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HPV Biomarkers in Oral and Blood-Derived Body Fluids in Head and Neck Cancer Patients

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ABSTRACT

Oral HPV DNA and circulating tumor (ct) HPV DNA in plasma were evaluated as potential biomarkers for HPV-associated head and neck cancer (HNC). Samples from HNC patients ($n = 132$), including 23 oropharyngeal cancers (OPC), and non-HNC controls ($n = 10$) were analyzed. HPV status was determined using a multiplex bead-based test (E7-MPG) applied to formalin-fixed paraffin-embedded (FFPE) tissues ($n = 90$), plasma ($n = 141$), gargle samples ($n = 141$), and oral swabs ($n = 142$). HPV DNA was detected in 25.8% of HNC tissues, 12% of plasma samples, 20.6% of gargles and 7% of oral swabs with HPV16 as the most prevalent genotype. Among OPC cases, HPV16 DNA was found in 71.4% of FFPE samples. High concordance was observed between paired OPC tissues and plasma (91.3%) or gargles (95.2%), with moderate concordance for oral swabs (59.1%). Gargle samples alone demonstrated a 100% detection rate for HPV16-positive OPC, regardless of the cT stage, outperforming plasma (86.7%). Combined oral gargle and plasma analyses detected all HPV-positive OPC cases (7/7) at the early cT1 stage. These findings highlight the limited involvement of HPV in non-oropharyngeal HNC compared to OPC, and support gargle and plasma samples as minimally invasive diagnostic tools for detecting HPV-associated OPC.

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1 | Introduction

Head and neck cancers (HNC) comprise several malignancies arising in the oral cavity, oropharynx, larynx, and hypopharynx. These neoplasms are associated with established risk factors such as tobacco, alcohol consumption and human papillomavirus (HPV) infections [1]. High-risk (HR) HPV genotypes—including HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59—are linked to a broad spectrum of human cancers [2], whereas low-risk (LR) genotypes like HPV6 and HPV11, are primarily responsible for benign genital warts [2, 3] and recurrent respiratory papillomatosis [3], a rare but debilitating condition, usually affecting the larynx.

HPV-associated HNC primarily occurring in the oropharynx, where the causal role of HPV is well-established [4]. Globally, the incidence of HPV-associated oropharyngeal cancer (OPC) is rising, [5, 6], with HPV16 detected in approximately 80% or more of these cases [4, 7].

To date, there are no validated screening protocols for the early detection of HPV-associated HNC, which impacts early diagnosis and clinical outcomes. Current methods for determining HPV status in cancer tissues include HPV DNA and E6/E7 mRNA assays, as well as $p16^{INK4a}$ immunohistochemistry (IHC) staining as a surrogate marker of HPV infection [6, 8]. The 8th Edition of the TNM Classification of Malignant Tumors [9] recommends $p16^{INK4a}$ detection alone or in combination with an HPV DNA assay to identify HPV-associated OPC for improved cancer classification and treatment planning.

Beyond traditional HPV diagnostic assays, additional HPV biomarkers traceable in body fluids, such as saliva and blood, are being explored to improve early detection of HPV-associated HNC [10–13]. Advantages of using body fluids over tumor biopsies include: (i) easier integration into diagnostic procedures, (ii) the absence of cancer tissues requirements, and (iii) minimally invasive sample collection.

Among blood-based biomarkers, circulating tumor (ct) HPV DNA has shown promise for the early diagnosis and relapse monitoring of HPV-associated OPC [11, 14–16].

Moreover, studies on oral HPV DNA in saliva have found a higher positivity rate in HNC HPV-associated patients compared to controls [10, 17–20], with some reports [18] detecting oral HPV DNA up to 3.9 years before an OPC diagnosis. Persistent oral HPV DNA positivity following treatment has been linked to poor prognosis and cancer recurrence [20, 21].

To date, the combined analysis of body fluid biomarkers in HPV-associated cancers remains mostly underexplored [12, 22, 23]. Only a limited number of studies have examined the combined analysis of viral biomarkers, but initial findings suggest potential for improved early detection of HPV-associated OPC at both initial diagnosis and recurrence [24, 25]. Therefore, further studies are needed to develop and validate screening methods and diagnostic algorithms based on body fluid biomarkers for early detection of HPV-associated HNC.

In this proof of principle study, we assessed whether HPV DNA positivity in body fluids could predict HPV positivity in OPC tissues and evaluated the effectiveness of each HPV biomarker in body fluids as a minimally invasive tool. These biomarkers could potentially enhance diagnostic algorithms for the early detection of HPV-associated OPC.

2 | Materials and Methods

2.1 | Study Group, Clinical Information, and Biological Samples

The study analyzed formalin-fixed paraffin-embedded (FFPE) tissues, plasma and oral (swabs and gargles) samples collected from 142 patients, referred to the Department of Otolaryngology and Head and Neck Surgery of the European Institute of Oncology, IEO, IRCCS, consecutively enrolled from 2019 to 2022.

Inclusion criteria: (i) age ≥ 18 years, (ii) suspected HN disease (iii) patient with clinical, radiological and/or cyto-histological diagnosis of HNC, and (iv) no previous treatments.

Specimens from 142 patients were analyzed for HPV DNA, including FFPE tissues (available for 90 patients), plasma ($n = 141$), gargles ($n = 141$), and exfoliated oral cells from swabs ($n = 142$) (Figure 1). Matched samples included 140 sets of oral gargles, swabs, and plasma, and 89 sets of oral gargles, swabs, plasma, and FFPE tissues. All cases were clinically (c) staged and, when surgery was performed as treatment, pathologically (p), according to the 8th TNM edition [9].

The study aimed to assess a new methodology as an early diagnostic tool to be applied before treatment. Therefore, clinical staging (cTNM) was used for analysis independently of the final treatment, rather than the pathological one (pTNM), as reported in Supporting Information S1: Table S1. Discrepancies between clinical and pathological staging (e.g., cTumor (T) > pT), and variations in the number of collected biological samples arose from instances where clinical suspicion of cancer was not confirmed by final histological examination (e.g., non-HNC cases). Additional discrepancies occurred in patients receiving radio-chemotherapy without surgery, where pTNM staging was not feasible. Furthermore, the collection of tissues, blood and oral gargle samples from all patients, was hindered by incomplete patient compliance and logical constraints imposed by the COVID-19 pandemic.

2.2 | Plasma and Oral Samples Collection

Ten milliliters of whole blood were collected from each patient into BD Vacutainer K2 EDTA tubes (BD Biosciences, San Jose, CA). For oral gargle specimens, 15 mL of sterile saline solution (0.9%: 9 g NaCl in 1000 mL of sterile water) was swirled in the oral cavity for 15 s and then collected in a 50 mL sterile falcon tube [26].

Exfoliated oral cells were also collected by swabbing the entire buccal surface (e.g, the alveolar ridges, lateral tongue, the tonsillar areas and base of the tongue) using a specific swab

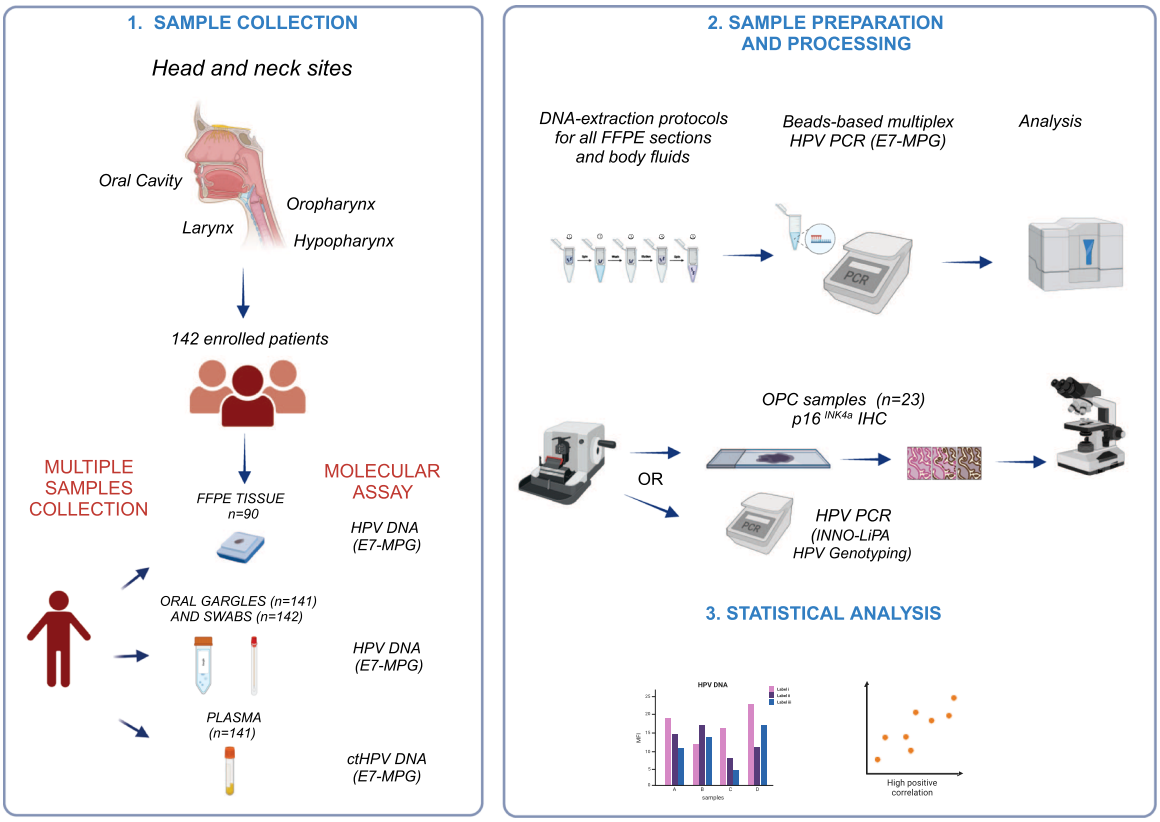


FIGURE 1 | Workflow of applied methodology. Left panel: Description of patients' sample collection and assays used for analysis. Right panel: Description of sample preparation, processing and analysis. Created with Biorender.

(ORAcollect-DNA kit; DNA-genotek Inc.). All samples, including blood, gargle and swab specimens were immediately frozen at −20°C. The collection process was conducted by two clinicians (MT and RDB) (see Supporting Information materials for further details and specifications).

2.3 | DNA Extraction From FFPE Tissues

FFPE tissue blocks (90 samples) were sectioned, as previously described [27] and the DNA was extracted as reported in Supporting Information material [27–29].

2.4 | Circulating Tumor DNA Extraction From Plasma Samples

Circulating cell free DNA was extracted from 500 μL of plasma using the QIAamp circulating nucleic acid kit (Qiagen, Hilden, Germany) as already described [22] (see Supporting Information materials for further details and specifications).

2.5 | DNA Extraction From Oral Samples

DNA from oral gargles and swabs was extracted using the Qiagen BioRobot EZ1 and the EZ1 DNA tissue kit according to instructions (Qiagen, Hilden, Germany) [26] (see Supporting Information materials for further details and specifications).

2.6 | HPV DNA Detection by Bead-Based Genotyping Luminex Assay (E7-MPG)

The E7-MPG assay was applied to analyze HPV DNA from 90 FFPE tissues, 141 plasma, 142 swabs, and 141 gargles as previously reported [30]. This well-validated molecular assay, which combines multiplex PCR and Luminex bead-based technology (Luminex Corp., Austin, TX, USA) using type specific primers, detects HR HPV genotypes [30–32] (see Supporting Information materials for further details and specifications).

2.7 | $P16^{INK4a}$ Immunohistochemical (IHC) Staining and HPV DNA Genotyping by INNO-LiPA HPV Genotyping Assay in OPC Samples

Since no universally accepted gold standard exists for assessing HR HPV in FFPE tissues, two commercial tests, widely applied in diagnostic settings of HPV-associated cancers, were employed to analyze the 23 OPC samples and evaluate their concordance with the E7-MPG molecular assay in identifying HPV-associated OPC. Unstained sections from each FFPE block, prepared as previously described [27], were processed for Hematoxylin and Eosin (H&E) and $p16^{INK4}$ staining [33]. Both assays were used as diagnostic tests to stratify HPV-associated OPC, as described in Table 1 and in Supporting Information S1: Table S2 (see Supporting Information materials for further details and specifications).

TABLE 1 | Pattern of direct (HPV DNA detection by INNO-LiPA genotyping or by beads based-Luminex assay E7-MPG) and surrogate ($p16^{INK4a}$ IHC) HPV biomarkers in matched oral, plasma, and tissues FFPE samples from 23 OPC patients.

				E7-MPG assay				IHC and HPV genotypingassay	
Sex	OPC subsite	Smoking packets/ year (p/y) [34]	cT Stage VIII ed [9].	FFPE tissue HPV DNA (MFI)	Plasma ctHPV DNA (MFI)	Gargle HPV DNA (MFI)	Oral swab HPV DNA (MFI)	$P16^{INK4a}$ IHC	HPV genotyping assay INNO-LiPA
M	Tonsil	Former 12 p/y	1	HPV16 (1685)	0	HPV16 (2236) HPV51 (23) HPV66 (637)	HPV66 (488)	Positive	
M	Tonsil	No	1	HPV16 (1028)	HPV16 (2600)	HPV16 (1549)	0	Positive	
M	Tonsil	No	2	HPV16 (1779)	HPV16 (1734)	HPV16 (1107) HPV58 (387) HPV68 (33)	0	Positive	
M	Tonsil	No	2	HPV16 (2122)	HPV16 (2307)	HPV16 (2510)	0	NA	HPV16 DNA positive
M	Tonsil	Former 12 p/y	1	HPV16 (2147)	HPV16 (1747)	HPV16 (1374)	0	Positive	
M	Tonsil	Former 12 p/y	1	HPV16 (2900)	HPV16 (1152)	HPV16 (2746) HPV39 (53)	0	Positive	
M	Tonsil	No	2	NA	HPV16 (2132)	HPV16 (2074) HPV33 (270)	0	Positive	
M	Tonsil	No	1	NA	0	HPV16 (2246)	0	Positive	
M	Tonsil	Former 20 p/y	1	NA	HPV16 (2226)	HPV68 (135)	0	Positive	
M	Tonsil	No	1	HPV16 (1826)	HPV16 (1184)	HPV16 (1362)	0	Positive	
M	Base of tongue	Former 12 p/y	3	HPV16 (2014)	HPV16 (2748)	HPV16 (879)	HPV16 (2380)	Positive	
M	Base of tongue	Yes 20 p/y	2	HPV16 (949)	HPV16 (2727)	HPV16 (1132)	HPV16 (1970)	Positive	
F	Tonsil	Former 30 p/y	1	NA	HPV16 (2080)	HPV16 (975) HPV70 (88)	HPV16 (2071) HPV70 (155)	Positive	
M	Base of tongue	Former 40 p/y	2	NA	HPV16 (2482)	HPV16 (1694) HPV51 (3)	HPV16 (1573)	Positive	
M	Base of tongue	No	4a	HPV16 (1760)	HPV16 (1677)	HPV16 (1823)	HPV16 (1457)	Positive	
F	Tonsil	Former 12 p/y	3	NA	0	HPV16 (8)	0	Negative	

(Continues)

TABLE 1 | (Continued)

				E7-MPG assay				IHC and HPV genotypingassay	
Sex	OPC subsite	Smoking packets/ year (p/y) [34]	cT Stage VIII ed [9].	FFPE tissue HPV DNA (MFI)	Plasma ctHPV DNA (MFI)	Gargle HPV DNA (MFI)	Oral swab HPV DNA (MFI)	$P16^{INK4a}$ IHC	HPV genotyping assay INNO-LiPA
M	Base of tongue	Former 40 p/y	1	NA	0	HPV56 (11)	0	Negative	
M	Tonsil	Yes 35 p/y	3	0	0	0	0	Negative	
M	Base of tongue	Yes 51 p/y	4a	0	0	0	0	Negative	
M	Base of tongue	No	3	NA	0	0	0	NA	HPV16 DNA negative
M	Tonsil	Yes 40 p/y	3	NA	0	0	0	Negative	
M	Tonsil	No	4b	0	0	0	0	Negative	
M	Tonsil	No	3	0	0	0	0	Negative	

2.8 | Statistical Analysis

HPV DNA prevalence was estimated as the proportion of FFPE tissue, plasma, and oral samples that tested positive for any HPV DNA genotype by multiplex PCR E7-MPG assay with corresponding binomial 95% confidence intervals (CIs). Demographic data were tabulated in percentage by HPV biomarkers and their combinations. Fisher's exact test with two-sided p-value < 0.05 was considered statistically significant.

The concordance of HPV16 DNA status determined by E7-MPG in body fluids with that of HPV status in FFPE OPC tissues, analysed by $p16^{INK4a}$ IHC or INNO-LIPA HPV genotyping tests, was estimated by means of the Cohen's kappa coefficient with corresponding 95% CI. To assess the level of agreement between HPV16 biomarkers in body fluids and in OPC FFPE tissues, interpretation of the Cohen's kappa statistics was established as follows: (i) < 0: poor, (ii) 0–0.20: slight, (iii) 0.21–0.40: fair, (iv) 0.41–0.60: moderate, (v) 0.61–0.80: substantial, (vi) 0.81–1.0: almost perfect [35].

Since a suggested reference test to identify HPV-associated HNC outside the oropharynx site has yet to be determined [6, 36, 37], the overall concordance between plasma, gargles and oral swabs versus FFPE tissues for HPV16 DNA detection in the other HNC sites (non-oropharynx) was evaluated by HPV DNA detection (E7-MPG assay).

Statistics was performed with GraphPad Prism (Version 10.1.1) and GraphPad online version (https://www.graphpad.com).

3 | Results

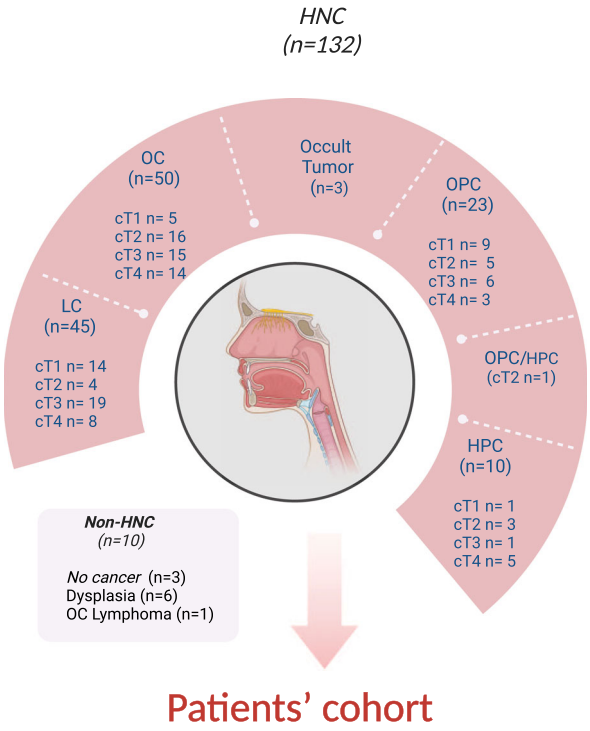
3.1 | Characteristic of the Patients' Cohort

A total of 142 patients were enrolled in the study, comprising 39 females (27.5%) and 103 males (72.5%). The mean age at diagnosis was 63.4 (SD ± 11) with females (mean, SD 67.4 ± 13) being older than males (mean, SD 61.9 ± 10).

Among the 142 patients, 43 (30%) were non-smokers, 52 (37%) were ex-smokers who had quit for at least 12 months before diagnosis and 47 (33%) were active smokers. Among the active smokers, 2 (2.1%) declared less than 10 packs/year (p/y) [34], 3 (6.4%) smoked between 10 and 20 p/y and 43 (91.5%) were heavy smokers with a smoking history of more than 20 p/y. In the group of the former smokers, 1 (2%) smoked less than 10 p/y, 12 (23%) between 10 and 20 p/y and 39 (75%) referred to more than 20 p/y.

Of the 142 patients, 132 were diagnosed with HNC, while 10 were non-HNC cases, which included squamous intraepithelial neoplasia (SIN) III/carcinoma in situ of the larynx ($n = 1$), dysplasia (SIN I–II) from the oral cavity ($n = 1$), oropharynx ($n = 1$) and larynx ($n = 3$), oral cavity lymphoma ($n = 1$), and non-cancer cases ($n = 3$). Figure 2 represents the patients' cohort.

Among the 132 HNC cases, 37.9% ($n = 50$) were oral cavity cancers, 34% ($n = 45$) laryngeal cancers, 17.4% ($n = 23$) OPCs, 7.5% ($n = 10$) hypopharyngeal cancer, 0.7% ($n = 1$) was



HNC: Head and neck cancer;OC: Oral cavity cancer;LC: Laryngeal cancer;OPC: Oropharyngealcancer;HPC: Hypopharyngeal cancer.

FIGURE 2 | Patient cohort and stratification of HNC cases by cancer sites and clinical tumor (cT) stages. Abbreviations are specified within the figure. Created with Biorender.

oropharyngeal/hypopharyngeal cancer and 2.3% ($n = 3$) were occult tumors (Figure 2).

These HNC cases ($n = 132$) were classified into different T stages according to the 8th TMN edition [9] and were staged both clinically and pathologically when possible (Supporting Information S1: Table S1).

Regarding the 23 oropharyngeal tumors, 7 were located at the base of the tongue, and 16 on the lateral wall (tonsil), as shown in Table 1. Examining smoking habits among the 23 OPC patients, 10 (43.5%) were non-smokers, 9 (39.1%) were ex-smokers who had quit smoking for at least 12 months before diagnosis, and 4 (17.4%) were active smokers (Table 1). Among the active smokers, all 4 (100%) patients reported a smoking history of more than 20 p/y [34] (Table 1).

3.2 | HPV DNA Prevalence in FFPE Tissues by Beads-Based Genotyping Luminex Assay (E7-MPG)

A total of 90 available FFPE tumor tissue samples were retrieved and analyzed for HPV DNA. One sample was beta-globin negative and was excluded. HPV positivity was detected in 25.8% (23/89) of the remaining samples (Tables 2 and 3). Overall, HR HPVs were found in the majority of positive cases (91.3%, $n = 21/23$). Among the HR HPVs, HPV16 DNA was identified in 22.4% of cancer specimens (20/89, 95% CI: 14.97–32.25) (Figure 3A). Specifically, HPV16 DNA was detected in 10 OPCs (5 cT1, 3 cT2, 1 cT3, and 1 cT4a), 5 oral cavity cancer (1 cT2, 3 cT3, and 1 cT4a), 4 laryngeal cancer (cT1, cT2, cT3, and cT4, one each) and 1 in hypopharyngeal cancer (cT2) (Figure 3A).

Additionally, three other genotypes, HPV18, 6 and 68 were detected in FFPE tissue specimens from oral cavity and larynx (Figure 3A). However, none of these genotypes was found positive by PCR in other biological samples, such as plasma or oral samples.

3.3 | ctHPV DNA Prevalence in Plasma by Beads-Based Genotyping Luminex Assay (E7-MPG)

A total of 141 plasma samples were analyzed for ctHPV DNA. All samples tested positive for beta-globin, confirming the successful DNA extraction and PCR amplification.

Among these, 124 out of 141 (88%) plasma samples were negative for HPV DNA, while 12% were HPV-positive (17/141; 95% CI: 7.57–18.55) (e.g., HPV16 and HPV35), as shown in Tables 2 and 3 and Figure 3B. All HPV-positive plasma samples presented single infections, exclusively with HR HPV genotypes. HPV16 was the predominant genotype detected in 16 out of 17 HPV-positive plasma samples. The majority of HPV16 ctDNA-positive plasma samples ($n = 13/16$; 81.2%) were from OPC patients. Among these, six were classified as cT1 ($n = 6/13$ 46.1%), five were cT2 (38.4%), one cT3 and one cT4a (7.7% each). The remaining three HPV16 ctDNA-positive plasma samples (18.8%) were collected from non-oropharyngeal sites (one hypopharyngeal, one laryngeal and one unknown primary tumor cancers (Tables 2 and 3 and Figure 3B).

TABLE 2 | Prevalence of any HPV DNA determined by beads-based Luminex multiplex PCR (E7-MPG) in oropharyngeal and non-oropharyngeal head and neck sites.

		Site					
		OP		non-OP		Occult tumor (Tx)	
HPV biomarker	Tested samples *N*	Positive *n* (%)	95% CI	Positive *n* (%)	95% CI	Positive *n* (%)	95% CI
ctHPV DNA (plasma)	141	13 (9.2%)	5.3–15.2	3 (2.1%)	0.45–6.35	1 (0.7)	0.01–4.3
Oral HPV DNA (gargle)	141	17 (12.1%)	7.5–18.5	12[a] (8.5%)	4.8–14.4	—	—
Oral HPV DNA (swab)	142	6 (4.2%)	1.75–9.11	4 (2.8%)	0.86–7.26	—	—
HPV DNA FFPE tissue	89[b]	10 (11.2%)	6.04–19.6	13 (14.6%)	8.6–23.5	—	—

[a]Oral cavity: $n = 6$, larynx: $n = 4$, of which 1 was dysplasia case; hypopharynx: $n = 2$.
[b]One FFPE tissue beta-globin negative sample was excluded from the analysis.

3.4 | HPV DNA Prevalence in Oral Specimens (Gargles and Swabs) by Beads-Based Genotyping Luminex Assay (E7-MPG)

A total of 141 gargle samples and 142 oral swabs were analyzed for the presence of HPV DNA. All samples tested positive for beta-globin amplification confirming adequate DNA quality.

HPV DNA was detected in 29 out of 141 gargle samples (20.6%, 95% CI: 14.67–28.02) (Table 2). Among these, 22 were single infections and 7 were multiple HPV infections.

Overall, HR HPV genotypes were prevalently identified (8 out of 14 HPV types) (Figure 3C), either as single or multiple infections. Details of the detected HPV genotypes are provided in Figure 3C for gargles and Figure 3D for oral swab samples.

HPV16 was by far the most prevalent type, found in 19 out of the 29 HPV-positive gargle specimens (65.5%), either alone ($n = 12$) or in coinfection with other HPV types ($n = 7$) (Figure 3C). By anatomic site, the majority of HPV16 positive gargle samples were found in OPC ($n = 15$) (Figure 3C). These were distributed across clinical stages cT1 ($n = 7$), followed by cT2 ($n = 5$), cT3 ($n = 2$), and cT4a ($n = 1$) (Table 1). The remaining HPV16 positive samples ($n = 4$) were detected at oral cavity ($n = 2$; cT1 and cT3), hypopharyngeal (cT2), and laryngeal (cT4a) cancers.

In addition to HPV16, single HPV infections with HPV6, 11, 31, 45, 51, 53, 58, and 68 were each detected in one sample, while HPV56 was found in two samples. Some HPV genotypes (e.g., 51, 58, and 68) were also detected in multiple HPV infections (Figure 3C).

For oral swab samples, 93% tested negative for any HPV type ($n = 132/142$) while 7% ($n = 10/142$, 95% CI: 3.73–12.62) were HPV DNA-positive (Tables 2 and 3). Among the positive samples HPV16 was identified in 70%, either as single infections ($n = 5$) or multiple infections ($n = 2$) (Figure 3D). Multiple infections involved HPV16/HPV70 and HPV16/HPV56. The coinfection HPV16/HPV70 in the oral swab was also identified in the corresponding paired gargle sample. HPV DNA of non-HPV16 types, namely HPV58, 66 and 70, were identified in swab specimens, with HPV58 and 66 also detected in the corresponding paired gargle specimens. The majority of HPV-positive oral swab samples were from OPC patients ($n = 6$) (Figure 3D), staged as cT1 ($n = 2$), cT2 ($n = 2$), cT3 ($n = 1$), cT4a ($n = 1$) (Table 1). The remaining HPV-positive swab samples ($n = 4$) were from oral cavity cancer (cT3), two hypopharyngeal (cT2 and cT4a) and one laryngeal (cT4a) cancers.

3.5 | Concordance of HPV16 DNA Between FFPE Tissues and Body Fluids in OPC and non-OPC

The agreement between the E7-MPG and the commercial tests ($p16^{INK4a}$ or HPV DNA) applied to FFPE OPC tissues for HPV16 detection was 100%, $k = 1$ (14/14, 95% CI: 100-100) (Supporting Information S1: Table S2).

The overall concordance for HPV16 detection between plasma ctHPV DNA tested with E7-MPG and FFPE OPC tissues, tested with $p16^{INK4a}$ or INNO-LiPA HPV assay, was 91.3%, $k = 0.81$ (21/23, 95% CI: 58.3–100). The sensitivity and specificity of ctHPV DNA in detecting HPV16 in plasma samples were 86.7% and 100%, respectively.

The concordance for HPV16 detection between oral gargles and FFPE OPC tissues tested by commercial assays, was 95.2%, $k = 0.88$ (20/21, 95% CI: 67.8–100), while the sensitivity and specificity in detecting HPV16 were 100% and 85.9%, respectively (Supporting Information S1: Table S2).

However, the concordance between oral swabs and FFPE OPC tissues for HPV16 detection was notably lower at 59.1%, $k = 0.28$ (13/22, 95% CI: 37–53.9), with sensitivity and specificity in detecting HPV16 of 35.7% and 100%, respectively (Supporting Information S1: Table S2).

Excluding OPC cases, the reported overall concordance rates for HPV16 DNA detection, matched to FFPE HNC tissues, were 87.3% for plasma, 87.7% for oral gargles, and 88.8% for oral swab (Supporting Information S1: Table S3). Analyzing their performance, the sensitivity and specificity of ctHPV DNA in detecting HPV16 were 10.0% and 100%, respectively (Supporting Information S1: Table S2). Sensitivity and specificity for HPV16 detection between oral gargle and FFPE tissues were 22.2% and 98.2%, respectively (Supporting Information S1: Table S2).

TABLE 3 | Characteristics of enrolled head and neck cancer patients ($n = 132$) stratified by sex, age, and anatomical sites, oropharyngeal cancer (OPC) and non-oropharyngeal cancer (non-OPC) head and neck cancer site according to HPV marker detected by beads-based Luminex assay (E7-MPG).

		Sex			Age			Site		
	HPV status	**M (*n*)**	**F (*n*)**	***p* value**[a]	**≤ 60 (*n*)**	**> 60 (*n*)**	***p* value**[a]	**OPC (*n*)**	**Non-OPC**[b] **(*n*)**	***p* value**[a]
HPV DNA marker										
ctHPV DNA (plasma)	Negative (n)	81	34	0.04	40	75	0.06	10	105	< 0.001
	Positive (n)	16	1		10	7		13	4	
Oral HPV DNA (gargle)[c]	Negative (n)	72	31	0.14	37	66	0.38	6	97	< 0.001
	Positive (n)	24	4		13	15		17	11	
Oral HPV DNA (swab)	Negative (n)	89	33	1.00	45	77	0.50	17	105	0.002
	Positive (n)	8	2		5	5		6	4	
HPV DNA FFPE Tissue[d]	Negative (n)	46	20	0.01	26	40	0.80	4	62	< 0.001
	Positive (n)	22	1		10	13		10	13	

Abbreviation: OPC, oropharyngeal cancer.
[a]Two-sided Fisher's Exact test.
[b]Occult tumor has been included in the non-OPC group.
[c]One gargle sample not available.
[d]One FFPE Tissue beta-globin negative sample has been excluded from the analysis.

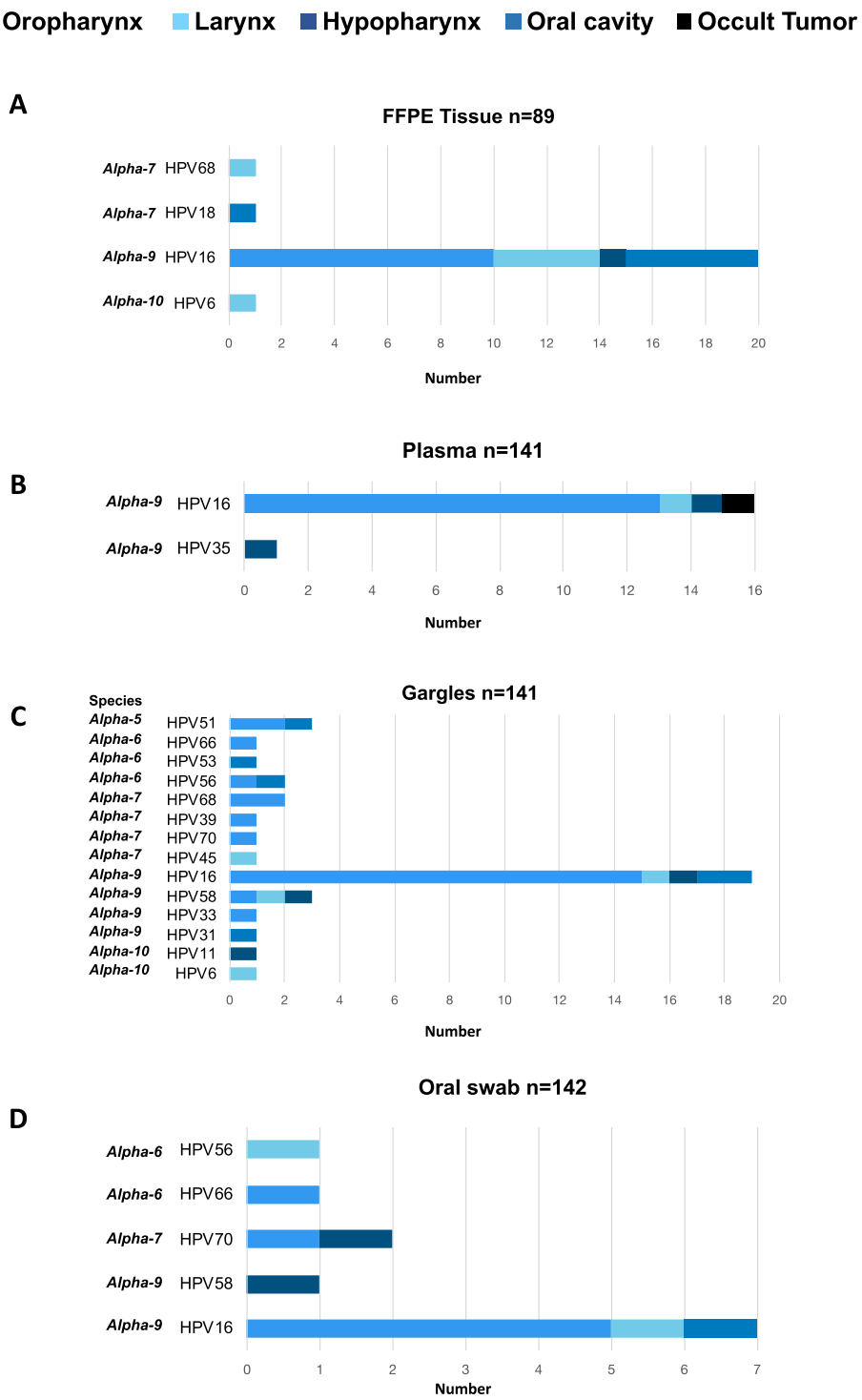


FIGURE 3 | Distribution of HPV genotypes detected by beads-based Luminex assay (E7-MPG) stratified by specimen types. (A) HPV genotypes in FFPE tissues. (B) HPV genotypes detected in plasma samples. (C) HPV types detected in gargles and (D) HPV genotypes detected in oral swabs.

Finally, the sensitivity and specificity of HPV16 between oral swab and FFPE tissues were 20.0% and 100%, respectively (Supporting Information S1: Table S2).

The majority of HPV DNA-positive plasma and oral samples, including any HPV genotype, were found in OPC patients: 76.5% ($n = 13/17$ total positives), 58.6% ($n = 17/29$ total positives), and 60% ($n = 6/10$ total positives) considering plasma, gargles and oral swab samples, respectively (Table 2). In OPC patients ($n = 23$), HPV16 was detected by E7-MPG in 21.7% ($n = 5/23$), 56.5% ($n = 13/23$), and 65.2% ($n = 15/23$) of oral swabs, plasma, and gargle specimens, respectively (Table 1). Thirteen out of 23 plasma samples from OPC patients (56.5%) tested positive for ctHPV16 DNA, and were staged as cT1 (6/9; 66.6%), cT2 (5/5; 100%), cT3 (1/6; 16.6%), and cT4 (1/3; 33.3%) (Table 1).

Thirteen out of 23 plasma samples from OPC patients (56.5%) tested positive for ctHPV16 DNA, and were staged as cT1 (6/9;

TABLE 4 | HPV biomarker combinations using detection of HPV16 DNA in plasma and oral samples (gargles) by beads-based Luminex assay (E7-MPG) multiplex PCR in oropharyngeal cancer (OPC) specimens according to cT stage.

HPV16 biomarkers in oral gargle and plasma	cT1 $n = 7$ (%)	cT2 $n = 5$ (%)	cT3 $n = 6$ (%)	cT4 $n = 3$ (%)	Total $n = 21$ (%)
Oral HPV16 DNA positive/ctHPV16 DNA positive	5 (71.4)	5 (100)	1 (16.7)	1 (33.3)	12 (57.1)
Oral HPV16 DNA positive/ctHPV16 DNA negative	2 (28.6)	—	1[a] (16.7)	—	3 (14.3)
Oral HPV16 negative/ctHPV16 DNA negative	—	—	4 (66.6)	2 (66.7)	6 (28.6)
Total	7 (100)	5 (100)	6 (100)	3 (100)	**21** (100)

Note: For this HPV biomarker combination, HPV DNA positive for genotype other than HPV16 were excluded. This combined analysis is based on data on OPC cases reported in Table 1.
Abbreviation: OPC, oropharyngeal cancer.
[a]One sample was $p16^{INK4a}$ negative in FFPE tissue counterpart, however, the HPV16 DNA MFI value, detected by beads-based Luminex assay (E7-MPG) multiplex PCR in oral gargle was very low (MFI = 8).

66.6%), cT2 (5/5; 100%), cT3 (1/6; 16.6%), and cT4 (1/3; 33.3%) (Table 1).

Of the gargle samples, 73.9% (17/23) showed HPV DNA positivity for any HPV type in samples from OPC patients, of which 88.2% (15/17) were HPV16-positive and stratified as follows: cT1: 77.7% (7/9), cT2: 100% (5/5), cT3: 33.3% (2/6), and cT4a 33.3% (1/3) (Table 1).

Regarding the oral swab samples, 26% (6/23) tested positive for any HPV genotype. Overall, 83.3% (5/6) were HPV16-positive and from patients with tumor stages cT1 (1/9), cT2 (2/5), cT3 (1/6), and cT4a (1/3) (Table 1).

Considering only HPV16, gargles alone detected all cT1 HPV16-positive OPC (100%, $n = 7/7$), compared to plasma alone 71.4% ($n = 5/7$) (Table 4). The combined analysis of ctHPV16 DNA in plasma and HPV16 DNA in gargles improved sensitivity to 100% ($n = 7/7$) for cT1.

When including any HPV genotype, the combined approach achieved a 100% detection rate for cT1 cases ($n = 8/8$), outperforming gargles alone (87.5%, 7/8) and plasma alone (75%, 6/8) (data calculated from Table 1).

4 | Discussion

Currently, no validated screening protocols exist for detecting HPV-associated HNC, which could significantly enhance early diagnosis and improve clinical outcomes. Notably, an increasing incidence of HPV-associated OPC has been observed in several high-income countries (HIC), predominantly among white-Caucasian males [4, 5, 38].

Geographical and demographic variations in HPV- associated OPC patients are apparent, particularly between Europe (EU) and the United States (US). European patients with HPV-associated OPC tend to be older and more likely to have a history of heavy smoking compared to their US counterparts [39, 40]. These factors, such as age and smoking are associated with poorer overall health, increased comorbidities, and more complex prognoses. In contrast, younger, less-smoking-prone US patients often experience better outcomes, likely due to fewer underlying health issues and a stronger immune response [41]. Additionally, smoking can reduce the effectiveness of treatments like radiotherapy, a common modality treatment for these patients, highlighting the need for tailored diagnostic and treatment protocols to address regional and lifestyle differences [41].

Despite the growing need for early identification of HPV-associated HNC, a consensus on diagnostic tests and screening protocols remains absent worldwide.

In recent years, several non-invasive HPV biomarkers, such as ctHPV DNA, oral HPV DNA and E6 antibodies have been explored as diagnostic tools for early diagnosis of HPV-associated HNC [23, 36, 38, 42]. These biomarkers offer significant advantages over tumor biopsies, including ease of collection, reduced invasiveness, and no requirement for tumor tissues.

In this study the potential of combining ctHPV DNA in plasma with oral HPV DNA from gargle and swab samples to detect HPV-associated HNC was evaluated.

Using a highly sensitive bead-based multiplex HPV genotyping assay, an HPV16 prevalence of 22.4% in HNC FFPE tissues was identified. Other studies have reported a higher prevalence in OPC, such as 40% in our previous investigation [43].

Regarding ctHPV DNA in plasma, HPV16-positive ctDNA was predominantly detected in OPC cases, representing 81.2% of ctHPV16-positive plasma samples. By contrast, only a minor fraction of HPV16 ctDNA-positive samples was found in occult tumors (6.2%) and hypopharyngeal and laryngeal cancers (12.5%). These findings are consistent with prior studies demonstrating the utility of ctHPV DNA in diagnosing and monitoring HPV-associated OPC recurrence [11, 14, 44].

Considering oral HPV DNA detection, gargle samples revealed an HPV prevalence of 20.6%, with HPV16 being the predominant genotype. Among HPV-positive OPC cases, 88.2% were HPV16 positive.

A previous follow-up study indicated that oral HPV16 DNA detection was associated with a 7.1-fold increase in the likelihood of HNC presence and with a 22.4-fold increase specifically for OPC [18]. A separate study reported 79.1% sensitivity of oral HPV test when $p16^{INK4a}$ IHC was used as the reference [45] also recently confirmed by Tang et al. [46]

Oral swabs showed lower sensitivity compared to gargles, likely due to: (i) operator dependence, (ii) variability in patient compliance and gag reflex, and (iii) difficulty accessing the oropharyngeal tumor site. Gargles, in contrast, offered a standardized and effective procedure, collecting saliva throughout the entire oral cavity and oropharynx using a 15 s rinse with a15 mL 9%-saline solution. Different protocols for gargles collection, which include gargling durations, volumes of solution or different solutions for conservation have been applied across epidemiological studies [26, 47].

Currently, it is not clear yet whether the combination of blood and oral HPV biomarkers could improve early detection of HPV-associated HNC. In OPC patients, around 90% of HPV16 DNA-positive plasma and gargle samples matched the HPV status of corresponding tumor tissues in this study.

Gargle samples alone demonstrated 100% sensitivity in detecting HPV16 DNA-positive OPC, outperforming plasma samples (86.7%). Including infection by genotype other than HPV16, combining gargle and plasma samples improved sensitivity to 100% for HPV16-positive OPC at early stages, compared to 87.5% for gargles alone and 75% for plasma alone.

Ahn et al. [25] reported sensitivities of 52.8% and 67.3%, for HPV16 DNA detection in saliva and plasma, respectively. Combining saliva and plasma increased sensitivity to 76% and specificity to 100%. Therefore, a combination of multiple samples can be a useful tool to identify patients with HPV-associated OPC [25].

The usefulness of a combined assays was also recently highlighted by Lewis et al. [48], who underlineed improved sensitivity by combining HPV serology with ctHPV DNA detection using ddPCR.

Of note, in two early-stage (cT1) cases, plasma samples were ctHPV16-negative, while gargle samples were positive. This discordance may be due to the absence or low release of ctHPV16 DNA in the bloodstream, as previously reported in several HPV-associated tumors [22, 49], possibly due to the small tumor size in early stage.

These data and other recent studies [48–50], suggest that non-invasive—or minimally invasive—tools, such as oral and plasma-based HPV biomarkers could complement current diagnostic strategies and improve the early detection of HPV-associated HNC [49, 50].

This study has several limitations. The relatively small cohort size and limited number of HPV-associated cases reduce the statistical power and generalizability of the findings. FFPE tissue was only available for a subset of patients, potentially limiting comprehensive comparisons across all samples. Additionally, the small number of HPV-associated cases precluded a detailed analysis of smoking status and specific oropharyngeal subsites in relation to HPV positivity, limiting insights into these factors' roles.

Future studies should address these limitations by including larger, more diverse cohorts and ensuring sufficient representation of HPV-associated cases. Further investigation into the impact of smoking and specific anatomical subsites on HPV positivity is warranted. Innovative biomarkers could enhance detection sensitivity, enabling earlier diagnosis and better stratification of HPV-associated HNC.

5 | Conclusion

These findings emphasize the limited role of HPV in non-oropharyngeal HNC compared to OPC.

Gargles appeared more sensitive than plasma for HPV16 DNA detection, particularly in early-stage OPC. Combining plasma and gargle assays enhanced sensitivity, reaching 100% for HPV16-positive OPC at early stages. However, further validation studies are crucial before these viral biomarkers can be implemented for early diagnosis and disease monitoring of HPV-associated OPC. Future efforts should also aim to develop comprehensive diagnostic algorithms that integrate these biomarkers into HNC routine clinical practice.

Author Contributions

Conceptualization and original draft preparation: Susanna Chiocca, Tarik Gheit, Maria Lina Tornesello, Luisa Galati and Marta Tagliabue. Supervised the study: Tarik Gheit, Mohssen Ansarin, and Susanna Chiocca. Methodology and analysis: Massimo Tommasino, Luisa Galati, Tarik Gheit, Sandrine McKay-Chopin, and Fausto Maffini. Study Design: Massimo Tommasino, Susanna Chiocca, Maria Lina Tornesello, Mohssen Ansarin, Luisa Galati, Giuseppe De Palma, Stefania Vecchio, Angelo Virgilio Paradiso, Laura Sichero, Luisa Lina Villa, and Giovanni Blandino. Patients enrollment and follow up: Marta Tagliabue, Rita De Berardinis, Francesco Chu, Francesco Bandi, Chiara Mossinelli, Jacopo Zocchi, Giacomo Pietrobon, Stefano Filippo Zorzi, Enrica Grosso, Stefano Riccio, Roberto Bruschini, and Gioacchino Giugliano. Clinical specimens (saliva, tissues and blood samples) collection and data curation: Marta Tagliabue and Rita De Berardinis. Draft revision and editing: Marta Tagliabue, Susanna Chiocca, Luisa Galati, Rita De Berardinis, Tarik Gheit and Maria Lina Tornesello. All authors have reviewed and approved the manuscript.

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Disclosure

Where authors are identified as personnel of the International Agency for Research on Cancer/World Health Organization and of the IRCCS Istituto Tumori "Giovanni Paolo II", Bari, the authors alone are responsible for the views expressed in this article and they do not necessarily represent the decisions, policy, or views of the Institute.

Ethics Statement

Ethical approval was obtained from IEO Ethical Committee (code IEO 1572), Milan, Italy.

Consent

All the included patients signed the informed consent.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All relevant data that support the findings of this study are included in the manuscript and are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.