Letters* 13 (5): 3809–16. https://doi.org/10.3892/ol.2017.5903.

Weyn, Christine, Jean-Marie Vanderwinden, Joanne Rasschaert, Yvon Englert, and Véronique Fontaine. 2011. "Regulation of Human Papillomavirus Type 16 Early Gene Expression in Trophoblastic and Cervical Cells." *Virology* 412 (1): 146–55. https://doi.org/10.1016/j.virol.2010.12.056.

Wittekindt, Claus, Steffen Wagner, Ayman Bushnak, Elena-Sophie Prigge, Magnus von Knebel Doeberitz, Nora Würdemann, Katharina Bernhardt, Jörn Pons-Kühnemann, Catharina Maulbecker-Armstrong, and Jens Peter Klussmann. 2019. "Increasing Incidence Rates of Oropharyngeal Squamous Cell Carcinoma in Germany and Significance of Disease Burden Attributed to Human Papillomavirus." *Cancer Prevention Research* 12 (6): 375–82. https://doi.org/10.1158/1940-6207.CAPR-19-0098.

Xiao, Zhengzheng, Qin Liu, Feng Mao, Jun Wu, and Ting Lei. 2011. "TNF-α-Induced VEGF and MMP-9 Expression Promotes Hemorrhagic Transformation in Pituitary Adenomas." *International Journal of Molecular Sciences* 12 (6): 4165–79. https://doi.org/10.3390/ijms12064165.

Xu, Hong-Fa, Tie-Jun Huang, Qin Yang, Liang Xu, Fen Lin, Yan-Hong Lang, Hao Hu, et al. 2019. "Candidate Tumor Suppressor Gene IRF6 Is Involved in Human Breast Cancer Pathogenesis via Modulating PI3K-Regulatory Subunit PIK3R2 Expression." *Cancer Management and Research* 11 (June): 5557–72. https://doi.org/10.2147/CMAR.S203060.

Yalcin-Ozuysal, Ö, M Fiche, M Guitierrez, K-U Wagner, W Raffoul, and C Brisken. 2010. "Antagonistic Roles of Notch and P63 in Controlling Mammary Epithelial Cell Fates." *Cell Death & Differentiation* 17 (10): 1600–1612. https://doi.org/10.1038/cdd.2010.37.

Yang, Ruwen, Jana Klimentová, Elke Göckel-Krzikalla, Regina Ly, Nadine Gmelin, Agnes Hotz-Wagenblatt, Helena Řehulková, Jiří Stulík, Frank Rösl, and Martina Niebler. 2019. "Combined Transcriptome and Proteome Analysis of Immortalized Human Keratinocytes Expressing Human Papillomavirus 16 (HPV16) Oncogenes Reveals Novel Key Factors and Networks in HPV-Induced Carcinogenesis." Edited by Laimonis A.

Laimins. *MSphere* 4 (2): e00129-19, /msphere/4/2/mSphere129-19.atom. https://doi.org/10.1128/mSphere.00129-19.

Yang, Yi-Shan, Karen Smith-McCune, Teresa M. Darragh, Yvonne Lai, Ju-Hwa Lin, Ting-Chang Chang, Hsiao-Yun Guo, et al. 2012. "Direct Human Papillomavirus E6 Whole-Cell Enzyme-Linked Immunosorbent Assay for Objective Measurement of E6 Oncoproteins in Cytology Samples." *Clinical and Vaccine Immunology: CVI* 19 (9): 1474–79. https://doi.org/10.1128/CVI.00388-12.

Yao, Yueting, Zhiling Yan, Shuying Dai, Chuanyin Li, Longyu Yang, Shuyuan Liu, Xinwen Zhang, Li Shi, and Yufeng Yao. 2019. "Human Papillomavirus Type 16 E1 Mutations Associated with Cervical Cancer in a Han Chinese Population." *International Journal of Medical Sciences* 16 (7): 1042–49. https://doi.org/10.7150/ijms.34279.

Zengin, Talip, Burcu Ekinci, Cansu Kucukkose, and Ozden Yalcin-Ozuysal. 2015. "IRF6 Is Involved in the Regulation of Cell Proliferation and Transformation in MCF10A Cells Downstream of Notch Signaling." Edited by Aamir Ahmad. *PLOS ONE* 10 (7): e0132757. https://doi.org/10.1371/journal.pone.0132757.

Zhang, Yanxiao, Lada A. Koneva, Shama Virani, Anna E. Arthur, Alisha Virani, Pelle B. Hall, Charles D. Warden, et al. 2016. "Subtypes of HPV-Positive Head and Neck Cancers Are Associated with HPV Characteristics, Copy Number Alterations, PIK3CA Mutation, and Pathway Signatures." *Clinical Cancer Research* 22 (18): 4735–45. https://doi.org/10.1158/1078-0432.CCR-16-0323.

Zhe, Xiangyi, Huizhen Xin, Zhenzhen Pan, Fuyuan Jin, Weinan Zheng, Hongtao Li, Dongmei Li, et al. 2019. "Genetic Variations in E6, E7 and the Long Control Region of Human Papillomavirus Type 16 among Patients with Cervical Lesions in Xinjiang, China." *Cancer Cell International* 19: 65. https://doi.org/10.1186/s12935-019-0774-5.

CHAPTER 6

Scientific contributions

During my PhD studies I have contributed to the publication of the following papers:

1. Rotondo JC, Aquila G, **Oton-Gonzalez L**, Selvatici R, Rizzo P, De Mattei M, Pavasini R, Tognon M, Campo GC and Martini F. *Methylation of SERPINA1 gene promoter may predict chronic obstructive pulmonary disease in patients affected by acute coronary syndrome*. Clinical Epigenetics. Accepted. doi: 10.1186/s13148-021-01066-w. IF= 5.028

2. Iaquinta MR, Lanzillotti C, Mazziotta C, Bononi I, Frontini F, Mazzoni E, **Oton-Gonzalez L**, Rotondo JC, Torreggiani E, Tognon M and Martini F. The role of microRNAs in the osteogenic and chondrogenic differentiation of mesenchymal stem cells and bone pathologies. Theranostics. 2021. In press. doi:10.7150/thno.55664. IF=8.063

3. Mazziotta C, Lanzillotti C, Iaquinta MR, Taraballi F, Torreggiani E, Rotondo JC, **Oton-Gonzalez L**, Mazzoni E, Frontini, Bononi I, De Mattei M, Tognon M and Martini F. MicroRNAs Modulate Signaling Pathways in Osteogenic Differentiation of Mesenchymal Stem Cells. International Journal of Molecular Sciences. 2021. 22(5): 2362. doi: 10.3390/ijms22052362. IF=4.556

4. **Oton-Gonzalez L#**, Rotondo JC#, Cerritelli L, Malagutti N, Lanzillotti C, Bononi I, Ciorba A, Bianchini C, Mazziotta C, De Mattei M, Pelucchi S, Tognon M and Martini F. *Association between oncogenic Human Papillomavirus type 16 and Killian polyp*. Infectious Agents and Cancer. doi: 10.1186/s13027-020-00342-3. IF=2.740

5. Lodhi N, Tun M, Nagpal P, Inamdar AA, Ayoub NM, Siyam N, **Oton-Gonzalez L**, Gerona A, Morris D, Sandhu R, Suh KS. *Biomarkers and novel therapeutic approaches for diffuse large B-cell lymphoma in the era of precision medicine.* Oncotarget. doi: 10.18632/oncotarget.27785. IF(2016)= 5.168

6. Rotondo J#, **Oton-Gonzalez L#**, Mazziotta C#, Lanzillotti C, Iaquinta MR, Tognon M and Martini F. *Simultaneous detection and viral DNA load quantification of different Human Papillomavirus types in clinical specimens by the high analytical droplet digital PCR method.* Front. Microbiol. 2020. doi: 10.3389/fmicb.2020.591452. IF=4.235

7. Corazza M#, **Oton-Gonzalez L#**, Scuderi V, Rotondo JC, Lanzillotti C, Di Mauro G, Tognon M, Martini F and Borghi A. *Tissue cytokine/chemokine profile in vulvar*

lichen sclerosus: An observational study on keratinocyte and fibroblast cultures. J Dermatol Sci. 2020. doi: 10.1016/j.jdermsci.2020.09.006, IF= 3.681

8. Rotondo JC#, **Oton-Gonzalez L#**, Selvatici R, Rizzo P, Pavasini R, Campo GC, Lanzillotti C, Mazziotta C, De Mattei M, Tognon M and Martini F. *SERPINA1 gene promoter is differentially methylated in peripheral blood mononuclear cells of pregnant women.* Front. Cell Dev. Biol. 2020. doi: 10.3389/fcell.2020.550543. IF=5.201
9. Tognon M, Tagliapietra A, Magagnoli F, Chiara Mazziotta C, **Oton-Gonzalez L**, Lanzillotti C, Contini C, Vesce F, Rotondo JC and Martini F. *Investigation on Spontaneous Abortion and Human Papillomavirus Infection.* Vaccines. 2020. 8(3), 473. doi: 10.3390/vaccines8030473. IF=3.269
10. Malagutti N^{+}, Rotondo JC^{+}, Cerritelli L, Melchiorri C, De Mattei M, Selvatici R, **Oton-Gonzalez L**, Stomeo F, Mazzoli M, Borin M, Mores B, Ciorba A, Tognon MG, Pelucchi S and Martini F. *High Human Papillomavirus DNA loads in Inflammatory Middle Ear Diseases*. Pathogens. 2020. 9(3), 224. doi: 10.3390/pathogens9030224. IF=3.405
11. Tagliapietra A, Rotondo JC, Bononi I, Mazzoni E, Magagnoli F, **Oton-Gonzalez L**, Contini C, Vesce F, Tognon M and Martini F. *Droplet digital PCR assay to detect Merkel cell polyomavirus sequences in chorionic villi from spontaneous abortion affected females*. J Cell Physiol. 2020. 1-7. doi:10.1002/jcp.29213. IF= 4.522

Remain in preparation, submitted or under review the following:

1. Mazziotta C, Lanzillotti C, Torreggiani E, **Oton-Gonzalez L**, Iaquinta MR, Mazzoni E, Gaboriaud P, Touzé A, Silvagni E, Govoni M, Martini F, Tognon M and RotondoJC. *Serum antibodies against the oncogenic Merkel Cell Polyomavirus detected by an innovative immunological assay with mimotopes in healthy subjects*. Science Translational Medicine. Submitted.
2. Hushcha Y, Blo I, **Oton-Gonzalez L**, Di Mauro G, Martini F, Tognon M and De Mattei M. *MicroRNAs in the regulation of melanogenesis*. IJMS. Submitted.
3. Frontini F, Bononi I, Torreggiani E, Di Mauro G, Iaquinta MR, **Oton-Gonzalez L**, Mazziotta C, Mazzoni E, Stendardo M, Boschetto P, Libener R, Guaschino R, Grosso

F, Guerra G, Martini F and Tognon M. *MiR-197-3p as a new marker of asbestos exposure and pleural mesothelioma*. In preparation.