

HUMAN PAPILLOMAVIRUS INFECTION IS A FAVORABLE PROGNOSTIC FACTOR FOR PATIENTS WITH STAGES I TO IVA ESOPHAGEAL SQUAMOUS CELL CARCINOMA BUT NOT ADENOCARCINOMA

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ABSTRACT

With its 2 distinct histological subtypes (squamous cell carcinoma [SCC] and adenocarcinoma [ADC]), its increasing incidence in both high- and low-income countries and its elevated 5-year mortality rate, esophageal cancer still represents a significant global health challenge. Although the implication of high-risk human papillomaviruses (HPV) in both anogenital and head and neck carcinogenesis is well-established, the association between these mucosotropic viruses and esophageal cancers has been a subject of debate for nearly 2 decades. In an effort to resolve this unclear situation and advance precision medicine, data from a large cohort of 378 patients diagnosed with locally advanced esophageal carcinoma (SCC [$n = 226$] and ADC [$n = 152$]) over a 20-year period were collected and thoroughly characterized (at clinical, histopathological, immunological, and virological levels). In total, about one-third (48/152, 31.58%) of ADC were positive for high-risk HPV DNA, but a transcriptionally active infection was only detected in 17.76% of the samples. Surprisingly, only a minority of malignant cells (typically <20%) showed viral

transcripts, and HPV positivity had no prognostic significance for patients with esophageal ADC. Regarding SCC, 12.39% (28/226) of tissue specimens were HPV-positive, with viral/transcriptional activity observed in virtually 100% of neoplastic cells. These HPV-positive neoplasms more frequently exhibited basaloid differentiation and nonaberrant p53 expression and were significantly less associated with tobacco/alcohol use than their virus-negative counterparts. Importantly, univariate analyses indicated that HPV positivity was a reliable predictor of improved progression-free survival in patients with esophageal SCC. Taken together, our findings indicate that unlike in ADC, where testing for HPV is unnecessary, a dual classification system for esophageal SCC based on HPV status could/should be considered, with potential implications for personalized and optimized treatment strategies.

Introduction

With an estimated 604,100 new cases diagnosed in 2020 worldwide and a projected increase of ~60% by 2040, esophageal cancer is (and will remain) a major contributor to the global cancer burden.¹ Classified into 2 main histological subtypes (squamous cell carcinoma [SCC] and adenocarcinoma [ADC]), considerable (and still not fully understood) variations in incidence and mortality are observed across continents. Representing ~85% of all esophageal malignancies and found throughout the esophagus, the SCC rates peak in Eastern Asia and Sub-Saharan Africa, where it is often the third leading cause of cancer death. As for ADC, which is exclusively detected in close proximity to the gastroesophageal junction, it is much more frequently diagnosed in high-income countries/regions (eg, the USA, Canada, Australia, and Northern/Western Europe). Similar to their counterparts arising from the oral cavity, esophageal SCC development has been linked to tobacco smoking and/or alcohol consumption.² Chronic acid reflux (with or without the occurrence of a specialized intestinal metaplasia [called Barrett's esophagus]), obesity, and, to a lesser extent, smoking are the main risk factors for esophageal ADC.³ Although a curative surgical resection is advisable to remove early stage tumors, the vast majority of patients already manifest a locally advanced disease at initial diagnosis and are, therefore, treated with chemotherapy or concurrent chemoradiotherapy (as the first-line regimen).⁴ Unfortunately, current patient management and treatment are clearly suboptimal, as evidenced by low 5-year overall survival rates (range: 10%-40%) for both cancer subtypes in most countries, and the identification of novel (and robust) prognostic biomarkers remains necessary.⁵

To date, >440 different human papillomaviruses (HPVs) have been identified and assigned into 5 distinct phylogenetic genera (α , β , γ , μ , and ν).⁶ Among these, only 13 mucosotropic HPV genotypes (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68, all belonging to the α genus) are classified as carcinogenic to humans (group 1) by the International Agency for Research on Cancer (IARC). These "high-risk" viruses (especially HPV16) are detected in 99.9% of the cervical SCCs and are also responsible for a substantial proportion of other squamous neoplasms diagnosed in the anogenital [anus (80%-90%), vagina (60%-80%), and vulva (25%-40%)] and upper aerodigestive [nasal cavity (10%-20%), pharynx (25%-40%), oral cavity (10%-20%), and larynx (10%-20%)] tracts.^{7, 8, 9, 10} In

addition, 60%-80% of ADC occurring at the anal and cervical squamocolumnar junctions are also etiologically linked to chronic HPV infections. It is noteworthy to mention that for still unclear reasons, HPV18 is up to 4 times more frequent in these glandular neoplasms compared with their squamous counterparts.^{11,12} Altogether, 4.5% of all cancers diagnosed each year worldwide are undoubtedly attributable to carcinogenic HPV.¹³ However, this percentage could be underestimated. Indeed, the link between some β HPV strains and nonmelanoma skin (pre)cancers remains somewhat unclear.¹⁴ The presence and the involvement of high-risk HPV in urothelial and esophageal carcinogenesis are other subjects of debate. In this latter context, huge divergences exist in the literature, which are most likely explained by the lack of in-depth multimodal characterization (at the virologic, histopathologic, immunologic, and epidemiologic levels) of tissue specimens in most (if not all) available studies.^{15, 16, 17, 18, 19}

In this work, after applying strict inclusion/exclusion criteria, 378 patients with nonmetastatic esophageal carcinoma [SCC ($n = 226$) and ADC ($n = 152$)] were selected. Although about one-third (48/152, 31.58%) of ADCs were found to be high-risk HPV (DNA) positive, only about half of these samples (27/152, 17.76%) displayed a transcriptionally active infection (E6/E7 mRNA detected by PCR and/or in situ hybridization). Unexpectedly, and in complete contrast to what has been reported for HPV-positive anal/cervical glandular neoplasms, only a small percentage of malignant cells (typically <20%) displayed viral transcripts. These observations (associated with the absence of prognostic value of HPV positivity) do not support the need to evaluate the presence of HPV in esophageal ADCs. The overall findings and conclusions regarding HPV in esophageal SCCs differed significantly. Indeed, carcinogenic HPV infection was detected in 28 (28/226, 12.39%) tumor specimens, but the large majority (26/28, 92.86%) displayed a diffuse p16^{ink4a} expression, and positive DNA/RNA in situ hybridization signals were detected in virtually 100% of the neoplastic cells, clearly arguing for a causal role of HPV in a minority of esophageal SCCs. Furthermore, univariate and multivariate analyses demonstrated that viral positivity was linked to improved progression-free survival, indicating that the assessment of HPV status could be clinically valuable in predicting the response of esophageal SCC patients to standard treatment (chemoradiotherapy).

Materials and Methods

PATIENT SELECTION AND CLINICOPATHOLOGICAL DATA RETRIEVAL

A total of 226 patients treated for stages I to IVA (according to the American Joint Committee on Cancer, eighth edition²⁰) esophageal SCC at 4 different University Hospital Centers (Centre Hospitalier Universitaire of Liege [Belgium], Centre Hospitalier Universitaire of Besançon [France], Centre Hospitalier Regional Universitaire of Tours [France], and Centre Hospitalier Universitaire Vaudois [Lausanne, Switzerland]) between January 2002 and December 2021 were selected. Diagnosed and treated at CHU of Liege or at Jules Bordet Institute (Brussels, Belgium) during the same period, 152 patients with nonmetastatic esophageal ADC were also included in the study. In order to avoid bias that could impact the analysis, the patients who did not have formalin-fixed

paraffin-embedded (FFPE) tumor sample(s) available, with missing follow-up information, or directly treated with noncurative intent treatments (palliative care) were not considered. Patients with high-grade dysplasia (stage 0) or metastatic (stage IVB) disease at the initial diagnosis were also excluded to ensure that the results were not unduly influenced by extreme cases, which may be treated with different protocols. Original diagnoses were confirmed by senior pathologists (Liege: P.D.; Brussels: D.L.; Tours: T.K.; Besançon: C.M. and F.B.; and Lausanne: F.F. and L.d.L.). Tumor differentiation was determined using established criteria (Supplementary Fig. S1). Two hundred twenty-four resections and 154 biopsies were included in the histopathological analysis. The patient characteristics (gender, age at diagnosis, body mass index [BMI], tobacco/ alcohol consumption), clinical tumor-node-metastasis staging, treatment details (eg, surgical procedures, radiotherapy doses, chemotherapeutic agents), and outcomes (follow-up data) were collected for all patients from personal medical records. For smoking and alcohol consumption, the cutoff points were set at 20 pack/year and 20 drinks/week, respectively. The present study (experimental protocol and data collection) was approved by the institutional review board of the respective institutions beforehand (Belgium: #2020/76; France: #F2162-HPVOESO; Switzerland: #2022-00437).

IMMUNOHISTOCHEMISTRY

As previously described,^{8,21} 5- μ m thick tissue sections were first deparaffinized in xylene and rehydrated in graded alcohols. The activity of endogenous peroxidases was blocked (4.5% H₂O₂ in methanol for 5 minutes) before the antigen retrieval step (10 mM citrate buffer [pH 6; Sigma-Aldrich] or 10 mM Tris/1mM EDTA solution [pH 9; Invitrogen] for 11 minutes at 120 °C in a pressure cooker [2100 Antigen Retriever, Aptum Biologics]). Nonspecific binding sites were blocked using animal-free blocking solution (Cell Signaling) for 10 minutes, and the tissue sections were then incubated with the following primary antibodies for 1 hour (at room temperature): anti-p16^{ink4a} (1/100, ENZABS377, Enzo Life Sciences), anti-p53 (ready to use, clone DO-7; Ventana Medical Systems), anti-Ki67 (ready to use, clone 30-9; Ventana Medical Systems), anti-PD-1 (1/200, clone NAT105; Abcam), and anti-CD8 (ready to use, clone SP57; Ventana Medical Systems). Mouse or rabbit EnVision detection system (Dako) was used for the secondary reaction (immunoperoxidase staining), and positive cells were visualized using the SignalStain DAB Substrate Kit (Cell Signaling). Mouse and rabbit control immunoglobulins G (Santa Cruz Biotechnology) were used as negative controls.

IMMUNOSTAINING ASSESSMENT

The expression of p53, Ki67, and p16^{ink4a} by esophageal malignant cells was evaluated by independent investigators (experienced pathologists). Disagreements were resolved by a group review. In each tissue sample, the entire neoplastic lesion was considered. As previously described,^{8,22} p53 expression was considered nonaberrant when a weak nuclear staining was detected in 1% to 59% of tumor cells. In the case of complete absence (0%) or moderate/strong positivity in \geq 60% of malignant cells, p53 staining was classified as aberrant. In line with interpretation criteria recently proposed in the context of HPV-positive oropharyngeal, vulvar, and anal cancers,²³⁻²⁵ esophageal SCC cases exhibiting cytoplasmic p53 immunoreactivity (with or

without accompanying nuclear staining), as well as those showing strong basal expression (in extensively keratinized tumors), were also considered aberrant (Supplementary Fig. S2). Based on nuclear Ki67 staining, the proliferation index of a given squamous or glandular malignancy was stratified as follows: 0% to 25%, 26% to 50%, 51% to 75%, and >75%. A similar classification (into 4 groups depending on the percentage of cancer cells displaying positive [both nuclear and cytoplasmic] immunoreactivity) was applied to p16^{ink4a} immunostainings. Regarding tumorinfiltrating T-cell subpopulations (CD8⁺ and PD-1⁺), the number of positive cells per mm² was precisely determined using QuPath software for digital pathology image analysis (version 0.3.2 or 0.4.0).²⁶ Manual verifications were performed for all scanned tissue samples.

HUMAN PAPILLOMAVIRUS GENOTYPING

To avoid contamination as much as possible, the microtome blade was changed after sectioning each FFPE block, and the microtome, tweezers, and brush were systematically cleaned with 1% isopropanol and 99% ethanol. As commonly practiced, DNA extractions (NucleoSpin DNA FFPE XS kit, Macherey Nagel) and PCR reactions were performed in separate rooms. HPV genotyping of SCC samples was performed using the Abbott RealTime High-Risk HPV assay (Abbott). Type-specific multiplex genotyping assay (combining multiplex PCR and bead-based Luminex technology [Luminex Corporation]) or the aforementioned Abbott assay was used to assess the presence of high-risk HPV in ADC specimens. Both assays are highly reliable and sensitive for the detection of all World Health Organization/ IARC-classified carcinogenic HPV genotypes (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) in FFPE cancer specimens.^{27,28} Notably, the bead-based genotyping Luminex assay enabled the detection of 8 additional alpha HPV types (low-risk: HPV 6, 11, and 70; probably carcinogenic to humans: HPV 26, 53, 66, 73, and 82).

DNA IN SITU HYBRIDIZATION

DNA in situ hybridization was performed according to the supplier's recommendations (Ventana Medical Systems). The INFORM HPV III Family 16 Probe cocktail (allowing the detection of HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66) and the Red Counterstain II (facilitating microscopic bright field observation of chromogenic products) were used.

DETECTION OF HUMAN PAPILLOMAVIRUS E6 TRANSCRIPTS BY PCR

Total RNA was extracted and purified using the ReliaPrep FFPE total RNA Miniprep system (Promega), according to the manufacturer's instructions. To ascertain that no remaining HPV DNA was present, DNase I treatment (1 U per 1 µg total RNA, 15 minutes at room temperature) was systematically performed. Five hundred nanograms of RNA were then reverse-transcribed using FastGene Scriptase II cDNA kit (Nippon Genetics) and random hexamers. Real-time PCR experiments (QuantStudio 3, Applied Biosystems) were performed using the FastStart Universal SYBR Green Master mix (Roche) and the following primer sequences: HPV16 E6 forward: 5' -GTT-ACT-GCG-ACG-TGA-GGT-ATA-TG-3'; HPV16 E6 reverse: 5'-CAT-TTA-TCA-CAT-ACA-GCA-TAT-GGA-TTC-3'; HPV18 E6

forward: 5'-CAG-AGG-TAT-TTG-AAT-TTG-CAT-TT-3'; and HPV18 E6 reverse: 5'-AAT-CTA-TAC-ATT-TAT-GGC-ATG-CAG-3'. 18S (forward: 5'-AGT-CCC-TGC-CCT-TTG-TAC-ACA-3'; reverse: 5'-GAT-CCG-AGG-GCC-TCA-CTA-AAC-3') was used as an internal control.

RNA IN SITU HYBRIDIZATION (RNASCOPE)

The detection of viral E6/E7 mRNAs on cancer sections was performed using the RNAscope 2.5 HD Detection kit (Advanced Cell Diagnostics), according to the manufacturer's instructions. All reagents (eg, wash buffers and target retrieval solution) as well as RNAscope probes specifically targeting HPV16 and 18 E6/E7 transcripts were also purchased from Advanced Cell Diagnostics. A negative control probe targeting diaminopimelate B (*DapB*, bacterial gene) and a positive control probe for peptidylprolyl isomerase B (*PPIB*, housekeeping gene) were included in each run to evaluate nonspecific signals and RNA integrity, respectively. All stained slides were reviewed by experienced pathologists, and the percentage of tumor cells displaying nuclear/cytoplasmic HPV mRNA signals was determined by computerized counts (QuPath software) and verified manually.

MUTATION ANALYSIS USING NEXT-GENERATION SEQUENCING

Following tissue lysis and DNA extraction (NucleoSpin DNA FFPE XS kit), regions of interest from 42 clinically relevant genes associated with solid tumors (*AKT1* [exon 3], *ALK* [exons 21-25], *BRAF* [exons 11 and 15], *CDK4* [exon 2], *CDKN2A* [exon 1-3], *CTNNB1* [exon 3], *DDR2* [exon 17], *DICER1* [exons 24 and 25], *EGFR* [exons 19-21], *ERBB2* [exons 8, 17 and 20], *ERBB4* [exons 10 and 12], *FBXW7* [exons 7-11], *FGFR1* [exons 12 and 14], *FGFR2* [exons 7, 12 and 14], *FGFR3* [exons 7, 9, 14 and 16], *FOXL2* [exon 1], *GNA11* [exons 4 and 5], *GNAQ* [exons 4 and 5], *GNAS* [exon 8], *H3F3A* [exon 2], *H3F3B* [exon 2], *HIST1H3B* [exon 1], *HRAS* [exons 2-4], *IDH1* [exon 4], *IDH2* [exon 4], *KIT* [exons 8-11, 13, 17 and 18], *KRAS* [exons 2-4], *MAP2K1* [exons 2 and 3], *MET* [exons 2, 14-20], *MYOD1* [exon 1], *NRAS* [exons 2-4], *PDGFRA* [exons 12, 14 and 18], *PIK3CA* [exons 2, 3, 6, 8, 10 and 21], *PTPN11* [exon 3], *RAC1* [exon 3], *RAF1* [exons 7, 10, 12-15], *RET* [exons 11, 13, 15 and 16], *ROS1* [exons 38 and 41], *SF3B1* [exons 15-17], *SMAD4* [exons 8-12], *TERT* [promoter, exons 1, 8, 9 and 13], and *TP53* [complete coding sequence]) were screened for somatic variants using the "Solid Tumor Solution" (Sophia Genetics), according to the manufacturer's recommendations. Sequencing was performed on a MiSeq platform using v3 chemistry (Illumina). Data analysis was carried out using the Sophia DDM Platform with Sophia Genetics' proprietary algorithms. As is common with paraffin-embedded samples, variants with a minor allele frequency <5% were not considered.

STATISTICAL ANALYSIS

All collected data were processed using GraphPad Prism 8.3 (GraphPad Software, LLC) or R (v4.2.1; Survminer v0.4.9 and Survival v3.5-5 packages) (R Core Team). Differences were considered statistically significant when $P < .05$. The comparisons of clinicopathological data between independent groups were performed using a Fisher exact test or the χ^2 test, depending on the number of variables involved (2 or more). Estimates for overall survival (OS) and progression-free

survival (PFS) were calculated using the Kaplan-Meier method. The OS was measured from the date of initial diagnosis or biopsy until the date of death (from any cause). For PFS, 3 clinical events were considered: (1) progression of persistent diseases, (2) local/primary site recurrences, and (3) distant metastases. For patients who remained alive (without any events) at the last follow-up visit, this date was used as the endpoint. Differences in survival distributions between groups were assessed using the log-rank (Mantel-Cox) test. The prognostic significance (hazard ratio [HR] with 95% CI) of each clinicopathological variable was assessed through univariate analysis. Variables/risk factors with $P < .25$ in univariate analysis were included in a subsequent multivariate analysis based on the Cox proportional hazards regression model. This analysis was then refined using a two-direction Akaike information criterion minimization stepwise approach, applying a $P < .1$ (cutoff) for variable exclusion.

Results

STUDY POPULATION: DEMOGRAPHIC AND CLINICAL CHARACTERISTICS

The general characteristics of the study population are illustrated in Figure 1. After applying strict inclusion/exclusion criteria (ie, invasive, nonmetastatic diseases at initial diagnosis treated with curative intent, with both available tissue samples and extensive clinicopathological data), 378 patients with stage I to IVA invasive esophageal cancer, either ADC (n = 152) or SCC (n = 226), were retained. No case displayed a mixed (adenosquamous) phenotype. For both patients with ADC and SCC, men significantly outnumbered women (male/female ratio: 5.61 [for ADC] and 2.23 [for SCC]), and the average age was around 65 years (ADC: 67 [range: 32-86]; SCC: 66 [range: 41-90]). In approximately 45% (71/152, 46.71%) of the cases, ADC had already spread to the lymph nodes at the time of diagnosis, and 3 quarters (114/152, 75%) of patients had stage IIA/B or III disease. A positive nodal status was also observed in ~45% (102/226, 45.13%) of SCC patients, with stage II (82/226, 36.28%) and III (60/226, 26.54%) neoplasms being the most common, as well. Due to insufficient/imprecise information regarding tumor size or nodal status, tumor stage was undetermined for 24 of 378 (6.35%) patients (6 with ADC and 18 with SCC). Most ADC patients were treated with chemotherapy with (54/152, 35.53%) or without (52/152, 34.21%) concomitant radiotherapy (41.4-50.4 Gy), and only a small proportion underwent surgery as the sole treatment (19/152, 12.5%) or received radiotherapy as monotherapy (8/152, 5.26%). Chemotherapy combinations varied considerably [eg, carboplatin-paclitaxel, 5-fluorouracil-leucovorin-oxaliplatin-docetaxel, or 5-fluorouracil-cisplatin-epirubicin], depending on tumor stage and treatment setting (neoadjuvant or adjuvant). For patients with SCC, neoadjuvant chemoradiotherapy [typically combining paclitaxel or 5-fluorouracil with a platinum-based agent (cisplatin or carboplatin) and 41.4 or 50.4 Gy of radiation delivered in 23 or 28 fractions of 1.8 Gy], followed by surgery, was the most commonly administered first-line regimen (78/226, 34.51%). Other standard approaches included primary surgery (51/226, 22.57%), definitive chemoradiotherapy (49/226, 21.68%), chemotherapy alone (21/226, 9.29%), and radiotherapy alone (8/226, 3.54%). The remaining

patients (with ADC or SCC) received alternative treatments such as neoadjuvant chemotherapy plus cetuximab (anti-epidermal growth factor receptor, $n = 3$), trastuzumab (anti-human epidermal growth factor receptor 2, $n = 2$), pembrolizumab (anti-PD-1, $n = 3$), or nivolumab (anti-PD1, $n = 3$). As anticipated, nearly half (69/152, 45.39%) of the patients with ADC were overweight (BMI ≥ 25), and >60% (138/226, 61.06%) of SCC patients were active smokers (>20 packs/year) and/or heavy alcohol consumers (>20 drinks/ week).

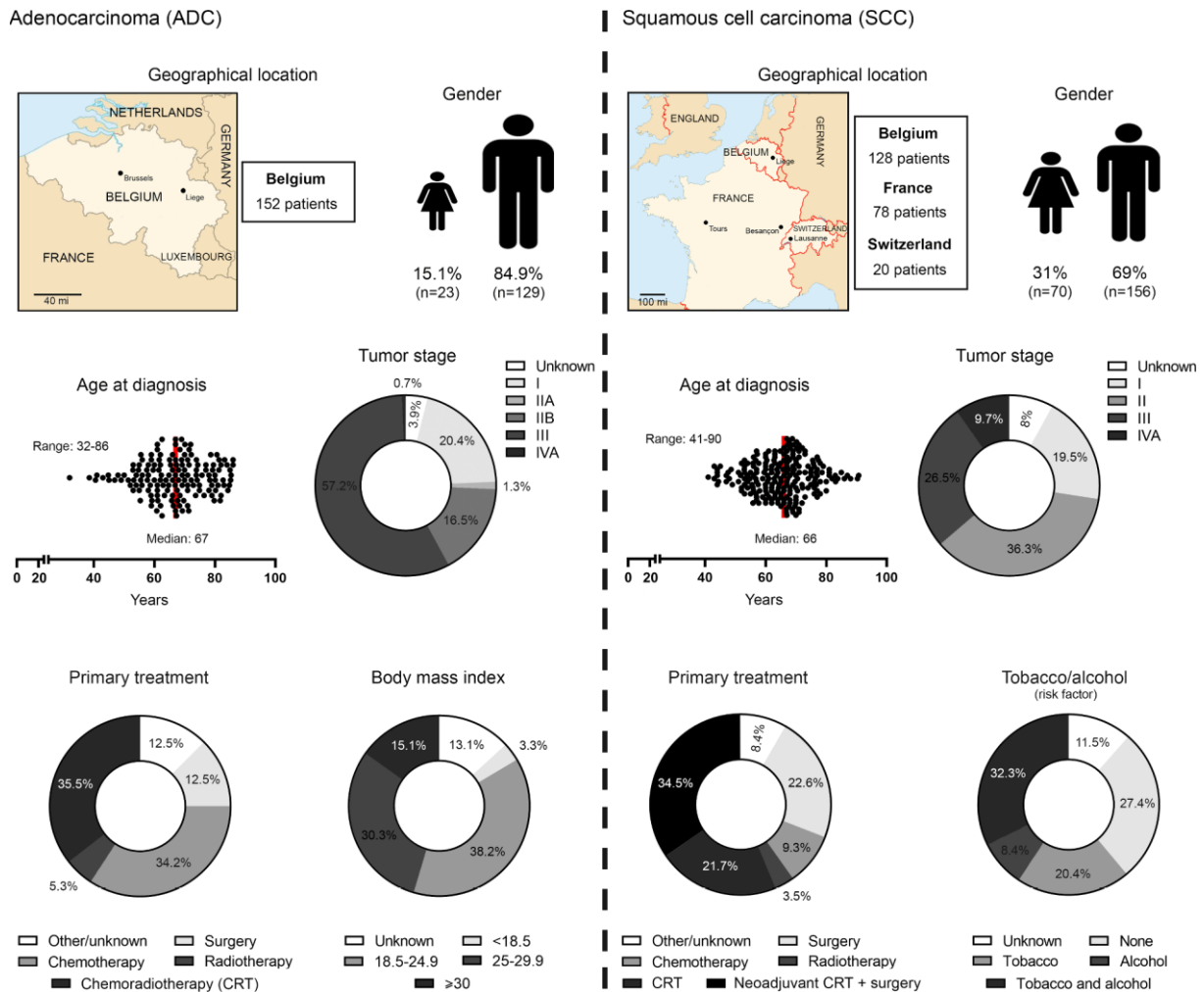


Figure 1. Description and general characteristics of the study population (esophageal ADC [$n = 152$] and SCC [$n = 226$]). ADC, adenocarcinoma; SCC, squamous cell carcinoma.

DETAILED VIRO-HISTOPATHOLOGICAL CHARACTERIZATION OF HUMAN PAPILLOMAVIRUS INFECTIONS IN ESOPHAGEAL CARCINOMAS

Confirming successful DNA extraction and PCR amplification, all 378 selected esophageal cancer cases showed amplification of the human β -globin gene (internal control for the 2 specific and sensitive HPV genotyping assays used in the present study [Abbott RealTime High-Risk HPV and type-specific multiplex genotyping]). Overall, carcinogenic HPV DNA was detected in 31.58% (48/152) and 12.39% (28/226) of ADC and SCC specimens, respectively. Infection with a single genotype was found in 83.33% (40/48) of ADC and in all (28/28) HPV-positive SCC cases. Regarding

HPV genotype distribution, HPV16 and HPV18 were by far the most commonly encountered in ADC, with HPV16 found in 66.67% (32/48) of cases and HPV18 in 41.67% (20/48). Notably, 100% (28/28) of virus-infected SCC were positive for HPV16. To thoroughly characterize these HPV infections and, in fine, determine a potential etiologic link between HPV and esophageal carcinogenesis, 4 additional assays were performed: (1) detection of viral transcripts by PCR, (2) p16^{ink4a} immunohistochemistry, (3) DNA in situ hybridization, and (4) RNAscope. The main results and representative images are shown in Figure 2. Taken together, 5 main observations were made for HPV-positive ADCs. First, despite positive PCR results, a positive in situ hybridization signal for HPV DNA (most often focal) was observed in only a minority (11/48, 22.92%) of cases. Second, nearly half (21/48, 43.75%) of HPV infections are inactive, as evidenced by the absence of viral mRNAs detected through both PCR and RNAscope. Third, only a minority of neoplastic cells (typically <20%) harbored viral RNA. Fourth, given the complete absence of p16^{ink4a} expression in 55.56% (15/27) of HPV DNA⁺/RNA⁺ specimens, this protein is not a reliable surrogate biomarker for detecting high-risk HPV in the context of esophageal ADC. Fifth, as shown in Table 1, no clinicopathological variables significantly differed based on HPV status. Strikingly, the findings from SCC samples were markedly different (Fig. 2; Table 2). Indeed, all (28/28) HPV infections were transcriptionally active, with both viral genome and mRNAs detected in nearly 100% of cancer cells, as demonstrated by DNA/ RNA in situ hybridization. These results were further confirmed by the diffuse p16^{ink4a} immunoreactivity detected in 26 of 28 (92.86%) HPV-positive SCCs, which were most often located in the upper (14/28, 50%) and middle (10/28, 35.71%) parts of the esophagus. Besides p16^{ink4a}, interestingly, p53 expression and tumor differentiation also showed significant differences according to the HPV status of tumors. Largely correlated with mutated/deleted TP53,²⁹ aberrant p53 expression (undetectable or ≥60% positive cells) was almost half as often noticed in HPV-related tumors (9/28, 32.14%), compared with their HPV-negative counterparts (130/198, 65.66%, $P = .0014$). As for basaloid differentiation, it was observed 3 times more frequently in the case of HPV positivity (32.14% vs 10.60%, $P = .0050$). Although the statistical significance was not reached ($P = .0677$), HPV-positive tumors also tended to exhibit higher intratumoral PD-1⁺ T-cell density compared with uninfected neoplasms. In parallel with the comprehensive virohistopathological characterization, the mutational landscape of 25 of 28 HPV-positive SCC was analyzed by next-generation sequencing. Three cases were excluded due to insufficient material ($n = 1$) or poor DNA quality/ high degradation ($n = 2$). The collected results confirmed the presence of TP53 mutation in all cases exhibiting aberrant p53 immunoreactivity. Consistent with findings previously reported in cervical and oropharyngeal tumors,^{30,31} PIK3CA mutations were detected in 4 cases (4/25, 16%), and no driver mutations were identified in nearly half of these HPV-positive malignancies (12/25, 48%; Table 3). Last but not least, patients with HPV-positive esophageal SCC were less frequently linked to tobacco use and/or excessive alcohol consumption compared with HPV-negative SCC (12/28, 42.86% vs 126/198, 63.64%, $P = .0021$).

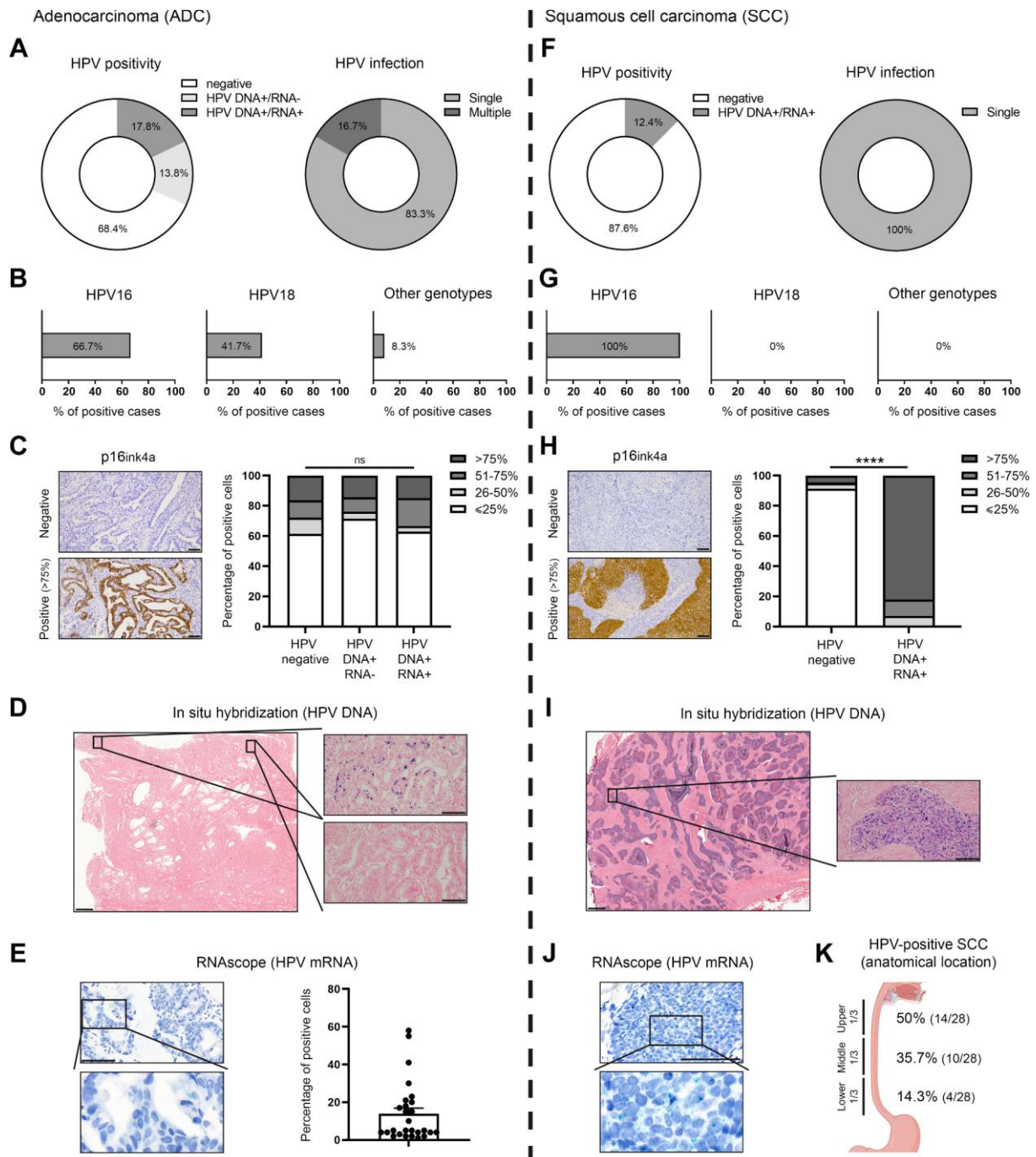


Figure 2. Comprehensive virohistopathological analysis of human papillomaviruses (HPV) infections in esophageal adenocarcinoma (ADC) and squamous cell carcinoma (SCC). HPV DNA/RNA status (A, F) and viral genotypes (B, G) identified in our cohort. Whereas single infections with HPV16 were reported in all HPV-positive SCC cases, a greater viral diversity was detected in HPV-infected ADC. (C, H) Representative examples of esophageal ADC and SCC stained for p16^{ink4a} and semiquantitative evaluation of this surrogate biomarker for HPV infection. (D, I) Representative HPV DNA staining patterns identified through in situ hybridization in esophageal cancers. Note the focal and diffuse positivity observed in ADC and SCC, respectively. (E, J) The transcriptional activity as well as the extent of viral infections were precisely investigated by RNAscope. Although viral/transcriptional activity was detected in virtually 100% of SCC cells, only a minority of ADC cells (typically <20%) exhibited viral mRNAs, confirming data previously collected by DNA in situ hybridization. (K)

Anatomical location of HPV-positive SCC. As commonly done, the esophagus was divided into 3 sections: upper, middle and lower. The scale bar represents 100 μ m or 1 mm (low-magnification scans). Asterisks indicate statistically significant differences (**** $P < .0001$). P values were determined using χ^2 test (C, H). ns, not significant.

Characteristics	HPV neg (n= 104) (68.42%)	HPV DNA+/RNA- (n = 21) (13.82%)	HPV DNA+/RNA+ (n = 27) (17.76%)	P value
<i>Age at diagnosis (32-86 y)</i>				.5081
<65	47 (45.19%)	7 (33.33%)	10 (37.04%)	
\geq 65	57 (54.81%)	14 (66.67%)	17 (62.96%)	
<i>Gender</i>				.8635
Male	89 (85.58%)	18 (85.71%)	22 (81.48%)	
Female	15 (14.42%)	3 (14.29%)	5 (18.52%)	
<i>cTNM</i>				.0327 ^a
<i>cT</i>				
T1-T2	50 (48.08%)	15 (71.43%)	9 (33.33%)	
T3-T4	51 (49.04%)	4 (19.05%)	17 (62.97%)	
Unknown	3 (2.88%)	2 (9.52%)	1 (3.70%)	
<i>cN</i>				.6702
N-	53 (50.96%)	12 (57.14%)	13 (48.15%)	
N+	50 (48.08%)	8 (38.10%)	13 (48.15%)	
Unknown	1 (0.96%)	1 (4.76%)	1 (3.70%)	
<i>cM</i>				/
M-	104 (100%)	21 (100%)	27 (100%)	
M+	0 (0%)	0 (0%)	0 (0%)	
<i>Clinical stage (AJCC 8th edition)</i>				.2460
Stage I-IIb	39 (37.50%)	11 (52.38%)	8 (29.63%)	
Stage III-IVA	62 (59.62%)	8 (38.10%)	18 (66.67%)	
Unknown	3 (2.88%)	2 (9.52%)	1 (3.70%)	
<i>HPV infection</i>				/
Single	/	16 (76.19%)	24 (88.89%)	
Multiple	/	5 (23.81%)	3 (11.11%)	
<i>HPV genotypes</i>				/
HPV16	/	15 (71.43%)	17 (62.96%)	
HPV18	/	8 (38.10%)	12 (44.44%)	
Others	/	3 (14.29%)	1 (3.70%)	
<i>Tumor differentiation</i>				.5150
Well differentiated	48 (46.15%)	11 (52.38%)	9 (33.33%)	
Moderately differentiated	30 (28.85%)	5 (23.81%)	12 (44.45%)	
Poorly differentiated	26 (25%)	5 (23.81%)	6 (22.22%)	
<i>Proliferative index (Ki67)</i>				.9677
<25%	19 (18.27%)	3 (14.28%)	4 (14.81%)	
26%-50%	36 (34.62%)	9 (42.86%)	10 (37.05%)	
51%-75%	28 (26.92%)	5 (23.81%)	9 (33.33%)	
>75%	21 (20.19%)	4 (19.05%)	4 (14.81%)	
<i>p53 staining</i>				.4086
Aberrant (0 or >60%)	68 (65.38%)	13 (61.90%)	21 (77.78%)	
Nonaberrant	36 (34.62%)	8 (38.10%)	6 (22.22%)	
<i>p16^{ink4a} staining</i>				.8165
<25%	64 (61.54%)	15 (71.43%)	17 (62.96%)	
26%-50%	11 (10.57%)	1 (4.76%)	1 (3.70%)	
51%-75%	12 (11.54%)	2 (9.52%)	5 (18.52%)	
>75%	17 (16.35%)	3 (14.28%)	4 (14.81%)	
<i>CD8⁺ cells</i> (median: 282 cells/mm ²)				.9586
<282 cells/mm ²	52 (50%)	10 (47.62%)	14 (51.85%)	
>282 cells/mm ²	52 (50%)	11 (52.38%)	13 (48.15%)	
<i>PD1⁺ cells</i> (median: 128 cells/mm ²)				.5746
<128 cells/mm ²	49 (47.12%)	12 (57.14%)	15 (55.55%)	
>128 cells/mm ²	55 (52.88%)	9 (42.86%)	12 (44.45%)	
<i>Body mass index</i>				.5585
\leq 18.5	4 (3.85%)	0 (0%)	1 (3.70%)	
18.6-24.9	41 (39.42%)	5 (23.81%)	12 (44.45%)	
25-29.9	32 (30.77%)	6 (28.57%)	8 (29.63%)	
\geq 30	16 (15.39%)	5 (23.81%)	2 (7.41%)	
Unknown	11 (10.57%)	5 (23.81%)	4 (14.81%)	

Characteristics	HPV neg (n= 104) (68.42%)	HPV DNA+/RNA- (n = 21) (13.82%)	HPV DNA+/RNA+ (n = 27) (17.76%)	P value
<i>Primary treatment</i>				.9954
Chemotherapy	36 (34.61%)	7 (33.33%)	9 (33.33%)	
Radiotherapy	5 (4.81%)	2 (9.52%)	1 (3.70%)	
Chemoradiotherapy	37 (35.58%)	7 (33.33%)	10 (37.05%)	
Surgery	13 (12.5%)	3 (14.28%)	3 (11.11%)	
Other/unknown	13 (12.5%)	2 (9.52%)	4 (14.81%)	
<i>Geographical location</i>				.1108
Liege (Belgium)	84 (80.77%)	19 (90.48%)	18 (66.67%)	
Brussels (Belgium)	20 (19.23%)	2 (9.52%)	9 (33.33%)	

Table 1. Demographic and characteristics of patients with esophageal adenocarcinoma according to human papillomavirus status. AJCC, American Joint Committee on Cancer; cM, clinical metastasis; cN, clinical node; cT, clinical tumor; cTNM, clinical tumor-node-metastasis; HPV, human papillomaviruses. ^a Statistically significant.

CLINICOPATHOLOGICAL FACTORS INFLUENCING OVERALL SURVIVAL

The median follow-up time was 34.8 months (range: 0.4203.5 months) for patients with ADC and 26.8 months (range: 0.2-184.3 months) for their counterparts with SCC. During this period, 196 of 378 (51.86%) patients died as a result of cancer or other causes (primarily due to old age). The mortality rates were identical for the 2 esophageal cancer subtypes: 51.97% for ADC (79 out of 152 patients) and 51.77% for SCC (117 of 226 patients). The OS data (Kaplan-Meier curves) as well as the results of both univariate and multivariate analyses are shown in Figure 3. In univariate analysis, a high T-classification (T3/T4) [HR = 2.292 for ADC ($P = .0004$); HR = 2.157 for SCC ($P = .0001$)], positive nodal status [HR = 2.614 for ADC ($P < .0001$); HR = 1.768 for SCC ($P = .0049$)] and advanced tumor stage (III-IVA) [HR = 2.305 for ADC ($P = .0003$); HR = 2.103 for SCC ($P = .0006$)] were significantly associated with decreased OS for patients with esophageal cancer, regardless of the cancer subtype. Although the statistical significance was not reached for ADC patients, as expected, the age at diagnosis (<65 vs ≥ 65) also influenced OS [HR = 1.438 for ADC ($P = .11$); HR = 1.826 for SCC ($P = .0014$)]. At last, in the specific context of SCC, a high intratumoral PD-1⁺ T-cell density was statistically related to improved OS (HR = 0.514; $P = .0004$). In multivariate analysis, tumor size ($P = .0481$) and lymph node status ($P = .0195$) were identified as independent prognostic factors for OS in patients with ADC. In parallel, advanced age (≥ 65 ; $P = .0014$) and high T-classification ($P < .0001$) emerged as independent predictors of reduced OS in patients with SCC. A similar tendency was also reported in the case of low densities of PD-1⁺ T cells within SCC microenvironment ($P = .057$).

RISK FACTORS PREDICTING DISEASE PROGRESSION/RECURRENCE

After completion of treatment, 53 (53/152, 34.87%) patients with ADC and 78 (78/226, 34.51%) patients with SCC experienced local recurrence/progression (in cases with residual disease) or metastatic spread. The median time to disease recurrence/progression was 14.9 months (15.1 months for ADC and 14.8 months for SCC patients). The Kaplan-Meier curves for PFS, along with the results from univariate and multivariate analyses, are shown in Figure 4. For both cancer subtypes, factors such as age at diagnosis, gender, tumor differentiation, proliferative index, and p53 expression by malignant cells had no impact on PFS. Involved in the pathogenesis, smoking and alcohol consumption did not alter PFS in patients with SCC

either. Likewise, although obesity is a well-established risk factor for the development of esophageal ADC, BMI did not affect PFS in ADC patients. In contrast, the following clinicopathological variables were significantly linked to reduced PFS in patients with either ADC or SCC: high T-classification (T3/T4) [HR = 2.269 for ADC ($P = .0038$); HR = 2.488 for SCC ($P < .0001$)], positive nodal status [HR = 3.767 for ADC ($P < .0001$); HR = 2.175 for SCC ($P = .0009$)], and advanced tumor stage (III-IVA) [HR = 2.808 for ADC ($P = .0003$); HR = 2.335 for SCC ($P = .0006$)]. In terms of T-cell subset densities within the tumor microenvironment, a high infiltration rate of CD8⁺ cells was significantly associated with decreased PFS in ADC patients (HR = 1.795; $P = .035$). Conversely, for SCC patients, a high density of intratumoral PD-1⁺ T cells showed a trend toward improved PFS (HR = 0.673; $P = .077$). The prognostic value of HPV status was also examined, and in contrast to observations in ADC patients, HPV-positive SCC patients showed significantly improved PFS compared with their virus-uninfected counterparts (HR = 0.489; $P = .016$). Similar findings were observed when analyzing p16^{ink4a} expression (HR = 0.510; $P = .016$). As shown in Figure 4C and F, the multivariate analysis indicated that a positive nodal status [for patients with ADC ($P < .0001$) or SCC ($P = .0152$)] as well as high T-classification ($P = .0014$) and negative HPV status ($P = .0221$; for SCC patients only) were strong independent predictors for reduced PFS.

Characteristics	HPV-negative (n = 198) (87.61%)	HPV-positive (n = 28) (12.39%)	P value
<i>Age at diagnosis (41-90 y)</i>			.4244
<65	95 (47.98%)	11 (39.28%)	
≥65	103 (52.02%)	17 (60.72%)	
<i>Gender</i>			.1892
Male	140 (70.71%)	16 (57.14%)	
Female	58 (29.29%)	12 (42.86%)	
<i>cTNM</i>			
<i>cT</i>			.0855
T1-T2	76 (38.39%)	16 (57.14%)	
T3-T4	106 (53.53%)	12 (42.86%)	
Unknown	16 (8.08%)	0 (0%)	
<i>cN</i>			.2589
N-	89 (44.95%)	17 (60.72%)	
N+	92 (46.46%)	10 (35.71%)	
Unknown	17 (8.59%)	1 (3.57%)	
<i>cM</i>			/
M-	198 (100%)	28 (100%)	
M+	0 (0%)	0 (0%)	
<i>Clinical stage (AJCC 8th edition)</i>			.1916
Stages I-II	106 (53.53%)	20 (71.43%)	
Stages III-IVA	75 (37.88%)	7 (25%)	
Unknown	17 (8.59%)	1 (3.57%)	
<i>HPV infection</i>			/
Single	/	28 (100%)	
Multiple	/	0 (0%)	
<i>HPV genotypes</i>			/
HPV16	/	28 (100%)	
HPV18	/	0 (0%)	
Others	/	0 (0%)	
<i>Tumor differentiation</i>			.0050 ^a
Well differentiated	49 (24.75%)	4 (14.29%)	
Moderately differentiated	93 (46.97%)	14 (50%)	
Poorly differentiated	35 (17.68%)	1 (3.57%)	
Basaloid	21 (10.60%)	9 (32.14%)	
<i>Proliferative index (Ki67)</i>			.6924
≤25%	31 (15.66%)	4 (14.29%)	
26%-50%	54 (27.27%)	7 (25%)	
51%-75%	63 (31.82%)	7 (25%)	
>75%	50 (25.25%)	10 (35.71%)	
<i>p53 staining</i>			.0014 ^a
Aberrant (0% or >60%)	130 (65.66%)	9 (32.14%)	
Nonaberrant	68 (34.34%)	19 (67.86%)	
<i>p16^{ink4a} staining</i>			.0001 ^b
≤25%	181 (91.41%)	0 (0%)	
26%-50%	7 (3.54%)	2 (7.14%)	
51%-75%	1 (0.50%)	3 (10.71%)	
>75%	9 (4.55%)	23 (82.15%)	
<i>CD8⁺ cells</i> (median: 310 cells/mm ²)			.9999
<310 cells/mm ²	99 (50%)	14 (50%)	
>310 cells/mm ²	99 (50%)	14 (50%)	
<i>PD1⁺ cells</i> (median: 204 cells/mm ²)			.0677
<204 cells/mm ²	104 (52.53%)	9 (32.14%)	
>204 cells/mm ²	94 (47.47%)	19 (67.86%)	
<i>Tobacco and alcohol consumption</i>			.0021 ^a
Tobacco alone	43 (21.72%)	3 (10.71%)	
Alcohol alone	16 (8.08%)	3 (10.71%)	
Tobacco and alcohol	67 (33.84%)	6 (21.43%)	

Characteristics	HPV-negative (n = 198)	HPV-positive (n = 28)	P value
	(87.61%)	(12.39%)	
None	46 (23.23%)	16 (57.14%)	
Unknown	26 (13.13%)	0 (0%)	
<i>Prior or synchronous diagnosis of head and neck cancer</i>			.4204
Yes	31 (15.66%)	6 (21.43%)	
No	167 (84.34%)	22 (78.57%)	
<i>Primary treatment</i>			.6676
Chemotherapy	19 (9.60%)	2 (7.14%)	
Radiotherapy	7 (3.54%)	1 (3.57%)	
Chemoradiotherapy	44 (22.22%)	5 (17.86%)	
Surgery	41 (20.71%)	10 (35.71%)	
Neoadjuvant chemo (radiotherapy) plus surgery	70 (35.35%)	8 (28.57%)	
Other/unknown	17 (8.58%)	2 (7.14%)	
<i>Geographical location</i>			.4995
Belgium	110 (55.56%)	18 (64.29%)	
France	69 (34.85%)	9 (32.14%)	
Switzerland	19 (9.59%)	1 (3.57%)	

Table 2. Demographic and attributes of esophageal squamous cell carcinoma patients according to human papillomavirus status. AJCC, American Joint Committee on Cancer; cM, clinical metastasis; cN, clinical node; cT, clinical tumor; cTNM, clinical tumor-node-metastasis; HPV, human papillomaviruses. ^a Statistically significant. ^b Statistically significant (P < .0001).

Discussion

Although some studies have suggested a potential link between HPV and esophageal carcinogenesis, the evidence is inconsistent and varies significantly, depending on both histological subtype of interest (ADC vs SCC) and geographic region (for a systematic review, see Li et al³²). This variability is further compounded by substantial methodological issues, particularly regarding HPV detection techniques. Indeed, most available studies rely on a single method to detect high-risk HPV infections (such as PCR using consensus primers, in situ hybridization, or anti-p16 immunohistochemistry), each of which has varying degrees of sensitivity and specificity. For example, DNA in situ hybridization is well known for its high specificity but low sensitivity compared with PCR-based assays or RNAscope, particularly when detecting low HPV copy numbers (1-5 copies per cell)^{33,34}. Notably, HPV detection sensitivity can also be affected by the intrinsic characteristics of the samples (eg, frozen vs paraffin-embedded, recent vs long-stored), as observed with RNAscope, where weaker signals (likely due to RNA degradation) are detected in tissue specimens older than 5 to 10 years (Supplementary Fig. S3). Consequently, reported HPV positivity rates in esophageal cancer samples vary widely, ranging from 0% to >70%. Furthermore, the presence of HPV DNA alone does not establish causality. Taken together, although 13 HPV strains are recognized as group 1 carcinogens to humans by IARC, none of these latter strains is regarded as being associated with esophageal cancer development yet due to limited and inconclusive evidence. The present study aimed to resolve the current uncertainties and contribute to the advancement of precision medicine. Given the substantial differences observed between ADC and SCC patients, it quickly became evident that these 2 histological subtypes needed to be clearly separated in our analyses.

Patient	Gene	Detected mutation	Effect on protein	Exon	Functional consequence
1	/	/	/	/	/
2	<i>CDKN2A</i>	c.250G>A	p.(Asp84Asn)	2	Probably pathogenic
	<i>FBXW7</i>	c.1393C>T	p.(Arg465Cys)	9	Pathogenic
	<i>TP53</i>	c.326T>G	p.(Phe109Cys)	4	Probably pathogenic
	<i>TP53</i>	c.661G>T	p.(Glu221*)	6	Probably pathogenic
3	<i>ERBB2</i>	c.2479A>C	p.(Met827Leu)	20	Uncertain significance
	<i>TP53</i>	c.743G>A	p.(Arg248Gln)	7	Pathogenic
	<i>TP53</i>	c.332T>A	p.(Leu111Gln)	26	Probably pathogenic
4	<i>TP53</i>	c.451C>T	p.(Pro151Ser)	5	Probably pathogenic
5	<i>TP53</i>	c.818G>C	p.(Arg273Pro)	8	Probably pathogenic
	<i>TP53</i>	c.41delinsCCTCG	p.(Leu14Profs*16)	2	Probably pathogenic
6	<i>TP53</i>	c.578A>G	p.(His193Arg)	6	Probably pathogenic
	<i>TP53</i>	c.523C>G	p.(Arg175Gly)	5	Probably pathogenic
7	<i>PIK3CA</i>	c.1633G>A	p.(Glu545Lys)	10	Pathogenic
8	/	/	/	/	/
9	/	/	/	/	/
10	/	/	/	/	/
11	<i>TP53</i>	c.742C>T	p.(Arg248Trp)	7	Pathogenic
12	<i>TP53</i>	c.310C>T	p.(Gln104*)	4	Pathogenic
13	/	/	/	/	/
14	/	/	/	/	/
15	<i>TERT</i>	c.-146C>t	Promoter	Promoter	Pathogenic
	<i>TP53</i>	c.743G>A	p.(Arg248Gln)	7	Pathogenic
16	<i>TP53</i>	c.707A>G	p.(Tyr236Cys)	7	Pathogenic
17	/	/	/	/	/
18	/	/	/	/	/
19	<i>PIK3CA</i>	c.1624G>A	p.(Glu542Lys)	10	Pathogenic
20	<i>PIK3CA</i>	c.1633G>A	p.(Glu545Lys)	10	Pathogenic
21	/	/	/	/	/
22	<i>PIK3CA</i>	c.1633G>A	p.(Glu545Lys)	10	Pathogenic
23	/	/	/	/	/
24	/	/	/	/	/
25	/	/	/	/	/
26	Poor DNA quality (high degradation)				
27	Poor DNA quality (high degradation)				
28	Unable to extract sufficient DNA due to the limited amount of remaining material				

Table 3. Mutational landscape of esophageal HPV-positive squamous cell carcinoma.

Focusing initially on ADC, we found that approximately onethird (31.6%) of the samples tested positive for high-risk HPV DNA by PCR. However, transcriptionally active infections were only detected in about half (17.8%) of these cases. Previously described (particularly in oropharyngeal cancers),^{35,36} the use of ultrasensitive PCR-based tests can lead to the detection of inactive HPV infections (or very low viral loads) that may not be biologically relevant. Consistent with this assumption, HPV positivity had no prognostic significance for ADC patients, showed no association with any clinicopathological variable, and was detected in only a minority of cancer cells (typically <20%). This latter observation made through RNA in situ hybridization is, however, of significant interest to the HPV research community. Indeed, it clearly reinforces in vitro data demonstrating that HPV does not solely target mucosal/cutaneous keratinocytes (as is still frequently mentioned in the literature) but can also infect multiple other types of epithelial cells (cancerous or not).³⁷ Although the production of new virions in cells other than keratinocytes remains questionable, our results also suggest that the HPV positivity rates of 10% to 20% observed in other malignancies (eg, breast and bladder cancers) are likely “real” and not, as often considered, related to technical issues such as false-positive samples or contaminations.^{38,39} Regarding the HPV genotypes identified, similar to data collected in the context of cervical ADC, where HPV infection is not incidental but actively contributes to malignancy,¹² HPV16 (66.67%) was the most frequently detected, followed by HPV18 (41.67%). At last, due to the frequent hypermethylation of the *CDKN2A* promoter (>50% of cases) during esophageal ADC development, 40 an important discordance was observed between

p16^{ink4a} expression and HPV DNA/RNA status (Supplementary Fig. S4), underscoring the indirect nature of this biomarker which should be used with relative caution and validated in the context of each specific cancer (sub)type.

The in-depth characterization of a large cohort of locally advanced esophageal SCC led to the unequivocal conclusion that a minority of cases (12.39% in the present study conducted in Western Europe) are etiologically linked to HPV infection. It is important to note that 6 of 28 patients (21.43%) with HPV-positive esophageal SCC had a history of cancer in the oral or pharyngeal region. Although the “coexistence” of esophageal and head and neck neoplasms (potentially due to the phenomenon of field cancerization) was also observed in patients with HPV-negative tumors (Table 2), this may have led to a slight overestimation of the incidence of HPV-positive esophageal SCC in our study population. Indeed, it remains uncertain whether these synchronous or metachronous tumors represent distinct primary malignancies or a single disease process with local metastatic spread. Overall, the true incidence of HPV-positive esophageal SCC in industrialized (high-income) countries likely ranges between 5% and 10%, similar to that observed in other regions of the upper aerodigestive tract (eg, oral cavity, hypopharynx, and larynx), with the notable exception of the oropharynx, where HPV prevalence is significantly higher for reasons that remain unclear. Although a clear confirmation using a multimodal approach is still needed, it appears reasonable to say that the proportion of HPV-driven SCC could be higher in regions such as Eastern Asia and Sub-Saharan Africa, where both HPV infections and esophageal SCC peak. From an anatomical perspective, it is noteworthy that HPV-positive SCC can be observed along the entire length of the esophagus but appears to occur more often in the upper (proximal) and middle parts. Despite 2 false-negative (p16^{ink4} negative/HPV-positive; 2/28, 7.14%) and a few false-positive (p16^{ink4} positive/HPV-negative; 10/198, 5.05%) results, which have also been previously documented in anal and oropharyngeal SCC,^{9,36} the excellent correlation between diffuse p16^{ink4} immunoreactivity and high-risk HPV infection was anticipated. Of note, in 7 cases (of 10), the strong expression of this tumor suppressor gene in the absence of viral infection was attributed to the complete loss of Rb protein (Supplementary Fig. S4), which is linked to a mutation in its gene (previously reported in 5 to 10% of esophageal SCC⁴¹). The unexpected result was that 9 HPV-positive tumors displayed an aberrant p53 staining (Supplementary Fig. S5). Although HPV positivity and TP53 mutation are not mutually exclusive and have been concomitantly observed in anal, cervical, and head and neck SCC specimens,^{8,30,42} the relatively high percentage (9/28, 32.14%) reported in this study is notable. Whether this observation can be attributed to the fact that 6 (of 9) of these HPV-positive SCC patients were active smokers and/or heavy alcohol consumers (lifestyle characteristics that are typically less common in HPV-infected patients compared with their uninfected counterparts) or simply reflects a small sample size bias (the sample size is too small to accurately represent the population of HPV-positive SCC) remains unknown. Although HPV status was not as rigorously defined as in our study (leading to a reported proportion of virus-associated cancers exceeding 40%), a high TP53 mutation rate in HPV-positive esophageal SCC patients was also reported in a recent study conducted in China.⁴³ However, it is noteworthy that demographic and clinicopathological features of HPV-positive esophageal SCC patients did not appear to differ significantly according to TP53 status or p16^{ink4a} expression (Supplementary Tables S1 and S2). Regarding the exclusive detection of HPV16

as well as the frequent basaloid differentiation and the high infiltration of PD-1⁺ cells within the tumor microenvironment of HPV-driven SCC, all these findings closely mimic those reported in both anogenital and oropharyngeal cancers over the past decade.^{8,9,44,45} Furthermore, consistent with a recent study by Sauer et al,⁴⁶ basaloid differentiation per se was not associated with a more/less aggressive behavior (Supplementary Fig. S6). In contrast to tumor differentiation, in agreement with a few smaller studies published in recent years,^{43,47,48} we clearly demonstrated that HPV/p16^{ink4a} - positive status was a robust predictor of favorable outcome in esophageal SCC patients. Similar to their counterparts diagnosed in the oral cavity and anogenital tract, the better prognosis of HPV-positive esophageal SCC is very likely related to their high radio/chemosensitivity explained, in part, by the low tumor hypoxia, the hijacking of host DNA repair pathways by viral E6/E7 oncoproteins as well as the elevated density of tumor-infiltrating (PD-1⁺) T cells.^{21,44,49} Supporting this hypothesis, 8 out of 10 HPV-positive SCC patients (80%), with available treatment response data who received chemoradiotherapy (from a total of 13), demonstrated a complete response to treatment.

In conclusion, there is compelling evidence suggesting that the presence of HPV in esophageal ADC represents fortuitous infections with no significant role in tumorigenesis or patient response to treatment. For the lack of a better alternative, treatment algorithms should continue to be dictated by traditional prognostic factors such as tumor size and nodal status. Conversely, similar to anal and oropharyngeal neoplasms, our findings suggest that a dual classification based on HPV status could/should be considered for esophageal SCC, potentially guiding the personalization and refinement of treatment strategies.

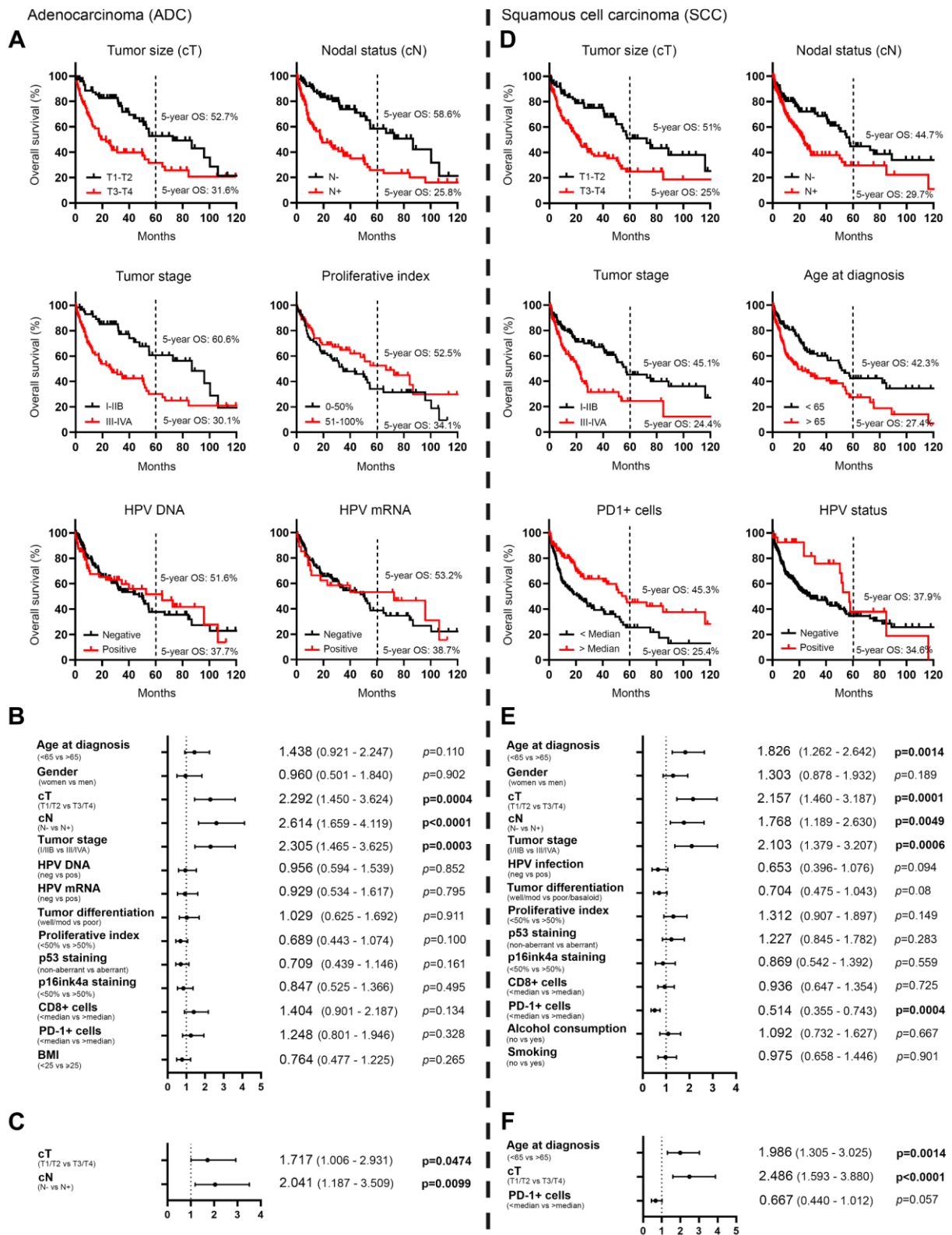


Figure 3. Analysis of prognostic factors associated with overall survival (OS). (A, D) Kaplan-Meier estimates of OS according to significant risk factors reported in univariate analysis (cutoff: $P < .1$). Kaplan-Meier OS curves according to human papillomavirus (HPV) status of tumors are also shown. Prognostic value of clinicopathological variables in univariate (B, E) and multivariate (C, F) analysis.

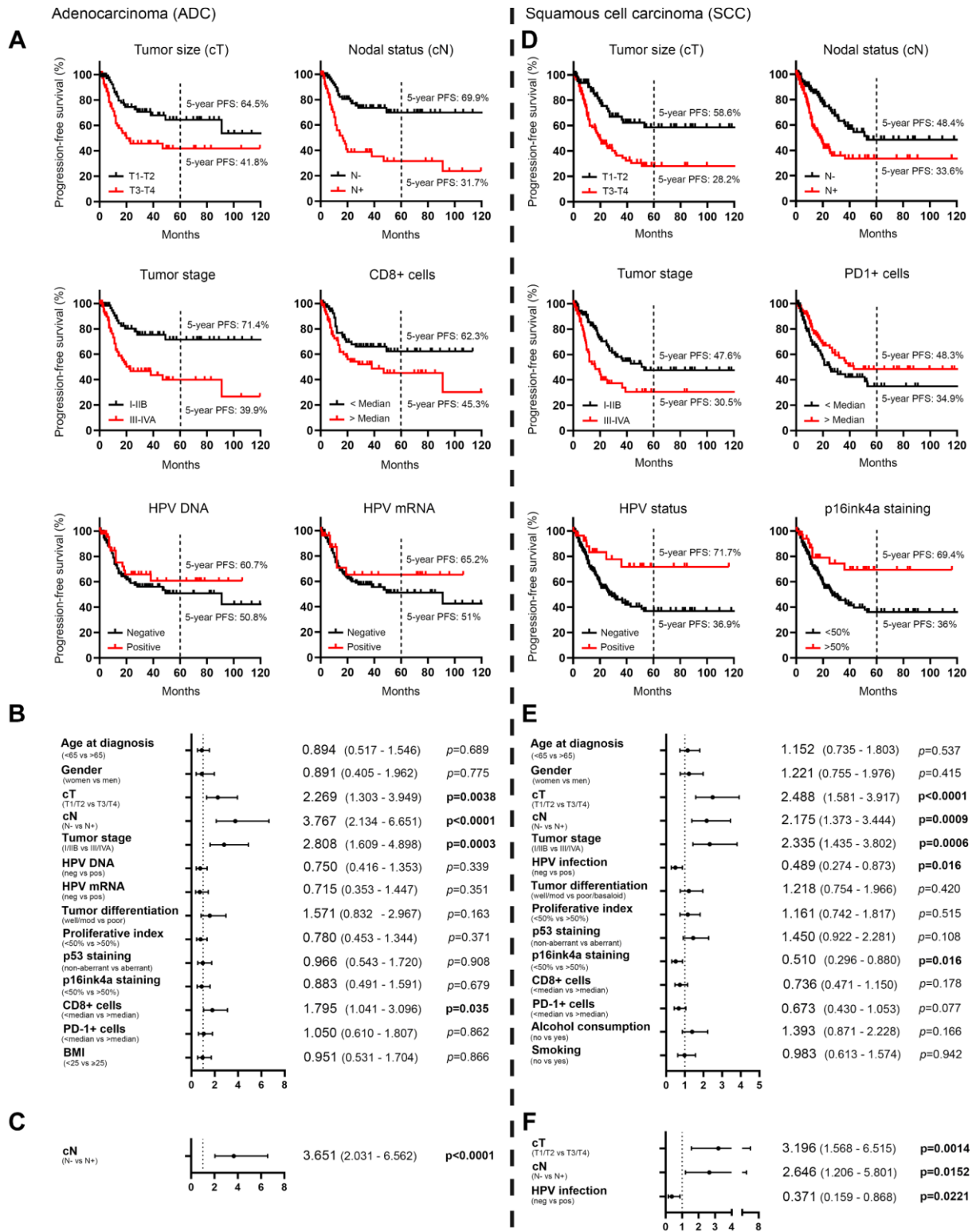


Figure 4. Analysis of prognostic factors associated with progression-free survival (PFS). (A, D) Kaplan-Meier PFS curves according to significant risk factors found in univariate analysis (cutoff: $P < .1$). Kaplan-Meier estimates of PFS according to human papillomavirus (HPV) status of tumors are also shown. Prognostic significance of clinicopathological variables in univariate (B, E) and multivariate (C, F) analysis. Although HPV

positivity has no significance in esophageal adenocarcinoma (ADC), its strong and independent predictive value in squamous cell carcinoma (SCC) patients should be noticed.

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AUTHOR CONTRIBUTIONS

M.H. designed the study; S.G., F.M., L.C., N.P., and T.K. collected clinicopathological data/tissue specimens; C.R., D.B., P.R., R.G., B. K., F.M., E.H., F.P., T.L., S.M., C.P., M.A., M.L., M.R., F.L., T.G., and P.H. performed experiments and/or quantifications; C.R., D.B., P.R., R. G., T.G., and M.H. interpreted the data; C.M., F.B., D.L., F.F., L.d.L., P. D., T.K., and M.H. reviewed the samples; C.R., O.P., and M.H. performed the statistical analysis; C.R. and M.H. generated the figures; M.H. wrote the paper. All authors had final approval of the submitted manuscript.

DATA AVAILABILITY

Most data relevant to this study are included in the article or provided as Supplementary Material online. Additional data are available upon reasonable request.

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DECLARATION OF COMPETING INTEREST

The authors declare no conflicts of interest.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Tissue/tumor samples (archival materials) and patient data used in this study were provided by 5 different University Hospital Centers (CHU of Liege [Belgium], Jules Bordet Institute [Brussels, Belgium], CHU of Besançon [France], CHRU of Tours [France] and CHUV [Lausanne, Switzerland]). The present study (experimental protocol and data collection) was approved by the institutional review board of the respective institutions beforehand (Belgium: #2020/76; France: #F2162-HPVOESO; Switzerland: #2022-00437).

Supplementary Material

The online version contains supplementary material available at <https://doi.org/10.1016/j.modpat.2025.100853>.

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