HPV involvement in head and neck cancers

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HPV involvement in head and neck cancers: comprehensive assessment of biomarkers in 3,680 cases

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ABSTRACT

Background: We conducted a large international study to estimate fractions of head and neck cancers (HNCs) attributable to HPV (HPV-AFs) using six HPV-related biomarkers of viral detection, transcription, and cellular transformation.

Methods: Formalin-fixed, paraffin-embedded cancer tissues of the oral cavity (OC), pharynx and larynx were collected from pathology archives in 29 countries. All samples were subject to histopathological evaluation, DNA quality control, and HPV-DNA detection. Samples containing HPV-DNA were further subject to HPV E6*I mRNA detection and to $p16^{INK4a}$, pRb, p53, and Cyclin D1 immunohistochemistry. Final estimates of HPV-AFs were based on HPV-DNA, HPV E6*I mRNA, and/or $p16^{INK4a}$ results.

Results: A total of 3,680 samples yielded valid results: 1,374 pharyngeal, 1,264 OC, and 1,042 laryngeal cancers. HPV-AF estimates based on positivity for HPV-DNA, and for either HPV E6*I mRNA or $p16^{INK4a}$ were 22.4%, 4.4%, and 3.5% for cancers of the oropharynx, OC, and larynx, respectively, and 18.5%, 3.0%, and 1.5% when requiring simultaneous positivity for all three markers. HPV16 was largely the most common type. Estimates of HPV-AF in the oropharynx were highest in South America, Central and Eastern Europe and Northern Europe, and lowest in Southern Europe. Women showed higher HPV-AFs than men for cancers of the oropharynx in Europe and for the larynx in South America.

Conclusions: HPV contribution to HNCs is substantial but highly heterogeneous by cancer site, region, and gender. This study, the largest exploring HPV attribution in HNCs, confirms the important role of HPVs in oropharyngeal cancer and drastically downplays the previously reported involvement of HPVs in the other HNCs.

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INTRODUCTION

Strong evidence has accumulated in the last 15 years showing that certain human papillomaviruses (HPVs) are etiologically involved in a subset of head and neck cancers (HNCs)[1]. While virtually all cervical cancers are considered HPV driven[2] the quantitative assessment of the etiological involvement of HPVs in HNCs is challenged by its multifactorial etiology largely attributed to tobacco and alcohol use[3-5]. Consequently, the unequivocal fraction of HPV-DNA-positive HNCs for which HPV infection is indeed the truly triggering carcinogenic event is unknown, and its estimation remains a challenge[6]. Further, the presence of HPV-DNA in HNCs is not sufficient to prove viral causation as it might just reflect a transient infection unrelated to the carcinogenic process[7,8]. It is thus crucial to explore the individual and combined expression patterns of other markers associated with HPV-induced carcinogenesis to assess the biological and oncogenic activities of HPVs identified in these cancers.

To that end we conducted a large international study in HNCs to assess levels of six markers associated with HPV carcinogenesis using a strict single protocol to standardize the entire process that spans from sample selection and processing to pathology review and testing. The ultimate goal of the study was to generate robust estimates of the HPV attributable fractions (AFs) in HNCs by anatomical site, gender, and geography.

METHODS

We carried out an international, cross-sectional study to assess the prevalence of viral DNA and other markers of HPV-related carcinogenesis in formalin-fixed paraffin-embedded (FFPE) samples of HNCs. Protocols were approved by the ethics committee of the Catalan Institute of Oncology (Comitè Ètic d'Investigació Clínica de l'Hospital Universitari de Bellvitge, L'Hospitalet de Llobregat, Spain), which required no informed consent to use archived tumor samples.

Selection of HNC cases and control tissue

HNC samples were selected from an international network of pathology departments identified in 44 centers in 29 countries in Europe, Africa, Asia, and America. Participating centers were requested to provide samples using a common protocol for sample selection, retrieval, processing, and shipping to ICO. Selected cancer cases had to fulfill pre-established inclusion criteria: to be diagnosed with primary invasive cancer of the oral cavity (OC), pharynx or larynx under specific codes of the International Classification of Diseases version 10 (ICD-10); to have complete data on year of diagnosis and site of the tumor; and to be selected in a consecutive or random manner from 1990 onwards. Centers were asked to contribute if possible a minimum of 50 samples per major anatomic HNC site. In order to assess potential carry over contamination at the local level we additionally requested tissue samples of patients with non-HPV related diagnoses processed in the same laboratory and close to the cases' diagnosis time. Cancers of the salivary glands, nasopharynx and external lip were initially not requested since they are *a priori* not considered to be related to HPVs. Nevertheless, we included a series of nasopharyngeal cancers from Europe (n=37), America (n=8), Africa (n=35) and Asia (n=21).

Based on previously published site-specific estimates of HPV-DNA prevalence[9], optimal sample size was set at around 1,000 cases per major cancer site in order to obtain HPV-DNA prevalence estimates with a ±2.5% precision.

FFPE blocks processing and histopathological evaluation

FFPE blocks were re-embedded at ICO whenever necessary. At least four paraffin sections were obtained for each block. First and last sections were used for histopathological evaluation and the in-between ones for HPV testing (sandwich method). Additional slides were obtained to assess expression of cellular proteins by immunohistochemistry (IHC). FFPE blocks were processed under strict pre/post PCR physical separation, and blank paraffin blocks were systematically tested in parallel to serve as sentinels for contamination as previously published[10]. Pathology review was performed blind with respect to the original local diagnosis and followed a pre-established algorithm for diagnostic consensus involving four pathologists. First, all cases were reviewed by a trained pathologist at ICO. Cases regarded as difficult to classify (n=668) were further reviewed by two senior expert pathologists also at ICO. Finally, cases having still an unclear histopathological diagnosis after the second review (n=67) as well as a random sample of approximately 10% of the first 2000 cases (n=182) were blindly re-evaluated by an external expert pathologist for a final evaluation. If there were discrepancies with the local collaborating center, the expert diagnosis prevailed. Pathological classification was based on the WHO pathological criteria for HNCs[11].

HPV-DNA detection and genotyping

The detailed methods used for HPV-DNA detection and genotyping have been reported elsewhere in a similar study on cervical cancer specimens[10]. Briefly, we used SPF-10 PCR and a DNA enzyme immunoassay (DEIA) to test for the presence of HPV-DNA. Virus genotyping was performed using reverse hybridization line probe assay (LiPA25_v1) on all samples testing positive for viral DNA, targeting 25 HPV types with different oncogenic potential. Specimens testing positive for HPV-DNA by DEIA but that could not be typed by LiPA25 were further analyzed by direct Sanger sequencing of PCR products[12]. HPV-DNA positive cases that could not be sequenced were labeled as "HPV undetermined". DNA quality was evaluated in all HPV-DNA negative samples by testing for the human tubulin gene[13]. All DEIA and LiPA25_v1 assays were performed at ICO. These assays were quality controlled and validated against an external HPV reference lab (DDL Diagnostic Laboratory, Rijswijk, The Netherlands) by cross-testing of 387 anogenital and head and cancer samples with overall percentage agreements and Kappa values of 92.8% (95% CI 89.7-95.1) and 0.78 (95% CI, 0.71-0.86), respectively, for DEIA, and 91.2% (95% CI 87.9-93.8) and 0.74 (95% CI 0.66-0.82), respectively, for HPV genotyping.

HPV E6*I mRNA detection

All HPV-DNA positive samples underwent RNA extraction and E6*I mRNA detection at DKFZ, Heidelberg, Germany, as developed by Halec and colleagues[14]. Briefly, the assays target a total of 20 HPV types. For each sample, type-specific E6*I mRNA RT-PCR was performed for all available HPV types detected at the DNA level, and additionally for HPV16. A random selection (0.6%) of HPV-DNA negative cases was tested for HPV16 E6*I mRNA. Detection of housekeeping gene ubiquitin C mRNA was used for RNA quality control in all tested cases.

Immunohistochemistry

Protein expression patterns were evaluated for $p16^{INK4a}$, pRb, p53, and Cyclin D1 in all HPV-DNA positive samples, and in a random selection of HPV-DNA negative cases in a ratio of 1:1, corresponding approximately to 12% of the negative cases. Stainings were all performed at Hospital General de L'Hospitalet, L'Hospitalet de Llobregat, Spain, under the manufacturer's standards: Roche mtm Laboratories AG (Heidelberg) for $p16^{INK4a}$, Vision Biosystems Novocastra (Newcastle) for pRb, and Dako (Denmark) for p53 and Cyclin D1. We used the predefined algorithm developed by Halec and colleagues[15] to determine the cut-off values for over- versus under-expression of each protein. The expected pattern for HPV-driven cases was over-expression of $p16^{INK4a}$ and under-expression of the other three markers.

Statistical analyses

Cases testing negative for both viral and human DNA were excluded from the analyses. HPV-DNA prevalence was calculated as the fraction of HPV-DNA positive cases by SPF-10 PCR/DEIA among all samples providing a valid HPV DNA result.

In line with work from several authors[15-17], we established that in order to explore algorithms to classify a HNC as HPV-driven we needed to consider markers of HPV infection (HPV-DNA detection), markers of transcriptional activity of HPV oncogenes (HPV E6*I mRNA), and surrogate markers of HPV-related cellular transformation ($p16^{INK4a}$, pRb, p53 and Cyclin D1). We used HPV-DNA and HPV-mRNA positivity as the gold standard to explore the additional value of the other four surrogate markers of cellular transformation by using statistical indicators such as sensitivity, specificity, odds ratios and area under the ROC curves. As shown in Supplementary Table 1, $p16^{INK4a}$ expression was the marker that showed the most consistent

association with the gold standard across anatomical sites. None of the other markers or combination of markers showed a statistically significant higher area under the ROC curve. Thus, we concluded that using $p16^{INK4a}$ and/or HPV-mRNA in addition to HPV-DNA yielded the most accurate approximation to judge HPV carcinogenicity in HNCs. Accordingly, we report ranges of estimated HPV-AFs by using different combinations of positivity by these three markers (Figure 2). HPV-AFs are expressed as the percentage of positive samples for the marker or combination of markers among all samples validly tested for the corresponding marker or markers, and 95% confidence interval (CI) around point estimates are presented.

We performed sensitivity analyses for $p16^{INK4a}$ positivity according to three different cutoff points of percentage of stained cells: >25%, >50%, and >75% (Supplementary Table 2). Since there were no statistically significant differences in the estimates across the three cutoff values, and for consistency sake, we used the >25% cutoff as used by Halec et al.[15]. For the geographical analyses, countries were grouped into world subregions according to the Globocan classification[18]. All statistical tests were two-sided and statistical significance was set at a P value of less than .05. All analyses were performed with STATA version 10.1 (StataCorp. 2007. Stata Statistical Software: Release 10. College Station, TX: StataCorp LP)

RESULTS

Figure 1 depicts the disposition of HNC samples collected, processed and finally tested. The laboratory at ICO received a total of 4,533 samples of which 4,022 were tested for HPV-DNA. A total of 3,680 HNC samples yielded a valid DNA result and were included in the final

272 analysis: 1,264 from the OC, 1,374 from the pharynx, and 1,042 from the larynx. As compared to
273 other regions, Africa and Asia proportionally contributed more invalid samples (i.e., those testing
274 both HPV-DNA and tubulin negative) than the other regions: 19.5% and 21.1%, respectively,
275 versus 8.5% in Central-South America, and 5% in Europe. Also, samples collected from older
276 periods (1990-2004) were more frequently invalid than those collected from more recent periods
277 (2005-2012): 12.6% versus 6.9%, respectively. In contrast, no differences in the percentage of
278 excluded samples were observed by age or gender. Figure 1 also shows the number of HPV-
279 DNA positive samples that were finally tested for the five additional markers and yielded a valid
280 result.

281 Table 1 summarizes the characteristics of HNC patients included in the analysis. Most
282 samples were recruited from centers in Europe (55.7%) and Central-South America (28.2%).
283 Patients were mostly men (76.2%) with a mean age at diagnosis of 61 years. Patients were
284 mainly diagnosed within the 2000-2009 decade (65.3%). The most frequent histological
285 diagnosis was squamous cell carcinoma (99.1%) of conventional keratinizing type (65.3%).

286 Table 2 shows HPV-DNA prevalence estimates and HPV type-specific distributions by
287 HNC site. Highest HPV prevalence was observed in the oropharynx (25.0%), followed by
288 pharynx unspecified (21.4%), nasopharynx (8.9%), OC (7.4%), larynx (5.9%), and hypopharynx
289 (3.9%). Supplementary Table 3 presents detailed HPV-DNA data for each anatomic sub-site.
290 Among sub-sites with at least 45 tested cases, cancer of the tonsils showed the highest HPV-
291 DNA prevalence (47%), followed by base of the tongue (18.5%), and oropharynx unspecified
292 (18.2%). HPV16 was by far the most frequently detected genotype among HPV-DNA positive
293 cases (76.2%), in particular in the oropharynx (83.2%). Table 2 also presents the results of the
294 HPV-driven expected patterns of the other markers. Among HPV-DNA positive cases, under-
295 expression of p53 and HPV E6*I mRNA detection showed the highest prevalence estimates for

the three major cancer sites. Among HPV-DNA negative cancer samples, $p16^{INK4a}$ over-expression was 13.2%, 10.5%, and 6.6% for OC, oropharynx and larynx, respectively (data not shown). Corresponding values for under-expression of pRb were 33.7%, 25.1%, and 24.0%; for p53: 59.3%, 48.8%, and 50.6%; and for Cyclin D1: 15.7%, 18.0%, and 23.7%. None of the randomly selected oropharyngeal HPV-DNA negative samples (n=20) tested positive for HPV E6*I mRNA (data not shown).

Figure 2 presents estimated HPV-AFs using HPV-DNA, HPV E6*I mRNA detection and over-expression of $p16^{INK4a}$. Ranges of AFs when considering HPV-DNA plus E6*I mRNA and/or $p16^{INK4a}$ were: 18.5 to 22.4% for the oropharynx, 3.0 to 4.4% for the OC, and 1.5 to 3.5% for the larynx. Corresponding values when considering positivity by both HPV-DNA and E6*I mRNA were 21.8%, 3.9%, and 3.1%, respectively. Full results by cancer subsite are provided in Supplementary Table 3. We observed that within both the oral cavity and the larynx, those subsites that were more proximal to the oropharynx showed higher HPV-AFs than those that were more distal to the oropharynx. Thus, HPV-AFs in combined oral cavity subsites that were proximal to the oropharynx ranged (when considering HPV-DNA plus E6*I mRNA and/or $p16^{INK4a}$) from 4.9% to 6.7%, as opposed to 1.4-2.3% in subsites that were distal to the oropharynx ($p<0.001$ for both comparisons). Corresponding values in the larynx were 4.2-4.2% versus 1.4-3.4% in combined subsites that were proximal versus distal to the oropharynx, but these differences were not statistically significant.

Table 3 shows prevalence estimates of the key HPV-related markers as well as the final HPV-AF estimates by selected patients' characteristics. Excluding strata with low numbers, HPV-AFs were highest in Central-South America, followed generally by Europe. Globally, women showed higher HPV-AFs than men for cancers of the oropharynx and larynx. For oropharyngeal cancer, HPV-AFs were higher in women as compared with men in all European

subregions -Central-Eastern Europe (61.5% versus 45.5%, p=0.09), Southern-Europe (22.6% versus 8.4%, p=.002), Western Europe (38.9% versus 13%, p=.02)-, except Northern Europe (50% versus 50%). HPV-AFs were also higher in women as compared with men for cancers of the larynx in South America (23.1% versus 4.2%, p<.0001), as well as in Southern Europe (5.9% versus 0.5%, p=.03). In contrast, in the oral cavity we found higher HPV-AFs in men than in women, but only in Northern Europe (10.9% versus 0%, p=.01). We did not identify a clear pattern of gender differences by calendar period within regions showing gender differences in HPV-AF estimates (data not shown). An inverse trend was observed between HPV-AFs and increasing age at diagnosis for each major site. Concerning time trends, HPV-AFs for the oropharynx clearly increased over time: 7.2%, 10.1%, 18.7%, 26.1% and 32.7% for calendar periods 1990-1994, 1995-1999, 2000-2004, 2005-2009, and 2010-2012, respectively. In contrast, no trends were observed for the other two major HNC sites.

HPV-AFs showed a marked geographic heterogeneity that was particularly evident for oropharyngeal cancer (Figure 3). For the oropharynx, AF estimates when considering HPV-DNA plus E6*I mRNA and/or $p16^{INK4a}$ were highest in South America (48.4%-53.6%), Central-Eastern Europe (44.9%-50%), and Northern Europe (25%-50%), and lowest in Southern Europe (7.6%-9.4%). For the oral cavity corresponding estimates were highest in South America (5.5%-7.3%), Northern Europe (4.2%-6.8%), and Central America (4.3%); and for the larynx, in South America (3.8%-6.5%), Central America (1.4%-5.6%), and Northern Europe (4.2%). Full results by geographic area are provided in Supplementary Table 4. Since the study was not powered to calculate precise country-specific estimates, AFs by country are not provided.

DISCUSSION

To our knowledge this study is the most focused and robust attempt to date to estimate the fraction of HNCs that might be driven by HPV infection. It is now well recognized that the mere detection of HPV-DNA is not sufficient to establish causality in HNCs. We have thus systematically assessed five additional markers related to HPV biological activity: HPV E6*I mRNA expression, and p16, pRb, p53, and Cyclin D1 protein detection. Each of these markers has advantages and limitations[5,19]. However, one of the key indicators of HPV-related carcinogenicity is HPV E6*I mRNA expression, a marker of transcriptional activity of HPV oncogenes[14,20]. Consequently, we have used both HPV-DNA and mRNA detection as the gold standard to assess the potential value of adding other surrogate markers of HPV-induced cellular transformation. As shown in Figure 2, using either or both E6*I mRNA or $p16^{INK4a}$ in addition to HPV-DNA yielded comparable AFs that were in the range of 18.5 to 22.4% for oropharyngeal cancer, 3.0 to 4.4% for OC cancer, and 1.5 to 3.5% for laryngeal cancer. The percentage point differences between the two methods ranged from 1.4 to 3.9, and they were basically due to lack of expression of $p16^{INK4a}$ in certain HPV-DNA+/mRNA+ samples. The loss of $p16^{INK4a}$ in these cancers might be a result of increasing genetic and epigenetic chromosomal instability induced by HPV oncoproteins.[20]

The first observation when assessing these HPV-AFs is the marked heterogeneity across anatomic sites, being highest in the oropharynx, substantially lower in the OC, and even lower in the larynx. The probability of an HPV-driven OC cancer was between 4 and 7 times lower than that of oropharyngeal cancer; and that of an HPV-driven laryngeal cancer between 5 and 15 times lower than that of oropharyngeal cancer. Even within a major site such as the oropharynx, AF estimates ranged from 4.0% in the posterior wall to 45.2% in the tonsil (Supplementary Table 3). Being an oropharyngeal subsite, we found an unexpected low HPV-AF for cancers of the

base of tongue (between 8.7% and 17.4%), but also realized that most of these cases (68/92, 74%) were from Spain, a country known to have low HPV-AFs for HNCs even for the oropharynx (6.7% to 8.6%). It is interesting to note that HPV-AFs for subsites within the oral cavity that were more proximal to the oropharynx were higher than those that were more distal from the oropharynx. Even though the same was observed for subsites in the larynx these differences did not reach statistical significance. This gradient of lower HPV involvement in more distant subsites from the oropharynx suggests either misclassification of anatomic subsite or a true biological gradient of HPV involvement.

It is important to note that our estimates of HPV-AFs are substantially lower than those published in the most recent meta-analysis of HPV in HNCs[8]: 39.8%, 16.3%, and 8.6% in the oropharynx, OC and larynx, respectively, when using HPV-mRNA and HPV-DNA positivity. The discrepancy may be due to the very low number of studies reporting on more than one marker, to differences in the geographic origin of the samples, as well as to the high heterogeneity in the laboratory procedures and assays used across studies. In contrast, our AF estimates for the oropharynx (18.5-22.4%) are relatively consistent with another review reporting a population HPV-AF of 25.6%[21].

As shown in Table 3 we also found important heterogeneity of HPV-AF estimates by geographical region, gender and age at diagnosis. Estimates ranged from 0% in Africa or Asia to 6.6% in Central-South America for OC; from 18.5% in Asia to 40.5% in Central-South America for the oropharynx; and from 0% in Asia to 6.1% in Central-South America for the larynx. Even within European sub-regions, wide variations were observed for each cancer site (Supplementary Table 4). Even though these estimates may seem low for some regions it is difficult to make fair comparisons as there are no large studies using several markers of HPV involvement. However, if we use just HPV DNA detection our estimates are substantially lower

391 that those recently reported for instance in a population-based study in the US in which HPV-
392 DNA was detected in 70.1% of oropharyngeal, 32.0% of oral cavity, and 20.9% of laryngeal
393 cancers[22]. Concerning gender, HPV-AFs estimates were substantially higher in women than in
394 men, but these differences were only statistically significant in Europe (in all European sub-
395 regions except Northern Europe) for oropharyngeal cancer, and in South America for laryngeal
396 cancer. Finally, we also found that the magnitude of AFs decreased with increasing age for each
397 of the three major HNC sites.

398 We speculate that globally, this large heterogeneity in HPV-AFs most likely reflects distinct
399 trends in temporal, geographical and sociodemographic shifts in population exposure to both
400 tobacco smoking and oral HPV infection, leading to a rapidly evolving epidemiology of HPV-
401 positive HNCs. Indeed, pronounced increasing trends in the incidence of HPV-positive HNCs
402 have been consistently observed in the last decade, in particular for HPV-positive oropharyngeal
403 cancers in young men in Northern Europe and North America[5,23-26]. It could be hypothesized that
404 the potential carcinogenic effects of highly prevalent tobacco smoking in the oropharynx
405 between the 60's and early 80's dominated over those induced by low prevalent oral HPV
406 infections. Since the 80's, at least in certain populations, the high smoking/HPV prevalence
407 ratios progressively diminished, and while population exposure to tobacco smoking decreased,
408 exposure rates to oral HPV simultaneously increased due to increasing use of oral sex practices.
409 Thus, the current burden of HPV-driven HNCs in a given population may substantially depend
410 on the prevalence and subsequent trends of these exposures starting 15 to 25 years before.
411 Given that our samples were gathered from diverse populations, age groups and time periods,
412 our estimates might be substantially underestimating the current true burden of HPV-driven
413 HNCs in some geographical areas of the world.

In contrast to previous reports, an important new finding of our data is the small HPV-AFs that we found for cancers of the oral cavity (<4.4%) and larynx (<3.5%). These small HPV-AFs could well be within the false-positive rate of triple-positivity by HPV-DNA, mRNA, and p16. We could also speculate that for these two cancers HPV might be a bystander infection taking advantage of a tumor that was caused by other means. Therefore, this study cannot rule out a potential effect of false-positivity, reverse-causation, misclassification of anatomical subsite, or some other artifact of our cross-sectional design, and thus conclude that HPV involvement in oral and laryngeal carcinogenesis is probably anecdotal. This could be also the case for the oropharynx, but since the HPV-AFs for this site are higher, the overall impact would be much lower than that in the oral cavity or the larynx.

Despite its strong design and large sample size, our study is not free of limitations. The main one is that while we tested all samples for the presence of HPV-DNA, the five additional markers were assessed in HPV-DNA positive samples and only in a small fraction of HPV-DNA negative cases. We therefore cannot completely rule out that we were missing some truly HPV-driven cases. However, our control testing for HPV16 mRNA among HPV-DNA negative samples was systematically negative. The effect of this potential misclassification would be towards underestimating the true role of HPV in head and neck carcinogenesis. Lack of representativeness of included samples from a given country or geographic region is also a potential limitation. The small number of samples included from North America and Africa, for instance, limits the validity of our results for these regions. It is clear also that the study is not population based, and as such, one cannot exclude some degree of referral or selection bias (i.e., centers could serve a biased population in a manner that might be associated with HPV-AFs). The fact that we required participating centers to provide unselected, consecutive HNC samples reduced to a certain degree the potential for selection bias within each center, but not

that in the country as a whole. Related to this, it is important to emphasize that the use of overall (i.e., worldwide) HPV-AF should not be used nor applied to any one geographic region for the purpose of establishing health policy (for example, cost effectiveness analysis of vaccination). Region- and cancer site-specific data should be used instead. There has been a problem in misuse of prior data, and having this incorrect use of HPV-AFs may have an erroneous impact.

In conclusion, this study presents robust evidence that the fraction of oropharyngeal cancers that are likely driven by HPV infection, mainly HPV16, is substantial (between 18.5% and 22.4%) but highly heterogeneous with anatomic subsite, geography and gender. In contrast, the etiological fraction of HPV in cancers of the OC and larynx is substantially lower than previously reported (<4.5%) and also less heterogeneous. Given the rapidly changing epidemiology of HPV-positive HNCs, our estimates might still be underestimating the true impact of HPV in oropharyngeal cancers, and it is likely that in the near future these AFs become even higher. Estimation of the real and evolving contribution of HPV to HNCs is key to forecast the future burden of these cancers as well as to inform on the global potential preventative impact of prophylactic HPV vaccination.

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Xavier Castellsagué: received institutional research funding from Merck, SPMSD, GSK Biologicals and Genticel, occasional speaker fees from Vianex, Merck and SPMSD, and occasional travel grants from Merck and SPMSD.

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574 **Table 1. Descriptive characteristics of head and neck cancer cases included in the study**

575

	Oral cavity n=1,264		Nasopharynx n=101		Oropharynx n=1,090		Hypopharynx n=127		Pharynx unspecified n=56		Larynx n=1,042	
	n	%	n	%	n	%	n	%	n	%	n	%
Geographical origin												
Europe	587	46.4	37	36.6	810	74.3	83	65.4	28	50.0	505	48.5
North America	32	2.5	0	0.0	13	1.2	5	3.9	0	0.0	34	3.3
Central-South America	488	38.6	8	7.9	158	14.5	12	9.4	12	21.4	359	34.5
Africa	58	4.6	35	34.7	6	0.6	26	20.5	4	7.1	73	7.0
Asia	99	7.8	21	20.8	103	9.4	1	0.8	12	21.4	71	6.8
Gender												
Male	781	62.4	75	74.3	884	83.2	99	78.6	37	74.0	929	89.5
Female	471	37.6	26	25.7	178	16.8	27	21.4	13	26.0	109	10.5
Missing	12	-	0	-	28	-	1	-	6	-	4	-
Year of diagnosis												
1990-1994	35	2.8	5	5.0	83	7.6	1	0.8	1	1.8	18	1.7
1995-1999	66	5.2	36	35.6	129	11.8	3	2.4	4	7.1	26	2.5
2000-2004	152	12.0	20	19.8	226	20.7	9	7.1	12	21.4	156	15.0
2005-2009	693	54.8	36	35.6	455	41.7	69	54.3	32	57.1	542	52.0
2010-2012	318	25.2	4	4.0	197	18.1	45	35.4	7	12.5	300	28.8

Range	1990-2012		1990-2011		1990-2012		1993-2012		1990-2011		1990-2012	
Age at diagnosis												
≤53	336	28.5	36	37.5	273	25.9	33	26.2	9	16.4	210	20.9
54-61	256	21.7	21	21.9	293	27.7	31	24.6	17	30.9	293	29.2
62-70	273	23.2	16	16.7	285	27.0	40	31.7	15	27.3	287	28.6
≥71	313	26.6	23	24.0	205	19.4	22	17.5	14	25.5	214	21.3
Missing	86	-	5	-	34	-	1	-	1	-	38	-
Mean age at diagnosis (SD)	61.4	(14.0)	56.6	(16.4)	61.0	(11.2)	58.1	(16.0)	62.3	(11.8)	61.8	(10.9)
Age range	17-98		16-93		20-92		17-91		26-87		18-89	
Histological diagnosis												
Squamous Cell Carcinoma	*1,257*	*99.4*	*95*	*94.1*	*1,083*	*99.4*	*124*	*97.6*	*54*	*96.4*	*1,033*	*99.1*
SCC NOS/Conventional non keratinizing	218	17.2	38	37.6	332	30.5	38	29.9	14	25.0	219	21.0
Conventional keratinizing	955	75.6	32	31.7	603	55.3	71	55.9	31	55.4	712	68.3
Conventional exophytic keratinizing	17	1.3	4	4.0	8	0.7	0	0.0	0	0.0	12	1.2
Basaloid/Papillary	39	3.1	20	19.8	129	11.8	12	9.4	9	16.1	70	6.7
Verrucous	8	0.6	0	0.0	1	0.1	0	0.0	0	0.0	4	0.4
Sarcomatoid	20	1.6	1	1.0	10	0.9	3	2.4	0	0.0	16	1.5
Undifferentiated Carcinoma	*2*	*0.2*	*6*	*5.9*	*5*	*0.5*	*3*	*2.4*	*1*	*1.8*	*8*	*0.8*
Adenosquamous carcinoma	*5*	*0.4*	*0*	*0.0*	*2*	*0.2*	*0*	*0.0*	*1*	*1.8*	*1*	*0.1*

"SCC": Squamous Cell Carcinoma; "SD": Standard Deviation; "NOS": Not Otherwise Specified.

579 **Table 2. HPV-DNA positivity and detected types, and E6*I mRNA, p16^{INK4a}, pRb, p53 and Cyclin D1 results among HPV DNA positive cases, by head and neck cancer site**

580

	Oral cavity n=1,264		Nasopharynx n=101		Oropharynx n=1,090		Hypopharynx n=127		Pharynx unspecified n=56		Larynx n=1,042	
	n	%	n	%	n	%	n	%	n	%	n	%
HPV DNA positivity *	93	7.4	9	8.9	273	25.0	5	3.9	12	21.4	61	5.9
Type of HPV infection †												
Single	81	87.1	9	100.0	269	98.5	5	100	11	91.7	58	95.1
Multiple ‡	5	5.4	0	0.0	1	0.4	0	0.0	0	0.0	1	1.6
Undetermined genotype §	7	7.5	0	0.0	3	1.1	0	0.0	1	8.3	2	3.3
HPV type distribution in single infection[2]												
HPV6	0	0.0	0	0.0	1	0.4	0	0.0	0	0.0	4	6.6
HPV11	1	1.1	0	0.0	0	0.0	0	0.0	0	0.0	1	1.6
HPV13‖	2	2.1	0	0.0	0	0.0	0	0.0	1	8.3	0	0.0
HPV16	64	68.8	7	77.8	227	83.2	4	80.0	8	66.7	32	52.6
HPV18	1	1.1	0	0.0	5	1.8	0	0.0	0	0.0	3	4.9
HPV19‖	0	0.0	0	0.0	1	0.4	0	0.0	0	0.0	0	0.0
HPV26‖	1	1.1	0	0.0	7	2.6	0	0.0	0	0.0	0	0.0
HPV30‖	0	0.0	0	0.0	1	0.4	0	0.0	0	0.0	0	0.0
HPV31	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	2	3.3
HPV33	0	0.0	0	0.0	9	3.4	1	20.0	0	0.0	2	3.3

HPV35	2	2.1	1	11.1	6	2.2	0	0.0	1	8.3	1	1.6
HPV39	1	1.1	0	0.0	1	0.4	0	0.0	0	0.0	3	4.9
HPV45	0	0.0	0	0.0	1	0.4	0	0.0	0	0.0	5	8.2
HPV51	2	2.1	0	0.0	2	0.7	0	0.0	0	0.0	0	0.0
HPV52	4	4.3	1	11.1	0	0.0	0	0.0	0	0.0	0	0.0
HPV53	1	1.1	0	0.0	1	0.4	0	0.0	0	0.0	0	0.0
HPV56	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	1.6
HPV58	1	1.1	0	0.0	2	0.7	0	0.0	0	0.0	1	1.6
HPV59	0	0.0	0	0.0	1	0.4	0	0.0	0	0.0	0	0.0
HPV66	0	0.0	0	0.0	1	0.4	0	0.0	0	0.0	0	0.0
HPV67‖	0	0.0	0	0.0	0	0.0	0	0.0	1	8.3	1	1.6
HPV68	0	0.0	0	0.0	1	0.4	0	0.0	0	0.0	2	3.3
HPV69‖	0	0.0	0	0.0	2	0.7	0	0.0	0	0.0	0	0.0
HPV90‖	1	1.1	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
HPV types grouped by risk and vaccine†¶												
Only high risk types	77	82.8	9	100.0	267	97.8	5	100.0	10	83.3	53	86.9
Only low risk types	4	4.3	0	0.0	2	0.7	0	0.0	1	8.3	5	8.2
Types in bivalent vaccine	65	69.9	7	77.8	232	85.0	4	80.0	8	66.7	35	57.4
Types in quadrivalent vaccine	66	71.0	7	77.8	233	85.3	4	80.0	8	66.7	40	65.6
Types in ninevalent vaccine	71	76.3	8	88.9	245	89.7	5	100.0	8	66.7	50	82.0
Contribution of other markers#	**+ / tested**	**%+**	**+ / tested**	**%+**	**+ / tested**	**%+**	**+ / tested**	**%+**	**+ / tested**	**%+**	**+ / tested**	**%+**

E6*I mRNA+	49/80	61.3	6/9	66.7	235/260	90.4	3/5	60.0	8/9	88.9	32/52	61.5
$p16^{INK4a}$+	44/91	48.4	1/3	33.3	207/267	77.5	3/5	60.0	5/12	41.7	20/61	32.8
pRb-**	55/93	59.1	2/3	66.7	221/267	82.8	3/5	60.0	8/12	66.7	35/60	58.3
p53-**	77/91	84.6	3/3	100	244/265	92.1	4/5	80.0	11/12	91.7	40/58	69.0
Cyclin D1-**	49/93	52.7	2/3	66.7	174/267	65.2	3/5	60.0	9/12	75.0	22/60	36.7
E6*I mRNA+ OR $p16^{INK4a}$ +	55/93	59.1	6/7	85.7	243/268	90.8	3/5	60.0	9/12	75.0	36/61	59.0
E6*I mRNA+ AND $p16^{INK4a}$ +	38/78	48.7	1/3	33.3	199/259	76.8	3/5	60.0	4/9	44.4	16/52	30.8

* Percentage of HPV-DNA positive cases among all cases tested by DEIA (see Methods).

† Percentages among HPV-DNA positive cases.

‡ Multiple infections were: Oral cavity: HPV 6&52 (n=2), HPV 16&52 (n=1), HPV 16&59 (n=1), and HPV 31&52 (n=1); Oropharynx: HPV 16&56 (n=1); Larynx: HPV18&44 (n=1).

§ HPV undetermined denotes cases that were DEIA positive but line probe assay $LiPA_{25}$ negative.

|| Genotype identified by sequencing.

¶ Multiple infections (n=7) are not included in these groups. Risk groups are defined according to the last IARC classification: we considered as high risk HPV types the types included in Group 1, Group 2A and Group 2B; other HPV types were classified as low risk HPV types[27].

Percentages among HPV DNA positive cases that were tested for each specific marker or combination of markers. Positivity for each individual marker refers to: detection of E6*I mRNA, over-expression of $p16^{INK4a}$ and under-expression of pRb, p53, or Cyclin D1.

** Under-expression

592 **Table 3. Prevalence of HPV-DNA, HPV types, E6*I mRNA and p16^{INK4a}, and estimates of HPV attributable fractions by head and neck cancer site and key patients'**
593 **characteristics**

	HPV-DNA prevalence		HPV16		Any HR HPV types		E6*I mRNA		p16^{INK4a}		HPV attributable fractions (%)	
	+/HPV-DNA tested	%	+/HPV-DNA tested	%	+/HPV-DNA tested	%	+/HPV-DNA &mRNA tested	%	+/HPV-DNA &p16 tested	%	HPV-DNA+ AND (mRNA+ OR p16+)	HPV-DNA+ AND mRNA+ AND p16+
Geographical origin												
Oral cavity *												
Europe	46/587	7.8	30/587	5.1	41/587	7.0	18/581	3.1	16/587	2.7	3.8	2.1
Central-South America	42/488	8.6	33/488	6.8	36/488	7.4	30/482	6.2	27/486	5.6	6.6	5.2
Africa	2/58	3.4	1/58	1.7	2/58	1.0	0/58	0.0	0/58	0.0	0.0	0.0
Asia	1/99	1.0	0/99	0.0	1/99	6.5	0/98	0.0	0/99	0.0	0.0	0.0
Oropharynx *†												
Europe	183/810	22.6	157/810	19.4	181/810	22.3	157/803	19.6	131/805	16.3	19.9	15.9
Central-South America	68/158	43.0	51/158	32.3	65/158	41.1	60/153	39.2	58/157	36.9	40.5	35.5
Asia	21/103	20.4	20/103	19.4	21/103	20.4	18/103	17.5	18/103	17.5	18.5	16.5
Larynx *												
Europe	25/505	5.0	17/505	3.4	24/505	4.8	11/504	2.2	7/505	1.4	2.4	1.2
Central-South America	30/359	8.4	13/359	3.6	26/359	7.2	19/354	5.4	13/359	3.6	6.1	2.8
Africa	5/73	6.8	2/73	2.7	4/73	5.5	2/71	2.8	0/73	0.0	2.7	0.0
Asia	1/71	1.4	0/71	0.0	0/71	0.0	0/70	0.0	0/71	0.0	0.0	0.0

	HPV-DNA prevalence		HPV16		Any HR HPV types		E6*I mRNA		$p16^{INK4a}$		HPV attributable fractions (%)	
	+/HPV-DNA tested	%	+/HPV-DNA tested	%	+/HPV-DNA tested	%	+/HPV-DNA &mRNA tested	%	+/HPV-DNA &p16 tested	%	HPV-DNA+ AND (mRNA+ OR p16+)	HPV-DNA+ AND mRNA+ AND p16+
Gender												
Oral cavity												
Male	58/781	7.4	43/781	5.5	50/781	6.4	32/772	4.2	30/781	3.8	4.7	3.2
Female	35/471	7.4	23/471	4.9	32/471	6.8	17/467	3.6	14/469	3.0	3.8	2.8
Oropharynx												
Male	198/884	22.4	168/884	19.0	194/884	21.9	170/876	19.4	147/880	16.7	19.9	16.2
Female	72/178	40.4	57/178	32.0	71/178	39.9	65/174	37.4	60/177	33.9	38.4	32.8
Larynx												
Male	47/929	5.1	26/929	2.8	42/929	4.5	23/922	2.5	14/929	1.5	2.8	1.2
Female	14/109	12.8	6/109	5.5	12/109	11.0	9/107	8.4	6/109	5.5	9.2	4.7
Year of diagnosis												
Oral cavity												
1990-1994	0/35	0.0	0/35	0.0	0/35	0.0	0/35	0.0	0/35	0.0	0.0	0.0
1995-1999	5/66	7.6	4/66	6.1	5/66	7.6	3/66	4.5	3/66	4.5	4.5	4.5
2000-2004	12/152	7.9	9/152	5.9	10/152	6.6	5/149	3.4	5/152	3.3	3.3	3.4
2005-2009	60/693	8.7	45/693	6.5	56/693	8.1	35/689	5.1	28/691	4.1	5.5	3.6

		HPV-DNA prevalence		HPV16		Any HR HPV types		E6*I mRNA		$p16^{INK4a}$		HPV attributable fractions (%)	
		+/HPV-DNA tested	%	+/HPV-DNA tested	%	+/HPV-DNA tested	%	+/HPV-DNA &mRNA tested	%	+/HPV-DNA &p16 tested	%	HPV-DNA+ AND (mRNA+ OR p16+)	HPV-DNA+ AND mRNA+ AND p16+
	2010-2012	16/318	5.0	8/318	2.5	11/318	3.5	6/312	1.9	8/318	2.5	2.8	1.6
Oropharynx													
	1990-1994	9/83	10.8	5/83	6.0	8/83	9.6	6/81	7.4	4/83	4.8	7.2	4.9
	1995-1999	13/129	10.1	11/129	8.5	13/129	10.1	13/129	10.1	12/129	9.3	10.1	9.3
	2000-2004	48/226	21.2	42/226	18.6	48/226	21.2	39/224	17.4	39/224	17.4	18.7	16.1
	2005-2009	136/455	29.9	118/455	25.9	132/455	29.0	114/447	25.5	96/452	21.2	26.1	20.6
	2010-2012	67/197	34.0	52/197	26.4	67/197	34.0	63/196	32.1	56/196	28.6	32.7	28.1
Larynx													
	1990-1994	0/18	0.0	0/18	0.0	0/18	0.0	0/18	0.0	0/18	0.0	0.0	0.0
	1995-1999	0/26	0.0	0/26	0.0	0/26	0.0	0/26	0.0	0/26	0.0	0.0	0.0
	2000-2004	9/156	5.8	4/156	2.6	7/156	4.5	4/153	2.6	4/156	2.6	3.2	2.0
	2005-2009	36/542	6.6	20/542	3.7	33/542	6.1	19/539	3.5	11/542	2.0	4.1	1.5
	2010-2012	16/300	5.3	8/300	2.7	14/300	4.7	9/297	3.0	5/300	1.7	3.0	1.7
Age at diagnosis													
Oral cavity													
	≤53	27/336	8.0	16/336	4.8	23/336	6.8	14/332	4.2	15/335	4.5	5.1	3.6

		HPV-DNA prevalence		HPV16		Any HR HPV types		E6*I mRNA		$p16^{INK4a}$		HPV attributable fractions (%)	
		+/HPV-DNA tested	%	+/HPV-DNA tested	%	+/HPV-DNA tested	%	+/HPV-DNA &mRNA tested	%	+/HPV-DNA &p16 tested	%	HPV-DNA+ AND (mRNA+ OR p16+)	HPV-DNA+ AND mRNA+ AND p16+
	54-61	16/256	6.3	13/256	5.1	14/256	5.5	10/252	4.0	9/255	3.5	4.3	3.2
	62-70	21/273	7.7	18/273	6.6	21/273	7.7	10/273	3.7	10/273	3.7	4.4	2.9
	≥71	25/313	8.0	15/313	4.8	20/313	6.4	12/308	3.9	8/313	2.6	3.8	2.6
Oropharynx													
	≤53	93/273	34.1	80/273	29.3	92/273	33.7	84/270	31.1	74/272	27.2	32.4	25.9
	54-61	81/293	27.6	70/293	23.9	79/293	27.0	72/290	24.8	63/292	21.6	25.0	21.4
	62-70	55/285	19.3	40/285	14.0	54/285	18.9	47/282	16.7	43/284	15.1	17.3	14.5
	≥71	41/205	20.0	36/205	17.6	40/205	19.5	32/203	15.8	27/203	13.3	16.2	12.9
Larynx													
	≤53	24/210	11.4	13/210	6.2	22/210	10.5	13/207	6.3	8/210	3.8	7.1	2.9
	54-61	15/293	5.1	7/293	2.4	13/293	4.4	8/291	2.8	7/293	2.4	3.1	2.1
	62-70	10/287	3.5	2/287	2.4	10/287	3.5	4/287	1.4	3/287	1.1	1.7	0.7
	≥71	10/214	4.7	5/214	2.3	9/214	4.2	7/212	3.3	2/214	0.9	3.3	0.9

* Excludes North America because of low number of cases tested (<45).

† Excludes Africa because of low number of cases tested (<45).

"HR": High Risk types; Risk groups are defined according to the last IARC classification: we considered as high risk HPV types those included in Group 1, Group 2A and Group 2B; other HPV types were classified as low risk HPV types [27]; Any HPV16 or any HPV HR types found in either single or multiple infections are included in the corresponding columns.

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Figures legends

Figure 1. Samples disposition and testing for HPV-related biomarkers. (*) Excludes samples that were too hemorrhagic or necrotic for appropriate assessment or processing. (†) Includes both cases that were HPV-DNA positive and cases that were HPV-DNA negative but tubulin positive; (‡) For E6*I mRNA, includes cases with available material that tested positive for an HPV type for which the type-specific mRNA detection assay was available; For immunohistochemistry assays, includes cases with available material. “H&E”: Haematoxylin and Eosin; “Uns.”: Unspecified.

Figure 2. HPV attributable fractions in head and neck cancers according to positivity and/or overexpression of selected biomarkers of HPV induced carcinogenesis. “n”: Number of positive cases; “N”: Number of tested cases for the specified markers; “CI”: 95% confidence interval; “Uns.”: Unspecified .

Figure 3. HPV attributable fractions in head and neck cancers by subregión according to positivity and/or overexpression of selected biomarkers of HPV induced carcinogenesis. (*) Excludes North America and Eastern-Southern Asia because of low number of cases tested (<45). (†) Excludes Western Africa, Northern America, Central-Southern Asia, and South-Eastern Asia because of low number of cases tested (<45). (‡) Excludes North America, Central-Southern Asia, Eastern Asia , and Western Asia because of low number of cases tested (<45). “AF”: attributable fraction.

Appendix

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