

## HPV involvement in head and neck cancers

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DOI:

[10.1093/jnci/djv403](https://doi.org/10.1093/jnci/djv403)

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Peer reviewed version

*Citation for published version (Harvard):*

ICO International HPV in Head and Neck Cancer Study Group & Rapozo, D 2016, 'HPV involvement in head and neck cancers: comprehensive assessment of biomarkers in 3,680 cases', *Journal of the National Cancer Institute*, vol. 108, no. 6. <https://doi.org/10.1093/jnci/djv403>

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This is a pre-copy-editing, author-produced PDF of an article accepted for publication in *Journal of the National Cancer Institute* following peer review. The definitive publisher-authenticated version Castellsagué et al. HPV Involvement in Head and Neck Cancers: Comprehensive Assessment of Biomarkers in 3680 Patients *JNCI J Natl Cancer Inst* (2016) 108 (6): djv403 doi:10.1093/jnci/djv403. First published online January 28, 2016 (12 pages) is available online at: [10.1093/jnci/djv403](https://doi.org/10.1093/jnci/djv403)

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

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93 *Study* is provided in Appendix at the end of the manuscript.

94

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102

103 Abstract word count: 255

104 Manuscript word count: 3,948

105 Tables: 7 (3 main, 4 supplementary)

106 Figures: 3

107

108 KEYWORDS: Human Papillomavirus, HPV, head and neck cancer, oral cancer, oropharyngeal  
109 cancer, laryngeal cancer, mRNA, p16, etiology, epidemiology, attributable fraction

110

111 RUNNING TITLE: HPV in head and neck cancers

112

## 113 **ABSTRACT**

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114

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

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

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

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



## 137 INTRODUCTION

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138

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

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

155



## 156 **METHODS**

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157

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

164

### 165         **Selection of HNC cases and control tissue**

166         HNC samples were selected from an international network of pathology departments  
167 identified in 44 centers in 29 countries in Europe, Africa, Asia, and America. Participating centers  
168 were requested to provide samples using a common protocol for sample selection, retrieval,  
169 processing, and shipping to ICO. Selected cancer cases had to fulfill pre-established inclusion  
170 criteria: to be diagnosed with primary invasive cancer of the oral cavity (OC), pharynx or larynx  
171 under specific codes of the International Classification of Diseases version 10 (ICD-10); to have  
172 complete data on year of diagnosis and site of the tumor; and to be selected in a consecutive or  
173 random manner from 1990 onwards. Centers were asked to contribute if possible a minimum of  
174 50 samples per major anatomic HNC site. In order to assess potential carry over contamination  
175 at the local level we additionally requested tissue samples of patients with non-HPV related  
176 diagnoses processed in the same laboratory and close to the cases' diagnosis time. Cancers of  
177 the salivary glands, nasopharynx and external lip were initially not requested since they are a  
178 *priori* not considered to be related to HPVs. Nevertheless, we included a series of  
179 nasopharyngeal cancers from Europe (n=37), America (n=8), Africa (n=35) and Asia (n=21).

180 Based on previously published site-specific estimates of HPV-DNA prevalence<sup>9</sup>, optimal  
181 sample size was set at around 1,000 cases per major cancer site in order to obtain HPV-DNA  
182 prevalence estimates with a  $\pm 2.5\%$  precision.

183

#### 184 **FFPE blocks processing and histopathological evaluation**

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

200

201

#### 202 **HPV-DNA detection and genotyping**

203 The detailed methods used for HPV-DNA detection and genotyping have been reported  
204 elsewhere in a similar study on cervical cancer specimens<sup>10</sup>. Briefly, we used SPF-10 PCR and  
205 a DNA enzyme immunoassay (DEIA) to test for the presence of HPV-DNA. Virus genotyping  
206 was performed using reverse hybridization line probe assay (LiPA25\_v1) on all samples testing  
207 positive for viral DNA, targeting 25 HPV types with different oncogenic potential. Specimens  
208 testing positive for HPV-DNA by DEIA but that could not be typed by LiPA25 were further  
209 analyzed by direct Sanger sequencing of PCR products<sup>12</sup>. HPV-DNA positive cases that could  
210 not be sequenced were labeled as "HPV undetermined". DNA quality was evaluated in all HPV-  
211 DNA negative samples by testing for the human tubulin gene<sup>13</sup>. All DEIA and LiPA25\_v1 assays  
212 were performed at ICO. These assays were quality controlled and validated against an external  
213 HPV reference lab (DDL Diagnostic Laboratory, Rijswijk, The Netherlands) by cross-testing of  
214 387 anogenital and head and cancer samples with overall percentage agreements and Kappa  
215 values of 92.8% (95% CI 89.7-95.1) and 0.78 (95% CI, 0.71-0.86), respectively, for DEIA, and  
216 91.2% (95% CI 87.9-93.8) and 0.74 (95% CI 0.66-0.82), respectively, for HPV genotyping.

217

#### 218 **HPV E6\*I mRNA detection**

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

225

## 226 **Immunohistochemistry**

227 Protein expression patterns were evaluated for p16<sup>INK4a</sup>, pRb, p53, and Cyclin D1 in all  
228 HPV-DNA positive samples, and in a random selection of HPV-DNA negative cases in a ratio of  
229 1:1, corresponding approximately to 12% of the negative cases. Stainings were all performed at  
230 Hospital General de L'Hospitalet, L'Hospitalet de Llobregat, Spain, under the manufacturer's  
231 standards: Roche mtm Laboratories AG (Heidelberg) for p16<sup>INK4a</sup>, Vision Biosystems Novocastra  
232 (Newcastle) for pRb, and Dako (Denmark) for p53 and Cyclin D1. We used the predefined  
233 algorithm developed by Halec and colleagues<sup>15</sup> to determine the cut-off values for over- versus  
234 under-expression of each protein. The expected pattern for HPV-driven cases was over-  
235 expression of p16<sup>INK4a</sup> and under-expression of the other three markers.

236

## 237 **Statistical analyses**

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

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

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

257 We performed sensitivity analyses for p16<sup>INK4a</sup> positivity according to three different cutoff  
258 points of percentage of stained cells: >25%, >50%, and >75% (Supplementary Table 2). Since  
259 there were no statistically significant differences in the estimates across the three cutoff values,  
260 and for consistency sake, we used the >25% cutoff as used by Halec et al.<sup>15</sup>. For the  
261 geographical analyses, countries were grouped into world subregions according to the Globocan  
262 classification<sup>18</sup>. All statistical tests were two-sided and statistical significance was set at a P  
263 value of less than .05. All analyses were performed with STATA version 10.1 (StataCorp.  
264 2007. Stata Statistical Software: Release 10. College Station, TX: StataCorp LP)

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266

## 267 RESULTS

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268

269 Figure 1 depicts the disposition of HNC samples collected, processed and finally tested.  
270 The laboratory at ICO received a total of 4,533 samples of which 4,022 were tested for HPV-  
271 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

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

302 **Figure 2** presents estimated HPV-AFs using HPV-DNA, HPV E6\*I mRNA detection and  
303 over-expression of p16<sup>INK4a</sup>. Ranges of AFs when considering HPV-DNA plus E6\*I mRNA and/or  
304 p16<sup>INK4a</sup> 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  
305 larynx. Corresponding values when considering positivity by both HPV-DNA and E6\*I mRNA  
306 were 21.8%, 3.9%, and 3.1%, respectively. Full results by cancer subsite are provided in  
307 **Supplementary Table 3**. We observed that within both the oral cavity and the larynx, those  
308 subsites that were more proximal to the oropharynx showed higher HPV-AFs than those that  
309 were more distal to the oropharynx. Thus, HPV-AFs in combined oral cavity subsites that were  
310 proximal to the oropharynx ranged (when considering HPV-DNA plus E6\*I mRNA and/or  
311 p16<sup>INK4a</sup>) from 4.9% to 6.7%, as opposed to 1.4-2.3% in subsites that were distal to the  
312 oropharynx (p<0.001 for both comparisons). Corresponding values in the larynx were 4.2-4.2%  
313 versus 1.4-3.4% in combined subsites that were proximal versus distal to the oropharynx, but  
314 these differences were not statistically significant.

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

320 subregions -Central-Eastern Europe (61.5% versus 45.5%,  $p=0.09$ ), Southern-Europe (22.6%  
321 versus 8.4%,  $p=.002$ ), Western Europe (38.9% versus 13%,  $p=.02$ )-, except Northern Europe  
322 (50% versus 50%). HPV-AFs were also higher in women as compared with men for cancers of  
323 the larynx in South America (23.1% versus 4.2%,  $p<.0001$ ), as well as in Southern Europe (5.9%  
324 versus 0.5%,  $p=.03$ ). In contrast, in the oral cavity we found higher HPV-AFs in men than in  
325 women, but only in Northern Europe (10.9% versus 0%,  $p=.01$ ). We did not identify a clear  
326 pattern of gender differences by calendar period within regions showing gender differences in  
327 HPV-AF estimates (data not shown). An inverse trend was observed between HPV-AFs and  
328 increasing age at diagnosis for each major site. Concerning time trends, HPV-AFs for the  
329 oropharynx clearly increased over time: 7.2%, 10.1%, 18.7%, 26.1% and 32.7% for calendar  
330 periods 1990-1994, 1995-1999, 2000-2004, 2005-2009, and 2010-2012, respectively. In  
331 contrast, no trends were observed for the other two major HNC sites.

332 HPV-AFs showed a marked geographic heterogeneity that was particularly evident for  
333 oropharyngeal cancer (Figure 3). For the oropharynx, AF estimates when considering HPV-DNA  
334 plus E6\*I mRNA and/or p16<sup>INK4a</sup> were highest in South America (48.4%-53.6%), Central-Eastern  
335 Europe (44.9%-50%), and Northern Europe (25%-50%), and lowest in Southern Europe (7.6%-  
336 9.4%). For the oral cavity corresponding estimates were highest in South America (5.5%-7.3%),  
337 Northern Europe (4.2%-6.8%), and Central America (4.3%); and for the larynx, in South America  
338 (3.8%-6.5%), Central America (1.4%-5.6%), and Northern Europe (4.2%). Full results by  
339 geographic area are provided in Supplementary Table 4. Since the study was not powered to  
340 calculate precise country-specific estimates, AFs by country are not provided.

341

## 342 DISCUSSION

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343

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

360 The first observation when assessing these HPV-AFs is the marked heterogeneity across  
361 anatomic sites, being highest in the oropharynx, substantially lower in the OC, and even lower in  
362 the larynx. The probability of an HPV-driven OC cancer was between 4 and 7 times lower than  
363 that of oropharyngeal cancer; and that of an HPV-driven laryngeal cancer between 5 and 15  
364 times lower than that of oropharyngeal cancer. Even within a major site such as the oropharynx,  
365 AF estimates ranged from 4.0% in the posterior wall to 45.2% in the tonsil (**Supplementary Table**  
366 **3**). Being an oropharyngeal subsite, we found an unexpected low HPV-AF for cancers of the

367 base of tongue (between 8.7% and 17.4%), but also realized that most of these cases (68/92,  
368 74%) were from Spain, a country known to have low HPV-AFs for HNCs even for the oropharynx  
369 (6.7% to 8.6%). It is interesting to note that HPV-AFs for subsites within the oral cavity that were  
370 more proximal to the oropharynx were higher than those that were more distal from the  
371 oropharynx. Even though the same was observed for subsites in the larynx these differences did  
372 not reach statistical significance. This gradient of lower HPV involvement in more distant  
373 subsites from the oropharynx suggests either misclassification of anatomic subsite or a true  
374 biological gradient of HPV involvement.

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

383 As shown in **Table 3** we also found important heterogeneity of HPV-AF estimates by  
384 geographical region, gender and age at diagnosis. Estimates ranged from 0% in Africa or Asia to  
385 6.6% in Central-South America for OC; from 18.5% in Asia to 40.5% in Central-South America  
386 for the oropharynx; and from 0% in Asia to 6.1% in Central-South America for the larynx. Even  
387 within European sub-regions, wide variations were observed for each cancer site  
388 (**Supplementary Table 4**). Even though these estimates may seem low for some regions it is  
389 difficult to make fair comparisons as there are no large studies using several markers of HPV  
390 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<sup>22</sup>. 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<sup>5,23-26</sup>. 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.

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

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

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

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

453

## 454 **ACKNOWLEDGEMENTS AND FUNDING**

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455

456 Partial support has been obtained from Spanish public grants from the Instituto de Salud Carlos  
457 III (grants FIS PI081535, FIS PI1102096, FIS PI1102104, RCESP C03/09, RTICESP C03/10,  
458 RTIC RD06/0020/0095, RD12/0036/0056 and CIBERESP), from the Agència de Gestió d'Ajuts  
459 Universitaris i de Recerca (AGAUR 2005SGR 00695, 2009SGR126, 2014SGR1077,  
460 2014SGR2016), from the European Commission (7<sup>th</sup> Framework Programme Grant HEALTH-  
461 F2-2011-282562 HPV-AHEAD), from the Lilly Foundation (Premio de Investigación Biomédica  
462 Preclínica 2012 F. Xavier Bosch), from the Asociación Española Contra el Cáncer (personal  
463 grant LA); from the Stichting Pathologie Ontwikkeling en Onderzoek (SPOO) foundation (The  
464 Netherlands); and from Sanofi Pasteur MSD and Merck & Co, Inc, who had no role in the  
465 design, analyses, interpretation and presentation of results.

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467

## 468 **CONFLICTS OF INTEREST**

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469

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

473 Hisham Mehanna: received research funding and speaker fees from GSK Biologicals and  
474 SPMSD

475 Nubia Muñoz: member of the HPV Global Advisory Board of Merck.

476 F. Xavier Bosch: received institutional research funding from Merck, SPMSD, GSK Biologicals,  
477 Qiagen, Roche and Genticel, occasional speaker fees from Merck, GSK, Roche and SPMSD,  
478 and occasional travel grants from Merck, GSK and SPMSD

479 Silvia de Sanjosé: received institutional research funding from Merck, SPMSD, GSK Biologicals,  
480 and Qiagen, and occasional travel assistance from Merck.

481 Laia Alemany: received institutional research funding from Merck and SPMSD, and occasional  
482 travel grants from Merck and SPMSD

483 Michael Pawlita: received institutional research funding from Roche and Qiagen

484

485 The following authors declare no conflict of interest:

486 Oluseyi F. Ajayi, Estela I. Albanesi, Maria Alejo, Llúcia Alòs, Isabel Alvarado-Cabrero, Maria  
487 Benevolo, Thorsten Biegner, Ignacio G. Bravo, Enrique Cadena, Edith Claros, Omar Clavero,  
488 Valérie Costes, Pablo Dabed, Flora Duarte, Marco A. Duran-Padilla, Ana Félix, Falco Fend,  
489 Annabelle Ferrera, Marcial Garcia-Rojo, Maria Julieta V. Germar, Manuel E. González, Marc  
490 Goodman, Núria Guimerà, Gordana Halec, Gillian Hall, Dana Holzinger, Ermina Iljazovic, Asha  
491 Jain, Chang-Suk Kang, Elena Kasamatsu, Jan Laco, Xavier León, Belen Lloveras, Luis Estuardo  
492 Lombardi, Rubén López-Revilla, Sani Malami, Václav Mandys, Ravi Mehrotra, Marisa Mena,  
493 Carla Molina, Hesler Morales, Cathy Ndiaye, Ashrafun Nessa, Jin-Kyoung Oh, Edyta C. Pirog,  
494 Mario Poljak, Miquel Quer, Wim Quint, Beatriz Quirós, Arzu Ruacan, Juan C. Ruiz-Cabezas,  
495 Tomasz Szafarowski, Leopoldo Tinoco, Sara Tous, Julio Velasco, and Halina Viarheichyk

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

575

	Oral cavity		Nasopharynx		Oropharynx		Hypopharynx		Pharynx unspecified		Larynx	
	n=1,264		n=101		n=1,090		n=127		n=56		n=1,042	
	n	%	n	%	n	%	n	%	n	%	n	%
<b>Geographical origin</b>												
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
<b>Gender</b>												
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	-
<b>Year of diagnosis</b>												
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	
<b>Age at diagnosis</b>													
	≤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	
<b>Histological diagnosis</b>													
	<i>Squamous Cell Carcinoma</i>	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
	<i>Undifferentiated Carcinoma</i>	2	0.2	6	5.9	5	0.5	3	2.4	1	1.8	8	0.8
	<i>Adenosquamous carcinoma</i>	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.

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578

579 Table 2. HPV-DNA positivity and detected types, and E6\*I mRNA, p16<sup>INK4a</sup>, pRb, p53 and Cyclin D1 results among HPV DNA positive cases, by head and neck cancer site

580

	Oral cavity		Nasopharynx		Oropharynx		Hypopharynx		Pharynx unspecified		Larynx	
	n=1,264		n=101		n=1,090		n=127		n=56		n=1,042	
	n	%	n	%	n	%	n	%	n	%	n	%
<b>HPV DNA positivity †</b>	93	7.4	9	8.9	273	25.0	5	3.9	12	21.4	61	5.9
<b>Type of HPV infection †</b>												
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
<b>HPV type distribution in single infection<sup>2</sup></b>												
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 <sup>INK4a</sup> +	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- <sup>**</sup>	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- <sup>**</sup>	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- <sup>**</sup>	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 <sup>INK4a</sup> +	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 <sup>INK4a</sup> +	38/78	48.7	1/3	33.3	199/259	76.8	3/5	60.0	4/9	44.4	16/52	30.8

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

582 † Percentages among HPV-DNA positive cases.

583 ‡ 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).

584 § HPV undetermined denotes cases that were DEIA positive but line probe assay LiPA<sub>25</sub> negative.

585 || Genotype identified by sequencing.

586 ¶ 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  
587 included in Group 1, Group 2A and Group 2B; other HPV types were classified as low risk HPV types<sup>27</sup>.

588 # 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  
589 mRNA, over-expression of p16<sup>INK4a</sup> and under-expression of pRb, p53, or Cyclin D1.

590 \*\* Under-expression

591



592  
593**Table 3. Prevalence of HPV-DNA, HPV types, E6\*1 mRNA and p16<sup>INK4a</sup>, and estimates of HPV attributable fractions by head and neck cancer site and key patients' characteristics**

	HPV-DNA prevalence		HPV16		Any HR HPV types		E6*1 mRNA		p16 <sup>INK4a</sup>		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+
<b>Geographical origin</b>												
<i>Oral cavity</i> *												
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
<i>Oropharynx</i> *†												
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
<i>Larynx</i> *												
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 <sup>INK4a</sup>		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+
<b>Gender</b>												
<i>Oral cavity</i>												
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
<i>Oropharynx</i>												
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
<i>Larynx</i>												
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
<b>Year of diagnosis</b>												
<i>Oral cavity</i>												
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 <sup>INK4a</sup>		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
<b>Oropharynx</b>												
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
<b>Larynx</b>												
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
<b>Age at diagnosis</b>												
<b>Oral cavity</b>												
≤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 <sup>INK4a</sup>		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
<b>Oropharynx</b>												
≤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
<b>Larynx</b>												
≤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

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

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

596 "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  
 597 classified as low risk HPV types<sup>27</sup>; Any HPV16 or any HPV HR types found in either single or multiple infections are included in the corresponding columns.

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600

## Figures legends

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

608

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

613

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

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**Appendix**

624

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