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IMMUNOPHENOTYPE OF OROPHARYNGEAL SQUAMOUS CELL CARCINOMA: FOXP3, IL-22 AND TREM-1 EXPRESSION IN THE TUMORAL ENVIRONMENT.

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To Edoardo and Ilaria.

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TABLE OF CONTENTS

Introduction	p. 4
Aim of the study	p. 58
Materials and Methods	p. 60
Results	p. 66
Discussion	p. 78
Conclusions	p. 85
Future research perspectives	p. 88
References	p. 89

INTRODUCTION

EPIDEMIOLOGY OF OROPHARYNGEAL AND ORAL SQUAMOUS CELL CARCINOMAS

Incidence and Mortality

Anatomically, the oral cavity and oropharynx are separate regions that border each other but do not overlap. The anatomic subsites of the oral cavity include the labial mucosa, buccal mucosa, floor of mouth, alveolar ridge and gingiva, anterior two-thirds of the tongue (anterior to the circumvallate papillae), hard palate, and retromolar trigone. The oropharynx consists of the soft palate, base (or posterior one-third) of tongue, palatine tonsils, palatoglossal folds, valleculae, and posterior pharyngeal wall. Distinct anatomic borders separate the two sites: from above, the junction of the hard and soft palate, and from below, the circumvallate papillae.

Reviewing the literature and surveillance data on oral and oropharyngeal cancers, with an epidemiological and statistical purpose, is difficult because these tumors often are reported in aggregate with other pharyngeal or head and neck malignancies, and anatomic subsite definitions are at times unclear or may not allow for distinction between the oral cavity and the oropharynx. For example, in the Surveillance, Epidemiology, and End Results (SEER) database, the "tongue" is considered a subsite of the oral cavity and pharynx; however, the tongue includes the base of tongue/lingual tonsils (which are part of the oropharynx) as well as the anterior two thirds of the tongue (which is part of the oral cavity). **[1]** Also, the SEER database lists the oropharynx and tonsils as distinct subsites, although the tonsils are part of the oropharynx. In the GLOBOCAN database, the oral cavity includes the base of tongue (which is part of the oropharynx) and palate (which may include both the hard palate [part of the oral cavity] and soft palate [part of the oropharynx]); also, "nasopharynx" and "other pharynx" are considered distinct subsites, with the latter referring not only to the oropharynx and tonsils but also to the hypopharynx, pyriform sinus, and "other and ill-defined sites of the lip, oral cavity, and pharynx". **[2]** In the Cancer Incidence in 5 Continents (CI5) and European Network of Cancer Registries (EUREG) databases, the "tongue" includes both the base of tongue (part of the oropharynx) and "other and unspecified parts of the tongue" (presumably the anterior two-thirds of the tongue, which is part of the oral cavity). **[3,4]**

These databases also list the "palate" as a subsite of the "mouth," although the palate may include both the hard palate (part of the oral cavity) and the soft palate (part of the oropharynx). Furthermore, some authors use the term "oral" in reference to both the oral cavity and the oropharynx, whereas others reserve this term solely for the oral cavity. Current evidence supports that tumors at these two sites are distinct and unique, with differing etiopathogenesis, treatment, and prognosis. **[5]**

According to the most recent GLOBOCAN estimates, worldwide in 2012, there were approximately 300,373 new cases of lip/oral cavity cancer (age-standardized rate [agestandardized to the world population] or ASR[W], 4.0 per 100,000) and 142,387 new cases of "other pharyngeal" (ie, excluding the nasopharynx) cancer (ASR[W], 1.9 per 100,000). **[2]** Notably, the estimated ASR(W) for lip/oral cavity cancer is highest for the World Health Organization (WHO) South-East Asia region (6.4 per 100,000), followed by the WHO Europe region (4.6 per 100,000), the WHO Eastern Mediterranean region (4.6 per 100,000), the WHO Americas region (4.1 per 100,000), the WHO Africa region (2.7 per 100,000), and the WHO Western Pacific region (2.0 per 100,000). For "other pharyngeal" cancer, the estimated ASR(W) is highest for the WHO South-East Asia region (3.6 per 100,000), followed by the WHO Europe region (2.7 per 100,000), the WHO Americas region (1.9 per 100,000), the WHO Eastern Mediterranean region (1.1 per 100,000), the WHO Africa region (0.8 per 100,000), and the WHO Western Pacific region (0.8 per 100,000). Worldwide mortality estimates for 2012 include an ASR(W) of 2.7 per 100,000 for lip/oral cavity cancer and 2.2 per 100,000 for "other pharyngeal" cancer. **[2]** In the United States, the American Cancer Society estimates that, in 2015, there will be 45,780 new cases of oral cavity and pharyngeal cancer (male-to female ratio, 2.5:1) and 8650 deaths from these tumors. **[6]** For oral cavity and oropharyngeal cancers combined, the SEER Program reports a median age at diagnosis of 62.0 years (for SEER 18 areas from 2008 through 2012), an age-adjusted incidence of 11.0 per 100,000 (for SEER 18 areas from 2008 through 2012; age-adjusted to the 2000 US standard population), a 0.8% average annual increase in delay-adjusted incidence (for SEER 13 areas from 2008 through 2012), and a 0.5% annual increase in age-adjusted incidence (for SEER 18 areas from 2003 through 2012). **[1]** With regard to epidemiologic trends, increasing oropharyngeal cancer incidence has been observed in numerous developed nations over the past few decades (eg, the US annual percentage change [APC]5 3.0 for SEER 9 areas from 1999 through 2012; Canada, APC52.7 from 1992 through 2009; Denmark, APC53.5 from 1978 through 2007;

Portugal, APC53.49 from 1998 through 2007; Netherlands, APC52.1 for males and APC52.7 for females from 1989 through 2011; Korea, APC52.35 from 1999 through 2009; and Australia, APC 51.2 for males and APC 50.8 for females from 1982 through 2008). **[1,7-13]** For oral cavity cancer, many regions have reported decreasing or stabilizing trends (eg, Canada, Australia, Bulgaria, Croatia, Slovenia, Ukraine, Slovakia, Netherlands, France, and Germany), whereas others have exhibited markedly increasing trends (eg, Iceland, Finland, and Ireland). **[4,9,13-15]** In India, oral cancer trends vary by region, although investigators estimate that the total number of new mouth cancer cases will increase from 45,859 in 2010 to 64,525 in 2020. **[16,17]** With regard to recent subsite trends in the United States, SEER 9 data from 2008 through 2012 show an average APC in age adjusted incidence of 3.0 for oropharyngeal cancer, 2.1 for tongue cancer, and 23.6 for floor of mouth/gum/other mouth cancer. **[1]** In Korea from 1999 through 2010, age-standardized incidence rates increased markedly for cancers of the oral tongue (APC52.2 for males, APC54.1 for females, and APC56.1 for individuals younger than age 40 years) and buccal mucosa (APC54.8). **[15]**

Etiology and Risk Factors

Significant epidemiologic shifts seem to reflect dynamic risk factor trends. Traditional modifiable risk factors include tobacco and alcohol use. In addition, in recent decades, human papillomavirus (HPV) has emerged as a major etiologic factor for OP-SCC. **[18-20]** These factors and others are discussed in more detail below. In regions such as North America, Australia, and parts of Europe, a dramatic increase in HPV-positive tumors accounts for rising OPSCC incidence; in contrast, regional variations in trends for OC-SCC and HPV-negative OP-SCC are largely consistent with tobacco use trends. **[20-21]** Nevertheless, the underlying cause for increased tongue cancer in the United States and other regions is unclear. In particular, a surprising increase in oral tongue cancer has been observed in young females, often with no significant tobacco and alcohol exposure. **[22-24]** Also, the vast majority of oral tongue cancers examined thus far have been negative for high risk HPV. **[25-35]** According to SEER 18 data in the United States from 2000 through 2012, the incidence of tongue cancer in adults aged 20 to 44 years increased among females (APC 51.0) but decreased among males (APC520.1). **[36,37]** In a pooled analysis of case-control studies by the International Head and Neck Cancer Epidemiology Consortium, adults aged 45 years and younger exhibited a higher proportion of oral tongue cancers compared with adults older than 45 years (16% in women/11% in men vs 10.3% in women/5.9% in

men, respectively). Also in that study, the associations of smoking and drinking with oral cavity cancer were weaker in young adults compared with older adults (ever-smokers: odds ratio [OR], 1.91 for young adults vs 2.18 for older adults; ever-drinkers: OR, 1.24 for young adults vs 1.61 for older adults). **[24]** In addition, in a study of 25 young adults diagnosed with oral tongue SCC at a single institution from 1989 through 2007, Harris et al reported that 60% were female and 52% were never-smokers/never-drinkers. **[28]**

Major Risk Factors

Tobacco

Tobacco consumption continues to be a major risk factor both for OC-SCC and OP-SCC. Based on sufficient evidence of carcinogenicity in humans, the International Agency for Research on Cancer classifies tobacco smoking as a group 1 carcinogen for both the oral cavity and the pharynx and classifies smokeless tobacco as a group 1 carcinogen for the oral cavity. **[38]** Although tobacco use has been declining or stabilizing in many high-income countries, it has been increasing in many low-income and middle-income countries, where nearly 80% of the world's one billion smokers currently reside. **[39]** A meta-analysis by Gandini et al noted a relative risk of 6.76 for OP-SSC and 3.43 for OC-SCC among current tobacco smokers compared with nonsmokers. **[40]** This smoking-associated risk appears to be dosedependent and correlates with daily or cumulative cigarette consumption. For patients who quit smoking, the risk for OC-SCC and OP-SCC declines over time and may approach that of nonsmokers after 10 or more years of cessation. **[41]** Although cigarettes represent the predominant form of tobacco used worldwide, tobacco types abound and vary in popularity by region. In the United States, there has been increased large cigar and pipe tobacco consumption over the past decade, likely in part because of federal excise tax increases in 2009, which made large cigars less expensive than small cigars and made pipe tobacco less expensive than roll-your-own tobacco and manufactured cigarettes. **[42]** The Centers for Disease Control and Prevention reported changes in the total annual number of these products consumed from 2008 to 2011 as follows: consumption increased for large cigars from 5.7 billion to 12.9 billion, decreased for small cigars from 5.8 billion to 0.8 billion, increased for pipe tobacco from 2.6 billion to 17.5 billion, and decreased for roll-your-own tobacco from 10.7 billion to 2.6 billion.43 Data are limited, but some studies suggest that the relative risk for head and neck SCC (HN-SCC) among pipe or cigar

smokers is comparable to or greater than that for cigarette smokers. **[41,43]** In parts of Asia, other popular forms of combustible tobacco include the bidi (tobacco hand-rolled in a tendu or temburni leaf), kretek (clove cigarette), and water pipe (hookah, nargile). Despite the need for further research regarding alternative combustible tobacco products, all forms of tobacco use are unsafe.

In Western countries, major types of smokeless tobacco include wet snuff, dry snuff, and chewing tobacco. The risk for OC-SCC appears to be greater with dry snuff (relative risk, 4-13) compared with moist snuff and chewing tobacco (relative risk, 0.6-1.7). **[44]** The development of oral cancer from long-term smokeless tobacco use has been largely attributed to tobacco-specific nitrosamines. However, tobaccospecific nitrosamine levels are relatively low in Swedish moist snuff (snus) and in contemporary American moist snuff, with recent analyses detecting no risk or a minimally elevated risk for HN-SCC among users of such products. **[45-48]** Nevertheless, the use of snus as a safer alternative to smoking and the effects of snus on initiation or cessation of smoking require further research. A recent meta-analysis found no statistically significant association between snus consumption and various cancer types, heart disease, or stroke **[48]**; however, in a cohort study of >40,000 Swedish male construction workers, an increased risk for cancer-specific death was observed both among exclusive smokers (hazard ratio, 1.15; 95% confidence interval [CI], 1.10-1.21) and never-smoking snus users (hazard ratio, 1.15; 95% CI, 1.05-1.26). **[49]** In a recent systematic review (based largely on Swedish males), dual use of snus and cigarettes was more common among adolescents than adults, more often began with cigarette than snus consumption, and was hypothesized to increase smoking quit rates. **[50]** In contrast, other investigators suggest that snus use may interfere with attempts to quit smoking. **[51]** In parts of Asia, smokeless tobacco often is combined with betel quid.

Alcohol

After adjusting for tobacco smoking and other confounding factors, most studies from the United States, Europe, and Asia have reported an increased risk for oral cavity/pharyngeal cancers in association with heavy alcohol consumption (typically defined as >60 grams [or 4 drinks] per day or >4 to 7 drinks per week), with point estimates of adjusted ORs ranging from 4.1 to 8.8.53 Alcohol also appears to be an independent risk factor, with studies of nonsmokers noting both a strong association and a dose-response relationship between alcohol consumption and oral cavity/pharyngeal SCC. **[52]** Recent meta-analyses have estimated that the

relative risk for HN-SCC is 1.3 for 10 grams of ethanol per day compared with 13.0 for 125 grams of ethanol per day, with higher risk estimates for OP-SCC than for OC-SCC. **[53]** Underlying carcinogenic mechanisms are not entirely clear, although several have been proposed. Ethanol is metabolized by epithelial cells and microflora into acetaldehyde, which is a known carcinogen. Accordingly, risk polymorphisms in alcohol-metabolizing genes (eg, alcohol dehydrogenase 1B gene [ADH1B], alcohol dehydrogenase 1C gene [ADH1C], aldehyde dehydrogenase 1 gene [ALDH1], and aldehyde dehydrogenase 2 gene [ALDH2]) have been identified; studies have reported reduced head and neck cancer risk with ADH1B*2 (meta-OR, 0.5; 95% CI, 0.37-0.68) and ADH1C*2 (meta-OR, 0.87; 95% CI, 0.76-0.99) alleles and an increased risk with ADH1B(*1/*11*1/*2) plus ALDH2(*1/*1) (OR, 2.31 for current regular drinkers; 95% CI, 0.77-6.95) and ADH1B(*1/*11*1/*2) plus ALDH2(*1/*21*2/*2) (OR, 4.01 for current regular drinkers; 95% CI, 2.06-7.81). **[54,55]** In addition, alcoholic beverages may contain aldehyde itself and various carcinogenic contaminants, such as polycyclic aromatic hydrocarbons and nitrosamines. **[56,57]** Nutritional deficiencies may contribute to an increased risk of HN-SCC in heavy drinkers as well.

Notably, combined cigarette smoking and alcohol consumption exhibits a synergistic effect, with a reported relative risk for HN-SCC of 15 or more among heavy users of both products. **[52]** Large-scale multicenter studies in Europe and Asia, as well as pooled analysis of European and American casecontrol studies, have attributed more than half of oral and oropharyngeal cancer cases to tobacco and/or alcohol. **[58-60]**

Betel Quid

Betel quid (paan) chewing is a common practice in many parts of Asia as well as in migrant Asian communities around the world, with 600 to 1200 million users estimated globally. **[61]** The habit produces pleasing psychostimulatory effects and is deeply entrenched in many cultures. **[60,62]** Betel quid consists of a mixture of areca nut, slaked lime, and betel leaf, which may be combined with tobacco, sweeteners, and/or spices. Regional variations include mawa, naswar, khaini, and zarda. In addition, prepackaged, freeze-dried betel quid substitutes (eg, gutka, pan masala) are widely available. The carcinogenicity of betel quid traditionally has been attributed to tobacco, although areca nut itself is carcinogenic. **[62]** Recent large-scale studies, meta-analyses, and systematic reviews have reported ORs for HN-SCC of approximately 7 to 8 for betel quid with tobacco and 3 to 6 for betel quid without tobacco. **[63-66]** Among individuals who smoke, drink alcohol, and

chew betel quid, OC-SCC risk is exceptionally high (approximate pooled OR, 40). Indeed, all three habits are prevalent in South-East Asia, where 75% of the approximately 59,000 males annually affected by oral cancer have a history of combined smoking-drinking-betel quid exposure. **[67]**

HPV

Over the past several decades, accumulating evidence from epidemiologic, clinicopathologic, and molecular studies has established HPV as a major etiologic factor in a subset of HN-SCC. The majority of HPV-related HN-SCC arises in the oropharynx, particularly the palatine and lingual tonsils. In contrast, only a small proportion of OC-SCC appears to be caused by HPV. Specifically, the high-risk genotype HPV-16 accounts for the vast majority (approximately 90% to 95%) of HPV-positive OP-SCCs, whereas greater variability in HPV types is seen in OC-SCC. **[68]** Interestingly, the prevalence of high-risk HPV DNA in oropharyngeal and oral cancers appears to vary by geographic region. For OP-SCC, prevalence has been reported to be highest (approximately 60%) in North America; intermediate (approximately 36% to 45%) in Asia, Oceania, and Europe; and low (approximately 15%) in South and Central America. **[69-71]** Also, prevalence within Europe varies by subregion from approximately 17% in Southern Europe to 38% to 39% in Northern, Western, and Eastern Europe. **[69,70]** In contrast, for OC-SCC, high-risk HPV DNA prevalence has been reported to be highest in Asia (25% for HPV-16). **[71]** Determining the HPV-attributable fraction of HN-SCC is somewhat problematic because of confounding factors (especially from tobacco use) and limitations in methodology. In particular, many large-scale studies have assessed the presence of high-risk HPV DNA without concurrently evaluating biomarkers of HPV carcinogenesis (ie, E6 and E7 messenger RNA [mRNA], p16 cellular protein), thereby failing to distinguish between “passenger” versus carcinogenic HPV infection. Nevertheless, with attempts to correct for some of these limitations, a recent systematic review and meta analysis of studies reported worldwide from 1990 to 2004 estimated that the HPV-attributable fraction is approximately 40% for OPSCC and 7% to 16% for OC-SCC.72 Similarly, in North America and Europe, transcriptionally active, high-risk HPV (as evidenced by either quantitative reverse-transcriptase polymerase chain reaction or in situ hybridization-based methods for high-risk HPV E6 and E7 mRNA) has been detected in only about 0% to 9% of OC-SCC cases examined.69 Particularly in developed nations, a recent dramatic rise in HPV-related oropharyngeal cancer incidence has raised concerns of an emerging cancer epidemic. **[5,7,72]** Remarkably, in

the United States, HPV has been estimated to account for approximately 16% of OP-SCCs in the early 1980s compared with >60% of cases in more recent studies. **[73]** In addition, recent data suggest that the HPV-positive fraction of OP-SCC in Europe is increasing at an especially rapid rate and, thus, may be approaching that of North America. **[74]** The risk profile for HPV-positive oropharyngeal carcinomas differs from that for HPV-negative tumors. In both groups, there is a male predilection. However, HPVpositive tumors are more likely to occur in patients who are white, somewhat younger (median age, 54 years vs 58 years), and of higher socioeconomic status. HPV-positive OP-SCC also is strongly associated with an increased number of lifetime sexual or oral sexual partners. **[75,76]** Compared with HPV-negative tumors, HPV-positive tumors are more likely to arise in individuals with a history of marijuana use and are less likely to arise in individuals with heavy tobacco and alcohol exposure. **[75]** Nevertheless, in various recent studies, 47% to 71% of patients with HPVpositive OP-SCC have had some history of tobacco use. **[77-81]** In addition, 61% to 75% of patients with HPVpositive OP-SCC have reported current alcohol use, although only 9% to 18% have been classified as daily or heavy consumers. **[78,79,81]** More research is needed to clarify interactions between HPV, tobacco, and alcohol. Molecular evidence in support of HPV-driven HN-SCC includes the following observations: 1) high-risk, tumorigenic HPV-16 is present in 90% of HPV-positive HNSCCs; 2) in situ hybridization demonstrates localization of HPV-16 within the nuclei of HN-SCC cells; 3) HPV-16 DNA is present in high copy numbers in HPV-positive HN-SCC cells; and 4) HPV-16 genomic DNA is frequently integrated into HPV-positive HN-SCC cells, with active transcription of the major viral oncoproteins E6 and E7. **[18,82]** Differences in molecular genetic profile support that HPVrelated HN-SCC is biologically distinct from HN-SCC related to tobacco and alcohol. In the early stages of HPVnegative carcinogenesis, there are frequent losses of chromosomes 9p, 3p, and 17p **[83]**; in particular, the tumor suppressor genes tumor protein 53 (TP53) (which encodes p53) and cyclin-dependent kinase inhibitor 2A (CDKN2A) (which encodes p16) are located at 17p13 and 9p21, respectively. Thus, frequent p53 and p16 mutations result in cell cycle dysregulation and genomic instability. In contrast, HPVrelated HN-SCC often lacks such chromosomal losses, exhibits decreased expression of wild-type p53 (because of inactivation and degradation by E6), and exhibits increased p16 (because of E7 binding retinoblastoma protein [pRb], thereby interfering with cell cycle arrest and allowing accumulation of the p16 tumor suppressor protein). **[84,85]** It is not entirely clear why HPV-related HN-SCC preferentially develops within the oropharynx. Traditionally, investigators have proposed that HPV infection

occurs via microtrauma and exposure of basal epithelial cells to viral entry. Notably, the oropharynx is analogous to the uterine cervix and anus, in that it exhibits a squamocolumnar transition zone. Thus, the accessibility of metaplastic basal/reserve cells within the transition zone may explain the susceptibility of these sites to carcinogenic HPV infection. **[86]** Others have theorized that the tendency for OP-SCC to originate specifically within the palatine and lingual tonsils may be related to the following: 1) the deep invaginations of the tonsillar crypts may function as a reservoir for HPV and other pathogens, 2) the reticulated epithelium in these sites is attenuated with a discontinuous basement membrane, and 3) the deep crypts within this lymphoid tissue represent immune-privileged sites that favor persistent HPV infection and allow tumors to evade immune surveillance. **[68,87]**

Minor Risk Factors

Microorganisms

With recent advances in high-throughput genetic-based assays, there has been a growing body of research concerning the relationship between the oral microbiome and OCSCC. Several studies have demonstrated differences in the oral microbiome between normal individuals and patients with OC-SCC. However, it is not entirely clear whether such microbial shifts play a direct role in carcinogenesis or merely reflect differences in adaptability of microbial species to the cancer microenvironment. **[88]** Possible mechanisms by which oral flora may contribute to cancer development include the following: 1) metabolism of procarcinogens (eg, conversion of ethanol to acetaldehyde by Candida, Neisseria, and streptococci), 2) production of carcinogens (eg, production of nitrosamine by Candida), 3) induction of chronic inflammation (eg, by periodontal disease-causing bacteria) with production of cytokines that enhance cell proliferation and inhibit apoptosis, 4) direct influences of bacteria on cell cycle signaling, and 5) direct DNA damage by bacterial toxins. **[88,89]** Although it is difficult to control for confounding factors (eg, tobacco use, alcohol consumption, nutrition, socioeconomic status), some studies suggest an association between oral/pharyngeal cancer and measures of bacterial load (eg, poor oral hygiene, poor dental status, chronic periodontitis). **[90-92]**

Dietary factors and vitamin/mineral deficiencies

Several epidemiologic studies have noted that a diet rich in fruits and vegetables and low in animal products is associated with a reduced risk for oral cavity, pharyngeal, and other cancers. **[93-95]** The protective effects of plant foods might be attributed to various substances, such as carotenoids, vitamins C and E, folate, flavonoids, fiber, and lycopene. In addition, there is an increased risk for SCC of the upper alimentary tract among iron-deficient patients—most notably those with untreated Plummer-Vinson syndrome. **[96]** Some investigators have noted high rates of vitamin D deficiency in oral/head and neck cancer patients; a weak inverse association between oral/pharyngeal cancer and dietary vitamin D intake; and correlations between smoking, alcohol, and vitamin D deficiency. **[97-99]** However, further research regarding the potential role of vitamin D metabolism in HN-SCC development is needed.

Immune status

Compared with the general population, HIV-positive patients and organ transplant recipients exhibit a higher incidence of lip, oral cavity, and pharyngeal cancer. **[100-102]** Interestingly, a few large-scale case-control studies have noted an inverse relationship between allergies and head and neck cancer risk. Some investigators have hypothesized that heightened T-helper 2 immunity in individuals with allergies and asthma might protect against tumor growth, although further studies are needed. **[103,104]**

Environmental pollutants

In parts of Taiwan with alarmingly high oral cancer rates, some researchers have noted elevated soil concentrations of carcinogenic heavy metals (such as arsenic, chromium, and nickel). However, the strength of association between regional oral cancer mortality rates and heavy metal soil concentrations has varied across studies. **[105-107]**

Occupational exposures

Some studies have reported an association between oral/pharyngeal cancer and various occupations (including construction, painting, carpentry, metalworking, and machine operating). **[108]** In such occupations, exposures to high levels

of solvents and metal/wood/cement dusts have been hypothesized to confer an increased risk for oral and/or pharyngeal cancer. However, supporting data are limited and often inconsistent, with likely a small contribution to the overall occurrence of these cancers.

Heritable conditions

There is an increased risk for oral/pharyngeal SCC in patients with certain rare heritable conditions, including Fanconi anemia, dyskeratosis congenita, and Bloom syndrome. **[109-114]**

PATHOGENESIS OF HEAD AND NECK SQUAMOUS CELL CARCINOMAS

Recent advances in our understanding of the molecular pathogenesis of HNSCC were provided by whole-exome sequencing (i.e., sequencing exons of all known protein-coding genes) conducted on a total of approximately 100 HNSCC specimens independently by two groups. **[115,116]** While the two studies analyzed etiologically similar tumors with related sequencing platforms, there was a five-fold difference in the average number of mutations reported per tumor. This difference likely reflects distinct bioinformatic and validation approaches used in the studies, and therefore a subset of identified changes may represent "passenger" mutations (as a result of increased mutation rates in cancer cells, or even mutation "miscalls") rather than true "driver" mutations with an etiologic role in HNSCC. Nevertheless, several key findings were shared by these studies. This work, together with a large body of previous genomic and functional analyses of HNSCC, highlights the relatively small number of oncogenes targeted by activating mutations and supports the fundamental roles of tumor suppressor pathways including p53, Rb/INK4/ARF, and Notch in disease pathogenesis. These and other bona fide HNSCC cancer genes play major roles in at least four key functional pathways: cellular proliferation, squamous epithelial differentiation, cell survival, and invasion/metastasis, with many of the genes impacting more than a single pathway. These pathways are critical to the pathogenesis of HNSCC and, not surprisingly, reflect normal developmental programs within the stratified squamous epithelium. Given the paucity of driver oncogenes in HNSCC, targeting these pathways therapeutically represents a substantial and critical challenge for improving outcomes of this disease.

Cellular proliferation and p53/Rb/CDKN2A/CCND1

Mutation of the TP53 tumor suppressor gene is the most common and among the earliest identified genetic alterations in HNSCC, occurring in more than half of all cases (2). As in other human cancers, missense mutations primarily within the DNA binding domain account for 75% of all mutations in the TP53 gene and confer both dominant negative and poorly understood gain-of-function properties. **[117-120]** In many of the remaining HNSCC tumors in which p53 is wild-type, p53 function may be inactivated by other mechanisms. These include expression of the HPV E6 protein (which binds p53 and targets it for proteasomal degradation), overexpression/amplification of MDM2 (which also mediates p53 proteasomal degradation), and deletion of CDKN2A, which may eliminate p14/ARF, a negative regulator of MDM2. **[121-124]** Overall, the data suggest that the p53 pathway is downregulated in at least 80% of HNSCCs. **[125]** The finding that TP53 is mutated in both leukoplakia (a histologically recognizable precursor lesion) and benign-appearing mucosa has led to a "patch-field" progression model of HNSCC development, in which the index squamous carcinoma (as well as subsequent tumors) develops from a field of genetically abnormal mucosa, itself the result of expansion of a clonal patch arising from a putative stem cell containing a mutated TP53 gene. **[126]** Interestingly, in some cases the TP53 mutations found in the tumor and adjacent mucosa are different, implying a distinct clonal origin for multiple patches and suggesting that metachronous tumors from the same patient (e.g., primary versus locally recurrent) could in fact develop from unique clones through independent acquisition of additional alterations. **[127]** In addition to tumor initiation, TP53 inactivation also contributes to the clinical behavior of tumors, at least in part independent of an influence on the response to genotoxic therapy. Thus, truncating and functiondisrupting mutations of TP53 are significantly associated with decreased survival — after primary surgery with or without postoperative radiotherapy — compared with either non-disruptive mutations or no mutation at all. **[128,129]**

The essential role of the retinoblastoma (Rb) pathway is evidenced by the finding of inactivation of CDKN2A, encoding the cell cycle regulators p16/INK4A and p14/Arf/INK4B, in HNSCC. CDKN2A mutations were found in approximately 7% of tumors by exome sequencing, with copy number losses in another 20%–30%. **[115,116]** It has been previously shown that CDKN2A inactivation by mutation is significantly more rare than deletion or epigenetic inactivation, which together account for inactivation of the gene in up to 75% of HNSCCs. **[130-132]** Although p16/INK4A loss (whether genetic or functional) has been repeatedly

demonstrated to correlate with indicators of worse prognosis, data on p14/Arf/INK4B loss (e.g., by methylation, when the genomic locus itself is not deleted) is conflicting, with one study suggesting worsened prognosis, while two others suggested improved prognosis, perhaps a result of increased radiation sensitivity. **[133-135]** In the case of HPV+ HNSCC, inactivation of the Rb pathway is achieved through expression of the HPV E7 protein, which binds RB1 and abrogates the requirement for p16/INK4A silencing. As a result, assaying p16 protein expression in tumor cells by immunohistochemistry (IHC) is of clinical value in determining HPV+ status. **[136]** Amplification of a discrete, approximately 5-Mb region of chromosome 11q13 containing the CCND1 gene (encoding cyclin D1) occurs in approximately one-third of HNSCCs, and perhaps even more frequently in HPV-negative tumors. **[137,138]** Furthermore, overexpression of cyclin D1 has been observed in up to 80% of HNSCCs. **[125]** This high frequency is remarkable given that CDKN2A loss or CCND1 amplification would seem to be redundant mechanisms to promote cell cycle progression through activation of G1 phase cyclin-dependent kinases (CDKs) 4 and 6. Nevertheless, these two genetic events are not mutually exclusive in HNSCC, potentially reflecting either qualitatively or quantitatively different effects. For example, cyclin D1 may indirectly stimulate CDK2 activity by sequestering the CDK2 inhibitors p21 and p27, and alternatively, cyclin D1 may function as a cofactor independent of its role in cell cycle regulation, through binding to transcription factors (e.g., PPARγ) or DNA repair proteins (e.g., BRCA2, Rad51). In keeping with their distinct contributions, loss of p16 expression and overexpression of cyclin D1 are independent predictors of death from tongue cancer, and the loss of p16 together with overexpression of cyclin D1 confers significantly worse 5 year survival than either condition observation alone. **[139,140]**

Terminal differentiation and the Notch/p63 axis

Perhaps the most novel finding to emerge from the whole-exome sequencing studies of HNSCC is the discovery of mutations within the NOTCH1 gene in 12%–15% of cases, and within additional NOTCH family members in 3%–5%. **[115,116]** Although Notch signaling had previously been implicated as pro-tumorigenic — by virtue of activating mutations and translocations observed in the genes for Notch receptors or their regulators in T cell acute lymphoblastic leukemia, chronic lymphocytic leukemia, and diffuse large B-cell lymphoma **[141-145]** — several of the NOTCH family mutations in HNSCC (and in chronic myelomonocytic leukemia, a rare myeloproliferative disease) encode inactivating mutations, suggesting a tumor suppressor

function. **[146]** The physiologic relevance of these findings is supported by animal models in which NOTCH activation in hematopoietic cells leads to T cell leukemias and inactivation in squamous epidermis promotes skin tumorigenesis. **[refs. 147-149; reviewed in ref. 150]** Notch signaling has been linked to multiple biological functions, including regulation of self-renewal capacity, cell cycle exit (in part through upregulation of p21/CDKN1A expression), and cell survival. **[151-153]** In the stratified epithelium, Notch has a central role in promoting terminal differentiation, **[153,154]** which is mediated through both direct effects (e.g., on activation of suprabasal keratins) and indirect effects on the Wnt, hedgehog, and interferon response pathways. **[148,152,155,156]** Additionally, Notch activity has been linked to suppression of HPV E6 and E7 protein expression, potentially providing additional selective pressure for loss of Notch in HPV+ HNSCC. **[157,158]** Further supporting a role for Notch in squamous epithelial differentiation is its control by the p53-related transcription factor p63, a master regulator of proliferative potential, lineage specification, and differentiation in stratified epithelia. Constitutive knockout of Tp63 in mouse models results in complete failure of normal epidermal development. **[159,160]** In mature epithelium, expression of p63 is highest in basal epithelial cells, where it functions as an inhibitor of NOTCH1 expression, and becomes downregulated during terminal differentiation coincident with NOTCH1 upregulation. **[161]** Reactivation of p63 expression is observed in the suprabasal layers of dysplastic mucosa, and overexpression and/or genomic amplification of the TP63 locus is observed in the majority of invasive HNSCCs. **[162,163]** TP63 gives rise to two major isoform classes, TAp63 and ΔNp63, which differ in the presence and absence, respectively, of an N-terminal transcriptional transactivation domain. Although tumor incidence data from Tp63-heterozygous mice are conflicting, ΔNp63 isoforms, which are selectively overexpressed in HNSCC, are likely oncogenic. **[164,165]** Importantly, TP63 was found to be mutated or amplified in 8% of samples in one of the sequencing studies. **[116]** Consistent with a contribution of ΔNp63 in these tumors, two of the mutations uncovered are predicted to alter the function of TAp63 (including a nonsense mutation) but not ΔNp63. In addition to its contribution through Notch suppression, ΔNp63 has been demonstrated to control other key tumor-relevant pathways, including cell survival (in part through suppression of the proapoptotic p53-related protein p73), senescence suppression (through suppression of p16/INK4A expression), and growth factor signaling (through induction of EGFR). **[166-169]**

Cell survival through EGFR/Ras/PIK3CA/PTEN/CASP8

The PI3K signaling pathway is commonly activated in HNSCC, as evidenced by recurrent alterations of two central regulators: PTEN, encoding a negative regulator, and PIK3CA, encoding a positive regulator of this pathway. PTEN is subject to frequent loss of heterozygosity in a variety of cancers, including up to 40% of HNSCCs, although biallelic inactivation occurs less frequently. **[170-173]** Loss of just a single PTEN allele in the remaining samples may contribute to tumorigenesis, however, since recent data suggesting a gene dosage effect for PTEN (69). Activating mutations in two "hot spot" regions of the PIK3CA gene occur in 6%–11% of HNSCCs, with a potential enrichment in tumors originating from the pharynx. **[174,175]** The latter finding is particularly noteworthy given the increased incidence of PIK3CA mutations in HPV-related versus non-HPV-related tumors observed in both exome sequencing studies. This observation suggests that PIK3CA mutations may cooperate with HPV E6 and E7 proteins in the development of invasive OPSCC, as has been suggested for cervical carcinomas. **[176]** The prominent role of the PI3K pathway in HNSCC has potentially important clinical implications, given that numerous targeted inhibitors of this pathway are currently being evaluated in clinical trials **[177]**. Activating missense mutations causing single amino acid substitutions in one of three positions (codon 12, codon 13, and codon 61) in the HRAS gene were uncovered in 3%–5% of samples in both whole-exome sequencing studies. While it is currently unknown whether HRAS-dependent signals function in collaboration with or independently of PI3K activation in HNSCC, several findings underscore the importance of this particular RAS family member to the pathogenesis of the disease. These include the more frequent occurrence of HRAS than KRAS mutations in HNSCC, particularly in relationship to tobacco history, whereas the reverse is true for several other malignancies **[178,179]**; the presence of HRAS mutations in HPV-driven tumors, suggesting potential cooperativity in tumor promotion; **[180]** and the more frequent association of HRAS versus KRAS mutation in squamous cell carcinomas arising in the setting of tobacco exposure in humans and chemical carcinogen exposure in mice. **[181]** Although Ras proteins themselves have proven difficult to target directly, therapeutic strategies that target downstream effectors of Ras proteins or the synthetic lethal dependencies that result from their mutational activation have already been successful in preclinical models. **[182-184]** Upstream signaling to both Ras and PI3K pathways may occur through activation or overexpression of receptor tyrosine kinases (RTKs) including EGFR. Although it is often considered to be among the most important therapeutic targets in HNSCC **[185]**, our understanding of the role

of EGFR is evolving with the appreciation that EGFR activating mutations are rare in HNSCC and that the reported frequency of EGFR gene amplification in HNSCC varies widely, in part due to varying definitions of the degree and size of copy number gain that constitute amplification. **[186,187]** Furthermore, although copy number gain of EGFR has been suggested to correlate with poor prognosis in HNSCC **[187,188]**, in general gain of EGFR has not been clearly demonstrated to predict improved outcomes following EGFR-directed therapy. **[189,190]** Similarly, therapeutic agents that inhibit EGFR, including the small molecule inhibitors gefitinib and erlotinib and the therapeutic

antibody cetuximab, have modest activity in HNSCC, with little or no correlation with EGFR status. **[191-195]** Two other genetic abnormalities affecting RTK signaling have received less attention but have potential near-term clinical impact. Expression of EGFRvIII, a variant EGFR protein that results from the in-frame genomic deletion of exons 2–7 and is present in gliomas and lung squamous cancers, was recently reported in 42% of HNSCCs. **[196]** Importantly, an antibody thought to be specific for EGFRvIII (e.g., rather than full-length EGFR) was used to initially identify cases; this finding was not reproduced in another study that sequenced the full-length EGFR cDNA. **[197]** It will be important to resolve whether EGFRvIII is expressed with any appreciable frequency in HNSCC, as EGFR kinase inhibitors have demonstrated clinical activity against tumors expressing this variant. **[198]** Mutation or amplification of the MET (c-Met) RTK gene has been reported in some HNSCC cases. **[199]** This finding is of clinical interest both because MET amplification is thought to confer resistance to EGFR-directed therapy **[200]** and because the small molecule therapeutic crizotinib, which inhibits both the MET and ALK kinases, has recently been FDA approved for use in lung cancers harboring ALK translocations. **[201]** While each of the above genes and pathways are associated with activities that may indirectly prevent programmed cell death, several constituents of the apoptotic signaling cascade may also have an important role in HNSCC. These include caspase-8 (CASP8), encoding the critical proapoptotic enzyme that initiates a cascade of proteolysis responsible for executing apoptosis and found to be mutated in 8% of samples in one exome sequencing study **[116,202,203]**; and BCL2, encoding a key antiapoptotic regulator reported to be overexpressed in a fraction of HNSCC cell lines, particularly those with reduced expression of p63. **[168]**

Adhesion and invasion signaling through TGF-β/SMAD and FAT1

Inactivation of TGF-β signaling components is well established in human cancer, including HNSCC, most commonly through loss of TGF-β receptor (TGFBR2) and SMAD genes as a result of chromosome 18q deletion. **[204]** Notably, although missense mutations in TGFBR2 have been previously described in primary HNSCCs **[205]**, and SMAD2 and SMAD4 mutations have been reported in HNSCC cell lines **[206]**, no point mutations in these genes were found through exome sequencing, perhaps due to the low frequency of these events. The TGF-β pathway is a pleiotropic regulator in human cancer, as mutational inactivation of its signaling components is associated with tumor initiation, while activation of the pathway is known to promote metastasis. Thus, genetic loss of TGF-β pathway factors would at first glance seem at odds with a contribution of this pathway to invasion and metastasis in HNSCC. Recent mouse models, however, suggest a more complex interaction. Conditional deletion of Smad4 in the mouse stratified epithelium led to HNSCC in association with increased genomic instability and increased inflammation, the latter attributed in part to elevation of TGF-β1 and activation of other SMADs in stroma, mucosal epithelia, and tumor cells. **[207]** In addition, Tgfbr2 deletion within the mouse head and neck epithelia is insufficient to cause HNSCC, but cooperates with activated Kras to promote squamous carcinomas that metastasize to local lymph nodes. **[208]** TGF-β1 itself has also been associated with epithelial mesenchymal transition (EMT) and metastasis, the latter in the absence of functional TGF-βRII. **[209]** New insight into potential mechanisms of HNSCC invasion and metastasis was provided by the identification of mutations in the FAT1 gene in nine HNSCC samples (12%) in one of the two exome sequencing studies. **[116]** Six nonsense mutations and a seventh frameshift were noted, suggesting FAT1 may function as a tumor suppressor. Notably, focal, intragenic homozygous deletions of FAT1 have previously been described in oral cancer. **[210]** As it is a member of the cadherin superfamily of cell membrane proteins that have demonstrated roles in the establishment of cell polarity and mediate cell-cell contacts, loss of FAT1 might be predicted to permit loosening of the adhesions that normally restrain growth and/or migration of cells in an epithelial sheet. Similarly, mutations in genes encoding other membrane-associated proteins with a role in the establishment of polarity and cell adhesion have been described in HNSCC. **[211]**

Additional genes/pathways

Several genes with unclear roles in HNSCC were found to be mutated at appreciable frequencies in at least one of the exome sequencing studies. Although the functional significance of the identified missense mutations is not clear (and some may merely represent passenger mutations), recurrent inactivating mutations were observed in several additional genes, suggestive of tumor suppressor activity. These include MLL2 and NSD1 **[116]**, both encoding histone methyltransferases, and SYNE1 **[115,116]**, a nuclear envelope protein. Mutations within MLL2 have recently been described in non-Hodgkin's lymphoma, and mutations within several other histone-modifying enzymes have been identified in renal cell carcinoma and diverse human cancers, suggesting a role for chromatin-mediated gene expression deregulation in cancer pathogenesis **[212-214]**. Although SYNE1 loss has been previously described in ovarian cancers and gliomas, this genomic locus spans more than 0.5 Mb (the longest isoform comprises 146 exons) and is subject to copy number variation in normal tissues. As such, this locus could be expected to accumulate relatively frequent passenger mutations, resulting in an overestimate of the significance of mutations if gene size is not taken into account. **[215-218]**.

IMMUNOLOGY OF HEAD AND NECK SQUAMOUS CELL CARCINOMAS

Recently, there has been a renaissance in the idea that nascent premalignant cells are destroyed by the immune system before tumor formation can occur (termed immune surveillance). Derangements in the immune system or alterations in the transformed cells may allow immune escape, which then enables the cancer to manifest. Tumors themselves produce cytokines, such as transforming growth factor-β (TGF-β), interleukin (IL)-6, and IL-10, which suppress cell-mediated antitumor immunity while activating STAT1 (signal transducer and activator of transcription 1) suppression. **[219,220]** Inflammatory transcription factors, such as NF-κB (nuclear factor κ-light chain-enhancer of activated B cells) and STAT3, are aberrantly activated in tumor cells and are intensively studied as possible targets for therapeutic intervention. Tumor progression depends on acquisition of traits that allow cancer cells to evade immune surveillance and an effective immune response. HNSCC is an immunosuppressive disease, with lower absolute lymphocyte counts than those found in healthy subjects, **[221]** impaired natural killer (NK) –cell activity, **[222,223]** and poor antigen-presenting function. **[224,225]** Impairment of tumor-infiltrating T lymphocytes has also been reported in HNSCC and other cancers, **[226,227]**

with a strong impact on clinical outcome. **[228]** In addition, suppressive regulatory T cells (Tregs) have been linked to HNSCC tumor progression. Tregs secrete suppressive cytokines such as TGF-β and IL-10, express cytotoxic T lymphocyte–associated protein 4 (CTLA-4), and correlate with tumor progression. **[229]** Therefore, immunomodulatory therapies that overcome immune suppressive signals in patients with HNSCC have therapeutic promise. These include cancer vaccines using tumor peptide antigens, or viral, bacterial, and DNA-based vectors as well as tumor antigen–specific monoclonal antibodies (moAbs). The recent clinical efficacy of US Foodand Drug Administration–approved moAbs targeting immune checkpoint receptors, including anti–CTLA-4 and anti–programmed death-1 (anti–PD-1), provide further promise for patient benefit from immunomodulatory therapies as positive clinical data emerge.

Cancer immunosurveillance and immunoediting

The idea of immune system control of malignant cells was first proposed by Ehrlich in 1908. The cancer immunosurveillance hypothesis was introduced about 50 years later by Burnet and Thomas, who suggested that tumor cells must have antigens recognizably different from normal cells, and therefore, have the potential for immune clearance. Conflicting experimental results led many to abandon the idea of cancer immune surveillance for several decades, until several key discoveries led to a revival of the hypothesis. First, in the 1970s, was the discovery of the NK cell by Herbermann, which seemed to provide innate immune protection from tumor. **[230]** The discovery of interferon-gamma (IFN-γ) and its proapoptotic effect on tumor growth gave additional support to the potential for immune clearance of cancer cells. **[231,232]** Mice with genetically induced immunodeficiency were found to be more susceptible to both spontaneous and chemically induced tumors. In immunodeficient patients with HIV-1 infection, a higher risk of human papillomavirus (HPV) – associated head and neck cancer (HNC) has been suggested. **[233,234]** In addition, pharmacologically immunosuppressed organ transplant recipients demonstrate increased risk of many tumors with no known viral etiology, such as lung, head and neck, **[235]** pancreatic, endocrine, colon, and melanoma tumors. **[236]** Cancer immune editing suggests a dynamic evolutionary progress whereby immune surveillance of cancers provides selective pressure on tumor cells and negatively selects for cells that can evade the immune system. **[237]** Thus,

successful tumor formation occurs only after the cancer has discovered a means by which it can evade the immune system.

Immune escape and immunosuppression

HNSCC cells reduce their inherent immunogenicity, and second, they actively suppress signals 1-4 of the antitumor immune response. A key component for the immune system's recognition of different or altered cells is the human leucocyte antigen (HLA) complex, which presents processed tumor antigenic peptides to T lymphocytes. **[225]** Tumor cells can reduce T-cell–mediated recognition by altering HLA class I expression. Recently, mutations in specific HLA alleles, β-2 microglobulin, and antigen processing machinery (APM) components have been observed in large-scale, next-generation HNSCC sequencing efforts, such as The Cancer Genome Atlas, **[238]** paralleling lung cancer mutations. Chromosomal **[239]** and regulatory expression defects **[224]** in the HLA/APM-encoding genes themselves can cause selective
loss of HLA and APM component expression in a substantial fraction of HNSCCs and are correlated with poor prognosis. **[240,241]** Cells with complete loss of HLA may evade immune response by T-cell recognition but are a strong trigger for NK-cell activation, as the absence of HLA removes a key inhibitory signal for NK cells. Therefore, tumor cells use multiple mechanisms to realize immunoevasion while avoiding total loss of HLA expression. Endogenous antigens are processed (degraded into peptides) through the cytoplasmic immunoproteosome. Antigenic peptides are transported to the endoplasmic reticulum by the transporter associated with antigen processing (TAP1/2) heterodimer of theAPM. In the reticulum, they associate with HLA class I heavy chains **[242]**. HNSCC cells that express HLA I and tumor antigen can still evadeT-cell recognition through decreased expression or mutation of APM components but still maintain moderate HLA I expression to avoid recognition by NK cells. In addition to oncogenic epidermal growth factor receptor (EGFR) expression and mitogenic signaling, immunosuppressive effects may result, including downregulation of HLA, APM components, and STAT1 activation, while leading to suppressive STAT3 signaling, cytokines, and ligands on HNSCC cells. Another important group of molecules that has emerged from the research is the group of immune checkpoint receptors. As part of the immune system's control mechanisms against over reactive functions during inflammatory responses and to limit autoimmunity, this mechanism can be exploited in the tumor microenvironment. Several receptors have been identified that are expressed on exhausted,

dysfunctional lymphocytes, including CTLA-4, lymphocyte-activation gene 3 (LAG-3; CD223), T cell immunoglobulin mucin protein-3 (TIM-3), and PD-1. The ligand for PD-1, PD-L1 (B7-H1, CD274), is upregulated in multiple tumor cell lines, including HNSCC, **[243]** and induces a loss of function of cytotoxic T lymphocytes (CTLs). **[244]** CTLA-4 is a member of the B7 receptor family expressed by $CD4^+$, $CD8^+$, and Tregs **[245]** and competes with CD28 to bind to stimulatory ligands CD80 and CD86. LAG-3 is another receptor that has been shown to enhance Treg function. **[246]** TIM-3 as a marker or a mediator for immunosuppression is still being investigated, **[247]** but studies have correlated TIM-3 expression levels with poor clinical outcome. **[248]** Understanding these mechanisms has facilitated further establishment of immunotherapies, as outlined below.

Establishment of a cancer-promoting tumor microenvironment

That some cancers arise at sites of chronic inflammation was first noted by Virchow over a century ago. Infiltration of inflammatory mediators and a complex milieu of cytokines, including TGF-β, IL-6, IL-10, GM-CSF, IL-1β, IL-23, and TNF-α, as well as chemokines, which are "chemotactic cytokines," may be exploited by tumor cells. More recent developments link many of those cytokines to the formation of suppressive immune cells such as myeloid-derived suppressor cells (MDSCs), Tregs, tumor associated macrophages (TAMs), and their effectors, which are exploited and promoted by the tumor microenvironment.

Cytokines

Cytokines, which suppress immune function, are known to be produced by HNSCC cells. **[249]** TGF-β suppresses NK and T-cell activation and is a key cytokine in the differentiation of Tregs. **[250]** IL-6 signals via STAT3 to inhibit dendritic cell (DC) maturation and NK-cell, T-cell, neutrophil, and macrophage activation **[251]** and has been correlated with recurrence and survival in HNSCC. **[252]** STAT3 is a transcription factor that is also involved in several other immunosuppressive pathways such as IL-10 signaling, **[253]** suppression of DCs, **[254]** downregulation of IL-12, **[255]** and generation of Tregs. **[256]** Prostaglandin E2 is a prosurvival, proangiogenic molecule that is produced by many cancers, including HNSCC. **[257-259]** Vascular endothelial growth factor, which is primarily thought of as a promoter of angiogenesis, is overexpressed in 90% of HNSCCs **[260]** and functions to increase the ratio of immature to mature DCs in the

tumor microenvironment, which is thought to lead to T-cell dysfunction and inactivation. **[261]** Toll-like receptors (TLRs) stimulate the production of proinflammatory cytokines such as TNF-α, IFN-γ with a T-cell–stimulating effect resulting in a type 1 helper response.

Cellular immune components of the tumor microenvironment: MDSCS, TREGS, and TAMS

MDSCs are a diverse cellular population of myeloid origin with T-cell suppressive functions. **[262]** Initial studies in HNSCC found that MDSCs inhibit activated T cells. Also, MDSCs produce nitric oxide and reactive oxygen species, which interact to catalyze the nitration of the T-cell receptor, which inhibits T-cell receptor and HLA interaction, signaling, and subsequent activation. **[263]** Treatments such as antibody depletion, retinoic acid, gemcitabine, and STAT3 blockade, which diminish MDSCs, restoreimmunesurveillance, increase T-cell activation, and improve efficacy of immunotherapy. The basal levels of MDSCs increase with age and may contribute to increased tumor frequency and growth rate increase with age. **[264]** A subset of suppressor Tregs that prevent autoimmunity was relatively recently identified. This subpopulation of CD4T cells also expressCD25, **[265,266]** CTLA-4 and CD39.Tregs promote cancer progression by causing anergy, apoptosis, and cell cycle arrest of activated T cells via production of IL-10, TGF-β, and direct cell-to-cell contact.49**[267]** They also inhibit the action of DCs, NKcells, and B cells. **[268]** In patients with HNSCCs, Tregs are increased in peripheral blood and are more potent among T cells infiltrating the tumor, resulting in an immunosuppressed state. **[245, 269,270]** Also, Treg numbers are inversely proportional to DC and CD8+ T-cell numbers in HNSCC. **[271,272]** In addition, Treg frequency is elevated in patients with HNSCC after treatment, indicating that oncologic treatment increases Treg numbers. **[245]** TAMs in the tumor microenvironment may be strongly antitumor and possess a so-called M1 phenotype, which is characterized by the production of IFN-γ and other type 1 cytokines. Alternatively activated macrophages force a Th2 response, with production of interleukins such as IL-4 and IL-13 that permit tumor growth. TAM infiltrating tumors correlate with worse clinical outcome and are closely associated with the alternatively activated (M2) phenotype. These TAMs have been demonstrated to produce EGF, IL-6, and IL-10 and have been associated with angiogenesis, local tumor progression, and metastasis. **[273]** Through these immune/inflammatory cells and mediators, HNSCC induces an immunosuppressed state via multiple potent mechanisms, which is a barrier to effective cancer immunotherapy. **[274]**

Immune evasion of HPV-associated HNSCC

HPV infection and immune evasion in HPV-associated cancers is a clinically relevant model for immunotherapy. A critical component in avoiding adaptive and innate immune response is HPV's interference with IFNs and other signaling pathways. IFNs link the innate immunity response to the adaptive immunity response by activating immature DCs and CD8+ T cells and producing virus-specific antibodies. **[275,276]** Interferon-alfa (IFN-α) and interferon-beta (IFN-β) have immunostimulatory properties, are produced by virally infected cells and execute their antiviral effects through inhibition of mRNA, NKcell stimulation, and inhibition of viral protein expression. **[275]** IFN-γ activates leukocyte migration, antigen presentation, and inflammation and is primarily produced by effector lymphocytes. Therefore, antiviral immune response critically depends on inflammatory signaling, as evidenced by the frequent inactivating mutations in the TNF receptor-associated factor 3, or the TRAF3 gene, found in The Cancer Genome Atlas. **[238]** Danger signals, such as TLRs, present on inflammatory cells can also help to detect so-called pathogen-associated molecular patterns **[277]** to stimulate these IFN's. Furthermore, HPV interacts with antigen presentation to reduce adaptive immune response and suppresses STAT1 signaling inhibition by IFN pathways, causing downregulation of HLA class I APM. **[277,278]** Genetic host polymorphisms, **[279]** and even mutations, such as the recently identified 10% to 12% frequency of genomic alterations in HLA/TAP/β2M antigen processing/presentation pathways, **[238]** may present an ultimate barrier to successful immunotherapy in these patients. During normal immune responses, the presence of checkpoint receptors, such as PD-1 or CTLA-4, limits an over robust immune response to protect from autoimmune reactivity. **[280,281]** In patients with HNSCC, elevated PD-1 expression has been observed on CD8+ HPV positive tumor-infiltrating lymphocytes **[282]** but, unexpectedly, patients with high numbers of PD-1 expressing T-cell infiltration have shown a better 5-year overall survival rate (93.9%) compared with those patients with low PD-1–expressing T-cell infiltration (63.6%). This potentially conflicting observation may reflect a quantitatively greater overall antitumor immune response, because proinflammatory conditions can stimulate PD-L1 expression. Interestingly, PD-L1 expression of tumor tissue was not correlated to clinical outcome. **[282]** As a result, the quality and quantity of tumor-infiltrating lymphocytes (TILs) determines the antitumor response. This is confirmed by recent studies correlating the number of TILs in patients with HPV-positive oropharyngeal squamous cell cancer with

disease prognosis. **[283,284]** Badoual et al **[282]** also observed a higher number of tumor infiltrations with Tregs in HPV-positive oropharyngeal squamous cell cancer. So far, the reasons for the better prognosis of HPV-positive patients despite all of the mentioned HPV- and non–HPV-associated immune evasion mechanisms remain unclear.

HPV-Specific Cancer Immunoprevention Strategies

The most successful HNSCC-targeted immunotherapy will likely be HPV-targeted immunoprevention vaccines. The aim of the preventive vaccines is to inhibit viral infection and thus hinder cancer formation. The immunization targets the L1 capsid proteins and is realized by using virus-like particles. These particles provoke a humoral antibody response and, interestingly, generate a significantly stronger humoral response than natural infection. **[285]** Several large randomized, double-blinded, placebo-controlled phase III trials demonstrated high efficacy (recombinant HPV vaccine [types 6, 11, 16, 18], 96.8% to 100%; recombinant HPV bivalent vaccine [types 16 and 18], 90.9% to 100%) in prevention of benign and malignant HPV-associated cervical lesions. **[286]** The effects of the vaccination on oropharyngeal lesion has not yet been fully evaluated but is expected to have promising results, considering the achieved antiviral results so far and the rising prevalence of HPV positive oropharynx carcinoma. **[278,287]** The GlaxoSmithKline vaccine delivered in a randomized, placebo-controlled Costa Rican cohort demonstrated significantly reduced (nearly eliminated) oral HPV infection in the vaccine group, **[288]** suggesting a potential benefit for reducing future oropharyngeal squamous cell cancer cases. Because these prevention vaccines induce L1 capsid–specific Abs 2 to 3 log-folds higher than natural infection, they prevent viral entry and initial infection. However, because established HPV infection leads to viral DNA integration and expression of intracellular E6 and E7 oncogenes and loss of L1 expression, these prevention vaccines are ineffective for previous infections and are not therapeutic tools for established HPV-associated cancers. **[289]**

CLINICAL FEATURES AND DIAGNOSTIC WORKUP OF OROPHARYNGEAL CARCINOMAS

Clinical presentation and evaluation

OP-SCC develops most frequently in the tonsillar region and base of the tongue, often appearing as an ulcerated mass, fullness, or irregular erythematous mucosal change. **[81]** Such tumors often present at a more advanced stage than OC-SCC because of their ability to grow undetected and their propensity for metastasis. The most common chief complaints are the presence of a neck mass (from metastatic disease), sore throat, dysphagia and change in voice quality (hot potato voice). However, significant differences are noted with respect to the HPV status of the tumor. **[290]** In patients with HPV-related OPSCC, the most common complaint is development of a neck mass (51%), followed by sore throat (28%), and dysphagia (10%). It is not unusual for a patient to present with significant metastatic neck disease yet to have a small primary tumor that remains hidden or undetectable. In contrast, the most common symptom in HPV-negative OPSCC is sore throat (53%), followed by dysphagia (41%), and neck mass (18%). **[114]**

An accurate ENT evaluation with high definition NBI video-endoscopy of the upper aerodigestive tract with image capture and storage is vital for assessing the limits of tumor spread, such as direct and through invasion of the soft palate from anterior to posterior surfaces, the inferior extent of lateral pharyngeal wall tumors into the vallecula and pyriform fossa, and the superior extension of tonsillar cancers into the postnasal space and skull base.

Examination under anesthetic and panendoscopy is strongly recommended to assess the extent and resectability of the primary tumor and to exclude second primaries, especially in hypopharynx and esophagus. Examination under anesthetic is mandatory if thorough endoscopic examination is not possible in the clinic as above and/or if no biopsy can be obtained. **[291]**

Narrow Band Imaging (NBI)

Technology

Narrow band imaging (NBI; Olympus Medical Systems Corporation, Tokyo, Japan) is an endoscopic optical imaging enhancement technology that improves the contrast of the mucosal surface texture and mucosal and submucosal vasculature. Utilising the principle that different wavelengths of light will penetrate at different

depths, the technology filters white light to emit two 30-nm narrow bands of blue and green light simultaneously. The blue light centred at 415 nm corresponds to the main peak absorption spectrum of haemoglobin and penetrates the superficial mucosal layer to a depth of 0.16 mm to enhance the intrapapillary capillary loops (IPCLs). Blood vessels in the deeper mucosal and submucosal layers are visible due to the deeper penetration of the green light centred at 540 nm, which reaches a depth of 0.24 mm **[292,293,294].** The manner in which white light is filtered and reproduced on a monitor differs slightly for the two types of commercially available NBI systems. For the sequential endoscopes (Evis Lucera Spectrum and Evis Lucera Elite), a rotating filter is positioned in the path of the white light emitted from a xenon lamp so that only the blue and green narrow bands of light are emitted when in NBI mode. Light reflected from the mucosa is captured by a black and white charged coupled device (CCD) at the end of the endoscope and then reconstructed by the video processor into a coloured image displayed on the monitor. By outputting the 415-nm light to B and G channels and the 540 nm to the R channel on the colour monitor, the superficial microvasculature can appear brown and deeper blood vessels, cyan. In contrast, the colour CCD endoscopes (Evis Exera II and Evis Exera III) do not have a rotating filter but instead, just an NBI filter when in NBI mode. The reflected light is captured by a colour CCD chip with colour filters in each pixel to separate the colours and then reconstructed in the same way as sequential systems **[295]**. Depending on the specifications and components of the NBI system used, switching between white light (WL) and NBI modes can be achieved via a button on the video endoscope, video camera or monitor console **[296]**. Magnifying endoscopy combined with NBI (NBI-ME) further improves the visualisation of the mucosal surface and underlying microvasculature. Whilst optical magnification of up to 80 times is possible with the sequential systems, colour CCD systems are capable of at least 50 times magnification due to the combined effect of 1.2–1.5 times digital zoom and a physical zoom property that allows the endoscope tip to be positioned up to 2 mm from the mucosal surface without affecting resolution **[295]**. NBI, with or without magnifying endoscopy, has been extensively used and studied in the gastrointestinal, aerodigestive and urinary tracts since it first became commercially available in 2006. However, after the coincidental finding of two cases of moderately differentiated oral squamous cell carcinomas (OSCCs) during gastrointestinal examination **[297]**, research of its use in the oral cavity and oropharynx has gained traction due to promising results of it improving the visualisation of potentially malignant and malignant diseases in the head and neck region.

Microvasculature

The ability of NBI to enhance the microvascular morphology enables clinicians to identify potentially malignant and malignant lesions, as angiogenesis is an intrinsic part of carcinogenesis. Early reports identified well-demarcated brownish areas with scattered spots as areas of neoplasia and areas with ill-demarcated borders as inflammatory lesions. These scattered brown spots represent intra papillary capillary loop (IPCL). The combined use of high-definition endoscopes and NBI technology, however, facilitates the visualisation of changes in the degree of dilation, tortuosity, meandering and caliber of IPCL. An increase in dilation, distortion, branching, elongation and density is more pronounced the further a lesion is along the carcinogenesis continuum, and this has been confirmed by histology and three-dimensional imaging **[298,299,295]**. When there is an increase in microvascular density, the lesion is inherently thickened and significantly correlated with a higher rate of subepithelial invasion **[298]**. Therefore, based on the changes of the IPCL parameters, classifications of IPCL patterns specific for particular regions have been developed, which can then be used to determine the severity of the disease. In general, IPCL pattern classifications are based on the stepwise changes in microvascular irregularities **[300-302]**. The first proposed IPCL classification was for the oesophagus **[300]**. In this classification by Inoue et al, there are two criteria – one is the changes in IPCL and the other is the staining pattern with iodine dye. Type I is normal tissue, which has a normal IPCL pattern and will stain positively with iodine. Type II lesions stain positively with iodine and are associated with inflammation and have one or two of four characteristic IPCL changes, with the most common being elongation and/or dilation. Type III has no changes or minimal changes in IPCL pattern, negatively stains with iodine and typically represents mild dysplasia. Type IV is associated with severe dysplasia and has two or three of four IPCL changes; there is no staining with iodine. Carcinoma is signified by type V, which has all four characteristic IPCL changes (dilation, tortuous weaving, and changes in calibre and shape), and is negatively stained with iodine. Type V is further subdivided into four types according to the degree of IPCL destruction and depth of invasion **[300]**. However, as iodine staining with Lugol's iodine cannot be performed in other areas of the head and neck due to the risk of aspiration and the irritant nature of iodine **[303,304]**, head and neck lesions are usually classified using only the IPCL criterion **[302]**. In the oral cavity, most studies use a variation of the IPCL classification for oral mucosa, which Takano et al simplified from Inoue's IPCL classification for

oesophageal mucosa **[300,301]**. This classification system consists of four progressive increases in IPCL pattern types, with a 5th (type 0) added by us recently when IPCL patterns are not visualised due to think keratosis or leukoplakia (Figure 1). Type I IPCL pattern is typically associated with normal mucosa and appears as regular brown dots when IPCLs are perpendicular to the mucosa, or waved lines when running parallel (Figure 2). Dilated and a crossing IPCL pattern is type II (Figure 3), and further elongation and meandering of IPCLs is type III (Figure 4). Both type II and type III are generally associated with non-neoplastic and inflammatory lesions **[305,306]**; however, type III has also been found to have a higher incidence of dysplasia and carcinoma in situ (CIS) **[307,294]**. In contrast, type IV is characterised by IPCL pattern destruction, large vessels and angiogenesis, and is indicative of neoplasia (Figure 5). The range for sensitivity and specificity for differentiating benign lesions from neoplastic ones when using types III and IV as the criterion is high, between 85% to 89% and 93% to 95%, respectively **[307,294]**. When more than one IPCL pattern type is present in a lesion, the most advanced is designated the IPCL type for that lesion **[301]**. Given the subjective nature of interpreting IPCL patterns, it is important to be aware of any habits and underlying diseases that the patient may have. Regular smoking causes vasoconstriction and convolution of blood vessels, and conditions such as uncontrolled diabetes may be associated with vascular complications. Both of these can affect the appearance of vessels **[294]**.

NBI of the oropharynx

Pharyngeal intraepithelial squamous dysplasia and carcinoma in situ (CIS) are usually asymptomatic and can be missed despite multiple passes of the endoscope during conventional endoscopy because images from laryngoscopes used in otolaryngology have poor definition **[308,309]**. Whilst Lugol staining has been shown to improve visualisation of these lesions in the oesophagus, Lugol chromoendoscopy is not possible in the pharyngeal region as it can cause severe mucosal irritation, chest pain, allergic shock and pulmonary aspiration **[310,311,303]**. The introduction of NBI in the head and neck provides an alternative technology that has the potential to enable clinicians to detect and diagnose neoplastic lesions at an early stage. In one of the first published papers on the use of NBI in the pharyngeal region, Muto et al identified 34 superficial oropharyngeal and hypopharyngeal lesions from 18 patients using a prototype version of NBI (Muto et al, 2004). All lesions

were histologically diagnosed as SCC and appeared as well-demarcated brown areas with scattered brown dots using NBI-ME. In contrast,

only four lesions had well-demarcated brownish areas and only one had scattered brown dots using conventional observation. These results indicate that detecting and monitoring head and neck lesions is significantly better by NBI than by conventional endoscopy **[308]**. Several other studies report that NBI improves the visualization of well-demarcated areas and irregular superficial microvascular patterns associated with SCC **[312,311,303]**. A study by Watanabe et al. **[310]** noted a considerable improvement in the contrast of the superficial microvascular pattern, such that the sensitivity of NBI was nearly two times better than conventional endoscopy. A later study by Watanabe et al. **[313]** evaluated the diagnostic accuracy of NBI for early head and neck cancers, and reported 97.7% sensitivity, 98.9% specificity, 86.3% PPV, 99.8% NPV and 98.8% accuracy. Of note is the significant difference between WL sensitivity (51.1%) and NBI sensitivity, which suggests that NBI is better at correctly detecting SCC than WL.

A multicentre, prospective, randomised controlled trial by Muto et al. **[314]** also reported significantly higher sensitivity, NPV and accuracy for the detection of head and neck squamous cell carcinoma (HNSCC) with NBI than by WL. In this study, 320 patients with oesophageal SCC were randomly allocated to either the primary WL followed by NBI or the primary NBI followed by WL imaging groups. All (100%; 15 of 15) superficial cancers in the head and neck, which included the oropharynx and hypopharynx, were detected with primary NBI, whereas only one of 13 (8%) was detected with primary WL imaging. The sensitivity, specificity, NPV, PPV and accuracy of primary NBI using 'well-demarcated brownish area and an irregular microvascular pattern (IMVP)' as the diagnostic criterion was 100%, 78.6%, 83.3%, 100% and 86.7%, respectively. In comparison, primary WL imaging had only 7.7%, 95.5%, 50%, 63.6% and 62.9%, respectively. The higher sensitivity, NPV and accuracy suggest that the number of false positives and false negatives is very low by NBI endoscopy. Therefore, the ability to correctly detect and diagnose HNSCC is better by primary NBI than by primary WL imaging. Interestingly, the detection rate of HNSCC can be significantly increased with secondary NBI after primary WL imaging, whilst a significant decrease will occur with secondary WL imaging after primary NBI **[314]**. Visualisation of superficial lesions and diagnostic accuracy is further improved with the use of NBI-ME. A retrospective study by Matsuba et al noted that the rate of clearly visualising well-demarcated areas associated with superficial oropharyngeal and hypopharyngeal cancers improved from 23%

with WL to 82% and 100% with NBI and NBI-ME, respectively **[303]**. The rate of visualising irregular microvascular patterns also improved considerably from 14% with WL endoscopy to 77% with NBI, and 100% with NBI-ME **[303]**. By providing clinicians images of the minute details of the superficial microvascular pattern, NBI can have an extremely high diagnostic accuracy for superficial cancer **[315,303]**. Fujii et al. **[298]** recognised the need to correlate NBI images with changes in IPCLs that occur over the clinical course of superficial SCC in order to make a precise pathological diagnosis and appropriate decision on optimal therapy. Even without magnification, small lesions less than 10 mm have a higher detection rate with NBI than with WL **[313]**. The addition of magnification to NBI improves the sensitivity for detecting abnormal microlesions that are 5 mm or less in diameter when compared to WL **[314,316]**. Kumamoto investigated the use of NBI-ME for diagnosing and treating minute pharyngeal neoplasias in 93 patients. NBI mode was used before WL as the contrast is improved with NBI. Under WL, lesions appeared faintly red, making it difficult to diagnose if NBI was not used first. All were IPCL type IV lesions and had similar features in both magnified and unmagnified NBI examinations despite the fact that different degrees of dysplasia were present. Low-grade dysplasia was present in lesions as small as 0.3 mm in diameter, and all lesions greater than 1.3 mm had dysplasia. Therefore, the authors recommended resection of all lesions 1.0 mm or greater in diameter, with an increase in diameter being associated with an increase in dysplasia ratio (Kumamoto et al, 2012)**[317,318]**.

Imaging

Cross-sectional imaging is required in all cases to complete assessment and staging. Magnetic resonance imaging (MRI) scanning with contrast is optimal for staging the primary tumour, particularly when assessing soft tissue spread, such as in the tongue base and/or body of the tongue. Computed tomography (CT) scanning may also be required, particularly to assess the extent of nodal disease and bony invasion, e.g. body of the mandible and skull base in tonsillar tumors and cervical spine in posterior pharyngeal wall tumors. The presence of nodal metastases should be evaluated by CT or MRI in all patients. Ultrasound with or without needle biopsy should be carried out for all patients presenting with a neck lump and is an accurate method of staging nodal disease in experienced hands. Distant metastases should be assessed by CT scanning of the chest and upper abdomen, to exclude metastatic disease to the lungs and liver. Magnetic resonance imaging scanning is not suitable for this due to the relatively slow acquisition process leading to movement artefact caused by

breathing. Fluoro-deoxy-glucose positron emission tomography combined with computed tomography (F-FDG PET–CT) scanning may be used to give additional staging information when it is available, particularly where staging is difficult clinically (e.g. patient with trismus) or where there is uncertainty on other imaging and/or equivocal findings that would preclude radical treatment. Positron emission tomography (PET) also has a role in the assessment of recurrent tumors and can detect recurrence at primary sites, neck nodes and/or distant metastases. Supported by the results of the UK PET-Neck randomized controlled trial (RCT) study, F-FDG PET–CT scanning is now also recommended for the assessment of response approximately three months post-chemoradiotherapy, particularly in patients with advanced nodal disease. PET-CT guided active surveillance showed similar survival outcomes to the planned neck dissection arm, but resulted in considerably fewer neck dissections, and fewer complications, and was cost effective, supporting its use in routine practice. **[319,320,321]**

Biopsy and pathology

Formal tissue biopsy of the primary cancer is one of the cornerstones of the management pathway in oropharyngeal cancer. Tumors can be biopsied under local or no anesthetic in the clinic. Otherwise, direct biopsy and staging under general anesthetic is necessary. In very few circumstances, a positive cancer diagnosis from fine needle aspiration (FNA) of involved nodes may suffice, provided the cytology result has been considered in conjunction with the clinical presentation and appropriate imaging at a head and neck cancer multidisciplinary team meeting. Such circumstances may arise in a person who is unfit to have an anesthetic for an open biopsy and in whom local anesthetic biopsies have not been successful. There is limited information on the reliability of p16 and HPV tests on FNA material and HPV testing is not currently routinely recommended on FNA samples. Most oropharyngeal cancers are squamous cell carcinomas. It is recommended that they are reported according to The Royal College of Pathologists UK Guidelines 2013 for the histopathology reporting of mucosal malignancies of the pharynx.

Because HPV-positive OP-SCCs have a better prognosis than HPV-negative tumors, HPV tumor status is routinely assessed at most institutions for patients who have oropharyngeal carcinoma or metastatic head and neck carcinoma with an unknown primary site. Upon histopathologic examination, HPV-related OP-SCC tends to be nonkeratinizing with a somewhat basaloid appearance recapitulating tonsillar crypt epithelium.

[322] Methods for evaluating HPV tumor status include quantitative reverse transcriptase polymerase chain reaction for high-risk HPV E6 and E7 mRNA, DNA or RNA in situ hybridization-based methods, and p16 immunohistochemistry. **[323]** The use of p16 immunohistochemistry as a surrogate marker for HPV status has been validated by many studies, albeit only for carcinomas of the oropharynx and mainly for tumors with nonkeratinizing morphology. **[324,325]** Accordingly, the College of American Pathologists recommends the following protocol for assessing HPV status in OP-SCC: 1) for entirely or predominantly nonkeratinizing tumors, strong and diffuse (ie, >70% cytoplasmic and nuclear) immunohistochemical expression of p16 is sufficient to indicate HPV positivity, and HPV DNA testing (ie, in situ hybridization or polymerase chain reaction) is not required; 2) for entirely or predominantly nonkeratinizing tumors with negative or focally positive immunohistochemical expression of p16, HPV DNA testing is required; 3) for keratinizing tumors with strong and diffuse immunohistochemical expression of p16, HPV DNA testing is required; 4) for keratinizing tumors, negative or focally positive immunohistochemical expression of p16 is sufficient to indicate negative HPV status, and HPV DNA testing is not required. **[324]** Also, the College of American Pathologists protocol advocates p16 immunohistochemistry or in situ hybridization as a reliable predictor of oropharyngeal origin in the evaluation of lymph node biopsies or fine-needle aspirations showing metastatic cervical carcinoma with an unknown primary.

Staging

The American Joint Committee on Cancer staging system for both oral and oropharyngeal cancers requires an assessment of the primary tumor (T), lymph nodes (N), and distant metastasis (M). **[326,327]** Prognosis traditionally has been linked to tumor stage. However, evidence supports that HPV-associated OP-SCC, despite often exhibiting lymph node disease at diagnosis, has a more favorable prognosis compared with HPV-negative disease. **[328,330]** Conversely, a history of cigarette smoking portends a worse prognosis. **[331,332]** Accordingly, some investigators propose that staging criteria for oropharyngeal cancer also should include HPV status and smoking history.164,165 For example, in a retrospective analysis of the effect of HPV tumor status on survival among patients with OP-SCC enrolled in a Radiation Therapy Oncology Group trial, Ang et al used recursive-partitioning analysis to identify factors (including HPV tumor status, pack-years of cigarette smoking, T classification, and N classification) that were most predictive of overall survival.78

Accordingly, patients were classified into the following categories: low-risk (HPV-positive tumors with ≤10 pack-years of smoking or N0-N2a HPV-positive tumors with >10 pack-years of smoking), intermediate-risk (N2b-N3 HPV-positive tumors with >10 pack-years of smoking or T2-T3 HPV-negative tumors with ≤10 packyears of smoking), and high-risk (T4 HPV-negative tumors or HPV-negative tumors with >10 pack-years of smoking). The 3-year overall survival rates for the low-risk, intermediate- risk, and high-risk groups were 93%, 70.8%, and 46.2%, respectively. Subsequently, investigators have confirmed these findings or have proposed other prognostic risk models. **[328,333-335]** Further validation studies for proposed risk models are needed, although clinical trials evaluating deintensified radiation and chemotherapy protocols are underway for patients considered to have a favorable prognosis based on HPV-positive tumor status and other parameters. Also of interest, retrospective studies by Mroz et al have found that increased mutant-allele tumor heterogeneity (MATH) (a quantitative measure of an individual tumor's genetic heterogeneity based on next-generation sequencing data) correlates with an adverse prognosis in HN-SCC patients, with high MATH values significantly associated with shorter overall survival (hazard ratio, 2.2-2.5), decreased survival among patients receiving primary or adjuvant chemoradiation therapy (hazard ratio, 5.2), HPVnegative tumor status, and disruptive TP53 mutations. **[336,337]** Despite a strong association between HPV positive tumors and low MATH values, bivariate Cox proportional hazards analysis suggests that the role of intratumor heterogeneity in HN-SCC mortality is independent of HPV tumor status. The investigators propose that MATH may be useful not only for clinical trials evaluating deintensified organ-preservation therapy for OP-SCC but also for the stratification of patients who have head and neck cancers unrelated to HPV.

Clinical and Pathologic T Category for HPV (p16-Positive) Oropharyngeal Cancer		**Clinical and Pathologic T Category for Non-HPV (p16-Negative) Oropharyngeal Cancer**	
T category	T criteria	T category	T criteria
T0	No primary identified	Tx	Primary tumor cannot be assessed
T1	Tumor 2 cm or smaller in greatest dimension	Tis	Carcinoma in situ
T2	Tumor larger than 2 cm but not larger than 4 cm in greatest dimension	T1	Tumor 2 cm or smaller in greatest dimension
T3	Tumor larger than 4 cm in greatest dimension or extension to lingual surface of epiglottis	T2	Tumor larger than 2 cm but not larger than 4 cm in greatest dimension

T4	Moderately advanced local disease; tumor invades the larynx, extrinsic muscle of tongue, medial pterygoid, hard palate, or mandible or beyond *	T3	Tumor larger than 4 cm in greatest dimension or extension to lingual surface of epiglottis
		T4	Moderately advanced or very advanced local disease
		T4a	Moderately advanced local disease; tumor invades the larynx, extrinsic muscle of tongue, medial pterygoid, hard palate, or mandible *
		T4b	Very advanced local disease; tumor invades lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, or skull base or encases carotid artery
Clinical N Category for HPV (p16-Positive) Oropharyngeal Cancer		**Clinical N Category for Non-HPV (p16-Negative) Oropharyngeal Cancer**	
N category	N criteria	N category	N criteria
Nx	Regional lymph nodes cannot be assessed	Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis	N0	No regional lymph node metastasis
N1	One or more ipsilateral lymph nodes, none larger than 6 cm	N1	Metastasis in a single ipsilateral lymph node, 3 cm or smaller in greatest dimension and ENE-negative
N2	Contralateral or bilateral lymph nodes, none larger than 6 cm	N2	Metastasis in a single ipsilateral lymph node larger than 3 cm but not larger than 6 cm in greatest dimension and ENE-negative; or metastases in multiple ipsilateral lymph nodes, none larger than 6 cm in greatest dimension and ENE-negative; or metastasis in bilateral or contralateral lymph nodes, none larger than 6 cm in greatest dimension and ENE-negative
N3	Lymph node(s) larger than 6 cm	N2a	Metastasis in a single ipsilateral lymph node larger than 3 cm but not larger than 6 cm in greatest dimension and ENE-negative
		N2b	Metastasis in multiple ipsilateral lymph nodes, none larger than 6 cm in greatest dimension and ENE-negative
		N2c	Metastasis in bilateral or contralateral lymph nodes, none larger than 6 cm in greatest dimension and ENE-negative
		N3	Metastasis in a lymph node larger than 6 cm in greatest dimension and ENE-negative; or metastasis in any lymph node(s) and clinically overt ENE-positive
		N3a	Metastasis in a lymph node larger than 6 cm in greatest dimension and ENE-negative
		N3b	Metastasis in any node(s) and clinically overt ENE-positive

Pathologic N Category for HPV (p16-Positive) Oropharyngeal Cancer		**LEGEND**	
N category	N criteria	*	*Mucosal extension to lingual surface of epiglottis from primary tumors of the base of the tongue and vallecula does not constitute invasion of the larynx.*
Nx	Regional lymph nodes cannot be assessed		
pN0	No regional lymph node metastasis	*ENE*	*Extranodal extension*
pN1	Metastasis in 4 or fewer lymph nodes		
pN2	Metastasis in more than 4 lymph nodes		

Adapted from: AJCC Cancer Staging Manual, Eighth Edition (2017) published by Springer Science and Business Media LLC (Amin MB, Edge SB, Greene FL, et al, eds. AJCC Cancer Staging Manual. 8th ed. New York: Springer; 2017).

THERAPEUTIC OPTIONS FOR OROPHARYNGEAL CARCINOMAS

General principles of surgery in HNSCC [338]

All patients should be evaluated by a head and neck surgical oncologist prior to treatment to review the adequacy of biopsy material, staging and imaging, to determine the extent of disease and exclude the presence of a synchronous primary tumor, to assess current functional status and to evaluate the potential surgical options, including those applicable if initial non-surgical treatment is unsuccessful.

Pre-treatment evaluation has the goal of maximizing survival with preservation of form and function and of developing a prospective surveillance plan that includes adequate dental, nutritional, and health behavior evaluation and intervention and any other ancillary evaluations that would provide for comprehensive rehabilitation.

For patients undergoing an operation, the surgical procedure, margins, and reconstructive plan should be developed and designed to resect all gross tumors with adequate tumor-free surgical margins. The surgical procedure should not be modified based on any response observed as a result of prior therapy except in instances of tumor progression that mandate a more extensive procedure in order to encompass the tumor at the time of definitive resection.

Tumor involvement of the following sites is associated with poor prognosis or function (in selected cases, surgery might still be considered) or with T4b cancer (ie, unresectable based on technical ability to obtain clear

margins). None of these sites of involvement is an absolute contraindication to resection in selected patients in whom total cancer removal is possible:

- Involvement of the pterygoid muscles, particularly when associated with severe trismus or pterygopalatine fossa involvement with cranial neuropathy;
- Gross extension of the tumor to the skull base (eg, erosion of the pterygoid plates or sphenoid bone, widening of the foramen ovale);
- Direct extension to the superior nasopharynx or deep extension into the Eustachian tube and lateral nasopharyngeal walls;
- Invasion (encasement) of the common or internal carotid artery: encasement is usually assessed radiographically and is defined as a tumor surrounding the carotid artery by 270 degrees or greater;
- Direct extension of neck disease to involve the external skin;
- Direct extension to mediastinal structures, prevertebral fascia, or cervical vertebrae; and presence of subdermal metastases.

Surgical resection of advanced tumors of the oral cavity, oropharynx, hypopharynx, larynx, or paranasal sinus will vary in extent depending on the structures involved. The primary tumor should be considered surgically curable by appropriate resection using accepted criteria for adequate excision, depending on the region involved. Surgery should be planned based on the extent of the primary tumor as ascertained by clinical examination and careful interpretation of appropriate radiographic images.

En bloc resection of the primary tumor should be attempted whenever feasible, in-continuity neck dissection is necessary when there is direct extension of the primary tumor into the neck. For oral cavity cancers, as thickness of the lesion increases, the risk of regional metastases and the need for adjuvant elective neck dissection also increases.

Perineural invasion should be suspected when tumors are adjacent to motor or sensory nerves. The goal is total cancer resection and when gross invasion is present and the nerve can be resected without significant morbidity, the nerve should be dissected both proximally and distally and should be resected to obtain clearance of disease;

frozen section determination of the proximal and distal nerve margins may prove helpful to facilitate tumor clearance.

Partial or segmental resection of the mandible may be necessary to adequately encompass the cancer with adequate tumor-free margins. Adequate resection may require partial, horizontal, or sagittal resection of the mandible for tumors involving or adherent to mandibular periosteum. Segmental or marginal resection should be considered in tumors that grossly involve mandibular periosteum (as determined by tumor fixation to the mandible) or show evidence of direct tumor involvement of the bone at the time of operation or through preoperative imaging (CT/MRI/Panorex). The extent of mandibular resection will depend on the degree of involvement accessed clinically and in the operating room. Medullary space invasion is an indication for segmental resection and frozen section examination of available marrow may be considered to guide resection.

For tumors of the larynx, the decision to perform either total laryngectomy or conservation laryngeal surgery (eg, transoral resection, hemilaryngectomy, supraglottic laryngectomy) will be decided by the surgeon but should adhere to the principles of complete tumor extirpation with curative intent and function preservation.

For maxillary sinus tumors, note that “Ohngren’s line” runs from the medial canthus of the eye to the angle of the mandible, helping to define a plane passing through the maxillary sinus. Tumors “below” or “before” this line involve the maxillary infrastructure. Those “above” or “behind” Ohngren’s line involve the suprastructure.

Transoral robotic surgery (TORS) or laser-assisted resections of primary cancers in the oral cavity, larynx, and pharynx are increasingly used approaches for cancer resection in selected patients with limited disease and accessible tumors. Oncologic principles are similar to open procedures. Successful application of these techniques requires specialized skills and experience.

The surgical management of regional lymphatics is dictated by the extent of the tumor at initial tumor staging. NCCN guidelines apply to the performance of neck dissections as part of treatment of the primary tumor. In general, patients undergoing surgery for resection of the primary tumor will undergo dissection of the ipsilateral side of the neck that is at greatest risk for metastases. Tumor sites that frequently have bilateral lymphatic drainage (eg, base of tongue, palate, supraglottic larynx, deep pre-epiglottic space involvement)

often should have both sides of the neck dissected with the extent of dissection determined as suggested below. For those patients with tumors at or approaching the midline, both sides of the neck are at risk for metastases, and bilateral neck dissections should be performed.

Patients with advanced lesions involving the anterior tongue, floor of the mouth or lip that approximate or cross the midline should undergo contralateral submandibular dissection as necessary to achieve adequate tumor resection.

Elective neck dissection should be based on risk of occult metastasis in the appropriate nodal basin. For oral cavity squamous cell carcinoma, sentinel lymph node biopsy or the evaluation of primary tumor depth of invasion is currently the best predictor of occult metastatic disease and should be used to guide decision making. For tumors with a depth greater than 4 mm, elective dissection should be strongly considered if RT is not already planned, for a depth less than 2 mm, elective dissection is only indicated in highly selective situations, for a depth of 2–4 mm, clinical judgment (as to reliability of follow-up, clinical suspicion, and other factors) must be utilized to determine appropriateness of elective dissection. Recent randomized trial evidence supports the effectiveness of elective neck dissection in patients with oral cavity cancers >3 mm in depth of invasion. Elective dissections are generally selective, preserving all major structures, unless operative findings dictate otherwise.

The type of neck dissection (comprehensive or selective) is defined according to preoperative clinical staging, is determined at the discretion of the surgeon, and is based on the initial preoperative staging as follows:

N0 Selective neck dissection

Oral cavity at least levels I-III

Oropharynx at least levels II-IV

Hypopharynx at least levels II-IV and level VI when appropriate

Larynx at least levels II-IV and level VI when appropriate

N1-N2a-c Selective or comprehensive neck dissection

N3 Comprehensive neck dissection

Level VI neck dissections are performed for certain primary sites (such as the larynx and hypopharynx) as required to resect the primary tumor and any clinically evident neck nodes. Elective dissection depends on primary tumor extent and site. Subglottic laryngeal cancers are sites where elective level VI dissections are often considered appropriate.

About management of recurrences, surgically resectable primary cancers should be re-resected with curative intent if feasible, and recurrences in a previously treated neck should undergo surgery as well. Neck disease in an untreated neck should be addressed by formal neck dissection or modification depending on the clinical situation. Non-surgical therapy may also be utilized as clinically appropriate.

***General principles of radiotherapy and chemotherapy in HNSCC* [338]**

Radiotherapy

Target delineation and optimal dose distribution require experience in head and neck imaging and a thorough understanding of patterns of disease spread. Standards for target definition, dose specification, fractionation (with and without concurrent chemotherapy), and normal tissue constraints are still evolving. IMRT or other conformal techniques (3-D conformal, helical tomotherapy, VMAT, and proton beam therapy [PBT]) may be used as appropriate depending on the stage, tumor location, physician training/experience, and available physics support. Close interplay exists between radiation technology, techniques, fractionation, and chemotherapy options resulting in a large number of combinations that may impact toxicity or tumor control. Close cooperation and interdisciplinary management are critical to treatment planning and radiation targeting, especially in the postoperative setting or after induction chemotherapy.9 FDG-PET/CT or MRI with contrast can be used for fusion in treatment planning.

Advanced radiation therapy technologies such as IMRT, IGRT (image-guided radiation therapy) and PBT may offer clinically relevant advantages in specific instances to spare important organs at risk (OARs) such as the brain, brain stem, cochlea, semicircular canals, optic chiasm and nerves, other cranial nerves, retina, lacrimal glands, cornea, spinal cord, brachial plexus, mucosa, salivary glands, bone (skull base and mandible), pharyngeal constrictors, larynx and esophagus; and decrease the risk for late, normal tissue damage while still

achieving the primary goal of local tumor control. The demonstration of significant dose-sparing of these OARs reflects best clinical practice.

Since the advantages of these techniques include tightly conformal doses and steep gradients next to normal tissues, target definition and delineation and treatment delivery verification require careful monitoring to avoid the risk of tumor geographic miss and subsequent decrease in local tumor control. Initial diagnostic imaging with contrast-enhanced CT, MRI, PET, and other imaging modalities facilitate target definition. Image guidance is required to provide assurance of accurate daily delivery.

Intensity-Modulated Radiation Therapy - IMRT has been shown to be useful in reducing long-term toxicity in oropharyngeal, paranasal sinus, and nasopharyngeal cancers by reducing the dose to salivary glands, temporal lobes, auditory structures (including cochlea), and optic structures. The application of IMRT to other sites (eg, oral cavity, larynx, hypopharynx, salivary glands) is evolving and may be used at the discretion of treating physicians. Helical tomotherapy and VMAT (volumetric modulated arc therapy) are advanced forms of IMRT.

Proton Beam Therapy - Achieving highly conformal dose distributions is especially important for patients whose primary tumors are periocular in location and/or invade the orbit, skull base, and/or cavernous sinus; extend intracranially or exhibit extensive perineural invasion; and who are being treated with curative intent and/or who have long life expectancies following treatment. Nonrandomized single institution clinical reports and systematic comparisons demonstrate safety and efficacy of proton beam therapy in the above mentioned specific clinical scenarios.

IMRT, PBT, and Fractionation - A number of ways exist to integrate IMRT or PBT, target volume dosing, and fractionation. The Simultaneous Integrated Boost (SIB) technique uses di erential “dose painting” (66–74 Gy to gross disease; 50–60 Gy to subclinical disease) for each fraction of treatment throughout the entire course of radiation.4 SIB is commonly used in the conventional (5 fractions/wk) and the “6 fractions/wk accelerated” schedule. The Sequential (SEQ) technique typically delivers the initial (lower dose) phase (weeks 1–5) followed by the high-dose boost volume phase (weeks 6–7) using 2–3 separate dose plans, and is commonly applied in standard fractionation and hyperfractionation. The Concomitant Boost Accelerated

schedule may utilize a “Modi ed SEQ” dose plan by delivering the dose to the subclinical targets once a day for 6 weeks, and a separate boost dose plan as a second daily fraction for the last 12 treatment days.

Palliative radiation should be considered in the advanced cancer setting when curative-intent treatment is not appropriate even if no general consensus exists for appropriate palliative RT regimens in head and neck cancer. For those who are either medically unsuitable for standard RT or who have widely metastatic disease, palliative RT should be considered for relief or prevention of locoregional symptoms if the RT toxicities are acceptable. RT regimens should be tailored individually; severe RT toxicities should be avoided when treatment is for palliation. While the use of shorter treatment courses is encouraged, the dose tolerance of the spinal cord and neural structures must be evaluated carefully in light of fraction size.

Reirradiation With 3-D Conformal RT, SBRT, PBT, or IMRT is strongly recommended and these patients must be evaluated by a multidisciplinary team at a high-volume head and neck center before treatment; prior radiotherapy should be more than 6 months from the appearance of new disease; before reirradiation, the patient should have a reasonable ECOG performance status of 0-1.The treatment team must be able to develop a reirradiation treatment plan that limits the cumulative dose of radiation to CNS tissues based on volume and the time interval between prior radiotherapy and anticipated retreatment. Radiation volumes should include known disease only. There is no need to treat prophylactic regions. When using SBRT techniques selection of patients who do not have circumferential carotid involvement is advised. Current SBRT schedules being used or investigated are in the range of 30–44 Gy using 5 fractions.

Chemotherapy

The choice of systemic therapy should be individualized based on patient characteristics (PS, goals of therapy) and the preferred chemoradiotherapy approach for fit patients with locally advanced disease remains concurrent cisplatin and radiotherapy.

Cisplatin-based induction chemotherapy can be used, followed by radiation-based locoregional treatment (i.e, sequential chemoRT). However, an improvement in overall survival with the incorporation of induction chemotherapy compared to proceeding directly to state-of- the-art concurrent chemoRT (cisplatin preferred, category 1) has not been established. Randomized phase III studies comparing sequential chemotherapy/RT

to concurrent chemotherapy/RT alone are ongoing and have not demonstrated a convincing survival bene t with the incorporation of induction chemotherapy. Cisplatin-based induction chemotherapy followed by high-dose, every-3-week cisplatin chemoradiotherapy is not recommended due to toxicity concerns. After induction chemotherapy, multiple options can be used for the radiation-based portion of therapy. Radiotherapy alone versus radiotherapy plus weekly carboplatin or cetuximab are among the options.

For SCC of lip, oral cavity, oropharynx, hypopharynx, glottic larynx, supraglottic larynx, ethmoid sinus, maxillary sinus, occult primary, the following protocols are recommended:

Primary systemic therapy + concurrent RT

- High-dose cisplatin (preferred) (category 1)
- Cetuximab (category 1 for oropharynx, hypopharynx, or larynx; category 2B for lip, oral cavity, ethmoid sinus, maxillary sinus, occult primary)
- Carboplatin/infusional 5-FU (category 1)
- 5-FU/hydroxyurea
- Cisplatin/paclitaxel
- Cisplatin/infusional 5-FU
- Carboplatin/paclitaxel10 (category 2B)
- Weekly cisplatin 40 mg/m2 (category 2B)

Postoperative chemoradiation

- Cisplatin13-17 (category 1 for non-oropharyngeal cancers with extracapsular nodal spread and/or positive margins)

Induction/Sequential chemotherapy

- Docetaxel/cisplatin/5-FU (category 1 if induction is chosen)
- Paclitaxel/cisplatin/infusional 5-FU
- Following induction, agents to be used with concurrent chemoradiation typically include weekly carboplatin or cetuximab

For SCC of nasopharynx the following protocols are recommended:

Chemoradiation followed by adjuvant chemotherapy

- Cisplatin + RT followed by cisplatin/5-FU or carboplatin/5-FU (category 2B for carboplatin/5-FU)
- Cisplatin + RT without adjuvant chemotherapy (category 2B)

Induction (category 3)/Sequential chemotherapy

- Docetaxel/cisplatin/5-FU
- Docetaxel/cisplatin (category 2B)
- Cisplatin/5-FU
- Cisplatin/epirubicin/paclitaxel

- Following induction, agents to be used with concurrent chemoradiation typically include weekly cisplatin or carboplatin

General principles of immunotherapy

Combinations of coinhibitory checkpoints

Targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) + PD-1/PD-L1 - Although both CTLA-4 and PD-1 are inhibitory coreceptors expressed on T cells, they have distinct ligands and functions. After antigen-driven T-cell receptor (TCR)-mediated T-cell activation, **[339]** CTLA-4 binds to ligands cluster of differentiation 80 (CD80) and cluster of differentiation 86 (CD86) **[340]** and inhibits effector T-cell activation and proliferation **[341-343]** by competitively inhibiting binding of B7 ligands to the costimulatory receptor cluster of differentiation 28 **[344-347]** and blockade of intracellular signaling pathways **[348-350]**; PD-1 is similarly located at the surface of effector T cell on activation, **[351]** where PD-1 binds to ligands PD-L1 **[352,353]** and PD-L2 **[354]** and prevents T-cell proliferation, **[355]** cytokine production **[356,357]** and survival, **[358,359]** which is typical of the state of T-cell exhaustion. **[360,361]** A recent study that evaluated blood and tissue specimens of patients undergoing monotherapy or combination therapies of anti-CTLA-4 and anti-PD-1 antibodies demonstrated that blockade of CTLA-4 induces a proliferative signature in a subset of memory T cells, whereas PD-1 blockade results in modification of genes that are involved in T-cell or natural killer (NK) functions. **[362]** Furthermore, anti-CTLA4 antibodies are more capable of inducing antibody-dependent cell-mediated cytotoxicity than PD-1 antibodies. **[363]** In this context, CTLA-4 and PD-1 can produce complementary effects on effector T cells, including inhibitory effects on early activation and differentiation by CTLA-4 and modulation of effector function by PD-1. **[364,365]** Preclinical observations that mice deficient for CTLA-4 **[366,367]** or PD-1 **[368-371]** had different toxicity patterns further highlighted their distinct properties and inspired efforts to examine the effects of the combined blockade of these pathways. In melanoma mouse model, the combination of CTLA-4 and PD-1 blockade significantly enhanced tumour rejection compared with either agent alone.**45** The first study testing the combination of T-cell checkpoint blockade was conducted in patients with advanced melanomas treated with the PD-1 inhibitor nivolumab and the CTLA-4 inhibitor ipilimumab. **[372]** Thirty-three of 86 patients enrolled in this phase I study had previously received ipilimumab within 12 weeks and were then treated sequentially with nivolumab

monotherapy (sequential regimen); 53 patients were ipilimumab naive and received ipilimumab and nivolumab combined (concurrent regimen). In patients treated with the concurrent regimen, 40% had objective partial response, while 65% derived clinical benefit. In patients treated with the sequential regimen, the ORR was 20% and 43% had clinical benefit. Importantly, the majority of responses seen in the concurrent arm were fast, deep (one-third achieving 80% reduction in tumour burden) and durable (78% of patients alive at 2 years). **[373]** Notably, there were some substantial toxicities. In the concurrent regimen, treatment-related grade 3–4 elevated liver enzymes were seen in 15%, gastrointestinal toxicities reported in 9%, rash in 4%, and pneumonitis and endocrinopathy occurred in 2% each. Still, toxicity was manageable and nivolumab 1 mg/kg plus ipilimumab 3 mg/kg every 3 weeks for four doses followed by nivolumab 3 mg/kg every 2 weeks was selected to be the optimal dosing regimen for further development.

A subsequent double-blind, phase II, randomised study of nivolumab plus ipilimumab compared with ipilimumab alone in advanced melanoma has confirmed the substantial activity of this combination. **[374]** Particularly, the ORR to nivolumab plus ipilimumab was 59%, versus 11% with ipilimumab alone. A more recent double-blind, phase III, randomized study of nivolumab plus ipilimumab versus nivolumab versus ipilimumab was performed in patients with treatment-naive advanced melanoma and confirmed the superiority of the combination versus ipilimumab or nivolumab alone (NCT01844505).

The predictive value of PD-L1 expression on tumour cells, which has been postulated to be a predictor of response to anti-PD-1/PD-L1 therapy **[375,376-379]** was also evaluated. Responses to both combination therapy and nivolumab monotherapy were enriched in PD-L1-positive patients (72.1% and 57.5%, respectively, compared with 54.8% and 41.3% in PD-L1-negative patients). Among PD-L1-positive patients, PFS was relatively similar in patients who received either combination therapy or nivolumab monotherapy, but follow-up is still short and many patients remain on treatment. Further follow-up will determine whether PD-L1 is useful for patient selection (combination vs PD-1 blockade monotherapy).

Building on the remarkable activity seen in patients with melanoma, several studies have begun to explore the combination of PD-1/PD-L1 and anti-CTLA-4 in other diseases including HNSCC. In HNSCC, several trials are currently assessing the efficacy of durvalumab, a selective high-affinity engineered human IgG1 mAb that blocks binding of PD-L1 to PD-1 and CD80, in combination with anti-CTLA-4 mAb tremelimumab. Durvalumab has yielded promising results (~14% response rate as per Response Evaluation Criteria in Solid

Tumours (RECIST) criteria, with 24% response rate in PD-L1-positive patients) in a phase I trial. **[380]** A phase II study is currently evaluating the efficacy of durvalumab monotherapy in PD-L1-positive R/M HNSCC (NCT02207530). The phase I, open-label, dose-escalation and expansion study evaluating durvalumab and tremelimumab in advanced solid tumours showed a 27% response rate (95% CI 13 to 46) in PD-L1-negative patients, with a disease control rate of 48% (95% CI 31 to 66) at ≥16 weeks after therapy. Notably, anti–PD-1/PD-L1 monotherapy yields an approximately 5%–10% response rate in PD-L1-negative patients; therefore, the addition of low-dose anti-CTLA-4 therapy may benefit these patients. Durvalumab at 20 mg/kg every 4 weeks plus tremelimumab at 1 mg/kg every 4 weeks was the dose level chosen for phase III development, and at this dose level, toxicity leading to discontinuation was <10%, while lower tremelimumab dosing did not affect clinical efficacy. The regimen of durvalumab 20 mg/kg plus tremelimumab 1 mg/kg given together every 4 weeks has been chosen for further development.

The phase III KESTREL study (NCT02551159) compares durvalumab alone and durvalumab plus tremelimumab with EXTREME standard of care regimen for first-line treatment of R/M HNSCC. KESTREL is an open-label, multicenter, global study of patients with R/M (oral cavity, oropharynx, hypopharynx or larynx) who have received no prior systemic chemotherapy (unless part of multimodality treatment for locally advanced disease). Patients will be stratified by PD-L1 expression status, tobacco history, tumour location, and then HPV status (oropharyngeal cancer) and randomized (2:1:1) to receive flat doses of tremelimumab 75 mg every 4 weeks (maximum four doses) plus durvalumab 1500 mg every 4 weeks; durvalumab 1500 mg every 4 weeks or EXTREME regimen (carboplatin or cisplatin + 5-fluorouracil + cetuximab), all until disease progression. The combination will be assessed versus standard of care in terms of coprimary endpoints, PFS and OS. Durvalumab plus tremelimumab versus standard of care will be further assessed in terms of overall response rate, duration of response, proportion of patients alive and PFS at 12 months, OS at 24 months, secondary progression, safety and tolerability, pharmacokinetics, immunogenicity and HR quality of life. The efficacy of durvalumab monotherapy versus both durvalumab/tremelimumab and EXTREME will also be tested. Exploratory endpoints include blinded independent central review of antitumour activity (immune-related RECIST v1.1) and potential biomarkers of progression/response.

EAGLE is a phase III trial designed to evaluate durvalumab alone or in conjunction with tremelimumab versus standard of care (cetuximab, taxane, methotrexate or fluoropyrimidine) in platinum-refractory HNSCC

(EAGLE-NCT02369874). CONDOR trial randomised patients to durvalumab alone, tremelimumab alone or the combination in patients with PD-L1-negative platinum refractory disease (NCT02319044).

Of note, US FDA has placed a clinical hold on the enrolment of new patients in clinical trials with durvalumab monotherapy or durvalumab and tremelimumab combination due to safety concerns (hemorrhagic complications). All trials are continuing with existing patients.

CheckMate 651 (NCT02741570) which recently opened to accrual is a phase III study of nivolumab in combination with ipilimumab compared with the standard of care (Extreme regimen) as first-line treatment in patients with R/M HNSCC.

Targeting lymphocyte activation group-3 or killer-cell immunoglobulin-like receptors + PD-1/PD-L1 or CTLA-4 - Another category of receptors with a modulating effect on immune cells includes other checkpoint receptors such as lymphocyte activation group-3 (LAG-3) or the killer-cell immunoglobulin-like receptors (KIRs). **[381]** They regulate immune response via interaction with major histocompatibility complex I molecules. Most of the receptors suppress cytotoxicity, mainly by turning off NK cells when human leukocyte antigen (HLA) is expressed on tumour cells. In combination with PD-1 blockade, murine data are suggestive of significant synergistic potential. Ongoing trials are testing an anti-KIR mAb in combination with ipilimumab (NCT01750580) or nivolumab (NCT01714739). A phase I trial is evaluating the efficacy of nivolumab in combination with anti-LAG-3 antibody BMS-986016 in advanced solid tumours including HNSCC (NCT01968109).

Targeting T-cell immunoglobulin and mucin domain 3 + PD-1/PD-L1 - T-cell immunoglobulin and mucin domain 3 (TIM-3) is a coinhibitory receptor expressed by interferon gamma (IFN-γ) secreting CD4 + helper T cells and cluster of differentiation 8 (CD8) + cytotoxic T cells. **[382]** High TIM-3 expression is a marker of T-cell exhaustion which is manifested by decreased T-cell proliferation, decreased IFN-γ, tumour necrosis factor-α (TNF-α) and interleukin-2 (IL-2) secretion, and increased IL-10 secretion. **[383-386]** In preclinical models, blockade of TIM-3 can enhance cytokine-producing, tumour-specific T cells and potentiate antitumour activity in combination with PD-L1 blockade. **[386,387]** A phase I study of TSR-022, an anti-TIM-3 mAb, in patients with advanced solid tumours is ongoing (NCT02817633).

Combinations with costimulatory checkpoints

Targeting glucocorticoid-induced TNF receptor + PD-1/PD-L1 - Glucocorticoid-induced TNF receptor (GITR)/GITR ligand axis is a pathway that functions by inhibiting T regulatory cells (Treg) function while activating CD8. T effector cells.61 Murine models have shown that GITR stimulation (with an agonistic antibody or with cognate ligand) promotes effector T-cell proliferation, cytokine production, **[388,389]** resistance to Treg suppression **[390-392]** and inhibition of Treg suppressive function. **[393]** In in vivo models, administration of a GITR agonist antibody is associated with reduction of intratumoural Treg accumulation and potentiation of antitumour CD8+ effector T-cell function, **[390,391,394]** as well as antitumour activity. **[390,394,395]** When given in combination with PD-1 blockade, increased activity was also seen. For example, when anti-GITR and anti-PD-1 administered to mice with ID8 ovarian cancer, 20% of mice were tumour-free after 90 days while either anti-PD-1 or anti-GITR antibody alone exhibited little antitumour effect. **[396]** Anti-GITR antibodies in clinical development (TRX518, MK4166) are being tested in solid tumours as single agents (NCT01239134) and in combination with PD-1 blockade (NCT02740270).

Targeting OX40 + CTLA-4 or PD-1/PD-L1 - OX40 (CD134) and its binding partner, OX40L (CD252), are members of the TNF receptor/TNF superfamily. OX40 is a costimulatory immune checkpoint molecule that is expressed on activated CD4 and CD8 T cells. **[397]** Costimulatory signals from OX40 lead to division and survival of T cells, enhancing the clonal evolution of effector and memory populations.**[398]** OX40 is also a regulator of Treg function. **[399]** In preclinical mouse models, agonist targeting OX40 can augment T-cell effector responses. **[400]** There is substantial preclinical evidence that anti-OX40 synergizes with immune checkpoint inhibitors and other immunotherapies. **[401-403]** In an ovarian cancer murine model, although treatment with either anti-OX40 or anti-PD-1 was ineffective, the combination of anti-OX40 and anti-PD-1 antibodies resulted in successful tumour growth inhibition. **[404]** Similarly, anti- OX40 and anti-PDL-1 antibodies have a synergistic effect in preclinical models. **[405]** In HNSCC patient samples, OX40 and CTLA-4 molecules have been shown to be expressed in tumour-infiltrating lymphocytes. **[406]** In a phase I study in patients with treatment refractory solid tumours, agonistic anti-OX40 antibody 9B12 showed mild toxicity and

good tumour control in 18/30 of patients treated. **[407]** A phase I study with anti-OX40 antibody MEDI6469 administered prior to surgical resection in patients with locally advanced HNSCC is currently recruiting patients (NCT02274155). Anti-OX40 antibodies (MOXR0916, MEDI6383) are currently being tested in combination with anti-PD-1/anti-PDL-1 agents in metastatic solid tumours (NCT02410512, NCT02221960).

Targeting 4-1BB (CD137) + CTLA-4 or PD-1/PD-L1 - 4-1BB is a costimulatory receptor that belongs to the TNF receptor family and is upregulated on CD8 T cells following activation. It is also expressed on CD4 T cells, NK cells and Tregs. **[408]** 4-1BB signalling enhances T-cell activation, provokes T-cell proliferation **[409]** and upregulates the expression of antiapoptotic molecules, **[410]** facilitating the formation of immunological memory. In preclinical models, anti-41BB agonistic antibodies have shown efficacy in combination with immune checkpoint inhibitors. In a melanoma murine model, concurrent administration of anti-41BB and anti-CTLA-4 antibodies resulted in prolonged survival. **[411]** In a phase I clinical trial, urelumab, a 4-1BB antibody, was evaluated in 83 patients with melanoma, renal cell carcinoma, ovarian and prostate cancer. Patients with melanoma showed good clinical response (three had partial responses and four stable disease) albeit with significant liver toxicity. 4-1BB has been found to be expressed in lower levels on CD4 T cells of patients with HNSCC. **[412]** Urelumab is being evaluated in combination with cetuximab (NCT02110082) and nivolumab (NCT02253992) in advanced solid tumours including HNSCC. Anti-41BB antibody PF-05082566 is being tested in combination with anti-OX40 antibody PF-04518600 in advanced solid tumours including HNSCC (NCT02315066).

Combinations with other molecules in the tumour microenvironment

Targeting indoleamine 2,3-dioxygenase + CTLA-4 or PD-1/ PD-L1 - Indoleamine 2,3-dioxygenase (IDO) is a haeme-containing enzyme involved in tryptophan catabolism, catalysing the degradation of amino acid l-tryptophan into kynurenine. **[413]** It is expressed in both tumour cells and infiltrating myeloid cells. IDO is an immunomodulatory enzyme that produces immunosuppressive effects, such as inhibition of T-cell activation and proliferation and decrease of TCR expression. **[414]** In preclinical models, IDO has been shown to inhibit immune responses through the depletion of l-tryptophan that is critical for anabolic functions in lymphocytes

or through the synthesis of specific ligands for cytosolic receptors that can alter lymphocyte functions. **[415]** In IDO knockout mice with melanomas, anti- CTLA4 targeting resulted in inhibition of tumour growth marked with increased infiltration of effector T cells. **[416]**

Preliminary results from a phase I/II study (NCT02178722) of IDO inhibitor epacadostat (INCB024360) with permbrolizumab in a variety of human malignancies including HNSCC were recently reported. **[417]** The combination of two immunotherapies showed an overall response rate of 53% and disease control rate of 74%; efficacy was greater in patients with melanoma. Toxicity was tolerable with very few patients experiencing grade 3/4 events. In one evaluable patient with HNSCC, a partial response was noted. A phase I/II study in which evaluated the combination of IDO inhibitor INCB024360 with ipilimumab in patients with melanoma showed a disease control rate of 755 in eight evaluable patients. Notably, patients had significant increase of liver function tests when treated with high doses of INCB024360. **[418]**

Other anticancer treatment modalities in combination with t-cell checkpoint blockade

Oncolytic viruses - Oncolytic viruses are natural or genetically altered viruses that preferentially infect and replicate in tumour cells and lead to immunogenic tumour cell death. Apart from direct tumour killing, oncolytic viruses promote the induction of antitumour T cells by the release of danger signals and tumour antigens following oncolysis. **[419]** Talimogene laherparepvec (TVEC) is an oncolytic immunotherapy that is furthest along in clinical development. It is derived from herpes simplex virus type-1 that has been engineered to selectively replicate within tumours and to produce granulocyte-macrophage colony stimulation factor (GM-CSF) to enhance systemic antitumour immune responses. In a randomized phase III clinical trial in patients with advanced melanoma, TVEC demonstrated statistically significant superior overall response rate compared with GM-CSF (26% vs 6%). **[420]**

TVEC is currently being tested in combination with immune checkpoint inhibitors. In a phase Ib trial, TVEC in combination with ipilimumab showed promising results (overall response rate 56%) in patients with melanoma, with tolerable toxicity. Another phase Ib/II is assessing the safety and efficacy of TVEC in combination with pembrolizumab versus permbrolizumab monotherapy in patients with stage IIIB/IV unresectable melanoma (NCT02263508).

In patients with HNSCC, TVEC was evaluated in a phase I/II study in combination with standard cisplatin and radiation for patients with locally advanced disease. All patients had post-treatment neck dissections. Median follow-up was 29 months with 100% patient free of locoregional disease and a disease-specific survival of 82.4% and overall survival rate of 70.5%. Pathological complete response in the neck dissections were 100%. **[421]** TVEC is currently being tested in combination with pemrolizumab in patients with R/M HNSCC in the phase Ib/III MASTERKEY232/KEYNOTE- 034 study (NCT02626000). Other oncolytic viruses, such as oncolytic reovirus and oncolytic adenoviruses H101 and Onyx 015 have been evaluated in advanced HNSCC as monotherapies or in combination with chemotherapy. **[422,423]** Recombinant vaccinia virus Pexa-Vec and recombinant avian fowlpox virus TRICOM are currently being assessed as monotherapies in HNSCC in phase I trials (NCT00625456 and NCT00021424).

Vaccines - Anticancer vaccine therapies include generating an antitumour immune response by presenting a tumour-associated antigen (TAA) plus an immunostimulatory adjuvant, resulting in immune sensitisation to tumour antigens. Several vaccination strategies have been evaluated, including the transfection of TAA expression plasmids into patient tissues (DNA vaccines), the administration of TAA peptides (peptide vaccines) and the use of cultured human or microbial cells to generate an antitumour immune response. **[424]**

In HNSCC, several vaccines, such as DNA vaccine INO-3112 and peptide vaccines Mucin-1 and Allo-Vax are currently under investigation in phase I/II clinical trials. In a phase I trial, five patients with advanced HNSCC were treated with peptide vaccines composed of HLA-I and HLA-II restricted melanoma antigen E-A3 or HPV-16 derived peptides, provoking a measurable immune response and acceptable toxicity. **[425]** Furthermore, a phase II trial evaluating the efficacy of HPV16 E6 and E7 peptide vaccines in patients with HPV-related tumours including HNSCC has been completed and results are expected shortly (NCT00019110).

Combination of vaccine therapy with immune checkpoint inhibitors is currently being assessed in a number of clinical trials. In a phase I trial in patients with advanced solid tumours including patients with HNSCC, a combination of pembrolizumab and modified vaccinia virus Ankara vaccine expressing p53 is being evaluated (NCT02432963). A phase I/II study of a live attenuated *Listeria monocytogenes* immunotherapy bioengineered to secrete an HPV-E7 tumour antigen as a truncated ListerioLysin O–E7 fusion protein in cells capable of

presenting antigen (ADXSII-001) is being tested alone or in combination with MEDI4736 in patients with R/M cervical or HPV+ HNSCC in a phase I/II study (NCT02291055). Ipilimumab is being evaluated in combination with vaccines in advanced pancreatic cancer and melanoma in ongoing clinical trials (NCT00836407, NCT01810016).

Treatment algorithm for OP-SCC

The most recent NCCN Guidelines for the management of OP-SCC divide the treatment algorithm into 3 staging categories: 1) T1-2, N0-1; 2) T3-4a, N0-1; and 3) any T, N2-3. Of note, the following categories are treated as advanced cancer: 1) T4b, any N; 2) unresectable nodal disease; 3) unfit for surgery; or 4) M1 disease at initial presentation.

Early-stage (T1-2, N0-1) oropharyngeal cancers may be treated with: 1) primary surgery—more specifically, transoral or open resection of the primary—(with or without neck dissection); or 2) definitive RT. **[426,427,428,429]** Panel members felt that the third option of RT plus systemic therapy (category 2B for systemic therapy) was only appropriate for T2, N. For patients with positive margins, re-resection is the preferred option for adjuvant treatment. RT is another option, and systemic therapy/RT may be considered. For patients with other risk features, options include RT or consideration of systemic therapy/RT. Adjuvant systemic therapy/RT is recommended for adverse pathologic features of extracapsular nodal spread with (or without) positive mucosal margins. **[430,431,432]**

For locally advanced resectable disease (T3-4a, N0-1; or any T, N2-3), 3 treatment options are recommended in addition to enrollment in multimodality clinical trials. The 3 options are: 1) concurrent systemic therapy/RT (surgery is used for managing residual or recurrent disease); [433]2) transoral or open resection of the primary and neck (with appropriate adjuvant therapy [systemic therapy/RT or RT]); or 3) induction chemotherapy (category 3) (followed by RT or systemic therapy/RT), although panel members had a major disagreement for induction therapy. **[426,427,434]** Concurrent systemic therapy/RT—with high-dose cisplatin as the preferred systemic agent—is recommended for treatment of locally or regionally advanced (T3-4a, N0-1, or any T, N2-3) cancer of the oropharynx. Many panel members did not agree that induction chemotherapy should be recommended for locally or regionally advanced cancer of the oropharynx. This disagreement is reflected by

the category 3 recommendations for oropharyngeal cancer. **[433,435-444]** Most panel members agree that concurrent systemic therapy with RT should be used to treat fit patients with locally advanced disease.

HPV and Treatment of Oropharyngeal Cancer

HPV status is a predictor of oropharyngeal cancer prognosis. A systematic review including 56 prospective or retrospective studies showed that patients with p16-positive oropharyngeal cancer had a better prognosis and fewer rates of adverse events, relative to patients with p16-negative disease. **[445]** Further, patients with p16-negative disease had worse outcomes following radiation treatment, relative to surgery (HR, 1.66; 95% CI, 1.26–2.18; P < .001), and this difference was not statistically significant for patients with p16-positive disease (HR, 1.33; 95% CI, 0.94–1.87; P = .114). There may also be an association between HPV status and survival in patients with recurrent or metastatic disease. **[446-449]**

Since patients with locally advanced HPV-positive oropharyngeal cancer may live longer, late toxicity and quality of life are concerns for these patients. **[450,451]** Therefore, consensus is increasing that HPV status should be used as a stratification factor or should be addressed in separate trials (HPV-related vs. unrelated disease) for which patients with oropharyngeal cancer are eligible. **[452-454]** Some clinicians have recently suggested that less-intense treatment may be adequate for HPV-positive oropharyngeal cancers (ie, deintensification) **[455]**; however, the available data supporting this assertion are limited by retrospective analyses, variability in HPV testing method used, and short follow-up periods. **[455,450,456,457]** Deintensification treatment protocols for HPV- associated locally advanced oropharyngeal cancer are being investigated in ongoing clinical trials. Strategies under active investigation include reducing or using response-stratified RT dose, using RT alone versus chemoradiation, using less invasive surgical procedures such as transoral robotic surgery, using sequential systemic therapy/RT, and using immunotherapy and targeted therapy agents such as cetuximab. **[450,451,458]** The ECOG-ACRIN phase II E1308 trial, in which patients with stage III-IV HPV16 and/or p16-positive oropharyngeal cancer (N = 80) received induction chemotherapy followed by reduced-dose RT and weekly cetuximab, recently reported results, showing that RT deintensification may result in equivalent or similar response in selected patients, compared to full-dose RT. **[459]**

The panel currently recommends adjuvant systemic therapy/RT in patients with squamous cell carcinoma of the oropharynx in the presence of the adverse pathologic features of extracapsular nodal spread with (or without) positive mucosal margins. This recommendation is primarily based on results from RTOG 9501 and EORTC 22931. **[430,431,432]** However, in a review of published data from these RCTs, it was noted that the panel's recommendations are based on studies that did not investigate the impact of HPV or p16 status. **[460]** In response to this review, the investigators from RTOG 9501 and EORTC 22931 pointed out that the prevalence of HPV-positive/p16-positive tumors was likely to be low in these trials. **[461]** Other limitations noted in this review included unplanned subgroup analyses, the grouping of multiple H&N subsites, inconsistent quantitative reporting and lack of reporting on tumor and lymph node classification, treatment effect sizes, multivariable analyses, and quality of life outcomes. Therefore, the investigators who carried out this review argued that these trials lack the generalizability necessary to rationalize the use of adjuvant systemic therapy/RT in patients with p16-positive disease.

Recent retrospective studies have not observed a statistically significant association between extracapsular spread and survival in patients with HPV-positive oropharyngeal cancer. **[462,452,463-466]** For example, a study of 220 patients with p16-positive oropharyngeal cancer who received surgical resection with or without adjuvant treatment showed that the presence of five or more metastatic nodes is associated with disease recurrence and survival, but extracapsular spread was not significantly associated with outcomes in this sample.361 Recent studies of patients with p16-positive oropharyngeal cancer treated with surgery show that soft tissue metastasis may be associated with poor survival outcomes, especially in patients with T3-T4 disease. **[462,467]** These results suggest that patients with p16-positive disease with extracapsular spread could potentially be treated differently than patients with p16-negative disease and extracapsular spread.

Adjuvant systemic therapy/RT in patients with oropharyngeal cancer who have extracapsular spread is recommended as a category 2A option, based on a lack of high-quality, prospective clinical evidence and controversy. Adjuvant systemic therapy/RT remains a category 1 recommendation for patients with other types of H&N cancer who have extracapsular spread, including HPV-negative oropharynx cancer. Deintensification treatment protocols for patients with HPV-related oropharyngeal cancer are currently being investigated (eg,

NCT01154920, NCT01706939, NCT01302834, NCT01855451). Panel members urge that patients with HPV-related cancers be enrolled in clinical trials evaluating biological and treatment-related questions. **[450,451,468]**

AIM OF THE STUDY

Despite advances in multimodality treatment, the 5-year progression-free survival (PFS) rates of patients with HPV-negative locally advanced disease do not exceed 40%–50% and survival rates in recurrent or metastatic (R/M) setting remain poor. **[469]**

Low survival outcomes in combination with substantial toxicities associated with current treatment strategies employed in HNSCC emphasize the necessity for novel treatment strategies. Immunotherapy has led to a paradigm shift in the treatment of several cancers, providing long-lasting, durable responses for patients with advanced cancers. **[375,470-472]** In July 2016, the Food and Drug Administration (FDA) has granted a priority review designation to nivolumab, an anti-programmed cell death protein-1 (anti-PD-1) monoclonal antibody (mAb) for the treatment of platinum-refractory recurrent and/or metastatic HNSCC **[473]** based on a pivotal phase III clinical trial which demonstrated improved overall survival (OS) compared with treatment with the investigator's choice of weekly methotrexate, docetaxel or cetuximab. **[474]** The anti-PD-1 pembrolizumab was also recently approved by the US FDA for the treatment of platinum-refractory recurrent and/or metastatic HNSCC based on the demonstration of a durable objective response rate (ORR) in a subgroup of patients in an international, multicenter, non-randomized, open-label, multi-cohort study. **[475]** Building on initial hypotheses **[476-478]** that the host immune system plays a pivotal role in shaping HNSCC, the recent successes of immunotherapies have confirmed the potential to harness the immune system for the treatment of patients with HNSCC. In particular, T-cell checkpoint inhibitors targeting PD-1 have demonstrated efficacy in HNSCC. **[474,475]** As single agents, these therapies have response rates in the range of 14%–32% in second-line setting in R/M HNSCC, with responses characterized by a durability that is rarely, if ever, attained with other types of anticancer therapy. However, only a minority of patients derives benefit from single-agent immunotherapies, with some patients not responding to treatment at all, and others attaining a limited response followed by tumour progression. One of the major challenges at present is the development of alternative treatment strategies that improve the subset of patients who may respond to immunotherapy. A better understanding of the mechanisms implicated in response to immune-based therapies may allow physicians to identify patients

likely to benefit from these therapies and will potentially provide insight into how other therapies may be used in combination to increase the number of patients who benefit from immunotherapy. **[473]**

Therefore, a better understanding and outlining of the immunological phenotype of head and neck cancer has a pivotal role in the identification of new prognostic markers and in the comprehension of the molecular pattern that determine the response to immunotherapy that to date, as previously debated, has obtained encouraging clinical results in the management of head and neck cancer.

Aim of this study, performed on a cohort of patient's affected by OP-SCC, is

1. to evaluate the expression of p16 and E6 as markers of HPV infection
2. to evaluate the expression of PD-1, PDL-1, CD4, CD8, FoxP3, IL22 and TREM-1
3. to correlate clinical features of patients with the expression of the investigated markers

MATERIALS AND METHODS

TISSUE SAMPLES

OP-SCC samples used for this retrospective monocentric study were stored in the archives of the Department of Pathology of the University of Turin at the "Città della Salute e della Scienza" Hospital of Turin and derived from patients surgically treated at the ENT Department of the same institution. They were consecutively extracted by the Pathologist and their suitability for study enrollment was determined after inclusion/exclusion criteria assessment analyzing medical records.

Inclusion criteria:

- advanced stage primitive OP-SCC surgically resected between 2004 and 2012
- availability of histologic specimen

Exclusion criteria:

- early stage OP-SCC
- bony invasion
- persistent or recurrent OP-SCC after surgery and/or chemotherapy and/or radiotherapy
- previous radiotherapy and/or chemotherapy for head and neck malignancies
- previous chemotherapy for solid tumors of other sites
- previous chemotherapy for hematological tumors
- immunosuppression following organ transplantation

Patient's and tumor's characteristics, risk factors, treatment modalities and follow-up data were collected from medical record analysis and from the informatics system of the above mentioned Hospital.

Specimen enrolled were sent to the Translational Medicine Laboratory and to the Applied Microbiology Laboratory of the Oriental Piedmont University for processing.

STAININGS

Haematoxylin - Eosin

Haematoxylin and eosin (H&E) staining was performed to provide a general overview of the lesions. Five µm thick sections were obtained from formalin-fixed paraffin-embedded (FFPE) surgical specimens stored in the archives of the Department of Pathology and placed onto superfrost ultra plus glass slides (Menzen-Gläser, Thermo Fisher Scientific, Runcorn, U.K.). They were de-paraffinized in xylenes and rehydrated in a descending scale of ethanols (100% x2, 95%, 90%, 70%). Sections were stained with haematoxylin and eosin, then mounted with an aqueous mounting medium (VectaMount AQ aqueous mounting medium, Vector Laboratories, Burlingame, distributed by DBA Italia Srl, Segrate, Milan, Italy) and evaluated by means of optical microscopy by an expert pathologist in order to have a general overview of the lesions **(Figure 1)**.

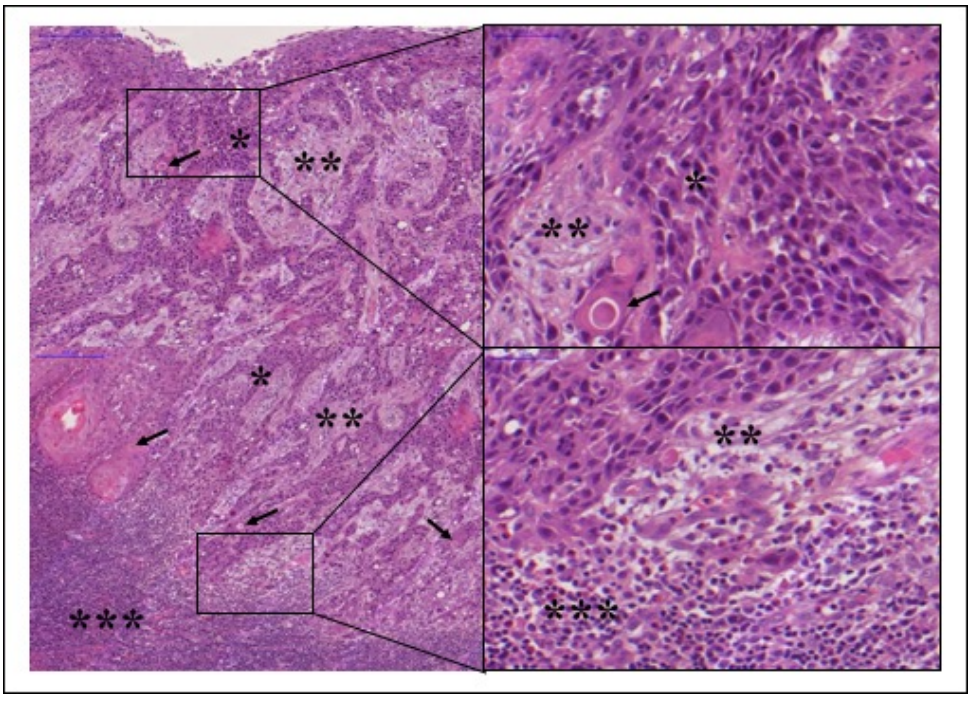


Figure 1. Haematoxilin and eosin staining of a representative case of OP-SCC. Left panels magnification is 10x, it is 40x in the left ones. The black boxes in 10x fields correspond to 40x magnification. Bar in the 10x fields = 200 μm, in the 40x fields = 50 μm. Tumor cells are indicated with *, tumor stroma with ** and tumor infiltrating lymphocytes (TILs), mixed with red blood cells, with ***. Black narrows indicate keratin pearls.

Immunohistochemistry

For the evaluation of the markers expression involved in this study (p16, E6, FOXP3, PD-1, PDL-1, CD4, CD8, T-REM and IL-22), an immunohistochemical (IHC) analysis was done by using the following primary mouse antibodies: cCDKN2A/p16INK4a (clone 2D9A12, working dilution 1:1000 in blocking solution, Abcam, Cambridge, U.K.); E6 HPV 16/18 (clone sc-460, working dilution 1:100 in PBG 1X, Santa Cruz Biotechnology, Segrate, Milan, Italy); FOXP3 (clone 236A/E7 ab20034, working dilution 1:100 in blocking solution, Abcam); PD-1 (clone NAT105, working dilution 1:200 in Monet blue diluent, Biocare Medical, Milan, Italy); PD-L1 (clone 130021, working dilution 1:150 in PBG 1X, R&D System, Milan, Italy); CD4 (clone 4B12, working dilution 1:50, Thermo Fisher Scientific, Runcorn, U.K.); CD8 (clone C8/144B, working dilution 1:50, Thermo Fisher Scientific) and TREM-1 (clone 174031, working dilution 1:100 in PBG 1X, R&D System). One polyclonal primary rabbit antibody anti IL-22 (Clone 18499 working dilution 1:100 in PBG 1X, Abcam, Cambridge, U.K.) was also used.

Sections were deparaffinized in xylene and stepwise rehydrated as above describe. Antigen unmasking was performed by heating in: i) EDTA buffer 1X at 95°C for 30 min (E6) or at 750W for 9 min (FOXP3); ii) sodium citrate buffer 10mM pH 6(Antigen Unmasking Solution, Vector Laboratories, Burlingame, distributed by DBA Italia Srl, Segrate, Milan, Italy), at 160W for 20 min (PD-1, PDL-1) or at 750W for 15 min (p16, IL-22 and T-REM). Endogenous peroxidase activity was blocked by incubating sections in 3% H_2O_2 in phosphate-buffered saline 1X (PBS, Sigma Aldrich, Milan, Italy) at pH 7.4 for 10 min. Unspecific binding was blocked by incubating samples with PBG 1X (phospate-buffered gelatin) composed of 0.2% gelatin (gelatin from cold water of fish skin, Sigma Aldrich Milan, Italy) and 0.5% bovin serum albumin (BSA, Sigma Aldrich, Milan, Italy)for 1 h in a humified chamber for IL-22, PD-1, PDL-1. For E6 and FOXP3 markers, unspecific binding was blocked by incubating samples with blocking solution made up by 1% gelatin (gelatin from cold water of

fish skin, Sigma Aldrich, Milan, Italy) and2% bovin serum albumin (BSA, Sigma Aldrich, Milan, Italy) for 1 h in a humified chamber. The respective primary antibodies were added to the slides and over-weekend (E6) or overnight (FOXP3, PD-1, PDL-1, IL-22) incubated at 4°C. The incubation with a secondary HRP conjugated anti-mouse antibody was performed with a MACH 4 probe (Biocare Medical, Milan, Italy) for 15 min followed by MACH 4 HRP-polymer for 20 min (E6, FOXP3) or DAKO REAL EnVision Detection System HRP (DAKO, Milan, Italy) for 30 min at room temperature (PD-1, PDL-1, IL-22). Dark brown positive signals were developed with 3,3'-diaminobenzidine (DAB) chromogen (DAKO, Milan, Italy). All sections were counterstained in Mayer's hematoxylin (Carlo Erba Reagenti S.p.A., Cornaredo, Milan, Italy) and then mounted with an aqueous mounting medium (Vectamount mounting medium, Vector, distributed by DBA Italia Srl, Segrate, Milan, Italy) and stored until analysis.

For CD4 and CD8 antigens, a Ventana benchmark XT Automated Platform was used (Ventana Medical System, Tucson, AZ, U.S.A.) by the Pathology Unit of the Sant' Andrea Hospital in Vercelli (Italy).

Interpretation of immunohistochemical reactivity

Images, markers expression and scores were captured by a virtual microscope through the Pannoramic Viewer software (3D Histech, distributed by Diapath, S.p.A., Martinengo, Bergamo, Italy). All specimens were evaluated by an expert pathologist (G.V.). The intensity and distribution patterns of the stainings were analyzed by two blinded, independent observers (G.V. and B.A.), with > 90% concordance. For each case, ten random representative high-power fields (HPF x400) were selected. When possible, for the analysis of the different markers, the same fields of consecutive sections were observed to limit the variability. Positive and total cells number was recorded both manually with a multichannel cell counter (ImageJ Cell Counter, distributed by NIH, Bethesda, U.S.A.) and by using Image-Pro Plus 6.0 software technology (Media Cybernetics, Rockville, MD, U.S.A.).

p16 and E6 staining - p16 and E6 nuclear and cytoplasmic presence inside tumoral epithelial cells was considered as a positive signal. Immunostaining scores were scaled from 0, negative, 1+, weak (1-30% positive cells) and 2+ strong (31-100% positive cells) according to the relative staining intensity and a mean of the percentages of labeled cancer cells/HPF.

CD4, CD8, Foxp3 staining - inflammatory infiltrate was analyzed for both its peritumoral (inflammatory cells just outside the tumour) and intratumoral (inflammatory cells within tumor nests) component and intratumoral infiltration was evaluated semi quantitatively. The degree of CD4, CD8 and FOXP3 expression was expressed as a mean of the number of reactive cells per HPF, and scored on a scale of 0-3+ as follows: 0, immune negativity; 1+, <10% positive cells; 2+, 10% -20% positive cells; 3+, >20% positive cells.

PD-1, PDL-1 staining - inflammatory PD-1 and PDL-1 positive infiltrate was also analyzed for both its peritumoral and intratumoral components. PD-1 and PDL-1 were examined on lymphocytic- and tumor microenvironment- (TME) cells, respectively. Count was made by counting and distinguishing between positive and negative cells per high-magnification field; the results were expressed as a mean of the percentage values of the analyzed fields. The expression degree of peritumoral and intratumoral PD-1 and PDL-1 was categorized quantitatively by the number of reactive cells per HPF. Immunostaining scores were scaled from 0 to 3+, with 0, immune negativity; 1+, < 20% positive cells; 2+, 21-50% positive cells; 3+, >50% positive cells.

IL-22 staining - cytoplasmic and/or cell membrane IL-22 immunoreactivity was evaluated both in the intratumoral and peritumoral compartments and quantitatively evaluated as a mean, according to criteria of staining percentages and intensities per HPF. For staining intensity, a four-points scale was used: 0+, immune negativity; 1+ (1-33% positive), 2+ mid staining (34-66% positive); 3+, strong staining (67-100% positive).

TREM-1 staining - for TREM-1, a semi-quantitative evaluation of the intratumoral and peritumoral signals was made and expressed as a mean of the number of reactive cells per HPF, by scaling the scores from 0 to 2+, with 0, negative, 1+, low (1-50 positive cells) and 2+, high (>50 positive cells).

The reaction specificity was confirmed with an antibody isotype control instead of the primary antiserum with an identical concentration of the respective non immunized serum.

Statistical analysis

The distribution of the patient' characteristics was summarized using frequency and percentage for qualitative variables and using median and interquartile range for continuous variables.

Overall survival was estimated with the Kaplan-Meier method. The cumulative incidence of death was calculated from the date of diagnosis of oropharyngeal squamous carcinoma to the date of death, or the completion of follow-up. The cause specific cumulative incidence function was estimated using the method proposed by Gooley et al. **[479]** in presence of competing event (death from other cause).

Differences within patients in terms of expression of tumoral markers (absence or presence) between peritumoral and intratumoral evaluation were tested performing McNemar Test.

Statistical analyses were performed using Stata version 13.1 (StataCorp, College Station, TX,USA).

RESULTS

PATIENTS AND TUMOR CHARACTERISTICS

After an accurate search of the archives of the Department of Pathology of the University of Turin at the "Città della Salute e della Scienza" Hospital of Turin we identified 79 specimens derived from the surgical resection of OP-SCC performed between 2004 and 2012. After a first evaluation by the pathologist, 4 specimens were excluded because the biological material was not suitable for further investigations due to its insufficiency.

We analyzed the medical records of 75 patients in order to evaluate their eligibility in the study and we excluded 21 patients because they were affected by early stage OP-SCC (stage I-II), 3 patients because the definitive histological examination demonstrated bony invasion, 19 patients because they were affected by persistent or recurrent OP-SCC after surgery and/or chemotherapy and/or radiotherapy, 2 patients because were previously treated with exclusive radiotherapy for laryngeal carcinoma, 11 patients because they previously underwent chemotherapy for solid tumors of other sites, 1 patient because of chemotherapy for a Hodgkin lymphoma and 1 patient because was kidney transplanted; at the end, only 17 specimens respected the inclusion criteria and were suitable for study enrollment.

Patient's and tumor's characteristics and risk factors of these 17 patients were collected from medical records analysis and from the informatic systems of "Città della Salute e della Scienza Hospital" and are summarized in **Table 1** and **Table 2**.

Table 1. Patient's characteristics and risk factors.

Demographic characteristics and risk factors	N (%)
Median age	62 yrs (IQR 55-66 yrs)
Sex	
• Male	16 (94%)
• Female	1 (6%)
Smoking	**12 (72%)**
• Heavy smoker (HS)	9 (54%)

• Medium smoker (MS) • Light smoker (LS) • No	1 (6%) 2 (12) 5 (30%)
Alcohol consumption • Harmful (HC) • High risk (HRC) • Low risk (LRC) • Absent	**11 (66%)** 7 (42) 2 (12%) 2 (12%) 6 (36%)
Smoking + Alcohol consumption	8 (47%)

LEGEND:

Alcohol consumption classification is based on the Reference Levels of Nutrients Intake adopted by Italian Health Ministry from 2014: **LRC** (*Male*: no more than 2 alcoholics unit/die - *Female*: no more than 1 alcoholic unit/die), **HRC** (*Male*: from 3 to 6 alcoholics unit/die - *Female*: from 2 to 4 alcoholics unit/die), **HC** (*Male*: more than 6 alcoholics unit/die - *Female*: more than 4 alcoholics unit/die). An alcoholic unit: 10-12 grams of ethanol corresponding to a glass of wine (125 mL) or a can of beer (330 mL) or a little glass of liquors (40 mL).

Smoking classification is based on the classification published by Neumann et al on the International Journal of Environmental Research and Public Health in 2013: **LS** (< 10 cigarettes/die or <10 packs/year), **MS** (10-20 cigarettes/die or 10-20 packs/year), **HS** (≥ 20 cigarettes/die or 20 packs/year).

Table 2. OP-SCCcharacteristics.

Characteristics of the resected tumor	**N (%)**
OP-SCC histotype	
Keratinizing	16 (94%)
Basaloid	1 (6%)
Tumor (pT)*	
T1	2 (12%)
T2	8 (47%)
T3	4 (24%)
T4a	3 (18%)
Nodes (pN)*	
N0	3 (18%)
N1	7 (41%)
N2	7 (41%)
N3	0 (0%)

Stage*	
I	0 (0%)
II	0 (0%)
III	9 (54%)
IV	8 (46%)
Grade	
G1	2 (12%)
G2	8 (46%)
G3	7 (42%)

LEGEND:

* Tumor stadiation was based on *AJCC Cancer Staging Manual, Seventh Edition (2010) published by Springer Science and Business Media LLC (Amin MB, Edge SB, Greene FL, et al, eds. AJCC Cancer Staging Manual. 8th ed. New York: Springer; 2010).*

The median follow up time is 9 years (IQR 8.5-11), the 5-yr Overall Survival (OS) was 64.7% (95%CI 37.7-82.3). Globally, from the diagnosi date to the end of the follow-up 10 (59%) patients are died and causes of death are as follows: 6 (60%) deaths are OP-SCC related (persistence/recurrence) and 4/10 are determined by other causes. Five-year cumulative incidence of death for OP-SCC is 29.5% (95%IC 10.7-51.1) and cumulative incidence of death from other cause is 5.9% (95%CI 0.4-23.5); survival analysis is reported in **Figure 2-4**.

Figure 2. Overall Survival (OS).

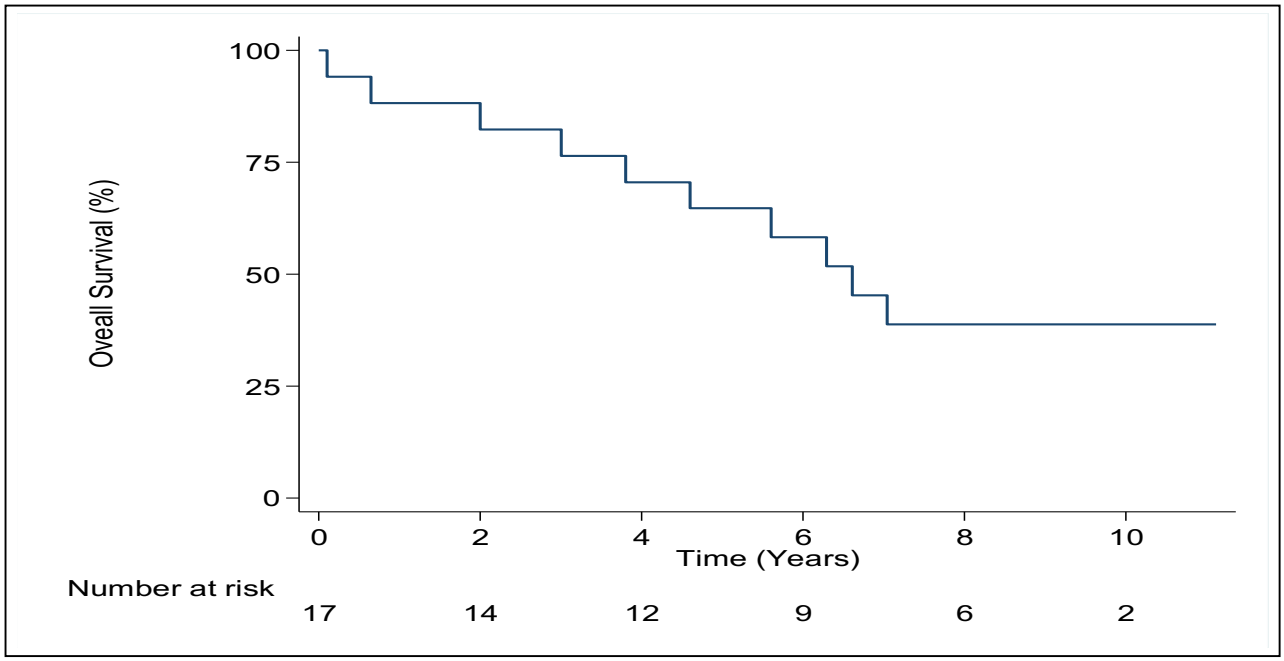


Figure 3. Cumulative incidence of death by any cause.

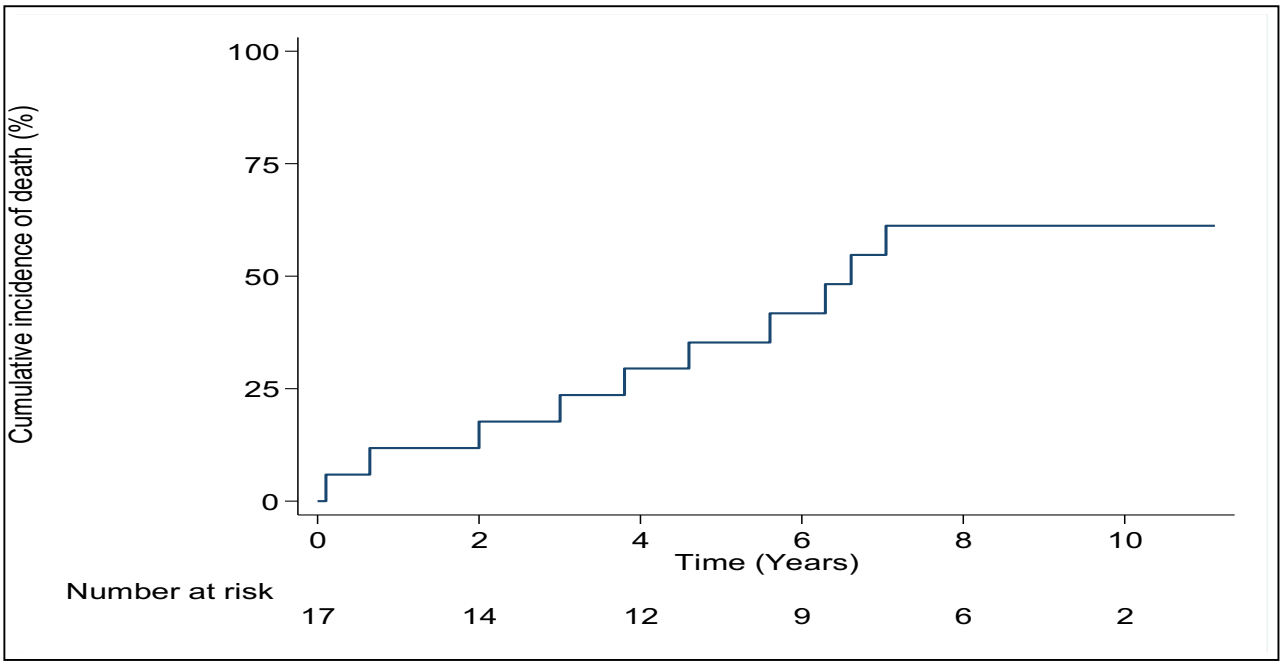
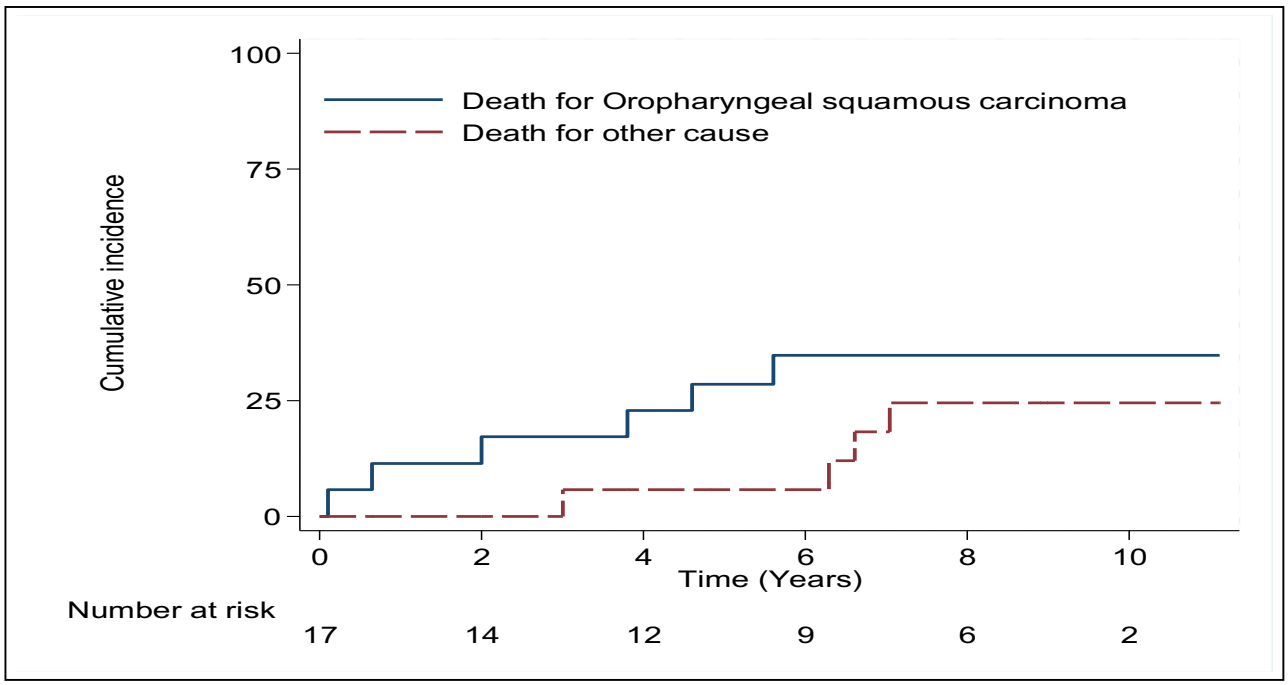


Figure 4. Cumulative incidence of death stratified by cause.



IMMUNOHISTOCHEMICAL DATA

p16 and E6 expression in OP-SCC specimens

The immunostaining for p16 is positive in 10/17 (60%) patients with a weak positivity (+ , 1-30% positive cells) in 4/17 (24%) patients and a strong positivity (++ , 31-100% positive cells) in 6/17 (36%) patients, while is negative in 7/17 (41%) patients; E6 is positively expressed in 17/17 (100%) patients with a weak positivity (+ , 1-30% positive cells) in 10/17 (69%) patients and a strong positivity (++ , 31-100% positive cells) in 7/17 (41%) patients.

The p16 antigen expression, when evaluated as ++, is uniformly distributed inside both cytoplasm and nuclei of almost all epithelial cells of tumoral nests **(Figure 5, panels a and a')**, while in + expression, positive cells are randomly spread in tumoral nests with a cytoplasmic or nuclear distribution (data not shown). In negative specimens p16 is occasionally present **(Figure 5, panels c and c')**. Where p16 is highly expressed the tissue is transformed and the epithelium of origin is not recognizable, while, where p16 is less expressed the original structural architecture of epithelium is more preserved and identifiable (data not shown).

The E6 oncoprotein expression, when present **(Figure 5, panels b and b')**, is uniformly distributed mainly inside the cytoplasm of all epithelial cells of tumoral nests; conversely a prevalent nuclear staining is evident in less transformed epithelia **(Figure 5, panels d and d')**. In 7 specimens p16 is negative and E6 is positive **(Figure 5, panels c-c' and d-d')**.

CD4, CD8 and Foxp3 expression in OP-SCC specimens

In the intratumoral environment CD4 immunostaining is positive in 17/17 (100%) patients with a + (< 10% positive cells) positivity in 2/17 (12%) patients, a ++ (10-20% positive cells) positivity in 4/17 (24%) patients and a +++ (> 20% positive cells) positivity in 11/17 (64%) patients; the same parameters were used to evaluate intratumoral CD8 and Foxp3 expression. CD8 immunostaining is positive in 17/17 (100%) patients with a + (< 10% positive cells) positivity in 2/17 (12%) patients, a ++ (10-20% positive cells) positivity in 6/17 (36%) patients and a +++ (> 20% positive cells) positivity in 9/17 (53%) patients. Foxp3 is positive in 7/17 (41%) patients with a + (< 10% positive cells) positivity in 5/17 (29%) patients, a ++ (10-20% positive cells) positivity in 2/17 (12%) patients and a +++ (> 20% positive cells) positivity in 0/17 (64%), it was negative in 10/17 (59%) patients.

In the peritumoral environment CD4 immunostaining is positive in 17/17 (100%) patients with a + (< 10% positive cells) positivity in 1/17 (6%) patients, a ++ (10-20% positive cells) positivity in 4/17 (24%) patients and a +++ (> 20% positive cells) positivity in 12/17 (71%) patients; CD8 immunostaining is positive in 17/17 (100%) patients with a + (< 10% positive cells) positivity in 8/17 (48%) patients, a ++ (10-20% positive cells) positivity in 7/17 (41%) patients and a +++ (> 20% positive cells) positivity in 2/17 (12%) patients. Foxp3 is positive in 10/17 (59%) patients with a + (< 10% positive cells) positivity in 4/17 (24%) patients, a ++ (10-20% positive cells) positivity in 5/17 (29%) patients and a +++ (> 20% positive cells) positivity in 1/17 (6%), it is negative in 7/17 (41%) patients.

CD4 and CD8 markers expression is uniformly distributed both in intratumoral and in peritumoral environment. **(Figure 6, panels a-a'-a*-a• and b-b'-b*-b•)**. Foxp3 distribution is dispersed both in intratumoral and peritumoral context **(Figure 6, panels c, c', c* and c•)**.

PD-1 and PDL-1 expression in OP-SCC specimens

In the intratumoral environment PD-1 immunostaining is positive in 12/17 (71%) patients with a + (< 20% positive cells) positivity in 12/17 (71%) patients, a ++ (21-50% positive cells) positivity in 0/17 (0%) patients and a +++ (> 50% positive cells) positivity in 0/17 (0%) patients, it is negative in 5/17 (29%) patients; in the peritumoral environment PD-1 immunostaining is positive in 7/17 (41%) patients with a + (< 20% positive cells) positivity in 7/17 (41%) patients, a ++ (21-50% positive cells) positivity in 0/17 (0%) patients and a +++ (> 50% positive cells) positivity in 0/17 (0%) patients, it is negative in 10/17 (59%) patients.

PD-1 positive cells in the peritumoral tissue are localized far from the invasive front of the carcinoma (data not shown), while in tumor nests they are quite uniformly distributed with the general tendency to gather together asymmetrically **(Figure 6, panels d and d')**.

The same parameters were used to evaluate PDL-1 expression on tumoral cells and in the tumoral microenvironment (TME). PDL-1 immunostaining is positive on tumoral cells in 17/17 (100%) patients with a + (< 20% positive cells) positivity in 9/17 (53%) patients, a ++ (21-50% positive cells) positivity in 8/17 (48%) patients and a +++ (> 50% positive cells) positivity in 0/17 (53%) patients, in the TME it is positive in

17/17 (100%) patients with a + (< 20% positive cells) positivity in 1/17 (6%) patients, a ++ (21-50% positive cells) positivity in 10/17 (59%) patients and a +++ (> 50% positive cells) positivity in 6/17 (36%).

PD-L1 positive cells are sparsely distributed intratumorally, while peritumorally they are homogeneously distributed surrounding tumoral nests. **(Figure 6, panels e, e', e* and e•)**.

TREM-1 expression in OP-SCC specimens

In the intratumoral environment TREM-1 immunostaining is positive in 3/17 (18%) patients with a + (1-50% positive cells) positivity in 2/17 (12%) patients, a ++ (> 50% positive cells) positivity in 1/17 (6%), it is negative in 14/17 (82%) patients; in the peritumoral environment TREM-1 is positive in 14/17 (82%) patients with a + (1-50% positive cells) positivity in 11/17 (64%) patients, a ++ (> 50% positive cells) positivity in 3/17 (18%) patients, it is negative in 3/17 (18%) patients.

TREM-1 expression is mainly negative (82% of patients) in the tumoral nests **(Figure 6, panels f and f')** while, when present, its pattern of distribution is not easily depictable (data not shown); conversely it is highly expressed in the peritumoral environment with a homogeneous spread even if a slight higher concentration near tumour nests can be described **(Figure 6, panels f* and f•)**.

IL-22 expression in OP-SCC specimens

In the intratumoral environment IL-22 immunostaining is positive in 17/17 (100%) patients with a + (1-33% positive cells) positivity in 10/17 (59%) patients, a ++ (34-67% positive cells) positivity in 7/17 (41%) patients and a +++ (> 67% positive cells) positivity in 0/17 (0%) patients; in the peritumoral environment it is positive in 17/17 (100%) patients with a + (1-33% positive cells) positivity in 5/17 (29%) patients, a ++ (34-67% positive cells) positivity in 8/17 (48%) patients and a +++ (> 67% positive cells) positivity in 4/17 (24%) patients.

In the peritumoral environment IL-22 expression is scattered with a predominant concentration near tumor nests where it is present, in the central part, with a multi-spot pattern (Figure 6, panels **g, g', g* and g•**).

The above illustrated results are graphically summarized in **Table 3** and were analyzed with the McNemar test in order to evaluate the difference between intratumoral and peritumoral environment in terms of expression

frequencies of tumoral markers (presence or absence; different intensities of expression were not considered). the expression of CD4, CD8, PDL-1 and IL-22 is concordant in both sites and the test is not applicable, while there is a discordance for TREM-1, PD-1 and Foxp3 that is statistically significant only for TREM-1 (p=0.001; McNemar test): it is more frequently expressed in the peritumoral tissue when compared with intratumoral one; for PD-1 and Foxp3 the p values were 0.1250 and 0.250 respectively.

After stratification of population for tumoral stage and causes of death, the same test was used to evaluate the presence of differences in TREM-1, PD-1 and Foxp3 expression in the peritumoral and tumoral environment: no statistically significant differences were observed.

Table 3. Markers expression in OP-SCC specimens.

	Intratumoral localization n/17 (%)				Peritumoral localization n/17 (%)			
CD4	**0**	+	++	+++	**0**	+	++	+++
	0	2 (12)	4 (24)	11 (64)	0	1 (6)	4 (24)	12 (71)
	Intratumoral localization n/17 (%)				Peritumoral localization n/17 (%)			
CD8	**0**	+	++	+++	**0**	+	++	+++
	0	2 (12)	6 (36)	9 (53)	0	8 (48)	7 (41)	2 (12)
	Intratumoral localization n/17 (%)				Peritumoral localization n/17 (%)			
Foxp3	**0**	+	++	+++	**0**	+	++	+++
	10 (59)	5 (29)	2 (12)	0	7 (41)	4 (24)	5 (29)	1 (6)
	Intratumoral localization n/17 (%)				Peritumoral localization n/17 (%)			
PD-1	**0**	+	++	+++	**0**	+	++	+++
	5 (29)	12 (71)	0	0	10 (59)	7 (41)	0	0
	Tumor cells localization n/17 (%)				Tumor Micro Environment localization n/17 (%)			
PDL-1	**0**	+	++	+++	**0**	+	++	+++
	0	9 (53)	8 (48)		0	1 (6)	10 (59)	6 (36)
	Intratumoral localization n/17 (%)				Peritumoral localization n/17 (%)			
TREM-1	**0**	+	++		**0**	+	++	
	14 (82)	2 (12)	1 (6)		3 (18)	11 (64)	3 (18)	
	Intratumoral localization n/17 (%)				Peritumoral localization n/17 (%)			
IL-22	**0**	+	++	+++	**0**	+	++	+++
	0	10 (59)	7 (41)	0	0	5 (29)	8 (48)	4 (24)
	n/17 (%)				n/17 (%)			
p16	**0**	+	++		**E6**	**0**	+	++
	7 (41)	4 (24)	6 (36)			0	10 (69)	7 (41)

EXPRESSION LEGEND:

p16 and E6: 0 negative, + low, ++ high; **CD4, CD8 and Foxp3:** 0 negative, + < 10% of positive cells, ++ 10% - 20% of positive cells, +++ > 20% of positive cells; **PD-1 and PDL-1:** 0 negative, + < 20% of positive cells, ++ 21% - 50% of positive cells, +++ > 50% of positive cells; **TREM-1:** 0 negative, + < 50% of positive cells, ++ > 50% of positive cells; **IL-22:** 0 negative, + 1% - 33%, ++ 34% - 67%, +++ > 67%.

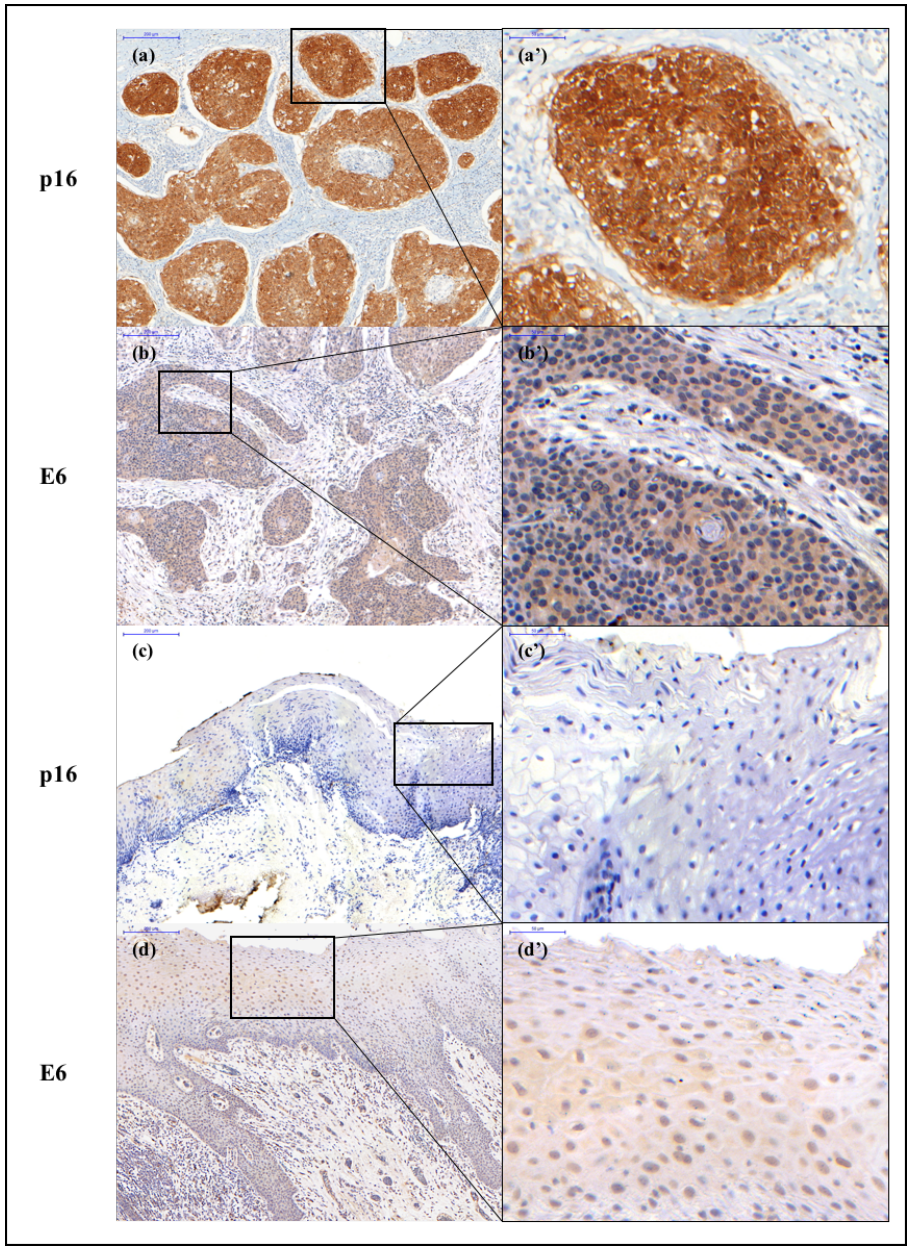


Figure 5. p16 and E6 expression in representative cases of OP-SCC. p16 staining: 10x (a, c) and 40x (a', c'). E6 staining: 10x (b, d) and 40x (b', d'). The black boxes in 10x fields correspond to 40x magnification. Bar in the 10x fields = 200 µm, in the 40x fields = 50 µm. Panels a,a' and b,b' as panels c,c' and d,d' belong to the same specimen.

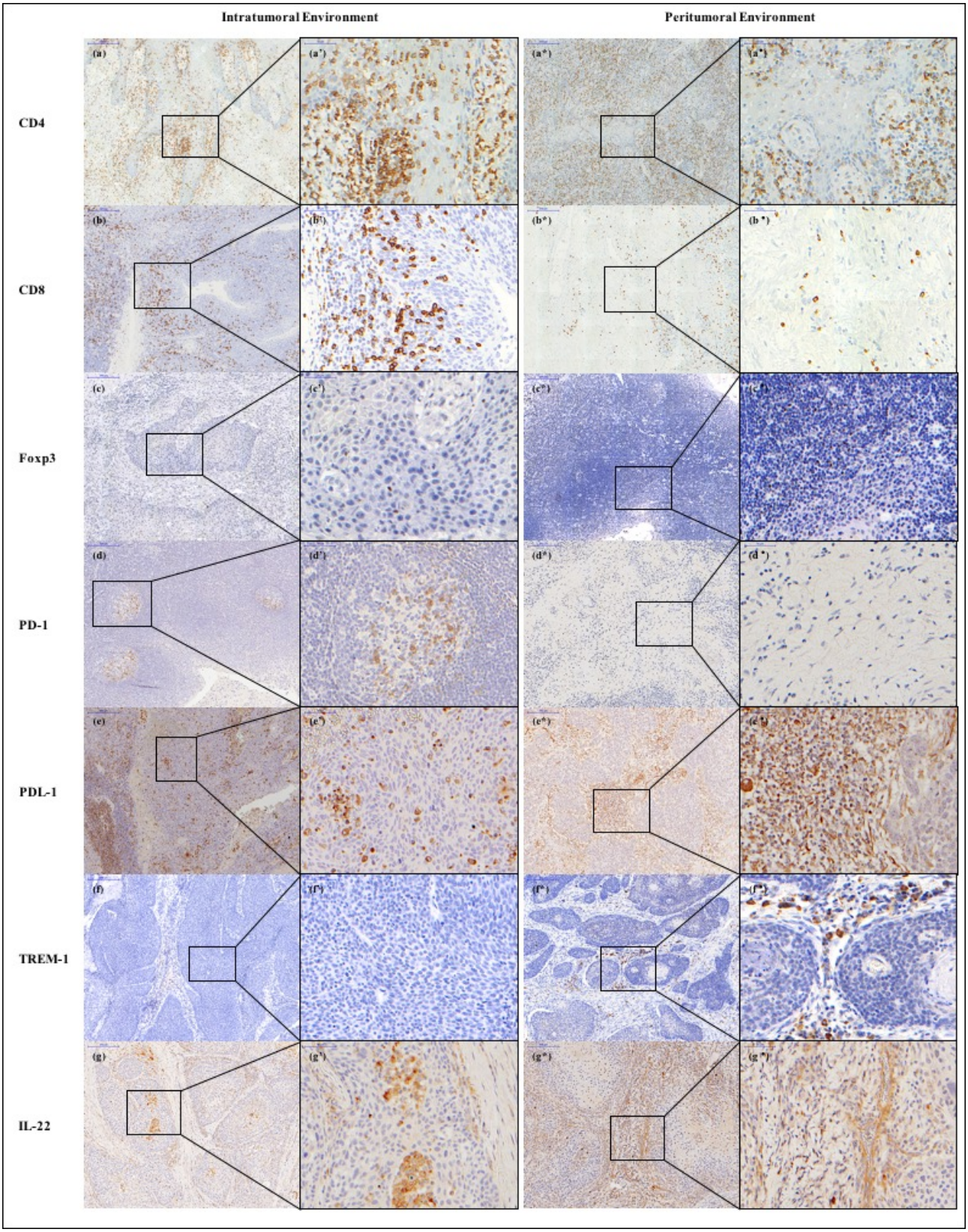

Intratumoral Environment
Peritumoral Environment
CD4
CD8
Foxp3
PD-1
PDL-1
TREM-1
IL-22

Figure 6. CD4, CD8, Foxp3, PD-1, PDL-1, TREM-1 and IL-22 expression in the intratumoral and peritumoral environment of representative cases of OP-SCC. CD4 staining: 10x (a, a*) and 40x (a', a•). CD8 staining: 10x (b, b*) and 40x (b', b•). Foxp3 staining: 10x (c, c*) and 40x (c', c•). PD-1 staining: 10x (d, d*) and 40x (d', d•). PDL-1 staining: 10x (e, e*) and 40x (e', e•). TREM-1 staining: 10x (f, f*) and 40x (f', f•). IL-22 staining: 10x (g, g*) and 40x (g', g•). The black boxes in 10x fields correspond to 40x magnification. Bar in the 10x fields = 200 μm, in the 40x fields = 50 μm. Representative expression levels of CD4 are +++ (a, a') and +++ (a*, a•), of CD8 are +++ (b, b') and + (b*, b•), of Foxp3 are + (c, c') and +++ (c*, c•), of PD-1 are ++ (d, d') and negative (d*, d•), of PDL-1 are ++ (e, e') and +++ (e*, e•), of TREM-1 are negative (f, f') and ++ (f*, f•) and of IL-22 are ++ (g, g') and +++ (g*, g•). PDL-1 expression in panel e and e' is referred to tumoral cells.

DISCUSSION

PATIENT'S CHARACTERISTICS

In this paper we studied a population of 17 patients (median age 62 yrs, 94% males, 6% females) affected by a HPV-related primitive squamous cell carcinoma of the oropharynx in advanced stage (54% stage III, 46% stage IV); risk factors evaluation showed that all patients presented at least one risk factor other than the HPV infection: 72% were smokers, 66% were alcohol consumer and in 47% of subjects alcohol and smoking were concomitant.

Survival analysis, based on a median follow up of 9 years (IQR 8.5-11), showed a 5-yr OS of 64.7% (95%CI 37.7-82.3), from the diagnosis date to the end of the follow-up 10 (59%) patients were died: 6 for persistence/recurrence of OP-SCC and 4 for other causes; 5-yr cumulative incidence of death for OP-SCC is 29.5% (95%IC 10.7-51.1) and cumulative incidence of death from other cause is 5.9% (95%CI 0.4-23.5); these findings mirror those reported by Nørregaard C et al. **[480]** in a study investigating a large cohort of Danish patients affected by OP-SCC: the 5-yr mortality was 27% due to OP-SCC and most of patients died within the first two years, only 9% died after 5 years, moreover The HPV- and p16-negative patients showed a greater risk of dying across all causes of death compared to the HPV-positive patients.

HPV status

HPV is a small (8-kb), nonenveloped circular DNA virus with epithelial tropism, its family is constituted by 200 viral strains approximatively and only 40 can be transmitted through direct skin or mucosa contact **[481-483]**. HPV infection is very common, indeed, almost all sexually active people acquire the virus during their life and most of them are able to clear the infection within 1 or 2 years without symptoms. However high-risk HPV (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) infection can persist for months to years and is associated to the development of SCC of several sites including oropharynx and oral cavity.

After infection and integration of viral DNA in the host DNA, the oncogenic activity is carried on by the expression of viral oncoproteins E6 and E7 which bind and promote degradation of p53 and retinoblastoma (pRb) tumor suppressor proteins respectively. After pRb loss of function there is, via a feedback mechanism, an upregulation p16 that is commonly not functional because of mutation or deletion or methylation of its promoter.

A copious number of studies support the concept that head and neck HPV related tumors are a clinically distinct subset characterized by better survival outcomes and by a better response to exclusive radiotherapy or radio-chemotherapy; in the clinical management of such tumors the HPV status evaluation is therefore fundamental and the detection of p16 represent an effective tool as demonstrated by several studies and meta-analyses. However, in several cases p16 expression is lower or its expression is only cytoplasmic and it is considered "negative", thus, the gold standard for HPV status evaluation is represented by E6 and E7 expression detection via quantitative reverse transcription polymerase chain reaction PCR (qRT-PCR), some authors used in- situ hybridization or immunohistochemistry tests if mRNA or DNA are not adequate.

For the above exposed reasons, in our study, we evaluated HPV status with immunohistochemistry both for p16 and E6 because viral mRNA and DNA were not detectable with qRT-PCR and in-situ hybridization: 41% of specimens were negative for p16 and 100% were positive for E6 confirming that all OP-SCC enrolled were HPV-related.

CD4+ and CD8+ lymphocytes infiltrate

The role of high density CD8+ lymphocytes infiltration in the tumoral mass has been identified as a favourable prognostic indicator in different types of cancer, including HNSCC, **[484-488]** because the ability of T cytotoxic cells to kill transformed cancer cells. The role of CD4+ lymphocytes is more controversial because most of studies focused the attention on Treg whose role seems to differ according to cancer type and etiology. In the intratumoral environment, we observed high levels of CD4+ and CD8+ infiltrating lymphocytes in most patients: 64% and 53% of patients had a +++ positivity for CD4 and CD8 respectively and a ++ positivity was detected in 24% and 36% respectively. A superimpose able pattern of expression was observed in the peritumoral environment. These data are confirmed by the findings of Partlova et al. **[489]** that, comparing

HPV-positive and HPV-negative head and neck squamous cell carcinomas, demonstrated a statistically significant higher number of CD8+ IFNγ + T cells, CD8+ IL-17+ T cells, CD4+ IFNγ + T cells (Th1), antigen presenting cells (dendritic cells and monocytes/macrophages) and proinflammatory chemokines in HPV related tumors.

Foxp3+ lymphocytes infiltrate

The transcription forkhead box p3 (Foxp3) is a key intracellular molecule for Tregs development and function **[490]** that can be considered as the most specific Treg marker. In normal conditions Foxp3+ Treg are involved in the maintenance of immunological tolerance, whereas, in pathological conditions, such as cancer, are essential suppressor of antitumor responses, thus a high infiltration of Foxp3+ Tregs in the tumor microenvironment is expected to be associated to an unfavorable outcome. This hypothesis was confirmed in several localized and metastatic cancers such as breast, ovarian, hepatocellular, lung, gastric and ovarian carcinomas **[491-496]**. In other tumors, like colorectal cancer, Foxp3+ Treg infiltration was associated with a prognosis improvement **[497,498]**. A systematic review and meta-analysis of literature regarding the prognostic value of Foxp3+ Treg infiltration in cancer, performed by Shang et al. in 2015, identified only two studies investigating this topic in head and neck cancer and data analysis showed that a high Foxp3+ Treg infiltrate correlates with an improved OS (OR 0.69, 95% CI 0.50 to 0.95, p=0.024). A more recent review and meta-analysis on the role of Foxp3+ Treg infiltration in HNSCC, performed by **[499]**, showed that high tumoral infiltration by Foxp3+ Treg predicts a better clinical outcome in this type of cancer. The authors explain these findings observing that Tregs suppress the ongoing and ineffective inflammatory response promoting tumor progression. **[500,501]** The main limit of this paper is represented by the absence of stratification for tumor subsite and HPV status: the only study, included in the analysis, that investigated Foxp3+ Treg infiltration in HPV+ tumors, observed a slightly and non-significantly better OS in patients with high infiltration. **[502]**
Sun et al., in a study performed on specimens derived from laryngeal squamous cell carcinoma, demonstrated a scarce infiltrate of Foxp3+ Treg in the intratumoral site and a more representative one in the peritumoral setting. Moreover they observed that M2 polarized macrophages, originated in the tumor environment, promote the differentiation of CD24+ CD25- T cells into aTreg; in turns these generated aTregs skew the

differentiation of monocytes toward M2 phenotype, forming a positive-feedback loop that contributes to immunosuppression. **[503]**

In the intratumoral environment, we observed levels of Foxp3 Tregs expression as follows: 12% and 29% of patients had a ++ and a + positivity respectively, whereas in the peritumoral site we registered levels of expression +++, ++ and + in 6%, 29% and 24% of patients respectively.

PD-1 and PDL-1 expression

Programmed cell Death protein 1 (PD-1) is a membrane protein that negatively regulate T cell activity and it is primarily believed to inhibit effector T cell activity in the effector phase within tissue and tumors. Its expression, on T cells and on other immunologic cells like B cells and NKs, is induced after the activation of peripheral T and B cells as well as monocytes. PD-1 has two ligands: PDL-1, expressed on resting T cells, B cells, DCs and macrophages and it is further up-regulated upon activation by several factors (IFNγ is one of its major cytokine inducer), and PDL-2 inducibly expressed only on DCs and macrophages. **[504]**

In our cohort of patients, we observed a + positivity of PD-1 in 71% and 41% of specimens in the intratumoral and peritumoral environment respectively; PD-1 positive cells were quite uniformly distributed with the general tendency to gather together asymmetrically. These data are in accord with Lyford-Pike's **[505]** study that evidenced a high expression of PD-1 on CD4+ and CD8+ in HPV related HNSCC, they did not report data on intra-/peritumoral expression.

On tumoral cells, we observed medium/low levels of PDL-1 expression in all patients: 48% and 53% of patients had a ++ and a + positivity respectively, in the tumor microenvironment we observed medium/high levels of PDL-1 expression in all patients: 36% and 59% of patients had a +++ and a ++ positivity respectively; PDL-1 positive cells were uniformly distributed in the peritumoral infiltrate and surrounding tumor nests where they were sparsely distributed. Our data on PDL-1 expression agree again with Lyford-Pike's **[505]** who observed, on HPV-related HNSCC, a mainly peritumoral peritumoral localization (13/14 samples) and only 1 case of diffuse PDL-1 positivity in tumor nests.

TREM-1 expression

Triggering Receptor Expressed on Myeloid cells-1 (TREM-1), expressed on monocytes, neutrophils, granulocytes, dendritic cells and natural killer cells, is a member of the Ig superfamily of immunoregolatory receptors, its ligands have not yet fully identified but some evidences indicate danger- and pathogen-associated molecular patterns (DAMPs and PAMPs) as possible activators of TREM signaling that is characterized by phosphorylation of DAP12. In vitro studies on neutrophils and macrophages demonstrate that DAP12 phosphorylation induces the expression of several inflammatory genes and, only in neutrophils, regulates their degranulation and production of ROS. Thus, it is evident that amplification of inflammation is the best-characterized function attributed to TREM-1. **[506]**

Macrophages, in tissues, acts as professional phagocytes and antigen-presenting cells and they are critically involved in tumor progression. The nature and the combination of activating stimula heavily impact on un-polarized macrophage (M0) polarization into proinflammatory type 1 macrophages (M1 induced by microbial factors and Th1 proinflammatory cytokines) and anti-inflammatory type 2 macrophages (M2 induced by Th2 cytokines, antinflammatory cytokines, glucocorticoids etc…). M1 cells have effector, proinflammatory and Th1-oriented immunostimulatory properties mediating antimicrobial defense, tissue destruction and antitumor resistance **[507,508]**, whereas M2 cells have Th2-type immunoregulation and resolution of inflammation properties mediating wound healing, angiogenesis and tumor growth. **[509-513]** Raggi et al. **[514]** demonstrated that M1 cells are characterized by a strong induction of CD80 and downregulation of CD206, conversely M2 cells presents low levels of CD80 and upregulation of CD2016. The same research group demonstrated that hypoxia (pO_2 0-20 mmHg), generated by disorganized or dysfunctional vascular network typical of pathological environments such as the tumoral one, acts on macrophages inducing the acquisition of some phenotypic and secretory features of M2 cells, moreover the hypoxic environment is able to modulate both M1 and M2 macrophages response pattern reducing the expression of molecules involved in migration, T cell activation, antigen presentation and increasing scavenger receptor expression and proangiogenic cytokines/chemokines production. They also demonstrated that TREM-1, constitutively expressed at low levels on M0, M1 and M2, in an hypoxic condition, is significantly induced on all macrophages subtypes and

that TREM-1 triggering reverses the M2-polarizing effect of hypoxia imparting a M1-skewed proinflammatory phenotype to macrophages.

We described for the first time the TREM-1 expression in a cohort oh HPV-related OP-SCC and immunohistochemical results showed, in the intratumoral environment, a + and a ++ positivity in 12% and 6% of specimens respectively, while, in the peritumoral infiltrate the positivity was ++ in 18% of tumors and + in 64% with a distribution surrounding tumoral nests.

IL-22 expression

IL-22 is a particular cytokine because it has a unidirectional signaling flow: immune cells are the only source of the cytokine and its target are non-hematopoietic cells, giving to IL-22 a key role in the immune-epithelial cross talk. The IL-22 membrane receptor, located on epithelial cells, renal tubular and pancreatic ductal cells, when triggered, activates a signaling patway that ends with the phosphorylation of STAT 3 and the upregulation of the expression of anti-apoptotic and mitogenic genes enhancing carcinogenesis. IL-22 role in the initiation and progression of cancer has already been demonstrated in lung, liver, gastric, colon, pancreatic and oral cavity tumor, but only few studies are available in the oncological fields and, as concluded by Lanfranca et al. the ultimate goal is to exploit the pathway to prevent cell proliferation and invasion in cancer. **[515,516]**

We described for the first time the IL-22 expression in a cohort oh HPV-related OP-SCC and immunohistochemical results showed, in the intratumoral environment, a + and a ++ positivity in 59% and 41% of specimens respectively, while, in the peritumoral infiltrate the positivity was +++ in 24% of tumors, ++ in 48% and + in 29% with a scattered localization in peritumoral environment with a predominant concentration near tumor nests where it is present, in the central part, with a multi-spot pattern.

Statistical analysis

We decided to use the McNemar test to assess the presence of differences in dichotomic data (presence or absence of the markers) because the analyzed sample is small and it does not allow the creation of a more detailed stratification of data (e.g. negative, +, ++ and +++ expression). The statistical analysis, performed to

assess the presence of differences between intratumoral and peritumoral environment in terms of expression frequencies of tumoral markers showed that CD4, CD8, PDL-1 and IL-22, being expressed in both settings in 100% of cases, are not evaluable with the above mentioned test, whereas TREM-1, PD-1 and Foxp3 are evaluable, because of the presence of a discordance in expression, and the test is statistically significant only for TREM-1 ($p=0.001$), for PD-1 and Foxp3 the p values were 0.1250 and 0.250 respectively. Correlations between markers expressions, risk factors and survival data are not possible because of the small size of enrolled patients.

Limits

The main limit of this study is represented by the small dimension of the sample that was determined by the strict inclusion criteria and their severe observance to exclude all potential conditions able to modify directly or indirectly the immunological environment of tumors. Moreover, in the last 15 years, the number of surgically resected tumors of the oropharynx decreased little by little because recent guidelines recommend radiochemotherapy as first line treatment, in particular for HPV-related tumors that show an higher radiosensitivity.

Other limits are represented by the monocentric design and HPV positivity of all the specimens.

CONCLUSIONS

Head and neck squamous cell carcinomas serves as a paradigm of immunosuppressive disease, as they are characterized by dysregulated cytokine profile, impaired function of immune effector cells and abnormalities in tumor associated antigens presentation.

Aware of the limits of the study and in the light of the immunohistochemical results we can conclude that the HPV-related OP-SCC, enrolled in this study, use different strategies to evade the immune surveillance of the host.

A persistent high risk HPV infection of oropharyngeal mucosa causes the integration of viral DNA into the host DNA with the consequent transformation of epithelial cells that lose the control on cell cycle and accumulate genetic mutations with the consequent expression of abnormal proteins: the tumor-associated antigens (TAAs). These molecules, presented by antigen presenting cells (DCs, macrophages etc…), are recognized by T cells and trigger the immune response.

Immune cells reach and infiltrate the tumor environment: we observed high levels of CD4+ T lymphocytes equally distributed between intra- and peritumoral environment and of CD8+ T lymphocytes mainly localized intratumorally. Partlova et al. observed also high levels of dendritic cells, monocytes/macrophages and proinflammatory chemokines.

A so rich inflammatory infiltrate should overcome cancer cells, however, thanks to several immunoescape mechanisms, it is ineffective. Our data and several recently published papers, demonstrate the concomitant high expression of PDL-1, on tumor cells and in tumor microenvironment, and of PD-1, mainly in the intratumoral site that leads to T lymphocytes anergy. Moreover, Lopez-Albaitero et al. and Ferris et al. observed that HNSCC cells inhibit T-cell-mediated recognition and activation by downregulating MHC I antigen presentation to endogenous TCR **[517,518]**; Woo et al. and Fourcade et al. demonstrated, on exhausted T cells isolated from solid malignancies, an upregulation of inhibitory co-stimulatory receptors other than PD-1, such as cytotoxic T-lymphocyte protein 4 (CTLA-4), T-cell immunoglobulin mucin-3 (Tim-3), lymphocyte activation gene 3, and T-cell tyrosine-based inhibitory motif domain (TIGIT). **[519,520]**

We observed also that the inflammatory infiltrate, in the enrolled OP-SCC, is populated by several Foxp3+ Tregs both in the intratumoral and peritumoral environment. Treg enrichment in tumour-infiltrating lymphocytes might be responsible for suppressing anti-tumour immunity in the TME. Mechanisms underlying Treg accumulation at tumour sites are not clear: Coombes et al. **[521]** and Sun et al. **[522]** suggested that it could be induced by the conversion of FOXP3− T cells into FOXP3+ T cells in the presence of TGF-β1 and retinoic acid, Darrasse-Jeze et al. **[523]** suggested a recruitment and expansion of Tregs in tumour site as a result of specific self-antigen recognition by memory Treg. Jie et al. showed that, in HNSCC, Foxp3+ CD4+ Tregs express high levels of CTLA-4, **[524]** a competitor of TCR for binding CD80/CD86 expressed on antigen presenting cells, the triggering of CTLA-4 induces the expression of the suppressive mediator Indoleamine 2,3-dioxygenase in DCs. Moreover the dialogue between Tregs and antigen presenting cells induce the downregulation of MHC class II and T cell co-stimulatory molecules and the upregulation of suppressive messengers, such as B7-H3 and IL-10. All these events, derived from the mutual interaction of Treg and APC enables Treg to sustain their immunosuppressive function that promote cancer progression. **[525]**

Other immunoescape tricks are provided by tumour environment hypoxia: Raggi et al. **[514]** demonstrated that low oxygen levels act on macrophages inducing the acquisition of some phenotypic and secretory features of M2, protumoral, cells (low CD80 expression and upregulation of CD206), moreover the hypoxic environment is able to modulate both M1 and M2 macrophages response pattern reducing the expression of molecules involved in migration, T cell activation, antigen presentation and increasing scavenger receptor expression and proangiogenic cytokines/chemokines production. Hypoxic conditions are also able to induce expression of TREM-1 on all macrophages subtypes.

We observed a high expression of TREM-1 in peritumoral environment and this finding, supported by Raggi et al. **[514]** results, clearly highlight the hypoxic condition of tumoral site and allows us to presume a high presence of M2-skewed macrophages that promote cancer growth. This condition is probably enhanced by a further mechanism observed by Sun et al. **[503]**in laryngeal cancers: M2 polarized macrophages, originated in the tumor environment, promote the differentiation of CD24+ CD25- T cells into aTreg; in turns these generated aTregs skew the differentiation of monocytes toward M2 phenotype, forming a positive-feedback loop that contributes to immunosuppression.

The last marker analyzed, IL-22, whose role in cancer promotion has been demonstrated in lung, liver, gastric, colon, pancreatic and oral cavity lesions, is highly expressed in the peritumoral environment and less in tumor nests. We did not analyzed the IL-22R expression, so our data can suggest the presence of high levels of Th22 cells in the lymphocyte infiltrate, while in tumor nests the IL-22 positivity can be interpreted as presence of Th22 but also as presence of IL-22 attached to its receptor expressed by epithelial tumour cells.

Based on our results, TREM-1, IL-22 and Foxp3+ CD4+ Tregs seems to be key markers in HPV-related OP-SCC development and, in the future, they could explain the different responses to treatment with anti PD-1/PDL-1 drugs and represent new targets, in particular TREM-1, for immunotherapy.

FUTURE RESEARCH PERSPECTIVES

- In order to increase the statistical power of the results of the present study, we are testing, with the same study protocol, the same panel of markers on 13 OP-SCC patients coming from A.O.U. "Maggiore della Carità" of Novara.

- An interesting investigation could be the evaluation of Foxp3+ Treg in HNSCC, stratifying the sample for tumoral site and identifying the possible presence of Treg subsets, to further detail the relationship between Foxp3+ Treg infiltration and survival data that now is quite ambiguous.

- A better quantitative, qualitative and topographic evaluation of TREM-1 expressing cells, both in the intratumoral and peritumoral environment of HPV+ and HPV- HNSCC can be useful to increase knowledges about cancer promotion.

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RELAZIONE FINALE PERCORSO DI DOTTORATO

PUBBLICAZIONI SCIENTIFICHE

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10. Garzaro M, Zenga F, Raimondo L, Pacca P, Pennacchietti V, Riva G, Ducati A, Pecorari G. Three-dimensional endoscopy in transnasal transsphenoidal approach to clival chordomas. Head and Neck. 2016 Apr;38 Suppl 1:E1814-9.

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PRESENTAZIONI A CONGRESSI NAZIONALI ED INTERNAZIONALI

1. Cisplatin + vinorelbine treatment of recurrent or metastatic salivary gland malignancies (RMSGM): a final report on 60 cases.

O. Ostellino, M. Garzaro, F. Pedani, M. Airoldi, E. Bellini, L. Raimondo, G. Pecorari.

ESMO 2014 Congress – Madrid 26-30 september 2014 (ATTI – POSTER)

2. Relationships between head and neck cancer patients and their caregivers:

focus on psychological distress and quality of life.

Raimondo L., Airoldi M., Garzaro M., Pedani F., Bellini E., Contu V., Torta R., Leombruni P., Bartoli C., Pecorari G.

XVI Congresso Nazionale AIOM - Roma, 24-25-26 ottobre 2014 (ATTI – POSTER)

3. 3D Endoscopic Endonasal Approach in Skull Base Surgery: Experience on 87 Consecutive Patients.

Giancarlo Pecorari, Francesco Zenga, Luca Raimondo, Paolo Pacca, Alessandro Ducati, and Massimiliano Garzaro.

AAO-HNS Annual Meeting – Orlando, 21-24 settembre 2014 (ATTI – PRESENTAZIONE ORALE)

4. Impiego del PRP nella parotidectomia esofacciale: valutazione di efficacia nella riduzione dell'incidenza di complicanze precoci.

Raimondo L.

II Congresso Nazionale ANTHEC – Cremona, 21-22 novembre 2014 (ATTI – PRESENTAZIONE ORALE)

5. How Alcoholism Influence HNSCC Patient-caregiver Dyad ? Massimiliano Garzaro, MD; Luca Raimondo, MD; Mario Airoldi, MD; Claudia Bartoli, MD; Paolo Leombruni; Giancarlo Pecorari. AAO – HNS Annual Meeting – Dallas, 27 – 30 Settembre 2015

6. HNC patient's alcohol abuse and caregiver's quality of life: What's wrong? Mario Airoldi, Luca Raimondo, Claudia Bartoli, Paolo Leombruni, Massimiliano Garzaro, Giancarlo Pecorari . ASCO Annual Meeting - Chicago, 29 Maggio – 02 Giugno 2015

7. Survival impact of different clinical characteristics of 108 cases of recurrent/metastatic salivary gland malignancies (RMSGM) treated with first line chemotherapy (CT). Mario Airoldi, Massimiliano Garzaro, Manuela Ceccarelli, Fulvia Pedani, Luca Raimondo, Claudia Bartoli, Elisa Bellini, Gianluca Fora, Chiara Monagheddu, Giancarlo Pecorari. ASCO Annual Meeting - Chicago, 29 Maggio – 02 Giugno 2015

8. La citologia nasale nell'ambito della Medicina Termale. Luca Raimondo. Attualità della Medicina Termale - Lurisia, 14 Maggio 2016

9. Cytological changes after radioactive fluoridated oligomineral water nasal irrigations in patients affected by non allergic rhinitis with neutrophils (NARNE). Raimondo Luca, Gestro Massimo, Zambianchi Cristina, Vigna Bruno, Garzaro Massimiliano, Pecorari Giancarlo.

Conventus Societas ORL Latina – Torino, 6-8 Luglio 2016

10. Effect of Radium 223 on remission of bone metastases in androgen receptor-positive (AR+) salivary duct carcinoma (SDC) of the parotid. Airoldi Mario, Pedani Fulvia, Bellini Elisa, Contu Viviana, Tucci Marcello, Podio Valerio, Raimondo Luca, Garzaro Massimiliano. ASCO Annual Meeting - Chicago, 03-07 Giugno 2016

11. Update sul trattamento degli acufeni. Luca Raimondo. Acufeni: aggiornamenti su ricerca diagnosi e trattamenti – Lurisia 15 Ottobre 2016

12. Squarzanti DF, Negro A, Raimondo L, Albera R, Airoldi M, Chiusa L, Pecorari G, Cappello P, Valente G, Azzimonti B. Immunophenotype of Oropharyngeal Squamous Cell Carcinoma: Foxp3, IL-22 and TREM-1 expression in the tumoral environment. Giornata IRCAD - Novara 11 Dicembre 2017

PARTECIPAZIONE A CORSI E CONGRESSI

1. Linee guida e loro applicazione in ambito di responsabilità professionale.
Torino, 19 Febbraio 2014

2. Appropriatezza e sicurezza degli interventi di adenotonsillectomia nell'adulto e nel bambino.
Torino, 9 Aprile 2014

3. Gestione delle patologie acute.
Torino, 11 Giugno 2014

4. Linee guida in ambito oncologico.
Torino, 24 Settembre 2014

5. Verso una condivisione delle modalità di definizione della priorità degli interventi in ambito orl.

Torino, 19 Novembre 2014

6. II Congresso Nazionale ANTHEC

Cremona, 21-22 novembre 2014

7. Attualità della medicina termale. Lurisia, 16 Maggio 2015

8. 102° Congresso Nazionale SIO. Roma, 27-30 Maggio 2015

9. Il consenso in ORL. Torino, 10 Giugno 2015

10. Il consenso nella chirurgia delle ghiandole salivari, cavo orale ed orofaringe. Torino, 23 Settembre 2015

11. AAO – HNS Annual Meeting. Dallas, 27 – 30 Settembre 2015

12. Il Consenso nella chirurgia naso-sinusale e base cranica, endoscopia chirurgico-diagnostica. Torino, 14 Ottobre 2015

13. Il consenso nella chirurgia di collo, laringe e tiroide. Torino, 04 Novembre 2015

14. Attualità della medicina termale. Lurisia, 14 Maggio 2016 (Relatore)

15. Conventus Societas ORL Latina. Torino, 6-8 Luglio 2016 (Relatore)

16. 55° Master di citologia nasale. Como 16-18 Giugno 2016 (Discente)

17. Acufeni: aggiornamenti su ricerca diagnosi e trattamenti. Lurisia, 15 Ottobre 2016 (Relatore)

18. 58° MASTER di Citologia Nasale – Pavone C.se, 8-10 Giugno 2017

19. Corso NBI e neoplasie delle VADS – Roma, 13-14 Luglio 2017

PROGETTI DI RICERCA ATTIVI

- Ruolo delle acque termali nel trattamento delle riniti croniche allergiche e non allergiche
- La citologia laringea: aspetti citologici della mucosa laringea nel paziente non fumatore e in quello fumatore
- Immunofenotipizzazione delle neoplasie squamose del distretto cervico-cefalico
- Ruolo della spettroscopia Raman nella diagnostica nasosinusale e oncologica ORL