



Original Research

HPV DNA/RNA detection in various oral and oropharyngeal biomaterials identifies active HPV infections also in non-neoplastic tonsils

Elgar Susanne Quabius^a, Silke Tribius^b, Alessa Heinrichs^c, Dirk Haaser^a, André Kühnel^d, Martin Laudien^a, Florian Hoppe^e, Robert Mlynski^c, Petra Ambrosch^a, Markus Hoffmann^{a,*}

^a Department of Otorhinolaryngology, Head and Neck Surgery, Christian-Albrechts-University Kiel, Arnold-Heller-Str. 3, Building 27, D24105 Kiel, Germany

^b Hermann-Holthusen-Institute for Radiation Oncology, Asklepios Hospital St. Georg, Hamburg, Germany

^c Department of Otorhinolaryngology, Head and Neck Surgery, University of Rostock, Germany

^d Department of Otorhinolaryngology, Head and Neck Surgery, Asklepios Hospital Harburg, Hamburg, Germany

^e Department of Otorhinolaryngology-Head and Neck Surgery, Klinikum Oldenburg, Hoppe, Germany

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ABSTRACT

Previous studies describe a correlation between HPV-positivity and non-smoking in TSCC; p16^{INK4A}-expression as surrogate-marker for HPV-DNA/RNA-positivity is discussed controversially. In the present study, these parameters are assessed prospectively. HPV-status of sputum and tonsillar-swabs was analyzed to determine their validity as surrogate-marker for tissue-HPV-status.

TSCC- (n = 52) and non-neoplastic tonsillar tissue (n = 163) were analyzed. HPV-DNA- and HPV-RNA-status of total sputum, cellular fraction and supernatants, tonsillar-swabs and -tissue was determined by (RT)-PCR. Immunohistochemistry determined p16^{INK4A}-expression.

23/163 (14.2%) non-neoplastic tonsils were HPV-DNA-positive; five patients (3 HPV16, 2 HPV11) had active HPV-infections (HPV-RNA-positive), in all biomaterials. 140/163 (85.9%) patients were either HPV-DNA-positive or HPV-DNA-negative in all samples. 21/52 (40.4%) TSCC-tonsils were HPV-DNA-positive; 17 patients were HPV-RNA-positive (14 HPV16; 4 HPV18). 40/52 (76.9%) TSCC-patients were congruent in all biomaterials. p16^{INK4A}-expression alone would have misclassified the HPV-status of 14/52 (26.2%) TSCC-patients.

This prospective study confirms the discrepancy between HPV-status and p16^{INK4A}-expression and the significant correlation between non-smoking and HPV-DNA-positivity. HPV-sputum- and/or swab-results do not consistently match tissue-results, possibly having (detrimental) consequences if those were used to assess tissue-HPV-status. In the 5 patients with active HPV infection in the non-neoplastic tonsils, tonsillectomy likely prevented subsequent development of TSCC.

Introduction

Infection with human papillomaviruses (HPV) are causing anogenital, specifically cervical cancers [1–3] and a subset of head and neck squamous cell carcinoma (HNSCC). In the head and neck, specifically SCC of the tonsils (TSCC), located in the anatomical region of the oropharynx, are often characterized by infection with predominantly HPV16 [4–8]. TSCC harboring active HPV infection, characterized by HPV-DNA and -RNA expression, are considered to be truly HPV-driven [9] with HPV prevalence rates in this tumor entity between 30% and 90% of cases dependent on the geographical region the patients live in [10–12].

HPV-driven HNSCC, especially TSCC, show significantly better survival times compared to HPV-negative TSCC [10,13]. This has generated enormous scientific and clinical interest in HPV-driven carcinomas. As a consequence, HPV status in oropharyngeal carcinomas has been included in the 8th edition of the TNM classification (AJCC/UICC) to reflect the favorable prognosis of HPV-positive TSCC [14]. On the other hand, numerous clinical studies have been initiated to test whether the same outcome can be achieved with de-escalated therapy in HPV-driven carcinomas [15]. The first two studies turned out to be negative and were recently published [16].

In contrast to cervical cancers with well-established precursor lesions (CIN I-III), similar precursor lesions are not known for TSCC [1]. The mechanisms leading to HPV-initiated carcinogenesis require the mucosa

* Corresponding author.

E-mail addresses: ElgarSusanne.Quabius@uksh.de (E.S. Quabius), s.tribius@asklepios.com (S. Tribius), alessa-heinrichs@gmx.de (A. Heinrichs), andrekuehnel@googlemail.com (A. Kühnel), Florian@klinikum-oldenburg.de (F. Hoppe), RobertArndt.Mlynski@med.uni-rostock.de (R. Mlynski), markus.hoffmann@uksh.de (M. Hoffmann).

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to be infected before clinically evident lesions can be detected. Thus, HPV initially infects neoplasia-free, clinically healthy mucosa, in the area of the upper aerodigestive tract as well as other anatomical sites. Therefore, clinically normal tissue of the tonsils is of particular interest because its HPV status provides information on the epidemiology of HPV in neoplasia-free tissues of the oral cavity and oropharynx. Moreover, precise HPV prevalence rates of clinically normal mucosa or tonsils can help to better understand HPV's natural history and the progression from HPV presence to carcinogenesis. HPV prevalence rates in non-neoplastic tonsils are reported to be between 0% and 12.5%. Negative HPV detection studies have been reported from non-neoplastic tonsils in children [17], children and adults [18–20] and adults only [21], while positive HPV detection studies come predominantly from children and adults [22–24].

Gargle and sputum samples are currently evaluated to determine whether HPV diagnostics on these minimally- or non-invasively obtained materials can provide reliable information regarding the HPV status of the tonsillar tissue [25,26]. Such a non-invasive surrogate method for detection of HPV infection could be suitable for developing screening programs, possibly to identify individuals who could particularly benefit from HPV vaccination or should be monitored more closely to prevent carcinogenesis. However, data obtained from tonsillar tissue, tonsillar brushes, gargle or sputum samples from the same subjects are currently not promising [24–26], regardless of whether neoplastic or non-neoplastic tissues have been examined. Franceschi and colleagues [24] conclude that brushes taken from non-malignant tonsillar tissues are not suitable for detecting precancerous lesions. Concordance rates in mouthwashes and tissue samples from the same patients with TSCC of only 58% were reported [24]. In addition, the same authors analyzed tonsillar swabs as well and found concordance rates between swabs and tissue samples of 65% only.

All these studies have the disadvantage of analyzing material of patients having either benign [24] or malignant lesions [27] or have enrolled only small numbers of patients with benign lesions with limited information about the HPV-status of these benign tissues [27]. Moreover, the vast majority of studies reviewed were performed in a retrospective setting while data from prospective studies are scarce.

Therefore, the aim of the present prospective study was to analyze the HPV-status of intraoperatively obtained tonsillar tissue specimens and swabs, and sputum samples of patients with either TSCC, tonsillar hyperplasia, or chronic or recurrent tonsillitis. The non-neoplastic and malignant tonsillar entities have been tested for HPV-DNA- and -RNA in order to compare their HPV prevalence rates and to possibly draw conclusions on aspects of their natural history. Tonsillar swabs and sputum samples from the same patients were analyzed to determine the validity of these biomaterials as surrogate markers for tissue HPV status. In addition, p16^{INK4A}-expression was determined in all tissue samples to further investigate the validity of this protein as a surrogate marker for HPV infection, a still highly controversial topic.

Patients and methods

Study design

In a prospective setting, patients with tonsillar squamous cell carcinoma (TSCC) or non-neoplastic tonsillar lesions, namely tonsillar hyperplasia (H) or chronic or recurrent tonsillitis (CRT), were enrolled when treated at the ENT Departments of the University Hospitals of Kiel and Rostock and the ENT Department of the Asklepios Klinik Hamburg-Harburg and Klinikum Oldenburg (all Germany). Smoking habit and age were documented. Each patient was asked to deliver 2 sputum samples directly preoperatively, which were immediately taken to the laboratories of the respective institution. For subsequent DNA and RNA isolation from the sputum, the first sputum sample was supplemented with 2 ml nucleic acid stabilizer (AmpTec, Hamburg, Germany). The second sample was centrifuged for 10 min (Heraeus Megafuge 1.0 or equivalent, at

600 g) to separate the cellular fraction. The resulting supernatant was supplemented with 2 ml of nucleic acid stabilizer and the cellular fraction was dissolved in 1.5 ml of the stabilizer. All samples were stored at room temperature until nucleic acid extraction, which was performed in laboratories in Kiel. Intraoperatively, patient material was extracted in different ways depending on the diagnosis: In CRT- and H-patients palatine tonsils of both sides were swabbed and resected; in TSCC-patients only the affected tonsil was swabbed and resected. From each tonsil eligible for analysis, 2 tonsillar swabs were taken with the Buccalyse swabs (Isohelix, Harrietsham, United Kingdom). One swab was used to extract DNA, the other swab was transferred into 700 µl of the above-mentioned stabilizer (AmpTec, Hamburg, Germany) to extract RNA. All swabs were stored at room temperature until nucleic acid extraction in Kiel. Tissue specimens (approx. 1 cm³) of each tonsil was shock frozen in liquid nitrogen and stored at –80 °C until nucleic acid extraction in Kiel. A second, adjacent piece of tissue was kept in formaldehyde for immunohistochemistry.

Nucleic acid extraction

DNA and RNA from all sputum samples and RNA from the tonsillar swabs was extracted using the ExpressArt Mag RNA+DNAready kit (AmpTec, Hamburg, Germany), according to the manufacturer's protocol. DNA of the tonsillar swabs was isolated using the Buccalyse DNA Release kit (Isohelix, Harrietsham, United Kingdom), according to the manufacturer's protocol. To isolate DNA and RNA from the tissues samples, 20–30 mg tissues were transferred into a Precellys[®] ceramic-Kit 1,4 mm tube (VWR International, Darmstadt, Germany), containing 600 µl RLT-buffer [part of the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany)]. The tubes were transferred in to the Precellys[®] tissue homogenizer and were homogenized using the program 1 × 5000 for 30 s. Afterwards the lysates were centrifuged in an Eppendorf centrifuge 5417R at maximum speed for 3 min at 4 °C. DNA and RNA was isolated from the supernatants using the AllPrep DNA/RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. All RNA samples were stored at –80 °C and all DNA sample at –20 °C until further analysis. Nucleic acid quantity and quality were assessed using the Nanodrop 1000 (peqlab, Erlangen, Germany) and the TapeStation 2200 (Agilent, Böblingen, Germany), respectively.

cDNA synthesis

RNA (200 ng) was transcribed into cDNA (TR-cDNA synthesis kit; AmpTec, Hamburg, Germany) under the following reaction conditions: 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C followed by 5 min storage on ice. To analyze cDNA integrity a PCR from each sample using 18S RNA primers (Promogene; Berlin, Germany) according to the manufacturer's protocol was performed.

HPV detection

HPV-DNA detection was performed by PCR amplifying 50 ng per sample using the primers GP5+/GP6+, as described previously [28]. For all (RT)-PCR reactions the Quanta Biosciences Perfecta PCR mix, utilizing the AccuStart[™] Taq DNA-Polymerase, was used. PCR conditions were as follows: initial denaturation: 15 min 95 °C, followed by 40 cycles of 1 min 95 °C, 1 min 40 °C and 1 min 72 °C, and a final elongation: 5 min 72 °C. DNA integrity was analyzed using genomic primers for the housekeeping gene β2-microglobulin (B2M; Promogene) according to the manufacturer's protocol; in brief, initial denaturation 10 min 95 °C; followed by 40 cycles: 20 s 95 °C, 20 s 60 °C and 20 s 72 °C. Additionally, a positive control (a synthetic oligonucleotide of the HPV L1 gene, covered by the GP5+/GP6+ primers; Eurofins; Ebersberg Germany) was amplified in the GP5+/GP6+ PCRs. Amplification products were sequenced by Sanger sequencing and alignments were obtained from the GenBank online BLAST server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). HPV-RNA

Table 1
Patient characteristics.

Pathology	n	Age					Sex		Smoking habit				HPV DNA tonsillar tissue	
		mean	std	median	min	max	male	female	active	never	former	N/A	positive	negative
CRT	107	24.05	10.78	23.17	3.48	57.80	42 (39.3)	65 (60.7)	40 (37.4)	66 (61.7)	1 (0.9)		12 (11.2)	95 (88.8)
H	56	29.30	21.47	29.34	2.07	70.05	37 (66.1)	19 (33.9)	23 (41.1)	31 (55.4)	1 (1.8)	1 (1.8)	11 (19.6)	45 (80.4)
TSCC	52	63.04	8.99	62.95	40.37	82.63	41 (78.8)	11 (21.2)	19 (36.5)	9 (17.3)	22 (42.3)	2 (3.8)	21 (40.4)	31 (59.6)

std: standard deviation, N/A no information available, numbers in parenthesis are percentage among disease type.

The entire patient population ($n=215$ patients) was comprised of patients with non-neoplastic chronic or recurrent tonsillitis (CRT; $n=107$), tonsillar hyperplasia (H; $n=56$) and patients with tonsillar squamous cell carcinoma (TSCC; $n=52$).

detection was performed by RT-PCR, using 10 ng cDNA, generated as described above, and HPV-type specific E6/E7 primers [29] under following PCR conditions: initial denaturation: 10 min 95 °C followed by 40 cycles: 15 s 95 °C, 45 s 60 °C.

Immunohistochemistry for p16^{INK4A}

Immunostaining of FFPE tonsillar tissue specimens (2- μ m sections) for p16^{INK4A}-expression was performed and evaluated according to Klaes and coworkers [30]. Depending on these criteria the results were classified as negative (<5% positive cells), weak (5–30%), moderate (31–75%), and strong (>75%), respectively. However, in accordance with previous own data [10] and those by others [30,31] only tissues with strong (>75%), diffuse immunohistochemical staining were considered positive. In addition, all tissues were stained for H&E to confirm the presence of cancerous tissue and tonsillar crypts, respectively.

Smoking history of the patients

Smoking habit was classified as never smoker, active smoker and former smoker, with the latter having stopped tobacco consumption at least 2 years prior to diagnosis. This classification has led to significant results published by our group [10,13]. In addition, information provided by patients regarding their substance abuse must be interpreted with caution and the meaning of pack years has become variable as cigarette packs, at least in Germany, contain between 10 and 40 cigarettes per pack.

Statistical analyses

Fisher's exact test (SPSS 20.0 software) followed by Bonferroni posttest, where appropriate was performed to correlate the patients' smoking habit and the tonsillar HPV-DNA-status, smoking habit and tonsillar p16^{INK4A}-expression as well as the correlation between tonsillar HPV-DNA-status and p16^{INK4A}-expression. Student's *t*-test was performed to analyze age related differences between 2 groups and one-way ANOVA followed by Bonferroni posttest was used to analyze age related differences when 3 or more groups were analyzed. *p*-values ≤ 0.05 were considered statistically significant.

Results

Patient demographics

The patient characteristics are presented in Table 1. A total of 215 patients were enrolled in the study. Patients in the participating departments were treated for non-neoplastic chronic or recurrent tonsillitis in 107 cases [(CRT); 65 (60.7%) female 42 (39.3%) male; 24.05 \pm 10.78 years (range: 3.48–57.80 years)]. Of the CRT-patients, 66 (61.7%) had never smoked and 40 (37.4%) were actively smoking at time of diagnosis; in one case (0.9%), the patient had stopped two years prior to diagnosis. Another 56 patients were diagnosed having tonsillar hyperplasia [(H); 19 (33.9%) female and 37 (66.1%) male; 29.30 \pm 21.47

years (range: 2.07–70.05 years)]. 31 (55.4%) patients never smoked and 23 (31.1%) actively smoked at time of diagnosis; in 1 case (1.8%) the patient had stopped smoking 2 years prior to diagnosis and in another case (1.8%) no data regarding smoking habit were available. The third group, 52 patients, was treated for tonsillar cancer [(TSCC); 11 (21.2%) female and 41 (78.8%) male; 63.04 \pm 8.99 years (range 40.37–82.63 years)]. Nine of these patients (17.2%) were never smoker, 19 (36.5%) were active smokers at time of diagnosis, and 22 (42.3%) had given up smoking at least two years before diagnosis; in two (3.8%) cases, data regarding smoking habit were missing.

HPV-status in tonsillar tissue

The HPV-DNA status of the patients' tonsillar tissue is also shown in Table 1. The tonsils of 12/107 (11.2%) CRT-patients were HPV-DNA positive (8=HPV11; 1=HPV16; 3=HPV6). An active HPV infection, as confirmed by detection of HPV-RNA, was detected in one patient with HPV16 and 2/8 patients with HPV11 infection, so that 2.8% of all and 25% of HPV-DNA-positive CRT-patients had active HPV infection. In the group of H-patients, 11/56 (19.6%) HPV-positive tonsils were detected (3=HPV11; 4=HPV16; 2=HPV18; 2=HPV6). Active HPV infection was found in 2 (both HPV16), i.e. 3.57% of all and 18.2% of HPV-positive H-patients. Patients with HPV positive CRT or H were significantly older than patients with HPV-negative non-neoplastic disease (HPV-positive CRT: 33.0 \pm 8.9 years, HPV-negative CRT: 22.9 \pm 10.5 years; $p=0.001$ and HPV-positive H: 43.8 \pm 13.6 years, HPV-negative H: 26.5 \pm 21.7 years; $p=0.035$). Of the 52 TSCC-patients, 31 (59.6%) were HPV-DNA-negative and 21 (40.4%) HPV-DNA-positive, with no age-related differences observed (HPV-positives: 62.3 \pm 9.3 years; HPV-negatives: 63.6 \pm 8.9 years; $p=0.612$). Of the 21 HPV-DNA-positive tissues, 14 were HPV16, all but one were also HPV-RNA-positive and 7 were HPV18, with 4 being HPV-RNA-positive. Thus, no active HPV infection was detected in only 4 (19%) of the 21 HPV-DNA-positive TSCC cases (active HPV infection in 32.69% of all and 80.95% of the HPV-positive cases). Therefore, the prevalence in the TSCC population examined is 40.4% HPV infection and 32.7% active HPV infection.

HPV-status and p16^{INK4A}-expression in tonsillar tissues

p16^{INK4A}-immunohistochemistry was performed and compared with the results of the tissue HPV status. Of the 52 TSCC-patients 31 (59.6%) were HPV-DNA negative of which 4 (7.7%) were p16^{INK4A}-positive (Table 2, cases 1–4). 21/52 TSCC cases (40.6%) were HPV-DNA positive. Of these 6 (11.5%) were HPV-RNA positive but p16^{INK4A}-negative (Table 2, case 5–10). Further 4 (19%) of the 21 HPV-DNA positive TSCC-patients were HPV-RNA-negative but p16^{INK4A}-positive (Table 2, case 11–14). Overall correlation between HPV-status and p16^{INK4A}-expression of the TSCC-patients is summarized in Table 3. p16^{INK4A}-expression was also analyzed in the non-neoplastic tonsils. Applying the mentioned algorithm classifying only tissue with strong (>75%) diffuse immunohistochemical staining as p16^{INK4A}-positive, resulted in 100% p16^{INK4A}-negative non-neoplastic cases. To make comparison to the literature on p16^{INK4A}-expression in non-neoplastic tonsillar tissue [32] possible, tis-

Table 2
Correlation between HPV-DNA, HPV-RNA and p16^{INK4A} expression in tonsillar tissue from TSCC patients.

Case	HPV-DNA	HPV-RNA	p16 ^{INK4A}
1	-	-	+
2	-	-	+
3	-	-	+
4	-	-	+
5	16	+	-
6	16	+	-
7	16	+	-
8	16	+	-
9	16	+	-
10	18	+	-
11	18	-	+
12	18	-	+
13	16	-	+
14	18	-	+

In 14 of the 52 TSCC tonsils HPV-DNA, HPV-RNA and p16^{INK4A}-staining did not show concordant results. Results of the remaining 38 patients were congruent in respect to their HPV-DNA, HPV-RNA and p16^{INK4A} expression. p16^{INK4A}-staining was scored positive (+) when more than 75% of the cells were stained [30]. In case of HPV-DNA positivity the HPV-type is given.

Table 3
Correlation between smoking habit and HPV-DNA expression in tonsillar tissue.

Pathology	Smoking habit	HPV DNA		p-value
		positive	negative	
CRT	active	0 (0.0)	40 (42.5)	0.001
	never	12 (100)	54 (57.5)	
H	active	2 (20.0)	21 (47.7)	>0.05
	Never	8 (80.0)	23 (52.3)	
TSCC	Active	1 (5.0)	18 (60.0)	<0.0001
	never	8 (40.0)	1 (3.3)	
	former	11 (55.0)	11 (36.7)	

numbers in parenthesis are percentage.

The patient population ($n=215$ patients) was comprised of patients with non-neoplastic chronic or recurrent tonsillitis (CRT; $n=107$; 1 HPV-negative former smoker was excluded), tonsillar hyperplasia (H; $n=56$; 1 HPV-positive former smoker was excluded; for 1 HPV-negative patient no information was available) and patients with tonsillar squamous cell carcinoma (TSCC; $n=52$; for 1 HPV-negative and 1 HPV-positive patient no information was available). p-values were calculated by Fisher's Exact test.

sues with moderate (31–75%) positive cells were considered p16^{INK4A}-positive as well. This altered algorithm did not change the result obtained in the H-patients. However, in 20/107 CRT-patients (18.7%) moderate p16^{INK4A}-expression was detected, but did not correlate with HPV-expression in the tissues (Table 3). Overall correlation between HPV-status and p16^{INK4A}-expression of the CT and H-patients is also given in Table 3.

HPV, p16^{INK4A} and smoking

The correlation between smoking habit of the patients and HPV-DNA-status of the patients' tonsillar tissue dependent on their disease is shown in Table 3. In TSCC and CRT-patients there is a significant correlation between a negative smoking history and a positive HPV-DNA status which was not the case in patients with tonsillar hyperplasia. However, 8 (72.7%) of the 11 HPV-positive patients with tonsillar hyperplasia are never smokers. Similarly, but only in the TSCC-patients a significant

correlation between a negative smoking history and a high (>75% of stained tumor cells) p16^{INK4A}-expression was seen.

HPV-infection in different biomaterials

Tonsillar tissue, sputum (total, supernatant, and cellular fraction) and swabs of the tonsillar tissue were analyzed for HPV-positivity in each patient. Noteworthy, when comparing HPV-status in tonsillar tissue, sputum or tonsillar swabs revealed HPV-infection of all affected biomaterials in a single patient were always infected with the same HPV-type.

HPV-infection of different biomaterials of the TSCC-patients

Correlating the HPV-status of tonsillar tissue with the HPV-status of the sputum and the tonsillar swabs of the TSCC-patients showed that in 13/21 (61.9%) of the HPV-DNA-positive TSCC-patients all examined biomaterial had congruent results. In 8/21 (38.1%) of patients with HPV-DNA positive tissue samples these results were not corroborated by the results of the sputum and/or swab samples. These 8 discordant cases are depicted in detail in Table 4 (cases 1–8). Among these 8 patients with HPV-DNA positive tissue samples there is a fair correlation between the tissue and the swab results; only 2/8 swabs are HPV-negative. In 27/31 (87.1%) of the TSCC-patients with HPV-DNA- and RNA-negative tonsils all other analyzed biomaterials were also HPV-DNA and RNA-negative. The remaining 4/31 (12.9%) TSCC-patients with HPV-DNA and RNA-negative tissue samples (Table 4, cases 9–12) had HPV-positive sputum and / or tonsillar swab samples. These 4 cases were reanalyzed by re-extracting DNA and RNA of the tissues and RT-PCR was performed using HPV16 and HPV18 specific primers. Again, no HPV-DNA or -RNA could be detected in these tissues, despite positive signals for the positive controls. The discordance between tissue and sputum was 50%. In one patient with HPV-DNA positive but -RNA negative tissue all other biomaterials were HPV-DNA and -RNA positive (Table 4, case 8). A further patient is positive for HPV-DNA and -RNA in all biomaterials except for the tissue specimens (Tab 4, case 9). In 2/17 HPV-positive sputum samples HPV-DNA could be detected in the cell free supernatant, the corresponding cellular fractions, were HPV-DNA negative.

HPV-infection in different biomaterials with non-neoplastic tonsillar lesions

Other than in cases with TSCC of patients with non-neoplastic lesions the palatal tonsils of both sides were removed and analyzed and tonsillar swabs were taken from both sides. Amongst the CRT-patients 12/107 (11.2%) had HPV-DNA-positive tissue samples. Three of these 12 patients were also HPV-RNA-positive, not only in their tissues but also in all biomaterials analyzed. Of the remaining 9/12 patients with HPV-DNA-positive but HPV-RNA-negative samples 5 patients had congruent results in all biomaterials. The discordant results of the remaining 4/12 CRT-patients with HPV-positive tissue samples are shown in Table 5 (case 1–4). In 89/95 (93.7%) CRT-patients with HPV-negative tissue samples these results were confirmed in patients' sputum and swab samples. The remaining 6/95 (6.3%) CRT-patients with HPV-negative tissue samples had HPV-positive swabs and/or sputum samples and are depicted in Table 5 (case 5–10). Among the H-patients with HPV-positive tonsillar tissues 8/11 (72.3%) showed congruent results in their swabs and sputum samples. 2 of these 8 patients were HPV-RNA positive, not only in their tissues but also in all other biomaterial analyzed. In 3/11 (27.3%) H-patients with HPV-positive tissue samples the results could not be confirmed in their swabs and/or sputum samples (Table 5, cases 11–13). Among the H-patients with HPV-negative tissue samples in 40/45 (88.9%) patients' swabs and sputum samples were also negative. The results of the remaining 5/45 (11.1%) H-patients are depicted in Table 5 (case 14–18); showing positive swab results in all cases and in 1 case (case 14) also a positive sputum result.

Table 4
HPV-DNA and -RNA results in the different biomaterial of TSCC patients.

Case	Smoking	Tonsillar tissue		Tonsillar swab		Sputum		Cellular fraction		Supernatant
		HPV DNA	HPV RNA	HPV DNA	HPV RNA	HPV DNA	HPV RNA	HPV DNA	HPV RNA	HPV DNA
1	never	16	+	16	+	-	-	-	-	-
2	former*	16	+	16	-	16	-	16	-	-
3	never	16	+	16	+	16	-	-	-	16
4	former*	16	+	16	+	16	-	-	-	16
5	former*	18	-	18	-	-	-	-	-	-
6	former*	18	-	-	-	-	-	-	-	-
7	never	16	-	-	-	-	-	-	-	-
8	never	18	-	18	+	18	+	18	+	-
9	active	-	-	18	+	18	+	18	+	-
10	active	-	-	16	-	16	-	16	-	-
11	former*	-	-	16	-	16	-	16	-	-
12	active	-	-	16	-	-	-	-	-	-

* stopped smoking at least 2 years prior to diagnosis. The results regarding HPV-DNA and -RNA-status of tonsillar tissue, the total sputum, the supernatant and the cellular fraction of the sputum and the tonsillar swabs of those 12 / 52 TSCC patients are depicted where the results between the biomaterials were not congruent. Results of the remaining 40 TSCC-patient were concordant in all biomaterials analyzed. Differences between tissue DNA status and the other biomaterials are indicated by bold characters. In case of HPV-positivity the HPV-type is given. Smoking habit of the patients is also shown.

Table 5
Discordant HPV DNA and -RNA results in patients with chronic or recurrent tonsillitis (CRT) and tonsillar hyperplasia (H).

Case	Smoking	Pathology	Tonsillar tissue		Tonsillar swab		Sputum*	
			Right	Left	Right	Left	Total sputum	Cellular fraction
1	never	CRT	6	6	-	-	6	6
2	never	CRT	6	6	6	6	-	-
3	never	CRT	11	11	-	11	11	11
4	never	CRT	11	11	11	11	-	-
5	former**	CRT	-	-	6	6	6	6
6	active	CRT	-	-	6	6	6	6
7	active	CRT	-	-	6	6	6	6
8	active	CRT	-	-	6	6	6	6
9	active	CRT	-	-	11	11	11	11
10	active	CRT	-	-	11	11	11	11
11	active	H	11	11	11	-	11	11
12	active	H	18	18	18	18	-	-
13	former**	H	6	6	6	6	-	-
14	active	H	-	-	11	11	11	11
15	active	H	-	-	6	6	-	-
16	active	H	-	-	11	11	-	-
17	active	H	-	-	11	11	-	-
18	never	H	-	-	18	18	-	-

* all supernatants were HPV-DNA negative; data not shown.

** stopped smoking at least 2 years prior to diagnosis. In patients with CRT and H both palatine tonsils were removed and swabs were also taken from both tonsils. Here those cases (10/107 CRT and 8/56 H) are shown, where differences between the tissue HPV-status, the sputum and swab results were detected. Results of the remaining 97 CRT and 48 H patient were concordant in all biomaterials analyzed. In case of HPV-positivity the HPV-type is given. All cases depicted here were consistently HPV-RNA-negative, hence only the HPV-DNA results are shown.

Discussion

The present study is unique in several aspects: Malignant and non-neoplastic lesions of palatal tonsils have been investigated in a prospective setting, with different patient biomaterials (tissue, swabs and sputum). All samples were analyzed not only for HPV DNA but also viral RNA, which was also performed in the swabs and sputum samples. In addition to the total sputum, the cellular fraction and the supernatant were tested separately for HPV. The findings obtained in this study are correspondingly diverse and are addressed in detail below. The high quality and validity of the results of the present study are based on the fact that if HPV was detected, the same HPV genotype has always been verified in different biomaterials of the same patients.

The immunohistochemically confirmed overexpression of the cellular protein p16^{INK4A} is considered a surrogate marker for active HPV

infection in HNSCC, which is based on a negative feedback mechanism by viral oncogene intervention in the cell cycle [8,33]. At least since the inclusion of p16^{INK4A}-immunochemistry in the 8th edition of the TNM Classification (AJCC/UICC) for the determination of HPV status in oropharyngeal carcinoma [14], p16^{INK4A}-analysis has become part of clinical routine. One aim of the study was to test the quality of p16^{INK4A}-expression in tonsillar tissue specimens as a surrogate marker for active HPV infection and to investigate to what extent other approaches such as HPV detection in tonsillar swabs or sputum might be better suited as surrogate markers for the true HPV status of tonsillar tissue. Along with other groups we have repeatedly demonstrated, that p16^{INK4A}-expression and HPV DNA/RNA detection show clear discrepancies [9,10,13,33]. Again, this was confirmed by the present study. Based on the p16^{INK4A}-analysis alone, 14/52 (26.2%) TSCC-patients would have been falsely classified as HPV positive or negative, with

possible (adverse) effects on therapy planning and outcome. In addition, p16^{INK4A}-expression was correlated with patients' smoking habit. The correlation between strong p16^{INK4A}-expression and negative smoking history of the TSCC patients adds to the controversial data. Similar to the here presented data, Mazul and coworkers found a significant correlation between strong p16^{INK4A}-staining and a negative smoking history [34], while no correlation between these parameters was seen by Pitak-Arnnop and coworkers [35]. Unfortunately, the present study's aim to establish sputum and tonsillar swabs as surrogate marker did not lead to a promising avenue. At least in our TSCC cohort, HPV sputum and/or swab results show similar high rates of discrepancies as seen for p16^{INK4A}-staining with possibly similar (adverse) consequences if sputum and/or tonsillar swabs were used to determine the HPV status of the tonsillar tissue itself.

HPV-DNA prevalence in malignant tonsillar samples examined in the present study were 40.4%. 81% of these, were active infections and were thus, as expected, in the range of data shown previously for our [8,10,13,33] and other comparable patient cohorts [5–7,12]. It is all the more remarkable, that of the 163 non-neoplastic tonsillar samples, not only 23 (14.2%) showed HPV-DNA infection (7 of them with high risk-HPV genotypes), but 5 of them [3.0% (3 HPV16, 2 HPV11)] carried active infections, based on RNA detected.

Viral activity was proven by the presence of viral RNA, since p16^{INK4A}-immunohistochemistry in non-neoplastic tonsillar samples showed - if at all - moderate staining (solely in CRT) in 18.7% and this did not correlate with HPV status. Despite the very low rate of active HPV infection in the non-neoplastic specimens investigated, this result is nonetheless very interesting. The question arises what information these cases can provide within the context of the hitherto poorly understood transition of the natural history of HPV in the oral cavity and oropharynx to carcinogenesis. In particular, will these 3 HPV16 positive non-neoplastic cases progress to carcinoma and has tonsillectomy spared these patients from this? This notion is supported by studies on TSCC-patients and healthy volunteers, who carry 2–3% virus-specific antibodies and these antibodies can sometimes be measured decades before the appearance of a tumor [36,37]. Moreover, a nation-wide cohort study in Sweden comparing cancer incidence in a tonsillectomy cohort ($n=225.718$) with Sweden's general population detected a reduced risk of tonsillar cancer but unrelated to other oropharyngeal or other head and neck cancers [34] which could be another argument for tonsillectomy potentially preventing TSCC.

Tables 4 and 5 show the discordant HPV DNA/RNA results in the different biomaterials of the respective patients. Case 3 and 4 as well as cases 8 and 9 in Table 4 are particularly noteworthy. The detection of HPV16 DNA in the supernatant of the sputum only, but not in the cell fraction of case 3 and 4, shows that HPV DNA can not only be localized extracellularly, but is also technically detectable in the cell free supernatant. It can therefore be concluded that not every HPV DNA detection in the total sputum is equivalent to the detection of a cellular infection. Given that in these cases tissues and swabs are HPV DNA/RNA positive, an experimental error cannot fully be excluded despite the accuracy of the methods used. Case 8 and 9 of Table 4 are positive for HPV RNA in all biomaterials with the exception of the tissue samples themselves despite repeated DNA/RNA extraction and analysis. In these cases, the tissue result is unlikely to be false negative and these patients possibly have active HPV infection at a different site of the upper aerodigestive tract.

Based on our results sputum- or swab-based screening tests do not seem suitable to identify individuals at increased risk for HNSCC nor as a screening instrument during surveillance following HNSCC therapy. This is in line with data from Martin-Gomez and co-workers [25] and Gipson and co-workers [26] who also addressed this topic, but either used gargle instead of sputum and did not test for RNA [25] or performed a meta-analysis summarizing existing data [26]. Therefore, at present the situation remains that HPV-associated diseases of the head

and neck only become evident and can be diagnosed when clinical symptoms and the tumor itself became apparent.

HPV-positive patients with non-neoplastic tonsils are significantly older than HPV-negative patients. This can most likely be attributed to observations from natural history studies, describing a continuous but with age increasing HPV detection rate in the oral cavity [38,39]. This is often explained by the increasing number of sexual partners with age [38]. We would like to refer to our recently published article on this topic, in which we discuss this correlation and its significance for the natural history of HPV and HPV-associated carcinogenesis in detail [40]. In the present study there is no age difference between HPV-positive and -negative TSCC patients. This phenomenon, which we have repeatedly seen in our analyses [8,10,13,33] contradicts a large part of the US-American literature [5,38,39,41]. The often significant age differences are explained by the fact that carcinogenesis initiated by the ingredients of tobacco smoke and/or alcohol takes longer, thus, patients being older at cancer diagnosis. However, there is increasing evidence that the observation that HPV-positive patients are significantly younger than HPV-negative patients [5,38,39,41] is not consistent in the literature, as different studies report no significant age-related differences between HPV-positive and HPV-negative patients, not only in European [10,42,43] but also in US-American study populations [44].

The well-established correlation of smoking and HPV negative and non-smoking and HPV positive tissue samples [10,13,19,39,45] is confirmed by the present study, both in TSCC and CRT cases. Among the H-cases, 8 of 11 HPV-positive patients are also non-smokers. It can be assumed that the association between smoking behavior and HPV status is based on the interaction between HPV and two proteins, SLPI and Annexin A2, both of which are increasingly expressed in smokers [46]. The authors currently analyze these parameters in this study population.

In summary, neither p16^{INK4A}-immunohistochemistry integrated into the TNM classification nor any other analytical procedure performed in the present study can be recommended as a surrogate for the true HPV status of the tonsillar tissue. The most reliable detection method of active HPV infections, which are responsible for neoplastic lesions, is the detection of viral RNA. All investigated HPV-positive TSCC patients are infected with high-risk HPV genotypes and the infections are active with few exceptions, while the majority of patients with non-neoplastic tonsillar disease are infected with low-risk HPV genotypes while infections are predominantly inactive. However, three percent of non-neoplastic tonsillar tissues show active mostly high-risk HPV infection. Future studies should try to answer the question, how the latter infections mark a potential transformation of oral HPV natural history to carcinogenesis and whether tonsillectomy may protect against (HPV-driven) TSCC.

Author Contributions

Conception and design: Hoffmann, Quabius
 Acquisition of data: Hoffmann, Quabius, Kühnel, Heinrichs, Haaser, Hoppe, Mlynski
 Analysis and interpretation of data: Hoffmann, Quabius, Laudien, Ambrosch
 Statistical analysis: Quabius, Hoffmann
 Writing, review and/or revision of the manuscript: Hoffmann, Quabius, Tribius
 Administrative, technical, or material support: T. Naujoks, C. Noack, G. Scherer, H. Clasen, M. Kunz
 Study supervision: Hoffmann, Ambrosch

Declaration of Competing Interest

No potential conflict of interest relevant to this article was reported.

Data for reference

Data available on request due to privacy/ethical restrictions.

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References

- [1] E.S. Prigge, D.M. von Knebel, M. Reuschenbach, Clinical relevance and implications of HPV-induced neoplasia in different anatomical locations, *Mutat. Res. Rev. Mutat. Res.* 772 (2017) 51–66.
- [2] N. Rakislova, A. Saco, A. Sierra, P.M. Del, J. Ordi, Role of Human Papillomavirus in Vulvar Cancer, *Adv. Anat. Pathol.* 24 (2017) 201–214.
- [3] I. Alkatout, M. Schubert, N. Garbrecht, M.T. Weigel, W. Jonat, C. Mundhenke, V. Gunther, Vulvar cancer: epidemiology, clinical presentation, and management options, *Int. J. Womens Health* 7 (2015) 305–313.
- [4] C. Fakhry, W.H. Westra, S. Li, A. Cmelak, J.A. Ridge, H. Pinto, A. Forastiere, M.L. Gillison, Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial, *J. Natl. Cancer Inst.* 100 (2008) 261–269.
- [5] M.L. Gillison, W.M. Koch, R.B. Capone, M. Spafford, W.H. Westra, L. Wu, M.L. Zahurak, R.W. Daniel, M. Viglione, D.E. Symer, K.V. Shah, D. Sidransky, Evidence for a causal association between human papillomavirus and a subset of head and neck cancers, *J. Natl. Cancer Inst.* 92 (2000) 709–720.
- [6] J.P. Klussmann, S.J. Weissenborn, U. Wieland, V. Dries, J. Kolligs, M. Jungehülsing, H.E. Eckel, H.P. Dienes, H.J. Pfister, P.G. Fuchs, Prevalence, distribution, and viral load of human papillomavirus 16 DNA in tonsillar carcinomas, *Cancer* 92 (2001) 2875–2884.
- [7] A.R. Kreimer, G.M. Clifford, P. Boyle, S. Franceschi, Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review, *Cancer Epidemiol. Biomarkers Prev.* 14 (2005) 467–475.
- [8] M. Hoffmann, A.S. Ihloff, T. Görög, J.B. Weise, A. Fazel, M. Krams, W. Rittgen, E. Schwarz, T. Kahn, p16(INK4a) overexpression predicts translational active human papillomavirus infection in tonsillar cancer, *Int. J. Cancer* 127 (2010) 1595–1602.
- [9] D. Holzinger, M. Schmitt, G. Dyckhoff, A. Benner, M. Pawlita, F.X. Bosch, Viral RNA patterns and high viral load reliably define oropharynx carcinomas with active HPV16 involvement, *Cancer Res.* 72 (2012) 4993–5003.
- [10] E.S. Quabius, J. Haag, A. Kühnel, H. Henry, A.S. Hoffmann, T. Görög, J. Hedderich, M. Evert, A.G. Beule, S. Maune, R. Knecht, A. Óvári, M. Durisin, F. Hoppe, S. Tribius, C. Röcken, P. Ambrosch, M. Hoffmann, Geographical and anatomical influences on human papillomavirus prevalence diversity in head and neck squamous cell carcinoma in Germany, *Int. J. Oncol.* 46 (2015) 414–422.
- [11] H. Mehanna, T. Beech, T. Nicholson, I. El-Hariry, C. McConkey, V. Paleri, S. Roberts, Prevalence of human papillomavirus in oropharyngeal and nonoropharyngeal head and neck cancer-systematic review and meta-analysis of trends by time and region, *Head Neck* 35 (2013) 747–755.
- [12] X. Castellsagué, L. Alemany, M. Quer, G. Halc, B. Quirós, S. Tous, O. Clavero, L. Alòs, T. Biegner, T. Szafarowski, M. Alejo, D. Holzinger, E. Cadena, E. Claros, G. Hall, J. Laco, M. Poljak, M. Benevolo, E. Kasamatsu, H. Mehanna, C. Ndiaye, N. Guimerà, B. Lloveras, X. León, J.C. Ruiz-Cabezas, I. Alvarado-Cabrero, C.S. Kang, J.K. Oh, M. Garcia-Rojo, E. Iljazovic, O.F. Ajayi, F. Duarte, A. Nessa, L. Tinoco, M.A. Duran-Padilla, E.C. Pirog, H. Viarheichyk, H. Morales, V. Costes, A. Félix, M.J. Germar, M. Mena, A. Ruacan, A. Jain, R. Mehrotra, M.T. Goodman, L.E. Lombardi, A. Ferrera, S. Malami, E.I. Albanesi, P. Dabed, C. Molina, R. López-Revilla, V. Mandys, M.E. González, J. Velasco, I.G. Bravo, W. Quint, M. Pawlita, N. Muñoz, S. de Sanjosé, F. Xavier Bosch, ICO International HPV in Head and Neck Cancer Study Group, HPV involvement in head and neck cancers: comprehensive assessment of biomarkers in 3680 patients, *J. Natl. Cancer Inst.* 108 (2016) djv403.
- [13] M... Hoffmann, E.S. Quabius, S. Tribius, S. Gebhardt, T. Görög, J. Hedderich, K. Huber, J. Dunst, P. Ambrosch, Influence of HPV-status on survival of patients with tonsillar carcinomas (TSCC) treated by CO2-laser surgery plus risk adapted therapy – a 10 year retrospective single centre study, *Cancer Lett.* 413 (2018) 59–68.
- [14] M.B. Amin, S.B. Edge, F.L. Greene, et al., *AJCC Cancer Staging Manual*, eighth ed., Springer, New York, 2017.
- [15] L. Masterson, D. Mואled, Z.W. Liu, J.E. Howard, R.C. Dwivedi, J.R. Tysome, R. Benson, J.C. Sterling, H. Sudhoff, P. Jani, P.K. Goon, De-escalation treatment protocols for human papillomavirus-associated oropharyngeal squamous cell carcinoma: a systematic review and meta-analysis of current clinical trials, *Eur. J. Cancer* 50 (2014) 2636–2648.
- [16] H. Mehanna, D. Rischin, S.J. Wong, V. Gregoire, R. Ferris, J. Waldron, Q.T. Le, M. Forster, M. Gillison, S. Laskar, M. Tahara, A. Psyrri, A. Vermorken, S. Porceddu, De-escalation after DE-ESCALATE and RTOG 1016: a head and neck cancer intergroup framework for future de-escalation studies, *J. Clin. Oncol.* (2020) JCO 2000056, doi:10.1200/JCO.20.00056.
- [17] K.M. Ribeiro, J.M. Alvez, S.S. Pignatari, L.L. Weck, Detection of human papilloma virus in the tonsils of children undergoing tonsillectomy, *Braz. J. Infect. Dis.* 10 (2006) 165–168.
- [18] M. Rusan, T.E. Klug, J.J. Henriksen, J.H. Bonde, K. Fuursted, T. Ovesen, Prevalence of tonsillar human papillomavirus infections in Denmark, *Eur. Arch. Otorhinolaryngol.* 272 (2015) 2505–2512.
- [19] E.S. Quabius, B. Bögershausen, L. Getzin, T. Görög, M.P. Gebhard, A.S. Hoffmann, M. Hoffmann, SLPI and AnxA2 expression in neoplasm-free palatine tonsils is associated with smoking habit of individuals, *Mol. Clin. Oncol.* 7 (2017) 427–434.
- [20] E. Palmer, R.G. Newcombe, A.C. Green, C. Kelly, O. Noel Gill, G. Hall, A.N. Fiander, E. Pirotte, S.J. Hibbitts, J. Homer, N.G. Powell, Human papillomavirus infection is rare in nonmalignant tonsil tissue in the UK: implications for tonsil cancer precursor lesions, *Int. J. Cancer* 135 (2014) 243743.
- [21] J.A. Ernster, C.G. Sciotto, M.M. O'Brien, L.J. Robinson, T. Willson, Prevalence of oncogenic human papillomavirus 16 and 18 in the palatine tonsils of the general adult population, *Arch. Otolaryngol. Head Neck Surg.* 135 (2009) 554–557.
- [22] I.N. Mammias, G. Sourvinos, C. Michael, D.A. Spandidos, Human papilloma virus in hyperplastic tonsillar and adenoid tissues in children, *Pediatr. Infect. Dis. J.* 25 (2006) 1158–1162.
- [23] A. Duray, G. Descamps, M. Bettonville, N. Sirtaine, P. Ernouf-Neufcoeur, S. Guenin, M. Mouallif, C.E. Depuydt, P. Delvenne, S. Saussez, High prevalence of high-risk human papillomavirus in palatine tonsils from healthy children and adults, *Otolaryngol. Head Neck Surg.* 145 (2011) 230–235.
- [24] S. Franceschi, J.D. Combes, V. Dalstein, S. Caudroy, G. Clifford, T. Gheit, M. Tommasino, C. Clavel, J. Lacau St Guily, P. Birembaut, Study of Natural History of Human Papillomavirus Infection and Precancerous Lesions in the Tonsils (SPLIT): deep brush-based cytology in tonsils resected for benign diseases, *Int J Cancer* 137 (2015) 2994–2999.
- [25] L. Martin-Gomez, W.J. Fulp, M.J. Schell, B. Sirak, M. Abrahamsen, K.A. Isaacs-Soriano, A. Lorincz, B. Wenig, C.H. Chung, J.J. Caudell, A.R. Giuliano, Oral gargle-tumor biopsy human papillomavirus (HPV) agreement and associated factors among oropharyngeal squamous cell carcinoma (OPSCC) cases, *Oral Oncol.* 92 (2019) 85–91.
- [26] B.J. Gipsen, H.A. Robbins, C. Fakhry, G. D'Souza, Sensitivity and specificity of oral HPV detection for HPV-positive head and neck cancer, *Oral Oncol.* 77 (2018) 52–56.
- [27] C. Nordfors, A. Vlastos, J. Du, A. Ahrlund-Richter, N. Tertipis, N. Grün, M. Romanitan, L. Haegglblom, A. Roosaar, G. Dahlöf, M.G. Donà, M. Benevolo, T. Ramqvist, E. Munck-Wikland, T. Dalianis, Human papillomavirus prevalence is high in oral samples of patients with tonsillar and base of tongue cancer, *Oral Oncol.* 50 (2014) 491–497.
- [28] T.W. Remmerbach, U.G. Brinckmann, A. Hemprich, M. Chekol, K. Kühndel, U.G. Liebert, PCR detection of human papillomavirus of the mucosa: comparison between MY09/11 and GP5+/6+ primer sets, *J. Clin. Virol.* 30 (2004) 302–308.
- [29] G. Halc, M. Schmitt, B. Dondog, E. Sharkhuu, N. Wentzensen, T. Gheit, M. Tommasino, F. Kommos, F.X. Bosch, S. Franceschi, G. Clifford, L. Gissmann, M. Pawlita, Biological activity of probable/possible high-risk human papillomavirus types in cervical cancer, *Int. J. Cancer* 132 (2013) 63–71.
- [30] R. Klaes, T. Friedrich, D. Spitzkovsky, R. Ridder, W. Rudy, U. Petry, G. Dallenbach-Hellweg, D. Schmidt, M. von Knebel Doberitz, Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri, *Int. J. Cancer* 92 (2001) 276–284.
- [31] J.K. Lilja-Fischer, J.G. Eriksen, J.B. Georgsen, T.T. Vo, S.R. Larsen, J. Cheng, M. Busch-Sørensen, D. Aurora-Garg, T. Steiniche, J. Overgaard, Prognostic impact of PD-L1 in oropharyngeal cancer after primary curative radiotherapy and relation to HPV and tobacco smoking, *Acta Oncol.* (2020) 666–672.
- [32] B. Klingenberg, H.C. Hafkamp, A. Haesevoets, J.J. Manni, P.J. Slootweg, S.J. Weissenborn, J.P. Klussmann, Speel EJ: p16^{INK4A} overexpression is frequently detected in tumour-free tonsil tissue without association with HPV, *Histopathology* 56 (2010) 957–6. 7.
- [33] M. Hoffmann, S. Tribius, E.S. Quabius, H. Henry, S. Pfannenschmidt, C. Burkhardt, T. Görög, G. Halc, A.S. Hoffmann, T. Kahn, C. Röcken, J. Haag, T. Waterboer, M. Schmitt, HPV DNA, E6*1-mRNA expression and p16^{INK4A} immunohistochemistry in head and neck cancer - how valid is p16^{INK4A} as surrogate marker? *Cancer Lett.* 323 (2012) 388–396.
- [34] A.L. Mazul, J.M. Taylor, K. Divaris, M.C. Weissler, P. Brennan, D. Anantharaman, B. Abedi-Ardekani, A.F. Olshan, Zevallos JP: oral health and human papillomavirus-associated head and neck squamous cell carcinoma, *Cancer* (2017) 71–80.
- [35] P. Pitak-Arnnop, L.K. Witohendro, J.P. Meningaud, K. Subbalekha, A. Iamaroon, N. Sirintawat, A. Klaisiri, A. Neff, Which characteristics can be expected from p16+ squamous cell carcinomas of the posterior oral cavity and oropharynx? – distinctive results from Central Germany, *J. Stomatol. Oral Maxillofac. Surg.* (2020) 213–218.
- [36] K.B. Ribeiro, J.E. Levi, M. Pawlita, S. Koifman, E. Matos, J. Eluf-Neto, V. Wunsch-Filho, M.P. Curado, O. Shangina, D. Zaridze, N. Szeszenia-Dabrowska, J. Lissowska, A. Daudt, A. Menezes, V. Bencko, D. Mates, L. Fernandez, E. Fabianova, T. Gheit, M. Tommasino, P. Boffetta, P. Brennan, T. Waterboer, Low human papillomavirus prevalence in head and neck cancer: results from two large case-control studies in high-incidence regions, *Int. J. Epidemiol.* 40 (2011) 489–502.
- [37] A.K. Chaturvedi, H. Song, P.S. Rosenberg, T. Ramqvist, W.F. Anderson, E. Munck-Wikland, W. Ye, T. Dalianis, Tonsillectomy and incidence of oropharyngeal cancers, *Cancer Epidemiol. Biomarkers Prev.* 25 (2016) 944–950.

- [38] G. D'Souza, K. Cullen, J. Bowie, R. Thorpe, C. Fakhry, Differences in oral sexual behaviors by gender, age, and race explain observed differences in prevalence of oral human papillomavirus infection, *PLoS One* 9 (2014) e86023.
- [39] A.K. Chaturvedi, B.I. Graubard, T. Broutian, R.K. Pickard, Z.Y. Tong, W. Xiao, L. Kahle, M.L. Gillison, NHANES 2009-2012 findings: association of sexual behaviors with higher prevalence of oral oncogenic human papillomavirus infections in U.S. Men, *Cancer Res.* 75 (2015) 2468–2477.
- [40] E.S. Quabius, A. Fazel, C. Knieling, S. Gebhardt, M. Laudien, C. Moore, A. Kühnel, F. Hoppe, R. Mlynski, A. Heinrichs, A. Fabian, M. Hoffmann, No association between HPV-status in tonsillar tissue and sexual behavior of the patients in a northern German population – critical view of the link between HPV natural history and HPV-driven carcinogenesis, *Papillomavirus Res.* (2020), doi:10.1016/j.pvr.2020.100207.
- [41] Z. Gooi, J.Y. Chan, C. Fakhry, The epidemiology of the human papillomavirus related to oropharyngeal head and neck cancer, *Laryngoscope* 126 (2016) 894–900.
- [42] S. Wagner, C. Wittekindt, S.J. Sharma, N. Wuerdemann, T. Jüttner, M. Reuschenbach, E.S. Prigge, M. von Knebel Doeberitz, S. Gattenlöhner, E. Burkhardt, J. Pons-Kühnemann, J.P. Klussmann, Human papillomavirus association is the most important predictor for surgically treated patients with oropharyngeal cancer, *Br. J. Cancer* 116 (2017) 1604–1611.
- [43] M.A. Broglie, W. Jochum, A. Michel, T. Waterboer, D. Foerbs, R. Schoenegg, S.J. Stoeckli, M. Pawlita, D. Holzinger, Evaluation of type-specific antibodies to high risk-human papillomavirus (HPV) proteins in patients with oropharyngeal cancer, *Oral Oncol.* 70 (2017) 43–50.
- [44] C. Fakhry, W.H. Westra, S.J. Wang, A. van Zante, Y. Zhang, E. Rettig, L.X. Yin, W.R. Ryan, P.K. Ha, A. Wentz, W. Koch, J.D. Richmon, D.W. Eisele, G. D'Souza, The prognostic role of sex, race, and human papillomavirus in oropharyngeal and nonoropharyngeal head and neck squamous cell cancer, *Cancer* 123 (2017) 1566–1575.
- [45] K.K. Ang, J. Harris, R. Wheeler, R. Weber, D.I. Rosenthal, P.F. Nguyen-Tân, W.H. Westra, C.H. Chung, R.C. Jordan, C. Lu, H. Kim, R. Axelrod, C.C. Silverman, K.P. Redmond, M.L. Gillison, Human papillomavirus and survival of patients with oropharyngeal cancer, *N. Engl. J. Med.* 363 (2010) 24–35.
- [46] E.S. Quabius, T. Görögh, G.S. Fischer, A.S. Hoffmann, M. Gebhard, M. Evert, A. Beule, S. Maune, R. Knecht, A. Óvári, M. Durisin, F. Hoppe, C. Röcken, J. Hedderich, P. Ambrosch, M. Hoffmann, The antileukoprotease secretory leukocyte protease inhibitor (SLPI) and its role in the prevention of HPV-infections in head and neck squamous cell carcinoma, *Cancer Lett.* 357 (2015) 339–345.