SQUAMOUS CELL CARCINOMA OF THE ORAL TONGUE: STUDIES OF BIOMARKERS CONNECTED TO HUMAN PAPILLOMAVIRUS INFECTION, EPITHELIAL TO MESENCHYMAL TRANSITION, AND LOCOREGIONAL METASTASIS

Nicola Sgaramella
TILL MIN UNDERBARA FRU OCH DOTTER
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Abstract

Background: Oral Tongue Squamous Cell Carcinoma (OTSCC) is the most frequent and aggressive carcinoma in the head and neck region. Its incidence has increased during the last decades, especially in young patients (≤40 years) mainly female. These young patients have either not been exposed to the traditional risk factors for this disease, or have a much reduced duration of exposure than the typical OTSCC patient. The reasons behind this increasing incidence remain unknown.

The aims of this thesis were to analyse the presence and possible role of human papillomavirus (HPV) in oral tongue cancer in correlation with its surrogate marker p16 and its receptor syndecan-1. Other aims were to evaluate expression of EMT (epithelial to mesenchymal transition) - related markers, such as E-cadherin, β-catenin, CK5 and CK19, and to address the potential predictive role of podoplanin in the loco-regional metastatic process.

Clinical parameters including age, sex, geographical distribution, relapse, tumour staging and grading were also investigated for a possible correlation with biomarker expression and prediction of survival rate and therapeutic strategy.

Materials and methods: More than one hundred samples of OTSCC coming from two University Hospitals of two different countries (Sweden and Italy) were analysed. HPV presence was evaluated by in situ hybridisation for detection of the high-risk HPV 16 and indirectly by immunohistochemistry (IHC) of its surrogate marker p16. Expression of the HPV receptor syndecan-1 and the EMT biomarkers E-cadherin, β-catenin, CK5, CK19 were also evaluated by immunohistochemistry.

Results: Tumour size and lymph node metastasis correlated to both overall and disease-free survival. Despite variable expression of the syndecan-1 receptor, HPV 16 was not detected in any sample analysed, excluding a possible association with p16, which was expressed in 33% of the cases.

All EMT-related markers were commonly expressed in tongue cancer. Data showed E-cadherin to be an independent prognostic factor with higher expression associated with poor overall survival. Notably, E-cadherin, β-catenin and CK5 directly correlated to each other.

Multivariate analysis of clinical data demonstrated that age of the patient is an independent prognostic factor with younger patients showing a worse survival rate. Patients younger than 40 years also showed significantly higher expression of podoplanin. Data for geographic distribution revealed a difference in expression of E-cadherin between Swedish and Italian patients.

Conclusions: In contrast to SCC of the base of the tongue and the tonsil, HPV is not present in OTSCC, excluding HPV infection as a risk factor. Higher levels of E-cadherin and young age is associated with poor survival in OTSCC patients. The different frequency of EMT markers seen between Swedish and Italian patients suggests an important role for the environment and the geographical area in the onset of different molecular patterns of OTSCC.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>CDKs</td>
<td>cyclin dependent kinases</td>
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<td>CKI</td>
<td>cyclin dependent kinase inhibitors</td>
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<td>CKs</td>
<td>cytokeratins</td>
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<td>CSCs</td>
<td>cancer stem cells</td>
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<td>DFS</td>
<td>disease-free survival</td>
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<td>E-cadherin</td>
<td>epithelial cadherin</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>EMT</td>
<td>epithelial to mesenchymal transition</td>
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<tr>
<td>FFPE</td>
<td>formalin-fixed and paraffin-embedded tissue</td>
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<td>HNSCC</td>
<td>head and neck squamous cell carcinoma</td>
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<td>HPV</td>
<td>human papillomavirus</td>
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<td>HR-HPV</td>
<td>high-risk human papillomavirus</td>
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<td>HSPGs</td>
<td>cell surface heparin sulfate proteoglycans</td>
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<td>IFPs</td>
<td>intermediate filament proteins</td>
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<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<td>ISH</td>
<td>in situ hybridization</td>
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<tr>
<td>LN/LNs</td>
<td>lymph node/lymph nodes</td>
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<tr>
<td>LOH</td>
<td>loss of heterozygosity</td>
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<tr>
<td>LR-HPV</td>
<td>low-risk human papillomavirus</td>
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<td>LVD</td>
<td>lymphatic vessel density</td>
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<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>MET</td>
<td>mesenchymal to epithelial transition</td>
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<td>N-cadherin</td>
<td>neural cadherin</td>
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<td>OPSCC</td>
<td>oropharyngeal squamous cell carcinoma</td>
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<td>OS</td>
<td>overall survival</td>
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<td>OSCC</td>
<td>oral squamous cell carcinoma</td>
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<td>OTSCC</td>
<td>oral tongue squamous cell carcinoma</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PDL-1</td>
<td>Programmed Death Ligand 1</td>
</tr>
<tr>
<td>pRb or Rb</td>
<td>retinoblastoma protein</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative or real time polymerase chain reaction</td>
</tr>
<tr>
<td>qRT/PCR</td>
<td>quantitative reverse-transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>QS</td>
<td>quick score</td>
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<tr>
<td>RT/PCR</td>
<td>reverse-transcriptase polymerase chain reaction</td>
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<tr>
<td>SCC</td>
<td>squamous cell carcinoma</td>
</tr>
<tr>
<td>SIN</td>
<td>squamous intraepithelial neoplasm</td>
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<tr>
<td>SLN</td>
<td>sentinel lymph node</td>
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<td>VEGFs</td>
<td>vascular endothelial growth factors</td>
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List of original papers

Paper I


Paper II


Paper III


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Ett syfte med detta forskningsarbete var att analysera den tänkbara rollen för HPV i oral tungcancer relaterad till dess surrogat-markör p16 och dess receptor syndecan-1.

Ett annat syfte med arbetet var att studera uttrycket av E-cadherin, β-catenin, CK5 and CK19, markörer relaterade till så kallad epitelial till mesenkymal omvandling (EMT), ett cancerrelaterat biologiskt fenomen som numera tycks spela en huvudroll i cancerinvasion och metastasering.

Ett tredje syfte var att analysera uttrycket av podoplanin, ett transmembranöst glykoprotein som är involverat i kollektiv såväl som i enstaka cellers migration och lymfkörtelmetastasering på halsen.

Material och metoder: Mer än hundra fall av oral tungcancer vilka samlats in vid två olika Universitetssjukhus i två olika länder (Sverige och Italien) analyserades.

HPV infektion studerades med så kallad situ hybridisering för detektion av HPV 16, samt med hjälp av immunohistokemi för uttryck av proteinerna p16 och syndecan-1. Med hjälp av immunohistokemi studerades också uttrycket av de tidigare nämnda EMT relatade proteinerna.

Preparaten poänggavs med hjälp av ett så kallat quick score (QS) system som utvärderade både mängden celler som uttryckte de olika proteinerna, samt intensiteten i uttrycket.

Podoplanin uttrycket analyserades både på protein och mRNA nivå.

Resultat: Storleken på tumören och förekomst av lymfkörtelmetastas associerade med overall och disease-free överlevnad. Trots att syndecan-1 uttrycktes i alla studerade preparat, kunde HPV 16 inte detekteras i något av preparaten. P16 uttrycktes i 33% av tumörerna.

Alla analyserade EMT relatade markörer uttrycktes i tuncancer. Uttrycket av E-cadherin var statistiskt associerat med sämre överlevnad. Ett direkt samband mellan förekomst av E-cadherin, β-catenin och CK5 kunde ses.

Den multivariata analysen av de kliniska uppgifterna visade att ung ålder vid diagnos är en fristående prognostisk faktor för lägre överlevnad. Unga patienter visade också statistiskt signifikant högre uttryck av podoplanin.
En skillnad i uttryck av E-cadherin sågs mellan svenska och italienska patienter.

**Slutsatser:** Till skillnad med orofarynx cancer så ses ingen HPV infektion i skivepitelcancer i oral tunga. P16 är ej en tillförlitlig biomarkör för HPV infektion i oral tungcancer, medan uttryck av proteinet är en fristående prognostisk markör associerad till mindre spridning av tumör till lymfkörtlar. Högre uttryck av E-cadherin i ung ålder är en fristående prognostisk faktor hos patienter med oral tungcancer. Skillnaden i uttryck av E-cadherin mellan svenska och italienska patienter indikerar en hypotetisk betydelse av geografisk lokalisation i utveckling av skivepitelcancer i tungan. Högre uttryck av podoplanin som sågs hos unga patienter betonar genetiska och epigenetiska möjliga skillnader mellan unga och medelålders/äldre patienter med oral tungcancer.
Introduction / Background

**Head and neck squamous cell carcinoma (HNSCC)**

Head and neck cancer is the malignancy occurring in the lip, oral cavity, salivary glands, tonsils, oropharynx, nasopharynx, hypopharynx, nasal cavity, middle ear, paranasal sinuses and larynx. Morphologically, squamous cell carcinomas predominate.

Head and neck squamous cell carcinoma (HNSCC) is reported to be the 6th most common cancer worldwide and, as a consequence of its highly invasive nature and proximity to vital structures, the 7th most common cause of cancer-induced mortality (Jemal et al., 2011). HNSCC is an extremely heterogeneous group of tumours both from a molecular (Huang et al., 2002; Bosch et al., 2004) and clinical point of view (Shah and Patel, 2003). The main clinical heterogeneity factor is the site of origin, which correlates with specific risk factors, symptoms, stage at diagnosis, tendency to local and distant metastasis, chemo- and radio-sensitivity and ultimately prognosis (Shah and Patel, 2003; De Vita et al., 2008). Tobacco and alcohol, which act synergistically, are the primary aetiological factors of HNSCC and account for the majority of head and neck tumours (Haddad and Shin, 2008), but also nutrition, poor oral hygiene, infections, family background and other anamnestic oncological diseases are commonly accepted risk factors for the development of head and neck tumours (Hooper et al., 2009; Wittekindt et al., 2012). Thereby several factors are involved in the carcinogenesis, such as age, gender, ethnicity, lifestyle, genetic background, status of health and exposure to one or more oncogenic factors.

Despite advances in diagnosis, surgical management, and chemo-radiotherapy regimens, the 5-year survival rate is estimated to be about 50% (Fuller et al., 2007) and it has improved only minimally over some decades. Several factors that contribute to these poor outcomes have been identified over the years, including access to care and delays in diagnosis and treatment (Paleri et al., 2010). Despite the declining use of tobacco, increasing trends in the incidence of HNSCC at specific sites, namely the occurrence of oral tongue SCC (OTSCC), increased during the past decades (Annertz et al., 2002; Carvalho et al., 2005; Chaturvedi et al., 2008; Harris et al., 2010).

**Oral tongue squamous cell carcinoma (OTSCC)**

Amongst all intra-oral sites, OTSCC is the most frequent and clinically behaves entirely differently. It seems to grow faster than cancer in other sites of the oral cavity, more frequently metastasises to cervical lymph nodes, and is associated with a poorer prognosis (Ridge et al., 2007). Indeed, despite overall
improvements in surgical and medical management, clinical outcomes in patients with OTSCC have remained largely unchanged (Salem, 2010; Saba et al., 2011). OTSCC shows a more aggressive phenotype with properties of split invasive growth pattern, rapid local invasion, a more intense inflammatory response at the tumour interface, high proportion of lymph node positivity at the time of diagnosis and high loco-regional relapse rate (Lundqvist et al., 2011; Shiboski et al., 2005). OTSCC metastasizes to regional lymph nodes rather than spreads haematogenously. Primary OTSCC spreads through lymphatic channels to the lymph nodes of the cervical region. The reason for the aggressive growth and the nodal disease in the early stage of OTSCC can depend on the unique anatomical features of this muscular organ within the oral cavity, namely the nine extrinsic and intrinsic muscles with no relevant anatomical barrier and the dense lymphatic network, with three main deep muscular lymphatic drainage pathways (Lindberg, 1972). Besides, it has been shown that the oral tongue sub-site differs from other sub-sites of the oral cavity in gene expression both in tumour-free and cancer tissue (Boldrup et al., 2011; Boldrup et al., 2012). The incidence of OTSCC seems to be increasing among young adults (≤40 years) (Koch et al., 1999; Llewellyn et al., 2003; Bonder et al., 2013; Troeltzsch et al., 2014), particularly women (Beena et al., 2011). Many authors attribute a more aggressive phenotype for OTSCC of young adults, with a high proportion of lymph node positivity at the time of diagnosis and a high recurrence rate after therapy (Atula et al., 1996; Knope et al., 2015).

Patients in the young age group often lack the traditional risk factors or are exposed to these factors for a limited time (Patel et al., 2011; Ng et al., 2017). These findings suggest that other aetiological factors are involved and diet, obesity (Lyengar et al., 2014), immune deficiency (Warnakulasuriya, 2008), non-smoking-related inflammation (Bektas-Kayhan, 2012; Vander Broek et al., 2013), dental trauma (Bektas-Kayhan et al., 2014; Perry et al., 2015), and oral microbiome have been speculated (Hooper et al., 2007; Dewhirst et al., 2010; Wade, 2013).

The most relevant and direct oncogenic microorganism in HNSCC is probably the human papillomavirus (HPV). Recently, the association between high-risk types of human papillomavirus and tongue cancer has been widely investigated (Herrero et al., 2003; Furniss et al., 2007; Harris et al, 2010; Patel et al, 2011; Wittekindt et al., 2012).

**Human papillomavirus (HPV)**

Human papillomavirus is a small, non-enveloped DNA virus, with a genome of around 8 kb that consists of double-stranded circular DNA. Overall, the HPV genome has the capacity to encode eight proteins: E1, E2, E4-E7, the non-structural proteins involved mainly in replication, transcription and
transformation, and L1 and L2, the structural proteins that compose the capsid (Syrjanen et al., 2011).

HPVs specifically target the undifferentiated proliferative basal cells of the epithelium. HPV infects the squamous epithelium of the skin, the genital mucosa and the upper aero-digestive airways. Infection with HPV is thought to require a minor abrasion or break in the epithelial covering. Initially, HPV infects undifferentiated proliferative basal cells, which are capable of dividing. Cell surface heparin sulfate proteoglycans (HSPGs) have been claimed as HPV primary receptors. Of these HSPGs, syndecan-1 is the most likely receptor for HPV (Rautava and Syrjänen, 2012). Over-expression of syndecan-1 is also found in basal and para-basal cells following a trauma (Shafti-Keramat et al., 2003).

Once inside the host cell, viral DNA localises into the nucleus and establishes itself as an episome. Only a portion of the virus reaching the nucleus seems to undergo replication and HPV replication remains confined to a small number of infected cells. At this stage, the viral proteins E1, E2, E6, and E7 transcribed from the early promotor are expressed at a low level. Mainly E6 and E7 may disturb normal terminal differentiation by stimulating cellular proliferation and DNA synthesis. After the onset of genome amplification, the capsid proteins L1 and L2 accumulate in the mature epithelial cells.

The assembly of infectious virions takes place in terminally differentiated cells of the upper epithelial layers, and the virions are shed to the environment when differentiating cells reach the surface (productive infection). There is no cytolysis or necrosis in this process and consequently no inflammation. In addition, there is no viremia in the HPV life cycle, and only very low levels of viral protein are presented to the immune system of the host. As a result, HPV is effective in evading detection by the immune system for long periods and generates only a weak immune response. Despite these immune evasion strategies, the majority of HPV infected patients become HPV DNA-negative within one year. In addition to active replication and productive infection, HPV may produce non-productive infection and persist in cells in low number episomic molecules. Of importance in the establishment of persistent infection is the immune escape of HPV mainly orchestrated by E5, E6, and E7 (Kanodia et al., 2007; Venuti et al., 2011). Persistent HPV infection (whether latent or chronic) is a key event for HPV-induced cellular transformation (transforming infection) which occurs through interactions of viral E6 and E7 proteins with p53 and the retinoblastoma protein (pRb or Rb) (Ljiang et al., 2008; Mollers et al., 2013; Sand and Jalouli, 2014) as a consequence of disruption of the E2 gene regulating the expression of E6 and E7. Different types of HPV have been identified based on DNA sequence variations. Human papillomaviruses are divided into low-risk (LR-HPV) and high-risk (HR-HPV) types, depending on the carcinogenic power (El-Mofty, 2007). As previously mentioned, during the normal HPV life cycle, viral DNA is maintained
episomally, a state predominantly associated with low-risk HPV such as HPV-6 and -11. In HR-HPVs, the oncoproteins E6 and E7 interact with different degrees of affinity with host cell proteins to disturb normal epithelial differentiation and apoptosis by stimulating cellular proliferation, DNA synthesis, and inhibition of cell cycle regulators (Doorbar, 2007). E6 binds and degrades p53 leading to substantial uncontrolled cell cycle progression, and E7 binds and inhibits the retinoblastoma protein resulting in deregulation of cell cycle control (Zur Hausen, 2006). Persistent infection with HR-HPVs with continued and aberrant expression of the E6 and E7 genes leads to genomic instability, and mutational events that can result in malignant transformation (Stanley et al., 2007, Syrjanen et al., 2011). Proteins of low-risk HPVs have a low affinity for tumour suppressor proteins. Thus, these viruses have low oncogenic potential and the infections are usually self-limited and associated with benign lesions (Syrjanen et al., 2011).

The aforementioned mechanism of HPV carcinogenesis, which implies the integration of HPV into a human chromosome, has been delineated in most cervical cancers, with almost 100% prevalence of an HPV-association with cervical carcinoma and approximately 70% of tumours associated with high risk HPV-16/18 as the primary aetiology (IARC 2007; de Sanjosé et al., 2007; IARC 2012; Guan et al., 2012). Of extra-cervical sites, ano-genital cancer has also been associated with high-risk HPV, possibly because of its route of infection (Narisawa-Saito and Kiyono, 2007; McLaughlin-Drubin and Munger, 2009). The full extent of this association in terms of age and onset of diagnosis is not well known (Chaux and Cubilla, 2012). Several studies have reported a role of HPV in the development of squamous cell carcinoma of the lungs, with a mean incidence of about 25% (Syrjanen, 1979; Klein et al., 2009). In the head and neck region, HPV association with HNSCC has been demonstrated for the oropharynx followed by larynx/hypopharynx and oral cavity (Kreimer et al., 2004).

**HPV and oropharyngeal squamous cell carcinoma (OPSCC)**

High-risk human papillomaviruses play an aetiologic role in between one third and one half of oropharyngeal squamous cell carcinomas (OPSCC) (Pannone et al., 2011). HPV16 was discovered to be the dominant HPV sub-type (Sand and Jalouli, 2014). The incidence of these virus-associated cancers has been rising rapidly, suggesting increased HPV exposure and infection rates. Indeed, OPSCC associated with HPV increased 225% from 1988 to 2004 and will surpass the incidence of cervical cancer, the primary HPV associated malignancy, by 2020 (Chaturvedi et al., 2011).

HPV-associated cancer typically occurs in non-smoking and non-drinking young or middle-aged patients (Carvalho et al., 2005; Sturgis and Cinciripini, 2007; Chaturvedi et al., 2011). The reason for the increase in HPV-positive OPSCC has
been suggested to depend on change in sexual behaviours e.g. early beginning of sex life, increased numbers of sexual encounters, and oral sex practice (Romanitan et al., 2008; Anaya-Saavedra et al., 2008; D’Souza et al., 2009). Nevertheless, alternative routes of infection are not excluded. Definitely for females, it was shown that an existing genital HPV-infection is an important predisposing factor for oropharyngeal HPV-infection (Termine et al., 2011). Further risk factors for infection with HR-HPV are young age, male sex, or HIV-infection (Kreimer et al., 2004).

HPV-positive cancers in the head and neck have well-established gross characteristics that differ from HPV-negative cancers. HPV-positive OPSCC is characterised as having smaller primary tumours, advanced N status with large and cystic nodal metastases, and poor pathologic tumour differentiation, being usually non-keratinising (Ang et al., 2010; O’Sullivan et al., 2012; Hong et al., 2013). Despite these clinico-pathological aspects, compared to the smoking and alcohol-associated tumours, HPV-positive OPSCC is associated with a “non-aggressive” shallow invasive phenotype and better survival due partially to increased sensitivity to chemotherapy and radiotherapy (Gillison et al., 2000; D’Souza et al., 2007; Muller et al., 2012; Rieckmann et al., 2013; Isayeva et al., 2015); HPV infection is indeed one major prognostic factor in OPSCC (Huang et al., 2012). Better loco-regional control and a lower incidence of second primary tumours are important parameters contributing to improved outcome in patients with an HPV-positive tumour (Posner et al., 2011; Huang et al., 2012).

Tumorigenesis in HPV-positive tumours is more dependent on pathway disruptions by viral oncoproteins than HPV-negative tumours, which are driven by genetic alterations that lead to oncogenesis in the more traditional multifactorial sequential process (Seiwert et al., 2015). Genetic alterations are indeed less frequently found in HPV-positive tumours (Stransky et al., 2011; Nichols et al., 2012). HPV positive HNSCC are generally associated with wild-type TP53, as opposed to tobacco-induced tumours that are characterised by genetic alterations of the TP53 pathway (Braakhuis et al., 2004).

A likely explanation for a lower number of loco-regional recurrences and second primary tumours in patients with HPV-positive OPSCC may indeed be related to the absence of field cancerisation (Begum et al., 2005; Fakhry and Gillison, 2006; Rietbergen et al., 2014) while in HPV-negative HNSCCs, at least 25% of the surgical margins contain mucosal tissue with tumour-associated genetic alterations (Brennan et al., 1995; Schaaij-Visser et al., 2009) and more than 30% of the local recurrences originate from a tumour-related genetically altered field (Tabor et al., 2004). The absence of an HPV infected field possibly also explains why almost no HPV-related lesions can be detected in the healthy population (Kreimer et al., 2004; Smith et al., 2007; D’souza et al., 2009). Definitive information regarding why HPV infection promotes treatment sensitivity of HPV-associated HNSCC is absent from the existing literature, but
enhanced radio-sensitivity has been related to the presence of wild type p53 in HPV+ tumours.

**HPV and OTSCC**

The incidence of HPV infection is very low in normal oral mucosa (Migaldi et al., 2012). The role of HPV in oral tongue cancer remains ambiguous because of the disparity of results published so far, which have reported HPV infection rates ranging from 0% to 100% (Matzow et al., 1998; Bouda et al., 2000; Mork et al., 2001; Ringström et al., 2002; Koskinen et al., 2003; Gillison and Shah, 2011; Syrjänen et al., 2011; Pannone et al., 2011; Marklund et al., 2012; Saito et al., 2013; Dahlgren et al., 2015). The prevalence of HPV infection in young patients suffering from OTSCC is controversial as well and is the object of ongoing studies: while some reports showed an increased incidence of HPV-positive OTSCC, more recent studies failed to demonstrate this association (Harris et al., 2010; Patel et al., 2011). The markedly different reports of HPV prevalence in OTSCC may be due to mixed samples with the oropharynx, study sample size, ethnic and geographical differences within the studies, and methodological differences in HPV detection (Hobbs et al., 2006). Together with methodological differences and bias in HPV detection, the topographic definition of oral/tongue cancer is likely of highest relevance in the discrepancy of the results.

Both the oropharynx and the oral cavity include portions of the tongue as one of their anatomical sites. Indeed, the anterior two-thirds of the tongue (the oral tongue) belongs to the oral cavity, while the posterior third (the base of tongue) is part of the oropharynx. HR-HPVs, especially type 16, are frequently detected in squamous cell carcinoma (SCC) of the base of the tongue and are associated with a favourable prognosis. However, the same does not necessarily apply to OTSCC (Dahlgren et al., 2004). Sub-site descriptions might therefore be erroneous in many studies, particularly in US-publications. There is evidence for misclassification of oropharyngeal cancers where the base of tongue or soft palate have been classified as cancers of the “tongue” or “palate” respectively (Myers and Sturgis, 2013). Another misclassification bias is to classify OTSCC as a more generic “oral” cancer, which is too broad a classification to allow causal agents to be specified.

In addition to these considerations of prevalence in different sub-sites, the correlation between HPV infection and prognosis remains unclear. Kozomara reported that more than 60% of OTSCC and SCCs of the floor of the mouth were HPV positive and that this positivity correlated with worse prognosis in terms of shorter overall survival (OS) (Kozomara et al., 2005). On the other hand, Lee et al. showed in their material that 36% of T1-T2 OTSCC were HPV positive and that a shallower stromal invasion could be noticed in these tumours (Lee et al., 2010). Besides, other studies showed a low incidence but significant correlation
between HPV infection and OTSCC, with HPV positivity being a favourable prognostic factor in terms of better overall and disease-specific survival (Mork et al., 2001; Ringström et al., 2002), while other authors showed an irrelevant low incidence and correlation (Koskinen et al., 2003; Liang et al., 2008).

**HPV DETECTION**

Currently, there is no widely accepted “gold standard” for HPV detection. Because cultivation of HPV is not possible up to now, evidence for an infection with HPV can only be found by detection of the viral DNA genome or virally-encoded transcripts. Current methods include HPV DNA direct hybridization (in situ hybridization, ISH), HPV E6/E7 mRNA ISH, consensus and type specific DNA amplification techniques (polymerase chain reaction, PCR), quantitative or real time PCR (qPCR), reverse-transcriptase PCR (RT/PCR), HPV E6/E7 mRNA quantitative reverse-transcriptase PCR (qRT/PCR), and immunohistochemical detection of surrogate HPV biomarkers (immunohistochemistry, IHC) (Singhi and Westra, 2010; Ramqvist and Dalianis, 2011; Bishop et al., 2012; Lewis et al., 2012). PCR technology is frequently used for the detection of HPV DNA and most meta-analyses on HPV and genital diseases are based on PCR as the gold standard. Screening for HPV can be performed using general or consensus PCR primers for HPV, which most frequently targets the \(L1\) gene, allowing detection of several HPV types, followed by type-specific PCRs or sequencing (Licirita et al., 2002; Van den Brule et al., 2002). In addition, in order to show sufficient amount and quality of HPV DNA, a PCR of a control cellular gene is used in parallel. Other more sensitive methods have also been developed such as that of Schmitt et al., who used a sensitive bead-based multiplex method set up for many different HPV types, where the HPV PCR products are coupled to type-specific probes on beads and analysed by Luminex (Schmitt et al., 2006). PCR methods are traditionally more sensitive than ISH but the latter technique is highly specific as it allows for the visualisation of hybridisation signals localised to the host cellular nuclei (Pannone et al., 2011). Compared with PCR methods, ISH is also a more practical tool for the pathologist due to several reasons: the introduction of non-
fluorescent chromogens nowadays allows visualization of DNA hybridization using conventional light microscopy, routinely used on formalin-fixed and paraffin-embedded tissue (FFPE) (Singhi and Westra, 2010), the development of different signal amplification methods has significantly improved the sensitivity of this technique, even to the point to be a more sensitive method than PCR in cases where only a few cells in the sample contain just one viral copy per cell, which is not detectable by PCR (Syrrjanen et al., 2011). In addition, information about morphological abnormalities associated with HPV can be provided (Sand and Jalouli, 2014). On the other hand, because consensus hybridisation probes lack sensitivity and are not usually employed, ISH detects only a single HPV genotype at a time and the use of ISH for prevalence studies is limited by the possible presence of HR-HPV types other than the specific HR-HPV type(s) that have been studied (most commonly HPV16 and HPV18).

However, when using very sensitive techniques for HPV DNA detection, it is important to also assay for the biological activity of HPV, for example by examining the presence of E6 and E7 mRNA, either through qRT/PCR on fresh tumour samples, because, while it is possible to perform this technique on FFPE samples, the maximum accuracy is found using fresh frozen tissue (Lindqvist et al., 2007; Braakhuis et al., 2009; Shi et al., 2009; Schlecht et al., 2011, Pannone et al., 2011), or through the newest and quite expensive HPV E6/E7 mRNA ISH on FFPE samples (Bishop et al., 2012; Lewis et al., 2012; Wang et al., 2012). These RNA-based tests, however, remain high cost, technically challenging and not widely available in clinical practice (Lewis, 2012; Mirghani et al., 2014). Nevertheless, the detection of viral transcripts or proteins is a primary method of detection of oncoviruses in tumours and may also be used to distinguish an active viral influence on tumour maintenance from incidental viral infection. Viral protein expression is indeed essential for viral oncogenesis in all known human oncoviruses.

In cancer samples, HPV can be found in episomal (extra-chromosomal) form, integrated, or in mixed forms of both. Viral integration into the host-cell genome correlates with dysfunction of E1 or E2 open reading frames which are active during HPV replication, and loss of E2 function allows up-regulation of E6 and E7 oncoproteins as E2 is a repressor of E6 and E7. In this way, DNA genotyping alone is not sufficient to clarify the oncogenic role of HPV, but the mRNA expression associated to DNA integration is necessary in the virus-related oncogenesis. Indeed HPV+ but E6/E7 mRNA- OPSCC show a prognosis similar to HPV- cancers (Holzinger et al., 2009).

In the majority of cervical cancers the hypothesis of carcinogenesis implies the integration of HPV into a human chromosome, however it has emerged recently that about 15-30% of cervical cancers may contain HPV episomal DNA as well. It has also been demonstrated that HR-HPV episomal DNA up-regulates expression of E6 and E7. While HPV DNA integration is a well studied topic for cancer in the
cervix, there are still few investigations in HPV-related HNSCC (Sand and Jalouli, 2014) and there are limited data showing viral integration into the genome in OPSCC (Begum et al., 2005): this may indicate an increased prevalence of HPV infection without integration probably due to higher amount of lymphoid tissue in Waldeyer’s ring.

Current literature shows that HPV 16, the genotype that has the highest ability to integrate into the host DNA in cervical cancer, is the almost exclusive genotype in HNSCC (El-Mofty and Lu, 2003). On the other hand, in HNSCCs, particularly tonsillar cancers, HPV DNA type 16 has been detected in integrated as well episomal form (Snijders et al., 1992), and expression of the oncoproteins E6, E7 occurs also when the virus is in the episomal form (Mellin et al., 2002). It is therefore reasonable to describe HPV-related cancerogenesis in HNSCC as heterogeneous and likely constituted of multiple pathways (Koskinen et al., 2003).

**P16**

As aforementioned, another method commonly used in the detection of HPV is immunohistochemistry. Because of the limited amount of antibodies against specific genotypes, IHC is utilised for HPV substitute markers, primarily p16INK4a (p16) since there is an association between the presence of HPV and p16 overexpression (Mellin et al., 2005; Singhi and Westra, 2010; Fisher et al., 2010).

The p16 protein, made by the p16INK4a (CDKN2A) gene located at 9p21, is a cyclin-dependent kinase inhibitor that inhibits pRb phosphorylation. A complex consisting of three different proteins, cyclin D and two cyclin dependent kinases (CDKs) CDK4 and CDK6, phosphorylates pRb. Among the so-called cyclin dependent kinase inhibitors (CKIs), p16 preferentially binds to CDK4 and CDK6, stopping their association with D-type cyclins and accordingly inhibiting phosphorylation of Rb. Inhibition of Rb blocks cell cycle progression at the G1 to S checkpoint (Zhang et al., 1999). When irreversible tumour-producing stimuli are present, some defence systems take place and enable cells to take the road of apoptosis (p14ARF/p53 pathway) or premature senescence (p16INK4a/Rb pathway) (Schmitt et al., 2002; Lleonart et al., 2009). In addition, overexpression of p16 is able to induce apoptosis in a p53-independent manner (Calbo et al., 2001; Modesitt et al., 2001; Calbo et al., 2004).

As previously mentioned, HPV-associated HNSCC depends on the biological roles of E6 and E7 oncoproteins, which inactivate p53 and Rb, respectively, resulting in dysregulation of cell cycle and altered protein expression. E7 inactivates the retinoblastoma protein resulting in p16 overexpression. The abnormally upregulated expression of p16 is a well-known, and, according to many investigators, the best single substitute biomarker for HPV-associated cervical
cancer (Klaes et al., 2001; Weinberger et al., 2006; Lewis, 2012; Narisawa-Saito and Kiyono, 2007).

Accumulation of p16 protein could happen in at least two ways. Firstly, viral oncoproteins target Rb, leaving an excess pool of p16, and, secondly, loss of Rb in itself is an oncogenic stress which can lead to activation of p16 (Witkiewicz et al., 2011).

p16 is expressed in a wide variety of SCCs other than those originating from cervix, such as HNSCC, however its reliability as substitute marker of HPV infection is controversial. Many authors consider it as a reliable surrogate marker for HR-HPV in oropharynx (Kim et al., 2007; Klussmann et al., 2003; Pannone et al., 2012) but not a specific marker of HPV status in non-oropharyngeal HNSCC (Doxtader and Katzenstein, 2012). Even regarding OPSCC, although highly sensitive, p16 specificity remains to be improved as compared to HPV DNA in situ hybridisation, varying according to different studies between 76% and 82% (Singhi and Westra, 2010; Lewis, 2012; Mirghani et al., 2014). Nevertheless, p16 overexpression is strongly correlated with transcriptionally active HPV, making it an attractive method of detection. In contrast, p16 status has been shown to be a poor surrogate for HPV status in oral SCC (Seiwert, 2014; Zafereo et al., 2016). Expression of p16 does not seem to be related to HPV infection in OTSCC either (Kabeya et al., 2012).

A possible reason for the discrepancies of the validity of p16 IHC found in HNSCC can be explained by variability in study protocols, staining methods, evaluation criteria and classification errors (Klingenberg et al., 2010; El-Naggar and Westra, 2012). But, first of all, the specificity of p16 seems to be limited by the presence of p16-positive cancers that are without evidence of HPV DNA or E6/7 expression (Jordan et al., 2012), meaning that some tumours, such as sino-nasal undifferentiated carcinoma (Hoffmann et al, 2012), express p16 in the absence of HPV (Mellin et al., 2005).

The multistep theory of carcinogenesis proposes that HNSCC results from the accumulation of genetic changes in mucosal cells resulting from sustained exposure to carcinogens such as tobacco and ethanol. The consequent genetic instability resulting from deregulation of certain cell cycle proteins results in the temporal transition of mucosal cells from a normal state to dysplasia and eventually to carcinoma. Although the temporal order of genetic alterations preceding overt malignancy is unknown, a genetic progression model of HNSCC (Califano et al., 1996) proposes that p16 (CDKN2A) is the earliest tumour suppressor gene to be inactivated in many human cancers, including HNSCC, whereas deregulation of TP53 and CCND1 (encoding cyclin D1) occurs later. Tobacco/alcohol-associated HNSCC appears to be associated with p16 downregulation (as an early event) and TP53 gene mutations leading to deregulation of cell proliferation and tumourigenesis (Psyrri and DiMaio, 2008; Chao et al., 2008; Paulson et al., 2008; Carnero and Lleonart, 2011). Close to 50%
of all human cancers indeed show CDKN2A gene inactivation (Gonzalez and Serrano, 2006).

The CDKN2A gene can be inactivated through point mutations, homozygous deletions of the locus, loss of heterozygosity of the locus or methylation of the promoter (Gazzeri et al., 1998; Andujar et al., 2010).

The loss of function of p16 at an early stage of carcinogenesis suggests a relevant role in the transformation of normal mucosa to preneoplastic lesions or carcinoma in situ (El-Naggar et al., 1999; Nagai, 1999; Tripathi et al., 2003). However, p16 inactivation has also been described as an intermediate or late event in tumour progression (Fukushima et al., 2002). The downregulation of p16 on one side highlights its role as a tumour suppressor. However, p16 can also be overexpressed in tumours, as in the case of HPV-related neoplasms.

As previously discussed, p16 is overexpressed in HPV-related cancers and significantly better prognosis and less local recurrence were reported for tumours of the head and neck region with overexpression of p16, whereas tumours without p16 overexpression have a sixfold increased risk of local recurrence (Weinberger et al., 2004; Vairaktaris et al., 2007). Moreover, inactivation of p16 is rarely found in HPV-associated tumours (Kim et al., 1998).

The correlation between p16 and prognosis is controversially debated for many HPV-negative tumours. Some studies show that overexpression of p16 relates to poor prognosis in late stage carcinogenesis in neuroblastoma, cervical, ovarian, breast, prostate and oral cancer due to dysfunction in the Rb pathway (Omura-Minamisawa et al., 2001; Li et al., 2011; Stephen et al., 2013). However, loss of p16 is associated with poor prognosis for tumours such as non-small cell carcinoma of the lung, melanoma, nasopharyngeal carcinoma, hepatocellular carcinoma and colorectal carcinoma (Yanagawa et al., 2002; Weinberger et al., 2004). Also for head and neck cancers, controversial results can be found in the literature regarding prognostic relevance of p16. In HPV-positive OPSCC, p16 overexpression is usually associated with improved outcome in terms of decreased local 5-year relapse rate and enhanced 5-year overall survival (Weinberger et al., 2004; Reimers et al., 2007; Liang et al., 2012; Jordan et al., 2012, Cai et al., 2014), while lack of p16 is associated with worse prognosis in HNSCC (Namazie et al., 2002; Worsham et al., 2006; Stephen et al., 2013). However, other studies could not confirm the same prognostic significance of p16 in HNSCC (Geisler et al., 2002; Koscielnly et al., 2007; Yuen et al., 2002).

Lack of p16 in association with poor prognosis (Henshall et al., 2001), p16 overexpression in association with better prognosis (Liang et al., 2012) as well as no prognostic relevance of p16 expression (Smith et al., 2008) have been reported for oral squamous cell carcinoma (OSCC).

It is then reasonable to believe that p16 overexpression can be indicative of two distinct situations: p16 overexpression in benign or pre-malignant lesions, in which overexpression is secondary to oncogene induced senescence; and p16 overexpression in malignant lesions, in which overexpression appears to be a
mechanism to arrest the uncontrolled proliferation caused by failure of the Rb pathway (secondary to viral infection, mutational silencing of the Rb gene, or other mechanisms) (Romagosa et al., 2011).

**EPITHELIAL TO MESENCHYMAL TRANSITION**

Epithelial to Mesenchymal Transition (EMT) is a reversible biological process by which epithelial cells lose their intercellular adhesion (cell-cell and cell-basal membrane contacts) and polarity and acquire features of mesenchymal cells, including increased migratory activity and enhanced production of extracellular matrix (ECM) (Sato et al., 2016; Yeung and Yang, 2017). At least theoretically, this process eases their passage and adjustment to tissue of mesenchymal origin, which is an essential step in the metastatic progression of human cancers. Indeed, these non-polarised mesenchymal cells are highly motile and invasive (Kalluri and Weinberg, 2009; Polyak and Weinberg, 2009) and are able to move to a distant site and re-differentiate to form a new structure.

EMT is classified into three subtypes, and can be seen in embryonic development, wound healing and cancer (Zeisberg and Neilson, 2009). Type 1 occurs in gastrulation and in migration of neural crest cells; some of the migrated cells undergo the reverse process, mesenchymal to epithelial transition (MET), to become epithelial cells in organs produced by the mesoderm and endoderm. Type 2 EMT occurs in wound healing and can result in fibrosis when there is persistent inflammation; cytokines generated by tissue injury induce the fibroblast phenotype from epithelial or endothelial cells. Type 3 occurs in subsets of invasive cancer cells by using some of the Type 2 EMT programme for migration and aggregation of epithelial cells in wound healing.

During type 3 EMT, cancer cells dedifferentiate and acquire motility and a stemness phenotype. Thus, these cells have been suggested as migrating cancer stem cells (CSCs) (Brabletz, 2012). The stemness phenotype comprises certain features such as apoptosis resistance, transient quiescence and self-renewal capacities, therefore long-term maintenance of this state, allowing survival during dissemination, colonisation at the metastatic site and resistance to chemoradiotherapy (Kalluri and Weinberg, 2009; Acloque et al., 2009; Thiery et al., 2009; Sun et al., 2012; Guo and Qin, 2015). In other words, during tumour progression, cancer cells take part in the process of EMT, which is a highly conserved biological mechanism that allows cancer cells to resemble stroma-like cells, like “wolfs in sheep’s clothing”. At the same time, in the process of EMT, cancer cells acquire a stronger ability to invade and metastasize, and to produce resistance to various treatments (Zhang et al., 2016).

A typical hallmark of EMT is the so called “cadherin switch”, characterised by downregulation of E-cadherin (epithelial cadherin) and cytokeratins (CKS), and
upregulation of N-cadherin (neural cadherin) (Gheldof and Berx, 2013). High expression of N-cadherin correlated with malignant behaviours such as a high-grade pattern of invasion, poorly differentiated cancer cells and lymph node metastasis. Thus, cadherin switching may be a critical event in the progression of HNSCC through EMT (Nguyen et al., 2011). In addition, up-regulation of mesenchymal proteins, such as fibronectin, alpha smooth muscle actin (αSMA) and vimentin guides the transition toward a mesenchymal state, facilitating both enhancement of cell motility and formation of new membrane protrusions such as invadopodia for ECM degradation (Sun et al., 2012; Leong et al., 2014). Finally, increased expression of metalloproteinases results in cell migration and invasive behaviour (Nieto and Cano, 2012). It is to note that disassembling adherens junctions, desmosomes and tight junctions leads to loss of apical-basal polarity, and also regulates a number of signaling pathways through their associated proteins (like β-catenin) that can further promote EMT and invasion (Gonzalez-Moles et al., 2014). Various signaling pathway such as TGF-β1/Smad, Wnt, NOTCH and growth factor receptor tyrosine kinases (EGF, FGF and HGF) have been implicated in the induction of EMT (Ghahhari and Babashah, 2015; Inamura et al., 2016; Malfettone et al., 2017). These signals are able to induce the activation of some EMT transcription factors, such as TWIST, SNAIL, Slug and ZEB1/2, that can bind to specific E-box regulatory regions that are present in several target genes, and regulate the expression of E-cadherin, vimentin, fibronectin and N-cadherin (Battle et al., 2000; Zhu et al., 2016; Diaz et al., 2016). These factors seem to play oncogenic roles such as promotion of tumour initiation and suppression of apoptosis and senescence, suggesting a potential intersection between EMT and other hallmarks of cancer (Puisieux et al., 2014). EMT events then require the coordinated expression of several sets of genes and signaling pathways, many of which have demonstrated the ability to regulate specific aspects of malignant transformation and cancer progression in preclinical models. In addition, although not fully understood, it seems that podoplanin, a small mucin-like transmembrane protein, is able to induce EMT in an independent manner, without the expression of conventional EMT transcription factors like SNAIL (Renart et al., 2015).

Although the EMT process has been extensively studied in laboratory procedures, some studies on human samples have raised questions about a possible use of EMT as a clinically relevant predictor on human samples because of its transient behaviour, lacking direct evidence for the existence of this process (Tarin et al., 2005; Savagner et al., 2010; Ledford, 2011). The evidence for the presence of EMT in cancer has anyway accumulated and most cancer cell biologists accept the EMT hypothesis, with only a few researchers arguing against the phenomenon. The main reasons for the difficulties in detection of EMT reside in cell plasticity and the transient nature of the EMT process. In addition, it is a challenge to distinguish EMT cells from stromal fibroblasts by light microscopy alone (Jensen et al., 2015).
E-CADHERIN

Epithelial-cadherin (E-cadherin) is a 120 kDa transmembrane glycoprotein that plays a main role in cell adhesion. It is a Ca$^{2+}$-dependent cell-surface protein characterized by long cytoplasmic and extracellular domains which create hemophilic interactions between adjacent cells to allow adhesion (Zeisberg and Neilson, 2009; Scanlon et al., 2013). The E-cadherin encoding gene (CDH1) is traditionally known as a tumour suppressor gene; its reduced expression is seen in the vast majority of epithelial cancers, causing tumour invasiveness and leading to worse patient prognosis. The reduction of E-cadherin expression can be caused by loss of heterozygosity (LOH), inactivating mutations, but mostly depends on hypermethylation of the CDH1 promoter or silencing of CHD1 expression by specific transcription factors (upregulation of transcriptional repressors: Snail, Twist and SIP1) (Sakamoto et al., 2012). Altered methylation patterns have been found in primary OSCCs as well as in their corresponding metastatic and recurrent tumours. An interesting characteristic of CDH1 methylation is that it is reversible: the hypermethylated gene can be demethylated returning to express E-cadherin (González-Moles et al., 2014). In this way, once cancer cell migration and stromal invasion is accomplished, E-cadherin can be re-expressed. This process can reverse the transformed mesenchymal cells to an epithelial phenotype (MET) which recovers cellular adhesiveness and facilitates cell proliferation and generation of secondary locations. Indeed, several reports have shown increased or similar E-cadherin levels in metastatic sites compared with their primary tumours (Scanlon et al., 2013; Gall and Frampton, 2013).

β-CATENIN

β-catenin is a protein belonging to the Armadillo family characterized by multiple cellular functions depending on its cellular localisation. As mentioned above, E-cadherin is linked to the cytoskeleton through β-catenin (Strumane et al., 2004; Nelson, 2008; Attramadal et al., 2015); such protein complex promotes cell to cell adhesion and prevents the cell dissociation required for cancer invasion and metastases. In oral epithelium therefore, detachment of this protein complex, as occurs in invasion, implies that E-cadherin is endocytosed and released into the cytoplasm, where it can be degraded by normal physiological mechanisms. In the cytoplasm, in the absence of WNT signals, β-catenin is phosphorylated by a multi-protein complex consisting of Axin, APC, CK1 and GSK3. The action of this complex prevents it reaching the nucleus and activating transcription of target genes. In this first scenario, the oncogenic activity associated with the loss of E-cadherin/β-catenin activity involves loss of cellular adhesiveness and gain of invasiveness, and it does not require aberrant activation of the canonical Wnt
pathway. These cases would show complete or partial loss of E-cadherin/β-catenin on the membrane with the absence of both cytoplasmic and nuclear β-catenin expression due to the lack of canonical Wnt activation (Lo Muzio et al., 2009).

Loss of membranous β-catenin expression can also be associated with its cytoplasmic/nuclear expression (Lo Muzio et al., 1999). In this situation, the causes of partial or complete loss of membranous β-catenin expression are identical to those reported above, while the cytoplasmic accumulation of β-catenin requires the failure of cytoplasmic degradation mechanisms for this protein. One of the acknowledged causes of β-catenin stabilisation in the cytoplasm is, as previously mentioned, the activation of the canonical Wnt pathway secondary to the interaction of Wnt proteins with their cell membrane receptors independently of E-cadherin expression. This activation takes place in the early stage of oral carcinogenesis and is able to disrupt the complex and inhibit the cytoplasmic phosphorylation of β-catenin avoiding its degradation (González-Moles et al., 2014).

β-catenin translocation from the cytoplasm to the nucleus is the final step of the canonical Wnt pathway and facilitates the transcription of β-catenin target genes, EMT genes, such as TWIST (Strumane et al., 2004). This is a transcription factor that plays a key role in the progression of a primary tumour to the metastatic stage. Twist promotes loss of cell-to-cell adhesion, causing an increase in cell motility, through an important negative regulation of the E-cadherin and the upregulation of mesenchymal markers like vimentin, fibronectin and N-cadherin (de Freitas Silva et al., 2014).

In OTSCC, it has been found that the cytoplasmic expression of β-catenin correlates with poor differentiation and advanced stages (Zhang et al., 2015). However, no correlations have been found between its expression and the occurrence of lymph-node metastasis (Freitas et al., 2010; Zhang et al., 2015).

**CYTOKERATIN 5 (CK5) and CYTOKERATIN 19 (CK19)**

The cytokeratin family comprises a group of more than 20 intermediate filament proteins (IFPs) expressed during normal epithelial differentiation as part of the cytoskeleton (Moll et al., 1982; Moll et al., 1990). IFPs are major components of the mammalian cytoskeleton and contribute to the physical strength and signal transduction in the cells (Lowery et al., 2015; Herrmann and Aebl, 2016). CKs 1-8 belong to the type 2 IFPs and are characterized by a molecular weight of 40-64 kDa, while CKs 9-20 belong to the type 1 group weighing 52-68 kDa (Kirfel et al., 2003).
CK5 is ubiquitously expressed in HNSCC paired with cytokeratin 14 and localised in the keratinised and non-keratinised epithelium (van der Velden et al., 1993; Vasca et al., 2014). It has been suggested that positive immunostaining for both p63 and CK5/6 in poorly differentiated metastatic carcinomas is highly predictive of a primary tumour of squamous epithelial origin (Kaufmann et al., 2001). CK5 expression seems also able to predict the difference between OSCC and squamous intraepithelial neoplasm (SIN) (Khanom et al., 2012). More interestingly, CK5 positivity seems to be helpful in the prediction of detection of cervical micrometastases in head and neck cancer samples (Becker et al., 2004).

CK19 is expressed in a wide range of epithelial tissues (Bloemendal and Pieper, 1989). Normally, it is expressed in the basal layer of stratified epithelia, but it seems to increase in dysplastic and neoplastic epithelia of the oral mucosa (Cintorino et al., 1990; van der Velden et al., 1993; Zhong et al., 2006). CK19 is expressed in about 30% of OTSCC with higher frequency in advanced stages and node positive patients (Vora et al., 2003). It is noteworthy that expression of CK19 in OTSCC has been identified as a negative predictor for overall survival and disease specific survival (Ernst et al., 2016).

LYMPH NODE METASTASIS

Despite advances in surgery and radiation therapy, the 5-year survival rate for oral cancer, and specifically OTSCC, has not improved significantly over the past three decades and remains at 50–55% (Reies et al., 1991; Silvermann, 2001). This is primarily because of the presence of lymph node metastases (Kalnins et al., 1977; Schuller et al., 1980; Snow et al., 1982; Grandi et al., 1985) though metastatic disease at distant sites, local recurrence and second primary tumours are also causes of death in these patients (Sano and Myers, 2007). The peculiar early and intense neck metastasis which characterizes OTSCC likely depends on the presence of a rich lymphatic network which also crosses the midline. This eases spread to the contralateral side as well as a high number of lymph nodes in the neck region favoured by the muscular structure of the tongue (Forastiere et al., 2001; Clark et al., 2006; Kuriakose and Trivedi, 2009). This frequent spread to lymph nodes from OTSCC emphasises the importance of early detection and/or stronger prognosis of nodal metastasis in order to reach a successful treatment.

Even without any clinical or instrumental signs of metastasis, up to 40% of all OTSCC show occult metastasis having histologically detectable spread to lymph nodes (Teichgraeber and Clairmont, 1984; Fakih et al., 1989; Cunningham et al., 1986; Ho et al., 1992; Lydiatt et al., 1993; Yuen et al., 1999; Po et al., 2002; Yu and
Recurrence occurs in approximately 23% of T1 OTSCCs and is primarily regional rather than local (Lim et al., 2006). Once regional disease occurs, prognosis is significantly worsened, decreasing to about 30% (Ho et al., 1992; Leemans et al., 1993). The finding of extracapsular spread of cervical lymph node metastasis of OTSCC is associated with even higher rates of regional and distant failure (Hirabayashi et al., 1991; Alvi and Johnson, 1996; Brasilino de Carvalho, 1998; Myers et al., 2001; Greenberg et al., 2003; Abu-serriah et al., 2015). Conceptually, the metastatic “cascade” is multifactorial and comprises the loss of cancer cell adherence at the primary site, cell motility, proteolysis of the extracellular matrix and basal membrane, invasion of local stroma, entry of vascular and lymphatic vessels (intravasation), extravasation, recolonisation, and expansion into distant sites (Cavallaro and Christofori, 2004; Howell and Grandis, 2005; Thompson et al., 2005). Gene products in these steps can be used as prognostic markers of neck metastasis.

Lymphatic invasion by tumour cells and tumour metastasis to regional lymph nodes (LN) indicate a poor prognosis, however, the biological behaviour of human lymphatic vessels within tumour lesions is controversial (Jain and Fenton, 2002). Whether de novo lymphangiogenesis is necessary for lymphatic spread of naturally occurring cancers is not known, because tumour cell invasion of pre-existing lymphatics at the tumour margins might also occur; besides cancer cells may invade into blood vessels and then reach lymphatic vessels via shunts (Koukourakis et al., 2005). Therefore, the moment when lymphangiogenesis (the generation of new lymphatic vessels from pre-existing lymphatics or lymphatic endothelial progenitors) occurs during the natural evolution of neoplasia is still incompletely described and in some studies lymphangiogenesis has been demonstrated as early as the stage of a premalignant lesion (Eichten et al., 2007).

Nevertheless, the use of relatively new specific lymphatic endothelium markers, such as podoplanin, lymphatic vessel endothelial hyaluronan receptor-1, and prox-1, enables differentiating lymphatic from blood vascular endothelium (Breiteneder-Geleff et al., 1999; Karaman and Detmar, 2014). This facilitates widespread investigations of lymphatic vessel involvement in human cancers, and it is universally accepted nowadays that lymphatic vessels and lymphangiogenesis do not only play a passive role in tumour metastasis, serving merely as channels for tissue-invading tumour cells (Matsumoto et al., 2010). Lymphatic vessels, conversely, contribute in many ways to metastasis through the vascular endothelial growth factors (VEGFs), predominantly lymphangiogenic, which are activated via the VEGF receptor 3, expressed in lymphatic endothelial cells (Ruddle, 2014), by several oncogenic products; targeted tumour cell recruitment and migration is in this way facilitated by lymphangiogenesis, which also contributes to modulating the anti-tumour immune responses at the level of
the primary tumour and the metastatic lymph node (Skobe et al., 2001; He et al., 2004).

Lymphatic vessels have been demonstrated in tumoural and peritumoural areas, and their number correlates with prognosis in malignant melanomas (Straume et al., 2003), breast cancers (Schoppmann et al., 2001), gastric adenocarcinomas (Yonemura et al., 2001), HNSCC (Beasley et al., 2002; Sun et al., 2012) and OSCC (Miyahara et al., 2007). Moreover, lymphatic vessel density (LVD) apparently represents a major prognostic element for lymph node metastasis.

Peri-tumoural lymphatics seem to be significantly larger than intra-tumoural ones, but with a significantly lower density (Ji, 2006), although other studies demonstrate that the number of intra-tumoural and peri-tumoural lymphatic vessels is similar in oral cancer (Kyzas et al., 2005; O’Donnell et al., 2008). The predictive value of lymphatic vessel localisation in SCC has been shown to be controversial. Despite the fact that a correlation between intra-tumoural LVD and LN metastasis has been demonstrated (Beasley et al., 2002; Muñoz-Guerra et al., 2004), it is a common opinion that the lymphatics located in the centre of a tumour are not functional (Padera et al., 2002) and that lymph node metastasis is achieved via an increased number and size of peri-tumoural lymphatic vessels. This provides more opportunities for lymphatic invasion of cancer cells and might also promote tumour spread by increased pumping and lymph flow, often mediated by VEGF-C (Schoppmann et al., 2001; Jain and Fenton, 2002; Franchi et al., 2004; Harrel et al., 2007; Breslin et al., 2007; Proulx et al., 2010; Alitalo and Detmar, 2012).

Tumour-derived secretory factors also promote the formation of a pre-metastatic niche for cancer cells with stem cell–like properties, a kind of protective and permissive microenvironment in the lymphatic endothelium for long-term tumour cell survival and growth leading to the manifestation of the so-called “in-transit metastases” prior to tumour cell colonisation and dissemination in the LN (Jurisic et al., 2012; Sceneay et al., 2013). Indeed, pre-metastatic niches, which are now widely accepted as true biological processes promoting metastatic growth (Dawson et al., 2009; Duda and Jain, 2010; Karaman and Detmar, 2014), depend on tumour mediated mobilisation and recruitment of bone marrow-derived cells transported by lymphatics that interact with the local stroma and extracellular matrix. Tumour secreted lymphangiogenic factors promote the so-called sinusoidal hyperplasia, the enlargement of lymphatic networks in the stroma and inside the sentinel lymph node (SLN), the first node of the locoregional LN district draining tumour cells (Hirakawa et al, 2007; Hirakawa, 2009).

Neo-lymphangiogenesis is therefore not restricted to the primary tumour microenvironment but also occurs in metastatic LNs, and, importantly, could be detected prior to the arrival of tumour cells and their actual direct visualisation in the lymphatic metastasis by standard methods; this assumption leads to a potential strategy for early detection of metastases through the utilisation of
antibodies against lymphatic vasculature markers (Hirakawa et al., 2005; Hirota et al., 2012; Wakisaka et al., 2015).

Tumour-induced loco-regional lymphangiogenesis then promotes further metastasis to distant LNs and beyond distant organs (Hirakawa et al., 2007). Still it remains controversial whether LN metastases represent mediators of systemic metastasis to distant organs or whether they are mere indicators of cancer aggressiveness (Sleeman et al., 2011).

The evidence for tumour and LN lymphangiogenesis representing important prognostic markers for the risk of future metastasis and lower overall survival (Alitalo and Detmar, 2012), makes it worthy to include the quantitative determination of lymphatic vessel activation within primary tumours and SLNs in routine pathological examinations. However, there are at present no standardised methods established for reproducible quantification of lymphatic vessels in tissue sections (Van der Auwera et al., 2006).

**PODOPLANIN**

Tumour cells are known to invade as single cells through the EMT process, or collectively. Whilst EMT has been thoroughly studied, collective invasion is less well understood. Podoplanin is a protein playing a role in EMT, making tumour cells acquire increased motility and a fibroblast-like phenotype through remodelling their actinic cytoskeleton (Martín-Villar et al., 2005; Moustakas and Heldin, 2007; Wu et al., 2016; Takemoto et al., 2017) but also in collective invasion, by inducing the formation of filopodia (Wicki et al., 2006). Podoplanin is a small mucin-like transmembrane heavy glycosylated protein which plays an important role during embryogenesis (Nose et al., 1990; Ugorski et al., 2016) and is involved in the physiology of the immune system (Astarita et al., 2012; Peters et al., 2015). In addition, it is involved in the development of kidneys and lungs as well as accurate formation of the lymphatic vascular system (Fukunaga, 2005; Gupta et al., 2014; Xia et al., 2016; Carrasco-Ramirez et al., 2016). Podoplanin is expressed in lymphatic endothelial cells, but not in blood endothelial cells, therefore, it is a specific marker for lymphatic endothelium (Agarwal et al., 2014). The use of antibodies against this lymphatic marker, such as the monoclonal anti-podoplanin clone D2-40, which was originally raised against an onco-fetal antigen in testicular germ cell tumours (Marks et al., 1999), represents a reliable approach for the identification of lymphatic vessels in most human tissues (Schacht et al., 2005; Yonemura et al., 2006; Michikawa et al., 2012), although it has to be kept in mind that podoplanin is also expressed by several non-vascular cells including myoepithelial cells (Cueni and Detmar, 2008).

A wide variety of tumours express podoplanin, both in the malignant cells and in the stroma, such as malignant mesothelioma (Kimura and Kimura, 2005), testicular carcinoma in situ and several germ cell tumours (Marks et al., 1999;
Kato et al., 2004; Mishima et al., 2006), some cerebral tumours (Mishima et al., 2006), cutaneous squamous cell carcinoma (Canueto et al., 2017) and vascular tumours including Kaposi’s sarcoma (Marks et al., 1999; Ordonez, 2014). In many cancers, the role of podoplanin has been associated with tumour progression and metastasis and, therefore, with poor prognosis (Swain et al., 2014).

Several studies have revealed altered podoplanin expression also in HNSCC (Chuang et al., 2013) and specifically in OSCC demonstrating a correlation between high expression level of podoplanin and lymph-node metastasis (Kato et al., 2005; Martin-Villar et al., 2005; Yuan et al., 2006). The overexpression of podoplanin has also been reported in oral hyperplastic and dysplastic lesions and premalignacies, such as oral leukoplakia, oral erythroleukoplakia, carcinoma in situ and lichen planus (Zhang et al., 2009; Funayama et al., 2011; Kreppel et al., 2012; Feng et al., 2012; de Vicente et al., 2013) but not in the normal epithelium, and interpreted as an event occurring early in tumourigenesis (Yuan et al., 2006).

Several investigators have reported that a high level of D2-40-positivity in the peri-tumoral area of oral carcinoma is associated with cervical lymph node metastasis (Longatto et al., 2007; Zhao et al., 2008). On the other hand, high rate of intra-tumoral lymphatic vessel density has shown to strongly correlate with the onset of lymph node metastasis (Chung et al., 2010).
Aims

- To address the potential role of the high-risk Human Papillomavirus types in development of OTSCC especially referring to OTSCC in young patients
- To evaluate the correlation of p16 expression and HPV infection in OTSCC
- To investigate the pattern of expression of the EMT related markers E-cadherin, β-catenin, CK5 and CK19 in OTSCC
- To address the significance of podoplanin expression in the loco-regional metastatic process of OTSCC
- To identify possible differences in tumour biology and patient outcome in correlation to age, sex, and geographical distribution
Materials and Methods

PATIENTS

The patient material comprised 131 patients with primary squamous cell carcinoma of the oral tongue (OTSCC). The majority of the patients, 96, were retrieved from the archives at the Pathology Unit at the University Hospital in Umeå, and the remaining 35 patients from the Second University of Naples. Of these 131 patients 109 were included in the study of p16, 129 in the study of podoplanin and 120 in the study of E-cadherin, β-catenin, CK-5 and CK-19 (Table 1). Ethical permission had been granted for inclusion in the studies.

<table>
<thead>
<tr>
<th></th>
<th>Country</th>
<th>Total number of patients</th>
<th>Age range in years, (mean)</th>
<th>Sex female/male</th>
<th>Follow up range in months, (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P16</td>
<td>96</td>
<td>13</td>
<td>19-93 (63.55)</td>
<td>55/54</td>
<td>1-179 (45.54)</td>
</tr>
<tr>
<td>EMT</td>
<td>87</td>
<td>33</td>
<td>19-93 (63.35)</td>
<td>60/60</td>
<td>1-179 (47.07)</td>
</tr>
<tr>
<td>Podoplanin</td>
<td>94</td>
<td>35</td>
<td>19-93 (63.42)</td>
<td>64/65</td>
<td>1-179 (46.56)</td>
</tr>
</tbody>
</table>

Table 1 Overview of clinical data including nationality of the patients in the different studies

In paper I, looking at expression of the syndecan-1 receptor, an additional 65 patients with primary squamous cell carcinoma of the tonsil were included. These patients were all retrieved from the Pathology Unit at the University Hospital in Umeå.

IMMUNOHISTOCHEMISTRY

All immunohistochemical stainings were performed using a Ventana staining machine (Ventana Medical Systems Inc, Tucson, AZ, USA) according to the supplier’s recommendations. Antibodies used, dilutions, and pretreatments are given in Table 2.
Table 2 Antibodies used, dilutions, and pretreatments

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Company</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>P16</td>
<td>1:200</td>
<td>Santa Cruz Technology, USA</td>
<td>Tris-EDTA, pH 8.0</td>
</tr>
<tr>
<td>Syndecan-1</td>
<td>1:100</td>
<td>Abcam, Cambridge, UK</td>
<td>Citrate buffer, pH 6.0</td>
</tr>
<tr>
<td>E-cadherin (M3612)</td>
<td>1:25</td>
<td>DAKO, Agilent, USA</td>
<td>Tris-EDTA, pH 8.0</td>
</tr>
<tr>
<td>Syndecan-1</td>
<td>1:100</td>
<td>Abcam, Cambridge, UK</td>
<td>Citrate buffer, pH 6.0</td>
</tr>
<tr>
<td>E-cadherin (M3612)</td>
<td>1:25</td>
<td>DAKO, Agilent, USA</td>
<td>Tris-EDTA, pH 8.0</td>
</tr>
<tr>
<td>Syndecan-1</td>
<td>1:100</td>
<td>Abcam, Cambridge, UK</td>
<td>Citrate buffer, pH 6.0</td>
</tr>
<tr>
<td>E-cadherin (M3612)</td>
<td>1:25</td>
<td>DAKO, Agilent, USA</td>
<td>Tris-EDTA, pH 8.0</td>
</tr>
<tr>
<td>β-catenin</td>
<td>1:1500</td>
<td>SIGMA-Aldrich, Tris-EDTA, pH 8.0</td>
<td>Tris-EDTA, pH 8.0</td>
</tr>
<tr>
<td>CK5</td>
<td>1:100</td>
<td>Novocastra, Germany</td>
<td>CC1-buffer</td>
</tr>
<tr>
<td>CK19</td>
<td>1:50</td>
<td>DAKO, Agilent, USA</td>
<td>TBE-buffer, pH 8.4</td>
</tr>
<tr>
<td>Podoplanin (D2-40)</td>
<td>1:25</td>
<td>Abcam, Cambridge, UK</td>
<td>Tris-EDTA, pH 8.0</td>
</tr>
</tbody>
</table>

Table 2 Antibodies used, dilutions, and pretreatments

For evaluation of staining results a quick score system (Detre et al., 1995) was used, see table below (Table 3).

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Percentage of tumour cells stained</strong></td>
<td><strong>Staining intensity</strong></td>
</tr>
<tr>
<td>0</td>
<td>Negative</td>
</tr>
<tr>
<td>1</td>
<td>0-4</td>
</tr>
<tr>
<td>2</td>
<td>5-19</td>
</tr>
<tr>
<td>3</td>
<td>20-39</td>
</tr>
<tr>
<td>4</td>
<td>40-59</td>
</tr>
<tr>
<td>5</td>
<td>60-79</td>
</tr>
<tr>
<td>6</td>
<td>80-100</td>
</tr>
</tbody>
</table>

Table 3 Quick score system by Detre *et al*.

A quick score (QS) was achieved for each case by multiplying results from A with B, giving a number between 0-18. Scoring for each factor was, with the exception of syndecan-1 and 40 cases for CK5 and CK19, made by several authors and results compared until agreement was achieved. The exceptional cases mentioned were scored by one H&N pathologist (KN) only (Figure 1).
IN SITU HYBRIDISATION OF HPV 16

All cases positive for p16, and 35 that were negative, were further analysed for presence of HPV 16 using in situ hybridisation. A cervical sample was included as positive control.
**QUANTITATIVE REVERSE TRANSCRIPTASE PCR**

In order to quantify the amount of podoplanin in the samples analysed, so called quantitative reverse transcriptase PCR (qRT/PCR) was used, amplifying the product of interest in parallel with a reference gene used for normalisation of the achieved values. In our case we used the mean of four reference genes to normalise against.

**STATISTICAL ANALYSIS**

For statistical analysis the SPSS (Statistical Pack for Social Sciences, Inc. Chicago, IL) version 22 was used. A threshold of p-value < 0.05 was considered statistically significant. For the analysis of correlation the Spearman coefficient and the Chi-squared test were used. For survival analysis Kaplan-Meier curves were plotted and Log Rank (Mantel Cox) test used for examining differences among groups in univariate analysis. While, for multivariate analysis the Cox Regression model was used. In addition, for the analysis of mRNA levels the Mann-Whitney test was used.
Results and Discussion

**TUMOUR STAGING, AGE AND SEX**

Results from our studies show a statistically significant correlation between patients suffering from an extended lesion and both lower survival rate and disease-free status. These data are totally in agreement with the well and universally accepted knowledge of the tumour stage grouping according to TNM categories as a reliable start point to predict prognosis of cancer patients (Wittekindt et al., 2012).

Analysing data on the basis of age at cancer onset, we have not found any statistical difference in univariate analysis (log Rank p-value = 0.322) for overall survival (Figure 2).

<table>
<thead>
<tr>
<th>Overall Comparisons</th>
<th>Chi-Square</th>
<th>df</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log Rank (Mantel-Cox)</td>
<td>2.264</td>
<td>2</td>
<td>.322</td>
</tr>
</tbody>
</table>

Test of equality of survival distributions for the different levels of Age.

Figure 2: Kaplan-Meier curve related to patients age at diagnosis and overall survival
However, adjusting for covariates as sex, lymph-node metastasis, relapse, staging and grading, in a multivariate Cox Regression analysis we have found that age could be considered an independent prognostic factor (p = 0.038; HR = 1.83, CI [1.03 - 3.27]), with younger patients showing a worse survival rate (Figure 3).

Figure 3: Cox Regression analysis for age

As previously mentioned, cancer databases from US and Europe show a clear increase during the past two decades of OTSCC among young patients, primarily of caucasian ethnicity. Thanks to the global anti-smoking campaigns, which are probably responsible for the decrease of the typical smoking-related middle- and older age cancers, younger patients suffering from OTSCC often lack the classical HNSCC risk factors such as tobacco and/or alcohol use (Annertz et al., 2002; Llewellyn et al., 2003; Patel et al., 2011).

The causes for this increasing incidence in young people still remain unknown. OTSCC may not genomically differ when comparing patients in young age (≤40 years) with older patients (Pickering et al., 2014), therefore, according to some researchers, there must be factors triggering a genomically and clinically similar disease both in young non-smoking patients as well as in older patients with a history of smoking and alcohol consumption. On the other hand, some authors hypothesize that OTSCC at younger age, an epidemiologically distinct disease, is also genomically distinct, especially with respect to alterations caused by smoking (Sebastian et al., 2014; Knopf et al., 2015). Carcinomas at young age tend to be clinically and histologically distinct from head and neck cancers in other age-groups, and are reported to have an aggressive clinical phenotype, with higher N stage and more perineural invasion often requiring intensive multimodality treatment (Garavello et al., 2007).

While in the whole group of HNSCC tumours there is no difference in prognosis between young and “normal aged” patients (Goldstein and Irish, 2005), patients
younger than 40 years affected by OTSCC seem to have a more advanced disease at presentation, and to be at higher risk for regional metastases, recurrence and death than matched older patients (Hilly et al., 2013). Besides, recurrent disease is more aggressive, with a fatality rate of 100% (Hilly et al., 2013).

In the present study, analysing the same regression model as for the variable age (Figure 3), we saw that sex is not an independent prognostic factor (p = 0.173; HR = 1.70, CI [0.79 - 3.66]).

**HPV16 INFECTION and SYNDECAN-1 and p16 EXPRESSION**

By utilizing *in situ* hybridisation technique for the detection of HPV16, none of the tongue samples analysed showed evidence of viral presence. Out of the 109 tumour specimens, 33% were positive for p16 expression, and 100% of the 89 OTSCCs analysed for syndecan expression were positive, with 82% having a QS of 6–18. The expression of the primary receptor for HPV, syndecan-1, did therefore not differ between these OTSCC and the previously studied group of tonsillar SCC, with a verified high percentage, 91%, of HPV infection (Loizou et al., 2015). Besides, there was no statistically significant correlation between expression of p16 and syndecan-1. These data therefore suggest that HPV16 is either not present or is present at extremely low levels in the majority of p16-positive OTSCCs, including those arising in young patients.

As previously underlined, HPV infection has been demonstrated in OPSCC, where its expression has also been associated with better survival (Lohaus et al., 2014). Despite the clinical presentation, HPV association with DNA integration, indeed, turned out to be an even better prognostic marker in comparison to neck metastasis (Straetmans et al., 2009). It has been suggested that HPV-related HNSCC may constitute a different type of HNSCCs than those related to alcohol and tobacco (Braakhuis et al., 2004; Blomberg et al., 2011; Pannone et al., 2011) with predominance of wild type of p53 (Wiest et al., 2002), and different gene signatures in the early phase of carcinogenesis (Braakhuis et al., 2004). The HPV-related HNSCC show a better response to radiotherapy. Notably, surgery followed by irradiation reveals no difference in prognosis when compared to chemoradiation alone (Straetmans et al., 2009).

The increase in HPV infection seen in industrialised countries occurring in the past few decades in younger generations seems to be related to changes in sexual behaviour and increased oral sex (D'Souza et al., 2009; Nguyen et al., 2010). Beside the now well-recognised and widely reported association between HR-HPV and tonsil SCC (Klussmann et al., 2001; Shiboski et al., 2005), HPV has recently been found in a small percentage of squamous cell carcinoma from the base of the tongue (Garnaes et al., 2015; Garnaes et al., 2016).
Considering the anatomical continuity between oropharynx and oral cavity with both including a portion of the tongue, and considering the increasing incidence of OTSCC in young adults cannot be explained by ordinary carcinogens for oral cancer, smoking and alcohol consumption, as young people have less cumulative exposure to carcinogens than older people (Atula et al., 1996; Myers et al., 2000; Salem et al., 2010), a role for HR-HPV has been hypothesised.

Many investigators have reported HPV infection rates in OTSCC ranging from 0% to 100% (Bouda et al., 2000; Mork et al., 2001; Kansky et al., 2003; Dahlgren et al., 2004; Liang et al., 2008; O'Regan et al., 2008, Lee et al., 2010). The remarkable difference in HPV prevalence among studies probably depends on methodological bias with non-uniform methods used for HPV detection. Different studies have used different experimental procedures such as ISH, southern blot hybridization, IHC and PCR. Brush, biopsy or surgical removal have been used for sampling and fresh-frozen or formalin fixed paraffin embedded (FFPE) tissue have been stored. Those assays that merely detect the presence of HPV, including passenger virus and viral contaminants, may yield incident rates that highly inflate the presence of clinically relevant virus. Furthermore, in the literature, differences exist in stringency for defining the anatomic tumour site. For example, the sub-site oral tongue has been investigated alone (not grouped into the larger oral cavity cancer field) just in a few publications. Besides, there is in literature a common practice of combining carcinomas of the oral tongue and base of tongue which would certainly increase the incidence of HPV-related oral tongue carcinoma. The higher viral prevalence in OTSCC thus probably derives from an overestimation. Interestingly, while Syrjänen in a recent meta-analysis showed a strong association between HPV and oral potentially malignant lesions and oral carcinoma (Syrjanen et al., 2011) and while Sand and Jalouli in a review published in 2014 stated that there seems to be enough evidence to claim that up to 35% of all OSCC are caused by HPV (Sand and Jalouli, 2014), the majority of publications investigating the oral tongue sub-site alone show just a low or absent HPV presence in OTSCC (Liang et al., 2008; Combes and Franceschi, 2014; Poling et al., 2014; Kabeya et al., 2012).

Indeed, unlike OPSCC, tumours of the oral cavity and in particular oral tongue carcinomas (which comprise the major subgroup of oral cavity tumours) seem to rarely harbour HPV, even though p16 expression is frequently present (Hilly et al., 2013). Particularly among young non-smoking patients, these tumours are not HPV-related (Myers et al., 2000; Patel et al., 2011; Harris et al., 2010; Harris et al., 2011). This finding reiterates the importance of investigating tumours of different sub-sites separately, for example base of tongue versus OTSCC.

The reason for the predominance of HPV infection in cancer of Waldeyer’s ring probably is due to the anatomo-physiological differences in the tonsils with their lymphoepitelial character in comparison to the squamous epithelial mucosa of
the oral cavity sites (Hoffmann et al., 2012). In fact, the mobile tongue is covered by both keratinised and non-keratinised squamous epithelium, whereas the tonsils are coated with stratified, non-keratinised squamous epithelium. The mucosa in the tonsillar area is morphologically in close contact with the lymphatic tissue of Waldeyer’s ring. A unique, reticular stratified epithelium with basaloid differentiation covers the invaginations in the depth of the crypts and enables transport and direct contact to lymphatic tissue. Basal cells and basal membrane are partially permeable and admit passage of lymphocytes and antigen presenting cells at the bottom of the epithelium (Pai and Westra, 2009). A breach in the continuity of the epithelium could explain the susceptibility of the tonsils to infection with viruses and bacteria. The relatively easy accessibility of the invaginated, mono-layered crypt epithelium and the presence of cytokines produced by the lymphoid tissue, which can stimulate viral transcription and cellular transformation, are the likely explanation for the high incidence of HPV infection in the oropharynx (Klussmann et al., 2001). HPV 16 is indeed found primarily in cancers originating from locations with inflammatory activity, such as the cervix, the tonsil and the base of tongue. To be speculative, one can suggest that HPV-infected cells, in the vicinity of immunologically competent cells that may respond frequently to various foreign antigens by inducing growth factors, may also be stimulated to divide, which could facilitate tumour development (Dahlgren et al., 2004).

The similar distribution of syndecan-1 expression in tonsil SCC and OTSCC may also lead to speculation that the high frequency of HPV-related OPSCC could depend on the rich lymphoid-rich environment of the oropharynx harbouring other types of viruses and thus some kind of viral coinfection such as HPV/Epstein-Barr virus (Hermann et al., 2004; Sand and Jalouli, 2014; Jiang et al., 2015).

In summary from today’s state of knowledge, the reason for a strong association between HPV-infection and tonsillar carcinoma seems to be primarily based on susceptibility, due to the special microanatomy of the tonsil and tongue base. The results of the present study indicate that a similar association cannot be shown in OTSCC.

However, due to the variable fixation, not necessarily complete digestion, and processing of these clinical samples, factors that influence the sensitivity of in situ hybridisation, we cannot completely exclude the presence of HPV16 in our samples. It is also possible that other high-risk HPV types are present, although several studies consistently demonstrate that HPV16 is the most prevalent type found in the oral cavity (Chaturvedi et al., 2011; Schlecht et al., 2011).

As aforementioned, p16 expression in OSCCs, particularly OTSCCs, seems to be independent of the presence of HPV (Hilly et al., 2013). In the present study, while no HPV16 DNA could be detected through ISH, p16 expression was seen in 33% (36/109) of the samples, with weak expression (defined as a quick score of 1-5) detected in 19% (21/109) and strong expression (QS 6-18) in 14% (15/109).
As earlier discussed, to date, no standard method is available for the verification of HPV-association in HNSCC. Basically, amplification-techniques for detection of viral DNA and mRNA transcripts and protein-labeling can be used. The choice of method depends on the material (fresh or fixed tissue), time available, human resources and financial aspects. Each method has its limitations, thus the combination of two or more methods is meaningful. Conveniently, immunohistochemical detection of the p16-protein is followed by an HPV-specific test such as DNA PCR or ISH on the p16 positive cases (Smeets et al., 2007; Singhi and Westra, 2010; Schache et al., 2011).

Expression of p16 occurs as a result of functional inactivation of the retinoblastoma protein by the HPV E7 protein. Thus, HPV positive tumours are characterized by high expression of p16 (Smeets et al., 2007; Fakhry et al., 2008). p16 expression analysed by immunohistochemistry has emerged as a practical surrogate marker of HPV status because of its high sensitivity and specificity in the detection of HPV in OPSCC (Westra, 2014). However, in non-oropharyngeal head and neck SCCs, in which the HPV positivity rate is much lower, p16 IHC alone as a surrogate marker for HPV status suffers from low specificity (Lingen et al., 2013; Chung et al., 2014). Indeed, it must be noted that the correlation between presence of HPV and p16 overexpression is not absolute (Ramqvist and Dalianis, 2011). Besides, p16 immunohistochemical positivity is unable to discriminate HPV integration in HNSCC (Pannone et al., 2011).

The poor concordance between p16 IHC and HPV ISH findings in our study is likely due to other mechanisms of p16 activation independent of HPV in oral tongue carcinogenesis. In the absence of HPV-16, p16 expression could be explained by infection with other HPV types (Jordan et al., 2012) or, most probable, is due to non-HPV related genetic or epigenetic loss of pRb or other molecular alterations in the p16 pathway independent of infection with high-risk HPV, which may include transcriptional upregulation by oncogenic transcription factors such as Ets and Myc and alterations of Ras-MAPK pathways (Li et al., 2011; Romagosa et al., 2011; Witkiewicz et al., 2011, Compton et al., 2011). Several studies illustrate similarities between HPV-positive and transcriptionally active OPSCC with cervical cancer, like non-mutated TP53, low Rb- and high p16-expression whereas oropharyngeal tumours not associated with HPV seldom are p16 positive (Knopf et al., 2015). OPSCC have indeed been grouped into three subsets: HPV16-positive/p16-positive (HPV-active), HPV16-positive/p16-negative (HPV-inactive) and HPV16-negative/p16-negative (HPV-negative) (Weinberger et al., 2006) characterised by a decreasing overall- and disease-free survival (DFS) (Stephen et al., 2013). Pannone stated that the methylation of CDKN2a/INK4a, and the consequent p16 inactivation, is a frequent epigenetic alteration in tobacco and alcohol-related oropharyngeal cancer and therefore the author suggested to divide OPSCC into two distinct groups: a) tobacco-alcohol-associated/HPV/DNA-negative/CDKN2a/INK4a methylated/p16-IHC-negative and b) tobacco-alcohol unassociated/HPVDNA-positive/CDKN2a/INK4a
unmethylated/p16-IHC-positive (Pannone et al., 2012). The lack of p16 expression defines a subgroup of oropharyngeal cancer patients with increased risk of local recurrence and worse clinical outcome (Shah et al., 2009). On the other hand, p16INK4a overexpression seems to be associated with better overall- and disease-free survival independent of HPV infection status (Weinberger et al., 2004; Reimers et al., 2007; Shah et al., 2009) likely because of decreased tumour invasiveness due to altering expression of extracellular matrix remodeling genes (Isayeva et al., 2015).

In the present study, a direct positive correlation was found between the expression of p16 and the presence/absence of lymph-node metastasis (r = 0.242; p=0.011). Loss of expression of p16 protein, as assessed by immunohistochemistry, has been reported in up to 47% of premalignant lesions (Papadimitrakopoulou et al., 1997) and has been associated with reduced disease-free and overall survival in invasive OTSCCs making it an independent prognostic variable on multivariate analysis (Bova et al., 1999; Shiboski et al., 2005).

While the protective role of p16 overexpression in HPV-positive OPSCC is most likely related to the HPV protective role in these cancers rather than to the p16 positivity as oncosuppressor gene in itself, it is presumable that in OTSCCs the opposite way is the predominant (Gröbe et al., 2013).

Although the mechanisms for improved prognosis in p16-positive OTSCC is unclear, it may relate to either the induction of a senescent phenotype and thereby slow tumour growth (Romagosa et al., 2011; Witkiewicz et al., 2011) or to an inherent radiosensitivity due to impaired DNA double-strand break repair capacity (Rieckmann et al., 2013), which in turn may relate to the ability of p16 to directly inhibit homologous recombination repair in HPV-positive SCCHN (Dok et al., 2014).

In this study, comparing p16 expression between age groups, 75% of tumours in patients aged ≤40 years were p16 negative, compared with 66% within the other two age groups. At 5-year follow-up, 4 of the 12 p16-negative young patients (33%) were alive, compared with 62% of the patients aged 41-65 years having passed 5-year follow-up.

The CDKN2a over-expression in the absence of a p16 protein refers most likely to CDKN2A deficiency possibly due to mutation, hypermethylation, and allelic alteration (loss of heterozygosity, microsatellite size alteration) which is associated with the development of dysplastic lesions (Wu et al., 1999; Rothenberg and Ellisen, 2012) and with the progression to carcinoma in situ and invasive cancer (Nagai, 1999; El-Naggar and Westra, 2012). The derogation of the TP53-CDKN2a pathways can enable carcinogenesis in young patients with OTSCC (where a longstanding tobacco or alcohol abuse is missing), characterised by a more severe outcome, as also the present results show (Knopf et al., 2015; Zafereo et al., 2016).
Obviously, in the present study, as well in many published reports, p16 is being expressed in the absence of HPV16 DNA. Its expression should thus not be analysed as a marker of HPV tumour infection, as is done for the oropharyngeal carcinoma (Gröbe et al., 2013).

Finally, it should be mentioned that loss of p16 is a potential druggable target as recombinant adenovirus capable of directing a high level of p16 protein expression (Ad5-p16) demonstrated a significant anti-tumour effect against human HNSCC in vivo (Rocco et al., 1998).

**EXPRESSION of E-CADHERIN, β-CATENIN, CK5 and CK19**

As is well known, the patterns of local invasion and distant spread of every individual tumour differ depending on intrinsic properties as well as on the local tumour microenvironment (Lundqvist et al., 2011). The TNM classification has been used for over 80 years and is valuable in estimating the outcome of patients for a variety of cancers. There has been a continual refinement of the staging system. Unfortunately, the predictive accuracy of the traditional staging system relies on the assumption that disease progression is a tumour cell-autonomous process. The focus of this classification is solely on the tumour cells and fails to incorporate the effects of the host response. The phenotype of a tumour is not governed only by the epithelial component but also by the tumour environment, that is, other cells in contact with the tumour, the mesenchyme and the inflammatory infiltrate of the immune response. Thus, tumour progression has to be considered as the result of a balance between an invasive tumour process and a defence system, and therefore many recent and ongoing studies focus on the epithelial to mesenchymal transition process which seems to play a significant role in cancer progression, including OTSCC (Wang C., et al., 2012).

The recurrence rate of early oral tongue cancer is high despite surgery with extensive histologic documentation of free resection margins, thus achieving histologically adequate margins does not avoid risk for relapse (Brandwein-Gensler et al., 2005; Li et al, 2013). This may be due to cancer cells being left behind in the surrounding tissue. These cells might be cancer stem cells or cells having undergone EMT. Frozen sections are often used to determine the completeness of a resection but may not be able to detect these cells in the specimens (Attramadal et al., 2015).

A key-role in in establishing and maintaining intercellular connections is played by E-cadherin, the main protein of adherens junctions anchoring oral epithelial cells to each other. In the present study, results of immunohistochemical analysis showed expression of E-cadherin in 98% (118/120) of the cases. Looking at age of
the patients did not show any statistical difference between young and middle-age/elderly patients (p = 0.37).

Higher expression of E-cadherin resulted in a worse overall survival rate both in univariate (log Rank p-value = 0.011) as well in multivariate analysis (p = 0.001; HR = 4.29; CI [1.78 - 10.34]) revealing that E-cadherin expression is an independent prognostic factor in patients with oral tongue cancer (Figure 4).

<table>
<thead>
<tr>
<th>Variables in the Equation</th>
<th>B</th>
<th>SE</th>
<th>Wald</th>
<th>df</th>
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<th>Upper</th>
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Figure 4: Cox Regression analysis for the marker E-Cadherin

Several studies have investigated the possible role of E-cadherin expression in prediction of overall survival and lymph node metastasis in patients suffering from oral squamous cells carcinoma (Pyo et al., 2007; Foschini et al., 2008; Pannone et al., 2014). Many immunohistochemical studies show aberrant E-cadherin expression in HNSCC and dysfunctional E-cadherin-mediated cell adhesion is often associated with cancer invasion and metastasis with a significant negative effect on overall survival (Chang et al., 2002). A meta-analysis of the literature on the topic showed that low expression could predict worse survival in patients with oral cancer (Luo et al., 2014). However, such results should be cautiously taken into consideration because of the presence of a high rate of heterogeneity and different cut-off values used. The results of the present work with higher expression of E-cadherin correlating with a poor disease-free survival and a higher number of deceased patients at two years overall survival, apparently are in contrast to what is mentioned above. However it is of note that not all the literature is in agreement on the protective role of E-cadherin expression, as in a published study on laryngeal carcinoma, an association between overexpression of E-cadherin and poor overall survival was seen (Greco et al., 2016). Looking only at the oral tongue sub-site, some studies found that downregulation of E-cadherin was associated with a higher rate of lymph-node metastasis compared to high expression (Chang et al., 2002; Han et
al., 2015). Mostaan and coworkers in a similar evaluation did not detect such a correlation (Mostaan et al., 2011). In addition, Sakamoto et al. found that loss of E-cadherin and overexpression of its transcriptional repressor SIP-1, was associated in T1/T2 No Mo oral tongue cancers with higher rates of delayed lymph-node metastasis after partial glossectomy (Sakamoto et al., 2012). In the same way, focusing on similar inclusion criteria, the study by Okamoto et al did not find such association (Okamoto et al., 2002).

Besides, according to the authors of the present study, possible explanations to the present results may reside in the well established concept of tumour heterogeneity. Previous works have shown that tumours consist of a heterogeneous population of cells with differences in their abilities for self-renewal, invasion and metastasis. Much of the heterogeneity visible in histological sections of tumours is due to a continuing, but often disturbed, maturation of tumour cells that reflects some persistence of the normal differentiation pattern of their tissue of origin (Gammon and Mackenzie, 2015). Most OSCCs are highly to moderately differentiated with major multicellular units having a differentiation pattern very similar to normal tissue and with regions of dedifferentiation with widespread cellular dissociation being found at the invasive front (Bryne, 1998). These small groups of cells are often referred to as tumour buds, and increased numbers of these appear to be a strong predictor of severe prognosis (Wang C. et al., 2011). It is known that invading and disseminating carcinoma cells often show great plasticity and undergo EMT. With the help of 3D confocal microscopy, it has been hypothesised that type 3 EMT in vivo means predominantly not a single cell migration (as seen in vitro) but a chain migration in multicellular groups, with the tip of the migrating cells undergoing partial EMT in terms of morphology (not elongated spindle-shaped cellular form) and expressed markers such as reduced E-cadherin not accompanied by an upregulation of N-cadherin (the so called cadherin switch) or vimentin and/or loss of keratin (Jensen et al., 2015). In other terms, EMT in cancer cells in vivo is not equal to the complete transition towards a pure mesenchymal phenotype, as is seen during development. Cancer cells in the tumour show a phenotypic plasticity in the expression of biomarkers as cells interconvert between individual and collective migration and vice versa in the different areas of the lesion likely depending on the amount of available oxygen (Gammon et al., 2016). These assumptions could therefore be the explanation of our results considering the fact that we have stained the whole specimens and not only the invasive front.

In addition, we know that hypermethylation of the promoter of the CDH1 gene which encodes E-cadherin is the most common way of quenching E-cadherin expression, but this hypermethylation in HNSCC has not been solely connected to poor prognosis but also to better prognosis (Marsit et al., 2008).
Hypermethylation is indeed not always correlated with advanced stage (Calmon et al., 2007), mortality, or second primary tumour (Dikshit et al., 2007). The hypothesis behind loss of E-cadherin through methylation as a favourable prognostic factor is explained by the fact that hypermethylation is thought to be an early event in tumourigenesis, leading to a tumour phenotype more susceptible to treatment. Loss of E-cadherin at a later stage, on the other hand, could be connected to a more aggressive tumour phenotype (Marsit et al., 2008).

A certain proportion of bias in the published reports on E-cadherin could also depend on the sample sizes of the studies and the method to detect E-cadherin. The analysis of E-cadherin involves many aspects, including protein, DNA, RNA levels, and/or gene hypermethylation status. To try to predict the prognosis of cancer patients, immunohistochemistry is a widely used method to detect the protein. Nevertheless different studies show different cut-off values for expression which may influence the final results and conclusions (Zhao et al., 2012). To overcome such differences, improvements in the standardisation of the studies should be performed to favour more accurate meta-analysis.

Finally, in the present study we analysed samples from oral tongue only. The authors believe that the inclusion of all head and neck sub-sites in studies of histopathological predictors of outcome could be a contributing factor to the conflicting findings in the literature (Kolokythas et al., 2015). Considering the substantial anatomo-physiological differences of the different head and neck sub-sites, further studies should pay attention to investigating E-cadherin expressions in tumours solely when the different primary sub-sites are distinguished (Zhao et al., 2012). Indeed, as tongue cancers also have a different and more aggressive clinical behaviour compared to cancer from other regions, this could explain certain differences in molecular expression (Gatta et al., 2015). Besides, because tumourigenesis is a multi-step process, any single molecule, such as E-cadherin, cannot be used to independently predict the prognosis of the patients. Combinations of prognostic biomarkers should always have increased prognostic power over individual markers themselves (Zou et al., 2010).

Future studies should also pay attention to control over influencing factors, such as tumour staging (very few studies have described the relationship between aberrant E-cadherin expression and patient prognosis in either early - stage I and II - or advanced - stage III and IV - disease), lymph node metastasis and therapeutic treatment.

As previously mentioned, the morbidity of OTSCC is the highest of the HNSCCs. Effective induction chemotherapy and/or adjuvant chemotherapy can improve local control rate and survival of patients with OTSCC. In this matter, the use of
E-cadherin expression to personalise anti-HNSCC therapy has been explored during past years (Eriksen et al., 2005; Huang et al., 2009).

Surprisingly, in the present work, a statistically significant ($p = 0.039$) higher expression of E-cadherin ($QS = 12-18$) was seen in Swedish (38%; 33/87 cases) compared to Italian patients (18%; 6/33 cases) (Table 4).

<table>
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<th>Nationality</th>
<th>E-cadherin</th>
<th>Total</th>
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<tr>
<td></td>
<td>0-10</td>
<td>12-18</td>
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<tr>
<td>swedish</td>
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<tr>
<td>italian</td>
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<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>101</td>
<td>19</td>
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</table>

Table 4: geographical distribution of E-cadherin expression

It is widely accepted that HNSCC shows differences in incidence and mortality rates between different ethnicities and geographic localisations, e.g. variable smoking habits with higher tobacco consumption in Southern Europe and possibly related higher incidence of HNSCC (Scully and Bedi, 2000).

In order to reduce the sources of bias, the present study included specimens from two different nations. Looking at the results, it can be speculated that possible geographical variations in the biology of tumours can make it difficult to identify a consistent pattern of tumour behaviour among different head and neck oncology units across the world.

The differential expression of E-Cadherin in Swedish compared to Italian patients is possibly a sign that the environment and the place of origin may lead to the onset of cancer with different molecular features. However, molecular differences among different sub-ethnicities have not been well investigated yet.

In oral cancer a differential expression of β-Catenin seems to play a major role in the involvement of oral carcinogenesis (Chaw et al., 2012) and can be considered a reliable prognostic marker in the induction of an invasive and metastatic behaviour (Moles et al., 2016), although other studies do not agree with such an assumption (Mahomed et al., 2007; Freitas et al., 2010; Balasundaram et al., 2014).
In the present study, an expression of 98% (the exact same value as for E-cadherin) was seen for β-catenin too. This direct correlation of immunohistochemical expression ($p < 0.05$) is a reasonable result taking into account that in the majority of the examined specimens, we observed β-catenin staining predominately at the cell membrane region (Figure 5).

![Figure 5: β-catenin staining being predominately at the cell membrane region](image)

Similar to E-cadherin expression, although not statistically significant ($p = 0.071$), a higher rate of β-catenin expression was more common in Swedish (17.5%; 17/87).

When evaluating the effect of anti-Programmed Death Ligand 1 (PDL-1) therapy in melanomas, it was found that β-catenin within the tumours caused exclusion of T-cells and thus also resistance to this therapy (Spranger et al., 2015). If results are valid also in other tumours, targeting of β-catenin could be used to gain an immunostimulatory effect in HNSCC.

As previously mentioned, epithelial cells that develop EMT like characteristics also show expression of basal cytokeratins such as CK5 and CK19. CK5 was expressed in all samples analysed, while CK19 was expressed in 63% (76/120 cases). No differences have been found between CK5 and CK19 expression in relation to overall survival rate and the analysed clinical-pathologic parameters. It was only possible to show an overall higher expression of CK5 (which has been
shown to be more ubiquitously expressed in HNSCC) than of CK19 (more related to tumours from pharynx and larynx). Expression of CK5 and E-cadherin were further directly correlated to each other (p < 0.05).

**PODOPLANIN EXPRESSION and mRNA LEVELS**

As earlier mentioned, OTSCC accounts for more than half of the total oral cancer cases diagnosed each year worldwide and is the most aggressive cancer in the oral cavity (Bello et al., 2010). Cancer metastasis, the dissemination of cancer cells from the primary tumour to organs where they initiate malignant growth, is the primary cause of cancer-related death. The most common reason for treatment failure following surgical extirpation of early-stage oral tongue cancer is regional recurrence (Yuen et al., 1997; Capote et al., 2007) and cervical lymph node metastasis has been shown to be the most significant prognostic factor of decreased survival and poor clinical outcome with salvage therapy (Fakih et al., 1989; Sparano et al., 2004). The presence of cervical nodal metastasis indeed significantly decreases survival for tongue carcinoma (Grandi et al., 1985) and about 40% of patients with tongue cancer have cervical lymph node metastases at diagnosis (Sano et al., 2007). Fifteen–30% of patients with clinical N0 neck harbour occult metastasis in the lymph nodes, suggesting that even small primary tumours in OTSCC carry a moderate risk for delayed development of clinically manifest cervical metastatic disease (Kerrebijn et al., 1999; Hao and Tsang, 2002; Crean et al., 2003; da Silva et al., 2012) while elective neck treatment is traditionally recommended when the risk of occult cervical metastasis exceeds 20%. Therefore elective management of the neck is an important consideration in patients undergoing treatment for primary OTSCC, but specifically the optimal management of a clinically-negative neck in stage I OTSCC, which should be considered a different disease entity from T2N0M0 OTSCC, has remained controversial over the past three decades. Surgical resection of the OTSCC has been accepted as the standard care (Shah and Gil, 2009). Besides, it is universally accepted that the neck has to be addressed by either surgery with or without adjuvant (chemo)radiation or by primary (chemo)radiation in case of overt lymph node metastases. However, elective neck dissection or watchful waiting policy of the neck after local excision are both viable options for the patient with a clinically negative neck. There is general agreement that elective treatment of the neck is indicated when the neck needs to be entered to resect the primary tumour or reconstruct the surgical defect, and when there is a high likelihood of occult (clinically and radiologically undetectable) lymph node metastasis. The decision to treat the neck in early-stage OTSCC should therefore consider on one side the diagnostic and therapeutic purposes of the procedure with the benefit to
avoid missing occult metastasis, and on the other side longer duration of surgery with the related unavoidable higher morbidity. The conventionally performed neck lymph node dissection could, mainly in low stage tumours, result in overtreatment, which can imply serious side effects and disfiguration. On the other hand, offering aggressive multimodality treatment (neo-adjuvant/adjuvant chemo-radiotherapy) to all low stage patients would expose a large subpopulation to unnecessary toxicities and debilitation such as acute patient discomfort (e.g. due to oral mucositis) as well as permanent sequelae (e.g. hyposalivation, shoulder dysfunction) or risk for development of radiation-induced neoplasms, and would expose the entire community to costs of unnecessary treatment (Shah and Gil, 2009; van Wouwe et al., 2009; Huang et al., 2010; Zhao et al., 2012).

Recently, sentinel node surgery has been introduced for detection of micrometastasis in lymph nodes. However, the accuracy of prediction is not perfect, and the predictive value of negative sentinel lymph nodes is about 90% (Civantos et al., 2010).

As the clinico-pathological characteristics of the patient and the disease most often guide the clinician’s choice of the elective therapeutic neck management strategies, it is mandatory to consider patient-related (coexisting conditions) as well as tumour-specific factors (growth pattern, grade, neuro-vascular invasion, invasive front histopathological features, tumour thickness and depth of invasion, mitotic figures and nuclear morphology, degree of inflammatory reaction surrounding the tumour, lymphatic vessels density, biomarkers) in the treatment planning (Spiro et al., 1986; Lim et al., 2004; Abu-serriah et al., 2015). Indeed, the size of the lymph node, the main indicator of metastasis in radiological investigation and physical examination of patients with HNSCC, is not trustworthy evidence of nodal spread (Remacle et al., 1988; Kau et al., 2000) and its reliability is reported to be between 50% and 80% (Kaya et al., 2001).

Consequently, it is desirable to provide other markers with high sensitivity and specificity as complementary prognostic indicators of lymph nodal engagement in order to reliably characterize OTSCC tumour behaviour and thereby guide clinical decision-making.

In this direction, the preoperative evaluation of cancer cell nuclear morphometry seems to be a reliable method (Sekine et al., 2003; Karino et al., 2014).

Tumour-related lymphangiogenesis may be regarded as a major prognostic element in this matter too, as high proportion of lymphatic vessels might enable invasion of tongue cancer and thus cervical node pre-metastatization, micrometastatization, and clinically manifest metastatization (Seppäla et al., 2016). As
a biomolecular prognostic marker, podoplanin, the aforementioned glycoprotein involved in the formation of lymphatic vessels, EMT process as well as in the collective cancer cell migration (Yu et al., 2004; Cueni et al., 2008; Martin-Villar et al., 2010; Inoue et al., 2012; Pula et al., 2013; Inoue et al., 2014) has been the object of several investigations including the one in the present study. Podoplanin likely plays a key role in aggressive tumours as there is a well-accepted cross-talk between invasive tumour, microenvironment and lymphangiogenesis (Mumprecht and Detmar, 2009; Schlereth et al., 2014).

Several reports indicate that podoplanin expression is notably increased in over 30% of pre-malignant oral lesions and in over 60% of oral cancers. Moreover, its expression has been shown in about 50% of T1 and T2 primary cancers, and this percentage increases to about 75% for T3 and T4 tumours. Besides, over 70% of primary OSCC with positive lymph node metastases express elevated levels of podoplanin (Funayama et al., 2011; Huber et al., 2011; Kreppel et al., 2012).

In the present study, evaluation of the expression of this protein revealed that it is expressed in 93.7% (121/129) of cases, showing low expression in 43% (55/129) and high expression in 51% (66/129). Compared to samples collected adjacent to the tumours and to tissues from healthy volunteers with no evidence of SCC or other disease, significantly higher levels of podoplanin mRNA were detected through qRT-PCR (p < 0.01). In turn, expression of podoplanin in clinically normal tongue adjacent to tumour was higher than in normal tongue taken from healthy control volunteers.

These results mirror the generally accepted higher expression of podoplanin in invasive SCC (Martín-Villar et al., 2005) and in hyperplastic and dysplastic areas of the oral mucosa (Kawaguchi et al., 2008; Inoue et al., 2011; Agarwal et al., 2014) as well in clinically normal mucosa adjacent to SCC indicating an early expression of this protein in tumourigenesis (de Vicente et al., 2013). Instead, podoplanin expression is not detected or is extremely low in normal epithelium (Agarwal et al., 2014).

While clinical studies indicate that overall- and disease-free survival rates continuously decrease in an indirect proportion to podoplanin expression (Kreppel et al., 2010; Kreppel et al., 2011), no such association could be detected in the present study. As previously mentioned, results of the predictive role of podoplanin as a marker of lymphatic vessel density have been shown to be controversial also in other studies. This might be due to differences in populations, tumour location, treatment and immunohistochemical or calculation techniques, such as podoplanin expression evaluating scales, and calculating vessels in intra- or peri-tumoral area. Besides, it has to be emphasised that the use of any single predicting factor alone is neither sensitive nor specific.
enough to reliably select patients who would benefit most from elective neck dissection (Johnson et al., 1980; Sparano et al., 2004) as a single morphological feature cannot accurately predict the biological behaviour of the tumour, which is decided by both morphological and cytogenetic factors.

It is worth underlining that, compared to many other published works, the number of patients in the present study is quite high and that also tongue tissue samples taken from healthy patients were analysed. Interestingly the results of the present work demonstrate that high expression (QS 6-18) of podoplanin was more frequent (78%) in young patients (age ≤40 years) compared to older (age > 40 years) patients (41%) showing an indirect correlation was found between the expression of podoplanin and age of patients (r = -0.204; p = 0.035). This once again remarks the intrinsic specificity of the group of young patients suffering from OTSCC compared to the majority of patients with oral tongue cancer, who are typically much older.

Finally, it is worth underlining that lymphangiogenesis may be regarded as a major prognostic element for nodal disease, and that the lymphatic endothelium, based on its potential contribution to cancer spread, cancer cell immune evasion, and maintenance of cancer stem cell–like properties, may represent an attractive therapeutic target for anti-neoplastic therapy. Blocking lymphangiogenesis of the tumour might be important in preventing the metastatic cascade of the cancer. Besides, preliminary studies performed in this direction suggest that inhibiting lymphangiogenesis might be easier than the ablation of pre-existent lymphatic vessels. One remaining challenge is the identification of rational combination regimens of anti-lymphangiogenic drugs with established chemotherapies and targeted anticancer therapies. In this matter, together with anti-growth factor antibodies (Stacker et al., 2004), podoplanin, a marker of lymphatic vessels associated to tumoural invasion and lymph node metastasis, may be considered not only a useful prognostic and diagnostic factor signaling a sub-group of aggressive tumours, but might also represent a chemotherapeutic target to combat cancer growth and progression. Indeed the bulk of this protein found outside of the cell could serve as an ideal target (Wickiewicz et al., 2006; Kunita et al., 2007). Some antibodies and lectins against podoplanin can therefore be used to inhibit tumour progression and lymphangiogenesis (Ochoa-Alvarez et al., 2015).
Conclusions

- In concert with the majority of published studies, the results of this study show that HPV16 infection is not present in OTSCC, making it unlikely that HPV has a role in the aetiology, pathogenesis and clinical outcome in this disease, and denying even the possibility to consider HPV infection as a risk factor for younger patients lacking traditional risk factors. Considering a similar expression of syndecan-1, the primary receptor for HPV, in both tonsillar SCC and OTSCC, it is possible to speculate that conditions for enabling entrance of the virus in the tissue are potentially the same in the oral tongue as in the oropharynx. Therefore the absence of clinically detectable high-risk HPV infection in OTSCC likely underlines the difference in the microanatomy and physiology between the two districts as well as gives room for speculation on the potential value of co-infection with other viruses in the tonsillar area.

- Data suggest that overexpression of p16 protein is not an appropriate biomarker for HPV association in oral tongue carcinogenesis. Fewer young patients compared to middle aged and elderly expressed the p16 protein. Age was an independent prognostic marker associated with less loco-regional metastasis.

- All the analysed EMT related markers, and in particular E-cadherin, β-catenin and CK5, were highly expressed in the samples. Higher expression of E-cadherin was an independent negative prognostic factor for overall survival, demonstrating the importance of investigating oral tongue cancer separately from the other sub-sites. Data for nationality revealed a difference in expression of E-cadherin between Swedish and Italian patients (p = 0.039), leaving a question open about the role of the geographical distribution in oral tongue carcinogenesis.

- In OTSCC significantly higher levels of podoplanin mRNA were detected compared to tumour free tissue and normal tongue from healthy controls. An indirect correlation was found between the expression of podoplanin and age of patients, once again underlying the peculiarities of OTSCC in young patients.
Results of the present study showed a statistically significant correlation between patients presenting an advanced stage of disease and survival. Patient age at diagnosis can be considered an independent prognostic factor, with patients of younger age showing a worse survival rate. Age represents an important factor to be investigated in the future, particularly since an increase in the incidence of OTSCC in young people is seen.

The geographical distribution of OTSCC samples in our study connoted a difference in E-cadherin expression between Italian and Swedish patients leaving the possibility to speculate about the possible influence of sub-ethnicity in cancer biology.
Limitations

This study has specific limitations, including its retrospective nature, lack of anamnestic information of smoking and alcohol habits, and relatively variable length of clinical follow up for individual patients. An even larger sample size would enable better understanding of the association between markers and OTSCC, therefore an even larger, appropriately powered, multi-centric prospective study would be the best choice in this field.
Future perspectives

HNSCC are a heterogeneous group of diseases, depending upon localisation. We therefore believe that HNSCC should not be regarded as a homogenous entity, but rather studied in separate groups according to anatomical origin in order to obtain a more homogeneous and reliable study cohort. Understanding more the aetiology, development and progression of the carcinoma of the tongue, specifically as a more aggressive juvenile variant is increasing, is mandatory considering that the response to treatment is highly variable among patients, and despite our efforts, mortality rates remain unacceptably high. Besides, a lack of treatment protocols in the management of cNo in the early stage of OTSCC still remains to be solved. Numerous studies using single markers to improve disease prognostication could not be validated. Despite an understanding of several of the clinic-pathological factors that are known to correlate with poor survival, there is currently no method to definitively determine the prognosis of OTSCC patients.
This likely depends on the fact that the clinical and histopathological characteristics of the tumour in addition to staging, molecular markers, and patient age and medical status, somehow contribute to response to treatment and outcome. Thus, efforts towards identifying predictive markers should be continued trying to build and adopt a risk assessment model, in other words a weighted comprehensive scoring system of both patient-related and tumour-related factors. Advances in imaging technology, the role of sentinel node biopsy, molecular biological diagnostic techniques, and gene expression profiles of tumour biopsies assessed by microarray hybridisation and/or deep sequencing will support this purpose in the near future.
Besides, just to emphasise the clinical relevance of bio-molecular studies on OTSCC, it would be important to find prognostic parameters to evaluate tumour aggressiveness and metastatic potential on incisional biopsies performed preoperatively in order to correctly plan the surgical management of the patient and to avoid unnecessary neck dissection and/or adjuvant therapies. To date, all the studies have contrarily focused on tissue samples obtained after complete surgical resection of the primary tumour.
Finally, it should be remembered that some of the studied bio-molecular markers, like p16, β-catenin, and podoplanin constitute also potentially attractive chemotherapeutic targets for anti-neoplastic therapy.
Acknowledgement

To me, this PhD thesis is not just a precious finish line after many years lovely spent in the in-depth analysis of oral tongue cancer biology, but is also a tangible sign of my work in Sweden, of my years in this wonderful country, my second homeland, to which I donated my young life. I actually wish so much to never end the connection with my mentor and co-workers here, and with the great research work they carry out. This PhD also shows the concrete achievement of a successful cooperation between different universities and different countries, even between those who apparently lie far from each other. As I believe that Science is something that can strongly unite people in the end, and that, especially nowadays that the world has become quite smaller, should strongly unite people regardless of their diverse background.

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