

HPV involvement in head and neck cancers

ICO International HPV in Head and Neck Cancer Study Group; Rapozo, Davy

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

Xavier Castellsagué, M.D.* (1,2), Laia Alemany, M.D.* (1,2), Miquel Quer, M.D. (3), Gordana Halec, Ph.D. (4), Beatriz Quirós, B.Sc (1), Sara Tous, B.Sc. (1), Omar Clavero, M.D. (1), Lúcia Alòs, M.D. (5), Thorsten Biegner, M.D. (6), Tomasz Szafarowski, M.D. (7), Maria Alejo, M.D. (8), Dana Holzinger, Ph.D. (4), Enrique Cadena, M.D.(9), Edith Claros, M.D. (10), Gillian Hall, FRCPATH (11), Jan Laco, M.D. (12), Mario Poljak, M.D. (13), Maria Benevolo, Ph.D. (14), Elena Kasamatsu, M.D. (15), Hisham Mehanna, M.D. (16), Cathy Ndiaye, Ph.D. (17), Núria Guimerà, Ph.D. (18), Belen Lloveras, M.D. (19), Xavier León, M.D. (3), Juan C. Ruiz-Cabezas, M.D. (20), Isabel Alvarado-Cabrero, M.D. (21), Chang-Suk Kang, M.D.(22), Jin-Kyoung Oh, Ph.D. (23), Marcial Garcia-Rojo, M.D. (24), Ermina Iljazovic, M.D. (25), Oluseyi F. Ajayi, FMCDs (26), Flora Duarte, M.D. (27), Ashrafun Nessa, Ph.D. (28), Leopoldo Tinoco, M.D. (29), Marco A. Duran-Padilla, M.D. (30), Edyta C. Pirog, M.D. (31), Halina Viarheichyk, M.D. (32), Hesler Morales, M.D. (33), Valérie Costes, M.D. (34), Ana Félix, M.D. (35), Maria Julieta V. Germar, M.D. (36), Marisa Mena, Ph.D. (1), Arzu Ruacan, M.D. (37,38), Asha Jain, M.D. (39), Ravi Mehrotra, M.D. (40,41), Marc T. Goodman, Ph.D. (42,43), Luis Estuardo Lombardi, M.D. (44), Annabelle Ferrera, Ph.D. (45), Sani Malami, M.D. (46), Estela I. Albanesi, M.Sc. (47), Pablo Dabed, M.D. (48), Carla Molina, M.D. (49), Rubén López-Revilla, M.D. (50), Václav Mandys, M.D. (51), Manuel E. González, M.D. (52), Julio Velasco, M.D. (53), Ignacio G. Bravo, Ph.D. (1), Wim Quint, Ph.D. (18), Michael Pawlita, M.D. (4), Nubia Muñoz, M.D. (9), Silvia de Sanjosé, M.D.(1,2), F. Xavier Bosch, M.D. (1) on behalf of the *ICO International HPV in Head and Neck Cancer Study Group*[†].

24

25 (1) Cancer Epidemiology Research Program, Catalan Institute of Oncology (ICO), IDIBELL,
26 L'Hospitalet de Llobregat, Catalonia (Spain)

27 (2) CIBER Epidemiología y Salud Pública (CIBERESP), Madrid, Spain

28 (3) Hospital Sant Pau, Barcelona, Spain

29 (4) German Cancer Research Center (DKFZ), Heidelberg, Germany

30 (5) Hospital Clínic, University of Barcelona, Spain

31 (6) Department of Pathology, University Hospital Tuebingen, Tuebingen, Germany

32 (7) Medical University of Warsaw, Warsaw, Poland

33 (8) Hospital General de l'Hospitalet, L'Hospitalet de Llobregat, Barcelona, Spain

34 (9) Instituto Nacional de Cancerología, Bogotá, Colombia

- 35 (10) Insituto Oncológico del Oriente Boliviano (IOOB), Laboratorio Privado de Patología Oncos,
36 Santa Cruz, Bolivia
- 37 (11) Manchester Royal Infirmary, Manchester, United Kingdom
- 38 (12) The Fingerland Department of Pathology, Charles University Faculty of Medicine and
39 University Hospital, Hradec Kralove, Czech Republic
- 40 (13) University of Ljubljana, Faculty of Medicine, Institute of Microbiology and Immunology,
41 Ljubljana, Slovenia
- 42 (14) Regina Elena National Cancer Institute, Rome, Italy
- 43 (15) Instituto de Investigaciones en Ciencias de la Salud, Universidad Nacional de Asunción,
44 Asunción, Paraguay
- 45 (16) Institute of Head and Neck Studies and Education, University of Birmingham, Birmingham,
46 United Kingdom
- 47 (17) Department of Social and Preventive Medicine, Université de Montréal, Montreal, Canada
- 48 (18) DDL Diagnostic Laboratory, Rijswijk, The Netherlands
- 49 (19) Pathology Department, Hospital del Mar IMIM, Universitat Pompeu Fabra, Barcelona, Spain
- 50 (20) Laboratorio Clínico y Molecular, Hospital SOLCA, Guayaquil, Ecuador
- 51 (21) Hospital de Oncología Centro Médico Nacional Siglo XXI, IMSS Mexico, D.F, Mexico
- 52 (22) Yeouido St. Mary's Hospital, The Catholic University of Korea, Seoul, Republic of Korea
- 53 (23) Graduate School of Cancer Science and Policy, and National Cancer Control Institute,
54 National Cancer Center, Goyang, Republic of Korea
- 55 (24) Hospital General de Jerez de la Frontera, Cádiz, Spain
- 56 (25) Pathology Department, University Clinical Center, Tuzla, Bosnia & Herzegovina
- 57 (26) Department of Oral Pathology, College of Medicine, University of Lagos, Lagos, Nigeria
- 58 (27) Centro de Cáncer Emma Romero de Callejas, Tegucigalpa, Honduras
- 59 (28) Department of Obstetrics and Gynecology, Bangabandhu Sheikh Mujib Medical University,
60 Shahbag, Dhaka, Bangladesh
- 61 (29) Hospital Oncológico SOLCA, Quito, Ecuador
- 62 (30) Pathology Department, Hospital General de Mexico, Mexico D.F., Mexico

- 63 (31) New York Hospital - Cornell Medical Centre, New York, United States of America
- 64 (32) Gomel State Medical University, Gomel, Belarus
- 65 (33) Instituto de Cancerología "Dr. Bernardo del Valle S." Guatemala, Guatemala
- 66 (34) CHRU Gui De Chauliac, Montpellier, France
- 67 (35) Instituto Portugues de Oncologia de Lisboa Francisco Gentil, Lisboa, Portugal
- 68 (36) University of the Philippines College of Medicine, Manila, Philippines
- 69 (37) Koç University School of Medicine, Istanbul, Turkey
- 70 (38) Hacettepe University School of Medicine, Ankara, Turkey
- 71 (39) Cancer Prevention & Relief Society, Raipur, India
- 72 (40) M.L.N Medical College, Allahabad, India
- 73 (41) Institute of Cytology and Preventive Oncology (ICMR), Noida, Uttar Pradesh, India
- 74 (42) Cancer Research Center, University of Hawaii, Hawaii, USA
- 75 (43) Cedars Sinai Medical Center, Los Angeles, California, USA
- 76 (44) Centro de Investigación Epidemiológica en Salud Sexual y Reproductiva - CIESAR,
77 Hospital General San Juan de Dios, Guatemala, Guatemala.
- 78 (45) Escuela de Microbiología, Universidad Nacional Autónoma de Honduras, Tegucigalpa,
79 Honduras
- 80 (46) Bayero University, Kano, Nigeria
- 81 (47) Ministerio de Salud de la provincia de Buenos Aires, Buenos Aires, Argentina
- 82 (48) Universidad Central de Venezuela, Caracas, Venezuela
- 83 (49) Centro de Oncología Preventiva, Universidad de Chile, Santiago, Chile
- 84 (50) Instituto Potosino de Investigación Científica y Tecnológica (IPICYT), San Luis Potosí,
85 Mexico
- 86 (51) Third Faculty of Medicine, Charles University and Faculty Hospital King's Vineyards,
87 Prague, Czech Republic
- 88 (52) IMAT Oncomédica, Montería, Colombia
- 89 (53) Hospital San Agustín, Asturias, Spain

90

91 *Indicates co-first authors

92 † A complete list of the investigators for the *ICO International HPV in Head and Neck Cancer*
93 *Study* is provided in Appendix at the end of the manuscript.

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95 Correspondence to: Xavier Castellsagué, MD, MPH, Ph.D.

96 Cancer Epidemiology Research Program

97 Catalan Institute of Oncology, Institut Català d'Oncologia (ICO)

98 Avda. Gran Via 199-203

99 08908 L'Hospitalet de Llobregat, Catalonia (Spain).

100 Tel.: (+34) 93 260-7812. FAX: (+34) 93 260-7787

101 E-mail: xcastellsague@iconcologia.net

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112

113 **ABSTRACT**

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^{INK4a}, 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^{INK4a} 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^{INK4a} 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

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)¹. While virtually all cervical cancers are considered HPV driven² 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 use³⁻⁵. 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 challenge⁶. 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^{7,8}. 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**

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 prevalence⁹, 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 published¹⁰. 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 HNCs¹¹.

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 specimens¹⁰. 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 products¹². 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 gene¹³. 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 colleagues¹⁴. 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^{INK4a}, 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^{INK4a}, 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 colleagues¹⁵ 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^{INK4a} 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 authors¹⁵⁻¹⁷, 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*I mRNA),
244 and surrogate markers of HPV-related cellular transformation (p16^{INK4a}, 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^{INK4a} 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^{INK4a} 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^{INK4a} 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.¹⁵. For the
261 geographical analyses, countries were grouped into world subregions according to the Globocan
262 classification¹⁸. 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)

265

266

267 RESULTS

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^{INK4a} 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^{INK4a}. Ranges of AFs when considering HPV-DNA plus E6*I mRNA and/or
304 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
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^{INK4a}) 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^{INK4a} 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**

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^{5,19}. 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^{14,20}. 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^{INK4a} 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^{INK4a} in certain HPV-DNA+/mRNA+ samples. The loss
358 of p16^{INK4a} in these cancers might be a result of increasing genetic and epigenetic chromosomal
359 instability induced by HPV oncoproteins.²⁰

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 HNCs⁸: 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%²¹.

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 cancers²². 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.

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

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466

467

468 **CONFLICTS OF INTEREST**

469

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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,
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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	%
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													
	<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

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

577

578

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		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	%
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 infection²												
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

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 LiPA₂₅ 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 types²⁷.

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^{INK4a} 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^{INK4a}, 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 ^{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												
<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 ^{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												
<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
Year of diagnosis												
<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 ^{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

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 types²⁷; Any HPV16 or any HPV HR types found in either single or multiple infections are included in the corresponding columns.

598

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*I 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.

621

622

623

Appendix

624

Members of the ICO International HPV in Head and Neck Cancer Study Group

625 Argentina: Estela Irene Albanesi (Ministerio de Salud de la Provincia de Buenos Aires); Virginia Estrada,
626 Sandra Erzi (Hospital San Juan de Dios); Susana Miciquevich (Facultad de Odontología de la UNLP);

627

628 Bangladesh: Ashrafun Nessa, AJE Nahar Rahman, Mohammed Kamal (Bangabandhu Sheikh Mujib
629 Medical University – BSMMU); Faruk Ahmed (Dhaka Medical College Hospital);

630

631 Belarus: Halina Viarheichyk, Sitnikov Valeriy (Gomel State Medical University); Achynovich Searhei
632 (Gomel Regional Clinical Oncological Hospital);

633

634 Bolivia: Edith Claros, Pedro A. Vega, Jannette Mollinedo (Servicio de Patología Instituto Oncológico del
635 Oriente Biliviano (IOOB); Laboratorio Privado de Patología Oncos); Pablo Sitic Vargas, Lily Marcela
636 Márquez (Servicio de Patología Instituto Oncológico del Oriente Biliviano (IOOB));

637

638 Bosnia & Herzegovina: Ermina Iljazovic (Pathology Department; University Clinical Center Tuzla; BiH);

639

640 Chile: Rodrigo Prado, Carla Molina, Rosa Muñoz (Centro de Oncología Preventiva, Facultad de Medicina,
641 Universidad de Chile); Ximena Rodriguez, Marisol Guerrero, Virginia Leiva, Elsa Olave, Claudia Ramis,
642 Viviana Toro (Hospital de San José);

643

644 Colombia: Enrique Cadena, Raúl Murillo, Gustavo Adolfo Hernández Suárez, Carlos Eduardo Pinzón
645 (Instituto Nacional de Cancerología); Manuel Enrique González, Sandra Aruachan, Marcos Torregrossa
646 (Instituto Médico de Alta Tecnología, IMAT Oncomédica, Montería);

647

648 Czech Republic: Václav Mandys (3rd Faculty of Medicine, Charles University and University Hospital
649 King's Wineyards); Jan Laco (Charles University Faculty of Medicine and University Hospital Hradec
650 Kralove);

651

652 Ecuador: Leopoldo Tinoco (Hospital Oncológico Solca-Quito); Juan Carlos Ruíz Cabezas, Fernando
653 Camacho, Rina Quinto, Leyda Jaramillo (Laboratorio Clínico y Molecular, Hospital de SOLCA Matriz
654 Guayaquil);

655

656 France: Valerie Costes, Renaoud Garre, Anne Sophie Ramayl (Hospital Gui de Chauliac-Department de
657 Pathologie); Massimo Tommasino, Tarik Gheit (International Agency for Research on Cancer);

658

659 Germany: Michael Pawlita, Gordana Halec, Dana Holzinger (Department Genome Changes and
660 Carcinogenesis, Heildelberg); Falko Fend, Thorsten Biegner (Institute of Pathology and Neuropathology
661 and Comprehensive Cancer Center, University Hospital Tuebingen);

662

663 Guatemala: Edgar Kestler , Luis Estuardo Lombardi, Obdulia Salic, Sergio Marroquin, Víctor Argueta,
664 Sergio Marroquin (Centro de Investigación Epidemiológica en Salud Sexual y Reproductiva - CIESAR,
665 Hospital General San Juan de Dios), Walter Guerra (Instituto de Cancerología, INCA); Hesler Morales
666 Mérida, Marcos Mauricio Siliezar Tala (Instituto de Cancerologia "Dr. Bernardo del Valle S");

667

668 Honduras: Annabelle Ferrera (Escuela de Microbiología, Universidad Nacional Autónoma de Honduras);
669 Flora Duarte Muñoz, Marco Molinero, Luxely Toledo, Eda Sofía Calix, Nelly Sarai Padilla (Centro de
670 Cáncer Emma Romero de Callejas, Tegucigalpa, Honduras);

671

672 India: Asha Jain (Cancer Prevention and Relief Society Raipur); Sushil K Giri (Regional Cancer Center,
673 Cuttack); Maheep Bhalla (JLN Hospital & Research Center, BSP, Bhillai); Bharat Patel (Lab One Raipur);
674 PSA Sarma (BSP Hospital); Ravi Mehrotra (M.L.N Medical College, Allahabad; Institute of Cytology and
675 Preventive Oncology (ICMR), Uttar Pradesh); Mamta Singh (M.L.N Medical College, Allahabad);

676

677 Italy: Maria Benevolo, Renato Covello, Giuseppe Spriano, Barbara Pichi (Regina Elena National Cancer
678 Institute), Maria Gabriella Donà (San Gallicano Dermatologic Institute);

679

680 Korea: Hai-Rim Shin (Western Pacific Regional Office, World Health Organization); Jin-Kyoung Oh
681 (National Cancer Center); Jung-il Suh (National Medical Center); Seo-Hee Rha (Dong-A University
682 Hospital); Dong-chul Kim (Kangnam St. Mary's Hospital); Kang Chang-Suk (Yeouli St. Mary's Hospital);

683

684

685 Mexico: Isabel Alvarado-Cabrero (Instituto Mexicano del Seguro Social); Patricia Alonso Viveros
686 (Pathology Department of Hospital General de México; Instituto Nacional de salud Pública), Marco
687 Antonio Duran Padilla, Maria Esther Gutiérrez Díaz Ceballos (Pathology Department of Hospital General
688 de México; Faculty of Medicine Universidad Nacional Autónoma de México); Claudia Magaña-León,
689 Rubén López-Revilla (Instituto Potosino de Investigación Científica y Tecnológica, AC); Cuahtémoc Oros

690 (Hospital Central Ignacio Morones Prieto, San Luis Potosí); Marco Antonio Duran Padilla, Patricia Alonso
691 Viveros, María Esther Gutiérrez Díaz Ceballos (HGM Unidad 310 (Patología), Col Doctores, Mexico DF,
692 Delegación Cauhutémoc);

693

694 Nigeria: A.A.F. Banjo, F.B. Abdulkareem, A.O. Daramola, C.C. Anunobi, R.U. Anorlu (Lagos University
695 Teaching Hospital Idi-Araba); Oluseyi Folake Ajayi (Department of Oral Pathology, College of Medicine,
696 University of Lagos, Lagos, Nigeria); Sani Malami, Ali Bala Umar (Faculty of Medicine, Bayero University);

697

698 Paraguay: Elena Kasamatsu, Antonio Leopoldo Cubilla, Francisco Perrota, Susy Figueredo, (Instituto de
699 Investigaciones en Ciencias de la Salud, Universidad Nacional de Asunción);

700

701 Philippines: Efren Javier Domingo, Jericho Thaddeus P. Luna, Maria Julieta V. Germar, Arnold M.
702 Fernandez, Carolyn Zalameda Castro, Roslyn Balacuit (University of the Philippine College of Medicine
703 General Hospital);

704

705 Poland: Tomasz Szafarowski (Ent Department, Division of Medicine and Dentistry, Warsaw Medical
706 University, Czerniakowski Hospital);

707

708 Portugal: Ana Felix, Jorge Manuel Soares (Instituto Portugues de Oncologia de Lisboa Francisco Gentil);

709

710 Senegal: Cathy Ndiaye, Nafissatou Ndiaye Ba, Victorino Mendes (HOGGY stands for Hôpital Général de
711 Grand Yoff ; DANTEC - Hôpital A. Le Dantec; FAC - Faculté de Médecine - Université Cheikh A. Diop);

712

713 Slovenia: Mario Poljak, Boštjan J. Kocjan, Nina Gale, Polona J. Maver (University of Ljubljana, Faculty of
714 Medicine, Institute of Microbiology and Immunology, Ljubljana, Slovenia);

715

716 Spain: Belén Lloveras, Gemma Martín (Hospital del Mar); Llúcia Alós (Hospital Clínic, Universidad de
717 Barcelona); Julio Velasco (Hospital San Agustín); Maria Alejo, Anna Mulero (Hospital General de
718 l'Hospitalet); Xavier León, Miquel Quer (Hospital de Sant Pau); Marcial García-Rojo, Lucía González
719 López, Matías Cuesta Gil (Hospital General Universitario de Ciudad Real); Laia Alemany, Francesc Xavier
720 Bosch, Ignacio G. Bravo, Vanesa Camón, Xavier Castellsagué, Omar Clavero, Silvia de Sanjosé, Anna
721 Esteban, Yolanda Florencia, Joellen Klaustermeier, Marisa Mena, Nubia Muñoz, Beatriz Quirós, Cristina
722 Rajo, Sara Tous, Marleny Vergara (IDIBELL, Institut Català d'Oncologia-Catalan Institute of Oncology);

723

- 724 The Netherlands: Wim G.V. Quint, Anco C. Molijn, Daan T. Geraets, Núria Guimerà (DDL Diagnostic
725 Laboratory); Chris J.L.M Meijer (Vrije Universiteit Medical Center);
- 726
- 727 Turkey: Alp Usubutun, Arzu Ruacan (Hacettepe University); Arzu Ruacan (Koc University School of
728 Medicine)
- 729
- 730 UK: Henry Kitchener, Gillian Hall (School of Medicine, University of Manchester); Godfrey Wilson
731 (Manchester Royal Infirmary); Mehanna Hisham, Davy Rapozo (University of Birmingham), Whitmore
732 Shelley, James Sean (UHCW NHS Trust);
- 733
- 734 USA: Wendy Cozen, Brenda Y. Hernández, Charles Lynch, Daniel B. Olson, Freda R. Selk (Cancer
735 Center, Hawaii-Iowa); Marc T. Goodman (Cancer Center, Hawaii-Iowa; Cedars Sinai Medical Center, Los
736 Angeles, California, USA); Edyta C. Pirog (New York Hospital - Cornell Medical Centre);
- 737
- 738 Venezuela: Pablo Dabed (Universidad Central de Venezuela).
- 739
- 740 The advisory committee members are: Chris J Meijer, Massimo Tommasino, Michael Pawlita, Wim Quint,
741 Lúcia Alós, and Nubia Muñoz.
- 742
- 743