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Human papillomavirus infection is rare in nonmalignant tonsil tissue in the UK: Implications for tonsil cancer precursor lesions

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The incidence of human papillomavirus (HPV)-associated tonsil cancer is increasing but the prevalence of HPV, and of premalignant precursors, in tonsil tissue is unknown. We aimed to assess prevalence of HPV infection in nonmalignant tonsillar crypt epithelia and to histopathologically characterise positive samples. Formalin-fixed paraffin-embedded (FFPE) tonsil tissue specimens were obtained from an age- and sex-stratified random sample of patients aged 0–69 years whose paired tonsils were archived following elective tonsillectomy at hospitals throughout England and Southern Scotland from 2004 to 2008. Homogenised fresh-frozen tonsil tissue was also obtained from archive for two random subsets of males aged 25–34 and over 44. HPV status was assessed in all samples for 20 mucosal HPV types by GP5+/6+ polymerase chain reaction (PCR) enzyme immunoassay and by HPV16 type-specific PCR targeting the E6 gene. In the homogenised material, HPV status was also assessed for 44 HPV types by SPF10-PCR enzyme immunoassay. Of 4,095 randomly sampled FFPE specimens, amplifiable DNA was extracted from 3,377 (82.5%) and from 511 of 524 (97.5%) homogenised tonsils. HPV DNA was identified in 0 of 3,377 (0%, 95% CI 0–0.089%) fixed samples and 0 of 511 (0%, 95% CI 0–0.58%) homogenised samples. This suggests HPV infection may be rare in tonsil reticulated crypt epithelia. Furthermore, we found no evidence of HPV-associated premalignant neoplasia. These data suggest that if HPV-associated premalignant lesions do occur, they are likely to be rare and may have a high risk of progression to carcinoma.

Epidemiology

Oropharyngeal squamous cell carcinoma (OPSCC) arises predominantly from the tonsils and base of the tongue. Tobacco and alcohol are known risk factors for OPSCC, but a growing proportion of cases are attributable to oncogenic human papillomavirus (HPV).[1–3] A meta-analysis of contemporary studies estimated the proportion of OPSCC associated with HPV to be 70% in North America and 73% in Europe,[4] and recent decades have seen sharp increases in the overall incidence of HPV-associated OPSCC.[5–7] In the USA, between 1988 and 2004, incidence rates for HPV-associated OPSCC

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What's new?

HPV, notorious for causing cervical cancer, also causes cancer of the tonsils. HPV-related tonsil cancers are on the rise in Britain, primarily in men. This study asked how common it is to find HPV in apparently healthy tonsils. The authors tested thousands of tonsils collected from elective tonsillectomies, but not a single one contained HPV, suggesting that HPV infects the tonsils only rarely. The authors also sought to uncover a pre-malignant lesion of the tonsils, analogous to the cervical intraepithelial neoplasia that afflicts the HPV-infected cervix, but found no such lesions in the tonsils.

rose from 0.8/100,000 to 2.6/100,000.[3] The incidence of tumours at specific subsites of the oropharynx may be underestimated owing to broad classification of many cases as ICD C10 "Malignant neoplasm of the oropharynx," but in England a minimum of 55% of OPSCC develop from the tonsils[8] and between 1995 and 2010 the age-standardised incidence rate (ASR) for ICD C09 "Malignant neoplasm of tonsil" increased threefold from 1.1 to 3.5/100,000.[8,9] In Sweden between 1970 and 2006, the incidence of HPV-associated tonsil carcinoma increased from 0.18/100,000 to 1.27/100,000.[7] Unlike other head and neck cancers, patients with HPV-positive OPSCC tend to be younger (usually <60 years) and often do not have a history of heavy alcohol and tobacco use.[5] Historically, HPV has been predominantly linked to cervical cancers in women; however, tonsil SCC predominantly affects men; in England in 2010, the ratio of male:female cases was 2.6:1.[8] A recent analysis suggests that in the USA by 2020, the number of HPV-positive OPSCC will exceed the number of cervical cancers.[3]

Despite now being recognised as the cause of the majority of OPSCC, the pathobiology and natural history of oropharyngeal HPV infection are not well understood. Data on the prevalence of HPV infection in the oral cavity, assessed by oral rinse, are accumulating,[10,11] and although such studies are essential for a full understanding of the epidemiology and aetiology of HPV-associated disease, they cannot be used to infer infection rates in the tonsillar reticulated crypt epithelia from which tonsillar carcinomas originate.[12] Determining infection rates in the tonsillar crypts requires studies based on invasively sampled tissue; however, to date the two largest studies in adults included only 212 and 229 cases and reported prevalence of 0 and 6.3%, respectively.[13,14] Among HPV-positive OPSCC, HPV16 is the predominant genotype, accounting for ~95% of cases[15]; however, it is not clear whether other HPV types also infect the tonsils but are not associated with malignancy. It is also unclear whether productive infection, resulting in release of infectious virions, can be established in the tonsils. The HPV life cycle is intimately linked to the differentiation of stratified squamous epithelium[16]; however, the squamous epithelium of the tonsillar crypts is reticulated (netlike) rather than stratified, and may not be compatible with completion of the viral life cycle. A final unanswered, and clinically very important question, is whether HPV infection is associated with a premalignant tonsillar lesion. In cervical epithelia, persistent viral infection leads to cervical intraepithelial neoplasia (CIN) followed by the accumulation of mutations that may lead to invasion and metastasis.[17] The premalignant CIN phase may last for months or decades, but whether an equivalent premalignant lesion occurs in the tonsils is unknown.

Our study aimed to address these questions by investigating HPV type-specific prevalence in a large nationally representative series of resected, clinically nonmalignant tonsils, followed by histopathological characterisation of HPV-positive cases, to determine whether productive infection and/or premalignant neoplasia were present. We also hypothesised that determination of HPV prevalence in noncancerous tonsils from males and females of different ages would allow inferences to be drawn regarding the age groups at risk of infection and potential routes of transmission.

Material and Methods

Study design

This was a cross-sectional study. Tonsil tissue was obtained from the National Anonymous Tonsil Archive. This archive was established to determine the prevalence of abnormal prion protein following the epidemic of bovine spongiform encephalopathy in cattle and related concerns regarding occurrence of variant Creutzfeldt–Jakob disease in humans. More than 100,000 paired tonsils were collected from children and adults following elective tonsillectomies performed at 134 hospitals throughout England and Southern Scotland between January 2004 and September 2008.[18] All tonsils were obtained with informed consent. One tonsil of each pair was collected as fresh tissue (chilled on wet ice during transit), then dissected, homogenised and stored at −80°C; the other tonsil was collected and transported in formalin, then embedded in paraffin at the study centre. Paired tonsils arrived at the study centre an average of 65 hr after operation.

For our study, 4,095 cases stratified for sex and geographical distribution were randomly sampled from the archive. As far as possible, these randomly sampled cases were equally distributed across the following age bands: 0–4, 5–9, 10–12, 13–15, 16–18, 19–21, 22–24, 25–29, 30–34, 35–39, 40–44, 45–49 and 50+ years (Table 1). The study had greater than 99% power to detect a difference between a prevalence rate of 1% and a prevalence rate of 5% between the two sexes and over 80% power to detect a difference between a prevalence rate of 1% and 5% between individual age bands. Research Ethics Committee approval was obtained before study commencement.

To control for the possibility that HPV present in very small foci of infection might be missed, two subsets of men

Table 1. Sample adequacy (beta-globin) and HPV testing for samples stratified by material type, age and gender

				Assay			
Material	Age (years)	Sex (M/F)	Samples (*n*)	Beta-globin	GP5+/6+	HPV16 E6	SPF10 DEIA
FFPE tonsils	0–4	M	180	154	0	0	–
		F	175	159	0	0	–
	5–9	M	165	136	0	0	–
		F	164	146	0	0	–
	10–12	M	164	143	0	0	–
		F	165	146	0	0	–
	13–15	M	164	148	0	0	–
		F	165	138	0	0	–
	16–18	M	163	149	0	0	–
		F	165	151	0	0	–
	19–21	M	165	142	0	0	–
		F	165	132	0	0	–
	22–24	M	165	135	0	0	–
		F	165	122	0	0	–
	25–29	M	168	131	0	0	–
		F	167	118	0	0	–
	30–34	M	168	129	0	0	–
		F	165	127	0	0	–
	35–39	M	169	138	0	0	–
		F	167	130	0	0	–
	40–44	M	165	133	0	0	–
		F	168	131	0	0	–
	45–49	M	116	101	0	0	–
		F	136	107	0	0	–
	50+	M	75	54	0	0	–
		F	101	77	0	0	–
	Total	M + F	4,095	3,377	0	0	–
Fresh homogenised tonsils	25–29	M	167	162	0	0	0
	30–34	M	168	162	0	0	0
	45–49	M	114	113	0	0	0
	50+	M	75	74	0	0	0
	Total	M	524	511	0	0	0

"–" denotes that the specified assay was not performed on this material.

from the original sample were selected for more intensive assessment of their tonsil tissue specimens. The results of oral rinse studies suggest that men in the age group 25–34 years are most likely to carry oral HPV infection followed by men over 44[10]; hence, archived homogenised fresh tonsil specimens from 335 and 189 men randomly sampled from these age groups were assayed for HPV DNA.

Laboratory analyses

For the main study sample HPV infection was initially assessed in sections cut from formalin-fixed paraffin-embedded (FFPE) blocks. This method was chosen as it allows further histopathological investigations in positive samples. FFPE blocks were sectioned with appropriate precautions to prevent block-to-block contamination (thorough cleaning of the microtome between samples, use of a fresh blade for every sample, use of Microsol wipes, *etc.*). Sections from blank (paraffin only) blocks were cut after every 20 samples and processed in parallel with cases. Sections from an FFPE HPV16-positive tonsil cancer were included as a positive control. DNA was extracted from 2 × 10 μm sections, taken through the centre of the tonsils, by overnight

digestion in 500 µl 50 mM Tris, 1 mM EDTA, 0.5% Tween with 1 mg/ml Proteinase K followed by heat inactivation (100°C, 5 min) and centrifugation. The supernatant was used as template in polymerase chain reactions (PCRs). Positive (HPV16-positive Caski cells) and negative controls were included in every extraction.

For the fresh tissue from the two subsets of adult males, DNA was extracted from homogenised tonsils by proteinase K (3 mg/ml) digestion overnight followed by phenol–chloroform extraction and ethanol precipitation. DNA purity and yield were determined by Nanodrop spectrophotometry. Positive (HPV16-positive Caski cells) and negative (water) controls were included for each extraction run. The adequacy of DNA for PCR analysis was assessed by amplification of a 209-bp region of the human beta-globin gene (PC03/PC05 primer set).[19] HPV typing was performed by PCR using GP5+/6+ primers (amplimer 142 bp) followed by enzyme immunoassay,[20] as previously described,[21] which detects 14 high-risk and six low-risk HPV types. Samples with absorbance greater than three times the mean of the negative controls were defined as positive[20]; in our laboratory this corresponded to 500 copies of the HPV16 genome per PCR (determined using WHO Labnet standards[22]). Positive (HPV16-positive Caski DNA) and negative (water) controls were included for each PCR run. Type-specific PCR to amplify a 161-bp fragment of the HPV16 E6 gene was performed using primers and conditions as previously described.[23] The HPV16 E6 type-specific PCR was an end-point assay and was assessed by visual examination of agarose gels including appropriate standards. The lower limit of sensitivity was identified as 500 genome equivalents using a dilution series of DNA extracted from a known number of Caski cells (each containing ~600 copies of HPV16). HPV testing was also performed using the SPF10-PCR Direct Enzyme Immuno Assay (DEIA) system (Labo Biomedical Products, Rijswijk, The Netherlands, based on licensed Innogenetics Technology),[24,25] as recently described (amplimer 65 bp).[26] Hundred nanograms of phenol–chloroform-extracted DNA was used as a template. The SPF10 DEIA is a licensed *in vitro* diagnostic assay and is validated for detection of 44 mucosal HPV types, including all 18 potentially carcinogenic HPV types (HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82) plus a further 26 low-risk types.[27,28] The kit includes a "borderline" standard and absorbance readings above borderline are interpreted as positive; the manufacturer's kit insert specifies the limit of detection as two HPV16 genomes per PCR.

Statistical analyses

HPV prevalence rates were estimated with 95% confidence intervals (CI) determined by the method of Lancaster.[29]

Results

FFPE tissue specimens for 4,095 patients, randomly sampled across age and sex categories, were retrievable from archive and included in the analysis (Table 1). Amplifiable DNA, assessed by PCR for the human beta-globin gene, was extracted from 3,377 of 4,095 (82.5%) tonsil samples from 1,693 males of mean age 23.3 years and 1,684 females with mean age 23.6 years. The DNA extracted from these FFPE samples was tested for HPV DNA using the GP5+/6+ EIA and HPV16 E6 PCR assays. None of the 3,377 samples tested positive for HPV by either assay, corresponding to a prevalence of HPV infection in nonmalignant tonsil of 0 of 3,377 (0%, 95% CI 0–0.089%) samples. The extraction and PCR-positive and -negative controls gave appropriate results in both assays. In samples that gave OD readings approaching the positive cut-off value, the assay was repeated to determine whether these samples represented low-level positives, but on repetition OD readings were close to the mean value observed for negative controls. To confirm that the reticulated crypt epithelia, from which tonsil SCC arises, had been sampled, sections adjacent to those used for DNA extraction were taken from 20 randomly selected blocks and subjected to H&E staining and histopathological examination. This confirmed that, as is normal for this tissue, around 20% of the cells on each slide comprised reticulated crypt epithelia and 80% were lymphoid stroma.

HPV prevalence was then determined in DNA extracted from homogenised fresh tonsils from individuals likely to have the highest prevalence of oral HPV infection (*i.e.*, men 25–34 years and >44 years).[10] This material was representative of the whole tonsil (in contrast to sections through centre of embedded material). The human beta-globin gene was successfully amplified in 511 of 524 cases (97.5%), 324 were aged 25–34, 187 were aged 45–69 and the overall mean age was 36.9 years. HPV infection was assessed using the GP5+/6+ EIA and HPV16 E6 PCR. All extraction controls and PCR controls produced the appropriate results, but all the 511 samples tested negative for HPV DNA. The analytical sensitivity of the GP5+/6+ EIA assay was determined using a standardized sample set obtained from the WHO HPV Labnet project,[22] which demonstrated that the GP5+/6+ PCR EIA was sensitive to 500 genome equivalents for HPV16. The sensitivity of the E6 HPV16 type-specific PCR was also 500 genome equivalents.

Finally, a highly sensitive commercial assay, the SPF10-PCR DEIA, was applied to DNA extracted from the fresh homogenised tonsils only. The SPF10-PCR DEIA has an analytical sensitivity of two HPV16 genome equivalents per PCR, and has been widely applied in studies of archival tissue.[26] All controls gave the appropriate results and all 511 fresh tonsil tissue samples tested negative, confirming 0% HPV prevalence (95% CI 0–0.58%).

Discussion

Despite rising incidence rates of HPV-associated tonsil cancer in the UK population, no HPV DNA was identified in benign tonsil tissue specimens from 3,377 English and Scottish males and females aged 0–69 years (average age 23). There are

three possible explanations for this finding. First, the sampling regimen could have been inadequate. To address this question, HPV infection was also assessed in homogenised whole tonsils from two subsets of men in the age groups at highest risk of HPV infection, *i.e.*, 25–34 and over 44. The increased risk of HPV infection in these groups was predicated on two observations: first, that HPV infection, detected in oral rinse samples, has been shown to have a bimodal distribution with highest prevalence among individuals aged 30–34 years, with men showing a significantly higher prevalence than women,[10] and second, that HPV-associated tonsil carcinomas are most common in men aged 45–75.[8] Given the absence of HPV in tissue homogenates, it did not appear that the negative results were due to inadequate sampling.

The second possible explanation was use of inappropriate or insensitive assays, but this was not the case here because the GP5/6 and SPF10 DEIA systems amplify 20 and 44 HPV types, with analytical sensitivities of 500 and two HPV16 genome equivalents, respectively. Furthermore, successful amplification of the human beta-globin gene in more than 80% of samples showed that PCR inhibition and excessive degradation of DNA were not significant factors, and inclusion of appropriate extraction and PCR-positive controls confirmed that the assays were performed correctly.

Therefore, we propose that the third explanation is the most likely, namely that HPV infection in nonmalignant tonsil is a very rare event. Given our large sample size, the greatest population prevalence for HPV consistent with our entirely negative result is 0.089%.

One small study of tonsillectomy samples from adults (USA, 1979–2001, $n = 212$) has previously reported an HPV prevalence of 0%.[13,14] This is highly consistent with our data, but owing to the small sample size, the USA study gave an upper confidence limit of 3.5% compared to 0.089% in our study. Smaller studies of predominantly juvenile tonsillectomy samples (Finland, 2001–2002, $n = 229$; Greece, 1995–2000, $n = 102$; Brazil, before 2005, $n = 100$; USA, before 2006, $n = 50$) have reported prevalence rates of 6.3, 8.5, 0 and 4%, respectively.[30–32] Methodological and quality control issues might underlie these differences; however, just as genital HPV prevalence varies between populations,[33,34] some differences between HPV prevalence in tonsillectomy studies might reflect genuine variation between populations. Hence, it is not clear how generalisable our findings may be outside the UK.

In relation to previous studies, it is important to distinguish between investigations of oral HPV infection ascertained from oral rinse samples containing cells primarily derived from the stratified squamous epithelium of the oral cavity and, studies such as ours, of resected tonsil tissue containing reticulated crypt epithelium from which OPSCC develop. This is relevant, as there is no evidence that HPV infection rates in the oropharynx can be inferred from oral HPV prevalence. In contrast to studies of benign tonsil tissue, where data are sparse, there is an emerging consensus regarding HPV infection in the oral cavity with substantial studies reporting significant levels of infection, especially in the USA where a prevalence of 6.9% (sampled 2009–2010) has been reported.[10] A recent meta-analysis estimated a pooled prevalence of 4.5% in oral samples collected in 11 countries between 1994 and 2007.[11]

The strengths of this study include the examination of nonmalignant tonsils from an unselected series of individuals during a recent period (2004–2008), when rates of tonsillar carcinoma had become relatively high (ASR 2.6/100,000) in the source population.[8] The large sample meant the study was highly powered (>99%) to detect prevalent HPV. The availability of homogenised whole tonsils and the use of multiple highly sensitive methods for HPV testing, targeting separate regions of the HPV genome, were additional strengths. The tonsillectomy specimens came from unselected people in regions across England and Southern Scotland[18] and these results should be broadly representative of the UK population. The primary weakness was that we could not rule out HPV infection present below the limits of detection of the assays used; hence, small foci of HPV-infected cells may have been missed. The predominance of lymphoid cells in tonsil tissue may also hinder the detection of small foci of HPV-infected epithelial cells. Our data are, however, certainly inconsistent with the presence of diffuse or productive HPV infections.

These findings have significant implications for understanding of the natural history of HPV in the tonsils, and suggest it differs substantially from the natural history of HPV in cervical tissue. In the cervix, HPV infection is common but progression is not; for tonsils, our data are more consistent with infection being rare, but the proportion of infections that progress being higher. Lower infection rates in tonsil tissue would be consistent with greater immunological exposure in tonsil epithelia relative to cervical epithelia, and with the recently reported low prevalence of HPV E6-directed antibodies in sera of people without HPV-associated disease.[35] Although detection of E6 antibodies >10 years before diagnosis of OPSCC suggests that a long duration of infection before diagnosis may be usual for oropharyngeal as well as for cervical cancers.[35] The lower prevalence of HPV in the tonsils might also be explained by cells of the reticulated crypt epithelium, from which most HPV-associated tonsil SCC develop,[12] being semireceptive to viral infection and semipermissive to expression of viral proteins. This could arise from a receptor required for viral entry into the cell being rare in the population, or from infrequent misrecognition of a common receptor. A semipermissive model of viral gene expression is potentially relevant as virus-induced cancers often arise at sites where productive viral infection cannot be properly supported.[16] The reticulated epithelial crypts comprise specialised epithelia with immune and secretory functions, not stratified epithelia,[36] hence they may not support the full HPV life cycle but could allow expression of the viral early genes including oncogenic E6 and E7.

One of the original aims of our study was to identify and characterise HPV-associated premalignant lesions in tonsil tissue, but the absence of any HPV-positive tonsils prevented this. The existence of such lesions was hypothesised largely by analogy to cervical neoplasia, where prolonged persistent HPV infection is often associated with CIN.[16] The absence of HPV-associated neoplasia in this study does not prove that it did not occur, but suggests that it may be uncommon without subsequent development of OPSCC, although HPV-associated field change has not been observed in tissue adjacent to OPSCC.[12] These data are highly relevant in the context of prevention of OPSCC. Because of the increasing incidence of OPSCC and the success of screening in preventing HPV-associated cervical cancer,[37] screening for OPSCC or its precursors has been considered.[38] However, effective prevention would depend on identification of a treatable HPV-associated premalignant neoplasia, which was not observed in our study. Similarly, the potential of tonsillar cytology appears limited by difficulty in sampling the correct mucosa.[39,40] Therefore, it appears that prophylactic vaccination or serological screening for E6 antibodies may be more promising options for long-term prevention of tonsil cancer.[35,41]

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References

1. D'Souza G, Kreimer AR, Viscidi R, et al. Case-control study of human papillomavirus and oropharyngeal cancer. *N Engl J Med* 2007;356:1944–56.
2. Gillison ML, D'Souza G, Westra W, et al. Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. *J Natl Cancer Inst* 2008;100:407–20.
3. Chaturvedi AK, Engels EA, Pfeiffer RM, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J Clin Oncol* 2011;29:4294–301.
4. Mehanna H, Beech T, Nicholson T, et al. 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* 2012;35:747–55.
5. Chaturvedi AK, Engels EA, Anderson WF, et al. Incidence trends for human papillomavirus-related and -unrelated oral squamous cell carcinomas in the United States. *J Clin Oncol* 2008;26:612–19.
6. Hocking JS, Stein A, Conway EL, et al. Head and neck cancer in Australia between 1982 and 2005 show increasing incidence of potentially HPV-associated oropharyngeal cancers. *Br J Cancer* 2011;104:886–91.
7. Nasman A, Attner P, Hammarstedt L, et al. Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma? *Int J Cancer* 2009;125:362–6.
8. Anon. Cancer Statistics Registrations, England (Series MB1), No. 41, 2010. Available at: http://www.ons.gov.uk/ons/taxonomy/index.html?nscl=Cancer+Registrations#tab-data-tables (Accessed 08/07/2013).
9. Anon. Cancer Statistics Registrations, England (Series MB1), No. 28, 1995–1997. Available at: http://www.ons.gov.uk/ons/rel/vsob1/cancer-statistics-registrations–england–series-mb1-/no–28–1995–1997/index.html (Accessed 08/07/2013).
10. Gillison ML, Broutian T, Pickard RK, et al. Prevalence of oral HPV infection in the United States, 2009–2010. *JAMA* 2012;307:693–703.
11. Kreimer AR, Bhatia RK, Messeguer AL, et al. Oral human papillomavirus in healthy individuals: a systematic review of the literature. *Sex Tranm Dis* 2010;37:386–91.
12. Begum S, Cao D, Gillison M, et al. Tissue distribution of human papillomavirus 16 DNA integration in patients with tonsillar carcinoma. *Clin Cancer Res* 2005;11:5694–9.
13. Ernster JA, Sciotto CG, O'Brien MM, et al. Prevalence of oncogenic human papillomavirus 16 and 18 in the palatine tonsils of the general adult population. *Arch Otolaryngol Head Neck Surg* 2009;135:554–7.
14. Chen R, Sehr P, Waterboer T, et al. Presence of DNA of human papillomavirus 16 but no other types in tumor-free tonsillar tissue. *J Clin Microbiol* 2005;43:1408–10.
15. Dayyani F, Etzel CJ, Liu M, et al. Meta-analysis of the impact of human papillomavirus (HPV) on cancer risk and overall survival in head and neck squamous cell carcinomas (HNSCC). *Head Neck Oncol* 2010;2:15.
16. Doorbar J. Molecular biology of human papillomavirus infection and cervical cancer. *Clin Sci* 2006;110:525–41.
17. Snijders PJ, Steenbergen RD, Heideman DA, et al. HPV-mediated cervical carcinogenesis: concepts and clinical implications. *J Pathol* 2006;208:152–64.
18. Clewley JP, Kelly CM, Andrews N, et al. Prevalence of disease related prion protein in anonymous tonsil specimens in Britain: cross sectional opportunistic survey. *BMJ* 2009;338:b1442.
19. de Roda Husman AM, Snijders PJ, Stel HV, et al. Processing of long-stored archival cervical smears for human papillomavirus detection by the polymerase chain reaction. *Br J Cancer* 1995;72:412–17.
20. Jacobs MV, Snijders PJ, van den Brule AJ, et al. A general primer GP5+/GP6(+)-mediated PCR-enzyme immunoassay method for rapid detection of 14 high-risk and 6 low-risk human papillomavirus genotypes in cervical scrapings. *J Clin Microbiol* 1997;35:791–5.
21. Powell NG, Boyde AM, Tristram AJ, et al. The potential impact of human papillomavirus vaccination in contemporary cytologically screened populations may be underestimated: an observational retrospective analysis of invasive cervical cancers. *Int J Cancer* 2009;125:2425–7.
22. Eklund C, Forslund O, Wallin KL, et al. The 2010 global proficiency study of human papillomavirus genotyping in vaccinology. *J Clin Microbiol* 2012;50:2289–98.
23. Collins SI, Constandinou-Williams C, Wen K, et al. Disruption of the E2 gene is a common and early event in the natural history of cervical human papillomavirus infection: a longitudinal cohort study. *Cancer Res* 2009;69:3828–32.
24. Kleter B, van Doorn LJ, Schrauwen L, et al. Development and clinical evaluation of a highly sensitive PCR-reverse hybridization line probe assay for detection and identification of anogenital human papillomavirus. *J Clin Microbiol* 1999;37:2508–17.
25. van Doorn LJ, Molijn A, Kleter B, et al. Highly effective detection of human papillomavirus 16 and 18 DNA by a testing algorithm combining broad-spectrum and type-specific PCR. *J Clin Microbiol* 2006;44:3292–8.
26. Tjalma WA, Fiander A, Reich O, et al. Differences in human papillomavirus type distribution in high-grade cervical intraepithelial neoplasia and invasive cervical cancer in Europe. *Int J Cancer* 2013;132:854–67.
27. de Villiers EM, Fauquet C, Broker TR, et al. Classification of papillomaviruses. *Virology* 2004;324:17–27.
28. Munoz N, Bosch FX, de Sanjose S, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* 2003;348:518–27.
29. Lancaster HO. The combination of probabilities arising from data in discrete distributions. *Biometrika* 1949;36:370–82.
30. Mammas IN, Sourvinos G, Michael C, et al. Human papilloma virus in hyperplastic tonsillar and adenoid tissues in children. *Pediatr Infect Dis J* 2006;25:1158–62.
31. Ribeiro KM, Alvez JM, Pignatari SS, et al. Detection of human papilloma virus in the tonsils of children undergoing tonsillectomy. *Braz J Infect Dis* 2006;10:165–8.
32. Sisk J, Schweinfurth JM, Wang XT, et al. Presence of human papillomavirus DNA in tonsillectomy specimens. *Laryngoscope* 2006;116:1372–4.
33. De Vuyst H, Clifford G, Li N, et al. HPV infection in Europe. *Eur J Cancer* 2009;45:2632–9.
34. Hariri S, Unger ER, Sternberg M, et al. Prevalence of genital human papillomavirus among females in the United States, the National Health And Nutrition Examination Survey, 2003–2006. *J Infect Dis* 2011;204:566–73.
35. Kreimer AR, Johansson M, Waterboer T, et al. Evaluation of human papillomavirus antibodies

and risk of subsequent head and neck cancer. *J Clin Oncol* 2013;31:2708–15.

36. Perry ME. The specialised structure of crypt epithelium in the human palatine tonsil and its functional significance. *J Anat* 1994;185 (Part 1): 111–27.
37. Peto J, Gilham C, Fletcher O, et al. The cervical cancer epidemic that screening has prevented in the UK. *Lancet* 2004;364:249–56.
38. Gillison ML, Castellsague X, Chaturvedi A, et al. Comparative epidemiology of HPV infection and associated cancers of the head and neck and cervix. *Int J Cancer* 2014;134:497–507.
39. Fakhry C, Rosenthal BT, Clark DP, et al. Associations between oral HPV16 infection and cytopathology: evaluation of an oropharyngeal "pap-test equivalent" in high-risk populations. *Cancer Prev Res* 2011;4:1378–84.
40. Lingen MW. Brush-based cytology screening in the tonsils and cervix: there is a difference! *Cancer Prev Res (Phila)* 2011;4: 1350–2.
41. Herrero R, Quint W, Hildesheim A, et al. Reduced prevalence of oral human papillomavirus (HPV) 4 years after bivalent HPV vaccination in a randomized clinical trial in Costa Rica. *PloS One* 2013;8:e68329.