Dissertation

Detection of serological HPV16 L1 status in patients with oropharyngeal carcinoma

Submitted by

Dr.med.univ. Thomas Weiland

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Ear, Nose and Throat University Hospital Graz

Under the Supervision of

Assoz.-Prof. PD. Dr. Valentin Tomazic, PhD

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of "Good Scientific Practice".

Thomas Weiland eh, May 2020

Disclosures

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List of co-authors and their institutions:

Alexander Eckert[3], Peter Valentin Tomazic[1], Axel Wolf[1], Prisca Pondorfer[1], Sarah Vasicek[1], Matthias Graupp[1], Clemens Holzmeister[1], Ulrich Moser[1], Alexandros Andrianakis[1], Georg Kangler[1], Peter Kiss[1], Luka Brcic[2], Matthias Kappler[3], Claudia Wickenhauser[4], Anja Haak[4], Maximilian Krüger[5], Bilal Al-Nawas[5], Sebastian Blatt[5], Norbert Brockmeyer[6], Adriane Skaletz-Rorowski[6], Anja Potthoff[6], Lars E. French[7], Sara Charnowski[7], Markus Reinholz[7], Andreas M. Kaufmann[8], Sarah Thies[8], Hans-Georg Lambrecht[9], Barbara Seliger[10], Dominik C. Wild[11] and Dietmar Thurnher[1]

[1]Department of Otorhinolaryngology-Head&Neck Surgery, Medical University of Graz, Graz, Austria

[2]Diagnostic and Research Institute of Pathology, Medical University of Graz, Graz, Austria

[3]Department of Oral and Maxillofacial Plastic Surgery, Martin Luther University Halle-Wittenberg, Halle, Germany

[4]Institute of Pathology, Martin Luther University Halle-Wittenberg, Halle, Germany

[5]Department of Oral and Maxillofacial Surgery-Plastic Surgery, University Medical Center Mainz, Mainz, Germany

[6]Department of Dermatology, Venerology, and Allergology, Center for Sexual Health and Medicine, WIR – Walk In Ruhr, Ruhr University Bochum, Bochum, Germany
[7]Department of Dermatology and Allergy, University Hospital, LMU Munich, Munich, Germany
[8]Clinic for Gynaecology, Gynaecological Tumor Immunology, Charité Campus Benjamin Franklin, Berlin, Germany
[9]Bioscientia Institute for Medical Diagnostics, Ingelheim, Germany
[10]Institue of Medical Immunology, Martin Luther University Halle-Wittenberg, Halle, Germany
[11]Department of Otorhinolaryngology-Head&Neck Surgery, General Hospital Ried im Innkreis, Ried, Austria

All co-authors have explicitly agreed to the use of their data in my thesis.

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Table of Contents

Abbreviations

CT	Computer tomography
CDK	Cyclin dependent kinase
DKFZ	Deutsches Krebsforschungszentrum
DNA	Desoxyribonuleic acid
E-Proteins	Early Proteins (E1-E7)
EGF	Epidermal growth factor
GST	Gluthathione-S-Transferase
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
IHC	Immunohistochemistry
L-Proteins	Late Oncoproteins (L1, L2)
LCR	Long control region
MRI	Magnetic resonance imaging
OPSCC	Oropharyngeal squamous cell carcinoma
ORF	Open reading frame
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
pRb	Retinoblastoma protein
RCT	Radiochemotherapy
RIT	Radioimmunotherapy
RT	Radiotherapy
SCC	Squamous cell carcinoma
URR	Upstream regulatory region
VLP	Virus like particle

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Abstract (English)

Introduction

Although demonstrably associated with better therapy response and survival rates, the majority of HPV-induced oropharyngeal squamous cell carcinomas (OPSCCs) are still primarily diagnosed at advanced tumor stages significantly reducing patients' prognosis. Since cell-based screening methods compared to the Pap-smear test in cervical carcinoma were proven to be unsuitable in OPSCC, attempts to develop reliable blood-based assays for detection of antibodies to relevant HPV-antigens became the focus of scientific interest. This study aims to assess the clinical performance of a newly developed HPV16-L1 DRH1 epitope-specific serological assay.

Material and Methods

In a prospective observational study, sera of 34 HNSCC patients and 1064 healthy controls were analyzed for the presence of HPV16-L1 antibodies using a novel subtype-specific competitive immunoassay based on the monoclonal mouse antibody DRH1. In the HNSCC cohort, serum samples were collected prior to treatment and in intervals of 3 to 6 months between september 2016 and November 2018 during clinical follow-up which was performed according to national guidelines including careful physical examination, soft tissue sonography of the neck and further imaging (CT/MRI) if indicated. HPV tumor status was analyzed by PCR-based detection of HPV-DNA including genotyping and p16 immunohistochemistry (ICH).

Results

The evaluated immunoassay showed a sensitivity of 95% (95% CI 77.2 – 99.9%) and an associated positive predictive value of 45.65% for HPV16-driven HNSCCs. On the basis of the healthy cohort, overall diagnostic specificity was calculated with 99.46% for men and 99.29% for women > 30 years of age. During serological follow-up, a decrease of antibody titers between 30 to 100% was observed in the majority of HPV16-driven HNSCC patients living disease free of up to 26 months. In one case, a sudden rise of antibody titer during follow-up was linked to tumor recurrence in the lungs.

Conclusion

It was shown for the first time that the specific detection of antibodies to HPV16-L1 is indicative for the course of HPV16-induced HNSCC. On the basis of its high sensitivity and specificity, the evaluated immunoassay seems to be a promising tool to reliably identify relevant HPV16-related disease and might be useful for measuring treatment response and disease control along follow-up.

Abstract (German)

Einführung

Trotz besseren Therapieansprechens und besseren Überlebensraten werden die meisten HPV-induzierten OPSCCs in fortgeschrittenen Tumorstadien diagnostiziert, was die Prognose signifikant verringert. Da sich zellbasierte Screening-Methoden bei OPSCC im Vergleich zum Pap-Test beim Zervixkarzinom nicht geeignet zeigten, waren blutbasierte Antikörperassays gegen relevante HPV-Antigene von großem wissenschaftlichen Interesse. Anhand dieser Studie soll ein neu entwickelter epitopspezifischer serologischer HPV16-L1 DRH1-Assay im klinischen Setting bewertet werden.

Material und Methoden

In einer prospektiven Studie wurden Seren von 34 Patienten mit HNSCCs und 1064 gesunden Kontrollen mittels neuem Immunoassay auf der Basis des monoklonalen Maus-Antikörpers DRH1 auf HPV16-L1-Antikörper untersucht. In der HNSCC-Kohorte wurden Seren vor Behandlung und in Intervallen von 3 bis 6 Monaten zwischen September 2016 und November 2018 während der Nachsorge entnommen, die gemäß den nationalen Richtlinien durchgeführt wurde, mittels körperlicher Untersuchung, Sonographie des Halses und weiterer Bildgebung (CT / MRT), falls angezeigt. Der HPV-Tumorstatus wurde durch PCR-basierten Nachweis von HPV-DNA inklusive Genotypisierung und p16-Immunhistochemie (ICH) erhoben.

Ergebnisse

Der Test zeigte eine Sensitivität von 95% (95% CI 77.2 – 99.9%) und einen PPR von 45.65% für HPV16-assoziierte HNSCCs. Anhand der gesunden Kohorte wurde die diagnostische Spezifität mit 99.46% für Männer und 99.29% für Frauen > 30 Jahre berechnet. Im Follow-up wurde den meisten der HPV16-induzierten HNSCC-Patienten eine Abnahme der Antikörpertiter zwischen 30 und 100% beobachtet. In einem Fall war ein plötzlicher Anstieg der Antikörper im Rahmen der Nachsorge mit dem Auftreten eines Rezidivtumors in der Lunge verbunden.

Schlussfolgerung

Zum ersten Mal konnte gezeigt werden, dass der spezifische Nachweis von Antikörpern gegen HPV16-L1 auf den Verlauf eines HPV16-induzierten HNSCCs hinweist. Aufgrund seiner hohen Sensitivität und Spezifität scheint der evaluierte Immunoassay ein vielversprechendes Instrument zur Identifizierung relevanter HPV16-bedingter Erkrankungen zu sein und könnte zur Messung des Therapieerfolgs und der Krankheitskontrolle während der Nachsorge nützlich sein.

Foreword

Human papilloma viruses are a large family of epitheliotropic DNA tumor viruses, representing one of the most common sexually transmitted infectious agents worldwide (1). The majority of HPV infections persist in the general population as commensals, causing inapparent or asymptomatic infections, rather than being associated with obvious disease (1). Ever since Prof. Zur Hausen was able to show the correlation between HPV infection and tumorigenesis of cervical carcinoma in women (2), we know to date, that HPV represents one of the most oncogenic viruses causing a wide range of malign tumors such as anogenital or oropharyngeal carcinomas.

Globally, 570,000 women and 60,000 men are suffering from HPV-induced cancers per year, implying 8.6% and 0.8% of all cancers occurring worldwide (3). With 530,000 new cases per year, cervical cancer accounts for the vast majority of all HPV-attributable cancer cases worldwide (3). Whereas more than two-thirds of cervical cancer cases are diagnosed in less developed countries, in contrary, the geographical distribution of HPV-induced cancers of the head and neck is diametrically different (3). The distinct majority occurs in more developed countries, with relatively high age-standardized incidence rates (over 1.25 per 100,000) in Northern America and Europe (3).

Over the last decades, it was observed that incidence rates of HPV-driven OPSCCs have been rising significantly in these countries (4). The prevalence of cervical cancer cases varies mainly due to differences in the occurrence of cervical HPV infection within a population and the presence or non-presence of adequate cervical cancer screening (3).

The collection and characterization of suspicious cells are in the focus of HPV-related screening strategies in cervical cancer. For squamous cell carcinomas, two areas of cancer origin are discussed in the literature: the squamous columnar junction and the cervical transformation zone, which are limited in size and simply accessible for collection of relevant cells by a smear or biopsy (5).

By contrast, there are no validated cell-dependent secondary prevention strategies for HPV-induced squamous cell carcinomas of the head and neck region (HNSCC) (6). Thus these tumors can be multifocal, the majority are to be found in the oropharynx, especially in the tonsils (7). Mainly for reasons of surface amplification the specific anatomical structure of tonsils shows

multiple deep crypts, where small tumors can easily hide. Therefore it is much less likely to gather representative cells by comparable smears in a screening attempt, where clinical signs of a tumor are missing. Currently, final diagnosis relies on morphological changes of tissue gathered by biopsy in cases where a neoplastic process is already macroscopically obvious. Often OPSCCs are confirmed in advanced stages when metastatic spreading to the local lymph nodes already took place (8). In fact, unilateral swelling of the neck due to lymph node metastasis is in many cases the first symptom to initiate the way to the doctor (8). These facts have been raising the question, whether there is a method for risk stratification for patients in addition to the survey of classic risk factors such as smoking and alcohol.

Attempts to develop blood-based assays to reveal relevant HPV-induced disease were hampered for several reasons: On the one hand, HPV-infection does not obligatorily lead to disease, on the other hand, there was no evidence that serum antibodies could differentiate between HPV-driven tumor disease and subclinical HPV-infection (5). A newly developed blood-based immunoassay for the detection of subtype-specific HPV-16 L1 antibodies promises to reliably distinguish between relevant HPV-induced disease and subclinical HPV-infection. The aim of the present study was to assess the clinical performance of the test used in a cohort of HNSCC patients and healthy controls.

1 Introduction

1.1 Human Papilloma Virus

The human papilloma virus is part of the family of papovaviridae (9). The non-encapsulated ikosaedric capsid measures between 50 to 55 nm in diameter consisting of 72 capsomers (9). Its circular double-stranded DNA composes of about 7,900 base pairs with mostly eight open reading frames (10). The genomic organization of all papilloma viruses is highly similar consisting of an "E"-region encoding for the "early"-proteins, an "L"-region encoding for the "late"-proteins and a preceding non-encoding region with regulatory function (Upstream Regulatory Region: URR) (1). The URR includes the binding sites for viral replication proteins E1 and E2 as well as for the intracellular transcription factor SP1 and p97, the promoter for early viral transcription (1). Adjoining, the E-region encodes with E1, E2, E4, E5, E6 and E7 for non-structural viral proteins (11). The L-region encodes for two structural proteins: the major capsid protein L1 representing about 80% of the virion and the minor capsid protein L2 (11). The structural relationship between those two hasn't been entirely resolved (11). Type-specific conformational antigen-determinants are located on the outer surface of the virion (11).

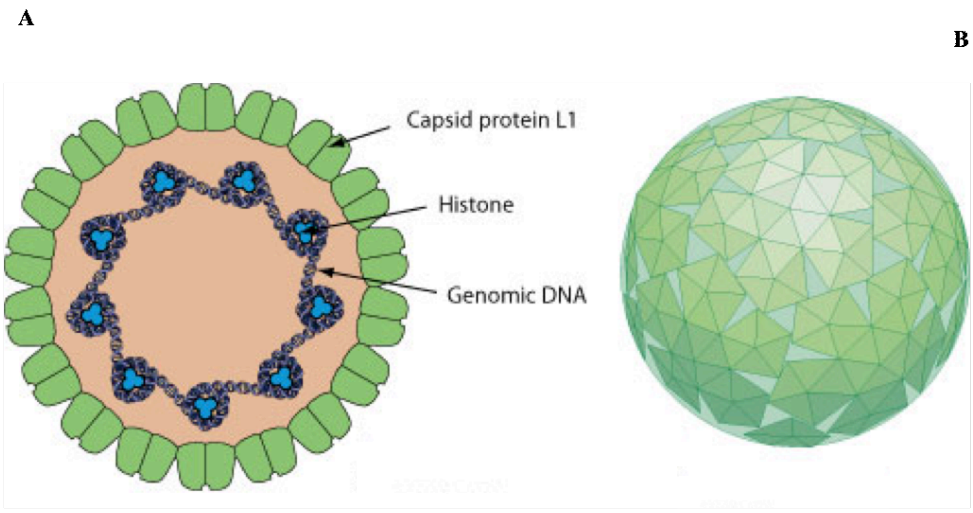

Figure 1: HPV structure

A: Cross-Section of the viral structure showing the L1 protein encapsulating the genomic DNA
B: 3-D-Modell of the capsid structure of a Human Papilloma Virus Particle
Source: ViralZone www.expasy.org/viralzone, Swiss Institute of Bioinformatics (12)

The papilloma virus was first discovered by Richard Shope in 1933 who managed to isolate the virus from a rabbit's tumor (13). First electro-microscopic examinations of the virion took place in 1968 (14). In the mid-seventies first molecular-biologic examinations and classifications were performed, hampered by lacking of appropriate cell cultural systems (15). To date, over 200 types of the papilloma virus were identified, whereas the majority is potentially human-infectious (1).

The accepted classification to differentiate between the subtypes is based on sequence homologies of the highly conserved open-reading frame of the L1 protein (16). To define a new type, an entirely cloned genome must show a deviation of at least 10% of the sequence homology of the L1 protein compared to the next relative (16). In general, genomic sequence homology between all HPV subtypes is with over 90% very high, whereas the viral genome seems to have a static character lacking of frequent sequence changes by mutation or recombination (16). Such mutational frequencies are comparable to those of the infected host's genome (16).

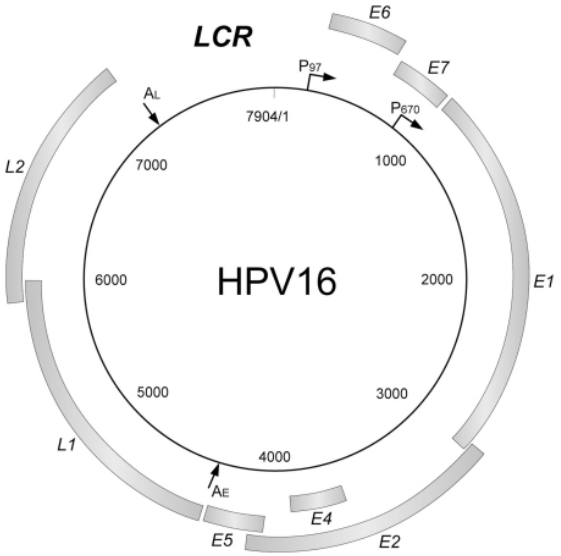

Figure 2: Structure of HPV16 genome

The genome of HPV16 consists of 7904 base pairs and encodes for 8 genes. It is organized in three subdivisions: a non-coding sequence, the early (E1, E2, E4, E5, E6, E7) and the late (L1, L2) genes. The early ORFs (E1-E7) are either expressed by the p97 or p670 promoter at various stages of epithelial differentiation. The late ORFs of the structure proteins L1 and L2 are expressed under the control of p670. The p97 promoter is located in a non-coding control area of the genome (Long Control Region = LCR). (17)

1.1.1 HPV Life Cycle

Like every virus, HPV is reliant on infecting a host to ensure replication of its genome and safeguard its viability (18). Its target locations are cells of the basal layer of keratinized or non-keratinized epithelium (18). Depending on the site of infection, it is distinguished between mucosal HPV-types causing mucosal infections and cutaneous HPV-types causing infections of the external skin (18). HPV-infections can have an asymptomatic clinical course, they can cause warts or be associated with other benign or malign neoplasms (1). Within the numerous HPV genotypes, depending on the associated risk of disease, a distinction is made between low-risk-HPV-types (e.g.: HPV-6, -11, -40, -42, -43, -44, -54, -61, -70, -72, -81) causing benign neoplasia and high-risk-HPV-types (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -73, -82) potentially inducing precancerous lesions respectively malign neoplasia (19). Micro traumas of the upper epithelial layers seem to be a precondition for infection, whereby the formation of a lesion is

related to an infection of epithelial stem cells (20). Several studies showed that for an efficient infection multiple cofactors like heparan sulfate proteoglycanes or laminin 5 play an important role for adhesion of the virus to the basal membrane or the cell surface (21-23). To date, the common hypothesis is that after docking to the cellular surface viral particles are actively being transferred to the body of the cell alongside filopodia followed by internalization into the host cell via endocytosis (24,25).

Function in viral lifecycle	Activities	Target factor
E1		
Replication of viral genome	DNA-binding activity, helicase activity, ATPase	RPA, topoisomerase, polymerase alpha-primase
E2		
Transcription of viral genes Replication of viral genome Maintenance of viral genome	Transactivation/transrepression, DNA-binding activity, DNA segregation in mitotic cell	Brd4, ChlR1
E4		
Unknown	Destruction of keratin network, induction of G2M arrest of cell cycle	Cytokeratin 8/18
E5		
Possibly involved in proliferation and/or inhibition of apoptosis	Affection of cellular signaling pathway	EGFR, PDGFR, V-ATPase, MHC1, TRAIL receptor, FAS receptor
E6		
Reactivation of cellular replication mechanisms Proliferation, immortalization, inhibition of apoptosis Maintenance of viral genome	Interaction with various cellular proteins	p53, ADA3, p300/CBP, E6AP, SP1, c-Myc, NFX1- 91,TERT, FAK, FADD, Caspase 8, BAX, BAK, IRF3, PDZ domain proteins
E7		
Reactivation of cellular replication mechanisms Proliferation, genomic instability, inhibition of apoptosis Maintenance of viral genome	Interaction with various cellular proteins	RB, p107, p130, HDAC, E2F6, p21, p27, CDK/cyclin, ATM, ATR, gamma-tubulin
L1		
Major capsid protein		
L2		
Minor capsid protein		

Table 1: HPV gene functions

Table based and changed after Kajitani et al. (17)

At first, minor copy numbers of viral DNA are present episomally in cells of the basal layer (26). Once an infected cell is divided, viral DNA is transferred to the daughter cell, which can subsequently produce further infected cells by proliferation (26). Early genes E1 and E2 and

further cellular factors initiate the replication of the viral genome to a copy number between 20-100 copies per infected basal cell (26).

The binding of the transcription factor E2 in the long control region (LCR) is the important step to recruit the DNA-helicase E1 to the origin of replication, enabling viral amplification independent from cellular DNA-synthesis (27). Viral replication and protein expression primarily take place in the spinous layer suprabasally (28).

Non-infected cells are divided asymmetrically whereby one cell stays in the basal layer and the daughter cell migrates to the suprabasal region (29). Here, the daughter cell switches to the G0-phase leaving the active cell cycle and starting the process of terminal differentiation to become a fully differentiated cell in the upper layers of the epithelium (29). There, the cell serves as a secure barrier to the environment and a shelter from pathogenic microorganisms (29).

Through expression of the early genes E6 and E7, basal cells switch from G1- to S-phase and are thereby stimulated to keep proliferation activity, initiating tissue growth and productive infection (30). The oncogenic character of the E6 protein relies mainly on the recruitment of the protein ligase E6-AP causing the degradation of the tumor suppressor protein p53 (31).

Due to its interaction with ubiquitin ligase Mdm2, p53 is normally present in merely minor quantities in undamaged cells (32). DNA damage leads to activation of protein kinases inducing phosphorylation of p53 reducing its affinity to Mdm2 (32). As a consequence, intracellular concentration of p53 rises being then able to promote transcription of several genes that prevent the switch from G1-phase to S-phase respectively initiating apoptosis (32). A dysfunction of this control mechanism leads to accumulation of damaged DNA in cell replicates promoting the emergence of mutations and malign transformation on the long hand (32).

Furthermore, E6 protein induces telomerase expression promoting telomerase activity, which leads to the immortalization of the cell (33). The E7 protein, however, is able to interact with cellular retinoblastoma protein (pRb) causing the release of cellular transcription factor E2F, which switches the cell to S-phase promoting the proliferation of the host cell (34).

Compared to the rest of the early proteins, the E4 protein is transcribed much later in the life cycle (35). It is produced, however, to the greatest amount and is said to be responsible for viral constitution and viral release (35).

While interacting with growth receptors like EGF or PDGF in the phase of infection, the E5 protein seems to play an important role for promoting proliferation by a distinct signal cascade (36). For mechanisms in the later phase of cancer genesis, the protein does not seem to be of important use anymore (36).

In suprabasal areas, the late promoter is activated leading to expression of L1, L2 and E4 genes enabling the release of mature viruses through mortifying upper epithelial layers (37). The late proteins L1 and L2 assemble to capsomers consequently forming the icosaedric capsid (37). Since L1 is able to interact with L2 and cellular receptors L2 manages to communicate with viral DNA (37).

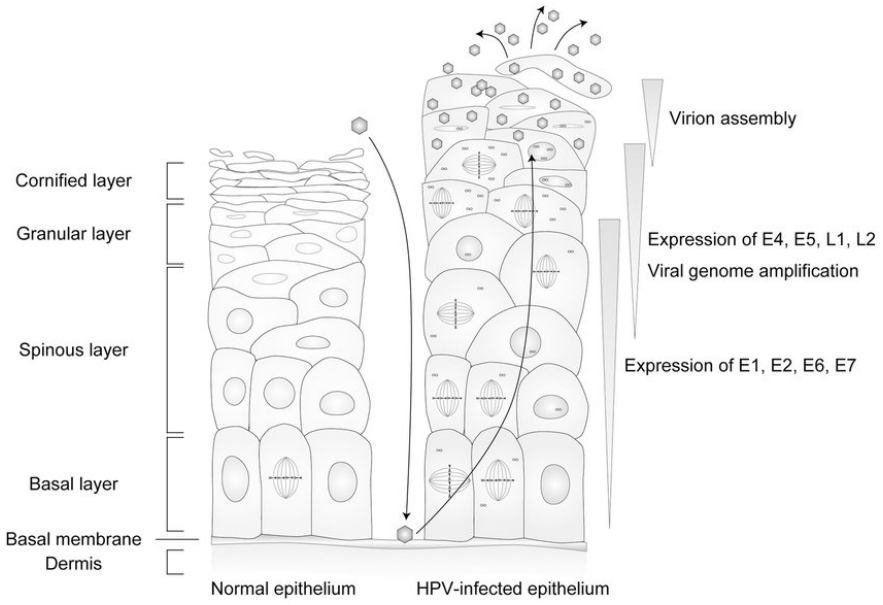

Figure 3: HPV life cycle

HPV specifically infects the cells in the basal layer of the stratified epithelium through epithelial lesions, where viral DNA is maintained episomally in its nuclei. The viral lifecycle is strictly controlled by host cell differentiation. The productive lifecycle in the late phase takes place in terminally differentiated cells in the upper layers of the epithelia. The progenitor virions are released from the keratinized epithelial cells. (17)

A coevolution of HPVs and their hosts enables a biological equilibrium, where the host does not

suffer from serious damage caused by the infection and the virus is just slightly limited by the immune system concerning reproduction (38). Such a productive viral life cycle was described above, where a coexistence of virus and host is possible. So the question is, which mechanisms lead to HPV-induced tumor growth and malignant transformation.

Normally, an infection with HPV-high-risk-types lasts 12 to 18 months before it is terminated by the immune system (39). Approximately 10% of the infections, however, cannot be cured by the immune system and might lead to tissue growth or malignant transformation through persistent infection over years (40). The development of an HPV-induced tumor can take quite some time, since studies showed a timespan between 10 to 15 years from HPV infection to tumor diagnosis (41). Beside cell deregulating and proliferation promoting mechanisms mainly carried out through oncogenes E6 and E7, modulating mechanisms to escape immune response play an important role for persistent infections (39).

In addition to age and subtype, the productive viral life cycle also depends heavily on the type of epithelium that is being infected. Within the five classified HPV genera α, β, γ, μ, ν, the vast majority of all studies on HPV derived from analyses of the α genus and therefor of predominantly mucosal HPVs causing either anogenital warts in children and adults or anogenital neoplasia in adults (42). Beta or cutaneous HPV types, which mainly cause skin warts, are characterized by the fact that they lack the E5 gene completely (43). In contrast to mucosal HPV subtypes, malign neoplasia is very rarely caused by cutaneous HPVs. The role of cancer development has been extensively studied particularly in cervical cancer, where tumorigenesis is closely associated with the cervical transformation zone, where the transition from the glandular epithelium to the squamous epithelium takes place (11). Although not being able to prove this assumption, the common hypothesis is that the virus reaches the basal layer through microlesions in the epithelium (20). In contrast, the tonsil tissue lacks this transformation zone, since this tissue, like other carcinomas of the skin, consists exclusively of squamous epithelium. For this reason, it is discussed that carcinogenesis in the tonsils and the cervix proceed differently.

However, the target locations for HPV infections are often areas of advanced immunologic activity as to be found in the cervix uteri or the tonsils. Nonetheless, the virus manages to successfully hide from immune response by various tricks (44). For example, its uncommon long binding and internalization phase of at least 24 hours keeps it from being terminated right away since transcription starts when wound healing is practically finished and immune response is already

fading (44).

Furthermore, minor viral expression in the basal layer in close vicinity to lymphatic tissue does not seriously attract immune system's attention compared to high viral expression rates in the upper epithelial layers would (45). HPV infected keratinocytes are even able to generate local immunosuppression showing notably smaller amounts of pro-inflammatory cytokines like IL-1, IL-6, TNF-α or TNF-β and higher anti-inflammatory IL-10 levels (46).

A key step for HPV-induced carcinogenesis is the integration of the viral genome in the host's genome causing a selective up-regulation of oncoproteins E6 and E7 and a notable proliferation advance compared to infected cells with episomal viral genome (47).

The basis of genome integration is presumably the consequence of double-strand breaks in the viral as well as in the host's genome and following ligation processes (48). Primary locations of double-strand breaks are the open-reading frames of E1, E2 and E4 causing deletions of adjacent genes including E5 and sometimes L2 (48). Locations of integration are well spread throughout the whole host's genome with a large amount of well-known fragile chromosomal areas (48). Deletion of E2 results in lacking of viral expression control of E6 and E7 enhancing oncogenic power (48).

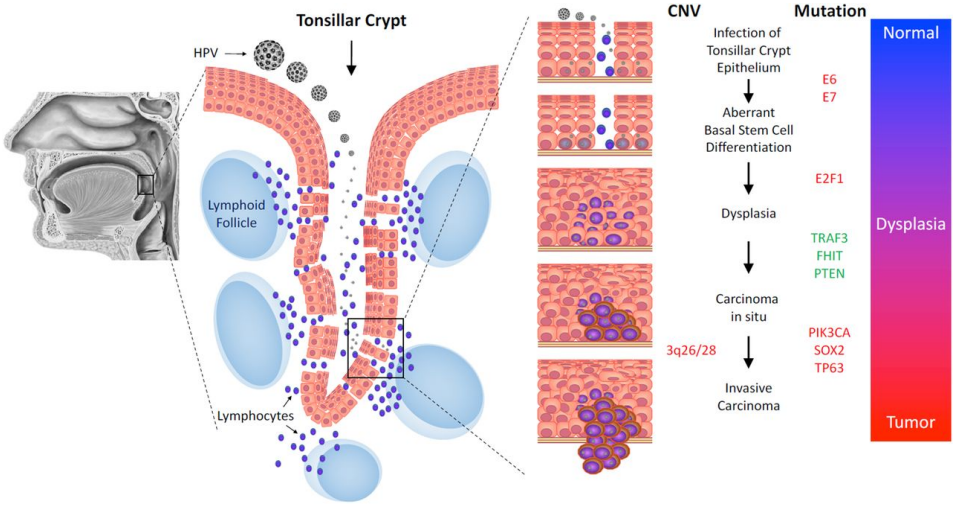

Figure 4: Tumor progression model for HPV-induced tonsil carcinoma

In the Head and Neck Region, HPV shows tropism for lymphoid-associated structures of the oropharynx, especially the palatine and lingual tonsils. Infection of the tonsillar epithelium causes aberrant basal cell differentiation, dysplasia, carcinoma in situ and eventually invasive carcinoma. In a hypothetical model for somatic mutations in a multistage tumor progression model, genes and loci in red are upregulated whereas genes in green loose their function due to mutation or deletion. Reprinted from Microbes and infection, 19(9-10), Faraji F, Zaidi M, Fakhry C, Gaykalova DA, Molecular mechanisms of human papillomavirus-related carcinogenesis in head and neck cancer, 464–75, 2017, with permission from Elsevier (49).

By this time, several risk factors that potentially trigger an HPV infection could be identified. Possibly through mechanisms of local and systemic immunosuppression, nicotine abuse is associated with a higher prevalence and incidence of HPV infections and is told to encourage the risk of persistent infections (50). Also the incidence of anogenital warts and cervical carcinoma is increased due to smoking (50).

Moreover, sexual behavior has a significant impact on the risk of HPV infection. Most importantly, promiscuity and sexual intercourse without a condom raise the incidence of infections (51,52).

Also a short time span between menarche and first sexual intercourse seems to enhance the risk of HPV infection (53). Since patients with impairment of cell-mediated immune defense like HIV-patients or transplant recipients often develop severe HPV infections, it is assumed that cell-mediated immune defense plays an important role for control of virus replication and disease (1).

Before the formation of an HPV-induced carcinoma, a transforming HPV infection takes place in the form of a precancerosis called intraepithelial neoplasia (54). In the case of cervical infection,

this is described as cervical intraepithelial neoplasia (CIN) based on histopathological findings (54). Histologically, it differs from healthy tissue by atypical mitosis, impairment of differentiation and abnormal cell proliferation with atypical cells and cell nuclei (54). For cytopathological findings, the terms LSIL (low-grade squamous intraepithelial lesion), which corresponds to CIN I, and HSIL (high-grade squamous intraepithelial lesion), which corresponds to CIN II and III are being used nowadays (55).

Dependent on the transformation in suprabasal cells triggered by E6 and E7 and the amplification of viral DNA, the grade of tissue alteration through an HPV-infection of the cervix uteri has been classified into the three groups CIN I-III (56). Since CIN I is characterized as a low-grad dysplasia with atypical cell nuclei just in the lower third of the epithelium, the advanced forms CIN II and III show a notably altered viral expression profile with higher grades of transformation and uncontrolled proliferation in suprabasal cells, leading to the formation of immature viruses in the upper epithelial layers (56).

To date, the common hypothesis is that tissue of high-grade lesions like CIN III predominantly show the expression of E6 and E7 genes throughout the entire epithelium with hardly any formation of capsid proteins (11).

1.2 Viral Infection and Tumor Disease

Despite striking developments concerning diagnostics and therapy over the last decades, cancer remains a major global health burden with estimated 14.1 million people developing disease annually worldwide, expected to rise to 19.3 million by 2025 (57). It is assumed that over 20% have a direct correlation between tumor genesis and biological involvement of viruses, bacteria or parasites (45). In the year 2009, the WHO declared 11 microorganisms, seven of them viruses, to be potentially carcinogenic for humans (58). To date, there is strong evidence that persistent viral infections with Ebstein-Barr-Virus (EBV), Hepatitis-B-Virus (HBV), Hepatitis-C-Virus (HCV), high risk types of HPV, Human-Immunodeficiency-Virus (HIV), Kaposi's-Sarcoma-Herpes-Virus (KSHV) and Human-T-cell-Lymphotropic-Virus (HTLV) potentially lead to cancerogenesis at multiple sites in the human body through a bunch of different complex mechanisms promoting uncontrolled tissue growth based on chronic inflammation (59). In 2002, approximately 1.9 million

infection-attributable cancer cases presented, whereby the amount in developing countries (26%) is about three times higher compared to industrial countries (8%) (60). After Helicobacter Pylori (5.5%), HPV is the second most common infectious agent responsible for cancerogenesis (60).

Despite of early indications of a causally coherence in studies of Rous 1911 (61) or Shope and Hearst 1933 (13), the correlation between infections with microorganisms and tumor genesis has long been a neglected field of science due to several reasons: On the one hand, infections suspected to be associated with human cancer development are ubiquitous (62). On the other hand, the time span between primary infection and tumor diagnosis is usually measured in several decades (62). Moreover, cancers present to be of monoclonal character and therefor cannot be caused by a systemic infection (62). Another important fact is the difficulty to differentiate between chemical and physical cancerogens and infections being potentially responsible for cancerogenesis in the same tumors (62).

In 1964, Tony Epstein and Yvonne Barr were able to correlate a virus infection with tumor genesis for the first time by detecting viral particles that were later named after them (EBV) in cells of a Burkitt's lymphoma (63). EBV was also the field of interest of an ambitious young scientist at the beginning of his career named Harald zur Hausen who was awarded with the Nobel Prize in 2008 for the discovery of the first types of HPV and the first assumption and later biological proof of its causal correlation with the development of cervical carcinoma (64).

1.3 Epidemiology

1.3.1 HPV Infection and its induced Pathologies

An infection caused by HPV represents the most common sexually transmitted disease worldwide (65). Both sexes contribute in the chain of transmission. The consequences, however, appear to be of unfair dispersion mostly concerning females. Globally, nearly every second person suffers from an infection with a genital HPV type in the course of a lifetime. In women until 50 years of age, the prevalence is even 80% (66). The major amount of infections is transient, lacking of any persistent pathology. Depending on the HPV type various clinical symptoms can be observed. Commonly occurring are infections with low-risk cutaneous types causing warts (1). There are just

a few exact studies in well-defined populations for the prevalence and incidence of human warts. Ordinary warts (Verruca vulgaris) are to be found in certain populations of up to 25%, especially in young children (1). Also plantar warts (Verruca plantaris) are mainly common in adolescents and young adults (1). The anogenital wart (Condyloma accuminatum), caused by infections with low-risk mucosal HPV types, constitutes one of the most frequent sexual transmitted diseases in the United States (1).

HPV-low-risk-type-6 and -11 are responsible for about 90% of the lesions (67). The common opinion is that approximately 1% of the sexually active population in the United States suffers from anogenital warts causing healthcare costs in significant measures (66). In 2001, the incidence of anogenital warts averaged 205 persons per 100,000 in the USA showing the highest levels in men between 20 and 29 years of age (68). Pirotta et al. found similar incident rates in Australia of 219 per 100,000 through all age groups with a peak for 20 to 29 year old women (69). A little lower incidence rate was found in a German cohort with an average of 169 per 100,000, again with the highest percentage in women from 20 to 29 years of age (70). Notably minor prevalence respectively incidence rates are found for respiratory papillomatosis caused by low-risk mucosal HPV types (71).

As mentioned above, first reports emerged in the late seventies postulating the occurrence and possible correlation of HPV and cervical cancer. Years later, biological evidence was undeniable and multiple HPV high-risk types with carcinogenic potential were identified.

To date, we know that about 4.5% of all cancer cases worldwide are related to HPV (8.6% in women, 0.8% in men) meaning approximately 630,000 new cancer diagnoses per year (3). The relative contribution of HPV high-risk types 16 and 18 presents to be 72% of all HPV-related cancers constituting 460,000 cases worldwide (3).

1.3.2 HPV and Cervical Cancer

Following breast cancer and skin cancer, cervical cancer is the third most common malignant tumor disease in women in the United states, whereby 99.7% of all cancer cases are attributable to HPV-infections (56). Only a few rare subtypes of cervical adenocarcinomas are not HPV-

associated (72).

With 530,000 new cases per year, cervical cancer accounts for the vast majority of all HPV attributable cancer cases globally (3). Regional incidence rates vary mainly due to differences in the population prevalence of cervical HPV infection and the presence or non-presence of adequate cervical cancer screening (3). These facts explain notably higher prevalence and incidence rates in developing countries, where cancer prevention programs are missing and health respectively hygiene standards are low (3). About two thirds of cervical carcinoma cases are presented in less developed countries (3).

As compared to European standards, an incidence rate of 8.6 per 100,000 was found in the German population in 2008 being assigned to the 12th place, concerning mortality rate (3.2) even on the 14th place of cancer diseases (73). On average, the incidence rate in the European Union was found to be 11.0 with a mortality rate of 4.1 per 100,000 (74).

HPV-high-risk-types-16 and -18 are together responsible for about 71% of all cervical cancer cases worldwide, whereby HPV-16 represents with an over three times higher share the major part (3). 90% of cervical cancers are caused by HPV-types-6, -11, -16, -18, -31, -33, -45, -52, -58 (3).

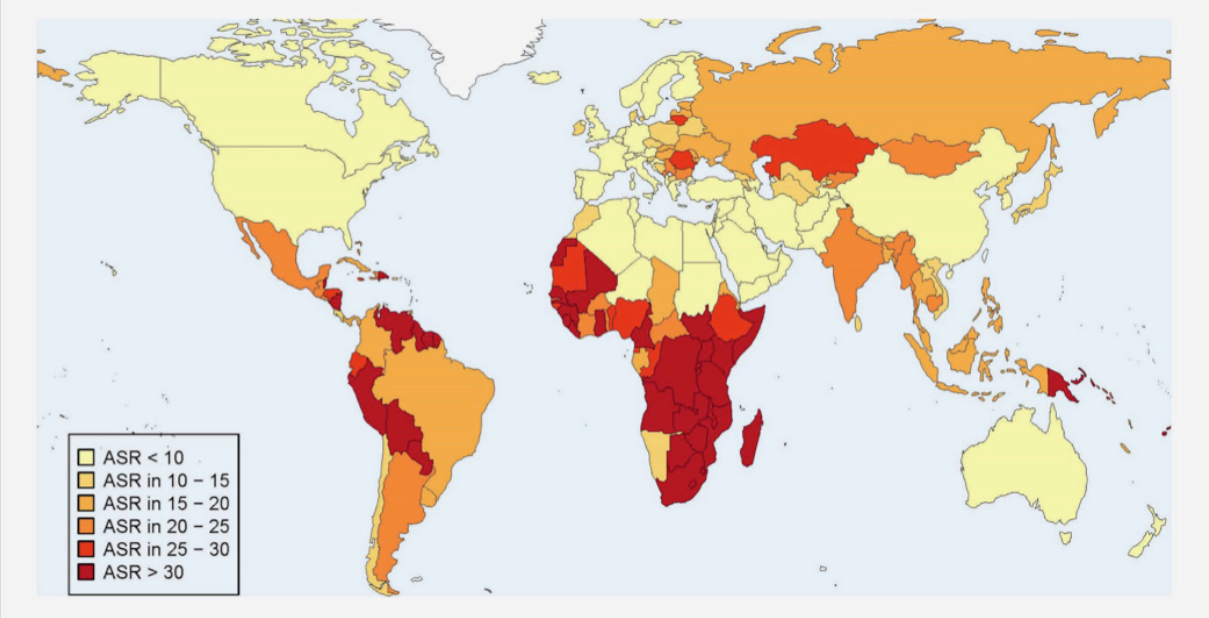

Figure 5: Global age standardized incidence rates (per 100,000) of cervical cancer cases attributable to HPV in 2012 (3)

Regarding precancerous lesions, it was shown that high-grade lesions (CIN III) present to be positive for at least one HPV-type (75). With regional differences, HPV-16 is in 45.4% of these cases worldwide attributable for disease. Europe, for instance, shows with 51.8% a notably higher incidence rate of HPV-16-related precancerous lesions compared to Oceania (33.3%) (75). The following common types are HPV-31 (8.7%) and -33 (7.3%), with, nevertheless, strong regional variations (75).

1.3.3 HPV and other anogenital Cancers: Anus, Vulva, Vagina and Penis

The incidences of other HPV-related anogenital tumors are comparatively rare. With 35,000 new cases per year, anal cancer represents the second common HPV-related anogenital disease behind cervical carcinoma appearing to be caused by an HPV-infection in 88% of the cases (3). The amount of HPV-16 or -18 positive cases is with 87% even higher compared to cervical carcinoma (3).

Risk factors are particularly unprotected anal sex, homosexuality in men and promiscuity (76). Therefor the regional highly varying incidence rates are notably higher in big cities with a large gay community like, for example, San Francisco (2.1 per 100,000) (76).

Generally, the incidence of anal carcinoma is quite balanced between the sexes with even a slight favor for females (77).

Globally seen, penile cancer (13,000 new cases per year), vaginal cancer (12,000 new cases per year) and vulvar cancer (8,500 new cases per year) play a comparably minor part (3). Also the proportions of attributed HPV-infections are significantly lower for penile carcinomas (50%) and vulvar carcinomas (25%) (3). Vaginal carcinomas, however, appear to have similar high HPV-associations (78%) in relation to cervical or anal carcinomas (3).

Countries, where the estimated amount of HPV-association in anogenital cancerogenesis is remarkably high, are primarily found in Latin and Northern America and Australia with a few outliers to be found in Europe and Africa (77).

1.3.4 HPV and Head and Neck Cancer

In the 1990s, increasing epidemiologic and molecular evidence supported the assumption that HPV could be attributed with cancerogenesis in the upper aero-digestive tract (78,79). Beside classical well-established risk factors like smoking and alcohol, epidemiologic data revealed over the last decades an emerging patient cohort of white men, under 50 years of age, non-drinkers and non-smokers developing squamous-cell-carcinoma in the head and neck region (HNSCC) particularly in the oropharynx (80). At the present day, we know that three tumor sites are potentially associated with a persistent HPV infection: the oropharynx and, to a much smaller extent, the oral cavity and the larynx (3). Globally, estimated 37,500 new HPV-induced cancer cases are occurring per year, whereby the majority of cases are OPSCCs, mainly proceeding from the mucosa of palatal tonsils and the base of tongue (3).

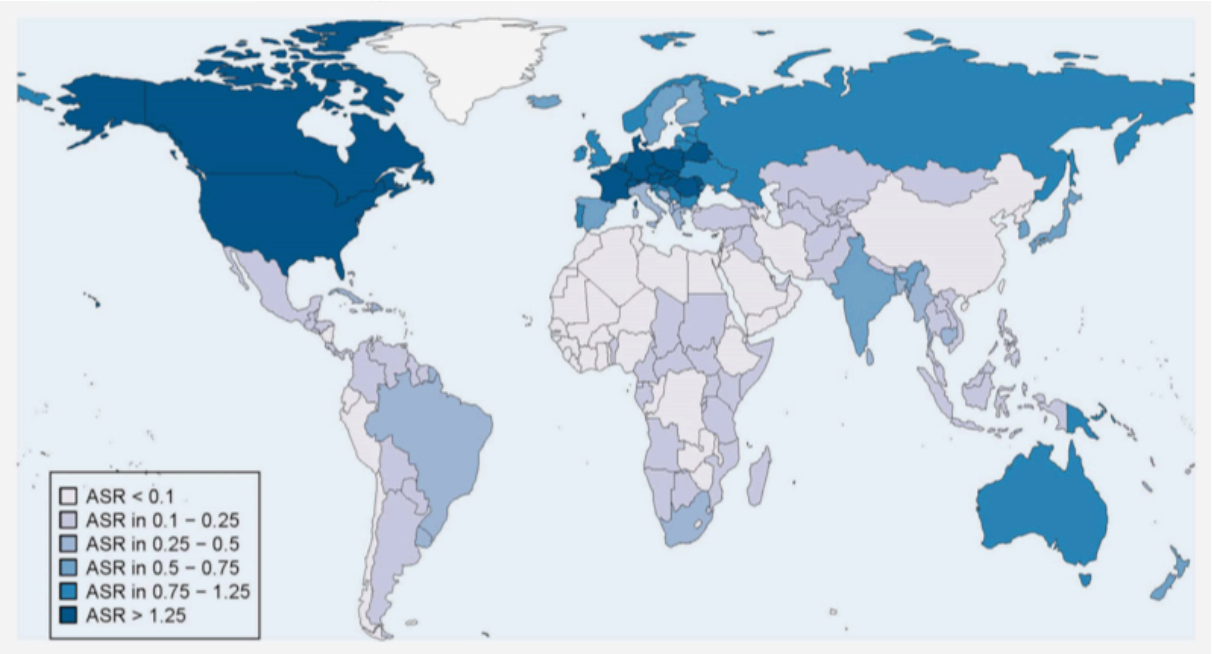

Figure 6: Global age standardized incidence rates (per 100,000) of head and neck cancer cases (oropharynx, oral cavity and larynx) attributable to HPV in 2012 (3)

Whereas more than two-thirds of cervical cancer cases are diagnosed in less developed countries, in contrary, the geographical distribution of HPV-induced cancers of the head and neck is diametrically different (3). The distinct majority of this entity emerges in more developed countries (27,500 new cases per year), with relatively high age-standardized incidence rates (over 1.25 per 100,000) in Northern America and Europe compared to low incidence rates in countries with poor socio-economic status (10,700 new cases per year) (3).

While incidence rates of HPV-negative cancer cases, mainly caused by alcohol and tobacco abuse, showed a steady decrease, in particular HPV-related OPSCC revealed increasing incidences over the last decades in industrial countries (80).
Furthermore, a significant difference in the appearance of tumor disease between the sexes is evident. With 30,000 new cases per year the vast majority of HPV-related HNSCCs is undisputedly in favor for the male sex, compared to 7,500 new cases in females worldwide (3). This might be explained by the fact that sexual behavior, especially oral sexual behavior, appears to be an important risk factor for the development of oral HPV-infection and consequently for cancerogenesis of OPSCC (81). Studies showed that men are significantly more likely to

participate in oral sexual behavior compared to women (82). The fact that oral sexual behavior mainly occurs in younger age groups might explain the rising incidence rates of HPV-related OPSCCs in patients less than 50 years of age in industrial countries (82). Nonetheless, both significant features, the favorable male sex and the younger-aged tumor-population, are not fully understood (82).

Globally, squamous cell carcinomas of the oropharynx are induced by a persistent infection with HPV in 30.8% of cases (3). In this aspect, remarkably varying regional incidences can be observed with the highest amounts (>40%) in countries with a good socio-economic status (Europe, Northern America, Australia, New Zealand, Japan, South Korea) compared to notably minor shares of HPV-association in poorer countries (<20%) (3).

Some studies even show incidence rates of about 70% for HPV-association in all HNSCCs in the United States (83).

With 85% of cases, HPV-high-risk-types-16 and -18 cause the vast majority of all HPV-related OPSCCs (3). HPV-16, however, constitutes the by far largest amount of HPV-types, expelling HPV-18 to a supremely low number of cases (3).

1.4 Head and Neck Squamous Cell Carcinoma

Head and Neck Carcinomas (HNCs) represent a heterogeneous group of malignant tumors in the upper aero-digestive tract. Referring to the UICC classification, the head and neck region is anatomically divided into the following areas: the lips, the oral cavity, the main nasal cavity and ethmoidal cells, the maxillary sinuses, the naso-, oro- and hypopharynx, the supraglottic, glottic and subglottic part of the larynx, the salivary glands and the skin of head and face (84).

With a prevalence of over 90%, the vast majority of histological entities are presented to be squamous cell carcinomas (HNSCC) (8). Adenocarcinomas or sarcomas are by far less frequent in occurrence (8).

In terms of the tumor localization, the anatomical attribution of the tumor is based on the area, where the main tumor body is to be found. In this term only, one anatomical area is eponymous.

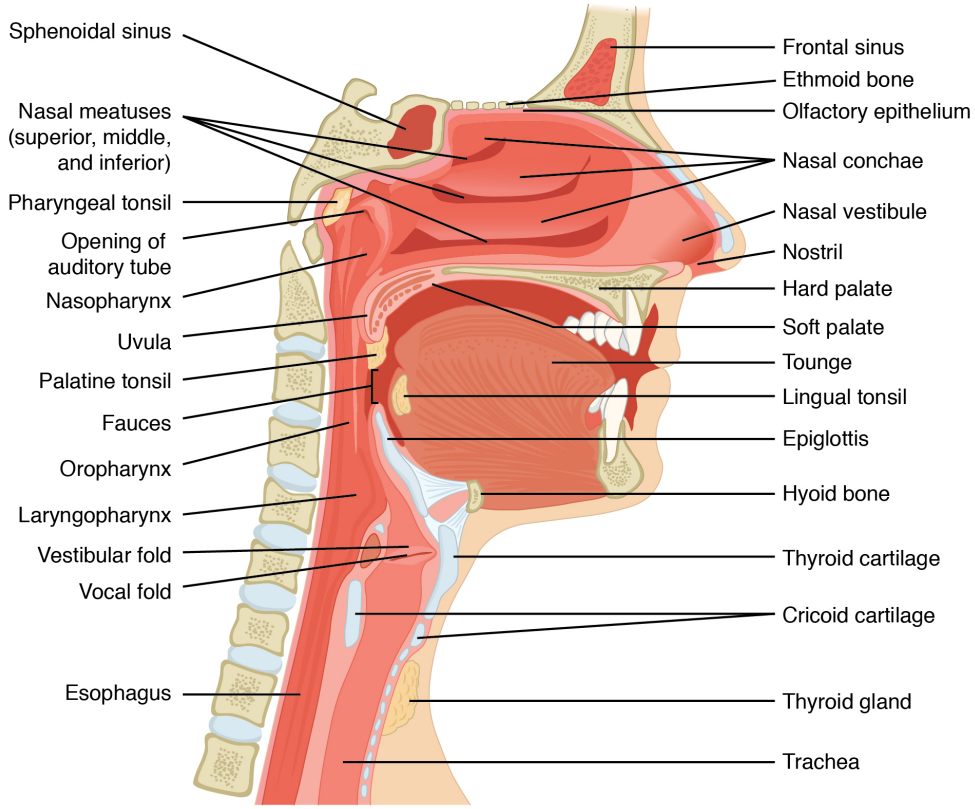

Figure 7: Anatomy of the head and neck region (85)

Guiding symptoms of HNSCCs are (86):

- Persistent pain in the head and neck area especially when swallowing possibly with radiation to the ears
- Chronic, non-healing, wounds on lips or oral cavity
- Nodal or exulcerating lesions on lips, oral cavity or pharynx
- Red or white stains on gingiva or tongue
- Chronic pharyngeal inflammation or globus sensation
- Impairment of chewing or swallowing
- Oral bleeding
- Trismus
- Dysfunctional tongue movement

- Persistent change of vocal sound or hoarseness
- Persistent or growing swelling of cervical lymph nodes

Cancers of the head and neck represent the sixth most common tumor disease worldwide with estimated 625,173 new cancer cases (including 354,864 cancers of the lips and oral cavity, 92,887 oropharyngeal cancers and 177,422 laryngeal cancers) and 323,160 deaths per year (77). Incidence rates show remarkably regional variations appearing to be the highest in regions with severe tobacco- and alcohol consumption (76).

1.4.1 Oral Cavity

The oral cavity is bounded by the lips, the gingiva covered alveolar processes and teeth, the cheeks as well as the hard and soft palate, whereas in the strict sense a division between oral vestibule and main oral cavity is made (87).

Hot spots for squamous cell carcinomas of the oral cavity are the anterior two-thirds of the tongue where tumors mainly develop at the lateral borders and may show features of deep infiltration and early metastasizing behavior (86). In particular the crossing lymph drainage systems enhances the risk of metastatic spread to the contra-lateral regional lymph nodes (86). Although quite obvious, tumors of the gingiva of the upper and lower chaw tend to be mistaken for local inflammation often causing delayed diagnosis (86). For reasons of possible surgical therapy evaluation of tumor relation to the jaw is inevitable (86).

1.4.2 Nasopharynx

The nasopharynx represents the upper part of the pharyngeal area bounded by the pharyngeal roof with close relation to the skull base, the pharyngeal sidewalls with the arising Eustachian tubes connecting the middle ear with the pharynx, the pharyngeal dorsal wall with close relation to the cervical spine, the choanae and the soft palate (87). WHO classification distinguishes between keratinized and non-keratinized squamous cell carcinoma of the nasopharynx whereby non-keratinized entities are further divided into those showing features of differentiation and undifferentiated ones appearing to be EBV-related and showing the worst prognosis (84).

Nasopharyngeal carcinomas tend to be diagnosed in advanced tumor stages because of long-term asymptomatic progression often showing lymph node swelling due to metastatic spread as a first symptom (88). The fact of a high percentage of tumor infiltration of the skull base at time of diagnosis reduces possible surgical therapy considerably (88).

1.4.3 Oropharynx

The oropharynx, as the main area of interest in this study, represents the center section of the pharyngeal region. It is separated from the oral cavity by the isthmus faucium (87). A virtual line at the level of the palatal arch is the cranial boundary (87). Caudally it reaches to the top edge of the epiglottis and the lingual tonsil presents the anterior border. Dorsally, it is bounded by the pharyngeal dorsal wall in close relation to the cervical spine (87). To the sides we find the palatopharyngeal arches with the palate tonsils in-between (87).

Hot spots for the development of oropharyngeal squamous cell carcinoma (OPSCC) are the tonsils and the base of tongue (86). Tonsil carcinomas tend to grow deeply invasive with possible progression to the base of tongue and the lateral pharyngeal wall (86). They can have an exophytic as well as an exulcerating process of growth (86). Trismus might appear in advanced tumor stages as a symptom for invasion of the pterygoid muscles (86).

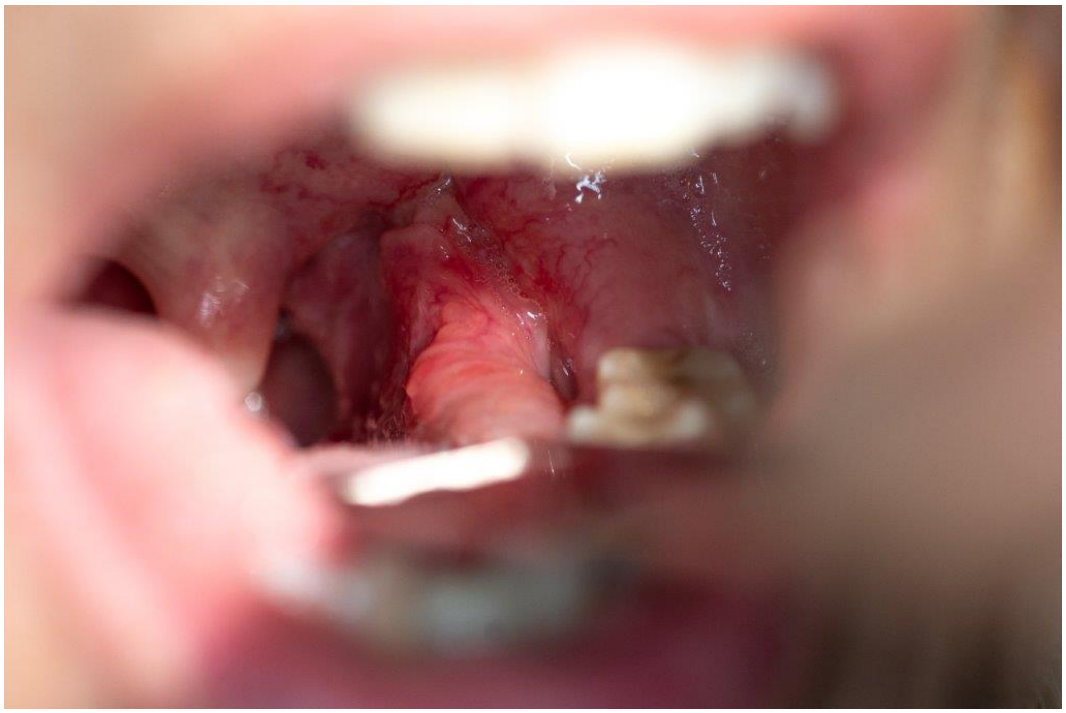

Figure 8: Clinical picture of a left tonsil oropharyngeal carcinoma

1.4.4 Hypopharynx

The hypopharynx constitutes the lower part of the pharynx reaching from the top edge of the epiglottis to the upper esophageal sphincter (87). It is divided into three areas: the piriform sinus, the dorsal wall of the hypopharynx and the post-cricoid region (87).

Hypopharyngeal squamous cell carcinomas are also characterized by a poor prognosis due to delayed diagnosis in advanced tumor stages because of long-term asymptomatic progression (88). Likewise, swelling of local lymph nodes, as a sign for metastatic spread, are common as a first symptom of the disease. Carcinomas in the post-cricoid area are correlated with Plummer-Vinson-Syndrome and malnutrition (89).

1.4.5 Larynx

The larynx, as part of the respiratory tract, forms the connection between pharynx and trachea (8). It is responsible for voice formation and protection of the respiratory tract (8). Topographically it can be divided into three areas: the supraglottic, glottic and subglottic area (8). About 70% of laryngeal squamous cell carcinomas are located in the glottic region primarily showing hoarseness as an early symptom of disease (8). Therefor clinical guidelines recommend an exploration by an ENT-specialist through laryngoscopy in cases of persistent hoarseness over 3 weeks (8). Tumor size, lymph node status and possible extra-capsular spread in cases of lymph node metastasis are of important prognostic value (8). The favored treatment option is surgical therapy up to radical approaches in form of total laryngectomy in advanced tumors (8).

1.4.6 Cancer of unknown primary (CUP)

Patients showing most commonly unilateral cervical lymph node metastasis primarily of a squamous cell carcinoma, lacking of any information concerning primary tumor origin, are suffering from a CUP-syndrome (86). Clinical guidelines recommend performing a PET-CT scan followed by a panendoscopy under general anesthesia including routine biopsies from the nasopharynx and the base of tongue as well as a diagnostic tonsillectomy (86). State of the art diagnostic confirmation is done through fine needle or core needle biopsy (86). An open approach for biopsy is not recommended due to the risk of dislocation of tumor cells (86). Some theories assume spontaneous remission of the primary tumor after metastatic spread (86).

Guidelines nowadays recommend staging CUP-syndromes like HPV-associated OPSCC if histology reveals p16-positivity (90). In cases of an EBV-association, it is assumed that the disease corresponds to nasopharyngeal carcinoma (90).

1.4.7 Etiology

In regard of molecular mechanisms, cancer is associated with genetic, progressive, multistep mutations caused by repeated DNA impairment and deregulation of cell-cycle-regulating proteins

(91). Major, well-explored, risk factors for cancerogenesis of HNSCCs are exogenous noxa, especially alcohol- and tobacco-consumption (8).

In the metabolism process of alcohol, acetaldehyde is being produced through oxidation (92). Local accumulation in the upper aero-digestive tract leads to notably enhanced incidences of HNSCCs underlining its multiple cancerogenous characteristics (92).

In terms of cigarette smoke, several carcinogenic contents like tar, nicotine, benzopyrenes, nitrosamines, hydrogen cyanides or formaldehyde are responsible for impairment of DNA repair mechanisms, cell cycle control mechanisms as well as for potential raise of cell proliferation rates and mutations (93). The combination of both exogenous noxa, which is renownedly quite common, multiplies the cancerogenous potential to a considerable degree (94).

Other significant risk factors for malignant degeneration are nutritional factors like the consumption of betel nuts, which is common in parts of South-East-Asia, poor oral hygiene and periodontitis, physical noxa like radioactive radiation, chronic mechanical irritation like through sharp-edged teeth, job-related toxic exposure (e.g. asbestos) and family-related as well as genetic dispositions (95-97).

As previously mentioned, growing epidemiological and biological evidence for active HPV-involvement in cancerogenesis of HNSCC, especially of OPSCC, was emerging in the 1990s. Today, we know that a persistent infection particularly caused by HPV-high-risk-type-16 is one of the major risk factors for developing OPSCC beside alcohol- and tobacco abuse (7,98,99). Primarily, the palate tonsils are the targets of interest in the oropharynx for the location of HPV-related SCCs (7,98,99).

By contrast to the epithelium of the cervix uteri, multiple deep crypts reaching far down to the lymphatic tissue and provide surface enhancement characterizing the epithelium of the tonsils (100). The top epithelial layer is very thin and fragile enabling direct contact to the pathogens for the lymphoid tissue in the crypt region (100). By this way, pathogens entering the oral cavity are easily presented to the immune system (100). The basal cell layer is not complete and the underlying basal membrane has a porous character allowing the direct passage for lymphocytes and antigen presenting cells (100). Given these facts, the tonsils are known to be the optimal environment for HPV-infection (100). Contrary to the cervical epithelium, micro-lesions are not

necessarily required for entering (100). The virus is able to get to the basal layer through the crypts directly (100).

In the late 1990s, first studies were able to show that HPV-positive tonsil carcinomas have a better overall- and progressive-free-survival despite the fact that they were characterized by a worse grade of differentiation and patients showed significantly advanced tumor stages compared to HPV-negative tumors (101). Following studies with larger sample sizes comparing HPV-positive against HPV-negative tumors in the oropharyngeal region revealed further distinctive features of this entity. In particular, affected patients were reported to be notably younger with a significant amount of non-smokers lacking of regular alcohol consumption (80). Since HPV is a sexually transmitted infection, sexual behaviors, especially the number of lifetime sexual partners respectively oral sexual behavior, seem to be significant risk factors (102). Incidence rates are strongly favorable for the male sex (80).

As already mentioned above, multiple epidemiologic studies could show, despite of regional differences, that incidence rates of HPV-positive OPSCCs are rising in industrial countries while those in countries with poor socio-economic status are dropping (4). Generally, incidence rates of HPV-negative tumors are declining in developed countries (4).

Clinically, HPV-related OPSCCs appear to present in advanced tumor stages, showing on the one hand lower T-stages but on the other hand progressed N stages (103). Lymph node metastases often have cystic character and present to be multilevel (104).

A further remarkable feature of HPV-positive OPSCCs is the better prognosis independent from cancer treatment. To date, we know that this tumor entity is significantly more sensitive to chemo- or radiation treatment particularly to combined treatment compared to their HPV-negative counterparts (105). Other studies also showed that the patient's prognosis is favorable for HPV-positive OPSCC cases when being surgically treated compared to their HPV-negative counterparts (106).

1.4.8 State of the Art Therapy of Head and Neck Cancer

In the case of primary diagnosis, HNCs in lower tumor stages AJCC I and II are primarily treated

in an unimodal approach either by surgery or radiotherapy (8). Tumors in advanced stages AJCC III and IV are being treated in a multimodal approach (8).

Multiple clinical factors are to be considered for choosing the optimal therapy protocol (86). Of particular importance considering surgical therapy are tumor size, histology, tumor's relation to critical structures, the expected post-therapeutic defect and loss of functionality, general state of health and most importantly the patient's wish (86).

In case of resectability, surgery is recommended as the primary treatment option potentially followed by post-surgical radiotherapy (8). As an alternative, primary radiotherapy with or without concomitant chemotherapy is recommended. For HNCs in higher tumor stages III and IV postoperative radiotherapy after primary surgery is indicated (8). In cases where an R0-resection could not be achieved or where postoperative histologic results show extra-capsular spread in affected lymph nodes, postoperative radiochemotherapy is indicated (8).

6 to 12 weeks after primary therapy, the first follow-up examination is performed to assess remission status (86). In cases of tumor persistence after primary therapy, salvage surgery is a further possible treatment option (86).

1.4.9 Surgery

Surgical treatment can either be curative or palliative (86). If the indication for surgery is chosen for curative treatment, the tumor must be resectable (86). Resectability means that the tumor and locoregional metastases can be removed with a high degree of probability with enough safety margins and morbidity acceptable for the patient (86).

An adequate safety margin should measure 5mm between tumor border and resection border in every direction (107). If this cannot be achieved, an R1-resection is stated meaning a significantly higher probability for local tumor recurrence (estimated 75%) compared to a R0-resection (estimated 30%) (107).

An R2-resection is stated when insufficient safety margins are macroscopically obvious (86). Additionally to the resection of the primary tumor, a neck dissection is usually performed (86). Neck dissections can be either elective or therapeutic depending on the N stage (86). In contrast to

a therapeutic approach, elective neck dissections are performed in a cN0 situation with the aim to reveal potential occult metastases (86). Estimated 33% of occult metastases occur without clinical hint for metastatic spread underlining the possible reduction of early cervical lymph node recurrence (108). For surgical approach in higher tumor stages, reconstructive procedures may be required (8). In the case of distant metastases, surgery is usually not performed (8).

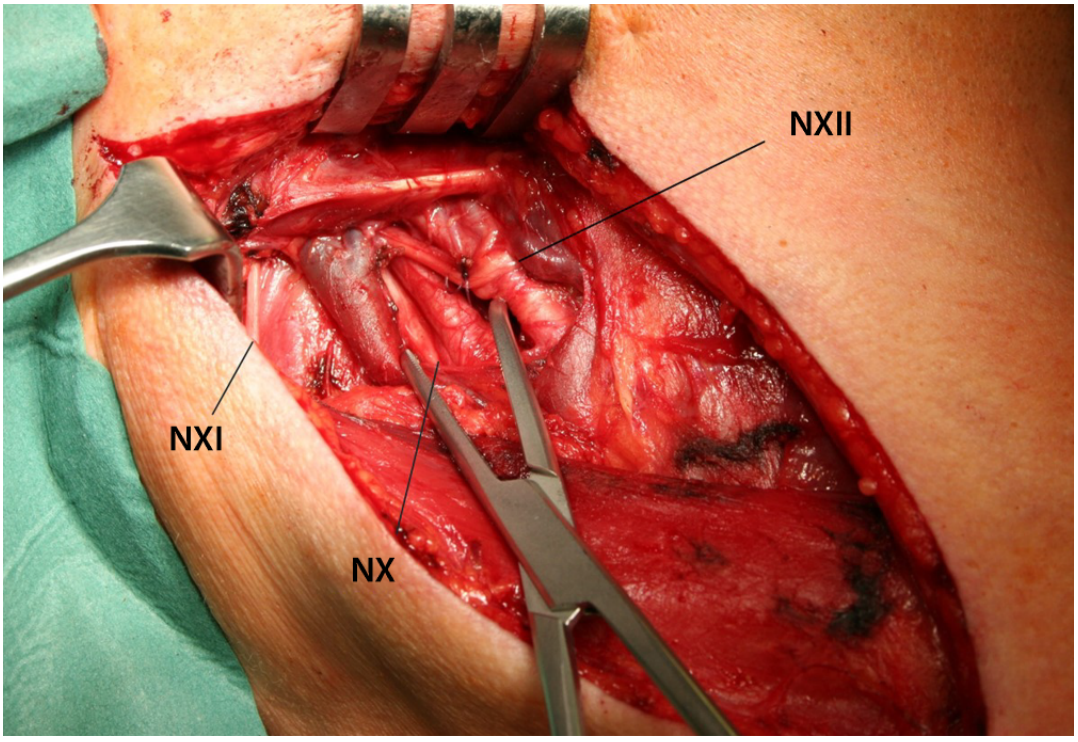

Figure 9: Surgical site of a neck dissection showing surgical preparation during a selective neck dissection of lymph node level II

Nerve vagus (X), accecorius nerve (XI) and hypoglossal nerve (XII) are demonstrated.
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1.4.10 Radiotherapy

As an alternative to primary surgery, the decision to use radiotherapy is based on comparably good healing rates and the possibility of a good functional and aesthetic result (86). It can either be

performed in a primary approach with or without concomitant chemotherapy or it can be added postoperatively in an adjuvant therapy approach (86).

1.4.11 Radiochemotherapy

Primary radiochemotherapy is usually indicated in case of advanced non-resectable HNC or with the aim of organ preservation (110). It was shown to be superior compared to primary radiotherapy alone in advanced tumor stages although toxicity is known to be significantly rising (110). Postoperative radiochemotherapy is indicated in case of R1-resection or extra capsular spread (86).

1.4.12 Radioimmunotherapy

As an alternative to radiochemotherapy, radioimmunotherapy with Cetuximab is a valid option in cases of advanced HNC (86). Cetuximab is a monoclonal antibody against the epidermal growth factor receptor (EGFR) authorized for the therapy in HNC (86). The combined therapy was also shown to be superior to radiotherapy alone but also associated with higher toxicity (86). Usually it is used for elder patients or patients with an impaired general state of health, where chemotherapy should be avoided with regard to potential toxicity (86).

1.4.13 Systemic Therapy

Systemic treatment in HNC is indicated in palliative settings where curative therapy options are lacking (8). The aim for palliative systemic therapy is to alleviate tumor associated symptoms, to raise quality of life and, secondary, to extend lifetime (8).

Depending on the general state of health and the age, several mono- or multidrug treatment regimes are available. With growing evidence through prospective clinical trials over the last years, new immunotherapy drugs are being approved for systemic treatment in HNC.

1.4.14 Therapy in HPV-positive OPSCC

The awareness of significantly superior sensitivity for radio- and chemotherapy led to prospective studies to evaluate dose-de-escalation designs concerning both chemo- and radiotherapy for HPV-positive OPSCCs with the intention to reduce acute and long-term toxicity without influencing therapy success and prognosis (111). Studies in different HPV-related tumor populations, like cervical or anal squamous cell carcinomas, already revealed promising data supporting this approach (112). To date, however, treatment of HPV-positive OPSCCs has not changed concerning therapy dosages. Nonetheless, clinical practice shows that the indications for primary radiochemotherapy in advanced HPV-related OPSCCs are made more generously, especially in cases, where primary surgery might cause significant functional impairment.

1.5 HPV detection in tumor tissue

Since multiple studies showed that HPV-association in oropharyngeal squamous cell carcinomas presents to be a significant prognostic marker, HPV-testing of tumor tissue is performed routinely nowadays and also recommended by multiple expert associations (113). The optimal method for HPV detection, however, is still controversial.

There are various well-described laboratory techniques available to evaluate the presence of HPV-DNA, mRNA or the $p16^{ink4a}$ protein in the clinical setting for head and neck cancer. As a fact, however, the United States Food and Drug Association (FDA) approved currently used tests for HPV-detection only for intended application in cervical carcinoma (112). HPV diagnostic tests for the use in oropharyngeal cancer are lacking of regular approval (112).

While the indirect proof of HPV-association through $p16^{ink4a}$ immunohistochemistry (IHC) is widely used in clinical routine, the direct detection of HPV E6 oncogene expression via PCR-based methods is regarded to be gold standard for the proof of HPV-presence (80). Nonetheless, there are multiple factors such as diagnostic performance, feasibility, cost factors and reproducibility that cause regular concerns (80).

1.5.1 P16^{ink4a} Immunohistochemistry (IHC)

Particularly in respect of the named factors cost and reproducibility, the established state-of –the-art routine for indirect HPV-detection in tumor tissue was chosen to be the immunohistochemical demonstration of p16^{ink4a} overexpression (113).

P16^{ink4a} is a tumor suppressor protein that protects cells against genomic instability (112). It is produced in cases of cellular stress such as DNA-impairment or the deregulation of cell-cycle-regulating proteins (112). P16^{ink4a} inhibits cyclin dependent kinase 4 and 6 (CDK 4/6) keeping it from the phosphorylation of the retinoblastoma protein (pRb) (112).

As already described previously, pRb plays a central role for cancerogenesis in HPV-associated OPSCCs. Being functionally inactivated by transcription of the viral oncoprotein E7, the cellular transcription factor E2F is consequently exposed, driving the cell from G1- to S-phase without brakes. In cells with pRb-impairment a significant overexpression of p16^{ink4a} is induced, without being able to take inhibition steps in the cell cycle (99). In this respect, p16^{ink4a} is synthesized to very high levels in nuclei and cytoplasm of tumor cells, so it can easily be detected through immunohistochemistry (114).

The correlation of p16^{ink4a} overexpression and HPV-association has been well described for cervical cancer for a long time leading to multiple studies that could reveal a similar correlation in HPV-positive OPSCCs. Since immunohistochemical p16^{ink4a} detection proves to be much more cost-effective and widely available, even in countries with low socio-economic status, compared to alternative methods such as PCR, p16^{ink4a} was advocated as a surrogate marker for the presence of HPV in oropharyngeal tumor tissue (80).

Nonetheless, several studies could show that the indirect marker p16^{ink4a} alone is not sufficient enough to reliably identify HPV-related OPSCCs. On the one hand, it was shown that a notably amount of HPV-negative tumors also revealed an overexpression of p16^{ink4a}, on the other hand, HPV-positive carcinomas were seen to be p16^{ink4a} -negative as well (115,116).

One reason that has been discussed for false positive p16^{ink4a} IHC results was the possible occurrence of mutations of the pRb gene without HPV involvement (117,118). Nevertheless, the incidence rates of these mutations appear to be rather low and therefor are probably not solely

responsible for all false positive test results (117,118).

In the context of potential misinterpretation of p16^{ink4a} IHC results, it is concerning that there has not been any consensus on the threshold for p16^{ink4a} positivity for a long time (119). High-risk-HPV-involvement in tumor cells has long been regarded as highly presumable in cases of significant p16^{ink4a} overexpression (119). The level of this significant overexpression has been debatable, however, for decades (119). Studies and laboratories have used different cutoff levels for defining p16^{ink4a} positivity lacking of consensus (119). Since cases where p16^{ink4a} expression is at a borderline level causing uncertainty in interpretation are rare, this issue has not been substantial for quite a long time (112). However, given the fact that test results might influence decisions for clinical treatment of patients, it became of undeniable relevance to define consistent threshold levels (112). Meanwhile several guidelines, like from the College of American Pathologists, recommend a p16^{ink4a} nuclear and cytoplasmic staining ≥70% of at least moderate or strong intensity to define a definitive positive result (113). In cases of borderline results (50-70%), additional HPV-testing using alternative techniques is recommended since small studies could show that most of such borderline cases actually had transcriptionally active high-risk-HPV in their tumors (112).

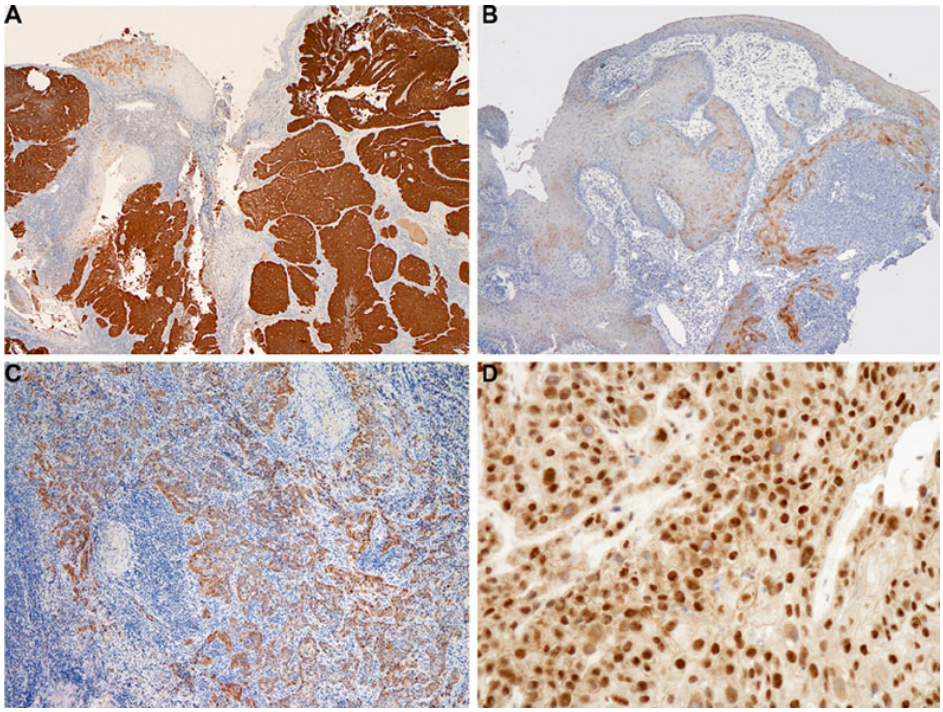

Figure 10: Diversity of p16 ^{ink4a} staining patterns

Strong diffuse staining in the nuclei and cytoplasm (A), patchy nuclear and cytoplasmic staining (B, C) and nuclear staining only (D). Only (A) should be regarded as significant p16 ^{ink4a} overexpression associated with HPV-driven tumor disease.
Reprinted by permission of Springer Nature: Histopathology ***(120)***, (2014).

Nevertheless, several publications found significantly high sensitivity for high-risk HPV of almost 100% (121), whereas specificity of p16 ^{ink4a} overexpression for proof of the presence of high-risk HPV-association appears to be lower with different findings in the literature between 72 and 95% (121-123).

Like for all diagnostic tests, the positive predictive value is highly correlated with the prevalence of the disease (124). Since the prevalence for HPV-positive OPSCCs is extraordinary high in some countries like in the United States, however, the positive predictive values are consequently high as well (124). Whereas in regions with notably minor prevalence rates like in certain European countries, the positive predictive values are to be found accordingly lower too (124). Therefor it is discussed whether the detection of p16 ^{ink4a} alone is sufficient for the HPV assessment for populations with a lower HPV prevalence (124).

Despite debates about the diagnostic performance considering p16 ^{ink4a} correlation with HPV-

association in cancerogenesis, there have been indications that p16 ink4a IHC positivity might predict clinical outcome independent from HPV status (125,126).

Since it was shown that patient survival curves of HPV-positive OPSCCs did not correlate with the traditional staging systems, the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) adapted their staging systems in 2017 now staging OPSCC-patients differently by their p16 ink4a status (127,128).

1.5.2 DNA-type-specific in-situ-hybridization (ISH)

For enhancing accuracy, it is repeatedly discussed to combine p16 ink4a detection with alternative methods such as DNA-type-specific in-situ hybridization (ISH) (129).

ISH is a molecular biological technique for detecting nucleic acids in tissue, cells or metaphase-chromosomes (119). For this reason, a labeled probe of a nucleic acid is used for binding to target HPV-DNA sequences in the nuclei of tumor cells (119). The probes can either be HPV-type-specific or be able to identify multiple HPV-types simultaneously (119).

The assay is known for its high specificity (130). Compared to alternative methods like PCR or p16 ink4a IHC, however, sensitivity appears to be minor, particularly when the viral load is low (130). Another weakness might be that signal interpretation shows inter-observer-discrepancies of up to 10% since staining signals are not of consistently clean quality (131).

Due to different test strengths, Smeets et al. underline the advantages of a two-stage testing approach, using p16 ink4a IHC as the first-line-method to eliminate HPV-negative cases from further analysis and performing HPV-16-ISH afterwards to significantly reduce the amount of false-positives (129).

1.5.3 DNA-type-specific PCR

Widely used alternative PCR-based methods aim for direct detection of viral DNA in tumor cells. For amplification of specific HPV DNA sequences, multiple available primer sets target consensus sequences of varying lengths within the HPV L1 gene (112). The advantage of these primers that

bind to a conserved region in multiple HPV types is the simultaneous testing of multiple HPV types (112).

Being known for its high sensitivity in the detection of HPV, the performance of available PCR assays varies depending on the choice on primer sets, the PCR protocol and the tissue condition being analyzed (119). A potential limitation might be the obtainment of false positive results while negative specimens could get contaminated from previously amplified specimens in the laboratory or from surrounding healthy cells being HPV-infected (119). What is also missing using this technique is the tissue context for interpretation of the results (112).

Nonetheless, still controversial is the question, in what way the detection of HPV-DNA in tumor cells proves that the tumor is necessarily HPV-driven. In this context, PCR assays probably show their most significant limitation, which is the lack of distinction between clinical significant HPV-infections, where the virus is actively involved in cancerogenesis and those infections without active involvement (132). To be able to identify clinical significant HPV-infections in tumor cells, the demonstration of transcriptional activity is necessary (133). Multiple studies showed that only OPSCCs with high levels of transcriptionally active high-risk HPV are associated with a significantly better prognosis (133).

1.5.4 RT-PCR and RNA-ISH

The gold standard for evaluating active involvement of HPV in carcinogenesis of OPSCCs is believed to be the detection of E6/E7 mRNA through reverse transcriptase polymerase chain reaction (RT-PCR) or the relatively newer technique RNA-ISH where the presence of transcriptionally active HPV can be verified (132,134).

Furthermore, attempts for HPV-antibody detection as an indirect proof for immune response against an HPV-driven tumor are taking place.

1.6 Prevention

1.6.1 Primary Prevention

Valid for all diseases, primary prevention strategies most importantly aim for the avoidance of triggering factors. In case of head and neck cancer, the most significant strategies for reduction of incidence rates are programs aiming for reduction of tobacco consumption since heavy smokers appear to have a 5- to 25-fold increased HNC-risk compared to non-smokers (135). This correlation is well documented in yearly cancer statistic reports. In particular, state-regulated strict laws could successfully reduce nicotine-related diseases such as HNSCCs by banishing smokers from public buildings and restaurants or by high tobacco taxation (77). Since regular alcohol consumption alone has been estimated to contribute 4% of HNCs globally, the reduction of daily alcohol drinking has also been a major issue for public health initiatives over decades (136). The consumption of ≥50 grams of alcohol per day is associated with at least a two or three times greater risk for developing HNC compared to non-drinkers (137). Nonetheless, attempts for reduction of tobacco- and alcohol abuse have been significantly hampered due to commonness in daily social routine and the powerful impact of advertising of the tobacco and liquor industry (138).

The third major risk factor, especially for cancerogenesis of OPSCCs, is HPV. Since the risk of infection is highly correlated with sexual behavior, avoidance of this triggering factor is practically not a considerable option. At least, monogamous sexual intercourse and avoidance of orogenital intercourse might potentially lower incidence rates. More efficient and much more practicable was the introduction of vaccines against certain types of HPV as a primary prevention strategy. State-regulated vaccination programs for countries with low and high socioeconomic status show promising efforts in reduction of anogenital cancer and highly likely for OPSCCs as well (6). FDA-approved vaccines Gardasil, Gardasil-9 and Cervarix target against most common HPV-high-risk-types aiming for the induction of antibody-mediated immunity against HPV-capsid-antigens (139). Four large randomized clinical phase-III trials performed in women ages 15-26 years could prove biologic efficacy of Gardasil and Cervarix vaccines in prevention of cervical HPV-infection and cancer and led to HPV-vaccination licensing in 2006 (6,140). By now, strong evidence was provided by several randomized clinical trials for a significant reduction of incidences of HPV-

related anogenital as well as oropharyngeal squamous cell carcinomas (6). Due to the insufficient latency period since the introduction, the full impact of current HPV-vaccines on oropharyngeal HPV-related cancer has not been presented yet (6).

In 2014, standard HPV-vaccination was established in the Austrian national vaccination plan, where boys and girls reaching the age of 9 years are being vaccinated in schools and public health institutions for free (141). Till the age 12, at least two partial vaccinations are necessary for sufficient immunity (141). For individuals aged over 12, three partial vaccinations are recommended (141).

1.6.2 Secondary Prevention

Secondary prevention strategies focus on two primary aims: the identification of high-risk-individuals and screening modalities (6). Even after life style modification, former HNC-patients have a high risk for developing a second primary tumor in the head and neck region, which is stated to be 2-7% per year (142). This elevated risk is caused by multiple reasons such as combined risk factors like tobacco- and alcohol-abuse, genetic instability or immunodeficiency following cancer therapy (143). Apart from hereditary risk factors, like in syndrome-patients associated with higher risk of cancerogenesis, the identification of high-risk-individuals based on exposition to triggering factors is of the greatest relevance (144). As already mentioned, the primary risk factors for HNC are tobacco- and alcohol-abuse as well as an HPV-high-risk-type-infection in particular HPV-type-16. Although often negated or trivialized, life-style factors like regular tobacco and alcohol consumption can simply be identified in anamnesis talk or through structured questionnaires. The identification of a relevant HPV-infection appears to be a major challenge. Though statements of non-monogamous relationship-status and promiscuous sexual behavior might give a hint for an elevated HPV-infection risk, a relevant infection cannot be identified through questionnaires or physical examination.

In order to reduce the HNC burden, attempts for secondary HNC prevention in the last decades hampered due to several factors: To date, strong evidence is missing that secondary prevention is able to effectively reduce HNC mortality (6). Another barrier is the lack of consensus on which population should be screened (6). Risk-based systematic screening protocols or algorithms for

HNCs are missing due to a lack of well-designed prospective clinical trials (6). According to the US Preventive Services Task Force, current evidence is insufficient to ensure benefits over harms for oral cancer screening in asymptomatic adults (145).

In regard to cervical carcinoma, with the Pap-smear-test, a successful secondary preventive tool, based on the collection and characterization of suspicious cells, was implemented decades ago (146). By contrast to oropharyngeal carcinoma, the area of cancer origin, the squamous columnar junction respectively the cervical transformation zone, is exactly known, limited in size and easily accessible, to collect relevant cells by smear or biopsy (5). Nowadays, the Pap-smear-test has been widely replaced by the direct proof of HPV presence via PCR, which is recommended for women over 30 years of age in Austria (147).

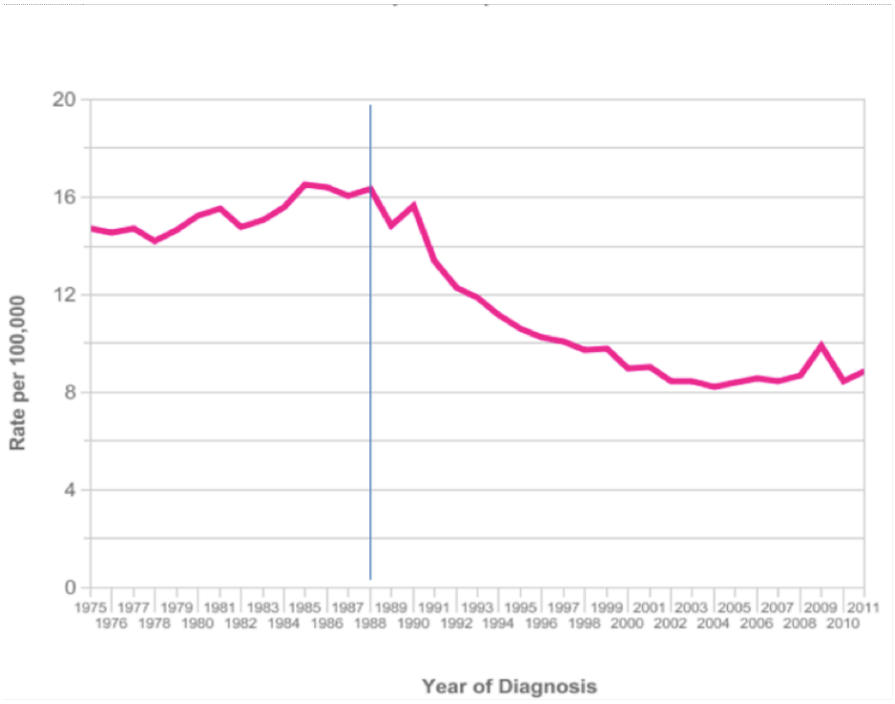

Figure 11: Incidence rates of cervical carcinoma cases in the UK

The figure shows the effect on incidence rates after implementation of the NHS cervical screening program based on the Pap-smear test in 1988. (148)

With OPSCC, current approaches of specimen collection reach their limits, since tumors can be

multifocal or simply not visible (5). As already mentioned above, the majority of OPSCCs are tonsil carcinomas. Mainly for reasons of surface amplification, the specific anatomical structure of tonsils shows multiple deep crypts, where small tumors can easily hide. Therefor, it is much less likely to gather representative cells by comparable smears in a screening attempt, where clinical signs of a tumor are missing (5). For now, final diagnosis relies on the identification of morphologic changes to determine the grade of disease meaning mucosal pathologies are already macroscopically obvious when biopsies are being taken.

The significance of HPV-association as a prognostic factor in OPSCCs pushed along further research on molecular-based modalities for earlier and less invasive OPSCC-detection. Nonetheless, there are no current guidelines for any HPV-specific primary screening tests for OPSCCs. (6)
Apart from direct HPV detection, through techniques like PCR or ISH, as already explained earlier, another major research focus lies on indirect tests for assessing circulating antibodies against HPV-oncoproteins in patient's serum, saliva or plasma (149).

However, the development of such antibody assays has been hampered by various factors: There was no evidence that identified antibodies could discriminate between HPV-associated tumor disease and subclinical infection (5). Another major barrier was the fact that HPV-subtypes are closely related to each other with DNA-sequence homologies of up to 90% (5). Therefor, a reliable discrimination between the subtypes appeared to be difficult because viral proteins share subtype-specific as well as functionally highly conserved and broadly cross-reactive adjacent epitopes (16). Classical serological antibody tests are not able to reliably differentiate between the serological responses to adjacent epitopes (150).

Subtype-specific conformational epitopes are located on the outer surface of so-called virus-like-particles (VLPs) consisting of L1-capsidproteins (5). VLPs are therefor used in current HPV-vaccines to guarantee subtype-specificity and are unable to induce a broadly cross-reactive HPV-protection (5). Nevertheless, HPV tests focusing on antibody detection against the L1-capsidprotein are said to be of poor diagnostic value and unreliable in current literature (149).

HPV-L1-related antibody levels were told to be mostly stable over time, correlating with sexual partners but not with HPV-associated malignant disease (150). Therefor HPV-L1-antibodies applied for markers of life-time-exposure to HPV but not for OPSCC-association (150). At the

moment, the detection of antibodies against early proteins, especially against HPV16-early-oncoprotein 6, is meant to be a reliable marker for relevant HPV-infection and OPSCC-association (149). HPV16 antibodies to the E6 oncoprotein has even been shown to be detectable 10 years prior to clinical manifestation of OPSCC (149,151,152). Furthermore, a nested case-control-study revealed that elevated serum antibody levels against HPV-E6 were common in patients who later developed anal carcinoma (153). Several studies showed a high sensitivity and specificity for the detection of antibodies to HPV16-early-proteins in $p16^{ink4a}$-positive-OPSCC (7,102,152,154-156). Nevertheless, what most of those studies have in common is the application of assays using bacterially expressed Gluthathione-S-Transferase (GST) fusion proteins for antibody detection. This test procedure was developed by the Deutsches Krebsforschungszentrum (DKFZ) in Heidelberg and is still the established method for HPV-antibody detection studies worldwide (157). A downside, however, seems to be the fact that these tests are not CE-marked yet and testing is mainly performed at one laboratory of the DKFZ, where test samples are usually being sent to. The establishment of this method in other research laboratories could not be achieved so far.

Since the year 2014, a newly available HPV16-L1 subtype-specific competitive serological assay based on the HPV16-L1-specific monoclonal antibody clone DRH1 is on the market. Developed and produced by company Abviris© in Germany, the assay is told to be able to reliably differentiate between relevant HPV16-infections associated with precancerous respectively malignant HPV16-induced disease and subclinical HPV16-infections. According to the manufacturer's information, this differentiation succeeds because the specific antibody clone 'anti-HPV16 L1 DRH1' is directed against a protein that is only formed by cells in which HPV16 has already actively intervened in cell division. So far, no published scientific literature is to be found that evaluates the clinical performance of this new test procedure.

1.7 Aim of the Doctoral Thesis

Since HPV-association in OPSCCs is demonstrably a reliable prognostic marker, indirect HPV-detection via $p16^{ink4a}$ IHC became standard in the OPSCC diagnostic workup. Nonetheless, the majority of OPSCC-patients are still diagnosed as late-stage tumors significantly affecting patient's outcome. Given this fact, a lot of scientific effort is put into research of diagnostic markers to

possibly enable early tumor diagnosis. The detection of antibodies against HPV-oncoproteins is a major field of interest in this regard. Despite of multiple promising study data, a consensual procedure agreement or screening standardization is still missing, primarily because specificity of classical serological assays is not sufficient enough to be reliably used in clinical routine.
This work aims to evaluate the clinical performance of a newly developed HPV16-L1-DRH1 competitive serological assay used in a cohort of patients with oropharyngeal squamous cell carcinoma at the Department of Otorhinolaryngology – Head and Neck Surgery at the Medical University of Graz. The primary focus lies on the association between HPV16-L1-antibody-titers and HPV16-induced OPSCCs as well as the antibody behavior under tumor-specific treatment and during follow-up. For definition of HPV-induction, tumor tissue specimens are examined for $p16^{ink4a}$ -overexpression and HPV-DNA-presence assessed through PCR. Furthermore, HPV-genotyping is performed.

2 Material and Methods

2.1 Patient Selection

In the present study, 34 patients presented with head and neck carcinoma at the Department of Otorhinolaryngology – Head and Neck Surgery, Medical University in Graz were included. Each patient signed informed consent forms after informational discussion about the planned course of the study. The ethics committee of the Medical University of Graz approved the study (EK-Nr.: 28-378 ex 15/16).

Inclusion criteria included patients ≥18 years of age, regardless of gender, presenting with a high suspicion for primary squamous cell carcinoma of the head and neck, preferably of the oropharynx.

Exclusion criteria included patients with recurrent or secondary tumors of the head and neck, patients in a reduced general state of health, not suitable for any curative tumor-specific therapy and patients with severe cognitive impairment, not able to fully understand the course of the study. Also patients who received vaccinations against HPV were excluded because elevated antibody levels against HPV-L1 would have been expected. Potential study participants were asked about their HPV vaccination status in the context of study enrollment and their medical history was

screened for other HPV-induced diseases like anogenital carcinoma. Patients, where histopathology revealed other tumor entities than squamous cell carcinomas, had to be excluded afterwards, which happened in one case explained later on. Patients, who already took part in a different study other than an observational study, were also excluded.

2.2 Diagnostic Workup

For the most part assigned by a resident ENT-specialist or in some cases by a general practitioner with suspected malignant disease, all patients underwent a detailed patient history including the investigation of type and duration of tumor-related symptoms and the evaluation of potential risk factors for cancerogenesis, especially regular tobacco- and alcohol consumption. Subsequently, a standardized physical examination including fiber optic endoscopy was carried out and radiologic imaging with CT or MRI of the head and neck region, chest x-ray and upper abdominal sonography was performed in the context of diagnostic workup. Consequently, all patients underwent panendoscopy under general anesthesia during inpatient stay. This standard procedure is primarily performed to evaluate tumor expansion and its relation to critical adjacent structures to consequently assess resectability. During inpatient stay, further standard examinations like blood tests, audiometry, tooth status, nutritional status and psychological talk were carried out, primarily to assess patient's individual suitability for therapy.

2.3 Tumor Specimen Collection

Partly, tumor biopsies were performed around primary presentation of the patient in the outpatient department under local anesthesia. In cases, where tumor origin was not obvious or tumor tissue was difficult to reach, biopsies were taken in the context of panendoscopy. In 31 patients, squamous cell carcinoma of the oropharynx was histologically proven. In two out of three patients primarily presented with cancer of unknown primary, histopathology as well revealed squamous cell carcinoma in biopsied cervical lymph node metastases. The third patient originally presented with CUP syndrome had to be excluded because histopathology surprisingly revealed malignant melanoma.

Tumor specimens were sent to the Institute of Pathology of the Medical University in Graz, where, after fixation in formalin and embedment in paraffin, the histopathological diagnostic workup was performed.

After completion of diagnostic workup and presented histopathological results, each case was presented to the tumor board, where an individual tumor-specific therapy was decided based on TNM status, sensible tumor resectability, age, general state of health and the patient's wish.

2.4 P16^{ink4a} Immunohistochemistry

After fixation in formalin and embedment in paraffin, all tumor specimens were immunohistochemically examined for p16^{ink4a} protein overexpression at the Diagnostic and Research Institute of Pathology of the Medical University in Graz, using the immunohistochemistry assay CINtec® p16 ^{ink4a} Histology KIT (Ventana Roche Diagnostics, Switzerland). According to international standards, the threshold for p16 ^{ink4a} positivity was determined by showing ≥70% p16 ^{ink4a} nuclear and cytoplasmic staining of at least moderate intensity.

2.5 PCR-based HPV-DNA Analysis and Multiplex HPV Genotyping

At the Diagnostic and Research Institute of Pathology, all tumor specimens were analyzed for the presence of HPV-DNA by means of broad-spectrum primer polymerase chain reaction (PCR) following HPV-genotyping using the HPV 3.5 LCD Array KIT (Chipron®, Germany).

PCR allows amplification of specific DNA sequences from the smallest quantities of raw material (158). In repeating cycles of DNA-denaturation, primer-hybridization and elongation, specific DNA sequences are being amplified (159). The reaction is catalyzed by a thermostable DNA-polymerase, additionally requiring desoxyribonucleotide triphosphates and two oligonucleotide primers (159). For HPV-DNA detection, primers are used, which are complimentary to conserved regions of the viral L1-gene (159). One primer is based on the commonly used MY11/MY09 system and a second one producing shorter amplicons of about 150 bp in length (160). Within one reaction, the amplification of HPV-type-specific amplimers of approximately the same length is

provided through characteristic polymorph sequences of the amplicon (160). Subtype specific capture probes fixed on an LCD chip surface are used for hybridization of amplified PCR-products that were labeled with biotin (160). Allowing parallel analysis of eight samples on one chip, each LCD chip contains eight identical micro arrays, separated in small reaction chambers, whereby each array can be addressed individually {Chipron LCD KIT}. Using the HPV 3.5 LCD Array KIT parallel specific identification of 32 different HPV subtypes was performed (160).

2.6 Serum Sample Collection

Patient sera were collected through venous blood draw in standardized serum tubes prior to tumor-specific therapy, mostly in the context of routine blood draw during inpatient stay, usually at a time when diagnosis of squamous cell carcinoma was histopathologically already verified. Serum tubes were centrifuged at 3000 rpm for 10 minutes. Subsequently, about 1 ml of patient's serum was transferred to standardized collection devices in a pouring technique. After labeling the devices with their consecutive ID number, they were stored at -20°C. For protection against contamination, gloves were worn at all times during laboratory work processes.

Serum collection was carried out during and after tumor-specific therapy as well as in the context of oncological follow-up meetings in standard intervals of three to six months from September 2016 to November 2018.

2.7 Follow-up

Oncological follow up was carried out in accordance to national guidelines including a meticulous physical examination, fiber optic endoscopy and soft tissue sonography of the neck in intervals of three months in the first two years. Thereafter, intervals were expanded to 6 months for the following two years.

The first oncological follow-up visit was carried out three months after primary tumor-specific therapy was completed. Including a full restaging examination by means of CT or MRI imaging of the head and neck, chest x-ray and upper abdominal sonography, therapy success was evaluated for the first time. Later on, full restaging imaging was only performed once a year if clinical suspicion

of tumor recurrence was missing.

In cases where tumor recurrence seemed possible, promptly CT or MRI imaging was performed and suspicious tissue was either biopsied in local or general anesthesia and the patient was again presented to the tumor board to determine further procedure.

2.8 Serological HPV16-L1 Antibody Detection

2.8.1 General Description of the used Test and its Components

Serological detection of specific HPV16-L1 antibodies was carried out using a competitive epitope-specific immunoassay (PrevoCheck®, Abviris, Germany) that is already CE-marked and commercially available in the form of a 'yes-or-no-test'. The assay is based on the purified monoclonal mouse antibody DRH1 that corresponds to a specific HPV16-L1 antibody. The test has the form of a lateral flow test, where DRH1 antibody equivalents in the serological test sample compete with gold-labeled DRH1 antibodies of the conjugate for binding sites of the DRH1-specific L1-reagent (ELL1).

2.8.2 Structure of the Cassette Test

The plastic case of the test cassette contains a test stripe with the opening for the sample (S) at the lower end. The opening of the reaction zone lies above. In the reaction zone, the test zone (T) in the middle part and the control zone (C) in the upper part is located. Reading the potential staining of the test zone is decisive to assess whether the test is positive or negative explained in more detail as follows.

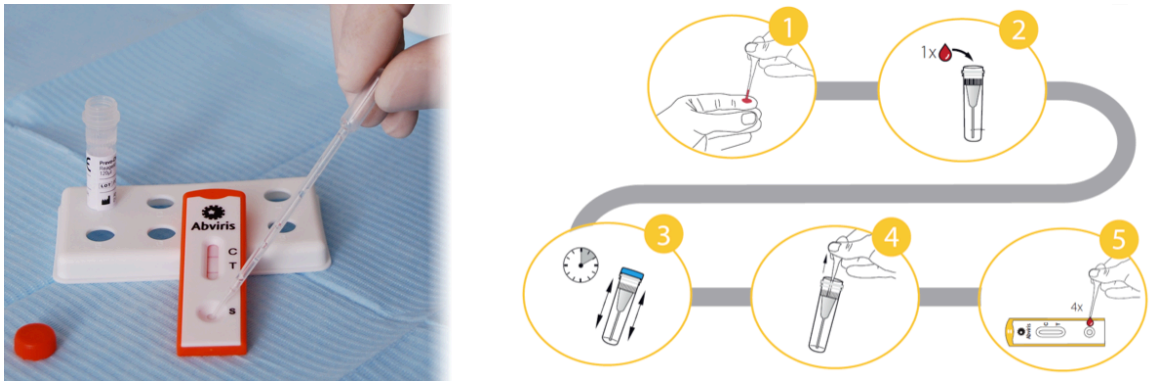

Figure 12: Prevocheck test kit and instruction for performing the test (shown here with the use of whole blood)

2.8.3 Components of the Test Kit

- Four cassette tests, separately packaged
- Four tubes with 120 µl of an HPV-ELL1 reagent (HPV16 L1 antibody specific surface protein in buffer that contains 0.09% sodium acid
- Eight pipettes
- One tube holder

2.8.4 Description of Mechanism of Action

After defrosting, each tube containing the serum sample was shaken before the needed amount for testing was extracted to provide a preferably high homogeneity of serum contents. First, about 25 µl oft the patient's serum sample were added to an ELL1-reagent and pre-incubated for 5 minutes. If DRH1 antibody equivalents are present in the serological sample, a complex of ELL1-reagent

and DRH1 antibody equivalents is formed, which inactivates the ELL1-reagent. The degree of inactivation depends on the concentration of the DRH1 antibody equivalents in the sample and increases proportionally.

If the patient's serum sample is lacking of any DRH1 antibody equivalents the ELL1-reagent stays fully reactive.

After pre-incubation, the mixture of patient's serum and reagent was transferred onto the opening zone for the sample (S) at the lower end of the test cassette. Beneath the sample zone gold-labeled DRH1 antibodies are located, which are dissolved by adding the aqueous ELL1-reagent mix. The mixture runs onto a polystyrene stripe of a lateral flow cassette to a reaction zone that is marked with a T for test line.

There, immobilized DRH1 antibodies bind the amount of still active ELL1-reagent and the formed complexes stick to the reaction zone on the test stripe.

The test system takes advantage of the fact that the ELL1 reagent has 360 binding sites for DRH1 antibodies. The test result line becomes visible since the gold-labeled DRH1 antibodies stick to free binding sites of the ELL1 reagent proving that it was still active after the preincubation with human serum.

HPV16-L1 antibodies, which would have inactivated the reagent, were not detected, the test is negative.

In cases, where the binding sites of the ELL1-reagent are fully saturated because of existent HPV16-L1 antibodies in the patient's serum, the test result line is not stained, the test is positive.

The staining of the C line at the top of the test cassette due to an independent antibody reaction proves that the sample volume was sufficient. Regardless of the test result, the C line must appear in any case proving the correct course of the test.

According to the manufacturer's instructions, the immunoassay, as commercially available, can also be performed with whole blood.

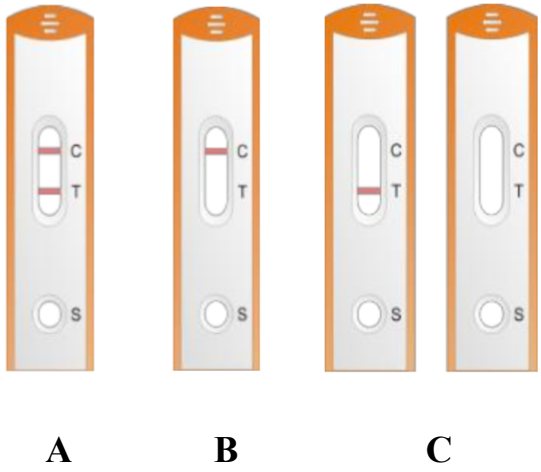

Figure 13: Test interpretation

(A) shows a staining of the C and T line according to a negative test result. There are no HPV16 L1 antibodies present in the sample or the antibody concentration is below the test's detection limit. (B) shows just the C line colored, which corresponds to a positive result. An HPV16 L1 antibody concentration above the test's threshold is detectable in the sample. (C) shows either a colored T line only or no staining at all, which corresponds to an invalid result. Insufficient sample volume, expired tests or incorrect procedures are the most likely reasons.

2.8.5 Measurement of Antibody Concentration using Photometry

Staining intensity of the T line after the reaction is inversely correlated to the HPV16-L1 concentration in the patient's serum. This intensity was measured using a spectrophotometer (EseQuant-Reader, QIAGEN, Germany). Since the immunoassay is a competitive test system, it allows absolute quantifications, like in our case in ng/ml. Other non-competitive test systems can only quantify in a semi-quantitative way, where the manufacturer chooses abstract units, without direct correlation to the absolute quantity. The possibility for absolute quantification in ng/ml is based on the fact that the used antibody equivalent DRH1 is a monoclonal antibody produced in cell culture systems. According to the manufacturer's information, these reference antibodies show a purity of 99.9% after purification process. By protein measurement of the purified antibodies, the absolute concentration in ng/ml can be determined. Based on a concentration curve, created by means of dilution series with defined concentrations measured at specific wavelengths, the total concentration can be deduced from each photometric measurement.

Each serum sample was tested twice. The mean of both determined antibody concentrations was selected as the real value and entered in the study database.

Figure 14: Work station setup for photometric measurement of antibody concentrations

2.8.6 Assessment Procedure

According to the manufacturer's information, the threshold level for a positive proof of present HPV16-L1 antibody levels that correspond with a precancerous respectively malignant HPV-16 induced disease is assumed to be 1000 ng/ml. This cut-off point was statistically estimated based on non-published study data by the company Abviris.

2.9 Diagnostic Specificity of a Healthy Control Group

As part of the multi-center study resulting from this work, serum samples from 1064 healthy volunteers were kindly provided by Bioscientia Laboratories (Ingelheim, Germany) to assess the

diagnostic specificity for the test. The “healthy status” of the probands was defined by normal CRP values, whereby, except for the non-presence of an HPV-related disease, no medical history data or data from recent physical examinations were provided. Using the exactly same test procedure including the measurement of absolute antibody titers, these serum samples were examined externally in the Bioscientia laboratory. The data was kindly provided to be used in this work.

2.10 Statistical Analysis

Statistical analysis and calculations of this work were performed with the software program SPSS Statistics 26. Statistical significance was determined for a p-value ≤ 0.05. Confidence intervals were determined with 95%.

In the course of the underlying published manuscript, parts of the statistical analysis were calculated externally (p-Wert, Jena).

Patient and tumor characteristics were calculated in dependence of their $p16^{ink4a}$ and HPV-DNA status. The differences between the groups were determined either by the chi-square test, Fisher’s exact test or the Wilcoxon rank test.

2.10.1 Crosstabs

For primary analysis, HPV16-induction for the present tumors was determined by $p16^{ink4a}$ positivity and the detection of HPV16 DNA in the tumor tissue. Crosstabs were created to illustrate the relation between HPV DNA status and $p16^{ink4a}$ overexpression. Separated by the cut-off level of 1000 ng/ml for definition of test positivity or negativity, the test results and the results concerning HPV16-DNA detection were entered in a four-field table. The data in this chart provides information in what frequency a positive test result correlates with the actual number of HPV16 induced tumors.

2.10.2 Sensitivity, Specificity, positive and negative predictive Value

By the creation of crosstabs, the calculation of sensitivity, specificity and positive and negative predictive value was possible. While the sensitivity indicates the proportion of HPV16-induced tumors classified as correct-positive by the test result, the specificity indicates the proportion of tumors classified as correct-negative by the test result. With the positive predictive value, the proportion of correct-positive among the total of the HPV16-induced tumors recognized by the test was calculated. The negative predictive value was used to determine the proportion of tumors that were not HPV16-induced recognized as correct-negative out of all of tumors negative for HPV16-induction.

2.10.3 Correlation Analysis (161)

By looking at the distribution of two variables, it is often obvious that the change of one variable has an influence in the change of the other variable. Correlation analysis describes the relationship between those two ore more variables expressed as a correlation coefficient ranging from -1 to +1. Low correlations are found if the result is near zero. A positive correlation is present if the result shows a value above zero, if the correlation coefficient shows a value under zero, the correlation is negative. Though often misinterpreted, the correlation coefficient does not suggest causality. It is not the degree of dependency that is determined, but only the degree of linear relationship. As for normally distributed variables, the Pearson correlation coefficient was calculated, for variables with a different scaling the Spearman rank correlation coefficient was calculated.

2.10.4 Receiver-Operating-Curve- (ROC), Area-under-the Curve- (AUC) Analysis (161)

ROC-curves were used to provide an overview of the diagnostic quality of the test. By determination of the relative frequency distributions in form of sensitivity (correct-positive) and false-positive rate (100-specificity) of the test results, a diagram is drawn, where the sensitivity is entered as the ordinate (y-axis) and the false-positive rate as the abscissa (x-axis). The result is typically a curved, ascending curve. Each point on the ROC curve represents a

sensitivity/specificity pair corresponding to a particular decision threshold. An ROC curve near the diagonal indicates a random process. The ideal ROC curve initially rises vertically showing a hit rate close to 100% with a course close to the upper left corner (the closer the better: 100% sensitivity, 100% specificity).

Additionally, the area-under-the-curve was calculated. Mathematically corresponding to the definite integral, it is a measure of how well a parameter can differentiate between two diagnostic groups (diseased/normal).

2.10.5 Cut-off Point Calculations

Calculated externally at P-Wert in Jena, by means of Youden-index, 'closest-to-(0,1) criterion' and calculations according to Liu, different measures for evaluating the ideal cut-off concentration to define whether a test result is positive or negative were performed.

Common for the determination of threshold decision points is the Youden-index, which is calculated by adding up sensitivity and specificity minus one, respectively minus 100 if the dimensions are given in %.

Alternatively referred to as the 'closest-to-(0,1) criterion', the point that is furthest away from the diagonal and therefor closest to point (0/1) or point (0/100) is used. This is the point at which sensitivity and specificity are equal to 100 (or equal to 1, depending on the scale).

As a third variant, the cut-off point was calculated using a method described by Liu et al., where, in contrary to the Youden-index, not the sum, but the product of sensitivity and specificity is maximized (162).

3 Results

34 patients highly suspicious for squamous cell carcinoma of the head and neck were recruited for this prospective non-interventional study at the Department of Otorhinolaryngology, Head and Neck Surgery in Graz. As to be expected in a Styrian hospital, all patients were of Caucasian ethnicity. In 31 patients squamous cell carcinoma in the oropharyngeal region was

histopathologically confirmed. Out of three patients presented with a clinical high suspicion for cervical lymph node metastasis of unknown primary, two of those were revealed to suffer from squamous cell carcinoma as well. In one of those three patients diagnosed with CUP syndrome, histopathology surprisingly revealed malignant melanoma. This is why he subsequently had to be excluded.

3.1 Epidemiology

The mean age of all 34 patients was 63.7 years with a range from 47 to 83 years. As to be expected, according to current epidemiological HNC data, the gender ratio of the study patients predominated for the male gender. The 26 recruited male patients with a mean age of 63.2 years ranging from 47 to 83 years presented to be slightly younger as the recruited 8 women with a mean age of 65.4 years ranging from 49 to 77 years. The vast majority of patients showed a largely good general condition of health. Only one patient was associated with an ECOG status two in the tumor board, meaning a confinement to bed of over 50% per day. The rest of the patient collective had ECOG status zero or one.

21 of the 31 OPSCC patients had their tumor origin in the tonsillar region, 9 OPSCCs were diagnosed as base of tongue carcinoma and one tumor was located at the soft palate. For one female patient, originally diagnosed as another CUP syndrome because of histopathologically confirmed squamous cell carcinoma in biopsied cervical lymph nodes being lacking of a hint for primary tumor location, the diagnosis had to be changed later on. Due to abnormalities in the performed CT-imaging after primary neck dissection was already performed, a second panendoscopy was carried out, where the tumor origin could finally be proved at the base of tongue. For the other three patients diagnosed as CUP syndrome, the primary tumor origin could not be ascertained in the course of disease, despite extensive diagnostics including PET CT. Also the colleagues at the Department of Dermatology, where the patient was subsequently transferred, could not reveal the origin of the primary malignant melanoma.

During the assessment of potential risk factors, 17 out of the 33 HNSCC patients reported regular tobacco use with ≥ 20 pack years. 12 of them were men and with 5 out of all 8 female patients, the majority of the women's group had a smoking habit. 11 out of 33 patients reported regular alcohol

consumption, which was defined as daily alcohol intake of at least two or more glasses of beer or wine, 8 of them were men. All 11 patients with a drinking habit were regular smokers too.

Using the current AJCC staging system 8, for 15 out of all 33 HNSCC patients, with a mean age of 63 (ranging from 51 to 79), an advanced tumor stage was found when first presented in the tumor board correlating to AJCC classification III and IV. 11 of them were male patients. The 18 patients, who appeared to be diagnosed at a minor tumor stage (AJCC I and II), were slightly older with a mean age of 65 years (range from 47 to 83).

10 out of the 15 advanced HNSCC patients showed regular tobacco consumption, whereas 7 patients out of these 15 reported additional regular alcohol intake.

Having a closer look at the impact of the new staging system, with 29 out of all 33 HNSCC patients, the vast majority would have been staged in advanced tumor stages III and IV using the former AJCC staging system 7.

Patient Characteristics				Tumour Characteristics					Serological Status						Surveillance	
Patient ID	Gender	Age at diagnosis	Smoker* /Alcohol**	Localisation	AJCC	HPV DNA	p16	Therapy***	positive/negative	Antibody Conc.	Decrease	Increase	Serum	Follow up	Disease free	Death
	M/W	in years	yes / no							in ng/ml			Samples	in month		
5	W	75	no/no	Tonsil	IV	HPV16	positive	S+RCT	positive	28,000	lost	lost	1	0	yes	no
24	M	62	yes/yes	Tonsil	III	HPV16	positive	RCT	positive	22,500	−60%	0	3	16	yes	no
19	M	67	no/no	Tonsil	I	HPV16	positive	S+RT	positive	11,100	−90%	0	7	16	yes	no
13	M	79	no/no	Base of Tongue	III	HPV16	positive	RT	positive	9000	−28%	0	6	15	yes	no
28	M	62	yes/yes	Tonsil	II	HPV16	positive	RCT	positive	8400	−63%	0	3	2	yes	no
10	M	57	no/no	Tonsil	II	HPV16	positive	S+RCT	positive	5800	−100%	0	8	20	yes	no
34	M	47	no/no	CUP	III	HPV16	positive	S+RT	positive	5000	−35%	0	3	1	yes	no
12	M	81	no/no	Base of Tongue	II	HPV16	positive	RIT	positive	4800	−43%	436%	7	19	no	no
15	M	57	no/no	Tonsil	II	HPV16	positive	RCT	positive	4000	−75%	0	7	18	yes	no
27	M	62	no/no	Tonsil	I	HPV16	positive	S	positive	2300	−76%	0	7	16	yes	no
4	W	66	no/no	Tonsil	II	HPV16	positive	S+RCT	positive	1900	−74%	0	7	17	yes	no
8	W	65	yes/no	Tonsil	II	HPV16	positive	RCT	positive	1740	−71%	0	7	19	yes	no
16	M	56	no/no	Tonsil	I	negative	positive	RCT	positive	1455	−100%	0	3	2	yes	no
29	W	65	yes/no	Tonsil	III	HPV16	positive	RCT	positive	1425	−60%	0	2	13	yes	no
25	M	70	yes/yes	Tonsil	II	HPV16	positive	RIT	positive	1300	−100%	0	5	16	yes	no
3	M	83	no/no	Tonsil	I	HPV16	positive	S	positive	1285	−53%	0	5	18	yes	no
20	W	77	no/no	Tonsil	I	HPV16	positive	RIT	positive	1250	−100%	0	5	14	yes	no
21	W	58	yes/yes	Base of Tongue	IV C	negative	negative	RCT	positive	1250	−100%	0	3	4	no	yes
11	W	49	yes/yes	Base of Tongue	II	HPV16	positive	S+RCT	positive	1184	−54%	0	6	16	yes	no
14	M	64	yes/no	Tonsil	III	HPV16	positive	RCT	positive	1120	0%	148%	6	15	yes	no
1	M	81	no/no	Tonsil	II	HPV16	positive	RT	positive	1070	0%	78%	4	11	yes	no
22	M	52	yes/yes	Base of Tongue	IV A	negative	negative	RCT	positive	1000	−100%	0	3	6	yes	no
7	M	72	no/no	Tonsil	III	negative	positive	RT	negative	965	−100%	0	7	15	yes	no
26	M	51	yes/yes	Soft Palate	IV A	negative	negative	RCT	negative	820	−50%	0	6	10	no	no
17	M	58	no/no	Tonsil	III	negative	negative	RCT	negative	710	−100%	0	6	16	yes	no
31	M	67	no/no	Base of Tongue	III	negative	negative	RCT	negative	700	lost	lost	1	0	lost	lost
32	M	56	yes/yes	CUP	IV A	negative	negative	S+RCT	negative	700	stable	stable	2	2	yes	no
9	M	72	yes/no	Base of Tongue	II	HPV33	positive	RCT	negative	630	stable	stable	6	16	yes	no
30	W	68	yes/yes	Base of Tongue	IV A	negative	negative	RCT	negative	600	lost	lost	1	0	yes	no
23	M	59	yes/yes	Tonsil	IV A	negative	negative	S+RCT	negative	400	stable	stable	3	6	yes	no
6	M	63	yes/yes	Tonsil	II	HPV33	positive	CT+RCT	negative	56	stable	stable	7	19	yes	no
2	M	55	yes/no	Tonsil	II	HPV16	positive	S+RCT	negative	0	stable	stable	7	26	yes	no
18	M	52	lost	lost	lost	lost	lost	lost	negative	0	lost	lost	1	0	lost	lost
33	M	59	yes/no	Base of Tongue	III	negative	negative	S+RT	negative	0	stable	stable	6	10	yes	no

Table 2: Baseline characteristics of 34 patients with head and neck cancer (Graz) (5)

Description of 34 tumor patients, 31 of them with OPSCC, 2 with CUP syndrome and one drop out, showing baseline characteristics, tumor description containing HPV DNA and p16 ^{ink4a} status from the tumor specimen and serological HPV16 L1 antibody status.
*≥ 20 pack years tobacco use **regular alcohol consumption ***S=Surgery, RT=Radiotherapy, CT=Chemotherapy, RCT=Radiochemotherapy, RIT=Radioimmunotherapy

3.2 P16^{ink4a} Immunohistochemistry and HPV DNA Detection

24 of the 33 HNSCC patients showed an overexpression of the p16 ^{ink4a} protein in the histopathological sections of the tumor biopsies. In 22 tumor specimens out of all 33, the presence of HPV DNA could be demonstrated. Using multiplex HPV genotyping, it was shown that for the vast majority of HPV DNA positive patients, specific DNA of HPV-subtype-16 was detected, namely in 20 cases (91%). For the other two HPV DNA positives, specific DNA of HPV-subtype-33 could be verified.

On the basis of these numbers, it is already obvious that the results of p16 ^{ink4a} IHC and PCR-based HPV DNA detection do not always correlate, as described in current literature. The details are described in table 3 below.

		p16 ink4a IHC		
		p16 ink4a positive	**p16 ink4a negative**	**All**
PCR-based HPV DNA Detection	**HPV DNA 16 pos.**	20	0	20
	HPV DNA 33 pos.	2	0	2
	HPV DNA neg.	2	9	11
	All	24	9	33

Table 3: Correlation between p16 ink4a IHC and PCR-based HPV DNA detection

For all of the patients in our cohort, where HPV DNA was detected in the examined tumor specimens, a p16 ink4a overexpression was shown too, meaning a sensitivity of 100% for p16 ink4a IHC. Specificity was with 82% notably lower because in two p16 ink4a positive patients the presence of HPV DNA was excluded in the PCR-based analysis. On the basis of these results, it was assumed that in 22 cases tumor disease was actually HPV-induced. Using the new TNM classification 8 that differentiates on the basis of the p16 status, 6 out of all 24 p16 ink4a positive patients presented in advanced tumor stages III and IV. By contrast, if these patients had been staged according to the former TNM classification 7, 20 patients would have been classified for advanced AJCC tumor stages III and IV. Out of the 22 HPV-DNA positives, 21 were diagnosed as OPSCCs, whereas in one case no primary tumor could be identified whereupon the diagnosis CUP syndrome was made. 17 out of the 21 p16 ink4a /HPV DNA positive OPSCCs were tonsil carcinomas, which is with 81% significantly more than in the p16 ink4a /HPV DNA negative group (25%). In the p16 ink4a /HPV DNA positive HNSCC group, 10 patients presented to have additional risk factors. 5 of them were smokers and the other 5 patients reported about additional regular alcohol intake.

All 9 p16 ink4a /HPV DNA negative HNSCC patients were diagnosed in advanced tumor stages, all of which were positive for the risk factor of regular smoking too, six of them reported about additional regular alcohol consumption. For one p16 ink4a negative patient presented with an advanced base of tongue carcinoma, however, none of the evaluated risk factors smoking, regular alcohol intake or HPV-DNA in the tumor sample could be ascertained. 8 p16 ink4a /HPV DNA negatives suffered from OPSCC, one patient was diagnosed with CUP syndrome. By contrast to

the p16 ink4a /HPV DNA positive patients, the negative ones diagnosed with OPSCC had their tumor origin to be mostly found at the base of tongue, namely in 5 cases. Only two patients suffered from tonsil carcinoma, whereas in one patient the tumor was located at the soft palate.

3.3 Therapy

According to the tumor board decision, all kinds of primary tumor-specific therapy concepts were found in our cohort, whereby the number of surgically treated patients was slightly lower compared to that of the conservatively treated patients.

12 out of all 33 HNSCC patients were primarily treated by means of surgery, mostly in the sense of primary tumor resection following uni- or bilateral neck dissection. In 2 of those cases, primary therapy was completed after surgery and patients went straight to follow-up. The other 10 patients received adjuvant therapy following surgery after being again discussed in the tumor board on the basis of histopathologic results, meaning post-operative radiotherapy (PORT) with or without concomitant chemotherapy.

21 patients were treated conservatively with primary radiotherapy, of which 15 got concomitant chemotherapy and 4 received concomitant immunotherapy with cetuximab. In two cases, neoadjuvant chemotherapy, according to the TCF scheme, was carried out based on tumor board's recommendation because of large tumor load.

In the group of the p16 ink4a negatives, only two patients were treated with primary surgery (22%), in contrast to the p16 ink4a positives, where, with 10 out of 24, a significantly higher amount of patients was treated surgically (42%). Due to the advanced tumor stages for all p16 ink4a negative patients, tumor-specific therapy was already limited at the time of diagnosis, in most cases leading to conservative treatment, although radiosensitivity is worse compared to p16 ink4a positives according to current literature.

3.4 Serological HPV16-L1 Antibody Detection

3.4.1 Test's Sensitivity

According to the manufacturer's cut-off concentration recommendation of 1000 ng/ml for defining test's positivity and negativity, 22 patients out of all 33 HNSCC patients showed a positive test result for the detection of tumor-related HPV16-L1 antibody titers. In 19 patients out of all 20 patients where HPV16 DNA could be ascertained in the tumor tissue, a positive result for an HPV16-L1 antibody concentration over 1000 ng/ml was shown. In three cases, the serological immunoassay was positive for HPV16-L1 antibody titers above the threshold of 1000 ng/ml, although no HPV DNA was detected in the multiplex PCR, yet in one of these cases $p16^{ink4a}$ overexpression was proven. Based on these results the test's sensitivity was ascertained with 95% for our patient collective.

3.4.2 Negative Test Results

For 11 patients out of all 33, the serological test was negative for HPV16-L1 antibody titers over 1000 ng/ml. Only in one case, the immunoassay showed negative results for HPV16-L1 antibodies in the patient's serum, although this patient, suffering from tonsil carcinoma, was proven to be positive for HPV16 DNA and additionally showing $p16^{ink4a}$ overexpression in the histopathologic tumor sections. The screening of the patient's medical history revealed no evidence of immune system disorders that could have explained the lack of HPV antibodies. For the other 10 patients, where the immunoassay showed negative results, 8 of them were also negative for any HPV DNA. 2 of them were positive for a different HPV-subtype, namely for HPV-subtype-33. The fact that the immunoassay was also negative for these two cases underlines the subtype-specificity of the test according to manufacturer's information. To sum it up, out of 13 HPV16 DNA negative patients (11 HPV DNA negatives and 2 HPV33 positives) 10 cases were determined 'test negative' (under the threshold concentration of 1000 ng/ml).

		Serological Antibody Status		
		HPV16-L1 pos. >1000 ng/ml	**HPV16-L1 neg. <1000 ng/ml**	**All**
PCR-based HPV DNA Detection	**HPV DNA 16 pos.**	19	1	20
	HPV DNA 33 pos.	0	2	2
	HPV DNA neg.	3	8	11
	All	22	11	33

Table 4: Correlation of serological HPV16-L1 antibody status and PCR-based HPV DNA detection

3.4.3 Serological Antibody Concentrations

The heights of the measured absolute antibody concentrations at the time of diagnosis highly varied with a mean antibody titer for the positive tests (above 1000 ng/ml) of 5313 ng/ml ranging from 28000 to 1000 ng/ml. The titer results for the negative tests (under 1000 ng/ml) showed in the majority of cases values above zero. The mean antibody titer for the negative tests was 507 ng/ml ranging from 965 to 0 ng/ml.

One might assume that the antibody titer heights of the HPV16 DNA positive tests would correlate positively with the tumor mass illustrated by the T stage, meaning the higher the tumor load the higher the antibody titer. This assumption could not be proven for our patient cohort. Using the spearman correlation, no significant correlation could be shown (p=ns).

Due to the fact that the barrier to the lymphatic tissue is particularly thin and fragile in the tonsillar crypts, enabling direct contact of the HP virus to the immunospecific tissue and beyond, current literature reports, that HPV-induced OPSCCs are more likely located in the tonsillar region. It could therefor be assumed that the height of the antibody titers might be higher in tonsillar carcinomas compared to other location in the oropharynx. Again, this assumption could not be proved for our patient cohort using a t-test (p=ns).

Also the assumption that a high metastatic spread to cervical lymph nodes, illustrated by the N

stage, might trigger augmented immunoresponse in the form of higher antibody titers because of increased presentation to immunospecific tissue, could not be ascertained using the spearman correlation (p=ns).

Looking at the patient's age, it was also assumed that the immunoresponse in younger patients might be stronger showing higher antibody titers compared to older patient groups. Again using the spearman correlation in this regard, no correlation was shown (p=ns).

3.4.4 Antibody Titers in the Course of Therapy and Follow up

Serum sample collection was carried out in the context of the routine oncologic follow up every 3 to 6 months until November 2018. On average, 5 serum samples per patient were gathered for a period of up to 24 months. The highest number of samples collected from one patient was 8. The duration of serological follow up varied mainly because some patients refused further blood draws on the long run, probably due to recurrent venous puncturing in the course of chemo- or immunotherapy. In some cases, a regular serum sample collection turned out to be difficult because appointments were canceled or postponed on short notice.

The majority of the 19 HPV16 DNA positive patients, who also showed HPV16-L1 antibody titers beyond the threshold of 1000 ng/ml, presented a "classical" trend of decreasing antibody concentrations in the course of tumor-specific therapy and in the serological follow up. For two patients, only the first serum sample around diagnosis was available because they refused to carry on with the study during therapy. With high antibody titers at the time of diagnosis, 16 out of 18 HPV16 DNA HPV16-L1 antibody positives showed a clear decrease of antibody concentrations under therapy and along follow up between 30 and 100%.

Three examples of "classical" antibody courses are illustrated below:

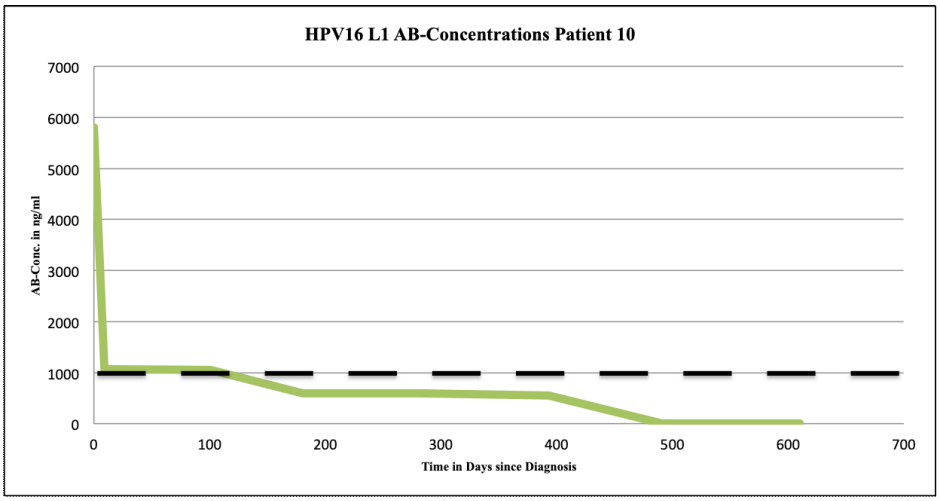

Figure 15: Classical antibody decrease during follow up, indicating a successful treatment understood as successful removal of tumor cells which is associated with disease free overall surveillance (5)

The black dotted bar at 1000 ng/ml represents the cut off antibody concentration discriminating positive and negative DRH1 test results.
Patient characteristics of patient 10 as described in table 2:
Male, 58 years old, carcinoma of the tonsils, cT3N1M0, HPV16 DNA positive, p16 ^{ink4a} positive
Therapy: Surgery + adjuvant radiotherapy
Decrease of antibody concentration by 90% within 6 months, and 100% within 18 months.

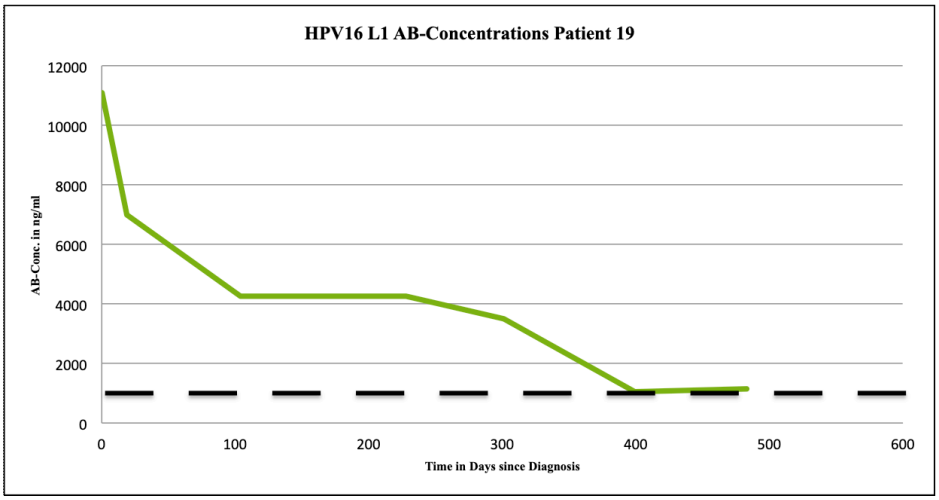

Figure 16: A decreasing trend of the HPV16-L1 antibody concentration under therapy and during serological follow up (5)

The black dotted bar at 1000 ng/ml represents the cut off antibody concentration.
Patient characteristics of patient 19 as described in table 2:
Male, 69 years old, tonsil carcinoma, cT2N0M0, HPV16 DNA positive, p16 ^{ink4a} positive
Therapy: Surgery + adjuvant radiotherapy
Decrease of antibody concentration by 90% within 16 months.

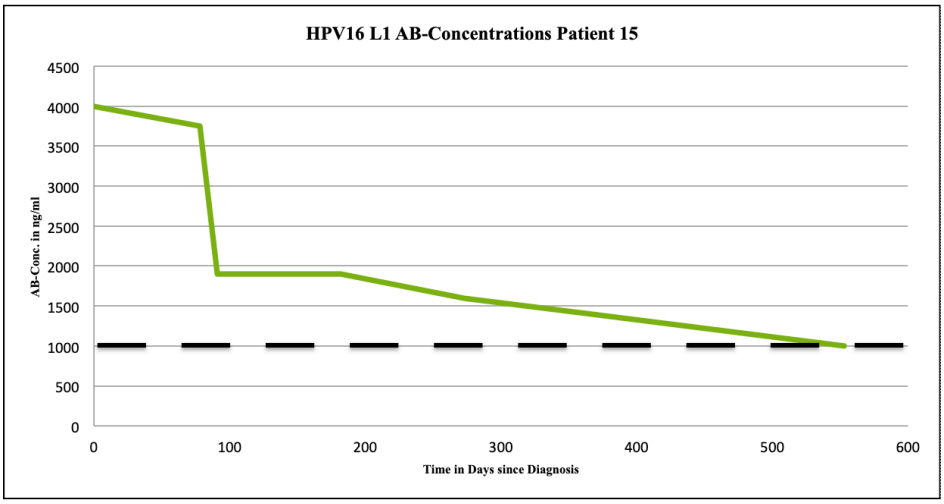

Figure 17: Another "classical" decrease of the HPV16-L1 antibody titer in a patient receiving conservative treatment (5)

The black dotted bar at 1000 ng/ml represents the cut off antibody concentration.
Patient characteristics of patient 15 as described in table 2:
Male, 60 years old, tonsil carcinoma, cT2N1M0, HPV16 DNA positive, p16 ^{ink4a} positive
Therapy: Primary radiochemotherapy
Decrease of antibody concentration by 75% within 18 months.

2 out of 18 HPV16 DNA HPV16-L1 antibody positive cases did not show a decrease at all. The serological trend even showed a steady increase right from the start. Both patients suffering from tonsil carcinoma were treated with radiotherapy. One of them additionally received concomitant chemotherapy. Although highly suspicious for an active rest respectively recurrent tumor triggering immunoresponse, this could not be clinically revealed in the follow up meetings.

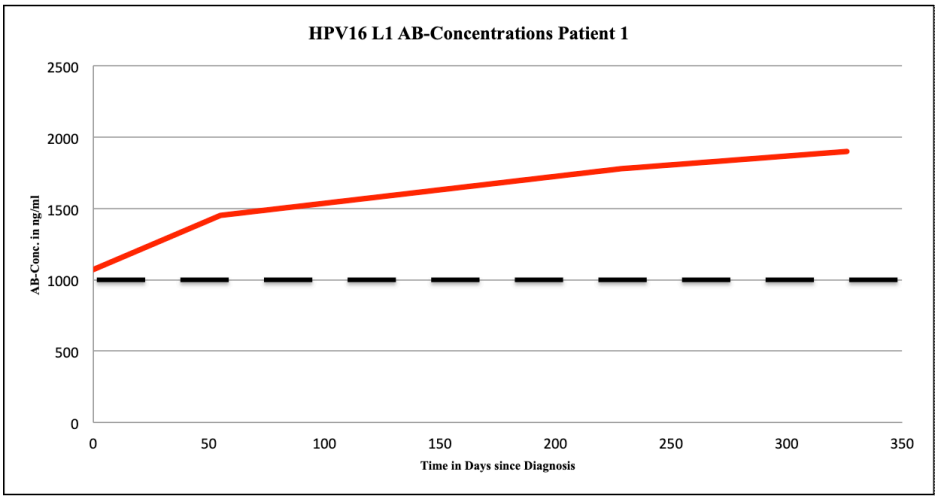

Figure 18: A continuous increase of the antibody titer in the course of tumor-specific therapy and serological follow up of unclear genesis (5)

Patient characteristics of patient 1 as described in table 2:
Male, 85 years old, tonsil carcinoma, cT3N0M0, HPV16 DNA positive, p16 ^{ink4a} positive
Therapy: Primary radiotherapy
Increase of antibody concentration by 78% within 11 months.

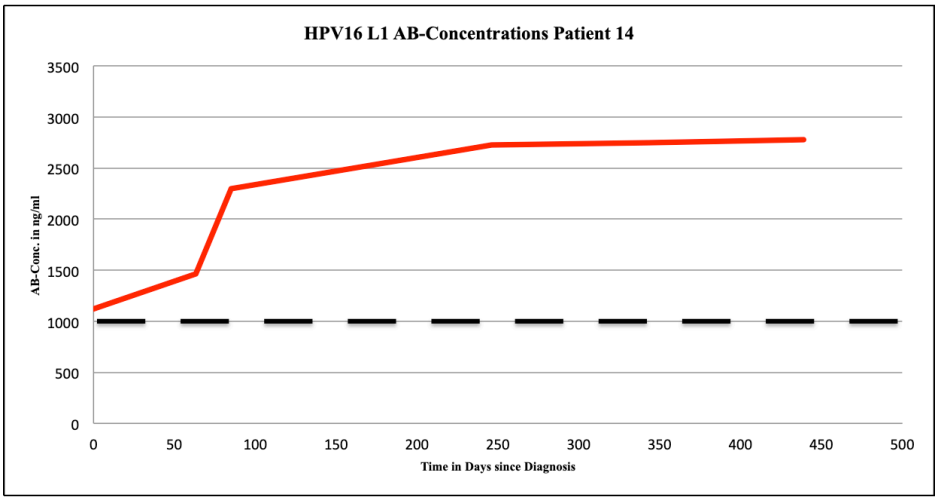

Figure 19: Again continuously increasing antibody concentration in the serological follow up (5)

Patient characteristics of patient 14 as described in table 2:
Male, 67 years old, tonsil carcinoma, cT4N1M0, HPV16 DNA positive, p16 ^{ink4a} positive
Therapy: Primary radiochemotherapy
Increase of antibody concentration by 148% within 15 months.

Probably the most interesting course of serological antibody follow up was to be found for patient 12. Suffering from an HPV16 DNA/p16 positive base of tongue carcinoma, the patient showed an initial decrease of HPV16-L1 antibodies of 43% after nine months, as to be expected under primary radioimmunotherapy. Three months later, however, a sudden rise of the antibody titer of unknown genesis was detected. Soon after, restaging imaging was carried out and revealed the occurrence of distant metastases in the lungs. In the further course, a continuous increase of the antibody titer of 436% within 10 months was protocoled. Until the end of serological follow up, it was the only observed case of tumor recurrence in the whole HPV16-induced cancer cohort.

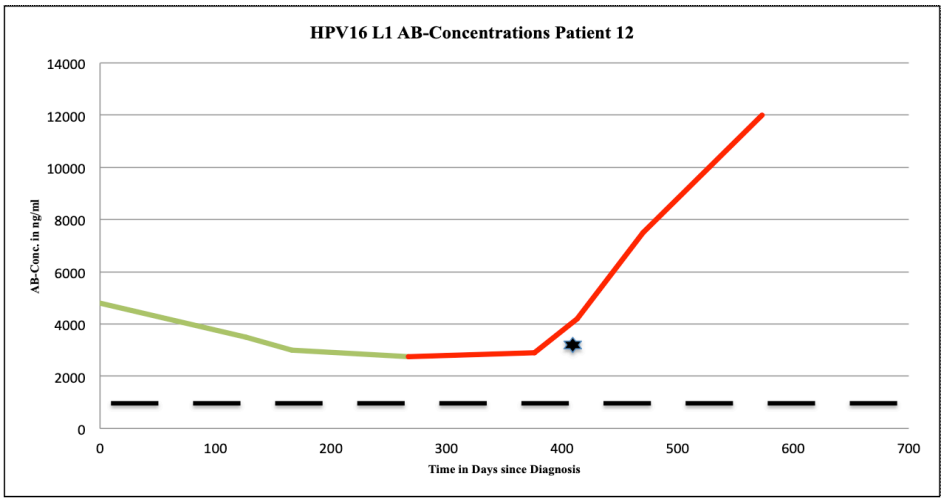

Figure 20: Antibody concentration during follow up of patient no 12 (5)

Initial antibody decrease during follow up by 43% after 9 months (in green), indicating a successful treatment, was followed by a sudden increase of antibody concentration (in red) 3 months later– soon after tumor recurrence in the lungs was diagnosed. The black spotted bar at 1000 ng/ml represents the cut off antibody concentration discriminating positive and negative DRH1 test results.
Patient characteristics of patient 12 as described in table 2:
Male, 81year old, base of tongue carcinoma, HPV16 DNA positive, p16 ^{ink4a} positive
Therapy: Primary radioimmunotherapie with Cetuximab
*Time of diagnosis of tumor recurrence

The 10 HPV16 DNA/immunoassay negative patients either showed a decrease in the HPV16-L1 antibody curve or a stable antibody course in the serological follow up exemplified below.

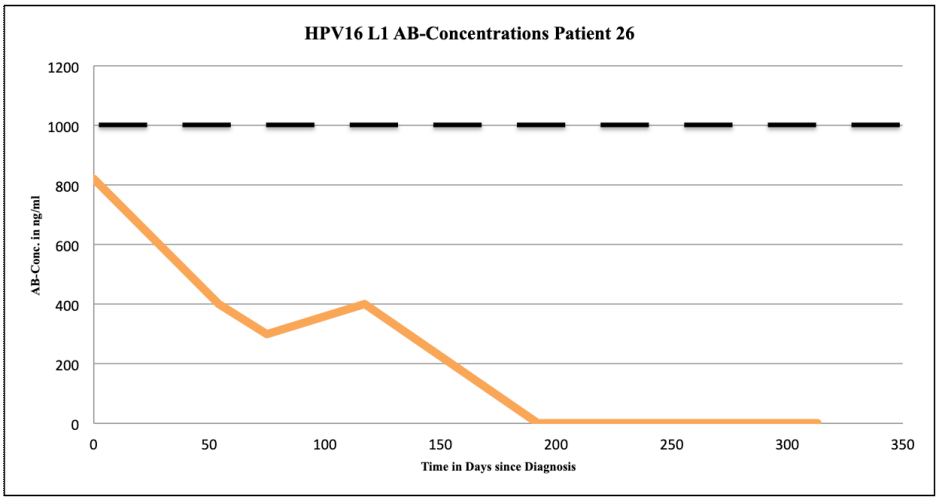

Figure 21: A decreasing antibody concentration in the serological follow up of an HPV DNA negative patient (5)

The immunoassay was also determined negative (< 1000 ng/ml) at the time of diagnosis.
Patient characteristics of patient 26 as described in table 2:
Male, 53 years old, carcinoma of the soft palate, cT3N2bM0, HPV DNA negative, p16 ^{ink4a} negative
Therapy: Primary radiochemotherapy
Decrease of antibody concentration by 100% within 10 months.

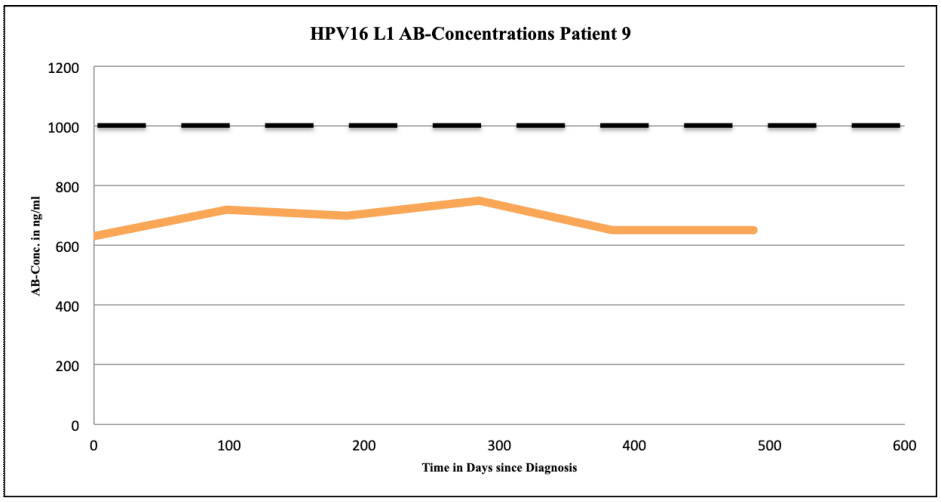

Figure 22: Stable antibody titers in the serological follow up of an HPV33 DNA positive patient (5)

The test revealed negative results (< 1000 ng/ml) at all times, underlining the subtype specificity of the HPV16-L1 specific immunoassay.
Patient characteristics of patient 9 as described in table 2:
Male, 76 years old, base of tongue carcinoma, cT3N1M0, HPV33 DNA positive, p16 ^{ink4a} positive
Therapy: Primary radiochemotherapy
Stable antibody concentrations throughout the whole serological follow up of 16 months.

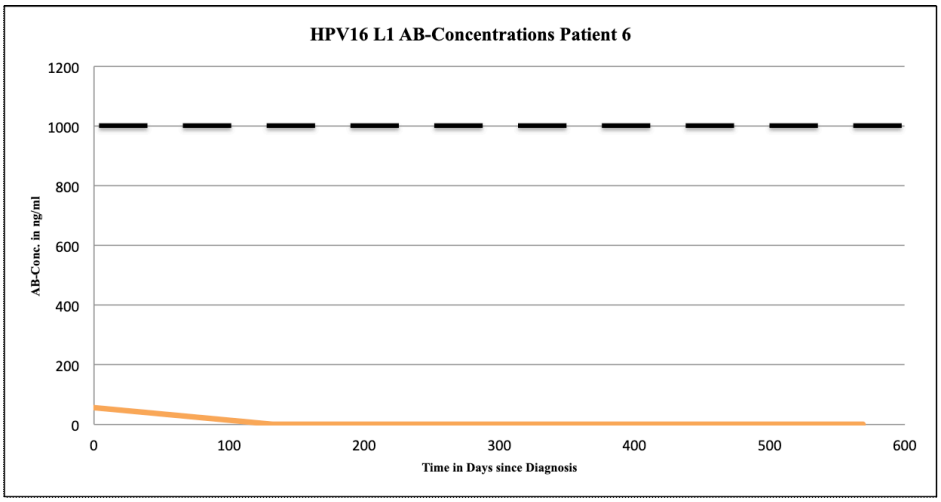

Figure 23: Again stable respectively negative antibody titers in the serological follow up of another HPV33 DNA positive patient (5)

The test showed practically no HPV16-L1 antibody titers at all for the whole duration of the serological follow up.
Patient characteristics of patient 6 as described in table 2:
Male, 66 years old, tonsil carcinoma, cT3N2bM0, HPV33 DNA positive, p16 ^{ink4a} positive
Therapy: Neoadjuvant chemotherapy following primary radiochemotherapy
Stable respectively negative antibody concentrations throughout the whole serological follow up of 19 months.

Until the end of serological follow up, one death was observed in a female patient who suffered from advanced base of tongue carcinoma with distant lung metastases being treated with primary radiochemotherapy following palliative chemoimmunotherapie because of local and distant rest tumor. Since she also suffered from severe COPD and the distant lung metastases seemed to be stable till the end, her death does not clearly seem cancer-related. According to the medical records, she died from lung failure. Since the tumor was HPV DNA/p16 ^{ink4a} negative, the first HPV16-L1 immunoassay revealed a positive result with an antibody titer of 1250 ng/ml, whereas the following serological tests were all negative.

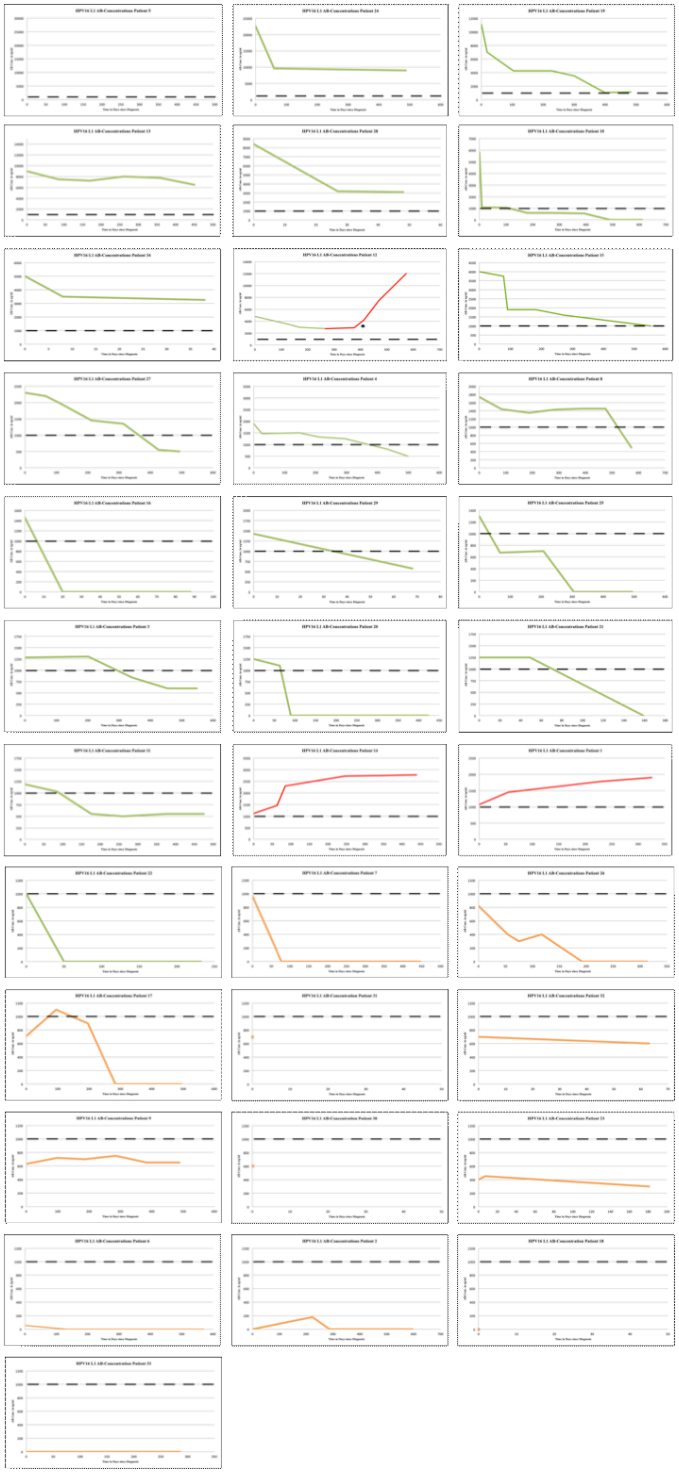

Figure 24: Overview of antibody concentrations of all HNSCC patients during follow-up (5)

Green curves: HPV16-L1 immunoassay positive
Orange curves: HPV16-L1 immunoassay negative
Red curves: Increasing antibody titers in three HPV16-L1 immunoassay positive patients
Baseline characteristics can be seen in table 2.

3.4.5 Analysis of a Healthy Control Group

Serum samples of 1064 randomly selected controls that were defined to be healthy because of negative CRP values were split into three age groups. 559 men showed a mean age of 48.1 years ranging from 1 to 93 years. In the whole male patient collective, three positive test results (0.54%) were obtained with an antibody titer above 1000 ng/ml. All three immunoassay positives presented to be in the age group of 30 years and older. In the younger patient groups (88 patients between 1 to 29 years of age) no positive test results over 1000 ng/ml were obtained at all, meaning a test specificity of 100% for these groups. Based on the results of the whole male patient collective, an overall specificity of 99.46% was calculated.

In the female patient collective, 505 women were found with a mean age of 49.4 years ranging from 1 to 92 years. In all serum samples collected from the female group, 22 positive test results with antibody concentrations above 1000 ng/ml were detected, whereby the distinct majority of the test positives were found in the younger patient cohort (under 30 years) showing 19 positive results in 81 patients. Only three positive test results were obtained from the 424 women aged 30 years and older, meaning a specificity of 99.29% for the older patient cohort. Based on the results of the whole female patient collective, an overall specificity of 95.6% was calculated.

Details of the control group analysis are presented in table 5 below:

	DRH1 negative		DRH1 positive		
female	0 ng/ml	1 – 999 ng/ml	> 1000 ng/ml	Spec. in %	in total
0 - 19 years	**19**	**8**	**10**		**37**
(in %)	(51.4)	(21.6)	(27.0)	(73.0)	(100)
mean in ng/ml	0	306	7345		2051
20 - 29 years	**25**	**10**	**9**		**44**
(in %)	(56.8)	(22.7)	(20.5)	(79.5)	(100)
mean in ng/ml	0	515	3892		913
30 y and older	**348**	**73**	**3**		**424**
(in %)	(82.1)	(17.2)	(0.71)	(99.3)	(100)
mean in ng/ml	0	220	1510		49
in total	**392**	**91**	**22**		**505**
(in %)	(77.6)	(18.0)	(4.4)	(95.6)	(100)
mean in ng/ml	0	260	5136		270

	DRH1 negative		DRH1 positive		
male	0 ng/ml	1 – 999 ng/ml	> 1000 ng/ml	Spec. in %	in total
0 - 19 years	**31**	**3**	**0**		**34**
(in %)	(91.2)	(8.8)	0	(100)	(100)
mean in ng/ml	0	217	-		19
20 - 29 years	**45**	**9**	**0**		**54**
(in %)	(83.3)	(16.7)	0	(100)	(100)
mean in ng/ml	0	128	-		21
30 y and older	**405**	**63**	**3**		**471**
(in %)	(86.0)	(13.4)	(0.6)	(99.4)	(100)
mean in ng/ml	0	168	6566		64
in total	**481**	**75**	**3**		**559**
(in %)	(86.1)	(13.4)	(0.5)	(99.5)	(100)
mean in ng/ml	0	165	6566		57

Table 5: Diagnostic specificity within healthy ´CRP negative´ blood donors (5)

3.4.6 ROC- and AUC-Analysis

For the ROC curve calculation, the test's sensitivity was determined based on the data that was

obtained from the HNSCC patient cohort in Graz. The false positive rate was ascertained on the basis of the data of the healthy control group of the Ingelheim patients.

In figure 25 below, the calculated ROC curve shows a fairly steep increase thus corresponding to an almost ideal course, which means a high diagnostic quality of the analyzed competitive immunoassay.

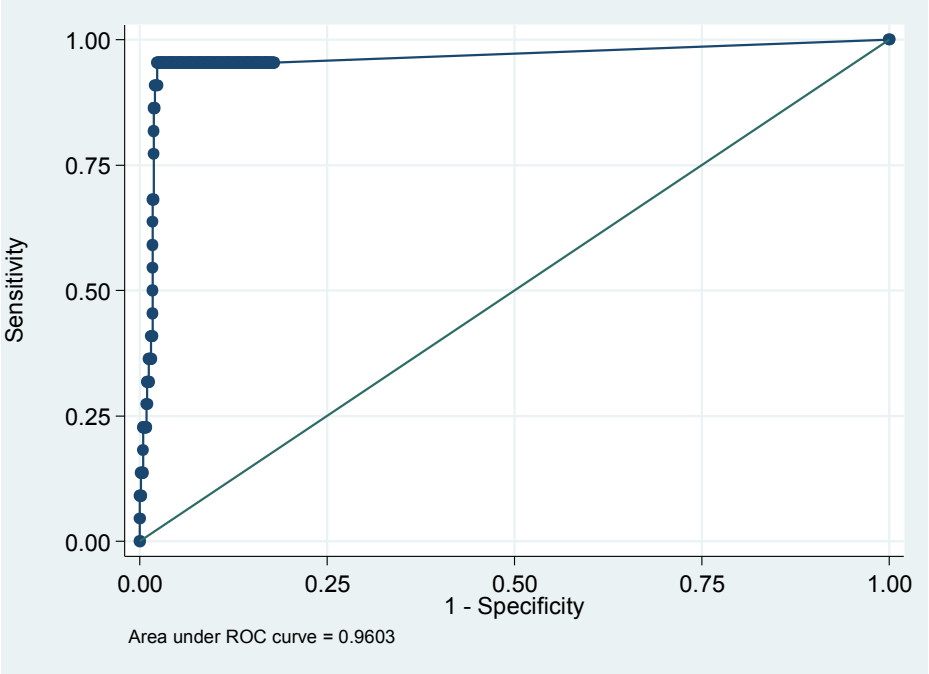

Figure 25: ROC curve analysis showing an almost ideal course and therefor a high diagnostic quality of the test (5)

The area under the curve was calculated with 0.96 underlining the high performance of the test in differentiating the positives from the negatives.

3.4.7 Cut-off Point Calculations

On the basis of the data used for the ROC curve calculations, further statistical methods, described in the statistical methods section, were applied for critical reevaluation of the used antibody

concentration threshold of 1000 ng/ml suggested by the manufacturer.

As shown in the table below, multiple possible cut points were calculated. It has to be noted that not all possible cut points were calculated, which would have been approximately 180 not regarding decimal places. In steps of 100, cut points were used.

Cutpoint	Sens	Spez.	PPW	NPW	LR+	LR-	Youden	Liu	Diff.
>=100	95.45	87.44	13.55	99.89	7.60	0.05	82.90	83.47	0.0178
>=200	95.45	91.28	18.42	99.90	10.95	0.05	86.74	87.13	0.0097
>=300	95.45	93.81	24.14	99.90	15.43	0.05	89.27	89.55	0.0059
>=400	95.45	95.97	32.81	99.90	23.69	0.05	91.42	91.61	0.0037
>=500	95.45	96.34	35.00	99.90	26.12	0.05	91.80	91.97	0.0034
>=600	95.45	96.63	36.84	99.90	28.29	0.05	92.08	92.23	0.0032
>=800	95.45	96.81	38.18	99.90	29.96	0.05	92.27	92.41	0.0031
>=900	95.45	97.38	42.86	99.90	36.37	0.05	92.83	92.95	0.0028
>=1000 Y L D	**95.45**	**97.66**	**45.65**	**99.90**	**40.74**	**0.05**	**93.11**	**93.22**	**0.0026**
>=1100	86.36	98.13	48.72	99.71	46.08	0.14	84.49	84.74	0.0189
>=1200	77.27	98.13	45.95	99.52	41.23	0.23	75.40	75.82	0.0520
>=1300	63.64	98.22	42.42	99.24	35.74	0.37	61.86	62.50	0.1325
>=1400	59.09	98.22	40.63	99.15	33.18	0.42	57.31	58.04	0.1677
>=1700	50.00	98.22	36.67	98.96	28.08	0.51	48.22	49.11	0.2503
>=1900	45.45	98.22	34.48	98.87	25.53	0.56	43.67	44.65	0.2978
>=2000	40.91	98.22	32.14	98.77	22.97	0.60	39.13	40.18	0.3495
>=2100	40.91	98.31	33.33	98.78	24.25	0.60	39.22	40.22	0.3495
>=2200	40.91	98.41	34.62	98.78	25.68	0.60	39.32	40.26	0.3494
>=2300	40.91	98.50	36.00	98.78	27.28	0.60	39.41	40.30	0.3494
>=2700	36.36	98.50	33.33	98.69	24.25	0.65	34.86	35.82	0.4052
>=3500	36.36	98.69	36.36	98.69	27.71	0.64	35.05	35.89	0.4051
>=4000	36.36	98.78	38.10	98.69	29.85	0.64	35.15	35.92	0.4051
>=4100	31.82	98.88	36.84	98.60	28.29	0.69	30.69	31.46	0.4650
>=4800	31.82	98.97	38.89	98.60	30.86	0.69	30.79	31.49	0.4650
>=5400	27.27	98.97	35.29	98.51	26.45	0.73	26.24	26.99	0.5290
>=5800	27.27	99.06	37.50	98.51	29.10	0.73	26.34	27.02	0.5290
>=6900	22.73	99.06	33.33	98.42	24.25	0.78	21.79	22.51	0.5972
>=7600	22.73	99.25	38.46	98.42	30.31	0.78	21.98	22.56	0.5972
>=7700	22.73	99.34	41.67	98.42	34.64	0.78	22.07	22.58	0.5972
>=7900	22.73	99.44	45.45	98.42	40.42	0.78	22.16	22.60	0.5971
>=8400	22.73	99.53	50.00	98.42	48.50	0.78	22.26	22.62	0.5971
>=9000	18.18	99.53	44.44	98.33	38.80	0.82	17.71	18.10	0.6694
>=10700	13.64	99.53	37.50	98.24	29.10	0.87	13.17	13.57	0.7459
>=10800	13.64	99.72	50.00	98.25	48.50	0.87	13.36	13.60	0.7459
>=11100	13.64	99.81	60.00	98.25	72.75	0.87	13.45	13.61	0.7459
>=12800	9.09	99.81	50.00	98.16	48.50	0.91	8.90	9.07	0.8264
>=14500	9.09	99.91	66.67	98.16	97.00	0.91	9.00	9.08	0.8264
>=22500	9.09	100	100	98.16	-	0.91	9.09	9.09	0.8264
>=28000	4.55	100	100	98.07	-	0.95	4.55	4.55	0.9112

Table 6: Showing different statistical parameters for the determination of the ideal cut-off point

From left to right the following parameters are listed: Sens. = Sensitivity, Spez. = Specificity, PPV = Positive Predictive value, NPV = Negative Predictive Value, LR+ = Positive Likelihood Ratio, LR- = Negative Likelihood Ratio, Youden = Youden Index*, Liu = Liu Index*, diff = closest-to-(0,1)-criterion*

*Statistical parameters are explained in the statistical methods section

For each measure, the ideal cut-off point of 1000 ng/ml as suggested by the manufacturer was confirmed. The correlating PPV was 45.65%, whereas the NPV was 99.9%. The associated LR+ was calculated with 40.74% and the LR- with 0.05%. Correlating overall sensitivity was calculated

with 95.45%, whereas the overall specificity was determined with 97.66%.

4 Discussion

Human Papillomaviruses are among the most common sexually transmitted diseases worldwide. Showing a high degree of infection in the general population, the virus mostly persists as a commensal in the human body, causing inapparent respectively asymptomatic infections (1). While the low-risk subtypes of the HPV family might cause benign lesions of the skin or mucosa, HPV-high-risk types are known to be among the most oncogenic virus entities, potentially causing a variety of different malign diseases (19). Ever since Prof. Zur Hausen was able to show the correlation between HPV infection and tumor genesis of cervical carcinoma in women (2), multiple studies followed proving the association of HPV infection and cancer development for several other tumor entities like anal cancer, vulvar cancer or HNSCC. Globally, 630,000 patients are suffering from HPV induced cancer disease per year, whereby women are affected in 90% of these cases (3). With 530,000 new cases per year, cervical cancer accounts for the vast majority of all HPV attributable cancer cases worldwide (3). Nonetheless, with the implementation of the Pap-smear-test as a successful secondary prevention tool decades ago, which is based on the collection and characterization of suspicious cells of the cervix uteri, a significant reduction of incidence rates of cervical cancer could be achieved, at least in more developed countries (163).

HPV-infections are estimated to cause about 30.8% of all 93,000 new OPSCC cases per year worldwide (3,77). Since the incident rates of HPV-induced anogenital carcinomas are notably higher in countries with a low socio-economic status, numbers of new HPV-induced OPSCC cases are demonstrably increasing in high-developed countries especially in the United States and in Western Europe (3). Among all HPV-high-risk subtypes identified so far, HPV16 accounts for the vast majority of HPV-induced OPSCC cases globally, with a share of about 80% (3). In contrast, the HPV16-attribution in cervical cancers is with about 60% significantly lower (3). With the introduction of the HPV-vaccines Gardasil, Gardasil-9 and Cervarix that target against the most common HPV-high-risk and some low-risk types, a promising primary prevention strategy was implemented in several state-regulated vaccination programs. Nevertheless, the full effect of this effort can only be seen after at least another decade and is of course limited to more developed countries that can afford it (6). As a fact however, secondary prevention strategies comparable to

the Pap-smear-test for cervical carcinoma are still missing for OPSCCs. While similar methods for the collection of relevant cells for OPSCC screening are not practicable, as already explained earlier, also efforts to develop a clinically relevant antibody test to identify HPV-induced malign disease has been hampered due to several reasons. The aim of this study was to evaluate the performance of a new competitive serological immunoassay to detect relevant HPV16-L1 antibody titers in a cohort of mainly OPSCC patients and healthy controls.

4.1 Epidemiologic Findings

Although the number of cancer cases was limited, the notable majority of HPV-associated OPSCCs (68%) in this study, defined by HPV DNA and $p16^{ink4a}$ positivity, underlines the high prevalence of this tumor entity in western countries. As we know from large epidemiologic reviews, the rate of cases, where HPV-infection supposingly leads to OPSCC development, was reported to reach up to 73% in the United States and even 93% in Sweden (83,164). In accordance with current literature, the amount of HPV-subtype-16 was with 91% by far the most common subtype in our cohort. Several epidemiologic studies confirmed the clear HPV-subtype-16 domination between 70 and 90% within HPV-related OPSCCs worldwide, of course with regional variations (3,98,152,165,166). Castellsague et al. describe a progressive drop in smoking/HPV prevalence ratios at least in certain populations since the 1980s (166). While tobacco consumption has been notably decreasing, exposure rates to oral HPV simultaneously increased, which was attributed to the increasing use of oral sex practices (166). This statement is based on several studies that could show the significant increase of incidence rates of HPV-induced OPSCCs especially in high-developed countries like the United States. Chaturvedi et al., for example, were able to demonstrate that HPV prevalence in OPSCCs changed from 16% in the late 1980s to 73% during the early 2000s in the US (83). The assumption that this extraordinary rise of HPV-induced OPSCCs is not just solely based on decreasing numbers of tobacco-related tumors, but is rather based on the change of sexual behavior is also well supported in current literature. In a review of D'Souza et al. studies from Australia, Sweden and the US are presented showing increasing markers of high-risk sexual behavior such as earlier ages of sexual debut, practice of premarital sex, average number of sexual life-time partners and practice of oral sex (164). Beside high-risk sexual behavior, also non-sexual transmission of oral HPV infection to the oral cavity is supported in some studies, like the correlating increase of oral HPV infection and number of open-mouth

kissing partners in college-aged men described as a possible salivary transmission by D'Souza et al (167).

The predominance of the male gender in our HNSCC patient cohort was no surprise as well, since several studies call this tumor entity a white men's disease (80). Independent from HPV status, a 3:1 sex ratio favorable for men is described for both groups, HPV-positives and negatives (80). This ratio largely corresponds to our cohort, where 23 males (74%) and 8 females (26%) out of all OPSCC patients were found. Also the gender distribution within the groups based on HPV status was similar. By having a closer look on our HPV DNA/p16^{ink4a} positives, 15 men (71%) and 6 women (29%) suffering from OPSCC were included, whereas, concerning the HPV DNA negative OPSCC patient group, 8 men (80%) and 2 women (20%) presented.

HPV-associated tumors of the oropharynx are reported to preferably arise from the tonsillar region since the tonsillar crypts represent the optimal environment for HPV-infection due to the close proximity to lymphatic tissue, as already explained earlier (80). According to these characteristic findings in current literature, the preferred location for tumor development of HPV-induced OPSCCs also presented to be the tonsillar region in our study cohort. 17 out of 22 HPV DNA/p16 ^{ink4a} positive patients (77.3%) were diagnosed as tonsillar carcinomas. 4 tumors were located at the base of tongue (18.2%), and in one case the tumor origin was not found leading to the diagnosis of CUP syndrome. In contrast, the majority of 11 HPV DNA negatives were found at the base of tongue (45.5%), 4 tumors aroused from the palatine tonsils (36.4%), one tumor developed at the soft palate, and again in one case, the primary tumor could not be detected.

Regarding the age at disease onset, a significant difference is described in the literature, whereby patients with HPV-negative OPSCCs are characterized by older age at time of diagnosis, typically in the seventh decade of life, whereas HPV-positive OPSCCs are seen at younger ages at onset, with a difference of 3-5 years (80). For our study cohort, this characteristic age distribution based on HPV status could not be confirmed. On the contrary, our 10 HPV DNA negative patients presented to be even younger at time of diagnosis with a mean age of 60 years (ranging from 51 to 72 years) compared to the 21 HPV-induced OPSCC patients, defined by HPV DNA and p16 ^{ink4a} positivity, showing a mean age of 67.2 years (ranging from 49 to 83 years). This discrepancy with current literature, however, is primarily due to the limited number of patients.

Beside the older age at disease onset, HPV-negative OPSCCs are significantly associated with

regular tobacco and alcohol use as the promoting risk factors for cancer development (168). Comparing HPV-positive to HPV-negative OPSCCs in our collective, a notably higher amount of patients with regular cigarette and alcohol consumption was accordingly detected. HPV DNA negative OPSCC patients in our cohort showed in 60% a positive smoker status and 50% reported to regularly drink alcohol, all of which were smokers too. In our HPV DNA/p16 ^{ink4a} positive OPSCC group, the share of regular smokers was with 48% notably less. Also the 24% in this collective reporting about regular alcohol consumption, again all of which were smokers too, were minor compared to the HPV-negatives. In general, regular tobacco and alcohol consumption are discussed to be risk factors for the development of HPV-induced OPSCCs as well, whereby inconsistent data can be found in the literature, where some studies support this association and others don't (7). Ribeiro et al., for example, found a correlation for smokers and HPV16 E6 positivity suggesting that after the inactivation of p53 and pRb by HPV proteins E6 and E7, different pathways independent from p53 and pRb for cancerogenesis are induced through tobacco and alcohol influence (156). Nevertheless, several other studies did not show strong evidence for tobacco and alcohol influence on cancerogenesis of HPV-induced OPSCCs. Due to inconsistent data, the effect of these risk factors on HPV-infection and following cancer development is not exactly understood (102,169). As a fact, it can be concluded that HPV-induced OPSCCs occur in patients with the additional risk factors tobacco and alcohol, as well as in those patients where these factors are missing. (7) By contrast however, cigarette smoking has been consistently described as promoting oral HPV infection (170,171). High-risk sexual behavior in this regard is not solely blamed for this association, rather, local immunosuppression through tobacco influence could play an important role (7). Also the influence of ongoing cigarette smoking on prognosis for patients suffering from HPV-induced OPSCCs is better understood. Ang et al. reported that cigarette smoking is the strongest modifier for prognosis in this tumor entity, with significantly better survival among HPV-positive never-smokers compared to HPV-positive smokers (172). Furthermore they found cigarette smoking to be the second most important predictor of patient survival after HPV infection.

An additional characteristic feature of HPV-positive OPSCCs well described in the literature is the fact that the majority of these cases are presented at a more advanced clinical stage at time of diagnosis compared to HPV-negatives (7). These tumors are reported to show minor T stages but advanced metastatic spread to cervical lymph nodes resulting in higher N stages when being diagnosed, however (80). Especially the impression of higher N stages diminished with the

introduction of the 8th edition of the AJCC/UICC staging system in 2017, where the weighting of the N stages was reduced for p16 ^{ink4a} positive OPSCCs aiming on a better separation of survival curves (128). In fact, our cohort also correlates with current literature using the former TNM staging system 7. More specifically, the median T stage of the HPV-negatives was 3 and the median N stage was 2, whereas the median T stage in the HPV DNA/p16 ^{ink4a} positive group presented to be 2 and the median N stage was 3 using former TNM staging system 7th edition. By use of the current staging system 8, the median N stage was 1 whereas the T stage stayed the same.

The most important characteristic of HPV-positive OPSCCs is the significant better prognosis compared to HPV-negatives. This fact has been examined and confirmed in numerous studies. The significant superiority has been described concerning both, overall and disease-free survival by several retrospective case-series (80,173,174). Though, the survival benefit for HPV-positive OPSCC patients for the reduction of risk of death varies between 20 and 80% in the literature (173). Also a significant reduction of 49% for the risk of disease recurrence was reported by Ragin et al. in a metaanalysis compared to HPV-negative OPSCCs (175). It was discussed that the survival benefit for HPV-related OPSCCs relies on observed patient characteristics such as a younger age at diagnosis, a better general state of health, fewer co-morbidities and less additional risk factors like tobacco consumption. Ang. et al., however, demonstrated that even after adjustment for these favorable prognostic factors, HPV-positive OPSCC patients still had a significantly better survival than their HPV-negative counterparts (172). They furthermore showed that only 10% of the observed survival benefit could be explained by these prognostic factors for the HPV-positive group (172). As already mentioned earlier, the same study could prove that cigarette smoking is the strongest modifier in prognosis among the HPV-positive cohort (172). Nevertheless, the underlying mechanisms for the favorable prognosis are still unclear. Partly it is explained by the fact of higher sensitivity to radio- or chemotherapy for HPV-related OPSCCs (176). One of the key aspects in this regard seems to be the lack of field carcinogenesis in the HPV-positive group showing significantly lower rates of genomic damage (98). Also the presence of an unmutated p53 gene was discussed to be a key modifier for the increased sensitivity to chemoradiation (177). Agrawal et al. demonstrated significantly lower genetic mutations in HPV-related OPSCCs compared to their HPV-negative counterparts by using whole exome sequencing (178). Furthermore, immune responses against HPV antigens, especially after incorporation of HPV E6/E7 oncoproteins, have also been mentioned as important contributors to better responses to radio- and/or chemotherapy (179,180). The first prospective study comparing treatment response

and survival rates in HPV-induced HNSCC patients and HPV-negative counterparts receiving neoadjuvant chemotherapy following radiochemotherapy was published in 2008 by Fakhry et al. (105). Clinical stage III and IV SCCs of the oropharynx and larynx were included. For all tumors, HPV status was determined by in-situ-hybridization and $p16^{ink4a}$ IHC. In the OPSCC cohort, 63% were detected to be HPV16 DNA positive, all of them showing significant overexpression of p16. All examined outcome variables were demonstrated to be significantly favorable in the HPV-positive group compared to the HPV-negatives. For example, response rates to induction therapy were 82% and 55% (p=0.01) and progression-free survival rates at 2 years were 86% versus 53% (p=0.02). After restriction to the OPSCC cohort alone, the outcome results were calculated to be even better compared to the combined cohort including laryngeal carcinomas. Several retrospective analyses of large phase III trials followed, where the significant better outcome rates for the HPV-associated OPSCCs could be confirmed (181,182). Based on these findings, a positive HPV-status in OPSCC patients was proved to be the most important prognostic factor. In our little more than two years follow-up phase, only one death was observed in an HPV DNA/$p16^{ink4a}$ negative patient in an advanced tumor stage AJCC IV with distant lung metastases at diagnosis. It is still unclear whether the death was cancer-related, since the patient died from lung failure in an external hospital, where the primary admission was due to exacerbated COPD. As a fact, the patient was not considered disease-free after primary radiochemotherapy and therefor underwent further palliative chemoimmunotherapy according to tumor board's recommendation. The last available staging, however, showed pulmonary metastases to be stable after palliative therapy. Even if the death was cancer-related, the prognosis of the patient was expected to be bad anyway independent from HPV-status with regard to the facts of advanced tumor stage, additional risk factors such as heavy cigarette and regular alcohol consumption and advanced COPD as concomitant disease. Since this case was the only observed death in our study cohort, no further information about HPV-influence on survival rates was available.

According to current literature, a positive HPV-status in OPSCCs is also a significant modifier for the reduction of risk of disease recurrence (175). In our study cohort, disease recurrence was observed in three cases, two of them occurring in HPV DNA/$p16^{ink4a}$ negative patients and one in a HPV16 DNA/$p16^{ink4a}$ positive patient. The two HPV-negatives presented in advanced tumor stages AJCC IVA and IVC at diagnosis, both with additional risk factors of regular tobacco and alcohol consumption underlining the poor prognosis at therapy onset. The third HPV-positive patient interestingly presented at a minor tumor stage AJCC II lacking of additional risk factors

like regular smoking or alcohol consumption. What probably was a key modifier for prognosis was the age of 81 years at therapy onset. Because of his advanced age, tumor board did not recommend a concomitant chemotherapy additional to primary radiotherapy. Instead, accompanying immunotherapy with cetuximab was decided. Since the patient showed severe skin reaction after first treatment, however, immunotherapy had to be cancelled and solely radiotherapy was performed. About one year after primary therapy was completed, distant lung metastases were diagnosed. Since adequate systemic tumor-specific therapy could not be performed in this case, therapy was restricted to locoregional control through radiotherapy only, whereby good therapy response was observed. Nevertheless, superior outcome rates are not observed in all HPV-positive OPSCC patients since this group also demonstrates certain heterogeneity especially in regard to prognosis. Ang et al., for example, could show that patients among the HPV-positive cohort with a history of regular tobacco consumption over 10 pack years and advanced nodal staging (N2b-3) have an increased risk of disease progression and death (3-year-overall-survival rate of 70.8%) compared to non-smokers (3-year-overall-survival rate of 93%) having the lowest risk of death (172). In their multivariate analysis, they could furthermore prove that also the patient's age had a significant impact on disease-free and overall survival rates. Comparing patients under and over 50 years of age, a hazard ratio of 1.73 (p=0.003) was calculated for the older group in regard to disease-free survival. Having again a closer look on our HPV-positive recurrence case, although presented in a comparable low tumor stage AJCC II, the patient showed two key modifiers for prognosis: the old age and an advanced nodal status N2b (according to former TNM staging 7). Based on these facts, a higher risk for metastatic spread can be assumed, what would have actually required the implementation of systemic therapy. In our experience, however, the possibilities of systemic therapy are often limited due to advanced age or comorbidity, and therefor treatment is usually restricted to primary radiotherapy for locoregional control. Like in our case, radiation alone might not be sufficient enough for patients more likely to develop distant metastases, despite the well-described increased radio sensitivity of HPV-positive tumors. Recent studies have shown that HPV-induced OPSCCs are not only restricted to the younger population and that incidence rates of this disease are rising in the older population (183). Windon et al. explored a cohort of 239 OPSCC patients by comparison of consecutive calendar periods (1995-2000, 2001-2006, 2007-2013) demonstrating that the prevalence rate of $p16^{ink4a}$ positive OPSCCs has been rising significantly not only in the younger population but also in the middle-aged (age 55-64 years) and in the older population (>65 years). For 58 elderly patients, it was shown that only 41% contributed to $p16^{ink4a}$

positive OPSCCs during 1995-2000, but the prevalence increased up to 75% for the period 2007-2013. Although it could further be shown that also in the older cohort, a positive HPV-status remained associated with a reduction to the risk of death, tumor-specific treatment for elderly patients will be a challenge in the future. Hopefully, rising knowledge about molecular specifics underlying carcinogenesis of HPV-induced tumors will lead to the development of new therapy pathways that might be less stressful than conventional chemotherapies in the future.

The combination of significantly better treatment response and survival rates with distinct patient characteristics like younger age and better fitness in HPV-positive OPSCCs led to de-intensified therapy trials with the aim to reduce toxicity without compromising therapy outcome (176). Different de-escalation regimes are currently the subject of several prospective clinical trials. Some, for example, examine the benefit for anti-epidermal growth factor receptor therapies (EGFR) especially for Cetuximab concomitant to radiotherapy instead of Cisplatin that is at the moment considered to be the gold standard (184). It is assumed that the immunoradiotherapy with Cetuximab will provide less toxicity and better quality of life (184). Although efficacy of Cetuximab has already been proven in this regard, strong biological evidence is lacking that anti-EGFR therapies are efficient in HPV-related tumor disease, since EGFR alterations are rare in HPV-positive OPSCCs (185). Hayes et al. reported about the cumulative effect of numerous mechanisms of biological alterations in HNSCCs and concluded that EGFR is a potential oncogenic target for HPV-negative tumors only (186). Despite several study limitations, some retrospective analyses also slowed the expectations on efficacy of Cetuximab therapy in HPV-positive tumors (187,188). Since advanced sensitivity to radiotherapy is already well documented in literature, trials examining the benefit and efficacy of reduced radiation doses in HPV-positive OPSCCs are also on the way (184). On the basis of the valid concept of induction chemotherapy leading to better responses to following radiotherapy, studies were designed, where HPV-positive patients undergo neoadjuvant chemotherapy and, provided they respond well, receive the following radiotherapy in lower doses (185). Since it is well documented that high-dose radiotherapy has a huge impact on swallowing dysfunction through impairment of the pharyngeal muscles, a reduction of the radiation dose is expected to lead to a significant increase of quality of life (189).

Primary radiochemotherapy is regarded to be the gold standard in terms of conservative oncologic therapy regimes in OPSCCs (8). We know to date that radiotherapy is responsible for the vast majority of therapy effect (8). Though chemotherapy is only regarded to play a subordinate role on

locoregional disease control, it is believed to be important for the avoidance of distant metastatic spread (8). Nevertheless, it is accompanied with acute and long-term toxicity such as nausea and vomiting, potential inner ear toxicity or development of polyneuropathy (8).

After stratifying HPV-positive OPSCC patients into two risk groups for the development of distant metastasis, O'Sullivan et al. examined the benefit of RT alone versus RCT (190). They found out that among the low-risk group, RT alone appeared to be equally effective compared to standard RCT in regard of distant control for N0-2a and for N2b minimal smokers (<10 packyears). However, in cases of an N2c situation, the therapy effect became inferior to concomitant chemotherapy. Although a shift from surgical to non-surgical oncologic therapy regimes for HPV-positive OPSCCs was observed in the last decades, current data also supports a better outcome in terms of survival after surgical therapy compared to HPV-negative patients (191). The subjects of on-going trials are less-invasive surgical procedures for HPV-positive OPSCCs such as the use of transoral robotic surgery (TORS), which allows the resection of selected pharyngeal tumors through the open mouth (192). Beside the advantage of less morbidity in comparison to open surgery, pathological staging after tumor resection could help de-intensifying adjuvant treatment (185). If clear surgical margins are achieved, it is a desirable goal to restrict oncologic therapy to surgery alone avoiding accompanied toxicity through adjuvant radio- or radiochemotherapy in HPV-positive patients with clinical N0 or N1 disease (184). Since several authors report that distant metastatic spread is the main cause of death in HPV-positive OPSCCs (193,194), primary surgery alone is only a treatment option for patients with low risk for distant metastases.

In conclusion several different de-escalating therapy regimes are currently subjects of ongoing clinical trials, all of which have the aim for increasing quality of life by reducing therapy related toxicities without compromising patients' outcome (184). To avoid metastatic relapse, the identification of subgroups with increased risk for metastatic spread within the HPV-positive OPSCC cohort will be essential in the future (190). Therefor, it is claimed that patients with advanced disease and heavy smokers should not be considered as potential candidates for safe dose-escalated treatment because current data show that those patients still have a relatively poor prognosis despite their positive HPV-status (184). Until today, the level of evidence for potential treatment de-escalation is still low and therefor should not be considered as standard. Results of the ongoing clinical studies must be awaited and hopefully bring clarity for upcoming therapy decisions for this distinct tumor entity (184).

4.2 Determination of HPV Status

The level of evidence for superior treatment response and survival rates in HPV-positive OPSCCs is high in current literature. Based on these facts, HPV became the most important prognostic factor in OPSCCs and will probably be key factor for treatment decisions in the future.

Since the incident rates of HPV-related OPSCCs rose constantly in the western world, it was shown that the former TNM staging system 7 could not separate survival curves of HPV-positive OPSCC patients properly (195). The numeric balance was skewed to advanced tumor stages III and IV, not matching the predictive features of any specific stage (195). It became obvious that the current staging system 7 lost the ability to reliably describe prognosis or behavior of HPV-positive OPSCCs (195).

This was taken into account by the new AJCC/UICC staging system 8 that was released in January 2017, where TNM staging of OPSCCs was changed on the basis of the p16 IHC tumor status (128). In the new edition, OPSCCs showing $p16^{ink4a}$ overexpression are characterized as HPV-induced and are therefor classified and staged differently compared to $p16^{ink4a}$ -negative OPSCCs (128). Whereas the T stages more or less stayed the same (except of former stage T4b was removed for p16 positives), one of the key changes compared to the former staging system was the downgrading of the cN stages, since it was demonstrated that clinical involved lymph nodes, whether one or multiple, as long as they were ipsilateral and less than 6cm in size, had the same influence on survival and therefor were staged in the same category N1 (127). It was furthermore revealed that contra- or bilateral involved lymph nodes less than 6cm in size had significant higher hazard ratios for the risk of death and thus were staged N2 (127). Worst survival rates were observed in lymph node metastasis greater than 6cm in size and were therefor staged in the highest category N3 (127). Another interesting change concerning our study cohort was the new recommendation to stage CUP patients showing $p16^{ink4a}$ overexpression in their tumor samples as T0 OPSCCs, because $p16^{ink4a}$ positive metastases of unknown primary tumors are reported to be high likely for the presence of occult primaries in the tonsil respectively the base of tongue (127). In one case indeed, being determined as an HPV-DNA/$p16^{ink4a}$ positive CUP syndrome at therapy onset, diagnosis had to be changed later on because the primary tumor was detected at the base of tongue. Two CUPs remained in our cohort, one being HPV16-DNA/$p16^{ink4a}$ positive, the other one being HPV-DNA/$p16^{ink4a}$ negative.

A major point of criticism in regard to TNM staging system 8th edition was, that the new classification is based on data of several single- and multicenter studies performed in the United States and Canada, where the amount of HPV-induced OPSCCs is known to be very high (195-197). Only two studies with small cohorts in Europe, where rates of HPV-induced OPSCCs are described to be notably lower (with regional differences), were performed to evaluate the new model (195). Another major point of criticism was that the indirect proof of HPV-association in OPSCCs through the detection of the surrogate marker $p16^{ink4a}$ in tumor tissue might not be reliable enough since several studies showed a relatively high false-positive rate of 10 to 20% for $p16^{ink4a}$ positive tumors, potentially causing an overestimation of HPV-relation and consequently also of the prognosis of these patients (198,199). Although based on a small cohort, Rietbergen et al., for instance, could show that those patients showing HPV DNA positivity besides $p16^{ink4a}$ overexpression in OPSCCs have a more favorable prognosis compared to $p16^{ink4a}$ positive patients where no HPV DNA could be detected (200). Nauta et al. demonstrated similar results where a $p16^{ink4a}$ positive/HPV DNA negative OPSCC patient group showed a worse prognosis compared to a cohort where additionally HPV DNA was detected in tumor tissue (124). Nevertheless, several studies demonstrated that $p16^{ink4a}$ overexpression alone is a positive prognosticator independent from HPV DNA status in OPSCC (116,152,172). In our cohort, 24 out of 33 patients (72.7%) were $p16^{ink4a}$ positive. In all 22 HPV DNA positive cases, $p16^{ink4a}$ overexpression was shown meaning a sensitivity of 100% for our study cohort. Out of the 11 HPV DNA negative cases, 2 tumor samples showed $p16^{ink4a}$ overexpression as well, meaning a specificity of 81.8%. These results correspond to the data found in the literature.

Especially in regard to potential de-escalated treatment regimens for HPV-positive patients in the future, the question if $p16^{ink4a}$ IHC alone is reliable enough to correctly identify suitable patients is the subject of on-going discussions (184). So far, however, there is no consensus on which test should be the gold standard in identification of high-risk HPV-related tumors (119). The risk of false-positive $p16^{ink4a}$ results could lead to an undertreatment of a proportion of patients falsely considered as HPV-driven (184). This could on the one hand be harmful for patients, and on the other hand might cause medico-legal consequences (184). Due to these considerations, many authors claim to combine different HPV-tests to compensate for the weaknesses of each individual one (129,201). In this work, the combination of $p16^{ink4a}$ positivity and positive PCR-based HPV DNA detection was chosen to reliably represent the group of HPV-driven tumors. This approach is supported by several studies in current literature (159,199,202). Nevertheless, a detection of HPV

DNA through PCR or ISH alone often arose the question, whether a positive result indicates that the examined tumor is actually HPV-induced. For the proof of active involvement of high-risk HPV in cancerogenesis, the detection of E6/E7 mRNA is considered by many to be the gold standard nowadays (119,132). However, this technique requires technical expertise that is not available in routine pathologic laboratories. Furthermore, the interpretation is reported to be to some extend subjective based on amplification curve analysis (112). The optimal examination material would be fresh frozen tissue, which is not routinely prepared in pathological workup for tumors of the head and neck. However, it is also possible to examine formalin-fixed paraffin embedded tissue blocks, which is technically challenging and only performed in some laboratories, where reproducible results can be achieved (132). Based on all this data, it was decided not to use this method in the presented study.

4.3 Serological HPV16-L1 Antibody Detection

Despite the fact that HPV-induced OPSCC as a distinct tumor entity is constantly increasing in the western world, the initial diagnosis is still made at an advanced stage in the majority of cases, which significantly reduce patients' prognosis. Often, the occurrence of a neck swelling due to regional lymph node metastases is only the primary symptom that causes the first way to the doctor. Nevertheless, to be able to make the diagnosis of HPV-induced OPSCC, biopsy material of actual tumor tissue is necessary meaning a tumor must be already macroscopically evident. Previous attempts to establish a comparable secondary prevention strategy to the cell-based Pap-smear test for cervical carcinoma failed due to several reasons. As explained earlier, the scientific community was concerned with the search for alternative screening methods. In this regard, the detection of relevant HPV antibodies became a big field of scientific interest. However, efforts to develop a reliable clinical test to detect relevant antibodies turned out to be difficult for various reasons. One of the biggest obstacles in this context was the very high sequence homology of up to 90% within the different HPV subtypes (16), which prevented type-specific proof for decades. All of the viral proteins, the early (E1-E7) as well as the late proteins (L1, L2), share subtype-specific and functionally highly conserved and broadly cross-reactive adjacent epitopes (16). A common assay for serological HPV-antibody detection used in multiple epidemiologic studies is based on the bacterially expressed Gluthathione-S-Transferase (GST) L1 fusion protein developed in 2001 at the DKFZ (203). Soon after publication, this ELISA was transferred to a fluorescent bead-based

multiplex format enabling the detection of antibodies to up to 100 different antigens simultaneously (204). Apart from the fact that this method is restricted to certain research laboratories at the DKFZ and, to the best of my knowledge, was not implemented in other laboratories so far, it might also bear the issue that it cannot reliable differentiate between the serological responses to adjacent epitopes (150). By contrast, other assays are based on virus like particles (VLPs), where subtype-specific conformational epitopes are known to be located on its outer surface (205). VLPs consist of HPV-L1 capsidproteins and are contained in prophylactic HPV vaccines to guarantee subtype-specific protection not inducing broadly cross-reactive protection against subsequent HPV-infections (205). VLP-based assays like the VLP-enzyme-linked-immunosorbent assay (ELISA) are therefor reported to feature a high sensitivity and specificity for subtype-specific HPV antibody detection (206). Several studies comparing the GST-L1 assay to VLP-based assays show largely just modest correlations supporting GST-L1 as a marker for cumulative infection. Scherpenisse et al., for example, compared GST-L1 to a VLP multiplex immunoassay, which is based on biologic similarities but is technically different than VLP-ELISAs, and found correlations for HPV16 and HPV18 detection to be 66% and 62% (207). Robbins et al. found these correlations to be somewhat higher with 85% and 77% respectively, though stating that a lack of concordance when comparing different assays probably results from the fact that cut-off points for seropositivity are not calibrated to one another (150).

In this work, we evaluated the clinical performance of a novel competitive immunoassay for the detection of antibodies to HPV-L1. Showing a high sensitivity of 95%, 19 out of 20 HPV16 DNA/p16^{ink4a} positive HNSCC cases that were categorized as HPV-driven, could be accurately identified using a cut-off point of 1000ng/ml recommended by the manufacturer. This cut-off value was confirmed on the basis of our study cohort, calculated by use of the three different methods: Youden-, Liu-index and closest-to-(0,1)-criterion. Based on our 1064 CRP-negative controls, a high specificity of 99.46% was calculated for the male group and 95.6% for the female group. By excluding the females under 30 years of age, where the notable highest amount of test-positive cases was observed, the specificity even rises to 99.29%. Though not being able to provide proof, the reason for the high share of test-positive cases (19 out of 81) among the young female cohort is probably due to distortion through HPV-vaccinated women. HPV-vaccination in Germany started in 2007 with girls at the age of 12 to 17 years (208) meaning these girls should be at least 25 years of age today. The recommendation for HPV-vaccination in boys followed much later in June 2018 (209).

Based on our results, the evaluated immunoassay seems to be a reliable tool to detect HPV-driven malignant disease. The positive predictive value was calculated to be 45.65% while the negative predictive value was 99.9%. Also based on the ROC-curve, it can be stated that the test is able to dependably separate HPV-associated tumor patients and healthy controls. The area-under-the-curve analysis in this regard was calculated with 0.96 (95% CI 0.91-1).

As of today, HPV-L1 detection was not considered to be a reliable marker for malignant HPV-induced disease, since HPV-L1-seropositivity was reported to reflect lifetime exposure correlating with lifetime sexual partners but not with acute disease (210-212). Rather, antibodies to HPV16 oncoproteins E6 and E7 are being viewed as markers for invasive HPV-related tumors in current literature (154,213). Multiple studies reported about high prevalence rates of HPV16 E6 and E7 proteins in HNSCCs. For example, D'Souza et al examined 100 OPSCC patients versus 200 controls in a case-control study and found a prevalence rate of 64% for HPV16 proteins E6 or E7 or both among the OPSCC patients (102). For this study cohort, a high odds ratio of 58.4 (95% CI 24.4-138.3) for the seroprevalence of HPV16 oncoproteins E6/7 and the development of OPSCC was calculated. In a publication of Smith et al. examining a cohort of 204 tumor cases and 326 controls, the presence of antibodies to HPV16 E6 (OR = 32.8; 95% CI 9.7–110.8) and E7 (OR = 37.5; 95% CI 8.7–161.2) was associated with a high risk for HNSCC (214). In a multicenter study, notably lower odds ratios were shown for the presence of antibodies to HPV16 E6/7 proteins and the development of both, oral (odds ratio 2.9; 95% CI 1.7-4.8) and oropharyngeal carcinomas (9.2; 95% CI 4.8-17.7) (213). Although an associated high risk for the development of HNSCC, especially of OPSCC, was shown by multiple studies, the fact that, for instance, the seropositivity of antibodies to HPV16 E6 was proven to be present several decades prior to tumor diagnosis arises the question of the clinical relevance of these findings. Kreimer et al., for example, showed that among a cohort of 135 OPSCC patients, a seroprevalence of antibodies to HPV16 E6 oncoprotein was detectable more than ten years before tumor diagnosis (215). Study authors discuss that it is unclear at what point HPV E6 antibodies are formed, and whether it is a sign of clinical important persistent oral infection, an HPV-induced intraepithelial neoplasia or a slowly progressing carcinoma. To evaluate antibody kinetics, Kreimer et al. examined HPV16 E6/7 antibody seroprevalence in the context of annual serial prediagnostic blood samples from the PLCO Cancer Screening Trial in a US cohort of 52 OPSCC patients in a following study (216). In 42.3%, HPV16 E6 antibodies were detected, all of which were demonstrated to be highly elevated and stable over time. Information about HPV-involvement in tumor specimen, however, was only

provided for nine cases. In a recent study, Kreimer et al. showed positive seroprevalence of HPV16 E6 antibodies even up to 28 years prior to diagnosis of OPSCC (217). The questions remain the same: What do we do with healthy patients showing HPV16 E6 antibody positivity but lacking of any signs of precancerous or even carcinogenic disease? And furthermore, do all these patients necessarily develop malignant disease? Another important information was missing on the kinetic behavior of HPV-related antibodies under tumor-specific therapy so far.

For the first time, we could demonstrate that an HPV-associated antibody is indicative of the course of HPV-induced tumor disease in a cohort of HNSCC patients (5). In 17 out of 19 immunoassay positive cases, declining antibody concentrations under therapy and in the course of follow-up between 30 and 100% were observed among our study cohort. The heights of antibody titers at time of diagnosis highly varied. Assumptions that high antibody concentrations might correlate with the tumor mass illustrated by the T stage could not be proved. Also the height of the N stages did not seem to have any influence on the height of antibody titers, although a stronger involvement of the immune system could have led to this assumption. In this regard, one could have assumed too that the immediate proximity to lymphatic tissue in cases of tonsillar carcinoma might have a positive influence on antibody titer heights, which was also cleared out.

The observed remarkable decreases of HPV-L1 antibody concentrations were associated with the successful elimination of antigen producing tumor cells. Although the number of cases was limited, all patients showing constantly declining antibody titers in the serological follow up were alive and free of disease over an observational period of up to 26 months (5). By contrast, all 8 HPV DNA negative cases as well as 2 HPV33 DNA positive cases showed stable respectively declining antibody titers along serological follow up. Even more interesting are two HPV16 DNA/p16^{ink4a} positive patients, where a constant rise of HPV-L1 antibody concentrations was observed, despite of a clinically successful tumor-specific treatment according to restaging examinations. For these cases, it is assumed that an ongoing release of antigen by hidden tumor cells might take place triggering immune response to produce HPV-related antibodies. To our knowledge, there is still no indication for a rest respectively recurrent tumor. Nevertheless, these patients are under careful observation. In one patient, primarily showing decreasing antibody concentrations after radiotherapy (adjuvant immunotherapy had to be stopped due to severe skin reaction), a sudden rise of antibody titers approximately one year after diagnosis was observed. The more than four-fold antibody increase was later linked to recurrent disease in the form of lung

metastases. By detecting an increase of the HPV-L1 antibody concentration after a constant decline along follow-up, the competitive immunoassay was able to indicate a relapse of disease much earlier before it became macroscopically evident. In our study published on the basis of this work, another case in a cohort of HIV positive anal carcinomas showed a sudden increase of antibody titers by 30% during follow-up and was later linked with disease recurrence (5). Although evidence is still limited, these observations hold the potential to start a tumor-specific therapy in the event of an urgent suspected recurrence before tumor disease becomes clinically obvious. In another case among 12 HIV positive anal cancer patients, a positive test result showing an antibody titer over 1000ng/ml was observed 293 days prior to tumor diagnosis (5).

Beside the general view of antibodies to HPV-L1 being just a ‘marker of infection’, which were reported to be stable over time, the current opinion was also that the L1-capsidprotein is just produced at the end of the viral life cycle for the encapsulation of the replicated viral DNA (218). Only highly-differentiated cells were believed to be able to produce the L1-capsidprotein (219). This statement was supported by several studies showing that L1-expression in precancerous cervical lesions was only restricted to fully differentiated superficial cells (220,221). Therefor L1-expression was demonstrated to be high in low-grade precancerous cervical lesions (CIN1) and restricted to just a small share in high-grade lesions (CIN III) (222). In accordance, a detection of L1-expression in tumor cells has so far been unsuccessful using traditional IHC and western blot staining protocols. In our publication that is based on this work, it was possible for the first time to directly prove L1-expression in different HPV DNA/p16^{ink4a} positive tumor cells using a modified staining protocol (5). In figure 26, the heterogeneously expression of the L1 protein is shown in HPV-induced tumor tissue.

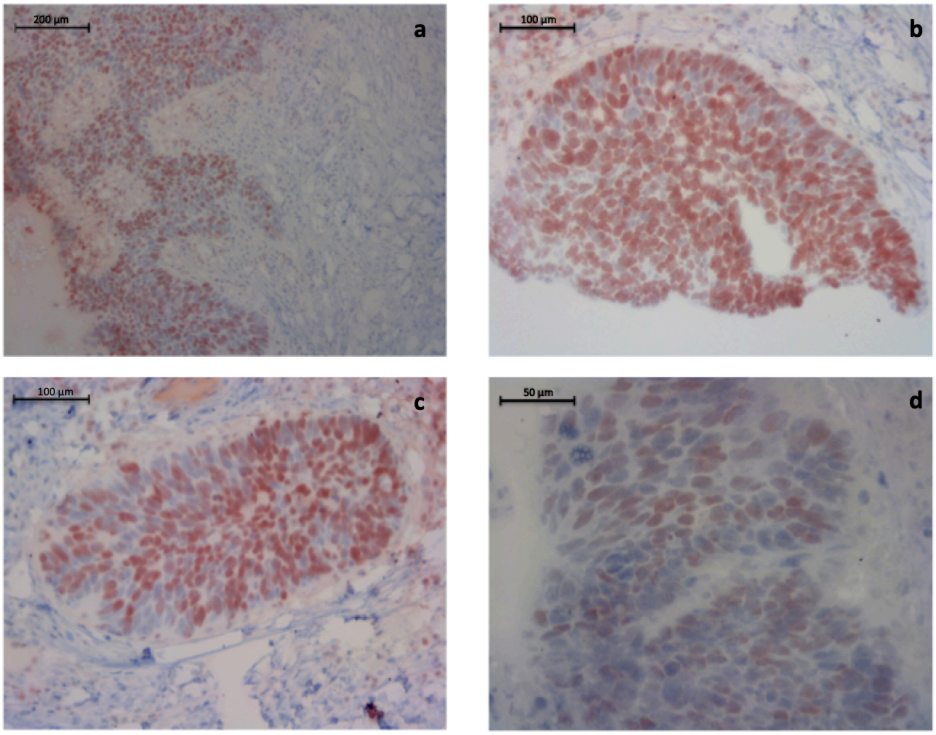

Figure 26: HPV L1 immunochemistry of different HPV driven HNSCC

As shown in pictures a - d, about half of the tumor cells show a nuclear staining in red color for the L1 capsid protein. L1 expression was confirmed by western blot analysis (data not shown). Magnification: 100x (a), 200x (b, c), 400x (d) (5)

Indications of active involvement of the L1 capsidprotein in cancerogenesis are rare but remarkable in current literature. Nevertheless, several studies supported this assumption. (5) For example, Bellone et al. and Schmitt et al. demonstrated that L1-mRNA expression was common in 29 HPV16-positive tumors, whereby copy numbers of L1-mRNA appeared to be even higher in a subset of patients compared to E6 (223,224). Since the assumption was that despite of L1-mRNA formation the L1 protein is not produced in tumor cells, the authors speculated that a translational control mechanism might exist to prevent L1 protein expression. However, that tumor cells are not only capable of producing L1-mRNA but the L1 protein itself was demonstrated by DeBrujin et al. who showed that L1-specific CD8+ cytotoxic T-cells could eliminate HPV16-L1-mRNA positive C3 tumour cells (225).

After being able to prove that the L1-capsidprotein is indeed produced in HPV-driven tumor cells, another important research question was, if the evaluated immunoassay could reliably detect the

actual course of antibody concentrations in the sense of rising and falling antibody titers. In this regard, a cohort of 29 female patients at a gynaecologic clinic in Berlin receiving three vaccinations with Gardasil-9 were tested before the first immunization and 3 to 6 weeks after the third immunization. All patients showed higher HPV16-L1 antibody concentrations after the third vaccination compared to the mearsurement before vaccination. A clear increase from an average preimmune antibody level of 364ng/ml to a more than 100-fold average level of 37500ng/ml was observed underlining the analytic specifity of the immunoassay. (5)

	Seronegative 0 ng/ml	**Seropositive 1-999 ng/ml**	**Seropositive ≥ 1000 ng/ml**	**in total**	
Pre-immune	n (%)	n (%)	n (%)	n (%)	95% CI
20 - 29 years	8 (40)	9 (45)	3 (15)	20 (100)	
mean (ng/ml)	0	382	2300	517	127 - 906
≥ 30 years	6 (66·6)	3 (33·3)	0	9 (100)	
mean (ng/ml)	0	75	0	25	0 - 201
in total	14 (48·3)	12 (41·4)	3 (10·3)	29 (100)	
mean (ng/ml)	0	305	2300	364	
Post-immune	n (%)	n (%)	n (%)	n (%)	
20 - 29 years	0	0	20 (100)	20 (100)	
mean (ng/ml)	0	0	42.470	42.470	15.544 – 69.396
≥ 30 years	0	0	9 (100)	9 (100)	
mean (ng/ml)	0	0	26.456	26.456	7.296 – 45.615
in total	0	0	29 (100)	29 (100)	
mean (ng/ml)	0	0	37.500	37.500	

Table 7: DRH1 pre- and post-immune test results of Gardasil-9 vaccinees (5)

Although the effect of established state-regulated HPV-vaccination programs will not be revealed for the next decades, it is for sure a hot topic already and might also distort the interpretation of positive test results in the future. This was best illustrated in our healthy female cohort under 30 years of age. Of course test positivity in HPV-vaccinated individuals cannot be reliably evaluated with regard to the detection of an HPV16-related malignant disease. With the intention to rule out

these insecurities for future test interpretations, a further type-specific competitive immunoassay targeting HPV-subtype 18 is currently being developed according to information of the manufacturer. If a postive test result for the presence of antibodies to HPV16-L1 is shown, an additional positive proof of HPV18-L1 antibodies would lead to the high likely conclusion of HPV-vaccination in cases where the vaccination status is unclear. To underpin these assumptions, further studies in HPV-vaccinated cohorts and non-vaccintated controls are needed.

By contrast to precancerous lesions of the cervix uteri, where, in cases of a positive DRH1 test result, morphologic atypia could adequately be diagnosed cyto- respectively histopathologically since the area of cancer origin is well known, this approach has not been proved a considerable option in cases of precancerous oropharyngeal lesions since those have not even been described in the literature yet. As mentioned above, the area of potential cancer origin in the oropharynx is highly multilocular and often difficult to reach. This is why cell-dependent diagnostic approaches have not been reliable enough to prove the presence of precancerous oropharyngeal lesions so far. Also based on our data, it cannot be concluded that the new immunoassay might potentially identify precancerous oropharyngeal lesions since this has not been examined by this work. In this regard, further projects where the new immunoassay will be combined with the attempt to gather relevant cells through suction- or brush techniques from the tonsils for cytopathological diagnostics are currently in planning.

Nowadays, direct HPV detection is recommended in cervical cancer prevention in Austria for women over 30 years of age. The question wether a direct HPV detection of high-risk subtypes through oral rinses could also be a suitable option for risk stratification in OPSCC screening was distracted by data from the US where a poor specificity of HPV-DNA detection in the oral cavity and pharyngeal region was shown. About 14.2 million US citizens present positive HPV DNA in the mouth and pharynx, 8.4 million of them are positive for high-risk subtypes (226). This number contrasts with 8900 HPV-attributed cases of head and neck cancer in Norther America per year (3), meaning that only approximately one person out of 1000 will not be able to control the HPV infection and subsequently develop cancer. Based on these data, direct HPV-detection in an OPSCC screening attempt seems not specific enough. In this regard, a combination with a reliable antibody assay might help for risk stratification, which could be examined in further studies.

A subject of regular discussions has been the question, if an antibody assay would be suitable as a screening tool for secondary prevention purposes. Based on our data, of course, this question

cannot be answered adequately. Nevertheless, we could show that the proportion of test-positives within a large healthy control cohort is very low underlining the specificity of the test, although further clinical respectively follow-up data is missing for these patients. Furthermore, a significant correlation of the test-positives with HPV-driven tumors could be proved illustrated by the high calculated sensitivity of 95%. Since former studies using GST-based assays showed that especially HPV16 E6 antibodies are seroprevalent decades before actual tumor diagnosis (215-217), further information about HPV-L1 antibody seroconversion examined with the evaluated immunoassay is needed. In this regard, additional studies on antibody kinetics in a healthy cohort with associated clinical follow-up data should be aimed for to bring more clarity to these uncertainties. Because a positive test, while used in a screening attempt, can occur years before diagnosis, this would likely cause great insecurity and anxiety in patients. Therefor, its clinical utility is highly questionnable. For this reason, it remains unclear, which patients should be screened with such an antibody assay. Kreimer et al. share the consideration that future algorithms to identify high-risk patients might make sense to define a meaningful screening population that should be explored by use of a reliable antibody assay (227). By this approach, the persons at highest risk to develop HPV-driven tumor disease could be identified.

With that in mind, we analyzed a cohort of 80 HIV positive patients from an HIV outpatient clinic in Munich without history of tumor disease representing a high-risk population in the context of our published study. 12 out of those 80 HIV positives (15%) were tested positive with the HPV16-L1 competitive immunoassay. This was considered to be 30 times higher than in the regular German population. Although clinical data for this cohort was not provided, the notable immune response to HPV-L1 antigen in the test positives was regarded to be an indication for the presence of precancerous disease or even for HPV16-induced invasive tumor disease (5).

This observation supports the idea that screening of a high-risk population in particular could be of benefit, although further studies are necessary to substantiate this consideration. Nevertheless, it remains unclear, how patients with a positive test result should be followed and whether screening programs are able to improve HPV-tumor-related outcomes. Kreimer et al. have been discussing in this regard that early detection of HPV-driven OPSCCs in symptom-free high-risk patients might possibly reduce treatment related morbidity by enabling less invasive therapy options (227).

An important point in the use of diagnostic tests especially in terms of the PPV is the incidence rate of the examined disease. In regard to a potential use in a screening approach, this would mean

for the DRH1 immunoassay that women with a positive test result lacking of any clinical signs of HPV-related cancer are being first sent to the gynaecologist for further exploration since the probability for a HPV16-associated malignant disease is by far the highest for cervical cancer in females. Whereas men with a positive test result would be first sent to the ENT-specialist since the probability for a HPV16-related malignant disease is highest in the head and neck region in males.

Undoubtedly of particular interest in the context of therapy options is the use of the HPV16-DRH1 immunoassy as a tool for monitoring the response to tumor-specific therapy and freedom of disease as part of follow-up examinations. In our study, we could demonstrate that the assay is capable of indicating the course of disease and potentially indentifying cancer recurrence before it becomes actually clinically evident. What should nonetheless always be kept in mind is that a sudden rise of the antibody titer during oncological follow-up could also be a hint for the development of a secondary HPV-induced carcinoma at a different location. Of course, further studies with larger patient cohorts and longer follow-up data are necessary to underpin the results of our work. Nevertheless, earlier, and therefor potentially less invasive, treatment might be an option for patients, where tumor recurrence associated with a sudden rise of HPV-L1 antibody levels is highly likely, even if the disease is not yet macroscopically clear.

Although the results of our work are encouraging, some limitations of the study are to be mentioned. First of all, the new DRH1 immunoassay was not compared to an alternative test procedure since a gold standard for reliable HPV antibody detection is not exactly defined. A direct comparison to other test methods is always difficult and harbors many possible sources of error, such as in terms of different cut off values. Nevertheless, further studies, where the novel HPV16-DRH1 immunoassay is directly compared to the much described GST-based assay, which has also been reported to show high correlations with HPV-driven OPSCCs in current literature, would be of high interest. This attempt may be hampered by the fact that the GST-based assay is not CE-marked and therefor not available for other research laboratories. Secondary, only clinical follow-up was provided for patients where a rise of the antibody titer was observed. In cases of a sudden increase of antibody level from a base level during aftercare, additional imaging e.g. a PET-CT scan should be carried out if the clinical examination does not reveal any sign of tumor recurrence. For patients, showing a continuous rise of antibody titers, additional examinations by other HPV-specialists like gynaecologists should be performed to rule out secondary HPV-related carcinoma at a different location. Furthermore, medical respectively clinical information and

follow-up data is missing for our large cohort of healthy controls. In this regard, the conclusion that the DRH1 assay can be recommended for a secondary preventive approach is not valid based on this data. Yet, the main focus of the study was not to examine the test's potential as a screening tool in the first place. Since this was the first study to evaluate its clinical performance the focus was to examine the correlation of positive test results and HPV16-positive OPSCCs and the antibody behaviour under therapy and during aftercare because it has never been described before. Further studies to examine a potential screening approach demand a large healthy study population with a long clinical follow-up and therefor extensive personel and financial resources, which are to be planned in the future if possible. In regard to consider the assay as a reliable post-treatment biomarker, further studies with larger patient cohorts and longer follow-up data will be necessary since we could prove the correlation of a sudden rise of HPV16-antibody titer and recurrent HPV16-induced carcinoma only in one case so far.

5 Conclusion

In summary, the results of our work are promising, since we were able to demonstrate a high clinical performance of the novel HPV16-DRH1 competitive immunoassay being used in a cohort of HNSCC patients and healthy controls. By revealing a high sensitivity of 95% and an associated positive predictive value of 45.65%, the assay was able to identify patients with HPV-induced OPSCCs with high probability. A high overall-specificity of 99.4% in 895 apparantly healthy individuals (the females under 30 were hereby excluded) underlines the assumption that the test may identify patients who actually suffer from an HPV16-driven tumor disease reliably.

We were able to demonstrate for the first time that an HPV-related antibody is indicative for HPV16-induced tumor disease. Furthermore, declining antibody concentrations were associated with successfull elimination of HPV16-related tumor cells due to tumor-specific therapy based on the results of our serological follow-up. Even in one case, tumor recurrence was associated with a sudden rise of HPV16-L1 antibody titer. Based on these observations that are the key value of our work, it can be concluded that the assay might be a reliable tool for measuring therapy response and disease control, although further studies with larger cohorts and longer follow-up data will be

necessary for validation.

The question if the DRH1 immunoassay could be used for secondary screening approaches in the future can of course not be adequately answered by this work. Nevertheless, based on the test's correlation with the control group of HPV-negatives and HPV-33-positives on the one hand and the high specificity of the test in our healthy control group on the other hand, it can be stated that the assay seems to dependably separate HPV-associated tumor patients and healthy controls. Particularly in comparison to the established Pap-smear test, being the most-effective cancer screening test so far that was able to significantly reduce HPV-induced cervical cancer with less impressive performance variables of about 50% sensitvity and 98% specificity, the novel immunoassay potentially demonstrates satisfactory results in terms of reliable informative value.

Nevertheless, the consideration to use this test as a secondary screening tool is associated with several unanswered questions. Most importantly, further information on HPV-L1 antibody kinetics based on the used assay are necessary to understand if a screening approach for the entire population makes sense at all, or if pre-test algorithms should be established to identify high-risk individuals for further antibody screening. Although we were able to prove in one case that a positive HPV16-L1 test result was detected 293 days before diagnosis of HPV-induced anal cancer, further trials with large healthy cohorts and associated clinical and follow-up data would be useful to gain more information on HPV16-L1 seroconversion before tumor diagnosis. On the other hand, further studies of high-risk patients most likely to develop HPV-driven cancer disease would make even more sense, because a greater amount of test-positives within such a cohort can be assumed.

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