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PROGNOSTIC BIOMARKERS OF HEAD AND NECK CANCER

PhD thesis

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1 List of abbreviations

AJCC	American Joint Committee on Cancer
CD	cluster of differentiation
CDKN2A	cyclin-dependent kinase inhibitor 2A
CRT	chemoradiotherapy
CTLA-4	cytotoxic T-lymhocyte-associated protein 4
CUL3	cullin-3 gene
DC	dendritic cell
DDX3X	DEAD-box helicase 3, X-linked
DNA	deoxyribonucleic acid
DOI	depth of invasion
DSS	disease-specific survival
EGFR	epidermal growth factor receptor
ENE	(tumor) extranodal extension
EORTC	European Organization for Research and Treatment of Cancer
ERK	extracellular signal–regulated kinase
EU	European Union
FFPE	formalin-fixed, paraffin-embedded
FGFR2/3	fibroblast growth factor receptor 2/3
5-FU	5-fluorouracil
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
HR	hazard ratio
IHC	immunohistochemistry
MLL2	histone-lysine N-methyltransferase 2B
MTX	methotrexate
NMSC	nonmelanoma skin cancer
NPC	nasopharyngeal cancer
NSD1	nuclear receptor binding SET domain protein 1

OCC	oral cavity cancer
OPC	oropharyngeal cancer
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
PD-L2	programmed death-ligand 2
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PNI	perineural invasion
RECIST	Response Evaluation Criteria in Solid Tumors
RTOG	Radiation Therapy Oncology Group
R/M	recurrent or metastatic
SHP-2	Src homology 2 domain-containing tyrosine phosphatase 2
TIL	tumor infiltrating lymphocyte
TIMC	tumor-infiltrating mononuclear cell
TNM	tumor, lymph node, metastasis (classification)
UICC	Union for International Cancer Control
VEGF	vascular endothelial growth factor

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2 INTRODUCTION

There are multiple definitions to grasp what head and neck cancer means both on-line and in written literature. A rather general interpretation from the Mayo Clinic reads as follows: *Head and neck cancers are a broad category of cancers that occur in the head and neck region.* (http://www.mayoclinic.org/diseases-conditions/head-and-neck-cancers/home/ovc-20246134) Another slightly more detailed definition originates from the National Cancer Institute of the United States that reads: *Head and neck cancers are cancers that start in the tissues and organs of the head and neck, including cancers of the larynx, throat, lips, mouth, nose, and salivary glands.* (https://www.cancer.gov/types/head-and-neck). However, my favorite definition can be found on Medline (National Library of Medicine, National Cancer Institute) which declares: *Head and neck cancer includes cancers of the mouth, nose, sinuses, salivary glands, throat, and lymph nodes in the neck. Most begin in the moist tissues that line the mouth, nose, and throat.* (https://medlineplus.gov/headandneckcancer.html)

By its nature, head and neck cancer often occurs at unveiled parts of the human body. It is generally associated with a certain degree of functional impairment causing significant mental and physical distress to its victims and a substantial economic burden to the whole society.

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2.1 Epidemiology of head and neck cancer

Head and neck cancer represents a major global health issue ranking as the 6th most common cause of cancer related deaths worldwide (Ferlay et al. 2010, Torre et al. 2015). There has been several estimations of head and neck cancer incidence. The GLOBOCAN Estimates of the worldwide incidence and mortality from several cancers including cancers of the head and neck region is widely considered a reliable calculation published by the International Agency for Research on Cancer (Ferlay et al. 2010). These statistics do not contain head and neck cancer as an independent category. Instead, data on cancers of anatomical subsets (e.g. larynx, nasopharynx, etc.) are presented separately. Based on figures from the GLOBOCAN 2012, cancers of the lip, oral cavity, nasopharynx, other pharynx and larynx taken together and regarded as head and neck cancer account for a substantial number of cancer incidence and mortality worldwide. Calculations were made in more developed and in less developed areas separately. The following numbers are age-standardized rates per 100,000 and are standardized to the world standard population (Torre et al. 2015).

Concerning incidence in male, head and neck cancer reached the figures of 17.4 and 13.3 per 100.000 in more developed and less developed areas, respectively. Mortality rates in male were 6.9 and 8.3 per 100.000 in more developed and less developed areas, respectively (Torre et al. 2015).

Looking at the data on females, a remarkably lower incidence and death rate could be seen. The incidence of head and neck cancer was 4.2 vs. 4.4 whereas mortality rate was found to be 1.2 vs. 2.7 per 100.000 in more developed and less developed areas, respectively. Compared with the most common causes of cancer-associated deaths in males, head and neck cancer occupies the 7th and 6th position in the ranking in more developed and less developed countries, respectively. Considering females, head and neck cancer-related mortality is the 15th in more developed areas and 9th in less developed ones on a worldwide scale.

International statistics show a very high occurrence rate of head and neck cancer in Hungary compared to other countries. In 2004, when 10 countries joined the European

Union (EU), Hungary led the total cancer-associated mortality statistics with 258.5 cancerous deaths/100.000 men. Concerning mouth or pharynx localization, Hungary topped the list of EU members with 21.9 deaths/100.0000 that was 27.5% higher than the figure of the second country (Slovakia). Similarly, Hungarian men led the larynx-related cancer mortality as well. (Levi et al. 2004). Hungarian women came out as second (133.5/100.000) after Denmark (136.7/100.000) with regard to total cancer related deaths, whereas Hungary proved to lead the mouth or pharynx-associated cancer and the laryngeal cancer mortality among women as well (Levi et al. 2004).

The contrast between HPV-related and non-HPV-related head and neck cancer cannot be disregarded when assessing data on epidemiology of this disease. As it unfolds in a much more detailed way in the following chapters, HPV-associated oropharyngeal cancer represents a distinct biological and clinical entity. Likewise, the occurrence of it has to be discussed separately from tobacco-related head and neck cancer.

During the last years, compelling evidence has accumulated that reflect a drastically increased incidence of oropharyngeal cancers (OPCs), especially in North America and in northern Europe (Gillison, Chaturvedi et al. 2015).

The proportion of HPV-related cancers within all oropharyngeal malignancies varies in different countries and regions. In Sweden it is 90%, in the United States 50% whereas a large, international study concluded that on average 24.3% of OPCs are HPV-associated (Nasman et al. 2016).

Worldwide cancer registry data (Cancer Incidence in Five Continents) can be used to further elucidate global incidence trends by comparing incidence trends from 1983 to 2002 for upper aerodigestive tract malignancies that are etiologically associated with HPV infection (e.g.: OPC) versus those associated with tobacco smoking (e.g.: oral cavity and lung squamous cell carcinomas) (Chaturvedi et al. 2015). This study found that OPC incidence increased especially among young men (< 60 years old) in developed countries, despite concomitant declines in incidence for oral cavity and lung squamous cell carcinomas. These contrasts suggest a role of HPV infection in increasing OPC incidence rates among men. However, among women, incidence rates increased for all three cancers, supporting a dominant effect of smoking. These figures are

consistent with a hypothesis of a greater impact of HPV infection on OPC incidence trends for men over the last several decades, in contrast to the effect of smoking for women (Chaturvedi et al. 2015).

Taken together, it seems that the growing number of HPV-associated OPCs boosts the incidence figures outweighing the beneficial effect of slightly decreased tobacco consumption.

Nevertheless, despite improving diagnostics and intense research the 5-year overall survival of head and neck cancer in general remains relatively poor, around 60%. (Jay et al. 2015).

2.2 Pathogenesis of head and neck squamous cell carcinoma (HNSCC)

2.2.1 Etiology and risk factors

The main risk factors of head and neck cancer are smoking, alcohol consumption, persistent high-risk HPV infection and poor oral hygiene.

Smoking

Smoking is an independent causative factor of head and neck cancer (Maasland et al. 2014). Patients who continue smoking during radiotherapy are thought to have a failure of local control (hazard ratio (HR) 1.5) and poorer survival (HR: 1.7), but recent data suggests that baseline smoking status may play a more important role (Zevallos et al. 2016). Smoking cessation before surgery reduces the risk of complications related to anesthetics and is taught to improve wound healing, especially after reconstructive surgery (Tang et al. 2016). After quitting tobacco usage, it takes 20 years until the risk of developing oral cavity cancer sinks to the level of non smokers (Marron et al. 2010).

Alcohol

Alcohol consumption is an other main risk factor of head and neck cancer. Simultaneous smoking and abusive drinking has a synergistic effect on deteriorating prognosis (Tan et al. 1997). Those patient who do not quit heavy drinking after

treatment for head and neck cancer have significantly worse survival (Mayne et al. 2009). The positive effect of alcohol cessation on the risk of head and neck cancer appears after 20 years (Marron et al. 2010).

High-risk HPV infection

The causative relation between infection by high-risk HPV subtypes and head and neck cancer was proved on the verge of the millennium (Gillison, Koch et al. 2000). Now, it is widely accepted that HPV infection is a causative agent in case of oropharyngeal malignancies only (Dillon and Harrington 2015, Castellsague et al. 2016).

The initial infection occurs during oro-genital sexual intercourse. However, there has been reports of oro-oral transmission as well (D'Souza et al. 2009). It is presumed that persistent oropharyngeal infection by high-risk HPV subtypes (mostly HPV-16) poses a risk of developing oropharyngeal cancer. Thus, persistent HPV infection and the transmission of it has drawn much attention recently.

Data of recent analysis (National Health and Nutrition Examination Survey, 2009 to 2012, https://www.cdc.gov/nchs/nhanes/index.htm) demonstrated a three-fold greater increase in high-risk oral HPV prevalence per sexual partner for men compared to that for women. That is consistent with reported higher transmission rates for HPV from female to male than vice versa (Gillison, Chaturvedi et al. 2015). The study found a plateau in prevalence among men at approximately 15 oral sexual partners in contrast to approximately five partners among women (Gillison Chaturvedi et al. 2015, Giuliano et al. 2015). Taking into consideration that the viral load on the surface of the infected cervix is higher than of the infected penis, male predominance could be explained by males acquiring a higher number of virus particles (assuming a heterosexual intercourse) (Marur et al. 2010). Ultimately, oropharyngeal cancer predominantly occurs in male.

Poor oral hygiene

In recent decades, many studies have concluded poor oral hygiene to be a significant risk factor for oral and oropharyngeal cancer (Maier et al. 2016). However, bad oral

condition often coexists with positive anamnesis for alcohol consumption and tobacco smoking.

Other risk factors

On one hand, minor risk factors include inherited diseases e.g.: Fanconi anaemia, ataxia telangiectasia, Bloom's syndrome and Li–Fraumeni syndrome (Shaw and Beasley 2016).

Secondly, acquired immunodeficiency because of poor nutrition, advanced age, immunosuppressive therapy after transplant or acquired immunodeficiency syndrome can increase the risk of developing head and neck cancer (Shaw and Beasley 2016).

2.2.2 Premalignant lesions

Leukoplakia and erythroplakia are well-known premalignant lesions of the upper respiratory and digestive tract. A large meta-analysis by Mehanna et al. assessed the malignant transformation rate of 992 patients with histologically confirmed oral dysplasia. They concluded the mean overall transformation rate to be 12.1% (Mehanna et al. 2009).

Others found that histologically confirmed dysplastic lesions that were not removed displayed a considerably higher transformation rate compared to those that were excised (Ho et al. 2013). An other large meta-analysis on laryngeal dysplastic lesions of 942 patients showed transformation in 14% after a mean interval of 5.8 years, adding that severity of dysplasia correlated with risk of transformation (Weller et al. 2010). However, in population-based studies of oral leukoplakia without histological inclusion criteria the risks are much lower; 40-50% vanish spontaneously and less than 1% transform (Lodi et al. 2006, Roosaar et al. 2016).

The transformation potential of oral lichen planus is controversial. On the contrary, proliferative verrucous leukoplakia presenting with exophytic widespread progressive leukoplakia is taught to have a very high (up to 50-80%) transformation rate, thus a poor overall prognosis (Shaw and Beasley 2016).

Surprisingly, no precursor lesion has been detected in connection with HPV-associated oropharyngeal cancer, that would enable screening of patients, despite the assumingely long latency that occurs between viral exposure and manifest tumor formation (Hayes et al. 2015).

2.2.3 The main molecular and genetic alterations in HNSCC

The idea that HPV-associated head and neck cancer is a distinct biological entity is supported by a compelling body of evidence and is widely accepted. The genetic landscape of HPV-negative and HPV-driven HNSCC was assessed by many large-scale studies such as the The Cancer Genome Atlas and Chicago HNC Genomics cohorts. Data show a surprisingly wide range of genetic alterations that are common in both non-HPV-related and HPV-related head and neck cancers. These common patterns are amplifications (e.g.: 1q, 3q, 5p, 8q) deletions (e.g.: 3p, 5q, 11q) and other similarities including the generally similar mutation rate and the number of copy number changes (Hayes et al. 2015). According to Seiwert et al. the overall mutational burden in HPV-negative and HPV-positive HNSCC was similar with an average of 15.2 versus 14.4 somatic exonic mutations in the targeted 617 cancer associated genes (Seiwert, Zuo et al. 2015).

However, the genetic landscape of HPV-negative and HPV-positive HNSCC differs significantly. The main mutational spectrum of HPV-negative tumors showed concordance with published lung squamous cell carcinoma analyses with enrichment for mutations in p53, cyclin-dependent kinase Inhibitor 2A (CDKN2A), histone-lysine N-methyltransferase 2B (MLL2), cullin-3 (CUL3), nuclear receptor binding SET domain protein 1 (NSD1), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), and NOTCH genes (Seiwert, Zuo et al. 2015). In contrast, HPV-positive tumors showed unique mutations in DEAD-box helicase 3, X-linked (DDX3X), fibroblast growth factor receptor 2/3 (FGFR2/3) and aberrations in PIK3CA, KRAS, MLL2, and enrichment in NOTCH1 (Seiwert, Zuo et al. 2015).

HPV-negative tumors display a clear prominence of amplification of 3q at the locus for oncogene PIC3CA and other transcription factors (Hayes et al. 2015). Although PIK3CA is altered in tumors without regard to viral association, two specific cytosine>thymine mutations in viral-associated tumors occur predominantly in two hotspots within the helical domain. These result in amino acid substitutions that are implicated in PIK3CA kinase and oncogene activation (Hayes et al. 2015).

Surprisingly, although squamous cell carcinoma of any site rarely demonstrates *KRAS* mutations, they are reported in HPV-positive HNSCC (Seiwert, Zuo et al. 2015). Although the data are sparse, the interaction between smoking and *KRAS* mutation suggests a mechanism through which tobacco might augment risk in HPV-positive HNSCC (Hayes et al. 2015).

Concerning epidermal growth factor receptor (EGFR) protein, HPV-positive tumors have generally shown low or absent levels of protein expression or EGFR gene amplification (Keck et al. 2015).

An other substantial structural difference is the event of viral DNA integration in HPV-positive tumors. Although the predicted impact of integration is generally to silence the gene, the nature of HPV DNA integration remains controversial and is the subject of ongoing investigation (Lawrence et al. 2015). There are data that suggest the most tumors have a single primary integration site, although the integration event itself may be complex at sites of gene amplification (Akagi et al. 2014). Nevertheless, an alternative possibility is that the gene disruption may be a passenger event only and the targeting of a gene may be nonspecific and may result from a stochastic event. The lack of recurrent events gives suggests a lower relevance of the integration site to tumor initiation and progression (Hayes et al. 2015).

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2.3 Clinical presentation of HNSCC

2.3.1 Recent changes in tumor, lymph node, metastasis (TNM) classification

Newly available data and the ever increasing number of HPV-positive oropharyngeal cancer cases worldwide produced an urging need for revision and reassessment of TNM classification system in head and neck oncology. This common interest resulted in the publication of the eighth edition of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual in the fall of 2016 (Lydiatt et al. 2017). The 8th manual incorporates significant changes compared to the previous one including a separate staging algorithm for HPV-associated cancer of the oropharynx; changes to the tumor T categories in the nasopharynx, oral cavity, and skin; and the addition of tumor extranodal extension (ENE) to the lymph node category for most anatomical sites (Lydiatt et al. 2017). Authors of both the AJCC and Union for International Cancer Control (UICC) guidelines strove to achieve global applicability and harmony between the two manuals. However, the manuals slightly differ from each other. In the AJCC version the non-HPV-associated pathologic category N criteria contains a group within N2, that is ENE-positive and less than 3 cm in greatest dimension that is considered N2a. On the other hand, the UICC manual does not contain this category but classifies all ENE-positive lymph nodes as N3b. Here, changes according to the AJCC guideline are to be briefly summarized. There are two major structural changes. First, HPV-associated OPC is addressed in a separate chapter based on p16 immunohistochemistry (IHC). On top of that, separate chapters for non-HPV-associated, p16-negative OPC/ hypopharyngeal cancer and for nasopharyngeal cancer are included (Lydiatt et al. 2017). Secondly, the head and neck section addresses nonmelanoma skin cancer (NMSCs) of the head and neck.

Changes related to HPV-associated oropharyngeal cancers

As mentioned above, distinct biological nature and recent clinical findings urged a change in OPC staging based on HPV status. The biomarker used by the manual to

determine HPV status is the p16 protein expression by IHC, that is surrogate marker of high-risk HPV-induced carcinogenesis with excellent prognostic capacity and nearly 100% sensitivity and 60-80% specificity (Kreimer et al. 2010). Furthermore, it is relatively cheap, widely accessible and is strait forward to interpret (Lydiatt et al. 2017). P16 positivity requires a diffuse, >75% cancer cell positivity with at least moderate intensity and positive nuclear staining (Lewis and Chernock 2014).

T categories remained merely the same except for 2 differences. Firstly, Tis (in situ carcinoma) does not exist in the p16-positive classification. Secondly, T4a and T4b melted into one category. N staging underwent significant modification and simplification. N1 encompasses any single or multiple ipsilateral lymph node metastases each smaller than 6 cm in greatest diameter. Contralateral or bilateral but smaller than 6 cm metastases on the neck were categorized as N2 without any further specification. N3 was kept for metastases greater than 6 cm.

In turn, N categories of non-HPV-associated OPC became somewhat more complex. One reason for that was the prognostic role of extranodal extension (ENE). The clinical N category for these cancers classifies all ENE-positive metastases as N3b, thus dissolving the previously homogenous N3 group. It is important to mention that ENE by imaging only is insufficient for ENE stratification. The unambiguous radiological finding has to be supported by physical examination signs as well, such as invasion of skin, infiltration of musculature, tethering to adjacent structures, or dysfunction of a nerve (Lydiatt et al. 2017).

Cancer of unknown primary tumor

Another change from prior editions of the TNM system is the elimination of the T0 category in sites other than the nasopharynx, HPV-associated OPC and salivary gland cancers. If no primary lesion can be identified, then the lymph node metastasis may have emerged from any mucosal site, meaning there is no point in retaining the T0 group except for the virally associated cancers of the oropharynx and nasopharynx (Lydiatt et al. 2017).

Changes in the T category of oral cavity cancers (OCCs)

The new T division of OCC introduced the term 'depth of invasion' (DOI) as a new prognostic factor. Recent data suggested that DOI is a better predictive parameter than tumor thickness (Shim et al. 2010). Although DOI was available for analysis in the sixth edition of TNM, the eight edition provided a precise definition. Clinically, DOI was divided into three categories: less invasive lesions (<5 mm), moderate depth lesions (from >5 to <10 mm) and deeply invasive cancers (>10 mm). Pathologically, DOI is measured from the level of the basement membrane of the closest adjacent normal mucosa. A "plumb line" is dropped from this plane to the deepest point of tumor invasion. The pathologic T category increases with every 5 mm (Lydiatt et al. 2017).

Staging of nonmelanoma skin cancer (NMSC)

As stated above, NMSC became part of head and neck chapter in the eight version of the manual. Most of the staging criteria remained the same except for the addition of DOI beyond 6 mm and perineural invasion (PNI) as parameters of the T category, both of which distinguish a lesion as T3, even if the tumor is of limited diameter (Lydiatt et al. 2017). DOI > 6 mm and PNI was associated with increased risk of recurrence and metastasis (Breuninger et al. 2013). Further on, a size criterion of 4 cm (instead of 5 cm as in the sixth edition) was reintroduced to distinguish between T2 and T3 similarly to other head and neck cancers (Lydiatt et al. 2017).

Changes in staging of nasopharyngeal cancers (NPCs)

There are 2 changes made in connection with NPCs. First, it gives a precise definition of the earlier somewhat ambiguous terms "masticator space" and "infratemporal fossa". Secondly, involvement of medial pterygoid, lateral pterygoid, and prevertebral muscles have been "down-staged" to T2 (Lydiatt et al. 2017). This was based on a recent analysis showing them to have a more favorable outcome using current treatment (Pan et al. 2016).

Concerning lymph node staging, modifications are to find as well. The unique term used for NPCs N category "supraclavicular fossa" was replaced by contemporary definitions

more suitable to axial cross-sectional imaging. In addition, the previously used low neck involvement (former N3a) and >6 cm size (former N3b) were unified into a single N3 group. Finally, both T4 and N3 would belong to stage IVA (formerly IVA and IVB) in stage categories (Pan et al. 2016, Lydiatt et al. 2017).

2.3.2 Presentation of HNSCC

The most common leading symptoms of head and neck cancers vary according to the anatomical site affected by the disease.

Cancer of the nasal cavity and paranasal sinuses

Any part of the nasal cavity and paranasal sinuses can be affected, but the lateral nasal wall, ethmoids and maxillary sinuses are the most common primary tumor sites. For unknown reasons, the frontal and sphenoid sinuses are rare primary locations (Lund et al. 2016).

The most common initial symptoms such as nasal blockage, blood-stained discharge and loss of smell are often overlooked though their often unilateral nature should raise suspicion. Delayed presentation is common. Subsequent extension to the surrounding structures can produce symptoms such as proptosis, diplopia and epiphora, trismus, facial pain, oro-antral fistula, paraesthesia or other neurological deficits and facial swelling or mass (Lund et al. 2016).

Nasopharyngeal carcinoma

It is more common in men than in women (gender ratio for men:women is 3:1), with a median age of 50 years at the time of presentation. The most common symptoms of nasopharyngeal carcinoma are nasal obstruction, epistaxis, conductive hearing loss due to otitis media with effusion, cranial nerve neuropathies caused by skull base invasion (commonly involved cranial nerves are III, IV, V and VI) (Simo et al. 2016).

Lip and oral cavity cancer

About 90 percent of lip cancers arise in the lower lip with 7 per cent occurring in the upper lip and 3 percent at the oral commissure (Kerawala et al. 2016). The clinical presentation of cancer of the lip is usually an exophytic, crusted lesion with or without invasion into underlying muscle. The adjacent lip often shows features of actinic sun damage such as color change, mucosal thinning and various associated areas of leukoplakia (Wolff et al. 2012).

In the oral cavity, the majority of squamous cell carcinomas are presented as ulcers or masses (Kerawala et al. 2016). Early lesions can appear as flat, discolored areas known as leukoplakia or erythroplakia (Rethman et al. 2010). Advanced tumors can present with additional symptoms because of invasion of neighboring structures causing tooth mobility, trismus, sensory changes and referred otalgia (Kerawala et al. 2016).

Oropharyngeal cancer

Although HNSCC of each anatomical region may firstly present with neck lumps, it is probably the most often seen in oropharyngeal cancer cases. These patients often present with painless, relatively large neck lumps. Other complains such as sore throat or tongue pain, referred ear pain, painful and/or difficult swallowing or a change in voice quality (often mentioned as hot potato voice) are the most common symptoms at presentation (Mehanna, Evans et al. 2016).

Laryngeal cancer

Presentation of laryngeal cancer is highly variable and depends on the site and size of the primary tumor. Tumors of the glottis typically present at an early stage as they cause hoarseness. In comparison, tumors of the supraglottis are likely to present later with symptoms of pain, hoarseness and swallowing difficulty. However, it is not uncommon for patients with laryngeal cancer to delay seeking medical advice and therefore presenting at a much later stage with symptoms of pain, swallowing difficulty, a palpable neck mass or even with airway obstruction and dyspnea (Jones et al. 2016).

Hypopharyngeal cancer

Late presentation is common. Commonly seen symptoms include sore throat, referred ear pain on swallowing and dysphagia that is often progressive, resulting in significant weight loss and malnutrition. Neck mass, hoarseness, voice change and/or upper airway obstruction are late symptoms indicating an advanced disease (Pracy et al. 2016).

Metastatic lymph node of the neck

As mentioned, metastatic lymph node in form of a neck lump may occur as a presenting symptom in HNSCCs independent of the site of primary tumor. For assessment and documentation purposes, the neck is divided into six anatomical regions. Level VII (superior mediastinum) is relevant for some head and neck cancers (Paleri et al. 2016). Clinical palpation alone is regarded as inaccurate (sensitivity and specificity 70–80 per cent) due to factors e.g. inter-observer variability, shape of neck, absence or presence of significant subcutaneous fat and varying size of involved cervical nodes (Paleri et al. 2016).

2.4 Management of head and neck cancer

The management of head and neck cancer inevitably involves professionals from various fields of medicine and thus it requires a close teamwork to provide the best possible care. The treatment of HNSCC patients is a rapidly evolving and changing area of oncology. A short summary of therapeutic modalities will be described on the following pages. Generally, early stage disease (stage I and II) can be treated by either surgery or radiation whereas patients with locally advanced disease (stage III, IVA and IVB) are candidates of multimodal treatment regimens. Those harboring a distant metastasis (M1 or stage IVC) need to be treated with a systemic approach such as chemotherapy and/or biological therapy.

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2.4.1 Surgery of head and neck cancer

A well-known Hungarian head and neck surgeon allegedly said once: “The last real chance of a head and neck cancer patient for cure is the first surgery”. Clearly, this work is not entitled to discuss head and neck surgery in detail. Nevertheless, a few key points have to be mentioned.

The main goal of surgery in head and neck oncology is to provide a complete and microscopic removal of tumorous tissue. Debulking surgery has little to no role in head and neck cancer except for airway preservation and symptom palliation. The quality of resection margin is a critical question and remains a profound prognostic factor (Hinni et al. 2013) and also influences the postoperative management.

The development of transoral, minimally invasive surgical approaches such as transoral robotic surgery (TORS) and transoral laser microsurgery (TLM) are one of the most prominent surgical advances of recent times (Homer and Fardy 2016). However, in case of transoral techniques, comparison is to be made to primary radiotherapy (RT) or chemoradiotherapy (Homer and Fardy 2016). In glottic cancer, it has only been shown that there is equal outcome using TLM or RT for T1a tumors in terms of local control (O'Hara et al. 2013). Evidence for T1b glottic cancers is less convincing (O'Hara et al. 2016) and there is clearly insufficient data for T2 glottic cancers and for supraglottic cancers (Homer and Fardy 2016).

TORS provides improved access to the upper aerodigestive tract such as the supraglottic larynx and the hypopharynx, with superior visibility and maneuverability to that of TLM and allows a multi-planar en bloc resection in the hypopharynx (Lorincz et al. 2015). Using TORS, adjuvant chemotherapy could be spared and adjuvant radiotherapy could be reduced in selected HNSCC patients without jeopardizing oncological outcome (Lorincz et al. 2015, Dabas et al. 2017). Given that TORS is a novel technique, larger studies and longer survival data are needed to establish its safety and role in the armamentarium of head and neck surgery.

For patients with T1/2 tumors (stage I and II), surgery and radiotherapy (RT) is most often efficient and applicable as a single modality. A single exception is the

nasopharyngeal carcinoma (NPC), where the role of surgery is mainly limited to diagnostic acquisition of tissue sample, and RT is the mainstay treatment of early stage disease.

For patients with advanced stage diseases (stage III or higher) multimodal approaches are implemented. Homer and Fardy summarize issues to consider when performing a radical resection for advanced disease as follows: “i.) Can a complete resection be achieved? If this is not realistic, then the morbidity of such surgery can rarely be justified. ii.) Even if complete resection can be achieved, is the mortality risk and morbidity justified by the chances of overall survival? iii.) If radical surgery is to be done, it should be done comprehensively. There should be no compromise in the extent of the resection, when the attendant morbidity is not materially affected by a more radical approach with appropriate reconstruction in expert hands. This may mean pharyngolaryngectomy instead of laryngectomy, mandibulectomy instead of soft tissue resection only in the oral cavity or extending a maxillectomy posteriorly or superiorly.” (Homer and Fardy 2016)

2.4.2 Radiation therapy

Radiotherapy is a key modality in head and neck ongology. The anti-tumor effect of it is reached by multiple mechanisms such as double fracture of DNA chains, production of free radicals, hypoxia, immunization, etc. However, radiation by its nature cannot differentiate between healthy and tumorous tissue hence it is associated with serious short term (e.g. mucositis, skin burnt, soft tissue damage/loss, etc.) and long term (e.g. decreased saliva production, lost or decreased smelling/gustation, strictures, swallowing impairment, stiffness of the neck, etc.) side effects. These conditions greatly affect patients’ quality of life. To minimize these side effects new techniques have been developed.

3D conformational radiotherapy (3DCRT)

3D conformational radiotherapy (3DCRT) is a sophisticated procedure that starts with the obtained, personalized CT scans. These images are utilized for treatment planning to deliver highly precise conformed dose distribution to the target region and to spare healthy tissues, thus this technique is used to treat patients with the complex tumor shapes (Hodapp 2012, Salimi et al. 2017).

Intensity modulated radiotherapy (IMRT)

Intensity modulated radiotherapy (IMRT) have been developed recently. IMRT techniques employ variable intensity across multiple radiation beams leading to the construction of highly conformal dose distributions (Teoh et al. 2011). Using IMRT, a better preservation of healthy tissues around the tumor can be achieved resulting in less therapy related acute and late toxicities (Staffurth 2010). Besides its advantages, IMRT has disadvantages as well. The planning and quality assurance processes needed for IMRT are more complex and time-consuming compared to conventional conformal RT (Miles et al. 2005, Teoh et al. 2011). In HNSCCs, randomised evidence showed that IMRT can reduce late toxicity parameters such as xerostomia by increasing sparing of the parotid glands compared to conventional techniques (Nutting, Morden et al. 2011).

Volumetric modulated arc therapy (VMAT)

Volumetric modulated arc therapy (VMAT) is another developing, novel radiation technique. It was introduced in 2007 as a method that allowed the simultaneous variation of three parameters during treatment e.g. gantry rotation speed, treatment aperture shape via movement of multileaf collimator leaves and dose rate (Otto 2008). One of the advantages is that VMAT has considerably shorter delivery time. Cilla et al. recently observed the swallowing organ sparing potential of VMAT and concluded that VMAT planning directed to spare swallowing structures is a feasible option, providing a significant reduction in normal tissue complication probability and swallowing dysfunction (Cilla et al. 2016).

Other forms of radiotherapy

Patient with tumors close to radiation sensitive essential structures (e.g. brain, spinal chord) and pediatric HNSCC patients may benefit from particle therapy such as protons and stereotactic radiotherapy (Nutting 2016).

Brachytherapy for radiotherapy-resistant HNSCCs can be a feasible option and enables good local control but keeping in mind that many advanced head and neck cancers develop regional or distant metastases, additional treatment should be considered (Hazkani et al. 2016).

The course of radiotherapy

Radiotherapy is delivered in fractions. With standard fractioning a total dose of 66-72 Gy is given. According to the standard regimen radiation is received on weekdays, 2 Gy per day for 7 weeks. In case of accelerated fractionation the therapy is given on each day including weekend in a non-stop manner. The rationale behind that is the inhibition of clonal selection and repopulation of cancer stem cells (Amdur et al. 1989).

However, a recent study observed accelerated fractionation plus concurrent cisplatin treatment versus standard fractionation concomitant chemoradiotherapy and found neither improved outcome nor increased late toxicity in patients with locally advanced head and neck cancer (Nguyen-Tan, et al. 2014).

Another novel delivery technique is the hyperfractionated delivery. This means the delivery of 1.1-1.2 Gy fractions twice a day. By doing so, the total dose can be boosted up to 74-82 Gy without increasing the risk of toxicities.

A phase III randomised study (RTOG 9003) reported that patients treated with hyperfractionation and accelerated fractionation with concomitant boost had significantly better local-regional control than those treated with standard fractionation. There was also a trend toward improved disease-free survival, although the difference in overall survival was not significant. The altered fractionation groups had significantly greater acute side effects compared to standard fractionation. However, there was no significant increase of late effects (Fu et al. 2000).

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2.4.3 Chemoradiotherapy and chemotherapy

In this chapter I intended to give a short list of chemotherapeutics used in head and neck oncology and than summarize the current state of combination therapies. A mention of palliative treatment regimens will take place as well.

Platinating agents

Cisplatin and carboplatin are alkylating-like drugs that preferentially bind to guanine nucleotids causing DNA crosslinks. This further interferes with mitosis, DNA repair, thus induces apoptosis. The main side effects are nephrotoxicity, ototoxicity and myelotoxicity. Carboplatin cause less harm to the kidney and therefore is a feasible option for patients with impaired kidney function.

Taxanes

Docetaxel and paclitaxel are taxanes that belong to alkaloid drugs. Taxanes target tubular proteins that leads to disruption of mitotic spindle assembly at the M-phase of mitosis. These agents are hydrophobic, thus are prone to unleash allergic reactions. Therefore a premedication (e.g. steroid iv. and antihistamine agent iv.) is given before administration.

5-fluorouracil (5-FU)

5-FU is an antimetabolite and a pyrimidine analog. When 5-FU is built into the DNA chain during DNA replication at the S-phase of mitosis it blocks the thymidylate synthase enzyme leading to DNA and RNA damage. The active form of the drug is a metabolite called ftorafur. The main side effects are: myelotoxicity, neurotoxicity and mucositis.

Methotrexate (MTX)

Methotrexate is an antifolate antimetabolite that impairs de novo biosynthesis of the nucleoside thymidine as well as purine and pyrimidine base biosynthesis, thus it

interferes with DNA synthesis. The main side effects are hepatotoxicity, myelosuppression and stomatitis. In case of intolerable toxicity leucovorin rescue can be administered within 24-36 hours of starting MTX therapy (Ackland and Schilsky 1987).

The role of chemoradiotherapy (CRT)

In 1987, a landmark phase II trial investigated the concomitant use of radiation and cisplatin in locally advanced, unresectable head and neck cancer (Al-Sarraf et al. 1987). Complete remission was achieved by 69% of patients and a comparison to radiotherapy alone arm suggested improved survival for those receiving the combined treatment. Since than, the superiority of concomitant CRT over radiotherapy alone in head and neck cancer found proof in numerous clinical trials (Calais et al. 1999, Jeremic et al. 2000, Adelstein et al. 2003). In 2009, a large meta-analysis of prospective clinical trials concluded that those receiving combined radiation and chemotherapy have better local tumor control and improved overall survival compared to those treated with radiation alone (Pignon et al. 2009). Thus, CRT became the standard of care for locally advanced, non-resectable HNSCC.

Nevertheless, the method of delivering CRT continues to be a matter of debate.

The same question arises in case of an adjuvant setting. Adjuvant chemotherapy is indicated in patients at high risk of recurrence after surgical resection, generally defined as having narrow or involved margins at the primary site, multiple nodal metastases, or extracapsular spread (Bernier et al. 2004, Cooper, Pajak et al. 2004).

Is there a benefit in administering a combined regimen postoperatively? Two phase III randomized trials observed this issue: Radiation Therapy Oncology Group (RTOG) 9501 (Cooper, Pajak et al. 2004) and European Organization for Research and Treatment of Cancer (EORTC) 22931 (Bernier et al. 2004). In RTOG 9501, improved locoregional control was observed compared with radiotherapy alone (hazard ratio (HR) for local or regional recurrence, 0.61; $P = .01$), but no survival benefit was observed (Cooper, Pajak et al. 2004). In EORTC 22931, the progression-free survival (HR, 0.75; $P = .04$) and the overall survival (HR, 0.70; $P = .02$) rates were better in the combined-

therapy group (Bernier et al. 2004). In both studies severe adverse events were more frequent in the combination arm.

The role of induction chemotherapy

Induction chemotherapy is the chemotherapy given prior to definitive local treatment (radiation, chemoradiation or surgery).

In 2007, two large-scale, international trials (TAX 323 and TAX 324) showed improved overall survival and locoregional control with taxane-platina-fluorouracil (TPF) triple combination compared to previously used platina-fluorouracil (PF) treatment. Both induction regimens were followed by chemoradiotherapy and in both trials the incidence of neutropenia and febrile neutropenia was higher in the TPF arm (Posner et al. 2007, Vermorken et al. 2007).

As mentioned before, superiority of CRT over induction chemotherapy plus radiation in terms of local control and survival was showed in 2009, although it was also observed that induction chemotherapy followed by radiation alone was associated with decreased rate of distant metastasis (Pignon et al. 2009). This lead to further studies investigating the potential benefit of induction chemotherapy plus CRT versus CRT alone. Both PARADIGM (Haddad et al. 2013) and DeCIDE (Cohen et al. 2014) phase III trials failed to prove a significant difference, leaving the question unresolved. Both studies failed to recruit the originally planned number of patients, hence they were underpowered.

Palliative chemotherapy

Selected patients with recurrent or metastatic (R/M) HNSCC may receive surgery in case of resectable disease or radiation therapy when the last radiation occurred more than 3 years ago. However in many cases these options are not feasible and palliative chemotherapy is the best therapeutic choice. Platinum-based chemotherapy consisting of either cisplatin or carboplatin is usually considered the first-line treatment for unresectable R/M HNSCC (Schantz et al. 2001). Cisplatin is often combined with fluorouracil. Platinum based chemotherapy showed improved response rate but did not

improve overall survival when compared with single agent methotrexate therapy (Forastiere et al. 1992). The first regimen that could improve overall survival was the combination of cetuximab, an anti-EGFR antibody with platinum based combination chemotherapy, as Vermorken et al. reported in 2008 (Vermorken, Mesia et al. 2008). Because of that, the first-line chemotherapy in R/M HNSCC is cisplatin or carboplatin plus 5-fluorouracil with or without cetuximab. The second line therapy is weekly given methotrexate.

For selected R/M HNSCC patients enrollment to clinical trials is another chance for improving survival and thus is highly recommended.

2.4.4 Biological therapies in HNSCC (other than immunotherapy)

2.4.4.1 GFR-RAS-RAF-MEK-ERK inhibitors

The GFR-RAS-RAF-MEK-ERK pathway plays an important role in the tumorigenesis and progression of HNSCC. Therefore various drugs have been developed to interrupt this signaling at different levels of signal transduction.

Cetuximab

Cetuximab is the only approved targeted therapy in head and neck cancer in the European Union. Cetuximab binds to EGFR inhibiting signal transduction from the cell membrane level. Unfortunately, reliable predictive biomarkers of cetuximab therapy are missing and further improvement of patient selection is needed. EGFR protein expression was not found to be a predictive of cetuximab therapy and there are several mechanisms assumed to be responsible for cetuximab resistance in HNSCC patients (Cooper and Cohen 2009).

In locoregionally advanced HNSCC radiotherapy plus concomitant cetuximab may be delivered as first line treatment since it improves locoregional control and reduces mortality without increasing toxic effects when compared to high-dose radiation alone (Bonner et al. 2006). As noted above, the combination of cetuximab with platinum

based combination chemotherapy has proven clinical benefit in the R/M setting (Vermorken, Mesia et al. 2008).

EGRF tyrosine kinase inhibitors (TKIs)

EGFR may be blocked at its tyrosine kinase enzyme activity as well. However, erlotinib (Soulieres, Senzer et al. 2004), gefitinib (Rodriguez et al. 2012) and lapatinib (de Souza et al. 2012) failed to show clinical benefit in head and neck cancer. Clinical trials investigating the use of afatinib in HNSCC are currently running (NCT01427478, NCT02979977, NCT01783587 and NCT01856478).

RAS inhibitors

A phase II study is running to investigate the effect of tipifartinib in HRAS mutant HNSCC (NCT02383927).

RAF inhibitors

Vemurafenib and dabrafenib are B-RAF inhibitors that have already showed clinical significance in late-stage melanoma. Their effectivity is investigated in numerous trials involving thyroid cancer but not HNSCC (e.g. NCT01709292, NCT03176485, etc. and NCT01723202, NCT01947023, respectively).

MEK inhibition

Trials investigating the MEK inhibitor trametinib involve oral cavity and pharyngeal cancer patients besides other primary tumor sites (NCT03065387 and NCT01553851).

2.4.4.2 PI3K-AKT-mTOR inhibitors

PI3K blocking is a promising therapeutic target. Buparlisib is a pan-PI3K inhibitor. In a multicentre, randomised, double-blind, placebo-controlled phase II trial (Beril-1) it has been suggested that buparlisib could be an effective second-line treatment for patients with platinum-pretreated recurrent or metastatic squamous cell carcinoma of the head and neck in combination with paclitaxel (Soulieres, Faivre et al. 2017).

Inhibitors of mTOR are not considered that effective yet. Temsirolimus failed to show clinical benefit (TEMHEAD study) in HNSCC (Grunwald et al. 2015).
Everolimus, an other mTOR inhibitor is still investigated in several clinical trials involving head and neck cancer patients (e.g. NCT00858663, NCT00935961, etc.).

2.4.4.3 Angiogenesis inhibitors

Bevacizumab, a vascular endothelial growth factor (VEGF) inhibitor is currently approved for the treatment of colorectal, breast, lung, renal, ovarian and cervical cancer in Hungary. Its clinical efficacy in HNSCC is a matter of question (NTC00588770).
Direct blockage of VEGF receptor by vandetanib did not fulfill expectations yet (Papadimitrakopoulou et al. 2016). However there are multiple clinical trials that investigate the feasibility of vandetanib in HNSCC (e.g. NCT00450138) or the preventive potential of it in patients with premalignant lesions of HNSCC (NCT01414426).

2.4.5 Immunotherapy

Immuno-oncology has brought a paradigm shift and an entirely new concept in the treatment of numerous malignancies including HNSCC. The immune system normally recognizes and eliminates cancer cells. However, immune evasion plays a key role in the development and evolution of malignancies including HNSCC (Economopoulou et al. 2016). Immune checkpoints were shown to play an important role in the tumor microenvironment serving as a mechanism of tumor immune evasion (Ramsay 2013). Immune checkpoint inhibitors, such as anti-PD-1, anti-PD-L1 and anti-CTLA-4 antibodies demonstrated clinical benefit and two PD-1 inhibitors were recently approved by the Food and Drug Administration (FDA) in the United States to treat patients with recurrent/metastatic HNSCC. There are several other methods under clinical or preclinical testing that aim to enhance anti-tumor immunity.

2.4.5.1 Checkpoint inhibitors

CTLA-4/B7 checkpoint

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is a member of the B7 receptor family expressed on CD4+ , CD8+ , and regulatory T cells (Strauss et al. 2007). CTLA-4 has two ligands, B7-1 and B7-2. CTLA-4 competes with CD28 to bind with B7-1 and B7-2, although CTLA-4 has much stronger binding affinity for the two ligands than CD28 (Grosso and Jure-Kunkel 2013). While CD28 is a costimulatory receptor, CTLA-4 signaling inhibits T cell activation via cell-cycle arrest and decreased cytokine production (Yu et al. 2015).

In humans, two isoforms of CTLA-4 is known to date. The full-length isoform contains an extracellular ligand-binding domain and an intracellular signal transducing domain; whereas the soluble isoform consists of the extracellular domain only (Perez-Garcia et al. 2013). Naive effector T cells and regulatory T cells express CTLA-4 at a low level on their surface, but after stimulation by T-cell receptor they upregulate membrane CTLA-4 and secrete soluble CTLA-4 as negative feedback to maintain immune self-tolerance (Greenwald et al. 2013). Therefore CTLA-4 is an early phase regulator of T-cell activation.

CTLA-4 inhibitor ipilimumab was the first checkpoint inhibitor approved by the FDA for the treatment of metastatic melanoma in 2010 (Hodi et al. 2010). In HNSCC there are currently no approved CTLA-4 inhibitors available. However numerous trials are investigating the feasibility of ipilimumab (e.g. NCT02812524, NCT01860430, NCT03003637, etc.) and tremelimumab, an other CTLA-4 inhibitor (e.g. NCT03019003, NCT02369874, NCT02319044, etc.) in R/M HNSCC.

PD-1/PD-L1 checkpoint

The PD-1/PD-L1 interaction is probably the best characterized immune checkpoint. PD-1 is expressed on T cells, dendritic cells (DCs), natural killer cells, macrophages and B cells (Chen 2004). PD-L1 can be expressed on T cells, antigen presenting cells such as B cells and myeloid DCs. At very low levels is expressed by tissue macrophages in the lung, kidney, liver, heart and placenta as well (Keir et al. 2008).

After binding to its ligand, PD-1 can recruit SHP-2 (Src homology 2 domain-containing tyrosine phosphatase 2) to the immunoreceptor tyrosine-based inhibitory motif domain of the intracellular part of PD-1, resulting in inhibition of downstream T cell receptor and CD28 signaling, mainly through PI3K/AKT pathway activation (Yokosuka et al. 2012).

Unlike CTLA-4, PD-1/PD-L1 inhibits the effector stage of T-cell activation (Pardoll 2012). However similarly to CTLA-4, activated T cells increase PD-1 expression on their surface (Pardoll 2012). Effects of PD-1/PD-L1 interaction include inhibition of T cell proliferation, survival and effector functions of T cells, induction of apoptosis of tumor-specific T cell and promotion of differentiation of CD4+ T cells into Treg cells. Furthermore, excessive induction of PD-1 on T cells can result in an exhausted state of T cells (Pardoll 2012, Santarpia et al. 2015).

Checkpoints are intended to regulate immune activation, thus protect the organism from excessive immune response to pathogens and maintain self-tolerance as well as immune homeostasis. However, this mechanism can be exported and is in fact used by cancer cells to hide from the immune system, a phenomenon that is commonly called immune-evasion. The rational of checkpoint inhibitors is that this hiding technique of tumor cells might be turned off unleashing the break on anti-cancer immune activity that may result in more effective fight against cancer cells.

Nivolumab, an anti-PD-1 monoclonal antibody (mAb) was the first checkpoint inhibitor that received approval in Europe for the treatment of late stage melanoma in 2015. The Checkmate 141 trial platinum refractory R/M HNSCC patient were given either nivolumab or the investigator's choice of treatment (either cetuximab, methotrexate or docetaxel monotherapy) (Szturz and Vermorken 2017). The study was terminated earlier than planned and the FDA gave breakthrough therapy title for nivolumab in R/M HNSCC. The decision was based on the fact that the 1 year overall survival of patients in the nivolumab arm was 36% compared to 16.6% in the other.

The Keynote-012 investigated the anti-PD-1 pemrolizumab in R/M HNSCC. 174 patients who progressed amid or during platinum based chemotherapy were recruited. Those receiving pembrolizumab displayed 18.2% overall response rate (partial or

complete remission) and 31.3% had a stable disease for at least 6 months. There were no difference based on HPV status (Seiwert, Gupta et al. 2015). Based on these results, the FDA accelerated the approval process and approved pembrolizumab for the therapy of R/M HNSCC in 2016.

There are over 50 different, mainly munticentre trials testing multiple checkpoint inhibitors in various settings of HNSCC.

2.4.5.2 Other immunotherapeutic approaches

HPV-driven tumors provide excellent target for the immune system by their nature. This is exploited by therapeutic vaccines. Numerous phase i/II clinical trials investigate the potential therapeutic use of anti-HPV DNA, peptide or bacteria vaccines. A phase II study researching E6 and E7 peptide vaccines in HPV associated tumors including oropharyngeal cancers is about to supply results (NCT00019110). Besides vaccines, there are phase I/II trials on the field of adoptive T cell transfer and T cell receptor transfer as well (Economopoulou et al. 2016).

There is hope that immunotherapy brings paradigm shift and revolution in medical oncology. For mankind, this would mean a rise of a new era in the long history of the battle against cancer.

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3 OBJECTIVES

At the time I joined our research team, there was no established prognostic or predictive marker for head and neck squamous cell carcinomas. Thus, our attention was focused on researching various biomarkers using retrospective analysis of clinical data and tissue samples provided by our institution. We published our results on the potential prognostic value of connexin 43 expression in HNSCCs in 2015 (Danos et al. 2015). Another field of interest was the prognostic role of the copy number gain of PIK3CA and MET (Brauswetter, Danos et al. 2016). Meanwhile, we turned our attention towards HPV associated oropharyngeal cancer. The question wether $p16^{INK4}$ immunohistochemistry is a reliable biomarker alone or HPV PCR detection is needed as well was unresolved as we started our investigation of this issue. In 2016 we published, that $p16^{INK4}$ status alone was an equally precise indicator of prognosis as $p16^{INK4}$/HPV DNA PCR double testing (Brauswetter, Birtalan et al. 2017). We confirmed that HPV-associated oropharyngeal cancer patients have significantly better disease-specific survival compared with non-HPV-associated cancers and gave a comprehensive analysis of the rate of HPV-associated oropharyngeal malignancies in Hungary (Brauswetter, Birtalan et al. 2017).

Our first question was weather HPV status is a predictive factor as well. In order to answer this question we compared the response rate of $p16^{INK4}$/HPV-positive versus $p16^{INK4}$/HPV-negative oropharyngeal cancer patients that were treated with induction chemotherapy.

Our second objective was to investigate the expression of checkpoint inhibitor proteins in HNSCC. In addition to that, we also wanted to find out weather checkpoint inhibitor protein expression is related to subsets of head and neck cancer, such as anatomical localization or subgroups based on $p16^{INK4}$/HPV status. Thus, we assessed expression of PD-1, PD-L1, PD-L2 and CTLA-4, just as markers of immune activation: CD8-expression and the rate of tumor infiltrating mononuclear cell infiltration.

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4 METHODS

4.1 Patients

We enrolled 124 therapy naive, consecutively diagnosed individuals with squamous cell carcinoma of the head and neck. We excluded tumors of nasopharyngeal or paranasal sinus localization. Out of this, 110 patients had available tumor blocks for immunohistochemical staining. For the research of immune checkpoint inhibitors we excluded oral cavity cancer patients (N=3) to increase homogeneity and one other patient whose archival tumor block was consumed by previous research, thus did not met the inclusion criteria any more. Doing so we left 106 individuals in the analysis. Each patient underwent treatment between 2012 and 2014 at the Department of Oto-Rhino-Laryngology, Head and Neck Surgery, Semmelweis University (Budapest, Hungary). Main characteristics of our cohort can be seen in ***Table 1***.

All procedures performed were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This research was approved by the Regional, Institutional Scientific and Research Ethics Committee of Semmelweis University (SE TUKEB 105/2014).

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Table 1. Patient characteristics (N=106)

Gender	**N (%)**
female	16 (15.1)
male	90 (84.9)
Age	**mean**
female	62.8 (50-79)
male	60.2 (41-91)
Localization and HPV status	**N (%)**
larynx	**42 (39.6)**
supraglottic	9 (8.49)
glottic	27 (25.5)
transglottic	6 (5.66)
oropharynx	**41 (38.7)**
HPV positive	9 (8.49)
HPV negative	32 (30.2)
hypopharynx	**23 (21.7)**
TNM stage	**N (%)**
I	15 (14.2)
II	15 (14.2)
III	18 (17)
IV A	41 (38.7)
IV B	10 (9.4)
IV C	7 (6.6)
Primary treatment	**N (%)**
surgery	44 (41.5)
radiotherapy alone	21 (19.8
chemoradiotherapy	20 (18.9)
other	6 (5.6)
best supportive care	15 (14.2)

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4.2 Study design

Predictive value of p16^{INK4} and HPV DNA PCR status

First, p16^{INK4} immunohistochemical staining was performed on each tumor sample (in details, see below). Those tested positive for p16^{INK4} underwent subsequent real-time high-risk HPV DNA PCR analysis. P16^{INK4} and HPV DNA PCR double positive samples were regarded as HPV positive. We selected patients who had an oropharyngeal tumor and received induction chemotherapy. Therapeutic response was assessed based on Response Evaluation Criteria in Solid Tumors (RECIST) 1.1. We sought association between p16^{INK4}/HPV status and therapeutic response (complete remission/partial remission/stable disease or progressive disease).

Expression of immune checkpoint inhibitors in subsets of HNSCC

We retrieved clinical parameters (localization, stage, grade, gender, smoking habits, alcohol consumption, response to induction chemotherapy, response to chemoradio-therapy) from our clinical database and utilized information on p16^{INK4}/HPV status gained from the previous analysis.

The expression of PD-1 on immune cells and the expression of PD-L1 and CTLA-4 on both tumor and immune cells were observed. We evaluated PD-L2 expression on tumor cells only. The rate of CD8+ mononuclear cells and the proportion of tumor infiltrating lymphocytes (TILs) was assessed as well.

We primarily aimed to investigate the prognostic impact of PD-1, PD-L1, PD-L2 and CTLA-4 expression as well as TIL density in HNSCC. In particular we focused on differences between subsets of this disease. Subsets were defined either as subgroups according to anatomical localization or subgroups based on p16^{INK4}/HPV status. Thus, our objective was to correlate these findings with clinicopathogical data as well as to analyze the link between these biomarkers and subsets of HNSCC. Through this we investigated whether HNSCC is an immunologically heterogenous disease or not.

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4.3 Tissue microarray construction

Formalin-fixed, paraffin-embedded (FFPE) tissue block were retrieved from archives of the 2nd Department of Pathology, Semmelweis University. Consequently, all blocks were created using uniform methods based on the local institutional protocol. TMA blocks containing 2 mm diameter cores were created using the TMA Master instrument (3DHISTECH Kft, Budapest, Hungary). TMA blocks contained 50 or 70 cores each.
To avoid misrepresentation of samples, 2-3 cores were acquired per tumor. Tissue sections (4 um) were cut on adhesion slides and used for immunohistochemical analysis. Similar sections were cut for DNA extraction and real-time PCR testing.

4.4 Immunohistochemistry and evaluation of slides

Immunohistochemistry was performed at the 2nd Department of Pathology, Semmelweis University. After immunostaining, slides were digitalized using a Pannoramic Scan instrument (3DHISTECH, Hungary). Three independent observers blinded to clinical data performed scoring of immunoreactions employing the Pannoramic Viewer software (3DHISTECH, Hungary). In case of inter-observer differences reevaluation took place by all 3 participants and a consensus was reached.
The type and dilution of antibodies used for immunohistochemical staining can be seen in **Table 2**.

4.4.1 $p16^{INK4}$ staining (Brauswetter, Birtalan et al. 2017)

BenchMark XT IHC/ISH (Roche, Germany) semi-automated device was used for $p16^{INK4}$ staining with the application of XT UltraView DAB v3 kit. The protocol of staining method was carried out as described previously (Vankos et al. 2015). Briefly, sections were incubated at 72 °C for 4 min. We used EZ Prep Solution (Ventana Medical Systems, Tucson, AZ, USA) three times to remove paraffin. Cell conditioning solution pH 8 (Ventana Medical Systems) was used for heat induced epitope retrieval at

Table 2. Monoclonal Mouse Antibodies Employed for Immunolabeling (PD-L1: programmed death-ligand 1, PD-L2: programmed death-ligand 2, PD-1: programmed cell death protein 1, CTLA-4: cytotoxic T-lymhocyte-associated protein 4, CD8: cluster of differentiation protein 8)

Antibody	**PD-L1**	**PD-L2**	**PD-1**
Clone	28-8	176611	UMAB199
Manufacturer	Abcam	R&D Systems	Origene
Positive control	lymph node	lymph node	tonsil
Dilution	1:200	1:100	1:100
Antigen retrieval	Ventana ULTRA CC1	Ventana Protease 1	Ventana ULTRA CC1
Antibody	**CTLA-4**	**CD8**	**p16^{INK4}**
Clone	F-8	C8/144B	CINtec E6H4
Manufacturer	Santa Cruz	Cell Marque	Ventana
Positive control	lymph node	tonsil	tonsil
Dilution	1:100	1:100	1:100
Antigen retrieval	Ventana ULTRA CC1	Ventana ULTRA CC1	Ventana ULTRA CC1

95 °C for 30 min followed by a heating at 100 °C for 4 min. Endogenous peroxidase activity was inhibited with one drop UV INHIBITOR (Ventana), which was applied at 37 °C for 6 min. Primary monoclonal antibody against p16^{INK4} (Clone CINtec E6H4, Ventana) was applied at 37 °C for 32 min in a dilution of 1:100. After incubation with UV HRP UNIV MULT secondary antibody solution (Ventana) at 37 °C for 8 min, peroxidase activity was visualized with diaminobenzidine (DAB) chromogen (Ventana). Nuclear counter-staining was done with hematoxylin II (Ventana). All washing steps were performed with diluted Reaction Buffer Concentrate (Ventana). In each core, nests of at least 200 tumor cells were analyzed by two independent assessors. Cut-off for p16^{INK4}-immunolabelling was set at 75% of cytoplasmic or nuclear staining (Lewis and Chernock 2014). Intensity of staining played no role in the evaluation. However, almost all p16^{INK4}-positive samples showed a remarkably strong intensity.

4.4.2 PD-L1 staining

Immunohistochemical staining of PD-L1, PD-1, PD-L2, CTLA-4 and CD-8 was performed similarly. Briefly, all slides were deparaffinized and immunostained with primary antibodies at 42°C for 32 minutes following antigen retrieval. PD-L1 labeling alone required 10 minutes longer antigen retrieval and for PD-L1 the primary antibody incubation time was 2 hours. Detection was performed using a secondary antibody for 60 min at room temperature following the protocols of ultraView™ Universal DAB Detection Kit (Ventana Benchmark Ultra, Ventana Medical Systems Inc., Tucson, Arizona, USA). Positive controls were included in each run showing appropriate results. Slides were counterstained with hematoxylin, washed in water, dehydrated, and cover-slipped before analysis. After initial manual calibration of the optimal dose of the primary antibody (Table 2), an automatic immunostainer (Ventana Benchmark Ultra, Ventana Medical Systems Inc., Tucson, Arizona, USA) was used for the serial immunolabeling.

PD-L1 score categories on tumor cells (PD-L1^{TC}) were formed based on the most relevant cut-off values of PD-L1 staining (Wu et al. 2015, Zhang et al. 2015). PD-L1^{TC} categories were: 0: no staining, 1: 0-1%, 2: 1-5%, 3: 6-10%, 4: 11-25%, 5: 26-100%. Scores greater than 1 were considered positive.

PD-L1 staining on immune cells (PD-L1^{IC}) was evaluated as follows: 0: no staining, 1: <1%, 2: 1-5%, 3: >5%. Staining scores higher than 0 were regarded positive.

4.4.3 PD-L2 staining

PD-L2, PD-1, CTLA-4 and CD-8 staining was performed as described above.

PD-L2 immunoscore was allotted to specimens as described elsewhere (Leng et al. 2016). The stained tumor cells percentage scores (0: no staining, 1: <10%, 2: 10-50%, 3: >50%) and intensity scores (0: no staining, 1: week, 2: moderate, 3: strong) were summed. The summed score was regarded negative when 0-4 and positive when it was 5 or 6.

4.4.4 PD-1 staining

PD-1 staining on immune cells was assessed: 0: <1%, 1: 1-5%, 2: >5%. Scores 1 and 2 considered to be positive.

4.4.5 CTLA-4 staining

CTLA-4 immunolabeling was investigated on both tumor cells (CTLA-4^{TC}) and immune cells (CTLA-4^{IC}). Given the scarcity of studies describing CTLA-4 expression on tumor cells, we decided to evaluate the samples per 10%; 0: no staining, 1: 1-10%, 2: 11-20%, etc. Positivity was defined when the score exceeded the median value as previously described (Salvi et al. 2012). The median score of CTLA-4^{TC} was 0 and >1% staining was regarded as positive. The same method was implemented in the assessment of CTLA-4^{IC} staining with >40% immune cell staining regarded as positive.

4.4.6 CD-8 staining

CD8 staining was evaluated as previously described (Marchevsky and Walts 2017): 0: <1%, 1: 1-5%, 2: 6-20%, 3: >20% High expression was declared if staining of lymphocytes was >20%.

4.5 High-risk HPV DNA real-time polymerase chain reaction (Brauswetter, Birtalan et al. 2017)

Human DNA was extracted from FFPE tissue sections in cases showing $p16^{INK4}$-immunolabelling by QIAmp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) in line with the manufacturer's instructions. High-risk HPV DNA detection was performed using CONFIDENCE™ HPV test (NEUMANN Diagnostics, Hungary) combined with genotyping for HPV 16, 18, 31, 33, 45, 52, 58. The sufficient amount of input DNA was controlled by fluorometric quantitation by Qubit™ Fluorometer (Invitrogen, Carlsbad,

CA, USA) using Qubit™ dsDNA HS Assay Kit (Invitrogen) according to the manufacturer's protocol.

CONFIDENCE™ HPV is a TaqMan®-based L1 region specific multiplex real-time PCR assay for viral DNA detection. The test detects HPV 16 and 18 separately and other high-risk types (HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) in a pooled manner. In case of high-risk HPV presence, genotyping was performed using type specific primers in separated reactions. The quantitative real-time PCR was carried out on QuantStudio™ 6 Flex platform (Thermo Fisher Scientific, Waltham, MA, USA) in 384-well plate format.

4.6 Tumor infiltrating lymphocyte (TIL) ratio

The proportion of TIL was assessed on hematoxylin and eosin (HE) stained whole tumor slides by an experienced pathologist (JH) according to the method described elsewhere (Vassilakopoulou et al. 2016). Briefly, TIL scoring was based on the area occupied by mononuclear cells relative to the entire stromal area. Scores were distributed as follows: low: 0-33%, moderate: 34-66%, high: 67-100%.

4.7 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Mac version 20.0.0 (SPSS Inc., Chicago, IL, USA). Pearson Chi-square tests and Fisher exact tests were used to test correlations between discrete variables. In case of survival analysis, Kaplan-Meier estimation with Log Rank test as well as univariate and multivariate regression were applied. All tests were 2-sided and p-values <0.05 were considered statistically significant. The following variables were used in the analysis: gender, tumor localization, HPV status, TNM stage, grade, response to induction chemotherapy, response to chemoradiotherapy, tobacco consumption, alcohol consumption and the biomarkers listed above. Disease-specific survival (DSS) time was calculated using the date of diagnosis and the date of death or last follow-up visit.

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5 RESULTS

5.1 P16^{INK4}-expression and high risk HPV DNA status

The result of p16^{INK4} immunohistochemistry was available in 110 patients. Out of the 110 tumor samples, 19 cases (17.3%) proved to be p16^{INK4}-positive. The highest proportion of p16^{INK4}-positive cases was observed in oropharyngeal tumors (38.1%), whereas other locations showed much lower (larynx: 4.8%, hypopharynx: 4.2%) or no (oral cavity: 0%) p16^{INK4}-immunolabelling rate.

The p16^{INK4}-positive cases were tested for 7 high-risk HPV subtypes (HPV 16, 18, 31, 33, 45, 52 and 58) using real-time DNA PCR method. Out of 19 cases, 9 tumors harboured HPV DNA (HPV-positive cases). HPV 16 was present in 8 cases, HPV 33 in one single case. All HPV-positive samples originated from the oropharynx. This means that the rate of HPV-associated oropharyngeal cancers was 21.4% (9/42 patients). Thus, the specificity of p16^{INK4} to detect oropharyngeal HPV presence was 56.3% (out of the 16 p16^{INK4}-positive oropharyngeal tumors, 9 cases tested positive for HPV as well).

5.2 P16^{INK4}/HPV DNA status and response to induction chemotherapy

Of the 110 patients with available immunohistochemical staining, 32 patients received induction chemotherapy (***Table 3***). TPF (docetaxel plus cisplatin plus 5-fluorouracil) was given in 30 cases and for 2 patients PF (cisplatin plus 5-fluorouracil) was the choice. P16^{INK4}-positive individuals showed a better response compared with the p16^{INK4}-negative group (Fisher's exact test: $p = 0.025$). There was a significant difference between groups based on HPV status as well (Fisher's exact test: $p = 0.009$).

Table 3. Response to induction chemotherapy based on p16INK4 and HPV status (PD: progressive disease, SD: stable disease, PR: partial remission, CR: complete remission, HPV: human papillomavirus. The therapeutic response was evaluated according to RECIST 1.1)

		therapeutic response				
		PD	**SD**	**PR**	**CR**	**Total**
p16^{INK4} status						
positive	**N (%)**	4 (18.2)	4 (18.2)	14 (63.6)	0 (0)	22 (100)
negative	**N (%)**	0 (0)	0 (0)	7 (70.0)	3 (30.0)	10 (100)
					p = 0.025	
HPV status						
positive	**N (%)**	4 (15,4)	4 (15,4)	18 (69,2)	0 (0)	26 (100)
negative	**N (%)**	0 (0)	0 (0)	3 (50.0)	3 (50.0)	6 (100)
					p = 0.009	

5.3 The impact of TIL rate

Our samples showed a high infiltration rate by TILs. A low TIL score was observed in 27.3% of cases whereas moderate and high infiltration was seen in 21.2% and 51.5%, respectively. Interestingly, PD-L1^{IC} positivity was associated with high TIL rate in the whole patient sample (Chi-square: p=0.016; ***Supplementary Table 1A*****)** and when observed in anatomical subsets separately in the hypopharynx only (Fisher's exact test p=0.006; ***Supplementary Table 1B***) TIL score correlated positively with CTLA-4^{IC} expression (Chi-square: p=0.013; ***Supplementary Table 1C***). Observing anatomical subgroups separately, TIL score and CTLA-4^{IC} expression correlated in the hypopharynx only (Fisher's exact test: p=0.028; ***Supplementary Table 1D***). In oropharyngeal tumors, TIL score was not associated with HPV status (Fisher's exact test: 0.474; data not published). We could not find differences in survival nor could we establish association with any other parameters and TIL score.

5.4 CD8 expression

The staining rate of CD8 and immune checkpoint proteins is summarized in ***Table 4***.
High CD8 expression was associated with the presence of PD-L1 on tumor cells (Chi-square: 0.001) and PD-L1^{IC} positivity showed a positive correlation with CD8 status (Chi-square: 0.023) as well. Interestingly none of the samples lacked entirely CD8+ T cell infiltration.

Table 4. Immunohistochemistry positivity rate on tumor cell and on immune cells (PD-L1: programmed death-ligand 1, PD-L2: programmed death-ligand 2, PD-1: programmed cell death protein 1, CTLA-4: cytotoxic T-lymhocyte-associated protein 4, CD8: cluster of differentiation protein 8)

Labeling	Tumor cells N (%)	Immune cells N (%)	Total N
PD-L1	31 (31.0)	68 (68.0)	100
PD-L2	71 (70.3)	-	101
PD-1	-	30 (29.4)	102
CTLA-4	20 (20.6)	48 (49.5)	97
CD8	-	60 (56.6)	101

5.5 PD-L1 expression on immune cells (PD-L1^{IC})

Representative images of PD-L1 and other immune checkpoint proteins can be seen on ***Figure 1***.
Considering HPV-negative tumors of all localizations PD-L1^{IC} positivity was proved to be associated with better DSS (HR=0.502; CI95%, 0.273-0.923; p=0.027) (***Figure 2***). In laryngeal tumors, PD-L1^{IC} positivity was associated with improved DSS (HR=0.222; CI95%, 0.062-0.795; p=0.021) compared to the PD-L1^{IC} negative group (***Figure 3***).

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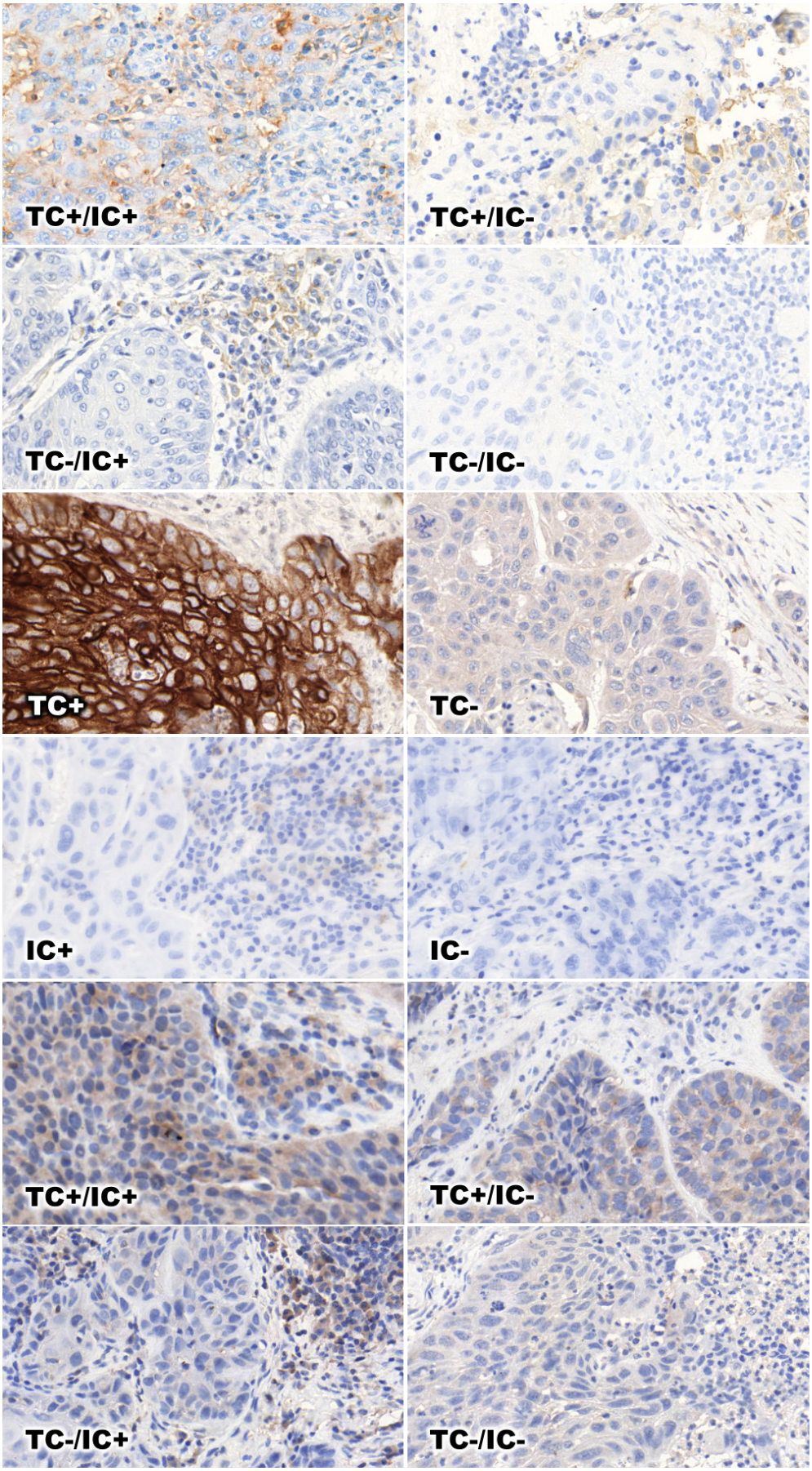

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Figure 1. Representative images of immunohistochemistry

(TC+/-: positive/negative in tumor cells, IC+/-: positive/negative in immune cells, PD-L1: programmed death-ligand 1, PD-L2: programmed death-ligand 2, PD-1: programmed cell death protein 1, CTLA-4: cytotoxic T-lymhocyte-associated protein 4, magnification: 200x)

In the multivariate analysis controlling for gender, tumor localization and stage, PD-L1^{IC} status did not prove to be an independent prognostic factor. Furthermore, PD-L1^{IC} positivity showed a positive correlation with CTLA-4^{IC} positivity (Chi-square: p=0.049; ***Supplementary Table 2A***). TIL score correlated positively with PD-L1^{IC} expression as stated above.

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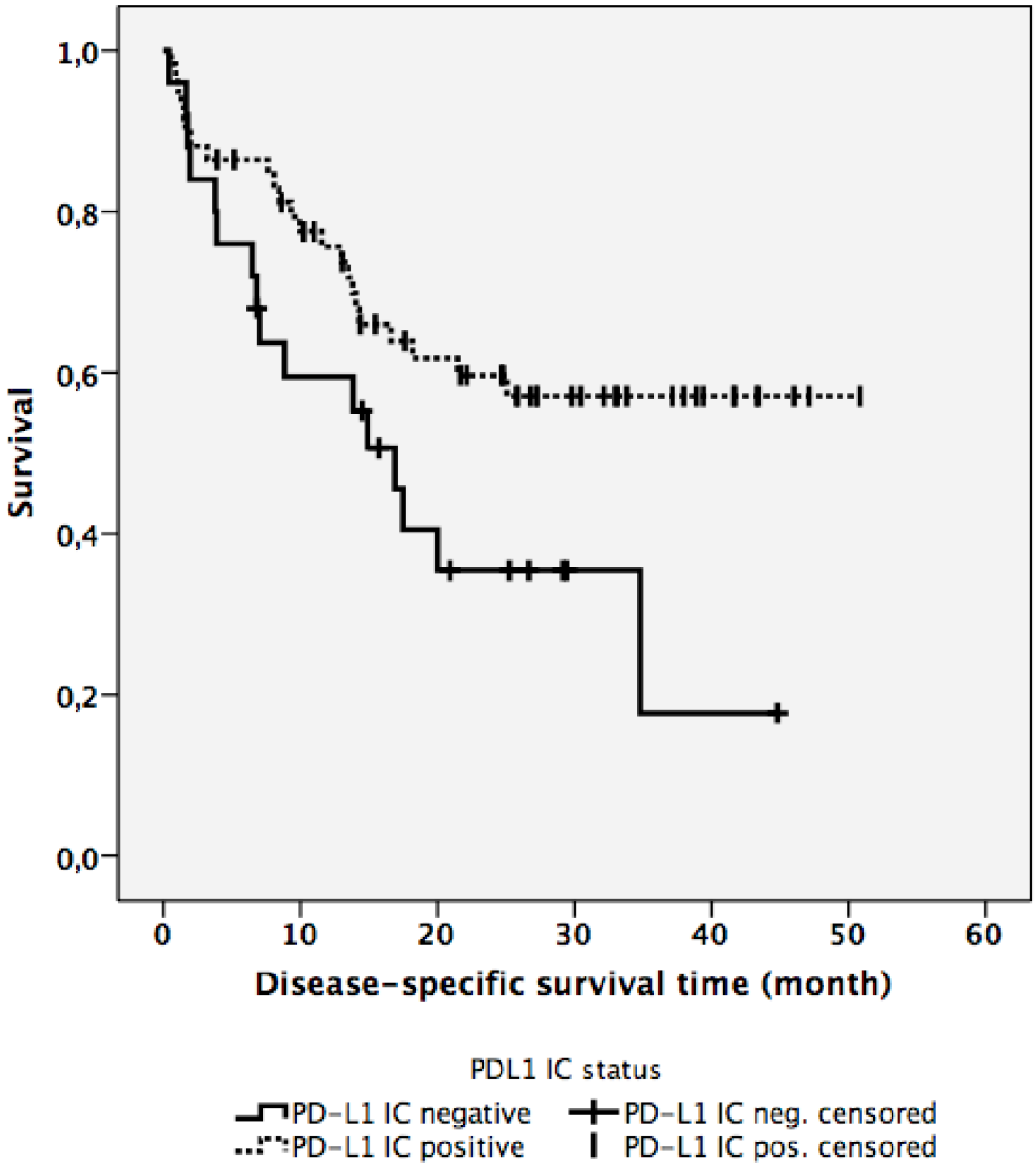

Figure 2. PD-L1 positivity on immune cells is followed by improved disease-specific survival in HPV-negative patients (HR=0.505; CI95%, 0.266-0.959; p=0.037).

(PD-L1: programmed death-ligand 1, IC: immune cell, HPV: human papillomavirus, HR: hazard ratio, CI: confidence interval)

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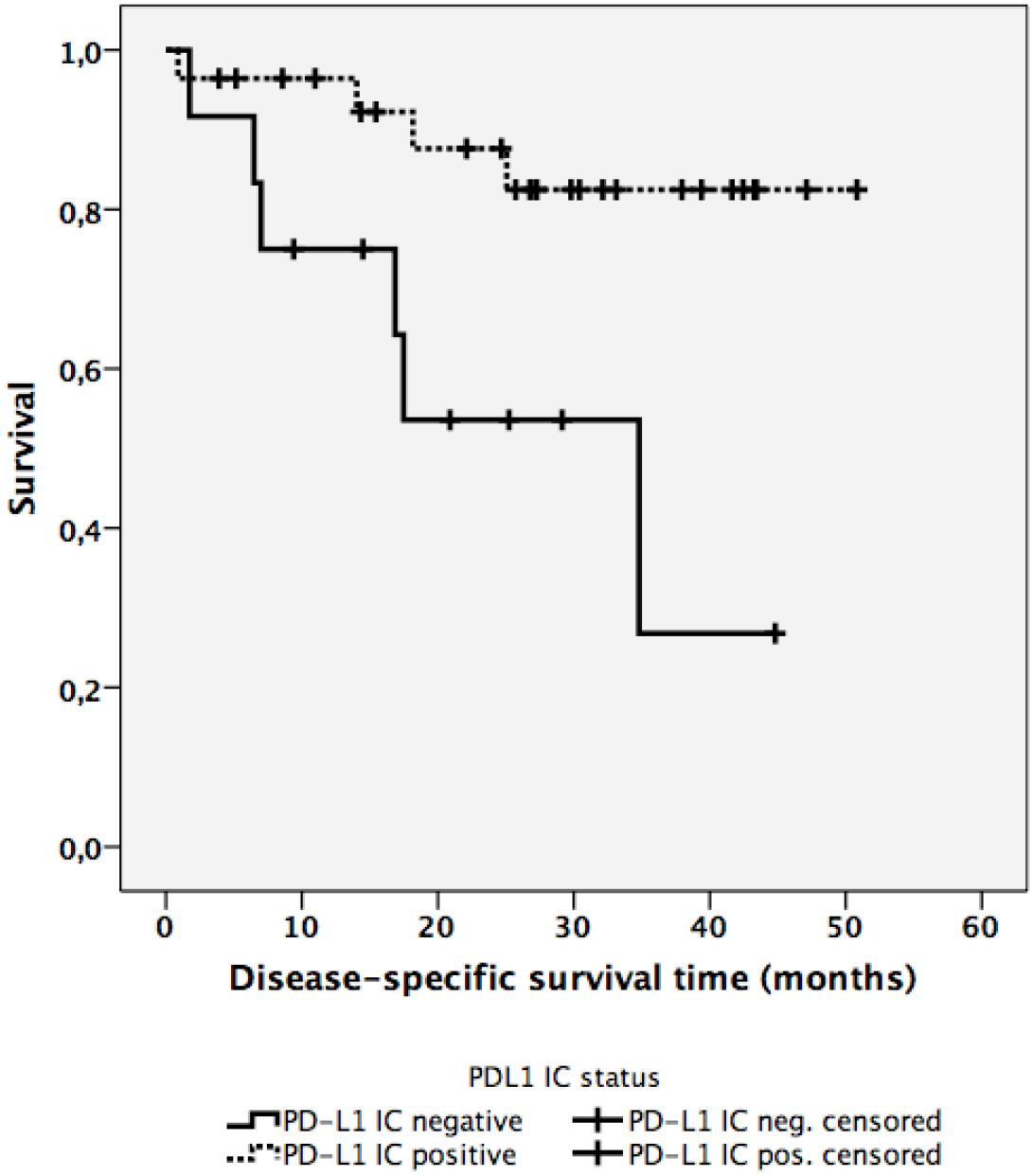

Figure 3. The prognostic role of PD-L1^{IC} status in laryngeal squamous cell carcinoma (HR=0.222; CI95%, 0.062-0.795; p=0.021)

(PD-L1: programmed death-ligand 1, IC: immune cell, HPV: human papillomavirus, HR: hazard ratio, CI: confidence interval)

5.6 PD-L1 expression on tumor cells (PD-L1^{TC}) was not associated with disease-specific survival.

PD-L1^{TC} and PD-1 status showed a remarkably strong positive correlation in all patients (Chi-square: p<0.001; ***Supplementary Table 2B***) and in oropharyngeal and laryngeal localization (Fisher's exact test for oropharynx: p<0.001, ***Supplementary Table 2C***; larynx: p=0.006, ***Supplementary Table 2D***), whereas in the hypopharynx the level of significance was not reached (Fisher's exact test: p=0.074, data not published). Negative HPV status was associated with negative PD-L1^{TC} expression in case of oropharyngeal malignancies (Fisher's exact test: p=0.019; ***Supplementary Table 2E***).

5.7 PD-1 expression

Besides the above mentioned data, PD-1 status did not correlate with anatomical subsets (Chi-square: 0.239; data not published), HPV status (Chi-square: p=0.601; data not published) or with any other parameters observed.

5.8 PD-L2 expression

Observing only HPV-negative tumors PD-L2 expression negatively correlated with the presence of PD-1 (Chi-square: p=0.027; ***Supplementary Table 3***). None of other biomarkers or clinicopathological parameters were associated with PD-L2 status.

5.9 CTLA-4 expression on immune cells (CTLA-4^{IC}) and tumor cells (CTLA-4^{TC})

TIL score correlated positively with CTLA-4^{IC} expression as described above. As mentioned earlier, CTLA-4^{IC} expression correlated with PD-L1^{IC} positivity. We have found that a proportion of HNSCC tumor cells showed faint cytoplasmic positivity for CTLA-4 (***Figure 1***). None of the biomarkers or clinicopathological parameters were associated with CTLA-4^{TC} expression.

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5.10 Anatomical localization and survival

Laryngeal cancer was characterized by superior DSS when compared to tumors of pharyngeal origin (HR=0.306; CI95%, 0.152-0.616; p=0.001; Log Rank p<0.001). However, this relation became tendential only when observation was limited to patients with locoregionally advanced (stage III-IV B) disease (Log Rank p=0.057).

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6 DISCUSSION

The predictive role of p16^{INK4} immunohistochemistry and HPV DNA PCR method regarding induction chemotherapy

Many studies have already reported the growing prevalence of human papillomavirus in head and neck squamous cell cancers. Chaturvedi et al. observed significantly increased OPC incidence during 1983 to 2002 predominantly in developed countries and at younger ages; results that underscore a potential role of HPV infection on increasing OPC incidence, particularly among men (Chaturvedi et al. 2013). Male predominance could be explained by the fact that during active orogenital encounter (assuming heterosexual relationship) males are exposed to a greater HPV load since the infected cervical secretes contain far more HPV virus particles than the infected penis (Marur et al. 2010).

Data published by Castellsague et al. shows that HPV-positivity rate of oropharyngeal cancers was 24.3% assessed by HPV DNA and p16^{INK4} dual testing (Castellsague et al. F. 2016). In our study, we found HPV-induced rate to be 21.4% in the oropharynx using p16^{INK4}/HPV DNA PCR co-testing (Brauswetter, Birtalan et al. 2017). Regarding other localizations, no p16^{INK4}-positive/ HPV DNA-positive tumor was detected.

Nevertheless, there was still a lack of consensus concerning the use diagnostic methods needed to detect HPV association (Jordan et al. 2012, Dreyer et al. 2013). Schache et al. recommended the combination of p16^{INK4} /DNA PCR method when analyzed eight possible assay/assay combinations (Schache et al. 2011).

In our study, we used both of the above recommended methods. First, p16^{INK4} immunohistochemistry was performed and those samples tested positive were further analyzed using high-risk HPV DNA real-time PCR. We assessed the rate of HPV-induced and p16^{INK4}-expressing tumors in Hungarian head and neck cancer patients. Both p16^{INK4}-positive and p16^{INK4}-positive/HPV DNA-containing (HPV-positive) tumors had a better response to induction chemotherapy. Although we worked with a fairly small sample size, we found a correlation between positive p16^{INK4} expression and better outcome of induction chemotherapy. Comparing groups according to HPV status, HPV

associated tumors were associated with better short-term oncological outcome. This correlation was somewhat stronger (p=0.009) that the one based on $p16^{INK4}$ expression (p=0.025).

Patients harboring HPV-associated oropharyngeal tumors are anticipated to have improved outcomes after induction chemotherapy or chemoradiation (Fakhry et al. 2008).

Considering the impact on quality of life that a curative surgery or radiochemotherapy can have and the relative younger age of patients with HPV-associated oropharyngeal malignancy, therapies offering organ preservation or less side-effects might be a feasible choice. Given the increased sensitivity of HPV-positive tumors to chemotherapy and radiation, several clinical trials seek to establish de-intensified treatment protocols for these patients without jeopardizing oncological outcome. These trials either lower radiation dose to spare radiation-associated early and late toxicities (e.g. NCT01084083 and NCT0189894) or omit cisplatin use to reduce acute toxicity and late renal and vascular complications (e.g. NCT01302834, NCT01855451, NCT01874171 and NCT02254278) (Bhatia and Burtness 2015).

Further multi-institutional, sufficiently large studies are needed to validate the independent predictive value of $p16^{INK4}$ protein expression and HPV-positivity with regard to response to induction chemotherapy.

Expression of checkpoint inhibitor proteins in subsets of head and neck cancer

Immunotherapy has evolved greatly during the last decade and holds the promise of a revolution in the treatment of cancer. There is great enthusiasm towards immunotherapeutic approaches of HNSCC, since it is a disease characterized by profound involvement of the immune system (Economopoulou et al. 2016). Checkpoint inhibitors such as anti-PD-1 monoclonal antibody nivolumab and pembrolizumab has recently gained FDA approval for the treatment of recurrent or metastatic HNSCC. Despite intense research, there is still an urging need for prognostic and predictive biomarkers. Besides tumor cell markers, the attention is now focused on markers of immune activation as well.

Teng et al. proposed a rather simplistic four tier classification of tumor microenvironments based on the presence of TILs and PD-L1 expression on tumor cells that might help tailoring immunotherapeutic treatments (Teng et al. 2015).

In this study we investigated the possibly existing differences between subsets of HNSCC based on clinicopathological data and correlations between particular biomarkers. The term subset referred to subgroups according to HPV status and anatomical localization. The rationale of this approach on one hand was the growing body of evidence that suggests differences in the expression of multiple biomarkers. On the other hand, it became clear in recent years, that HPV associated and non-HPV associated HNSCCs are distinct biologic entities (Dillon and Harrington 2015).

There is conflicting data on the prognostic role of PD-L1 expression on tumor cells. In our study we could not identify any connection between PD-L1^{TC} expression and survival. The attention has somewhat shifted towards PD-L1^{IC} expression recently that might serve as a prognostic factor in HNSCC as well. The prognostic role of PD-L1 expression on tumor infiltrating mononuclear cells has been known in other tumors such as urothelial carcinoma (Bellmunt et al. 2015) and spinal chordoma (Zou et al. 2016). Until recent days, no such a correlation was found in HNSCC. Kim et al. showed first that there is a survival benefit for those patients with PD-L1 positivity on TIMCs (Kim et al. 2016). Our findings reflect that data, however we could confirm this in the HPV-negative population only. Nevertheless, we showed a vast survival benefit in favor of laryngeal cancer patients with PD-L1^{IC} positive tumors. That aligns with previous findings on increased TIL density and quantitative PD-L1 protein levels associated with better outcome in laryngeal squamous cell cancer (Vassilakopoulou et al. 2016). This can be explained by the phenomenon, that activated T cells produce IFN gamma which consequently induce PD-L1 expression of surrounding immune and tumor cells thus indicating immune activity (Bellmunt et al. 2016). PD-L1^{IC} expression was found to coexist with enhanced TIL density, CD8 infiltration and CTLA-4^{IC} expression. This might reflect the same immune activation e.g. by IFN gamma but the presence of CTLA-4^{IC} expression perhaps mirrors a negative regulatory mechanism.

PD-L1^{IC} and PD-L1^{TC} status did not appear to correlate but this might be caused by sample size. There was a strong correlation between PD-L1^{TC} and PD-1 in all regions as well as laryngeal and oropharyngeal localization separately but it has not reached statistical significance in the hypopharynx. This underlines the efficacy of checkpoint inhibitors in HNSCC blocking the PD-1/PD-L1 signal transmission. PD-L1^{TC} positivity correlated with high CD8 expression that might reflect an enhanced immune activation underscoring the relation of PD-L1^{TC}/PD-1 coexistence. Interestingly, when analyzing anatomical regions separately, this relation remained significant in the hypopharynx only.

We demonstrated that PD-L1 expression on tumor cells was associated with HPV status in oropharyngeal tumors. That might well be a protective mechanism on behalf of cancer cells, caused by increased immune activation against HPV-infected tumor cells.

We found no survival differences based on TIL density. However, a correlation between TIL and PD-L1^{IC} / CTLA-4^{IC} was observed. This was true observing all locations and interestingly when analyzing anatomical subsets separately it remained true in the hypopharynx only. Although the survival of hypopharyngeal and oropharyngeal cancer patients was not distinct from each other (data not published), this might indicate a special role of hypopharyngeal localization in terms of cancer immunity. However, this sample size is not suitable for either supporting or refuting this statement with confidence.

There is paucity of data on PD-L2 expression in HNSCC. Derks et al. first described PD-L2 in Barret's esophagus and in 51.7% of esophageal adenocarcinoma cancer cells. They hypothetized that a shift from Th1 to Th2 immune response and the consequently changed cytokin milieu contributed to PD-L2 induction (Derks et al. 2015). To our knowledge, there has been no systematic evaluation of PD-L2 expression in HNSCC cancer cells to date. Our data reflects, that in HPV-negative HNSCC PD-L2 is negatively associated with PD-1 expression in TIMCs. That was the only inverse correlation we found in our study. Thus, PD-L2 might have no role or plays an insignificant role in PD-1 associated immune evasion. We found no correlation between PD-L2 and other markers or clinicopathological features.

An other interesting aspect could be the role of CTLA-4 expression in both immune and cancer cells. In fact, anti-CTLA-4 immunotherapeutics preceded those blocking the PD-1/PD-L1 signaling as ipilimumab was approved by the FDA for patients with metastatic melanoma in 2010 (Hodi et al. 2010). Cytotoxic T-lymphocyte-associated antigen 4, as its name reflects is primarily expressed in T cells. However, there is compelling evidence, that expression in tumor cells occurs as well (Queirolo et al. 2009). Chakravarti et al. reported that high expression levels of CTLA-4 in immune or tumor cells was followed by decreased progression-free survival and poor overall survival in melanoma patients (Chakravarti et al. 2016). Yu et al. found that CTLA-4 expression on lymphocytes was associated with better prognosis, but CTLA-4 positivity on tumor cells was associated with worse prognosis in breast cancer (Yu et al. 2015). The prognostic and predictive potential of CTLA-4^{TC} expression in HNSCC is not clear yet. In our study, we observed 20.6% of HNSCC samples expressing CTLA-4 on tumor cells. Staining occurred predominantly in the cytoplasm that might question its specificity.

Despite, percentage of stained tumor cells in CTLA-4^{TC} expressing samples ranged between 1-50%. Secondly, the use of positive and negative controls, just as staining immune cells as an internal control seem to support our results. Nevertheless, none of the other markers or clinicopathological data correlated with this finding.

On the contrary, CTLA-4 expression on TIMCs was associated with high TIL density in the whole study population and in the hypopharynx alone as well when analyzed in separate anatomical localizations. Furthermore, a coexistence of PD-L1-positive and CTLA-4-positive immune cells was observed. This finding supports the feasibility of combined checkpoint inhibitor treatment in HNSCC.

Nevertheless, our study has limitations. First of all, tissue microarray construction bears a certain risk for misrepresentating the whole tumor. Given there is a heterogeneous expression of immune markers including PD-L1, TMA construction is an issue worth to consider. To avoid incorrect sampling, possibly 2 or 3 cores per patient were obtained in this study. Another limitation could be that our samples originated from both surgical specimens and diagnostic probe excision materials representing rather intratumoral and

rather peritumoral tissues, respectively. Finally, the relatively small sample size was a major limiting factor as mentioned before.

In contrary, the patient population can be regarded as representative. This is indicated by the above mentioned figures that reflect the findings of previously published data.

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7 CONCLUSIONS

The predictive role of p16^{INK4} immunohistochemistry and HPV DNA PCR method regarding induction chemotherapy

1. P16^{INK4} immunohistochemistry can be considered a possible, precise and widely affordable tool in predictive characterization of oropharyngeal squamous cell cancers in term of response to induction chemotherapy.
2. In comparison with p16^{INK4}/HPV DNA PCR double testing, p16^{INK4} status alone proved to be an equivocally precise indicator of clinical outcome.

Expression of checkpoint inhibitor proteins in subsets of head and neck cancer

3. Our results showed a survival benefit of PD-L1 expression on immune cells in HPV-negative HNSCC.
4. PD-L1^{IC} expression was found to indicate a better prognosis in laryngeal squamous cell carcinoma as well.
5. PD-L2 expression showed no correlation with any parameters observed, except for a negative correlation with PD-1-positive status.
6. We found a proportion of HNSCC expressing CTLA-4 in tumor cell but failed to prove any clinical significance or correlation with other markers.
7. We have not found any remarkable differences between anatomical subgroups of HNSCC.
8. HPV status clearly divided subsets of this disease in terms of cancer immunity.
9. A possibly distinct role of hypopharyngeal localization with regard to immune activity requires further clarification by larger studies.

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8 SUMMARY

Squamous cell carcinoma of the head and neck is a major burden on a global scale with over 600.000 new cases annually. Since HPV associated oropharyngeal carcinoma is a distinct biological entity with often better oncological outcome, de-intensification protocols are pursued. Similarly to melanoma and lung cancer, immunotherapy has drawn great attention recently in head and neck oncology as well. Nevertheless, there is an urgent need for prognostic and predictive biomarkers.

Our studies aimed to explore and compare the predictive value of $p16^{INK4}$ expression and HPV DNA PCR method with regard to induction chemotherapy. On the other hand, we investigated the expression of checkpoint inhibitor proteins in subsets of head and neck cancer to find out wether this is a homogenous disease in terms of immune activation or not.

$P16^{INK4}$, PD-L1, PD-L2, PD-1, CTLA-4 and CD8 expression was observed in archival tumor samples of head and neck cancer patients. High-risk HPV DNA real-time PCR was performed. Proportion of infiltrating lymphocytes (TILs) was assessed as well.

We found a better therapeutic response of $p16^{INK4}$-positive as well as of HPV-positive patients to induction chemotherapy. Both single and double testing were significant predictive markers of induction chemotherapy outcome with the double testing being slightly more precise than the $p16^{INK4}$ expression only.

We showed the prognostic significance of PD-L1 immune cell positivity in HPV-negative tumors and in laryngeal localization. PD-$L1^{IC}$ positivity was associated with better disease-specific survival and it was correlated with CTLA-4^{IC} expression and was accompanied by high TIL rate. PD-L1 expression on tumor cells and PD-1 status showed strong correlation. $p16^{INK4}$ expression was associated with PD-$L1^{TC}$ status in oropharyngeal cancers. CTLA-4^{IC} and CTLA-4^{TC} positivity was observed in 49.5% and 20.6%, respectively. We have not found any essential differences between anatomical subgroups. However, a possibly distinct role of hypopharyngeal localization regarding immune activity requires further clarification. On the contrary, $p16^{INK4}$ expression/HPV status clearly divided subgroups in terms of immune checkpoint protein expression.

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9 ÖSSZEFOGLALÁS

A fej-nyaki laphámsejtes daganatok világszinten jelentős tehertételt jelentenek. Évente mintegy 600.000 új eset kerül felfedezésre. A HPV indukálta szájgarati daganatok önálló entitást képeznek, rendszerint jobb prognózissal társulnak, ami új protokollok megalkotását teszi szükségessé. Napjainkban a melanomához és a tüdőrákhoz hasonlóan a fej-nyaki onkológiában is nagy figyelem övezi az immunterápiát. Mindazonáltal sürgető szükség lenne megbízható prognosztikai és prediktív biomarkerekre.

Vizsgálataink során a $p16^{INK4}$ expresszió és a HPV DNS PCR módszer indukciós kemoterápiára vonatkozó prediktív szerepét vizsgáltuk és hasonlítottunk össze. Ugyanakkor célul tűztük ki az immun ellenőrzőpont fehérjék expressziójának összehasonlítását a fej-nyaki laphámrák alcsoportjaiban, hogy kiderítsük, homogén betegségcsoporttal állunk-e szemben az immunaktiváció szempontjából.

$P16^{INK4}$, PD-L1, PD-L2, PD-1, CTLA-4 és CD8 expresszót vizsgáltunk fej-nyaki daganatos betegek archív szövetmintáin. Magas rizikújó HPV DNS valósidejű PCR-t végeztünk. A tumorinfiltráló limfociták arányát is meghatároztuk.

Jobb terápiás választ találtunk indukciós kemoterápiára a $p16^{INK4}$-pozitív és a HPV-pozitív betegek esetén is. Bár mindkét módszer szignifikáns prediktív markernek bizonyult, a kettős tesztelés valamivel precízebb ereményt adott a csak $p16^{INK4}$ expresszió alapján meghatározott csoportokhoz képest.

Kimutattuk a PD-L1 immun sejt pozitivitás prognosztikai szerepét HPV negatív ill. gége lokalizációjú fej-nyaki laphámrák esetén. A $PD\text{-}L1^{IC}$ pozitivitás jobb betegségspecifikus túléléshez társult és korreleált a $CTLA\text{-}4^{IC}$ expresszióval és a magasabb TIL aránnyal. A PD-L1 tumorsejteken való expressziója és a PD-1 status között szoros összefüggés mutatkozott. Szájgarat esetén a $p16^{INK4}$ expresszió kapcsolatot mutatott a $PD\text{-}L1^{TC}$ státusszal. A $CTLA\text{-}4^{IC}$ and $CTLA\text{-}4^{TC}$ pozitivitás 49.5% és 20.6%-ban volt megfigyelhető. Nem találtunk összefüggést az anatómiai lokalizációval. Mindazonáltal az algarati elhelyezkedés potenciális szerepe további vizsgálatokat igényelhet. Ezzel szemben a $p16^{INK4}$/HPV státusz egyértelmű alcsoportokat elkülönített el az immun ellenőrzőpont fehérjék expressziója szempontjából.

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10 BIBLIOGRAPHY

Ackland SP, Schilsky RL. (1987) High-dose methotrexate: a critical reappraisal. J Clin Oncol, 5: 2017-2031.

Adelstein DJ, Li Y, Adams GL, Wagner H, Jr Kish JA, Ensley JF, Schuller DE, Forastiere AA. (2003) An intergroup phase III comparison of standard radiation therapy and two schedules of concurrent chemoradiotherapy in patients with unresectable squamous cell head and neck cancer. J Clin Oncol, 21: 92-98.

Akagi K., Li J, Broutian TR, Padilla-Nash H, Xiao W, Jiang B, Rocco JW, Teknos TN, Kumar B, Wangsa D, He D, Ried T, Symer DE, Gillison ML. (2014) Genome-wide analysis of HPV integration in human cancers reveals recurrent, focal genomic instability. Genome Res, 24: 185-199.

Al-Sarraf M, Pajak TF, Marcial VA, Mowry P, Cooper JS, Stetz J, Ensley JF, Velez-Garcia E. (1987) Concurrent radiotherapy and chemotherapy with cisplatin in inoperable squamous cell carcinoma of the head and neck. An RTOG Study. Cancer, 59: 259-265.

Amdur RJ, Parsons JT, Mendenhall WM, Million RR, Cassisi NJ. (1989) Split-course versus continuous-course irradiation in the postoperative setting for squamous cell carcinoma of the head and neck. Int J Radiat Oncol Biol Phys, 17: 279-285.

Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, Westra, WH, Chung CH, Jordan RC, Lu C, Kim H, Axelrod R, Silverman CC, Redmond KP, Gillison ML. (2010) Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med, 363: 24-35.

Bellmunt J, Mullane SA, Werner L, Fay AP, Callea M, Leow JJ, Taplin ME, Choueiri TK, Hodi FS, Freeman GJ, Signoretti S. (2015) Association of PD-L1 expression on tumor-infiltrating mononuclear cells and overall survival in patients with urothelial carcinoma. Ann Oncol, 26: 812-817.

Bernier, J., Domenge, C., Ozsahin, M., Matuszewska, K., Lefebvre, J.L., Greiner, R.H., Giralt J, Maingon P, Rolland F, Bolla M, Cognetti F, Bourhis J, Kirkpatrick A, van Glabbeke M. (2004) Postoperative irradiation with or without concomitant

chemotherapy for locally advanced head and neck cancer. N Engl J Med, 350: 1945-1952.

Bhatia A, Burtness B. (2015) Human Papillomavirus-Associated Oropharyngeal Cancer: Defining Risk Groups and Clinical Trials. J Clin Oncol, 33: 3243-3250.

Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, Jones CU, Sur R, Raben D, Jassem J, Ove R, Kies MS, Baselga J, Youssoufian H, Amellal N, Rowinsky EK, Ang KK. (2006) Radiotherapy plus Cetuximab for Squamous-Cell Carcinoma of the Head and Neck. N Engl J Med, 354: 567-578.

Botti G, Scognamiglio G, Cantile M. (2016) PD-L1 Immunohistochemical Detection in Tumor Cells and Tumor Microenvironment: Main Considerations on the Use of Tissue Micro Arrays. Int J Mol Sci, 17: 7

Brantsch KD, Meisner C, Schonfisch B, Trilling B, Wehner-Caroli J, Rocken M, Breuninger H. (2008) Analysis of risk factors determining prognosis of cutaneous squamous-cell carcinoma: a prospective study. Lancet Oncol, 9: 713-720.

Brauswetter D, Birtalan E, Danos K, Kocsis A, Krenacs T, Timar J, Mihalyi R, Horcsik D, Polony G, Tamas L, Petak I. (2017) p16INK4 expression is of prognostic and predictive value in oropharyngeal cancers independent of human papillomavirus status: a Hungarian study. Eur Arch Otorhinolaryngol, 274: 1959-1965.

Brauswetter D, Danos K, Gurbi B, Felegyhazi EF, Birtalan E, Meggyeshazi N, Krenacs T, Tamas L, Petak I. (2016) Copy number gain of PIK3CA and MET is associated with poor prognosis in head and neck squamous cell carcinoma. Virchows Arch, 468: 579-587.

Breuninger H, Black B, Rassner G. (1990) Microstaging of squamous cell carcinomas. Am J Clin Pathol, 94: 624-627.

Brougham ND, Dennett ER, Cameron R, Tan ST. (2012) The incidence of metastasis from cutaneous squamous cell carcinoma and the impact of its risk factors. J Surg Oncol, 106: 811-815.

Calais G, Alfonsi M, Bardet E, Sire C, Germain T, Bergerot P, Rhein B, Tortochaux J, Oudinot P, Bertrand P. (1999) Randomized trial of radiation therapy versus concomitant

chemotherapy and radiation therapy for advanced-stage oropharynx carcinoma. J Natl Cancer Inst, 91: 2081-2086.

Castellsague X, Alemany L, Quer M, Halec G, Quiros B, Tous S, Clavero O, Alos L, Biegner T, Szafarowski T, Alejo M, Holzinger D, Cadena E, Claros E, Hall G, Laco J, Poljak M, Benevolo M, Kasamatsu E, Mehanna H, Ndiaye C, Guimera N, Lloveras B, Leon X, Ruiz-Cabezas JC, Alvarado-Cabrero I, Kang CS, Oh JK, Garcia-Rojo M, Iljazovic E, Ajayi OF, Duarte F, Nessa A, Tinoco L, Duran-Padilla MA, Pirog EC, Viarheichyk H, Morales H, Costes V, Felix A, Germar MJ, Mena M, Ruacan A, Jain A, Mehrotra R, Goodman MT, Lombardi LE, Ferrera A, Malami S, Albanesi EI, Dabed P, Molina C, Lopez-Revilla R, Mandys V, Gonzalez ME, Velasco J, Bravo IG, Quint W, Pawlita M, Munoz N, Sanjose S, Xavier Bosch F. (2016) HPV Involvement in Head and Neck Cancers: Comprehensive Assessment of Biomarkers in 3680 Patients. J Natl Cancer Inst, 108(6).

Chakravarti N, Ivan D, Trinh VA, Glitza IC, Curry JL, Torres-Cabala C, Tetzlaff MT, Bassett RL, Prieto VG, Hwu WJ. (2016) High cytotoxic T-lymphocyte-associated antigen 4 and phospho-Akt expression in tumor samples predicts poor clinical outcomes in ipilimumab-treated melanoma patients. Melanoma Res, 27: 24-31.

Chaturvedi AK, Anderson WF, Lortet-Tieulent J, Curado MP, Ferlay J, Franceschi S, Rosenberg PS, Bray F, Gillison ML. (2013) Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers. J Clin Oncol, 31: 4550-4559.

Chen L, (2004) Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. Nat Rev Immunol, 4: 336-347.

Cilla S, Deodato F, Macchia G, Digesu C, Ianiro A, Piermattei A, Valentini V, Morganti AG. (2016) Volumetric modulated arc therapy (VMAT) and simultaneous integrated boost in head-and-neck cancer: is there a place for critical swallowing structures dose sparing? Br J Radiol, 89: 20150764.

Cohen EE, Karrison TG, Kocherginsky M, Mueller J, Egan R, Huang CH, Brockstein BE, Agulnik MB, Mittal BB, Yunus F, Samant S, Raez LE, Mehra R, Kumar P, Ondrey F, Marchand P, Braegas B, Seiwert TY, Villaflor VM, Haraf DJ, Vokes EE. (2014) Phase

III randomized trial of induction chemotherapy in patients with N2 or N3 locally advanced head and neck cancer. J Clin Oncol, 32: 2735-2743.

Cooper JB, Cohen EEW. (2009) Mechanisms of resistance to EGFR inhibitors in head and neck cancer. Head Neck, 31: 1086-1094.

Cooper JS, Pajak TF, Forastiere AA, Jacobs J, Campbell BH, Saxman SB, Kish JA, Kim HE, Cmelak AJ, Rotman M, Machtay M, Ensley JF, Chao KS, Schultz CJ, Lee N, Fu KK. (2004) Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck. N Engl J Med, 350: 1937-1944.

D'Souza G, Agrawal Y, Halpern J, Bodison S, Gillison, ML. (2009) Oral sexual behaviors associated with prevalent oral human papillomavirus infection. J Infect Dis, 199: 1263-1269.

Dabas S, Gupta K, Ranjan R, Sharma AK, Shukla H, Dinesh A. (2017) Oncological outcome following de-intensification of treatment for stage I and II HPV negative oropharyngeal cancers with transoral robotic surgery (TORS): A prospective trial. Oral Oncol, 69: 80-83.

Danos K, Brauswetter D, Birtalan E, Pato A, Bencsik G, Krenacs T, Petak I, Tamas L, (2015) The Potential Prognostic Value of Connexin 43 Expression in Head and Neck Squamous Cell Carcinomas. Appl Immunohistochem Mol Morphol, 24: 476-481.

de Souza JA, Davis DW, Zhang Y, Khattri A, Seiwert TY, Aktolga S, Wong SJ, Kozloff MF, Nattam S, Lingen MW, Kunnavakkam R, Stenson KM, Blair EA, Bozeman J, Dancey JE, Vokes EE, Cohen EE. (2012) A phase II study of lapatinib in recurrent/ metastatic squamous cell carcinoma of the head and neck. Clin Cancer Res, 18: 2336-2343.

Derks S, Nason KS, Liao X, Stachler MD, Liu KX, Liu JB, Sicinska E, Goldberg MS, Freeman GJ, Rodig SJ, Davison JM, Bass AJ. (2015) Epithelial PD-L2 expression marks Barrett's Esophagus and Esophageal Adenocarcinoma. Cancer Immunol Res, 3: 1123-1129.

Dhar PK, Rao TR, Sreekumaran Nair N, Mohan S, Chandra S, Bhat KR, Rao K. (2000) Identification of risk factors for specific subsites within the oral and oropharyngeal region--a study of 647 cancer patients. Indian J Cancer, 37: 114-122.

Dholam KP, Chouksey GC. (2016) Squamous cell carcinoma of the oral cavity and oropharynx in patients aged 18-45 years: A case-control study to evaluate the risk factors with emphasis on stress, diet, oral hygiene, and family history. Indian J Cancer, 53: 244-251.

Dillon MT, Harrington KJ. (2015) Human Papillomavirus-Negative Pharyngeal Cancer. J Clin Oncol, 33: 3251-3261.

Dreyer JH, Hauck F, Oliveira-Silva M, Barros MH, Niedobitek G. (2013) Detection of HPV infection in head and neck squamous cell carcinoma: a practical proposal. Virchows Arch, 462: 381-389.

Economopoulou P, Agelaki S, Perisanidis C, Giotakis EI, Psyrri A. (2016) The promise of immunotherapy in head and neck squamous cell carcinoma. Ann Oncol, 27: 1675-1685.

Fakhry C, Westra WH, Li S, Cmelak A, Ridge JA, Pinto H, Forastiere A, Gillison ML. (2008) Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. J Natl Cancer Inst, 100: 261-269.

Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer, 127: 2893-2917.

Forastiere AA, Metch B, Schuller DE, Ensley JF, Hutchins LF, Triozzi P, Kish JA, McClure S, VonFeldt E, Williamson SK, von Hoff DD. (1992) Randomized comparison of cisplatin plus fluorouracil and carboplatin plus fluorouracil versus methotrexate in advanced squamous-cell carcinoma of the head and neck: a Southwest Oncology Group study. J Clin Oncol, 10: 1245-1251.

Fu KK, Pajak TF, Trotti A, Jones CU, Spencer SA, Phillips TL, Garden AS, Ridge JA, Cooper JS, Ang KK. (2000) A Radiation Therapy Oncology Group (RTOG) phase III randomized study to compare hyperfractionation and two variants of accelerated fractionation to standard fractionation radiotherapy for head and neck squamous cell carcinomas: first report of RTOG 9003. Int J Radiat Oncol Biol Phys, 48: 7-16.

Gillison ML, Chaturvedi AK, Anderson WF, Fakhry C. (2015) Epidemiology of Human Papillomavirus-Positive Head and Neck Squamous Cell Carcinoma. J Clin Oncol, 33: 3235-3242.

Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, Zahurak ML, Daniel RW, Viglione M, Symer DE, Shah KV, Sidransky D. (2000) Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst, 92: 709-720.

Giuliano AR, Nyitray AG, Kreimer AR, Pierce Campbell CM, Goodman MT, Sudenga SL, Monsonego J, Franceschi S. (2015) EUROGIN 2014 roadmap: differences in human papillomavirus infection natural history, transmission and human papillomavirus-related cancer incidence by gender and anatomic site of infection. International journal of cancer. Int J Cancer, 136: 2752-2760.

Greenwald RJ, Latchman YE, Sharpe AH. (2002) Negative co-receptors on lymphocytes. Curr Opin Immunol, 14: 391-396.

Grosso JF, Jure-Kunkel MN. (2013) CTLA-4 blockade in tumor models: an overview of preclinical and translational research. Cancer Immun 13: 5.

Grunwald V, Keilholz U, Boehm A, Guntinas-Lichius O, Hennemann B, Schmoll HJ, Ivanyi P, Abbas M, Lehmann U, Koch A, Karch A, Zorner A, Gauler TC. (2015) TEMHEAD: a single-arm multicentre phase II study of temsirolimus in platin- and cetuximab refractory recurrent and/or metastatic squamous cell carcinoma of the head and neck (SCCHN) of the German SCCHN Group (AIO). Ann Oncol, 26: 561-567.

Haddad R, O'Neill A, Rabinowits G, Tishler R, Khuri F, Adkins D, Clark J, Sarlis N, Lorch J, Beitler JJ, Limaye S, Riley S, Posner M. (2013) Induction chemotherapy followed by concurrent chemoradiotherapy (sequential chemoradiotherapy) versus concurrent chemoradiotherapy alone in locally advanced head and neck cancer (PARADIGM): a randomised phase 3 trial. Lancet Oncol, 14: 257-264.

Hayes DN, Van Waes C, Seiwert TY. (2015) Genetic Landscape of Human Papillomavirus-Associated Head and Neck Cancer and Comparison to Tobacco-Related Tumors. J Clin Oncol, 33: 3227-3234.

Hazkani I, Rabinovics N, Limon D, Silvern D, Koren S, Hadar T, Bachar G, Shpitzer T, Popovtzer A. (2016) Brachytherapy for radiotherapy-resistant head and neck cancer: A review of a single center experience. Laryngoscope 126: 2246-2251.

Hinni ML, Ferlito A, Brandwein-Gensler MS, Takes RP, Silver CE, Westra WH, Seethala RR, Rodrigo JP, Corry J, Bradford CR. Hunt JL, Strojan P, Devaney KO, Gnepp DR, Hartl DM, Kowalski LP. Rinaldo A, Barnes L. (2013) Surgical margins in head and neck cancer: a contemporary review. Head Neck 35: 1362-1370.

Ho MW, Field EA, Field JK, Risk JM, Rajlawat BP, Rogers SN, Steele JC, Triantafyllou A, Woolgar JA, Lowe D, Shaw RJ. (2013) Outcomes of oral squamous cell carcinoma arising from oral epithelial dysplasia: rationale for monitoring premalignant oral lesions in a multidisciplinary clinic. Br J Oral Maxillofac Surg, 51: 594-599.

Hodapp N. (2012) [The ICRU Report 83: prescribing, recording and reporting photon-beam intensity-modulated radiation therapy (IMRT)]. Strahlenther Onkol, 188: 97-99.

Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbe C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ. (2010) Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med, 363: 711-723.

Hoffmann C, Cornu N, Hans S, Sadoughi B, Badoual C, Brasnu D. (2016) Early glottic cancer involving the anterior commissure treated by transoral laser cordectomy. Laryngoscope, 126: 1817-1822.

Homer JJ, Fardy MJ. (2016) Surgery in head and neck cancer: United Kingdom National Multidisciplinary Guidelines. J Laryngol Otol, 130: 68-70.

Jay FP, Costas I, Reichman ME. Chapter 2: Cancers of the Head and Neck. In: Ries LAG, Young JL, Keel GE, Eisner MP, Lin YD, Horner M-J (editors). SEER Survival Monograph: Cancer Survival Among Adults: U.S. SEER Program, 1988-2001, Patient and Tumor Characteristics. National Cancer Institute, SEER Program, NIH Pub. Bethesda, MD, USA, 2007: 07-6215.

Jeremic B, Shibamoto Y, Milicic B, Nikolic N, Dagovic A, Aleksandrovic J, Vaskovic Z. Tadic L. (2000) Hyperfractionated radiation therapy with or without concurrent low-dose daily cisplatin in locally advanced squamous cell carcinoma of the head and neck: a prospective randomized trial. J Clin Oncol, 18: 1458-1464.

Jones TM, De M, Foran B, Harrington K, Mortimore S. (2016) Laryngeal cancer: United Kingdom National Multidisciplinary guidelines. J Laryngol Otol, 130: 75-82.

Jordan RC, Lingen MW, Perez-Ordonez B, He X, Pickard R, Koluder M, Jiang B, Wakely P, Xiao W, Gillison ML. (2012) Validation of methods for oropharyngeal cancer HPV status determination in US cooperative group trials. Am J Surg Pathol, 36: 945-954.

Keck MK, Zuo Z, Khattri A, Stricker TP, Brown CD, Imanguli M, Rieke D, Endhardt K, Fang P, Bragelmann J, DeBoer R, El-Dinali M, Aktolga S, Lei Z, Tan P, Rozen SG, Salgia R, Weichselbaum RR, Lingen MW, Story MD, Ang KK, Cohen EE, White KP, Vokes EE, Seiwert TY. (2015) Integrative analysis of head and neck cancer identifies two biologically distinct HPV and three non-HPV subtypes. Clin Cancer Res, 21: 870-881.

Keir ME, Butte MJ, Freeman GJ, Sharpe AH. (2008) PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol, 26: 677-704.

Kerawala C, Roques T, Jeannon JP, Bisase B. (2016) Oral cavity and lip cancer: United Kingdom National Multidisciplinary Guidelines. J Laryngol Otol, 130: 83-89.

Kim HR, Ha SJ, Hong MH, Heo SJ, Koh YW, Choi EC, Kim EK, Pyo KH, Jung I, Seo D, Choi J, Cho BC, Yoon SO. (2016) PD-L1 expression on immune cells, but not on tumor cells, is a favorable prognostic factor for head and neck cancer patients. Sci Rep, 6: 36956.

Kreimer AR, Clifford GM, Boyle P, Franceschi S. (2005) Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. Cancer Epidemiol Biomarkers Prev, 14: 467-475.

Lawrence MS, Sougnez C, Lichtenstein L, Cibulskis K, Lander E, Gabriel SB. (2015) Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature, 517: 576-582.

Leng C, Li Y, Qin J, Ma J, Liu X, Cui Y, Sun H, Wang Z, Hua X, Yu Y, Li H, Zhang J, Zheng Y, Wang W, Zhu J, Wang Q. (2016) Relationship between expression of PD-L1 and PD-L2 on esophageal squamous cell carcinoma and the antitumor effects of CD8+ T cells. Oncol Rep, 35: 699-708.

Levi F, Lucchini F, Negri E, La Vecchia C. (2004) Trends in mortality from major cancers in the European Union, including acceding countries, in 2004. Cancer 101: 2843-2850.

Lewis JS Jr, Chernock RD. (2014) Human papillomavirus and Epstein Barr virus in head and neck carcinomas: suggestions for the new WHO classification. Head Neck Pathol, 8: 50-58.

Lodi G, Sardella A, Bez C, Demarosi F, Carrassi A, (2006) Interventions for treating oral leukoplakia. Cochrane Database Syst Rev, 18: CD001829.

Lorincz BB, Busch CJ, Mockelmann N, Knecht R. (2015) Feasibility and safety of transoral robotic surgery (TORS) for early hypopharyngeal cancer: a subset analysis of the Hamburg University TORS-trial. Eur Arch Otorhinolaryngol, 272: 2993-2998.

Lorincz BB, Mockelmann N, Busch CJ, Munscher A, Sehner S. Dalchow CV, Knecht R. (2015) Two-Year Survival Analysis of 50 Consecutive Head and Neck Cancer Patients Treated with Transoral Robotic Surgery in a Single European Centre. Ann Surg Oncol, 22: 1028-1033.

Lund VJ, Clarke PM, Swift AC, McGarry GW, Kerawala C, Carnell D. (2016) Nose and paranasal sinus tumours: United Kingdom National Multidisciplinary Guidelines. J Laryngol Otol, 130: 111-118.

Lydiatt WM, Patel SG, O'Sullivan B, Brandwein MS, Ridge JA, Migliacci JC, Loomis AM, Shah JP. (2017) Head and Neck cancers-major changes in the American Joint Committee on cancer eighth edition cancer staging manual. CA Cancer J Clin, 67: 122-137.

Maasland DH, van den Brandt PA, Kremer B, Goldbohm RA, Schouten LJ, (2014) Alcohol consumption, cigarette smoking and the risk of subtypes of head-neck cancer: results from the Netherlands Cohort Study. BMC Cancer, 14: 187.

Maier H, Zoeller J, Kreiss M, Sennewald E, Heller WD. (1991) [Oral hygiene and dental status in patients with squamous cell cancers of the upper aerodigestive tract]. HNO, 39: 227-232.

Mandai M, Hamanishi J, Abiko K, Matsumura N, Baba T, Konishi I. (2016) Dual faces of IFN-gamma in cancer progression: a role of PD-L1 induction in the determination of pro- and anti-tumor immunity. Clin Cancer Res, 22: 2329-2334.

Marchevsky AM, Walts AE, (2017) PD-L1, PD-1, CD4 and CD8 Expression in Neoplastic and Non-Neoplastic Thymus. Hum Pathol, 60:16-23.

Marron M, Boffetta P, Zhang ZF, Zaridze D, Wunsch-Filho V, Winn DM, Wei Q, Talamini R, Szeszenia-Dabrowska N, Sturgis EM, Smith E, Schwartz SM, Rudnai P, Purdue MP, Olshan AF, Eluf-Neto J, Muscat J, Morgenstern, H, Menezes A, McClean M, Matos E, Mates IN, Lissowska J, Levi F, Lazarus P, La Vecchia C, Koifman S, Kelsey K, Herrero R, Hayes RB, Franceschi S, Fernandez L, Fabianova E, Daudt AW, Dal Maso L, Curado MP, Cadoni G, Chen C, Castellsague X, Boccia S, Benhamou S, Ferro G, Berthiller J, Brennan P, Moller H, Hashibe M. (2010) Cessation of alcohol drinking, tobacco smoking and the reversal of head and neck cancer risk. Int J Epidemiol, 39: 182-196.

Marur S, D'Souza G, Westra WH, Forastiere AA. (2010) HPV-associated head and neck cancer: a virus-related cancer epidemic. Lancet Oncol, 11: 781-789.

Mayne ST, Cartmel B, Kirsh V, Goodwin WJ Jr. (2009) Alcohol and tobacco use prediagnosis and postdiagnosis, and survival in a cohort of patients with early stage cancers of the oral cavity, pharynx, and larynx. Cancer Epidemiol Biomarkers Prev, 18: 3368-3374.

Mehanna H, Evans M, Beasley M, Chatterjee S, Dilkes M, Homer J, O'Hara J, Robinson M, Shaw R, Sloan P. (2016) Oropharyngeal cancer: United Kingdom National Multidisciplinary Guidelines. J Laryngol Otol, 130: 90-96.

Mehanna HM, Rattay T, Smith J, McConkey CC. (2009) Treatment and follow-up of oral dysplasia - a systematic review and meta-analysis. Head Neck, 31: 1600-1609.

Miles EA, Clark CH, Urbano MT, Bidmead M, Dearnaley DP, Harrington KJ, A'Hern R, Nutting CM. (2005) The impact of introducing intensity modulated radiotherapy into routine clinical practice. Radiother Oncol, 77: 241-246.

Nasman A, Attner P, Hammarstedt L, Du J, Eriksson M, Giraud G, Ahrlund-Richter S, Marklund L, Romanitan M, Lindquist D, Ramqvist T, Lindholm J, Sparen P, Ye W, Dahlstrand H, Munck-Wikland E, Dalianis T. (2009) Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma? Int J Cancer, 125: 362-366.

Nguyen-Tan PF, Zhang Q, Ang KK, Weber RS, Rosenthal DI, Soulieres D, Kim H, Silverman C, Raben A, Galloway TJ, Fortin A, Gore E, Westra WH, Chung CH, Jordan RC, Gillison ML, List M, Le QT. (2014) Randomized phase III trial to test accelerated versus standard fractionation in combination with concurrent cisplatin for head and neck carcinomas in the Radiation Therapy Oncology Group 0129 trial: long-term report of efficacy and toxicity. J Clin Oncol, 32: 3858-3866.

Nutting C, (2016) Radiotherapy in head and neck cancer management: United Kingdom National Multidisciplinary Guidelines. J Laryngol Otol, 130: 66-67.

Nutting CM, Morden JP, Harrington KJ, Urbano TG, Bhide SA, Clark C, Miles EA, Miah AB, Newbold K, Tanay M, Adab F, Jefferies SJ, Scrase C, Yap BK, A'Hern RP, Sydenham MA, Emson M, Hall E. (2011) Parotid-sparing intensity modulated versus conventional radiotherapy in head and neck cancer (PARSPORT): a phase 3 multicentre randomised controlled trial. Lancet Oncol, 12: 127-136.

O'Hara J, Markey A, Homer JJ. (2013) Transoral laser surgery versus radiotherapy for tumour stage 1a or 1b glottic squamous cell carcinoma: systematic review of local control outcomes. J Laryngol Otol, 127: 732-738.

Otto K. (2008) Volumetric modulated arc therapy: IMRT in a single gantry arc. Med Phys, 35: 310-317.

Paleri V, Urbano TG, Mehanna H, Repanos C, Lancaster J, Roques T, Patel M, Sen M. (2016) Management of neck metastases in head and neck cancer: United Kingdom National Multidisciplinary Guidelines. J Laryngol Otol, 130: 161-169.

Pan JJ, Ng WT, Zong JF, Chan LL, O'Sullivan B, Lin SJ, Sze HC, Chen YB, Choi HC, Guo QJ, Kan WK, Xiao YP, Wei X, Le QT, Glastonbury CM, Colevas AD, Weber RS, Shah JP, Lee AW. (2016) Proposal for the 8th edition of the AJCC/UICC staging system for nasopharyngeal cancer in the era of intensity-modulated radiotherapy. Cancer, 122: 546-558.

Papadimitrakopoulou VA, Frank, SJ, Cohen EW, Hirsch FR, Myers JN, Heymach JV, Lin H, Tran HT, Chen CR, Jimeno A, Nedzi L, Vasselli JR, Lowe ES, Raben D. (2016) Phase I study of vandetanib with radiation therapy with or without cisplatin in locally advanced head and neck squamous cell carcinoma. Head Neck, 38: 439-447.

Pardoll DM. (2012) The blockade of immune checkpoints in cancer immunotherapy. Nature reviews. Cancer, 12: 252-264.

Perez-Garcia A, Osca, G, Bosch-Vizcaya A, Kelleher N, Santos NY, Rodriguez R, Gonzalez Y, Roncero JM, Coll R, Serrando M, Lloveras N, Tuset E, Gallardo D. (2013) Kinetics of the CTLA-4 isoforms expression after T-lymphocyte activation and role of the promoter polymorphisms on CTLA-4 gene transcription. Hum Immunol, 74: 1219-1224.

Pignon JP, le Maitre A, Maillard E, Bourhis J. (2009) Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): an update on 93 randomised trials and 17,346 patients. Radiother Oncol, 92: 4-14.

Posner MR, Hershock DM, Blajman CR, Mickiewicz E, Winquist E, Gorbounova V, Tjulandin S, Shin DM, Cullen K, Ervin TJ, Murphy BA, Raez LE, Cohen RB, Spaulding M, Tishler RB, Roth B, Viroglio Rdel C, Venkatesan V, Romanov I, Agarwala S, Harter KW, Dugan M, Cmelak A, Markoe AM, Read PW, Steinbrenner L, Colevas AD, Norris CM Jr, Haddad RI. (2007) Cisplatin and fluorouracil alone or with docetaxel in head and neck cancer. N Engl J Med, 357: 1705-1715.

Pracy P, Loughran S, Good J, Parmar S, Goranova R. (2016) Hypopharyngeal cancer: United Kingdom National Multidisciplinary Guidelines. J Laryngol Otol, 130: 104-110.

Queirolo P, Laurent S, Boitano M, Carrega P, Saverino D, Alviano F, Caielli A, Camoriano M, Ferlazzo G, Pistillo MP. (2009) Targeting ctla-4 directly on melanoma

cells: A possible novel perspective in the immunotherapy of cutaneous melanoma. J Clin Oncol, 27: e22138.

Ramsay AG. (2013) Immune checkpoint blockade immunotherapy to activate anti-tumour T-cell immunity. Br J Haematol, 162: 313-325.

Rethman MP, Carpenter W, Cohen EE, Epstein J, Evans CA, Flaitz CM, Graham FJ, Hujoel PP, Kalmar JR, Koch WM, Lambert PM, Lingen MW, Oettmeier BW Jr, Patton LL, Perkins D, Reid BC, Sciubba JJ, Tomar SL, Wyatt AD Jr, Aravamudhan K, Frantsve-Hawley J, Cleveland JL, Meyer DM. (2010) Evidence-based clinical recommendations regarding screening for oral squamous cell carcinomas. J Am Dent Assoc, 141: 509-520.

Rischin D, Young RJ, Fisher R, Fox SB, Le QT, Peters LJ, Solomon B, Choi J, O'Sullivan B, Kenny LM, McArthur GA. (2010) Prognostic significance of p16INK4A and human papillomavirus in patients with oropharyngeal cancer treated on TROG 02.02 phase III trial. J Clin Oncol, 28: 4142-4148.

Rodriguez CP, Adelstein DJ, Rybicki LA, Saxton JP, Lorenz RR, Wood BG, Scharpf J, Ives DI. (2012) Single-arm phase II study of multiagent concurrent chemoradiotherapy and gefitinib in locoregionally advanced squamous cell carcinoma of the head and neck. Head Neck, 34: 1517-1523.

Roosaar A, Yin L, Johansson AL, Sandborgh-Englund G, Nyren O, Axell T. (2007) A long-term follow-up study on the natural course of oral leukoplakia in a Swedish population-based sample. J Oral Pathol Med, 36: 78-82.

Rosenquist K, Wennerberg J, Schildt EB, Bladstrom A, Goran Hansson B, Andersson G. (2005) Oral status, oral infections and some lifestyle factors as risk factors for oral and oropharyngeal squamous cell carcinoma. A population-based case-control study in southern Sweden. Acta Otolaryngol, 125: 1327-1336.

Salimi M, Abi KST, Nedaie HA, Hassani H, Gharaati H, Samei M, Shahi R, Zarei H. (2017) Assessment and Comparison of Homogeneity and Conformity Indexes in Step-and-Shoot and Compensator-Based Intensity Modulated Radiation Therapy (IMRT) and Three-Dimensional Conformal Radiation Therapy (3D CRT) in Prostate Cancer. J Med Signals Sens, 7: 102-107.

Salvi S, Fontana V, Boccardo S, Merlo DF, Margallo E, Laurent S, Morabito A, Rijavec E, Dal Bello MG, Mora M, Ratto GB, Grossi F, Truini M, Pistillo MP. (2012) Evaluation of CTLA-4 expression and relevance as a novel prognostic factor in patients with non-small cell lung cancer. Cancer Immunol Immunother, 61: 1463-1472.

Santarpia M, Gonzalez-Cao M, Viteri S, Karachaliou N, Altavilla G, Rosell R. (2015) Programmed cell death protein-1/programmed cell death ligand-1 pathway inhibition and predictive biomarkers: understanding transforming growth factor-beta role. Transl Lung Cancer Res, 4: 728-742.

Schache AG, Liloglou T, Risk JM, Filia A, Jones TM, Sheard J, Woolgar JA, Helliwell TR, Triantafyllou A, Robinson M, Sloan P, Harvey-Woodworth C, Sisson D, Shaw RJ. (2011) Evaluation of human papilloma virus diagnostic testing in oropharyngeal squamous cell carcinoma: sensitivity, specificity, and prognostic discrimination. Clin Cancer Res, 17: 6262-6271.

Schantz SP, Harrison LB, Forastiere A. Tumors of the nasal cavity and paranasal sinuses, nasopharynx, oral cavity, and oropharynx. In: DeVita VT, Hellman SA, Rosenberg SA (editors). Cancer: principles and practice of oncology. 6th ed. Lippincott Williams & Wilkins, Philadelphia, PA, USA, 2001: 797-860.

Schmults CD, Karia PS, Carter JB, Han J, Qureshi AA. (2013) Factors predictive of recurrence and death from cutaneous squamous cell carcinoma: a 10-year, single-institution cohort study. JAMA dermatol, 149: 541-547.

Seiwert TY, Haddad RI, Gupta S. (2015) Antitumor activity and safety of pembrolizumab in patients (pts) with advanced squamous cell carcinoma of the head and neck (SCCHN): preliminary results from KEYNOTE-012 expansion cohort. J Clin Oncol, 33: LBA6008.

Seiwert TY, Zuo Z, Keck MK, Khattri A, Pedamallu CS, Stricker T, Brown C, Pugh TJ, Stojanov P, Cho J, Lawrence MS, Getz G, Bragelmann J, DeBoer R, Weichselbaum RR, Langerman A, Portugal L, Blair E, Stenson K, Lingen MW, Cohen EE, Vokes EE, White KP, Hammerman PS. (2015) Integrative and comparative genomic analysis of HPV-positive and HPV-negative head and neck squamous cell carcinomas. Clin Cancer Res, 21: 632-641.

Shaw R, Beasley N. (2016) Aetiology and risk factors for head and neck cancer: United Kingdom National Multidisciplinary Guidelines. J Laryngol Otol, 130: 9-12.

Shim SJ, Cha J, Koom WS, Kim GE, Lee CG, Choi EC, Keum KC. (2010) Clinical outcomes for T1-2N0-1 oral tongue cancer patients underwent surgery with and without postoperative radiotherapy. Radiat Oncol, 5: 43.

Simo R, Robinson M, Lei M, Sibtain A, Hickey S. (2016) Nasopharyngeal carcinoma: United Kingdom National Multidisciplinary Guidelines. J Laryngol Otol, 130: 97-103.

Soulieres D, Faivre S, Mesia R, Remenar E, Li SH, Karpenko A, Dechaphunkul A, Ochsenreither S, Kiss LA, Lin JC, Nagarkar R, Tamas L, Kim SB, Erfan J, Alyasova A, Kasper S, Barone C, Turri S, Chakravartty A, Chol M, Aimone P, Hirawat S, Licitra L. (2017) Buparlisib and paclitaxel in patients with platinum-pretreated recurrent or metastatic squamous cell carcinoma of the head and neck (BERIL-1): a randomised, double-blind, placebo-controlled phase 2 trial. Lancet Oncol, 18: 323-335.

Soulieres D, Senzer NN, Vokes EE, Hidalgo M, Agarwala SS, Siu LL. (2004) Multicenter phase II study of erlotinib, an oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with recurrent or metastatic squamous cell cancer of the head and neck. J Clin Oncol, 22: 77-85.

Staffurth J. (2010) A review of the clinical evidence for intensity-modulated radiotherapy. Clin Oncol (R Coll Radiol), 22: 643-657.

Strauss L, Bergmann C, Gooding W, Johnson JT, Whiteside TL. (2007) The frequency and suppressor function of CD4+CD25highFoxp3+ T cells in the circulation of patients with squamous cell carcinoma of the head and neck. Clin Cancer Res, 13: 6301-6311.

Szturz P, Vermorken JB. (2017) Immunotherapy in head and neck cancer: aiming at EXTREME precision. BMC Med, 15: 110.

Tan EH, Adelstein DJ, Droughton ML, Van Kirk MA, Lavertu P. (1997) Squamous cell head and neck cancer in nonsmokers. Am J Clin Oncol, 20: 146-150.

Tang MW, Oakley R, Dale C, Purushotham A, Moller H, Gallagher JE. (2014) A surgeon led smoking cessation intervention in a head and neck cancer centre. BMC Health Serv Res, 14: 636.

Teng MW, Ngiow SF, Ribas A, Smyth MJ. (2015) Classifying Cancers Based on T-cell Infiltration and PD-L1. Cancer Res, 75: 2139-2145.

Teoh M, Clark CH, Wood K, Whitaker S, Nisbet A. (2011) Volumetric modulated arc therapy: a review of current literature and clinical use in practice. Br J Radiol, 84: 967-996.

Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. (2015) Global cancer statistics, 2012. CA Cancer J Clin, 65: 87-108.

Vankos JB, Piurko V, Suba Z, Nemeth Z, Timar J, Kenessey I. (2015) [The prognostic role of expression of p16 tumor suppressor gene in Hungarian patients with oral squamous cell carcinoma]. Magy Onkol, 59: 352-359.

Vassilakopoulou M, Avgeris M, Velcheti V, Kotoula V, Rampias T, Chatzopoulos K, Perisanidis C, Kontos CK, Giotakis AI, Scorilas A, Rimm D, Sasaki C, Fountzilas G, Psyrri A. (2016) Evaluation of PD-L1 Expression and Associated Tumor-Infiltrating Lymphocytes in Laryngeal Squamous Cell Carcinoma. Clin Cancer Res, 22: 704-713.

Vermorken JB, Mesia R, Rivera F, Remenar E, Kawecki A, Rottey S, Erfan J, Zabolotnyy D, Kienzer HR, Cupissol D, Peyrade F, Benasso M, Vynnychenko I, De Raucourt D, Bokemeyer C, Schueler A, Amellal N, Hitt R. (2008) Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med, 359: 1116-1127.

Vermorken JB, Remenar E, van Herpen C, Gorlia T, Mesia R, Degardin M, Stewart JS, Jelic S, Betka J, Preiss JH, van den Weyngaert D, Awada A, Cupissol D, Kienzer HR, Rey A, Desaunois I, Bernier J, Lefebvre JL. (2007) Cisplatin, fluorouracil, and docetaxel in unresectable head and neck cancer. N Engl J Med, 357: 1695-1704.

Ward FJ, Dahal LN, Wijesekera SK, Abdul-Jawad SK, Kaewarpai T, Xu H, Vickers MA, Barker RN. (2013) The soluble isoform of CTLA-4 as a regulator of T-cell responses. Eur J Immunol, 43: 1274-1285.

Weller MD, Nankivell PC, McConkey C, Paleri V, Mehanna HM. (2010) The risk and interval to malignancy of patients with laryngeal dysplasia; a systematic review of case series and meta-analysis. Clin Otolaryngol, 35: 364-372.

Wolff KD, Follmann M, Nast A. (2012) The diagnosis and treatment of oral cavity cancer. Dtsch Arztebl Int, 109: 829-835.

Wu P, Wu D, Li L, Chai Y, Huang J. (2015) PD-L1 and Survival in Solid Tumors: A Meta-Analysis. PLoS One, 10: e0131403.

Yokosuka T, Takamatsu M, Kobayashi-Imanishi W, Hashimoto-Tane A, Azuma M, Saito T. (2012) Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. J Exp Med, 209: 1201-1217.

Yu H, Yang J, Jiao S, Li Y, Zhang W, Wang J, (2015) Cytotoxic T lymphocyte antigen 4 expression in human breast cancer: implications for prognosis. Cancer Immunol Immunother, 64: 853-860.

Zevallos JP, Mallen MJ, Lam CY, Karam-Hage M, Blalock J, Wetter DW, Garden AS, Sturgis EM, Cinciripini PM. (2009) Complications of radiotherapy in laryngopharyngeal cancer: effects of a prospective smoking cessation program. Cancer, 115: 4636-4644.

Zhang Y, Kang S, Shen J, He J, Jiang L, Wang W, Guo Z, Peng G, Chen G, He J, Liang W. (2015) Prognostic significance of programmed cell death 1 (PD-1) or PD-1 ligand 1 (PD-L1) Expression in epithelial-originated cancer: a meta-analysis. Medicine, 94: e515.

Zou MX, Peng AB, Lv GH, Wang XB, Li J, She XL, Jiang Y. (2016) Expression of programmed death-1 ligand (PD-L1) in tumor-infiltrating lymphocytes is associated with favorable spinal chordoma prognosis. Am J Transl Res, 8: 3274-3287.

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11 BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

Publications related to the thesis

1. **Birtalan E**, Danos K, Gurbi B, Brauswetter, Halasz J, Kalocsane Piurko V, Acs P, Antal B, Mihalyi R, Pato A, Fent Z, Polony G, Timar J, Tamas L. (2017) Expression of PD-L1 on immune cells shows better prognosis in laryngeal, oropharygeal and hypopharyngeal cancer. *Appl Immunohistochem Mol Morphol,* doi: 10.1097/PAI. 0000000000000590. *(IF:1.634)*
2. Brauswetter D, **Birtalan E**, Danos K, Kocsis A, Krenacs T, Timar J, Mihalyi R, Horcsik D, Polony G, Tamas L, Petak I (2017). $p16^{INK4}$ expression is of prognostic and predictive value in oropharyngeal cancers independent of human papillomavirus status: a Hungarian study. *Eur Arch Otorhinolaryngol,* 274: 1959-1965. *(IF: 1,660)*

Publications not related to the thesis

1. Acs B, Madaras L, Tokes AM, Kovacs AK, Kovacs E, Ozsvari-Vidakovich M, Karaszi A, **Birtalan E**, Dank M, Szasz AM, Kulka J. (2017) PD-1, PD-L1 and CTLA-4 in pregnancy-related - and in early-onset breast cancer: A comparative study. *Breast,* 35: 69-77. *(IF: 2.801)*
2. **Birtalan E**, Dános K, Tamás L. (2016) Immunológiai megfontolások a humán papillomavírus asszociált fej-nyaki daganatok megelőzésében és kezelésében. [Hungarian] *Otorhinolaryngologia Hungarica,* 62: 54–x. *(IF: 0)*
3. Danos K, Brauswetter D, **Birtalan E**, Pato A, Bencsik G, Krenacs T, Petak I, Tamas L. (2016) The Potential Prognostic Value of Connexin 43 Expression in Head and Neck Squamous Cell Carcinomas. *Appl Immunohistochem Mol Morphol,* 24: 476-481. *(IF: 1,634)*
4. Brauswetter D, Danos K, Gurbi B, Felegyhazi EF, **Birtalan E**, Meggyeshazi N, Krenacs T, Tamas L, Petak I. (2016) Copy number gain of PIK3CA and MET is associated with poor prognosis in head and neck squamous cell carcinoma. *Virchows Archiv : an international journal of pathology* 468, 579-587. *(IF: 2,848)*

5. Dános K, **Birtalan E**, Brauswetter D, Tamás L. (2015) A humán (emberi) papillomavírus (HPV) szerepe a szájgarat daganatainak kialakulásában, kórismézében és kezelésében. [Hungarian] - review *Nőgyógyászati Onkológia,* 20,: 8-11. *(IF: 0)*
6. Szentkúti G, Brauswetter D, Dános K, **Birtalan E**, Tamás L. (2014) A fej-nyaki daganatok magas incidenciájának és mortalitásának okai hazánkban. [Hungarian] *Otorhinolaryngologia Hungarica,* 60: 77-80. *(IF: 0)*
7. Kovacs A, Olah A, Lux A, Matyas C, Nemeth BT, Kellermayer D, Ruppert M, Torok M, Szabo L, Meltzer A, Assabiny A, **Birtalan E**, Merkely B, Radovits T. (2015) Strain and strain rate by speckle-tracking echocardiography correlate with pressure-volume loop-derived contractility indices in a rat model of athlete's heart. *American journal of physiology Heart and circulatory physiology,* 308: 743-748. *(IF: 3.348)*
8. Matyas C, Nemeth BT, Olah A, Hidi L, **Birtalan E**, Kellermayer D, Ruppert M, Korkmaz-Icoz S, Kokeny G, Horvath EM, Szabo G, Merkely B, Radovits T. (2015) The soluble guanylate cyclase activator cinaciguat prevents cardiac dysfunction in a rat model of type-1 diabetes mellitus. *Cardiovascular diabetology,* 14:145. *(IF: 4.752)*
9. Oláh A, Németh BT, Mátyás C, Horváth EM, Hidi L, **Birtalan E**, Kellermayer D, Ruppert M, Merkely G, Szabó G, Merkely B, Radovits T. (2014) Cardiac effects of acute exhaustive exercise in a rat model. *International journal of cardiology,* 182: 258-266. *(IF: 6.189)*
10. Radovits T, Olah A, Lux A, Nemeth BT, Hidi L, **Birtalan E**, Kellermayer D, Matyas Cs, Szabo G, Merkely B. (2013) Rat model of exercise-induced cardiac hypertrophy: hemodynamic characterization using left ventricular pressure-volume analysis. *American Journal of Physiology-Heart and Circulatory Physiology,* 305: 124-134. *(IF: 3.348)*

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13 SUPPLEMENTARY TABLES

Supplementary Table 1A. PD-L1 IC x TIL Crosstabulation
(PD-L1: programmed death-ligand 1, IC: immune cell, TIL: tumor infiltrating lymphocyte)

		TIL			Total
		1	**2**	**3**	
PD-L1 IC negative	**N**	13	3	14	30
	%	43.30%	10.00%	46.70%	100.00%
PD-L1 IC positive	**N**	11	17	35	63
	%	17.50%	27.00%	55.60%	100.00%
Total	**N**	24	20	49	93
	%	25.80%	21.50%	52.70%	100.00%

Chi-square: p=0.016

Supplementary Table 1B. PD-L1 IC x TIL Crosstabulation (in hypopharynx)
(PD-L1: programmed death-ligand 1, IC: immune cell, TIL: tumor infiltrating lymphocyte)

		TIL			Total
		1	**2**	**3**	
PD-L1 IC negative	**N**	5	0	1	6
	%	83.30%	0.00%	16.70%	100.00%
PD-L1 IC positive	**N**	2	2	11	15
	%	13.30%	13.30%	73.30%	100.00%
Total	**N**	7	2	12	21
	%	33.30%	9.50%	57.10%	100.00%

Fisher's test: p=0.006

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Supplementary Table 1C. CTLA-4 IC x TIL Crosstabulation
(CTLA-4: cytotoxic T-lymhocyte-associated protein 4, TIL: tumor infiltrating lymphocyte, IC: immune cell)

		TIL			Total
		1	**2**	**3**	
CTLA-4 IC negative	**N**	18	9	18	45
	%	40.00%	20.00%	40.00%	100.00%
CTLA-4 IC positive	**N**	6	10	29	45
	%	13.30%	22.20%	64.40%	100.00%
Total	**N**	24	19	47	90
	%	26.70%	21.10%	52.20%	100.00%

Chi-square: p=0.013

Supplementary Table 1D. CTLA4 IC x TIL Crosstabulation (in hypopharynx)
(CTLA4: cytotoxic T-lymhocyte-associated protein 4, TIL: tumor infiltrating lymphocyte, IC: immune cell)

		TIL			Total
		1	**2**	**3**	
CTLA-4 IC negative	**N**	6	0	4	10
	%	60.00%	0.00%	40.00%	100.00%
CTLA-4 IC positive	**N**	1	2	8	11
	%	9.10%	18.20%	72.70%	100.00%
Total	**N**	7	2	12	21
	%	33.30%	9.50%	57.10%	100.00%

Fisher's test: p=0.028

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Supplementary Table 2A. PD-L1 IC x CTLA4 IC Crosstabulation

(PD-L1: programmed death-ligand 1, CTLA4: cytotoxic T-lymhocyte-associated protein 4, IC: immune cell)

		PD-L1 IC negative	PD-L1 IC positive	Total
CTLA-4 IC negative	**N**	20	28	48
	%	41.70%	58.30%	100.00%
CTLA-4 IC positive	**N**	11	37	48
	%	22.90%	77.10%	100.00%
Total	**N**	31	65	96
	%	32.30%	67.70%	100.00%

Chi-square: p=0.049

Supplementary Table 2B. PD-L1 TC x PD-1 Crosstabulation

(PD-L1: programmed death-ligand 1, TC: tumor cell, PD-1: programmed cell death protein 1)

		PD-1 negative	PD-1 positive	Total
PD-L1 TC negative	**N**	**59**	**9**	**68**
	%	86.80%	13.20%	100.00%
PD-L1 TC positive	**N**	11	20	31
	%	35.50%	64.50%	100.00%
Total	**N**	70	29	99
	%	70.70%	29.30%	100.00%

Chi-square: p<0.001

Supplementary Table 2C. PD-L1 TC x PD-1 Crosstabulation (in oropharynx)

(PD-L1: programmed death-ligand 1, TC: tumor cell, PD-1: programmed cell death protein 1)

		PD-1 negative	PD-1 positive	Total
PD-L1 TC negative	**N**	25	2	27
	%	92.60%	7.40%	100,00%
PD-L1 TC positive	**N**	3	7	10
	%	30.00%	70.00%	100.00%
Total	**N**	28	9	37
	%	75.70%	24.30%	100.00%

Fisher's test: p<0.001

Supplementary Table 2D. PD-L1 TC x PD-1 Crosstabulation (in larynx)

(PD-L1: programmed death-ligand 1, TC: tumor cell, PD-1: programmed cell death protein 1)

		PD-1 negative	PD-1 positive	Total
PD-L1 TC negative	**N**	24	3	27
	%	88.90%	11.10%	100,00%
PD-L1 TC positive	**N**	6	7	13
	%	46.20%	53.80%	10.00%
Total	**N**	30	10	40
	%	75.00%	25.00%	100.00%

Fisher's test: p=0.006

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Supplementary Table 2E. PD-L1 TC x HPV Status Crosstabulation (in oropharynx)

(PD-L1: programmed death-ligand 1, TC: tumor cell, HPV: human papillomavirus)

		HPV negative	HPV positive	Total
PD-L1 TC negative	**N**	25	3	28
	%	89.00%	10.70%	100,00%
PD-L1 TC positive	**N**	5	5	10
	%	50.00%	50.00%	100.00%
Total	**N**	30	8	38
	%	78.90%	21.10%	100.00%

Fisher's test: p=0.019

Supplementary Table 3. PD-L2 x PD-1 Crosstabulation (in HPV negative tumors)

(PD-L2: programmed death-ligand 2, PD-1: programmed cell death protein 1, HPV: human papillomavirus)

		PD-1 negative	PD-1 positive	Total
PD-L2 negative	**N**	16	13	29
	%	55.20%	44.80%	100,00%
PD-L2 positive	**N**	49	14	63
	%	77.80%	22.20%	100.00%
Total	**N**	65	27	92
	%	70.70%	29.30%	100.00%

Chi-square: p=0.027