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Development and validation of new biomarkers for the early diagnosis and personalised management of patients with HPV-related head and neck cancers

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ABSTRACT

The escalating global incidence of Human Papillomavirus (HPV) related Head and Neck Squamous Cell Carcinoma (HNSCC) highlights the urgent need for improved diagnostic and surveillance tools, especially given the absence of screening programs. Liquid biopsies, such as blood and saliva, are a good source of circulating tumour DNA, which contains tumour-specific genetic information which are promising for HPV-related HNSCC screening programs and post-treatment surveillance. Unfortunately, the lack of a standardised workflow from pre-analytical to analytical stages for the use of liquid biopsy in HPV testing for HNSCC has hampered consistent and reliable diagnostic outcomes.

This thesis aimed to address these critical gaps by contributing to the technical development and validation of the OncoPredict HPV Quantitative Typing assay. Its primary objectives included assessing the assay's ability to detect and quantify HPV DNA in blood and saliva samples from patients with HPV-related HNSCC, evaluating its accuracy for quantifying HPV 16 viral loads against digital droplet PCR (ddPCR), and assessing its clinical performance for post-treatment monitoring of disease recurrence or relapse among oropharyngeal squamous cell carcinoma (OPSCC) patients. Additionally, the thesis aimed to evaluate the molecular target CCR5 for reliable normalisation of viral load in saliva samples.

The work involved a clinical, prospective, multicentre, non-profit study (IDENTIFY study), 74 patients with newly diagnosed HNSCC were enrolled, with 67 patients (51/67 (76.1%) had OPSCC) included in the final analysis. Blood and saliva samples were collected at baseline and during longitudinal follow-up. The OncoPredict HPV QT assay demonstrated high analytical accuracy and sensitivity, showing moderate to strong concordance with p16 immunohistochemistry (p16 IHC), a widely accepted surrogate marker, achieving Cohen's kappa values of 0.65 for plasma and 0.60 for saliva, and a combined value of 0.77. This concordance between the OncoPredict HPV QT assay and p16 IHC suggests its utility as a complementary test, as relying solely on p16 IHC can lead to false positives and false negatives, making it insufficient for HPV status determination. The assay's agreement with ddPCR for HPV 16 viral loads quantification, its ability to detect and quantify all 12 high-risk HPV genotypes, and its cost-effectiveness with shorter turnaround time and standard input sample volume position it as a standardised tool for quantitative viral load assessment. Furthermore, longitudinal monitoring using the OncoPredict HPV QT assay exhibited potential for detecting cancer recurrence weeks to months earlier than conventional clinical imaging, with persistent or increasing HPV DNA levels serving as strong indicators of relapse.

In conclusion, the validation of the OncoPredict HPV QT assay confirms its reliability and potential as a valuable tool for HPV DNA detection and quantification in HPV-related OPSCC patients. Its robust analytical performance, strong concordance with p16 IHC, and high agreement with ddPCR affirm its utility in advancing HPV diagnostics. The integration of this validated, commercially available and standardised CE-marked assay into a comprehensive liquid biopsy strategy offers an accessible tool to facilitate the development of screening programs, advance early detection, personalise management, and enhance continuous post-treatment surveillance of this challenging disease, ultimately improving patient care.

CHAPTER 1: INTRODUCTION

1.1 Epidemiology of Head and Neck Squamous Cell Carcinomas

Head and neck squamous cell carcinomas (HNSCC) represent a diverse group of diseases, with squamous cell carcinoma accounting for approximately 90% of cases. These cancers originate from the epithelial lining of their distinct anatomical sites, such as the oral cavity, oropharynx, pharynx, and larynx [1,2]. The World Health Organisation (WHO) categorises HNSCC based on these distinct anatomical sites as defined by the International Classification of Diseases (ICD-10), recognising their differences in presenting symptoms, treatment strategies, and prognoses [2,3]. For example, human papillomavirus-related HNSCC (HPV-related HNSCC), which is mostly associated with the oropharynx, has specific ICD-10 codes such as C01 for the base of the tongue, C09 for the tonsil, and C10 for other parts of the oropharynx [2]. These codes help in precisely identifying and classifying the specific locations affected by HPV-related HNSCC.

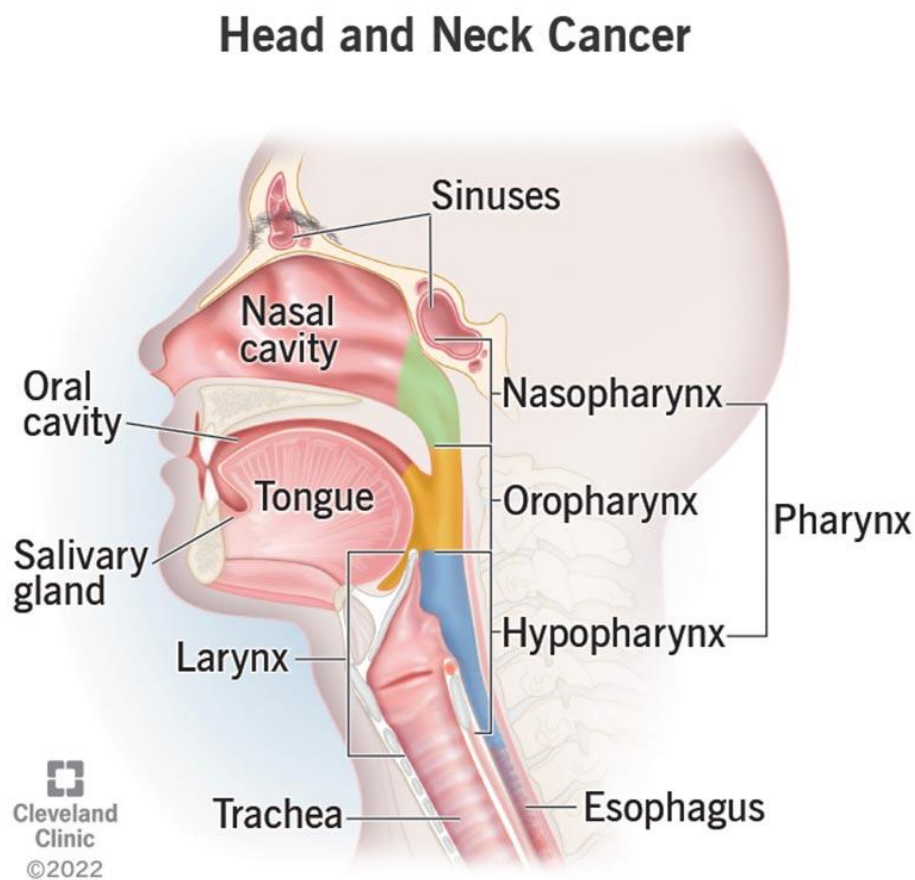


Figure 1: Major anatomical sites of HNC (photo collected from the Cleveland Clinic website [4])

1.1a Global Incidence Trend of HNSCC

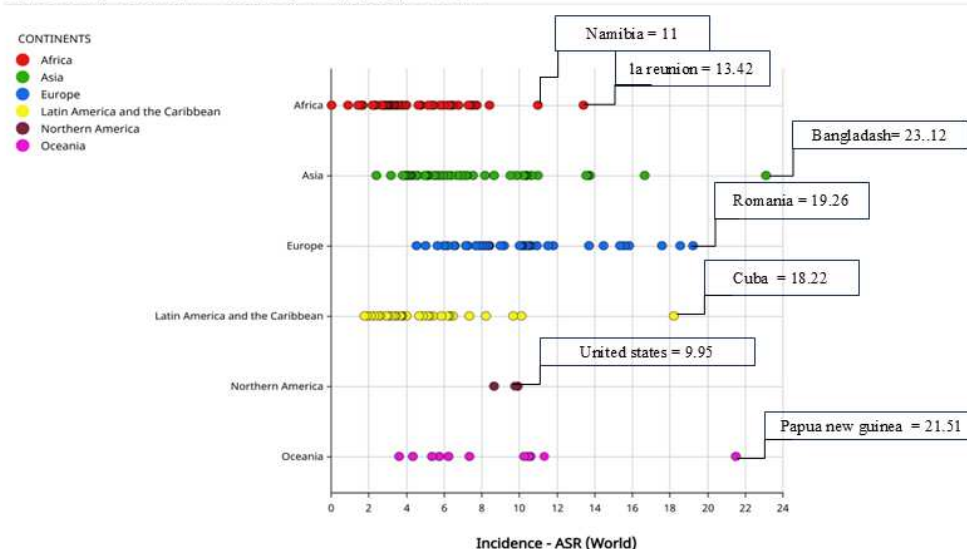
HNSCC, which could be considered a significant global health challenge, is the sixth most common cancer worldwide. According to the latest data from GLOBOCAN 2022, there are approximately 892,128 new cases and 458,486 deaths annually [5].

Asia currently bears the highest burden with 575,411 new cases, corresponding to an incidence rate of 9.9 per 100,000 population. However, Oceania reports the highest age-standardised rate (ASR) at 11.3 per 100,000, despite having the lowest absolute number of new cases (6,996) (Table 1). Bangladesh records the highest ASR among individual countries (23.13/100,000), followed by Papua New Guinea (21.51), Romania (19.26), Cuba (18.22), La Réunion (13.42), Namibia (11), and the United States (9.95) (Figure 2). The International Agency for Research on Cancer (IARC) forecasts a substantial 49.8% increase in new cases by 2045 [6].

Country	Number	ASR (World) Per 100,000	Crude rate	Cumulative risk
Africa	41204	4.7	2.9	0.54
Latin America and the Caribbean	50882	6.2	7.7	0.72
Northern America	64860	9.8	17.4	1.2
Europe	152775	10.7	20.4	1.3
Oceania	6996	11.3	16.0	1.3
Asia	575411	9.9	12.4	1.1
Total	892128	9.2	11.3	1.1

Table 1: HNSCC incidence across different continents. Data collected from the Global Cancer Observatory [6]

Region grouping vs Incidence - ASR (World), Both sexes, in 2022
Lip, oral cavity + Oropharynx + Nasopharynx + Hypopharynx + Larynx



Cancer TODAY | IARC - <https://gco.iarc.who.int/today>
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Figure 2: Countries with the highest ASR across different continents. Data collected from the Global Cancer Observatory [6]

Within Europe, 152,775 new HNSCC cases were reported, with Russia recording the highest number of new cases. Romania, however, exhibited the highest incidence rate at 19.26 per 100,000 population, as depicted in Figure 2, which illustrates the varying incidence rates across different continents. Italy, for instance, according to the latest data, had 11,811 new HNSCC cases annually, and this is projected to rise to 40,200 by 2045, with an incidence rate of 8.9 per 100,000 and a mortality rate of 2.86 per 100,000 (Figures 3a and 3b) [6].

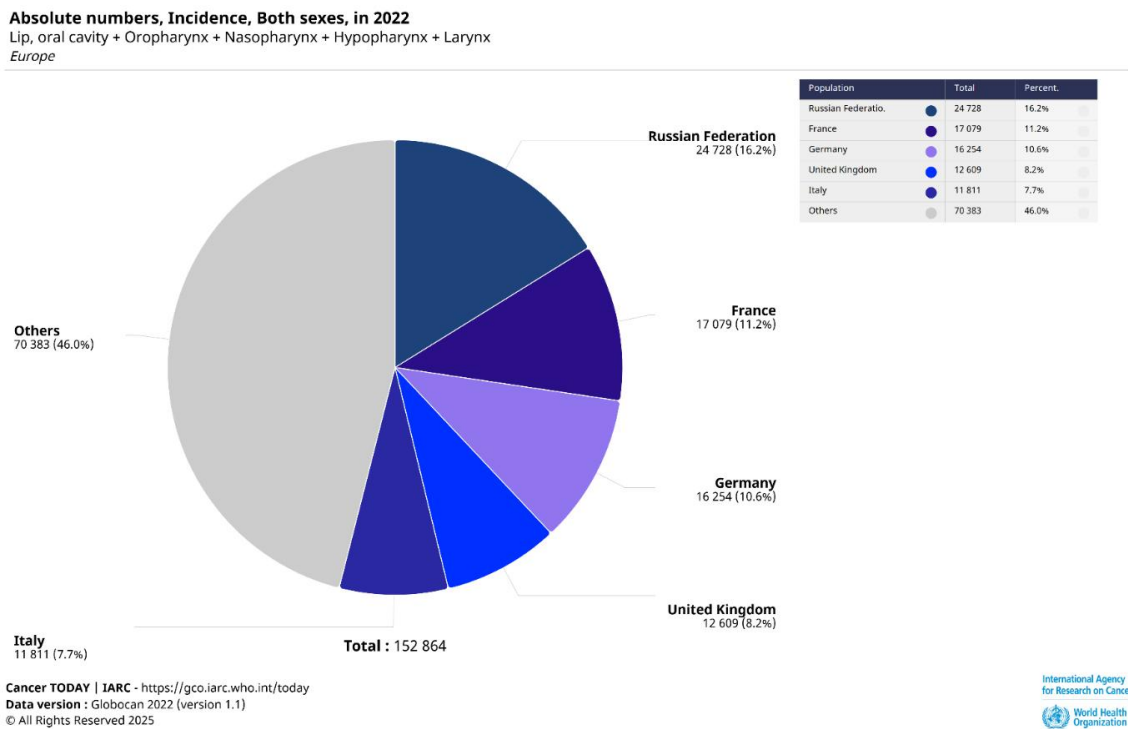
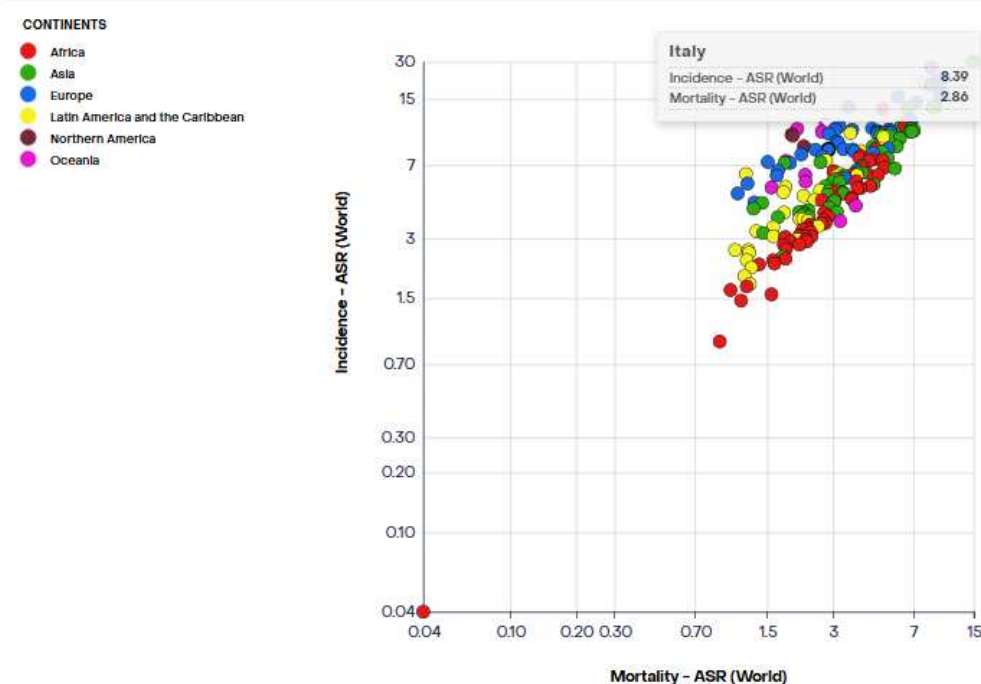


Figure 3a: Annual new cases of HNSCC in Italy according to the latest IARC data[6]

Incidence - ASR (World) vs Mortality - ASR (World), Both sexes, in 2022
 Lip, oral cavity + Oropharynx + Nasopharynx + Hypopharynx + Larynx



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Figure 3b: Italy incidence and mortality rate according to the 2022 IARC data[6]

1.1b Risk Factors

The International Head and Neck Cancer Epidemiology (INHANCE) consortium has conducted extensive pooled analysis using data from studies worldwide to better identify the different risk factors associated with HNSCC [7]. These include well-established factors such as tobacco smoking, alcohol drinking, diet and nutrition, anthropometric and physical activities, oral health/hygiene, medical history, sexual history, genetics, occupation and socioeconomics [2,7–9]. Additional risk factors that are implicated include betel quid chewing, ultraviolet light exposure, herpes simplex virus, and Epstein-Barr virus [10]. Traditionally, HNSCC are more prevalent in older individuals with a history of tobacco and alcohol consumption. However, a decline has been observed, particularly in developed countries, which may be attributed to decreased tobacco use. On the other hand, there has been a concerning increase in cases of HPV-related HNSCC among younger people, especially HPV-related oropharyngeal squamous cell carcinomas (HPV-related OPSCC) in North America and northern Europe [11,12]. Research carried out by the INHANCE consortium highlighted

the crucial role that the number of lifetime sexual partners in an individual's life plays in the risk of developing HPV-related HNSCC [13]. The findings indicated that a higher number of lifetime sexual partners and the practice of oral sex increase the risk of oropharyngeal cancer. For instance, a correlation was observed between oropharyngeal cancer and individuals reporting six or more lifetime sexual partners, as well as oral sex with four or more partners. Similarly, tonsil cancer was found to be associated with at least four lifetime oral sex partners. Among men, the risk is amplified by a history of oral sex and an earlier age at first sexual experience. Similarly, women who have engaged in oral sex and individuals with multiple sexual partners show an elevated risk for cancer at the base of the tongue. Additionally, a history of same-sex sexual contact among men is strongly associated with an increased risk [13].

1.1c Diagnosis and staging

Upon suspicion of HNSCC, a comprehensive medical history is taken, followed by a thorough physical examination that includes assessing the mouth, head, and neck for abnormalities such as lumps, swelling, changes in the oral cavity or throat, and any nerve-related issues in these areas. Ideally, this is followed by radiological imaging, such as positron emission tomography (PET), computed tomography (CT), nuclear magnetic resonance imaging (NMRI), and X-rays, before obtaining large biopsy specimens. This approach helps prevent biopsy-induced anatomical distortion or false-positive PET scan results [10,14]. Following cancer detection, staging is performed as it is essential for predicting survival rates and guiding the management of HNSCC. The American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) use the TNM staging system, which assesses Tumour size (T), lymph Node involvement (N), and the presence of distant Metastasis (M) (Table 2). This system is widely used in oncology to determine the extent of the disease at various stages: before treatment (clinical staging, cTNM), after surgical treatment (pathological staging, pTNM), and at disease recurrence (rTNM). TNM staging is fundamental for treatment planning and prognosis assessment, with prognosis evaluation being one of its most significant applications [10,11,14,15].

T – This category describes the extent of the tumour
○ TX: Primary tumour cannot be evaluated
○ T0: No evidence of primary tumour
○ Tis: Carcinoma in situ (early cancer that has not spread to neighbouring tissue)
○ T1–T4: Size and/or extent of the primary tumour
N – This category describes whether nearby lymph nodes are involved
○ NX: Regional lymph nodes cannot be evaluated
○ N0: No regional lymph node involvement (no cancer found in the lymph nodes)
○ N1–N3: Involvement of regional lymph nodes (number and/or extent of spread)
M – This category indicates whether the cancer has spread to distant parts of the body
○ M0: No distant metastasis (cancer has not spread to other parts of the body)
○ M1: Distant metastasis (cancer has spread to distant parts of the body)

Table 2: staging of HNSCC

In general, early-stage HNSCC (Stages I-II) is characterised by small, localised tumours that show little to no lymph node involvement and without distant metastasis. As the disease progresses to Stages III and IV, the tumour increases in size, invading surrounding tissues and structures and/or involving a significant number of lymph nodes. Stage IV is further defined by the presence of distant metastatic spread [11]. The Staging criteria usually vary depending on the anatomical site; for example, oropharyngeal cancer is further staged based on its HPV status.

1.1d Treatment and Disease Recurrences

The management of HNSCC is usually guided by a multidisciplinary team, as treatment strategies depend on several factors such as the disease stage, HPV status, tumour location, and surgical feasibility. This collaborative approach integrates expertise from head and neck surgeons, radiotherapists, and medical oncologists, along with support from dental care, nutritionists, speech and language therapists, audiologists, and occupational and physical therapists. Psychosocial services also play a vital role in patient care. The primary goal is to preserve both structural and functional integrity while ensuring long-term quality of life for patients [11,16]. Currently, the therapeutic options for HNSCC typically involve surgery, radiotherapy, chemotherapy, and chemoradiotherapy. In cases of early-stage disease, treatment usually consists of a single treatment approach, either surgery or radiotherapy. However, in more advanced-stage disease, a multimodal treatment plan is required, such as initial surgery followed by (chemo)radiotherapy, to optimise disease control [11,16–18]. Despite oncological advancements, the overall 5-year survival rate for HNSCC patients remains around 60%,

largely due to late diagnoses. Furthermore, more than 65% of patients experience disease recurrence or relapse within two years of primary treatment. This highlights the urgent need for improved prognostic and predictive biomarkers for screening, early cancer diagnosis and post-treatment follow-up [2,11,18–21].

1.2 Human papillomavirus (HPV)

HPV Papillomaviruses are small, non-enveloped icosahedral viruses about 50–60 nm in diameter. They have a circular, double-stranded DNA genome (~7000-8000 bp) that is chromatinized. HPV is associated with approximately 5% of all cancers globally, accounting for an estimated 730,000 new cases, and 31.1% of all infection-attributable cancer cases (Fig. 4) [22–25]. HPV is responsible for the majority of cervical cancer cases, accounting for approximately 90% of these cases. Beyond this, HPV is also implicated in other cancers to various degrees, such as head and neck cancer, specifically oropharyngeal, vaginal, vulvar, penile and anal. In addition to its oncogenic potential, HPV can also cause non-cancerous lesions such as warts and benign mucosal papillomas [26,27].

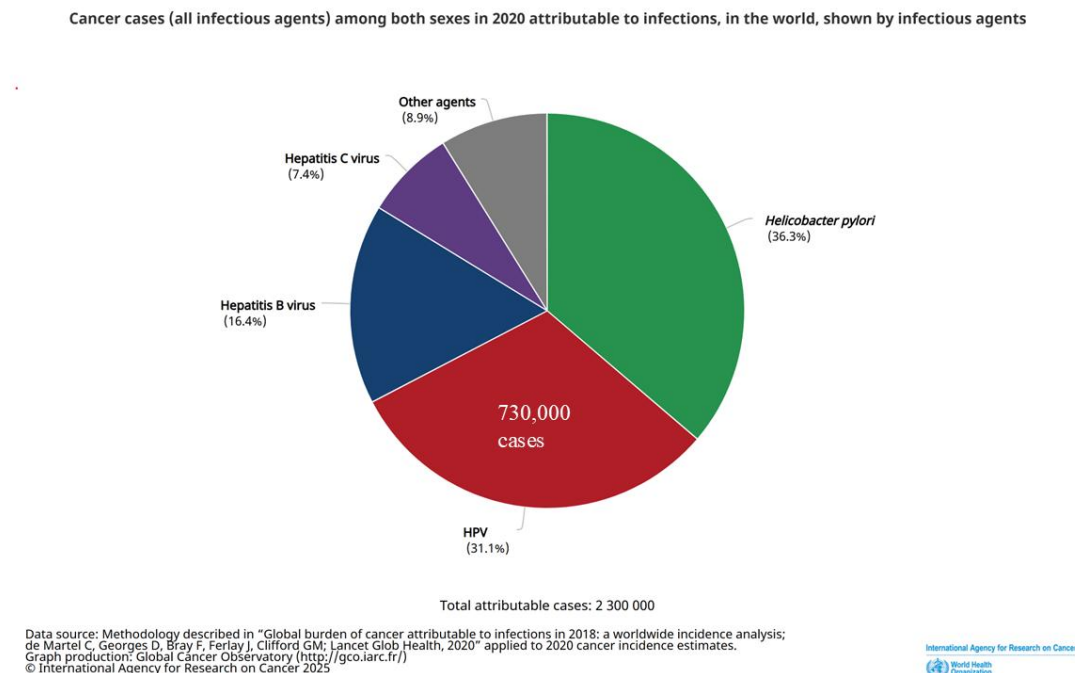


Figure 4: Number of cancer cases attributable to HPV infection and other infectious agents (figure collected from the Global Cancer Observatory [25])

1.2a. HPV Classification

HPV belong to the *Papillomaviridae* family, which encompasses 16 different genera (Figure 5) [28–30]. To date, over 200 HPV genotypes have been identified and grouped in different genera (Alpha-, Nu-/Mu-, Beta- and Gamma-papillomavirus) according to the viral genome structure and tropism to human epithelial tissues [28–30]. The alpha genus comprises viruses linked to the development of mucosal tumours in humans, whereas the beta genus contains viruses associated with the development of cutaneous tumours (Figure 5) [31–33].

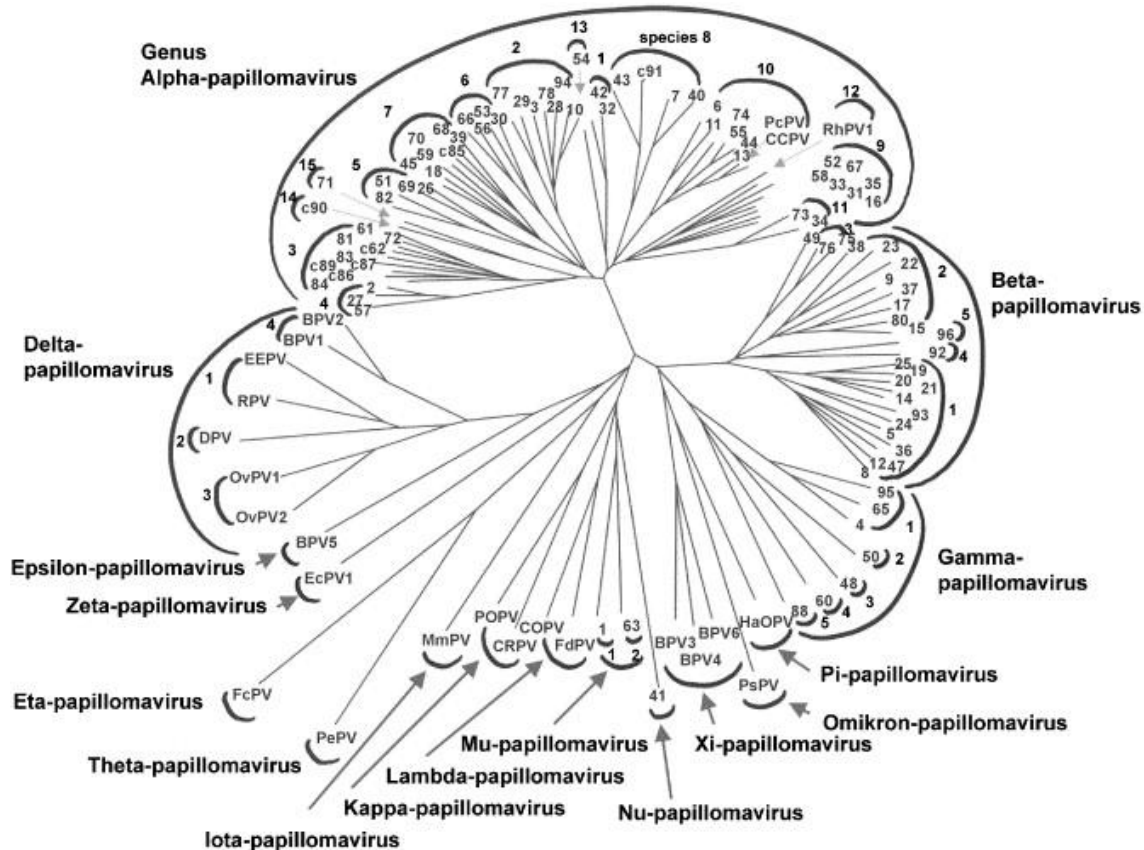


Figure 5: Phylogenetic classification of Papillomaviruses [28]

The IARC has classified 12 HPV types as high-risk (HR) due to their carcinogenic potential: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59. These HR-HPVs are implicated in various cancers, as detailed in Table 3. HPV6 or HPV11, considered low-risk HPV (LR-HPV), generally cause benign diseases such as genital warts [34], while other types are classified as probably or possibly carcinogenic [31].

Human papillomavirus	Genotypes	Associated disease
High risk or oncogenic	HPV 16, 18, 31, 33, 35, 39,45, 51, 52, 56, 58, 59	Cervical, anal, vaginal, vulvar, penile, and oropharyngeal cancer and associated precursor lesions
Low-risk types	HPV 6, 11	Genital warts, recurrent respiratory papillomatosis
Probable carcinogenic	HPV 68	Cervical
Possible carcinogenic	HPV 5,8	Squamous cell carcinoma of the skin in patients affected by epidermodysplasia verruciformis
Possible carcinogenic	HPV 26, 30, 34, 53, 66, 67,69, 70, 73, 82, 85, and 97	Uncertain

Table 3: Classification of the oncogenic properties of different HPV types according to IARC [27]

1.2b. HPV genome

Despite the heterogeneous nature of Papillomaviruses, they share a conserved genome structure and organisation. HPV possesses a circular double-stranded DNA genome divided into three main regions: the Long Control Region, the Early (E) region, and the Late (L) region. The designations 'E' and 'L' refer to the phase in the viral life cycle when these proteins are initially expressed (Figure 6). The genome encodes eight genes, among which E6 and E7 are the most critical oncogenes, playing central roles in HPV-driven carcinogenesis (Table 4) [27,31,32]

Functions	
E1	Drives DNA replication and transcription
E2	Regulates DNA replication and transcription
E4	Disrupt the cell's cytoskeleton.
E5	Interacts with cellular proteins
E6	Induces DNA synthesis and degrades p53
E7	Promotes Rb protein binding
L1 and L2	Encodes viral capsid proteins to produce a new virus

Table 4: Summary of the functions of key genes involved in HPV infection and its life cycle

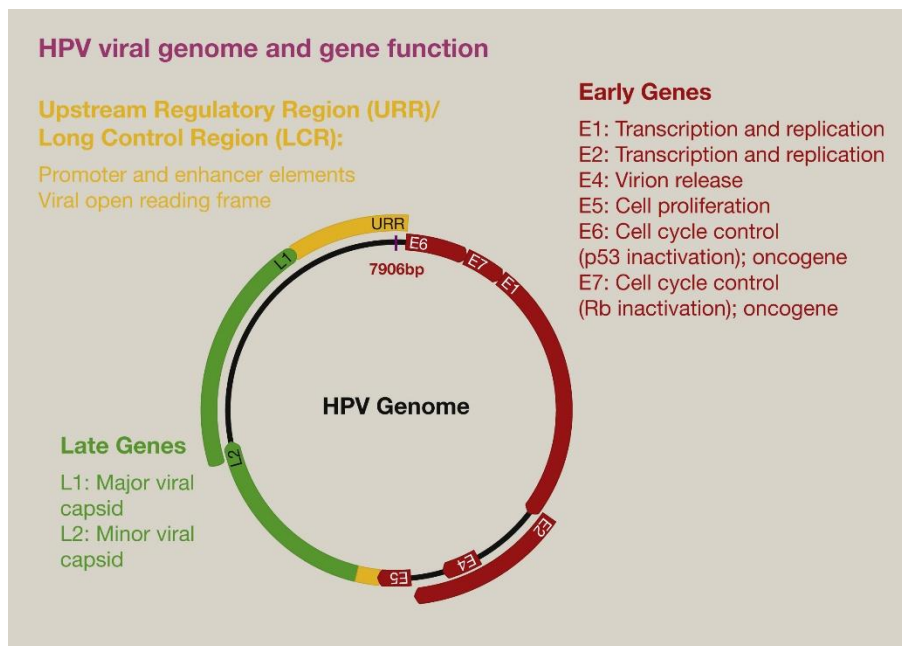


Figure 6: Virtual depiction of the HPV viral genome and gene function. [35]

1.2c. HPV Life Cycle

HPVs are epitheliotropic, and their life cycle is exclusively confined to stratified squamous epithelia. The HPV life cycle begins with the infection of the basal layer through microtraumas that compromise the epithelial barrier (Figure 7). HR-HPVs are characterised by their ability to drive continuous cell proliferation in the basal and parabasal layers. Once within the basal cells, the HPV genome uses the host's cellular machinery for replication, proceeding through four stages: initial infection, maintenance, vegetative amplification, and finally, virus assembly and release. Initially, the HPV genome is present in low copy numbers in infected basal cells. However, as these cells undergo differentiation, viral replication intensifies, leading to a high copy number and the expression of the L1 and L2 capsid proteins. These L1 and L2 proteins are essential for the production of new virions, which are subsequently released from the epithelial surface. These newly released virions proceed to infect other basal cells and enter their nuclei. Upon entering the nucleus, the E1 and E2 proteins are expressed, initiating viral DNA replication and facilitating the stable integration or episomal maintenance of HPV particles within cellular chromosomes. The E6 and E7 proteins then play a critical role in maintaining the HPV life cycle by promoting cell proliferation and survival [27,36].

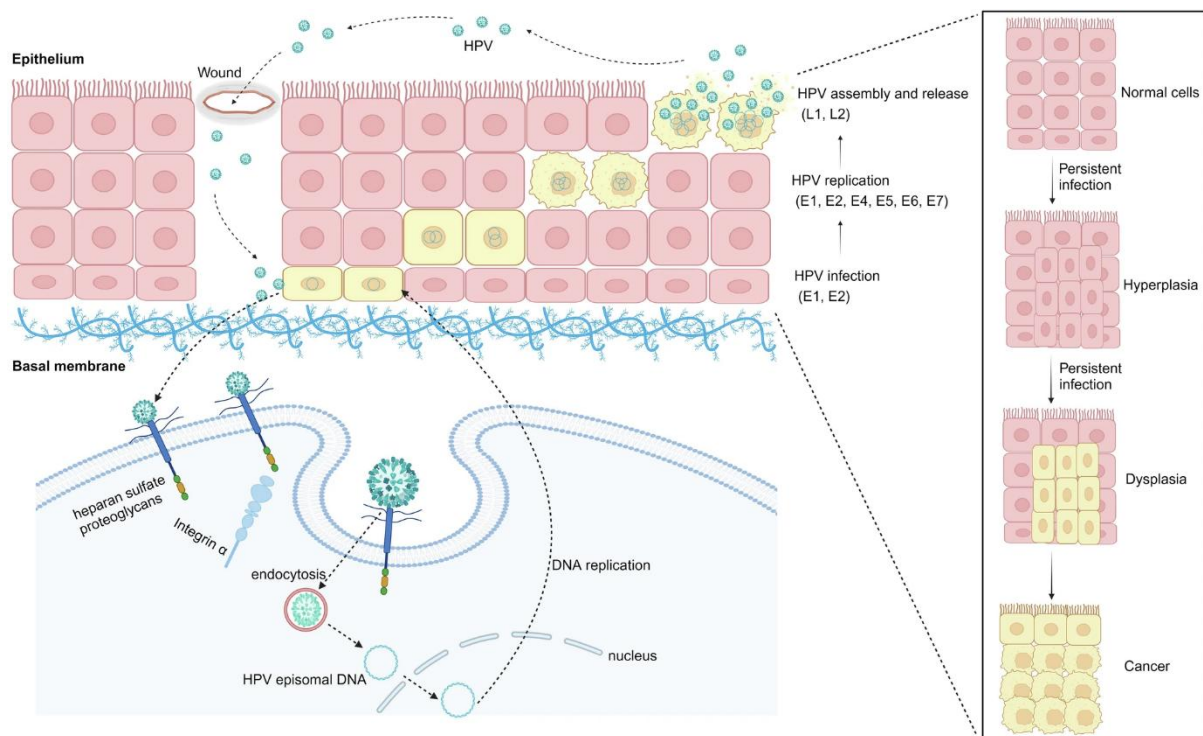


Figure 7: HPV life cycle: regulation of HPV cell cycle entry and proliferation[36]

1.2d. HPV Transmission

HPV infections are primarily transmitted through direct skin-to-skin or skin-to-mucosa contact, with most infections typically clearing within 1-2 years [37,38]. To better understand HPV transmission dynamics, several studies have been carried out, most notably the HITCH (HPV Infection and Transmission among Couples through Heterosexual activity) cohort study [39]. According to a study of 179 couples from the HITCH cohort, HPV transmission occurred in 73 partnerships. The majority (83.6%) of these transmissions involved a single HPV genotype, while 13.7% involved 2 HPV genotypes, and an even smaller fraction (2.7% (2/73)) involved 3 HPV genotypes. The study found comparable transmission rates from male to female and female to male partners, although factors like sexual habits, condom use, and male partner circumcision can affect these rates [40]. Another study using data from the HITCH cohort demonstrated that HPV prevalence is associated with sexual habits, lifetime number of vaginal sexual partners, and the type of relationship an individual is involved in (i.e., monogamous or non-monogamous). HPV was detected in 96% of individuals with concurrent partners, and those with 10 or more lifetime sexual partners had a 100% HPV detection rate [41].

1.3 HPV-related Oropharyngeal Squamous Cell Carcinoma (HPV-OPSCC)

Oropharyngeal squamous cell carcinoma (OPSCC), a subset of HNSCC, which includes cancer of the tonsils, base of tongue, soft palate and uvula [42]. Historically linked to smoking and alcohol consumption, but this has gradually changed, particularly in North America and Europe. The emergence of HPV as a significant risk factor has driven an increase in OPSCC, initially observed in younger populations but now also increasingly among individuals over 70 years of age [43–46]. HPV-related OPSCC is rapidly becoming a global pandemic, with about 42000 cases in 2012 (males = 34000; females = 8000), with projections that its incidence may surpass cervical cancer as the most prevalent HPV-related cancer [23]. The WHO classifies OPSCC as either HPV-dependent or non-HPV-dependent[47].

The HPV-OPSCC typically exhibits a characteristic basaloid morphology, thought to originate from non-keratinising tonsillar crypt epithelium [17,48]. A key distinguishing feature between HPV-related and non-HPV-related OPSCC is the overexpression of the p16 protein in HPV-related cases, routinely detected via immunohistochemistry (IHC). Despite indications that HPV-related OPSCC generally carries a more favourable prognosis [46], its exact impact on overall survival is still a matter of debate, as several studies present conflicting results. For example, a study by Lewis et al., which analysed tissue biopsy samples from p16-positive OPSCC for HPV DNA, found that p16-positive/HPVDNA-negative patients had a notably better OS than p16-negative patients. However, the study did not find a difference in OS and disease-free survival rates between p16-positive/HPVDNA-negative and p16-positive/HPVDNA-positive patient cohorts [49]. In another study, it was reported that patients who were p16-positive and HPVDNA-negative experienced worse OS than those who were p16-positive/HPVDNA-positive [50]. On the contrary, another group found no significant difference between p16-positive and p16-negative OPSCC patients, suggesting that p16 positivity does not necessarily equate to a favourable outcome [51]. Sharma et al. additionally demonstrated that 5-year OS was about 32% lower in groups with discordant HPV status (p16-positive/HPV-negative and p16-negative/HPV-positive) compared to the concordant p16-positive/HPV-positive group [52]. These disparities in results are often attributed to differences in study design, patient populations, geographical origin, specific HPV detection methodologies, length of follow-up, or treatment protocols, highlighting the complexity of accurately stratifying risk and prognosis in HPV-related OPSCC [53,54].

1.3a Global Incidence of HPV-related OPSCC

A recent systematic review by Fonsêca et al., observed that HPV- related OPSCC is indeed emerging as a global pandemic, with a notable male predominance, HPV prevalence was about 66% in male compared to 19% in women [55], This aligns with a study by Yong Lu et al. where it was highlighted that the attributable fraction (AF) of HPV in OPSCC was approximately four times higher in male than in female [56]. Both studies reported a similar overall HPV positivity rate of about 42% - 42.7% in OPSCC. The incidence of HPV-related OPSCC tends to be higher in more developed nations, with countries in Northern Europe, North America and Oceania showing increasing HPV prevalence. Specifically, Fonsêca et al. found that Finland and Sweden have a prevalence of 82% each, followed by New Zealand with 76%. In North America, HPV prevalence in the United States escalated from 17% in 1997 to 60% in 2018, with Canada showing even higher prevalence rates between 72%–74%. Interestingly, South America and Africa consistently show lower to moderate HPV prevalence, possibly due to a lower number of published research and inadequate sample sizes in existing studies from these regions [55].

Over the last few decades, Italy has seen a steady increase in HPV-related OPSCC. A multicentre study by Del Mistro et al., analysing data from 130 OPSCC patients from northern Italy, reported an increased prevalence of HPV positivity, increasing from 17% in the 2000s to 46% in 2013–2018. This upward trend was observed in both genders, though more pronounced in females ($p = 0.010$) and in younger patients ($p = 0.004$) [57]. These findings align with a previous Italian cancer registry-based study covering the period from 1988 to 2012, which also reported a rising trend in HPV-related HNSCC, particularly in the oropharynx [58].

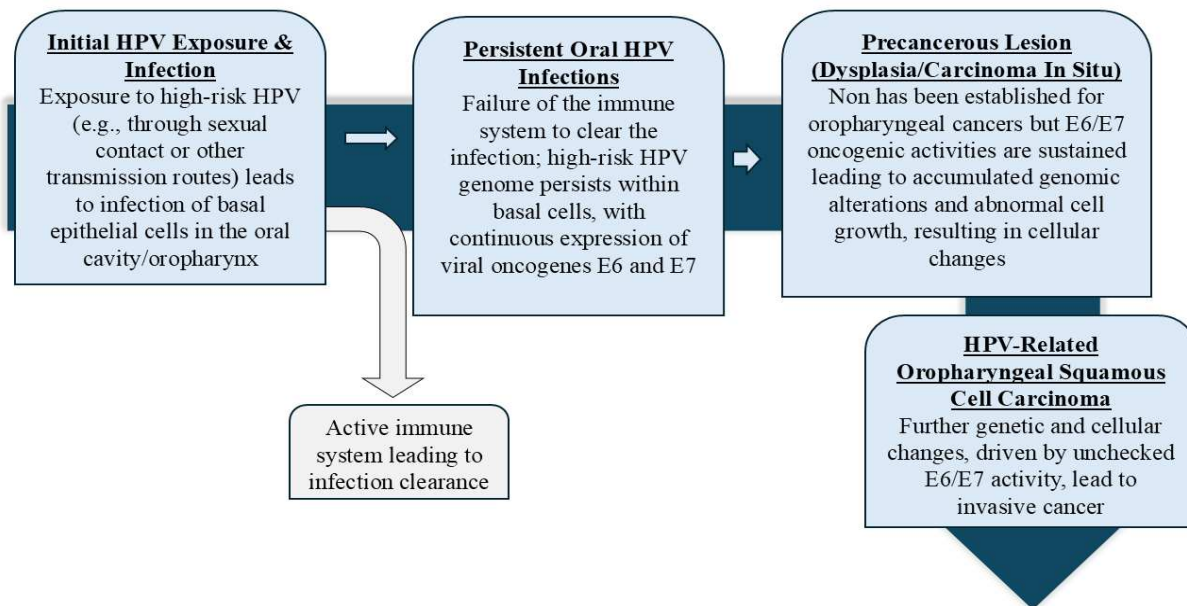


Figure 8: HPV carcinogenesis

1.3b Carcinogenesis of HPV-related OPSCC

The exact mechanism of carcinogenesis that transforms HPV infection into overt OPSCC is still largely unknown, with most current understanding extrapolated from the well-established cervical cancer model. The unique microenvironment of the tonsils and tonsillar crypts facilitates the direct passage of immune cells and pathogens like HPV. Persistent HPV infection can lead to precancerous lesions, although none have been clearly established for OPSCC (Figure 8). The development from HPV infection to invasive cancer can take up to a decade [59]. In HPV-related OPSCC carcinogenesis, the early proteins E6 and E7 of HPV play an important role. E6 primarily acts by suppressing p53, a vital tumour suppressor, while E7 forms a complex with retinoblastoma protein (pRb). This interaction leads to the degradation of pRb and the subsequent release of E2F transcription factors, which result in the deregulation of the G1/S cell cycle checkpoint and activate the re-entry of S-phase, thereby supporting viral replication. E7 activation also drives the overexpression of p16INK4a by disrupting the pRb/E2F complex, removing inhibitory control and enabling epithelial cells to bypass oncogene-induced senescence and activate survival pathways. The overexpression of p16INK4a is crucial for the survival of HPV-associated tumours and is commonly lost in non-

HPV-related OPSCC, making p16INK4a a reliable surrogate marker for identifying transcriptionally active HPV in oropharyngeal carcinogenesis [59].

1.3c Diagnosis of HPV-related OPSCC.

HPV-related OPSCC are usually diagnosed in advanced stages, with small tumour size (T) and high nodal dissemination. Current clinical practice guidelines, such as those developed by the European Head and Neck Society, the European Society for Medical Oncology, and the European Society for Radiotherapy & Oncology, mandate HPV evaluation using p16-IHC for all newly diagnosed OPSCC patients. In some cases, additional HPV testing using molecular PCR-based methods (RT-PCR, qPCR, ddPCR) or in situ hybridisation (ISH) is advised to confirm HPV positivity [60]. This HPV evaluation technique (p16-IHC), though effective, relies on invasive tissue biopsies, which can be challenging, painful, and time-consuming procedures, especially when it comes to post-treatment surveillance [61]. The use of tissue biopsy might fall short in capturing the full extent of tumour heterogeneity (inter-tumoral, intra-tumoral, or metastatic), thereby impacting the sensitivity, specificity, and overall accuracy of diagnostic and prognostic assessments [62–64]. These drew the attention of researchers to the use of liquid biopsy as a non-invasive complementary or alternative diagnostic tool, offering potential advantages of real-time tumour characterisation, early detection, and post-treatment surveillance of HPV-related HNSCC [65].

1.4 Liquid Biopsy in HNSCC

In recent years, the concept of liquid biopsy has emerged as a transformative, non-invasive approach to cancer diagnosis and monitoring. This approach offers a cost-effective alternative to traditional tissue biopsies and also allows the detection of tumour-derived components in blood, saliva, and urine (Figure 9) [62,66]. This technique does not just help in early detection and disease surveillance but also in understanding tumour resistance mechanisms and detecting tumour recurrence or metastasis after treatment, thereby advancing personalised medicine [66,67]. The term “liquid biopsy” refers to the analysis of biomarkers found in various biological fluids, such as blood, saliva, or urine and these biomarkers includes circulating tumour cells (CTCs), circulating cell-free nucleic acids (ccfNAs)—such as cell-free DNA (cfDNA), circulating tumour DNA (ctDNA), as well as metabolites, exosomes (EXOs), mRNA, and miRNAs [62,68]. These biomarkers usually carry tumour-specific molecular alterations, thereby providing a real-time snapshot of tumour heterogeneity and dynamic biological behaviour [69,70]. In the context of HNSCC, particularly HPV-OPSCC, liquid

biopsy is gaining increasing clinical and research relevance [65]. Blood and saliva are currently the most utilised fluids for liquid biopsy in HNSCC.

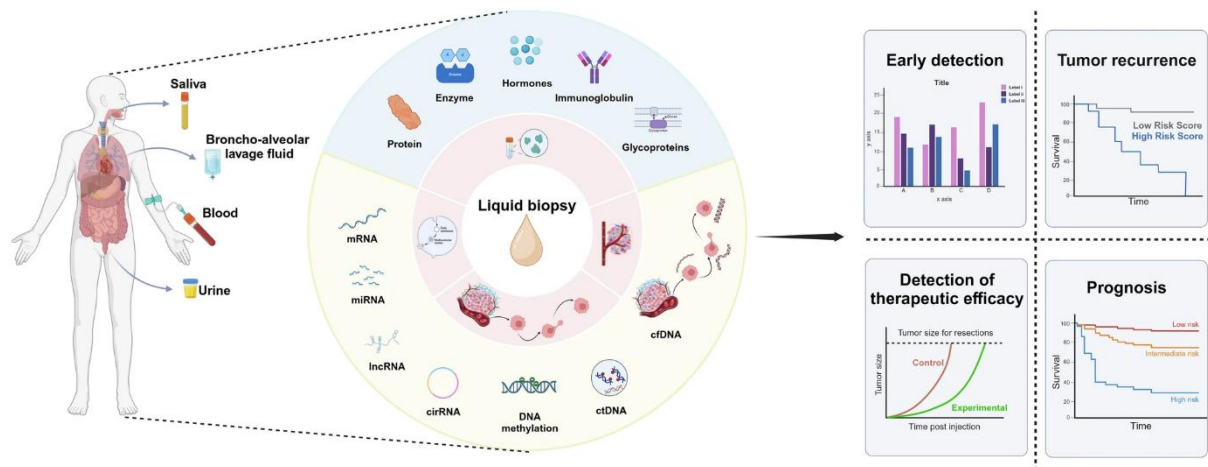


Figure 9: Overview of liquid biopsy in cancer: key biomarker types (e.g., ctDNA, CTCs, exosomes, and cfDNA), their sources (blood, saliva, urine), and clinical applications including early detection, prognosis, treatment monitoring, and recurrence surveillance [40].

1.4a Types of liquid biopsy biomarkers in HNSCC

A variety of tumour-derived components can be isolated from both blood and saliva in patients with HNSCC. Molecular analysis of these distinct elements provides complementary and valuable insights into the tumour's biological characteristics.

Circulating tumour cells (CTC)

CTCs are tumour cells that are passively being shed from the primary tumour or metastatic lesions into the bloodstream or saliva. Its presence was first reported by Thomas Ashworth in 1869 [71]. They can exist as single cells or clusters of cells, and they can offer unique and early evidence of tumour aggressiveness, potentially preceding the clinical detection of metastases. In HNSCC, CTCs have been linked to disease severity and poor outcomes [72,73], serving as biomarkers for disease aggressiveness, detection of metastatic disease, monitoring treatment response, and post-treatment surveillance. CTCs are more frequently detected in patients with late-stage or metastatic cancer compared to those with Stage I–III disease, and their clearance can be associated with treatment response. A major challenge in the use of CTCs as a biomarker is their low numbers in blood circulation (approximately 1 cell per 1×10^9 blood cells), which makes them difficult to detect, though this varies by tumour type [69,74]. Technological advances have been made to overcome this pitfall. CTC detection and quantification are usually

achieved by: immunofluorescence using anti-CK antibodies, molecular assays - including RT-qPCR, fluorescent in situ hybridisation (FISH), next generation sequencing (NGS), and detection of tumour-specific proteins released by CTCs (Enzyme-linked immunosorbent spot (EpiSpot) assay) [75]. Up to date, CellSearch System (Veridex) is the first and only in vitro diagnostic kit approved by the FDA for the detection and quantification of CTCs in certain malignancies such as breast, colorectal and prostate cancers [75].

Exosomes

Exosomes are a subset of extracellular vesicles (EVs) released through exocytosis following the fusion of multivesicular bodies (MVBs) with the plasma membrane. First described by Pan and Johnstone in 1983 [76], exosomes typically range in size from 40 to 100 nm and can be secreted by various cell types, including blood cells, epithelial cells, immune cells, platelets, and smooth muscle cells. They carry a complex cargo that includes DNA, messenger RNAs (mRNAs), microRNAs (miRNAs), long non-coding RNAs (lncRNAs), circular RNAs (circRNAs), proteins, and metabolites. Exosomes hold significant potential for clinical applications such as monitoring disease progression, detecting mutations, splice variants, gene fusions, and performing gene expression profiling. Various isolation techniques have been developed based on the biophysical characteristics and immunoaffinity of exosomes. In the context of HNSCC, exosomes are emerging as a promising biomarker. For example, a study by Theodoraki et al. investigated circulating plasma exosomes in HNSCC patients being treated with a combination of cetuximab, ipilimumab, and radiation therapy. Through serial monitoring, it was observed that patients with disease recurrence had higher levels of both total exosomes (TEX) and T cell-derived exosomes compared to those who remained disease-free. This supports that exosomes could potentially be valuable biomarkers for post-treatment surveillance in HNSCC [77]. However, the clinical application of exosomes is currently hindered by technical difficulties in isolation, purification, and quantification. Further technological advancements are required to fully determine their true sensitivity [74].

Circulating tumour DNA (ctDNA)

ctDNA represents a subset of cell-free DNA (cfDNA) that is released into the bloodstream and saliva from cancerous cells, through both pathological and physiological mechanisms, including cellular apoptosis and necrosis from rapidly proliferating cancer cells and thus carries the mutations of the original tumour [62,78]. CfDNA was first identified in human plasma by Mandel and Métais in 1948 [79]. A few decades later, studies demonstrated that cfDNA

concentrations are significantly higher in the blood of cancer patients compared to healthy individuals [80]. Building upon these findings, Stroun et al. in 1989 provided evidence of neoplastic characteristics within cfDNA, marking a pivotal moment in the understanding of tumour-derived nucleic acids [81]. The precise mechanism of ctDNA release remains unclear, but apoptosis and necrosis are considered primary processes, with macrophages also potentially contributing through phagocytosis of necrotic neoplastic cells [68,69,75]. Studies have shown that the half-life of cfDNA in the circulation is between 16 minutes. This contributes to the proposed clinical potential of ctDNA as a non-invasive biomarker for characterising tumour molecular features (tumour profiling), assessing tumour burden, monitoring treatment response in real-time, and detecting disease relapse/recurrence [68,82].

Saliva is also a valuable source of biomarkers (enzymes, hormones, cytokines, antibodies, microorganisms, and cell-free DNA (cfDNA)), because it can be described as an ultrafiltrate of blood, including. Many of the components found in saliva are from the salivary glands, while others can also be transported from the bloodstream due to the proximity of the salivary glands and blood vessels. This transportation of materials between the salivary glands and blood vessels is a result of processes such as diffusion, active transport, and ultrafiltration. Consequently, saliva is a promising non-invasive liquid biopsy medium, which is capable of mirroring the physiological and pathological status of various conditions, including cancer, cardiovascular disease, autoimmune disorders, and infections. This makes it valuable for early diagnosis and post-treatment monitoring [83–85].

There is currently no standardised method for the isolation and analysis ctDNA and this due to various issues like: choosing the best sample matrix to use (plasma, serum, whole saliva, oral swab, oral rinse), sample collection technique (types of collection tubes – with or without preservative and techniques), time between sample collection and analysis, ctDNA isolations and detection technologies. This lack of standardisation is a major hindrance in the adoption of ctDNA for routine clinical practice [75]. The traditional Sanger sequencing is usually used for the detection of ctDNA, but its inability to detect low-abundance somatic mutations in plasma ctDNA from cancer patients led to the adoption of PCR-based methods like quantitative PCR (qPCR) and droplet digital PCR (ddPCR). Notably, ddPCR offers high analytical sensitivity and specificity for ctDNA detection and quantification, achieving detection limits of approximately 0.0001% [86]. Other quantification methods of ctDNA include UV spectrophotometry, electrophoresis-based assays, double-stranded DNA assay, PicoGreen, and

single-stranded OliGreen DNA assay [69]. The recent introduction of next-generation sequencing has profoundly impacted the liquid biopsy field, enabling comprehensive genomic profiling and identification of tumour-specific alterations [69,75].

The potential of ctDNA as a valuable marker in HNSCC has been demonstrated through studies utilising plasma and saliva samples. A study of 93 HNSCC patients found that plasma ctDNA was more effective for detecting tumours in the oropharynx, hypopharynx, and larynx, while salivary ctDNA proved more sensitive for oral cancer due to its direct proximity to the tumour site. Combining analysis of plasma and saliva samples further improved detection sensitivity. Stratification by disease stage showed ctDNA in 100% of patients with initial disease cases and 95% of advanced disease cases. Anatomical site-specific detection rates were 100% for the oral cavity, 91% for the oropharynx, and 100% for both the larynx and hypopharynx. [87]. Additionally, another study reported that HNSCC patients had higher cfDNA levels than the control group, although this increase was not statistically significant. However, patients with oropharyngeal tumours showed significantly higher levels of ctDNA compared to those with other HNSCC subtypes [88].

1.4b Circulating HPV DNA (ctHPV DNA) biomarker in HPV-related OPSCC

As previously mentioned, patients with HPV-related OPSCC exhibit significantly higher levels of ctDNA than those with other HNSCC subtypes, primarily due to the distinct viral aetiology of their tumours. In HPV-related OPSCC, the detection of HPV-specific circulating tumour DNA (ctHPV DNA), especially fragments of the E6 and E7 oncogenes, has demonstrated high sensitivity and specificity both for diagnosis and post-treatment monitoring [89]. The detection of ctHPV DNA in blood (plasma and serum) and saliva (including whole saliva, oral rinses, and oral swabs) has demonstrated diagnostic accuracy comparable to the use of p16 IHC in tissue samples for HPV detection. These studies have reported sensitivity for ctHPV DNA ranging from 90% to 95%, with specificity exceeding 98% [90–92]. Additionally, a systematic review by Wuerdemann et al. reported a pooled sensitivity of 0.81 for diagnosis with a pooled specificity of 0.98, while the pooled sensitivity and specificity for post-treatment monitoring were 0.73 and 1.00, respectively. This finding further supports ctHPV DNA as a diagnostic and surveillance tool [93].

1.4c. ctHPVDNA detection methods

The detection and analysis of ctHPVDNA in HPV-related OPSCC employ a range of molecular techniques, each with specific strengths and limitations. The most commonly used approaches include quantitative polymerase chain reaction (qPCR), droplet digital PCR (ddPCR), and next-generation sequencing (NGS). A recent review by Parisi et al summarised this quite well in Table 5.

Method	Sensitivity	Specificity	Cost	Turnaround Time	Clinical Use	Economic Feasibility
qPCR	75–85%	>90%	Low	24–48 h	Limited due to lower sensitivity	Widely available and cost-effective
ddPCR	>95%	>98%	Medium	24–72 h	Recommended for real-time monitoring	Cost-effective for clinical use
NGS (hybrid capture)	>99%	97%	Very high	7–10 days	Comprehensive profiling, mutation analysis	Expensive, requires bioinformatics support
NGS (amplicon-based)	95%	96%	High	5–7 days	Targeted mutation detection	Costly but clinically relevant for resistance monitoring

Table 5: Comparative analysis of different diagnostic methods [92].

For the analysis of ctHPVDNA in HPV-related OPSCC, qPCR is widely used due to its accessibility and cost efficiency; however, it's been reported to be modest in detecting low-abundance ctHPVDNA, therefore making its sensitivity limited. In contrast, the novel ddPCR technologies enhance the ctHPVDNA detection rate by enabling absolute quantification and superior sensitivity, making it especially effective for identifying minimal residual disease. The detection of HPV DNA in plasma using ddPCR has shown impressive diagnostic capability with a pooled sensitivity of 96% and a specificity of 98% [92]. Also, the use of Next-generation sequencing (NGS) approaches (like hybrid capture and amplicon-based techniques), though it allows for a broader genomic profiling while identifying viral and somatic mutations, it is, however, more expensive and has longer processing times.

Currently, there is only one clinically validated ctHPVDNA blood assay commercially available, and that is the NavDx assay, developed by Naveris (Natick, MA, USA). This assay utilises droplet digital PCR (ddPCR) and specifically detects tumour-tissue modified viral (TTMV) HPV DNA fragments circulating in plasma cell-free DNA. It targets the five high-

risk HPV genotypes (HPV 16, HPV 18, HPV 31, HPV 33, and HPV 35) most commonly associated with OPSCC. This test has proven valuable for non-invasive disease monitoring, enabling early detection of recurrence and assessing treatment response[94–96].

1.5. In Vitro Diagnostic Device (IVD) Development

The development of *in vitro* diagnostic devices (IVD) has become a cornerstone of modern medical diagnostics, providing rapid, accurate, and non-invasive tools for disease detection and personalised disease monitoring. IVD assays function by analysing biological samples (e.g., blood, saliva, urine, swabs) outside the human body to identify specific biomarkers indicative of physiological or pathological conditions. These advanced diagnostic tools have considerably improved clinical decision-making by facilitating early diagnosis, guiding personalised treatment strategies, and enabling continuous patient monitoring. With the help of advancements in molecular biology, automation of technologies, and bioinformatics, substantial progress has been seen in IVD development. This has led to the development of highly sensitive, specific, and multiplexed diagnostic platforms. This breakthrough has helped to reshape disease management across various fields, including infectious diseases, oncology, and genetic disorders.

The development of an IVD assay is an intricate process starting from biomarker identification, the design of the assay, rigorous analytical and clinical validation, and continuous post-market surveillance. Throughout this process, the IVD manufacturer must strictly comply with international standards and regulatory frameworks, combined with scientific and technical precision, to ensure the efficacy, safety, and quality of the diagnostic products. Two fundamental frameworks in this area are the ISO 13485:2016 quality management standard and the In Vitro Diagnostic Regulation EU 2017/746, which fully replaced the former IVDD in May 2022.

ISO 13485:2016 is a globally recognised standard that outlines the requirements for a quality management system (QMS) specifically designed for the medical device sector, including IVDs. It promotes a risk-oriented approach across all stages of the product development and manufacturing, outlining the necessity for design controls, comprehensive risk management, and formal corrective and preventive actions (CAPA) processes. Furthermore, it highlights the importance of thorough documentation, process validation, traceability, and post-market surveillance to ensure that the products consistently remain safe, effective, and compliant

throughout their entire lifecycle. Compliance with ISO 13485 is a prerequisite for regulatory approval and a crucial requirement for achieving CE marking under the EU IVDR and compliance with the FDA Quality System Regulation (QSR) in the United States.

The IVDR, on the other hand, introduces a more stringent risk-based classification system (Classes A to D) for IVDs. It necessitates greater involvement of notified bodies, imposes significantly higher expectations for clinical evidence, post-market surveillance, and overall transparency. A major component of IVDR is the Performance Evaluation Report (PER), which must include comprehensive data on the scientific validity of the intended use, analytical performance, and clinical performance.

For an IVD to be legally placed in the market in the EU, manufacturers are required to obtain CE certification, confirming the product's compliance with the IVDR. The CE marking process involves several critical steps:

1. **Determine the Risk Class**

The IVD must be classified into one of four risk classes (A–D), with Class D representing the highest risk (e.g., tests for blood-borne pathogens). This classification determines the conformity assessment route.

2. **Implement a QMS**

Establish and maintain a QMS compliant with ISO 13485:2016, covering all aspects from design and development to production and post-market surveillance.

3. **Compile Technical Documentation**

Technical files must include device description, intended use, design and manufacturing information, risk analysis, verification and validation data, labelling, and instructions for use.

4. **Conduct a Performance Evaluation**

Prepare a PER that includes:

- Scientific validity of the marker–disease relationship.
 - Analytical performance (e.g., sensitivity, specificity, stability, reproducibility, repeatability).
 - Clinical performance (e.g., diagnostic accuracy, clinical sensitivity, specificity, and reproducibility).
- **Involving a Notified Body**

Except for Class A non-sterile devices, a Notified Body is required to review the technical documentation and QMS. A successful audit leads to the issuance of a CE certificate.

5. Device and Unique Device Identifier (UDI) Registration

The manufacturer is obligated to register the device, the UDI, and relevant documents in the EUDAMED (European database on medical devices), the EU's central database for medical devices.

6. Affixing the CE Mark

Upon confirmation of conformity, the CE mark can be placed on the device, indicating its compliance with the IVDR and authorisation for distribution within the EU.

This stringent regulatory framework goes to show the critical requirements for introducing new diagnostic tools to the market. The present thesis, through its validation of the OncoPredict HPV QT assay, directly contributes to addressing clinical performance aspects required for such IVD development, paving the way for its potential future clinical translation within these stringent frameworks.

CHAPTER 2: SCOPE

Globally, over 264 distinct HPV tests (and 511 test variants) are commercially available, with a primary intended use in cervical cancer screening [97]. A review by Poljak et al demonstrated that only about 21% of these available HPV assays have been validated in accordance with the internationally recognised validation criteria defined by Meijer et al in 2009 [97,98]. The majority of the validated assays offer partial genotyping, typically for HPV 16, HPV 18, and HPV 45, with fewer providing comprehensive genotyping for all 12 high-risk HPV types. Furthermore, only a very limited number of assays provide quantitative genotype-specific viral load determination [97].

The WHO recently published a document entitled “Target product profiles for human papillomavirus screening tests to detect cervical pre-cancer and cancer” (TPP) [99], defining the preferred and minimal requirements of HPV molecular screening assays; according to a panel of WHO Experts, an ideal HPV assay should detect a minimum of 8 and a maximum of 12 carcinogenic HPV genotypes. Currently, only approximately 20 HPV tests fulfil both the minimal clinical criteria for HPV test validation for use in cervical cancer screening as well as the WHO TPP accuracy conditions [99–101]. Most HPV assays, however, allow detection of 14 “high-risk” HPV types, whilst OncoPredict HPV QT is the only clinically validated assay which targets only the 12 carcinogenic HPV types, as recommended by WHO. Complementary international initiatives such as VALGENT (VALidation of HPV GENotyping Tests) and VALHUDES (VALidation of HUMAN papillomavirus assays and Collection DEVICES for HPV testing on Self-samples and Urine Samples) have been introduced to evaluate the clinical performance validation of HPV assays on cervical and self-collected samples [102,103].

Unfortunately, all these guidelines and validation criteria are mostly targeted towards cervical cancer screening assays. There is a significant lack of established validation frameworks for assays used in other HPV-related cancers, including those affecting the oropharynx, anal canal, vulva, and penis. This represents a critical gap in the field, necessitating dedicated validation efforts for these distinct clinical applications.

This thesis directly addresses this unmet need by undertaking further validation of the qPCR-based OncoPredict HPV QT assay, which is notably one of the 20 assays currently compliant with the international validation criteria and the WHO TPP accuracy conditions for cervical cancer screening on both cervical and self-collected samples [102,104–106]. The present work

seeks to validate its potential as a quantitative HPV DNA detection tool, specifically in both plasma and saliva samples obtained from patients with HPV-related OPSCC. This validation has been conducted as part of the present PhD through a clinical study, performed in strict compliance with ISO 20916:2019, the international standard regulation for "In vitro diagnostic medical devices — Clinical performance studies using specimens from human subjects — Good study practice." [107].

The objectives of this thesis have been to:

- Contribute to the technical product development of the OncoPredict HPV QT assay, working in collaboration with the manufacturing company Hiantis Srl.
- Evaluate the accuracy of the OncoPredict HPV QT assay in quantifying HPV 16 viral loads, in comparison to an established reference method for the absolute quantification of viral load, specifically digital droplet PCR (ddPCR);
- Evaluate the clinical performance of the OncoPredict HPV QT assay in carcinogenic HPV viral load determination in blood and saliva liquid biopsies as part of a longitudinal clinical study of patients presenting with a new diagnosis of OPSCC and being followed up during treatment for the early detection of disease recurrence or relapse.
- Evaluate the molecular target CCR5, incorporated into the OncoPredict QC module of the OncoPredict HPV QT assay, for the reliable normalisation of viral load in saliva samples through the quantification of human cell numbers, by comparing its results with cellularity assessments derived from a Thoma cell-counting chamber and Fluorescence-activated Cell Sorting.

CHAPTER 3: “IDENTIFY” CLINICAL STUDY - IDENTIFICATION OF CIRCULATING MARKERS TO CUSTOMIZE THE FOLLOW-UP OF HEAD AND NECK CANCER PATIENTS FOR EARLY DETECTION OF RECURRENCES/SECOND TUMOURS

Samples used for this thesis work were collected from the participants as part of a multicentre clinical study titled: “*Identification of circulating markers to customise the follow-up of head and neck cancer patients for early detection of recurrences/second tumours*”, with the acronym: “IDENTIFY”

3.1 Study Description

This is a clinical, non-pharmacological, interventional, cohort, prospective, multicentre, non-profit study. Data of patients who met the inclusion criteria were collected consecutively at baseline and during subsequent clinical evaluations at 3, 6, 12 and 18 months after treatment for a newly diagnosed HNSCC (for at least 36 months of patient observation after enrolment; Figure 10). 200 patients (178 + dropout of about 10%) were estimated to be enrolled for the study.

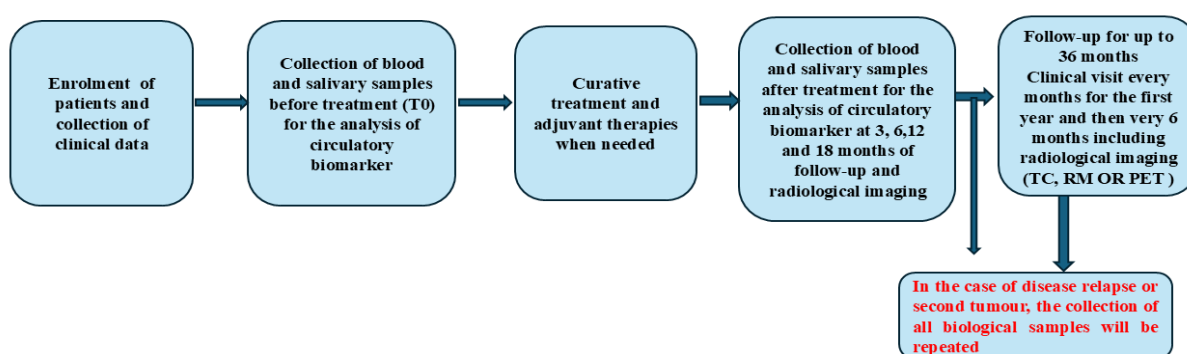


Figure 10: An overview of the Identify Study

3.2 Study Objectives

Primary Objectives

- To identify a profile of circulating biomarkers (from blood plasma, saliva) with adequate sensitivity and specificity to identify patients with squamous cell carcinoma of the head and neck area at higher risk of disease recurrence and/or second tumour.

- To identify cohorts prone to relapse based on highly sensitive and specific diagnostic measures of metabolic, methylation markers, viral markers, clinical parameters and a radiomic-combinatorial approach based on liquid biopsy, imaging and rich clinical parameters.

Secondary Objectives

- To identify a recurrence/second tumour earlier than clinical and radiological investigations.
- To identify potential pathways associated with rich metabolic genes that can be targeted for effective therapeutics.
- To identify, in the relapse cohort, any subgroups with distinct characteristics such as genetics, metabolic, pathological, immunological, or radiological.
- To identify pathways potentially targetable with adjuvant therapy in the group of patients at greatest risk of relapse/second tumour.
- To identify how radiation treatment impacts the immune system and the lymphocyte subpopulation.

Inclusion criteria:

- Age $\geq$ 18 years.
- Written informed consent.
- Squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx and larynx confirmed by biopsy and histological examination.
- Clinical stage II-IV according to the VIII edition of the AJCC staging system, regardless of the state of p16.
- Willingness to supply blood, saliva and wound drainage fluid (WDFs) samples for study (WDFs limited to a subgroup of patients at the Coordinating Centre: ASST Spedali Civili of Brescia and University of Brescia).

Exclusion criteria:

- Relapsing or metastatic disease.
- Locally advanced disease not amenable to treatment with curative intent.
- Previous local or systemic treatment for head and neck cancer.
- Patient with sinonasal or nasopharyngeal carcinoma.

- Patient with lateral neck metastasis from unknown primary, except for HPV-positive nodes, stage II according to the VIII edition of the AJCC staging system.
- Any concomitant chemotherapy, experimental protocol, biological or hormonal therapy for cancer treatment.
- Subjects with previous malignancies (except non-melanoma skin cancers and the following in situ tumours: bladder, stomach, oesophagus, colon, endometrium, cervix/dysplasia, melanoma or breast), unless complete remission has been achieved at least 2 years before entering the study, AND no additional therapy is required during the study period.
- Psychiatric disorder or known substance abuse that could interfere with cooperation in the study protocol.
- History or recent onset of any condition which, in the investigator's opinion, could interfere with the participation of the subject for the entire duration of the protocol.

3.3 Participating Centres

The whole study is coordinated by ASST Spedali Civili of Brescia and the University of Brescia.

Table 6 reports participating centres that have met the feasibility criteria of the study and are involved in the enrolment of patients who meet the inclusion criteria, collect main clinical-biological prognostic factors, perform biological sampling as required by the flowchart (Table 7) and follow up patients as per clinical practice.

Enrolment centres
ASST Spedali Civili of Brescia
IRCCS Humanitas di Rozzano (Milano)
Fondazione IRCCS Policlinico S. Matteo Pavia
AO Padova
Fondazione IRCCS San Gerardo Dei Tintori (Monza)
AOU Sassari
Policlinico S. Orsola-Malpighi (Bologna)
IRCCS Candiolo
Istituto Nazionale Dei Tumori (Milan)
AO Verona
Policlinico S. Martino di Genova

Table 6: Enrolment centres for the “Identify” Study

Table 7: Flowchart of the “Identify” Study

Period (duration)	Screening and Biological Samples Collection (≤ 7 days)	Baseline	Follow-up period										
Visit	0	1	2	3	4	5	6	7	8	9	10	11	12
Week	-1 to 0	1	12	24	36	48	72	96	120	144	168	192	240
Month	0	1	3	6	9	12	18	24	30	36	42	48	60
Informed Consent (a)	X												
Inclusion/Exclusion Criteria	X												
Demography	X												
Clinical-biological prognostic factors (b)	X												
HPV assessment and tissue collection for analysis (c)	X*												
Blood Sample for Methylation (d)	X		X	X		X	X						
Plasma Sample for Metabolomic assays (e)	X		X	X		X	X						
Blood and Saliva Samples for HPV analysis (f)	X		X	X		X	X						
Saliva Sample for Biomarker assessments (g)	X		X	X		X	X						
Curative treatment (i)		X											
Radiological imaging (j)			X	X		X	X						
Disease status assessment (i)***			X	X	X	X	X	X	X	X	X	X	X
Assessment of second tumours (i)***			X	X	X	X	X	X	X	X	X	X	X
Survival status (i)			X	X	X	X	X	X	X	X	X	X	X

* For oropharyngeal tumours only;

** In case of surgical neck dissection and only at the coordinating centre;

*** In case of recurrence/second primary between the 18th and 36th month of follow-up, the whole set of biomarker analysis will be repeated before treatment starts.

(a) A signed, written informed consent form must be obtained from the patient before any study-specific procedures or assessments;

(b) Including age, sex, stage, smoke/alcohol status, comorbidities, immune conditions, environmental exposure, COVID-19 exposure, liver function, inflammatory markers, and type of treatment.

- (c) Assessment of p16 status will be performed only in oropharyngeal cancer patients using the CINtec p16 histological test (Ventana Medical Systems, Tucson, AZ, USA) with strong and widespread nuclear and cytoplasmic staining in at least 70% of the cells used as a reference for positivity and assessment of HPV DNA status by PCR or ISH, HPV DNA viral load and E6/E7 HPV mRNA detection by genotype-specific Real-time PCR/RT-PCR/ddPCR assays
- (d) Peripheral blood (10 mL) samples will be collected in an EDTA tube to assess the level of methylation of circulating DNA of the SEPT9 and SHOX2 genes;
- (e) Plasma (300 µL) samples collected on which a fully quantitative targeted mass spectrometry (MS) analysis (targeting over 180 metabolites) will be performed;
- (f) Peripheral blood (10 mL Cell-Free DNA BCT tube (Streck)) and saliva (FLOQSwab resuspended in 1 mL eNAT) samples will be collected to: detect and quantify HPV 16, 18, 31, 33, 45, 51 and 52 DNA, quantify HPV-16 DNA viral load and detect HPV-16, 18, 31, 45 and 58 oncogenic transcripts (mRNA);
- (g) Saliva (about 5 mL) will be collected to test hypermethylation of circulating SEPT9 and SHOX2 DNA in saliva and the level of miR-423-5p and miR-626;
- (h) WDFs (both cervical and flap donor site, about 30 mL) will be collected at postoperative days 1 and 3 to test hypermethylation of SEPT9 and SHOX2 in cell-free circulating DNA (ccfDNA);
- (i) Curative treatment (surgical approach or chemo/radiation therapy), disease status, second tumour development assessment and survival status (cancer-free survival and overall survival) will be evaluated over time.
- Standard follow-up will be requested to comply with the following schedule:
- clinical visits: every 3 months for the 1st year; every 4 months for the 2nd year; every 6 months for the 3rd and 4th year, then annually
- (j) Both CT scan and MRI of head and neck are accepted (+/- 1 month); In case of already foreseen PET-CT scan, it may substitute analysis of locoregional area. Imaging of distant sites in case of smoking history ≥ 20 pack/years, annually with CT scan of thorax (according to NCCN guidelines) or with PET-CT scan.

All patients enrolled in the study by the participating centres with newly diagnosed primary squamous cell carcinoma of the head and neck region (oral cavity, oropharynx, hypopharynx, larynx), Stage II–III–IV (8th AJCC edition), undergo:

- **Collection of key clinical-biological prognostic factors:** age, sex, stage, smoking/alcohol status, comorbidities, immune conditions, environmental exposures, COVID exposure, liver function, inflammatory markers, and type of treatment.
- **For oropharyngeal tumours:** assessment of p16 status using the CINtec® p16 histological test (Ventana Medical Systems, Tucson, AZ, USA), with strong and diffuse nuclear and cytoplasmic staining in at least 70% of tumour cells as the criterion for positivity. HPV DNA status will be assessed by a real-time-based quantitative assay and an in-house droplet digital PCR assay.
- **Collection of blood and saliva samples** for the analysis of various circulating biomarkers.

All collected samples are then transported to the participating laboratories for analysis (see Table 8 and Figure 11 for the list of laboratories and circulating targets).

Participating Laboratories	Analysis Carried Out
Laboratory of Applied Biology, University of Brescia	Analysis of the methylation of the DNA circulating in the plasma, saliva and WDFs
Laboratory of Immunology, University of Brescia	Analysis of the immune cell population
Institute of Molecular Medicine, A. Nocivelli, ASST Spedali Civili Brescia	Analysis of salivary miRNAs

Laboratory of Clinical Microbiology and Virology, University of Milano-Bicocca	Analysis of circulating HPV genotype-specific DNA and viral load in blood and saliva
Dr Wishart's The Metabolomics Innovation Centre, University of Alberta, and revalidated at CHU de Québec- Université Laval, Quebec City, QC, Canada	Metabolomics Assay using the Phytonix platform
BioMark Diagnostics Solutions Inc. lab	SSAT1 Amantadine assay for metabolomic analysis

Table 8: List of participating laboratories and analysis carried out.

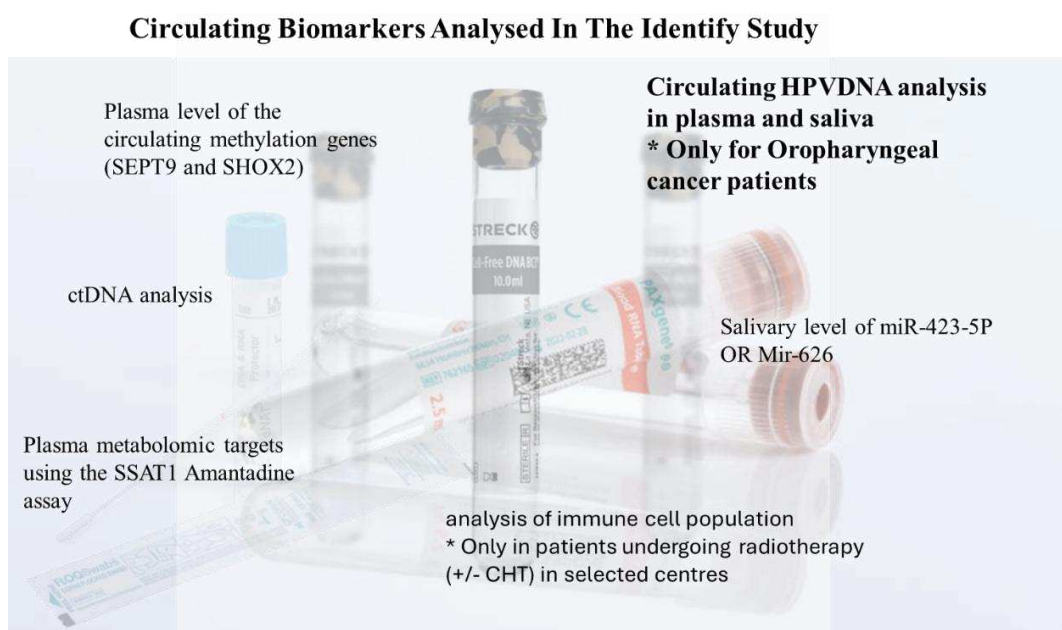


Figure 11: various circulating biomarkers analysed for the IDENTIFY study

For this thesis, presumed HPV-positive head and neck cancer patients were considered. All samples were sent to the **Laboratory of Clinical Microbiology and Virology, University of Milano-Bicocca, for the analysis of circulating HPV genotype-specific DNA and viral load in plasma and saliva.**

CHAPTER 4: MATERIALS AND METHODS

4.1 Study population

The study population included all patients enrolled in the multicentre IDENTIFY study who had a newly diagnosed primary oropharyngeal squamous cell carcinoma (OPSCC). For these patients, p16 status was evaluated using the CINtec® p16 Histology test (Ventana Medical Systems, Tucson, AZ, USA) by the respective enrolling centres, while disease staging was determined according to the 8th edition of the AJCC classification. A case was classified as p16-positive when strong and diffuse nuclear and cytoplasmic staining was observed in $\geq 70\%$ of tumour cells.

In addition to OPSCC patients, blood and saliva samples were also collected from patients with newly diagnosed primary cancers of other head and neck sites (larynx, oral cavity, and hypopharynx). All collected samples were subsequently sent to the Laboratory of Clinical Microbiology and Virology at the University of Milano-Bicocca for analysis.

4.2 Sample collection

Blood and saliva samples were collected from all participating patients. Blood samples were drawn into Cell-Free DNA BCT® CE tubes (Streck) for the preservation of cell-free DNA (Figure 12a), and Saliva samples were collected using FLOQSwabs® (Copan), which were then immediately resuspended in eNAT® (Copan) medium to stabilise the nucleic acids (Figure 12b).

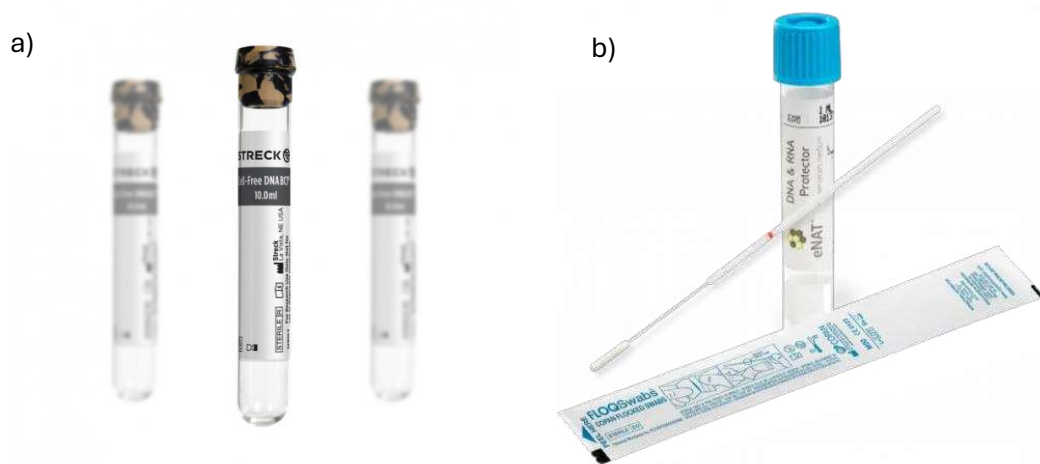


Figure 12: a) Cell-Free DNA BCT® CE (Streck); b) FLOQSwabs® resuspended in eNAT®(Copan)

4.2.1 Cell-Free DNA BCT® CE (Streck) Tube

The Cell-Free DNA BCT® CE tube is specifically designed for the collection and preservation of blood samples intended for cell-free DNA (cfDNA) analysis. It stabilises cfDNA in plasma and also preserves cellular genomic DNA from nucleated blood cells and circulating epithelial or tumour cells present in whole blood. The tube contains a specialised preservative reagent that:

- Stabilises nucleated blood cells
- Prevents the release of genomic DNA
- Inhibits nuclease-mediated degradation of cfDNA

This ensures the overall integrity and stability of cfDNA in the sample.

Samples collected in Cell-Free DNA BCT® CE tubes remain stable for up to 14 days at temperatures ranging from 6 °C to 37 °C, enabling flexible sample collection, transport, and storage without immediate processing [108].

Blood is drawn via venipuncture. Immediately after collection, the tube must be gently inverted 10 times to mix the blood with the preservative (Figure 13). One complete inversion involves a full 180-degree rotation of the wrist and return to the starting position, as illustrated below.

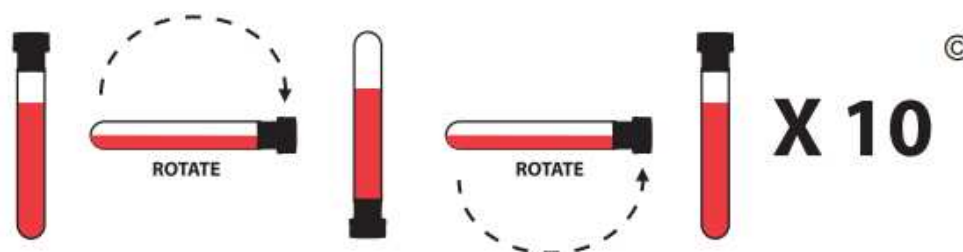


Figure 13: inversion method after sample collection

4.2.1a Plasma collection and processing

1. Sample Transport:
 - Blood samples were processed using a double-spin method within three days of collection to isolate plasma and minimise cellular contamination.
2. First Centrifugation:
 - Whole blood samples were centrifuged at $1,600 \times g$ for 10 minutes at room temperature.
 - After centrifugation, the plasma layer was carefully transferred to a 15 mL tube.

- The buffy coat was separated and stored in a cryovial for future use.
3. Second Centrifugation:
 - The separated plasma in the 15 mL tube underwent a second centrifugation step at $1,600 \times g$ for an additional 10 minutes at room temperature to remove residual cellular debris.
 - During this step, cryovials were prepared and labelled with the patient ID, timepoint, and collection date.
 4. Aliquoting and Storage:
 - After the second centrifugation, the purified plasma was aliquoted into labelled cryovials, with each vial containing 1 mL of plasma, for subsequent analysis and storage at -80°C

4.2.2 The eNAT® system paired with FLOQSwabs® (Copan)

Copan eNAT® system is designed for the collection, transport, and preservation of clinical specimens intended for analysis via nucleic acid amplification techniques (NAATs). The eNAT® medium stabilises and preserves both RNA and DNA for extended periods and is compatible with most commercially available nucleic acid extraction and amplification platforms. eNAT® is a detergent- and guanidine thiocyanate-based medium that rapidly inactivates nucleases and stabilises nucleic acids from viruses, bacteria, *Chlamydia*, protozoa, and *Mycoplasma*. Its combination with FLOQSwabs®, the system enables efficient, capillary-driven sample uptake and enhanced elution of biological material, thereby improving the quality and sensitivity of downstream molecular diagnostics.

eNAT® medium preserves nucleic acids for:

- Up to 4 weeks at room temperature (RT) or 4°C
- Up to 6 months at -20°C to -80°C [109,110].

4.2.2a Saliva collection and processing

1. Pre-sample Collection: Patients are advised to avoid eating or drinking for at least 1–2 hours before sample collection.
2. Sample Collection: Using a FLOQSwab®, the inside of the mouth and tongue surfaces are gently swabbed to collect sufficient cellular material.

3. Sample Stabilisation: Immediately after collection, the swab is placed into 1 mL of eNAT® medium and mixed thoroughly.
4. Laboratory Processing:
 - Upon arrival at the laboratory, each sample was vortexed vigorously for 30 seconds.
 - The sample is then aliquoted into two cryovials, each containing 500 µL.
 - Cryovials are labelled with the patient ID, collection timepoint, and date
5. Storage: Samples are stored at –80 °C until further analysis.

4.3 Nucleic Acid Extraction

Nucleic acid extraction was performed using the NucliSENS® easyMAG® system (bioMérieux, Marcy-l'Étoile, France) (Figure 14). This is an automated system that utilises BOOM® technology, an innovative method developed by bioMérieux for solid-phase nucleic acid extraction using silica beads. The principle behind this methodology is based on electrostatic interaction, allowing negatively charged DNA and RNA molecules to bind and adhere to the surface of positively charged silica beads.



Figure 14: The NucliSENS® easyMAG® system (bioMérieux, Marcy-l'Étoile, France)

The NucliSENS easyMAG nucleic acid extraction system operates on a generic and robust method for isolating total nucleic acids (RNA and DNA) from complex biological specimens using magnetic silica particles, based on Boom chemistry. In this process (Figure 15), liquid samples - such as plasma, saliva, or swab eluates - are first mixed with a lysis buffer containing a chaotropic agent, guanidinium thiocyanate (GuSCN). This agent disrupts viral particles, bacteria, fungi, and any cellular material present, thereby releasing nucleic acids while simultaneously inactivating nucleases that could degrade them. Magnetic silica particles are then added to the lysate, where nucleic acids selectively bind to the silica under high-salt conditions provided by the lysis buffer. The bound nucleic acids are subsequently purified

through a series of washes using two different wash buffers that remove proteins, inhibitors, and other contaminants. The final step involves eluting the nucleic acids from the magnetic silica using an elution buffer, typically at an elevated temperature to enhance release. The magnetic silica is then separated from the eluate using the magnetic device, leaving behind a purified and concentrated nucleic acid solution suitable for downstream molecular applications, such as PCR or next-generation sequencing.

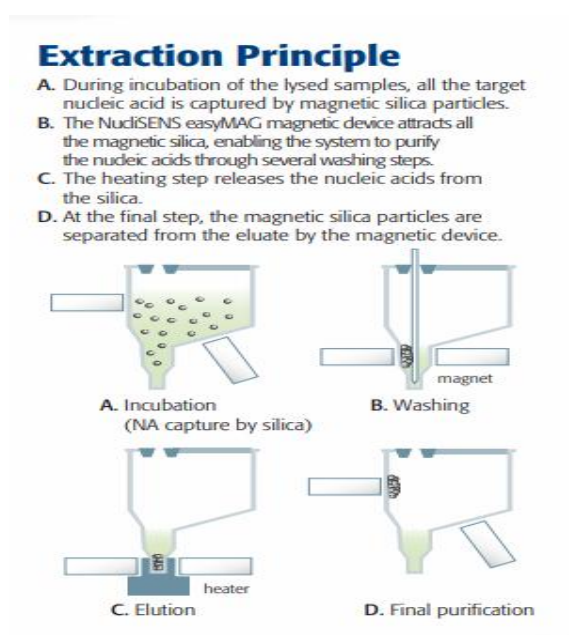


Figure 15: Operating principle of the NucliSens easyMAG automatic extractor, by BioMérieux

The NucliSENS easyMAG (Figure 16) nucleic acid extraction system is equipped with a user-friendly touchscreen interface that facilitates efficient management of samples, reagents, and consumables. The integrated software provides systematic guidance throughout the workflow, enabling accurate data input and protocol selection. At the beginning of the extraction process, key sample parameters are entered into the system, including the sample matrix, input volume, desired elution volume, and the specific extraction protocol to be used.

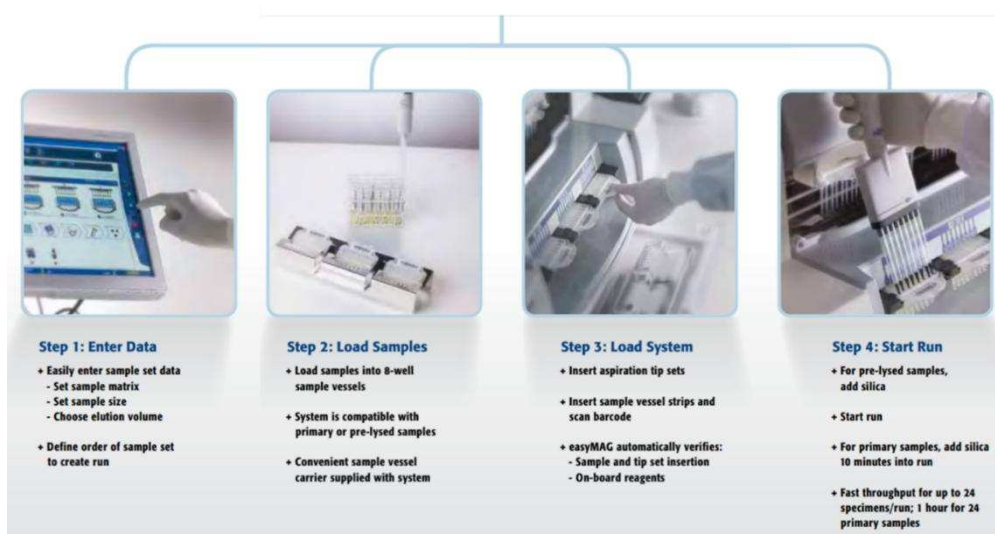


Figure 16: Workflow of the Nucleic Acid Extraction Process Using the NucliSENS easyMAG System

The system offers two protocol options: Generic and Specific B Protocol 2.0.1. The Generic protocol typically requires 30–40 minutes, while the Specific B Protocol 2.0.1 takes approximately 50–60 minutes, depending on sample load. The Specific B Protocol 2.0.1 includes additional wash steps and utilises elevated temperatures of about 70°C during the elution phase to enhance nucleic acid purity and yield.

- For this study, the Specific B Protocol 2.0.1 was used.
- Once the protocol was selected (Specific B Protocol 2.0.1),
- The biological samples (plasma and saliva) were loaded into eight-well carriers, each uniquely barcoded for traceability (Table 9).
- The system is compatible with both pre-lysed samples and primary specimens. Primary samples like ours must undergo a lysis step before processing, which was 10 minutes of incubation in the lysis buffer for our sample types.
- Crucially for quality control and validation, an additional 10 µl of an exogenous control gene target (synthetic firefly luciferase DNA), provided with the OncoPredict HPV Quantitative Typing kit, was added to each sample during the incubation step.
- After the incubation time, 125 µL of diluted (a ratio of 1:2) magnetic silica is added to bind nucleic acids.
- Once samples were prepared and loaded, the automated isolation and purification of nucleic acids were initiated via the system's touchscreen interface.

Sample type	Starting volume	Elution volume
Plasma	1ml (1000 µl)	50 µl
Saliva	0.5ml (500 µl)	50 µl

Table 9: Starting and elution volumes of each sample type

4.4 HPV Detection and quantification using the OncoPredict HPV Quantitative Typing assay (QT) and digital droplet PCR (ddPCR)

4.4.1 OncoPredict HPV Quantitative Typing assay (QT)

The OncoPredict HPV QT assay (Hiantis Srl, Milan, Italy) is a CE-marked, quantitative, multiplex real-time PCR assay designed for the detection and full-genotyping of high-risk human papillomavirus. This assay has an initial intended use for cervical screening on various sample types, such as cervical, self-collected vaginal samples, and urine.

- A. Target Genes: The assay targets type-specific sequences within the E6 and E7 oncogenes of 12 HR-HPV genotypes: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59. Resulting amplicon sizes range from 60 to 128 base pairs, varying by genotype.
 - B. Assay Setup and Reaction Components: The assay contains five reaction wells (illustrated in Figure 17): a quality control well (QC) and four quantitative detection wells (QT1–QT4).
 - Quality Control Module (QC): This reaction well serves as a comprehensive quality control module to assess sample adequacy and processing integrity throughout the analytical process. The QC module specifically evaluates:
 - Sample cellularity by quantifying the single-copy human CCR5 gene
 - Extraction efficiency through the detection of a synthetic firefly luciferase DNA control added prior to extraction
 - PCR inhibition, monitored via amplification of an additional synthetic, non-human target.
 - Quantitative Modules (QT1-QT4): Four wells (QT1 to QT4) are for quantitative detection, with each well able to detect three specific HR-HPV genotypes. Each of these four wells also includes an external amplification control, which is a synthetic, non-human DNA sequence to monitor for PCR inhibition.
- In addition to the incorporated controls, which are important for the preanalytical process, the assay also includes external controls to ensure proper PCR reaction performance.

- Negative Control or Non-template control (NTC)
- Positive Control (PC) - (synthetic DNA containing the target sequences of 13 HPV types)
- Sets of calibrators (Cal 1- Cal 4)

Both HPV viral load and sample cellularity are determined using standard calibration curves. Results are expressed as viral copies/mL for plasma samples and viral copies per 10⁴ human cells, normalised to the cell count derived from CCR5 quantification.

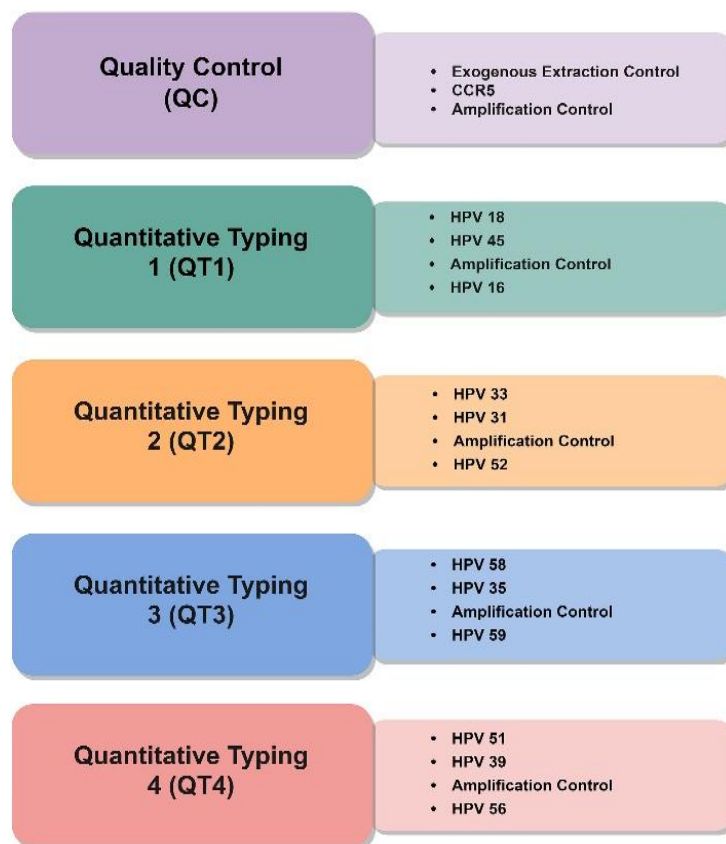


Figure 17: the set-up of the OncoPredict HPV QT assay, composed of quality control (QC) for the evaluation of sample adequacy and 4 Quantitative modules (QT1 to QT4) for the detection of type-specific viral loads.

PCR plate preparation

10 µl master mix (this comprises reaction mix, each module (QC and QT1-QT4) and enzymatic solution) is added to the corresponding reaction wells of a PCR plate, after which 5 µl of sample

template or calibrators 1-4 was added, resulting in a final reaction volume of 15 µl per reaction well. Similarly, 5 µl of positive and negative controls are added to their respective wells (Table 10)

	Samples	PC QT	NTC
Wells	A1 -G10	A11-G13	H2-H10
QC and QT1-QT4 master mix	10µl	10µl	10µl
Samples	5µl	-	-
PC QT	-	5µl	
NTC	-	-	5µl
Total	15µl	15µl	15µl

Table 10: sample of plate preparation and volume of master mix and sample to be added to each plate well

Following preparation and dispensing, the reaction plate was loaded into the CFX96 Touch Real-Time PCR thermocycler (Bio-Rad) for amplification. Four fluorophores were used for detection: FAM, HEX, ROX, and Cy5 (Table 11).

QC	QT1	QT2	QT3	QT4	Fluorophores channel
CCR5	HPV 16	HPV 52	HPV 59	HPV 56	CY5
Exogenous control	HPV 18	HPV 33	HPV 58	HPV 51	FAM
-	HPV 45	HPV 31	HPV 35	HPV 39	ROX
IAC	IAC	IAC	IAC	IAC	HEX

Table 11: Each HPV genotype in each reaction well and its corresponding fluorophore channel

The thermal cycling protocol applied is outlined in Table 12 below.

cycle	repetition	step	Time	temperature
1	1	1	15:00	95°
2	50	1	00:20	95°
		2 (*)	01:00	60°

Table 12: The OncoPredict thermal profile

All HPV16-positive samples were further quantified through the use of our in-house optimised droplet digital PCR (ddPCR) using the QX200 Droplet Digital PCR System (Bio-Rad, Hercules, CA).

4.4.2 In-house optimised droplet digital PCR (ddPCR)

ddPCR has recently emerged as a highly effective method for the detection of cancer-related biomarkers in liquid biopsy samples, due to its remarkable sensitivity and specificity. When compared to quantitative PCR (qPCR), ddPCR demonstrates significantly higher sensitivity, achieving detection levels as low as 0.01%. This ability makes it particularly advantageous for applications such as absolute allele quantification, identification of ctDNA somatic mutations, DNA methylation profiling, and gene rearrangement analysis. The ddPCR methodology involves the partitioning of the reaction mixture into approximately 20,000 nanolitre-sized droplets via a water-oil emulsion system. Within each droplet, PCR amplification occurs independently, and the resulting fluorescence signals generated by target-specific probes or intercalating dyes are subsequently analysed to differentiate between positive and negative droplets, thereby facilitating accurate quantification of target nucleic acids [111]. A major turn-off in the use of ddPCR for routine analysis is its turnaround time (4- 5 hours) and cost.

ddPCR Workflow Protocol

- Preparation of Total Mix (per sample)

Reagent	Volume (Per sample)
Master mix (ddpcr supermix no DUTP)	10 µl
Forward primers (QT)	0.9 µl
Reverse primers (QT)	0.9 µl
probe	0.25 µl
H2O*	7 or 9µl

Table 13: Preparation of the Total mix for each sample. *The volume of H2O added is based on the volume of nucleic acid extract added, which is 3ul for plasma and 1ul for saliva

Workflow steps:

1. Plate Loading:
 - Dispense 21 µL (saliva) or 19 µL (plasma) of the Total Mix into each well of the ddPCR 96-well transparent plate.
 - Add 1 µL or 3 µL of the DNA sample to each well.
2. Cartridge Preparation (Figure 18a and 18b):
 - Transfer 20 µL of the reaction mixture into the middle row (**red**) of the droplet generation cartridge (Figure 18b).
 - Add **70 µL of droplet generation oil** into the **bottom row (green)** of the cartridge (Figure 18b).

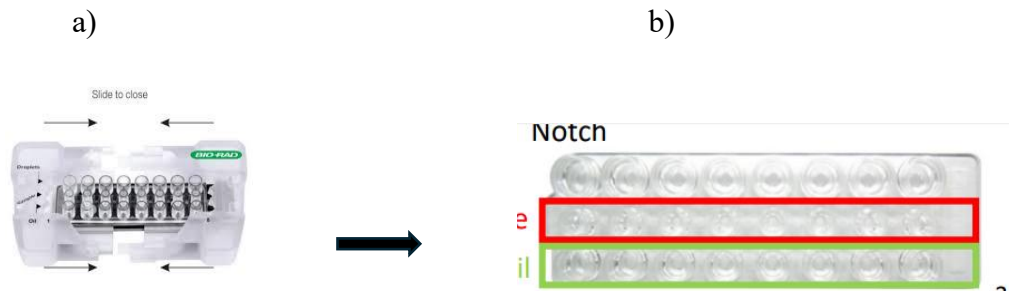


Figure 18: a) the ddPCR droplet generation cartridge and b) the designated wells for both samples and droplet generation oil

3. Droplet Generation:

- Secure the gasket over the cartridge (Figure 19a).



Figure 19: a) preparing the droplet generator cartridge to be placed into the droplet generator by securing a gasket over it, b) Bio-Rad droplet generator

4. After Post-Emulsification:

- Carefully transfer 40 μ L of the generated droplets back into the corresponding wells of the ddPCR plate.

5. Sealing:

- Seal the plate with a pierceable foil seal.
- Place the sealed plate in the Bio-Rad Plate Sealer.

6. PCR Amplification:

- Transfer the sealed plate to the T100 Thermal Cycler (Bio-Rad) (Figure 20).
- Run the following thermal cycling protocol:
 - Initial denaturation: 95°C for 10 minutes
 - 40 cycles of:
 - Denaturation: 94°C for 15 seconds
 - Annealing/Extension: 60°C for 1 minute



Figure 20: Bio-Rad thermal cycle

7. Droplet Reading & Analysis:

- Load the completed PCR plate into the QX200 Droplet Reader (Bio-Rad) (Figure 21) to measure the droplet absorbance.
- Analyse the fluorescence signal using QuantaSoft Software v1.7.4.0917.



Figure 21: Bio-Rad QX200 Droplet Reader

4.5 Statistical Analysis

HPV DNA prevalence was estimated as the proportion of blood and saliva samples that tested positive for any HPV DNA genotype using the OncoPredict QT assay, with corresponding binomial 95% confidence intervals (CIs). Demographic data were tabulated and stratified by sex, age, and disease site (OPSCC vs. non-OPSCC) according to HPV biomarker status as determined by the OncoPredict QT assay and Fisher's exact test will be used to assess their associations, with a two-sided p-value < 0.05 considered statistically significant.

Agreement between p16 status and HPV DNA detection was evaluated using Cohen's Kappa coefficient (κ), with corresponding 95% CIs. The level of agreement was defined as:

- None - $\kappa < 0.01$,
- Poor - $0.01 < \kappa < 0.20$,

- Fair – $0.21 < k < 0.40$
- Moderate – $0.41 < k < 0.60$,
- Good – $0.61 < k < 0.80$,
- Very good/excellent – $0.81 < k < 1.00$.

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) will be calculated considering all p16-positive samples as diseased and p16-negative as non-diseased

The correlation between disease stage and HPV viral load in blood and saliva was assessed using statistical analysis performed in **R**.

CHAPTER 5: RESULTS

5.1 Study Cohort Characterisation

5.1.1 Patient Enrolment and Sample Collection

As part of the Identify Study, plasma and saliva samples from 74 patients were sent to the Laboratory of Clinical Microbiology and Virology for HPV DNA detection and quantification. Of these, 7 patients were excluded from the analysis due to either missing baseline (T0) samples, withdrawal from the study, or death (see Figure 22).

The final analysis included samples from 67 patients at baseline (T0); 37/67 patients at the 3-month follow-up (FU3M), 33/67 at FU6M, 26/67 at FU12M and 13/67 patients at FU18M. 62 plasma samples and 60 saliva samples were obtained at T0 from the 67 patients, while at FU3M, 37 plasma and 31 saliva samples were collected; patients and samples collected at subsequent follow-ups are illustrated in Figure 22.

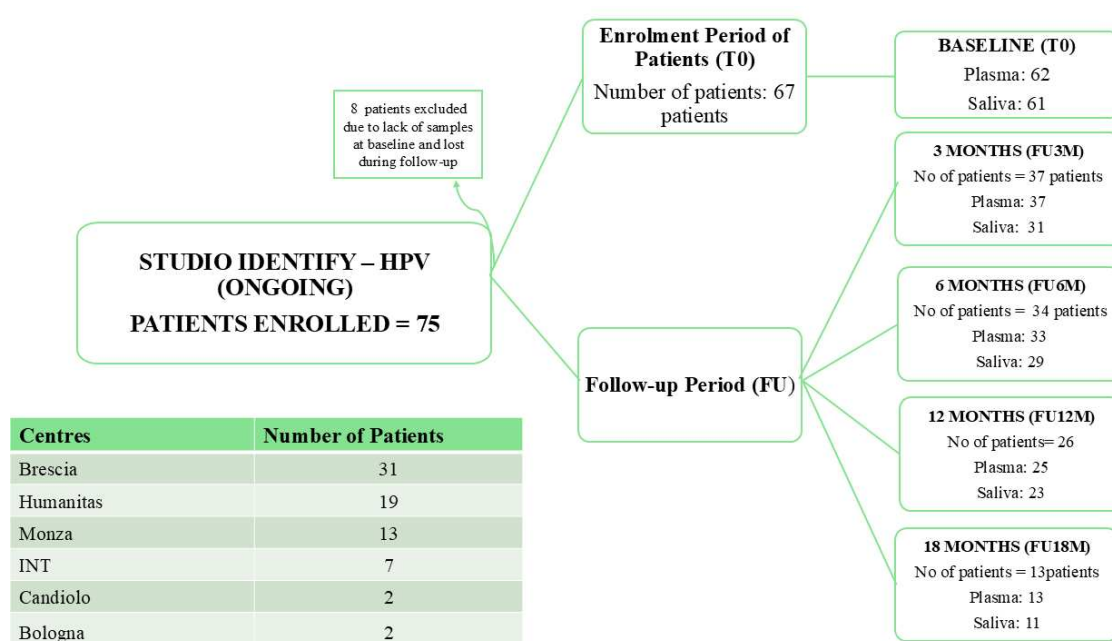


Figure 22: Flowchart of patients enrolled, and samples collected at Time 0 (T0), and at subsequent follow-up timepoints

5.1.2 Demographic and Clinical Characteristics of the Study Population

The cohort consisted of 80.6% males and 19.4% females, with a median age of 63 years (range: 39–88). The oropharynx was the most common primary tumour site, accounting for 76.3% of all cases, followed by the oral cavity and larynx (8.9% each) and the hypopharynx (5.9%). Most patients were diagnosed with the disease at stage 1 (32.8%) and stage III (34.3%). Regarding smoking status, 46.3% were ex-smokers, 16.4% were current smokers, and 29.9%

were non-smokers. Forty-five patients had p16-positive tumours, while 21 were p16-negative. Demographic and clinical characteristics of the study population are summarised in Tables 14 and 15.

	Percentage (n)
Total number of patients	100% (n=67)
Gender distribution	
Male	80.6% (n=54)
Female	19.4% (n=13)
Median age (range)	63 (39–88) years
Primary Tumour Site	
Oropharynx	76.1% (n= 51)
Oral cavity (Tongue)	8.9% (n= 6)
Larynx	8.9% (n=6)
Hypopharynx	5.9% (n=4)
AJCC Stage	
I	32.8% (n=22)
II	14.9% (n=10)
III	34.3% (n=23)
IV	17.9% (n=12)
Smoking History	
Smoker	16.4% (n=11)
Ex-smoker	46.3% (n=31)
Non-smoker	29.9% (n=20)
Unknown*	7.4% (n=5)
Comorbidities	
Yes	67.2% (n=45)
No	31.3%(n=21)
Unknown*	1.5% (n=1)
HPV Status (p16 Status)	
p16 positive	67.1% (n=45)
p16 negative	31.3% (n=21)
Unknown*	1.5% (n=1)
ctHPVDNA Status with OncoPredict QT assay	
Positive	64.2% (n=43)
Negative	35.8% (n=24)
Treatment Modalities	
Radiotherapy alone (RT)	7.5% (n=5)
Radiotherapy + Chemotherapy (RT-CHT)	73.1% (n= 49)
Surgery alone	11.9% (n=8)
Surgery + Radiotherapy (Surgery+RT)	7.5% (n=5)

*Unknown means that the data are not available or missing

Table 14: A summarised description of patients’ demographics

Patients ID	Age	Sex	Disease Site	TNM Classification	AJCC Stage	Smoking Status	Treatment	p16 Status (IHC)
BS008	47	M	Oropharynx	T4N0	III	Yes, 30P/Y	RT-CHT	Positive
BS013	77	M	Oropharynx	T4N1	III	Ex, 120P/Y	RT-CHT	Positive
BS017	69	M	Oropharynx	T1N1	I	Ex, 55P/Y	RT-CHT	Positive
BS018	75	M	Oropharynx	T1N3	II	Ex, 2P/Y	RT-CHT	Positive
BS020	73	M	Oropharynx	T4N1	III	Yes, 35P/Y	RT-CHT	Positive
BS023	66	M	Oropharynx	T2N1	I	Ex, 4P/Y	RT-CHT	Positive
BS024	57	F	Oropharynx	T1N1	I	Ex, 40P/Y	RT-CHT	Positive
BS031	66	M	Oropharynx	T3N2	II	Ex, 15P/Y	RT-CHT	Positive
BS034	80	M	Oropharynx	T2N0	I	Ex, 30P/Y	RT	Positive
BS039	39	F	Oropharynx	T4N2	III	No	RT-CHT	Positive
BS043	54	M	Oropharynx	T4N2	III	Ex, 40P/Y	RT-CHT	Positive
BS044	70	M	Oropharynx	T1N2	II	Ex, 2.5P/Y	RT-CHT	Positive
BS053	64	M	Oropharynx	T3N1	II	No	RT-CHT	Positive
BS058	66	F	Oropharynx	T1N1	I	Ex, 17P/Y	RT-CHT	Positive
BS062	45	F	Oropharynx	T4N2	III	Ex, 15P/Y	RT-CHT	Positive
BS063	69	M	Oropharynx	T2N1	I	Ex, 22P/Y	RT-CHT	Positive
BS070	54	M	Oropharynx	T2N1	I	Ex, 20P/Y	RT-CHT	Positive
BS071	53	M	Oropharynx	T2N2	II	Yes, 30P/Y	RT-CHT	Positive
BS072	58	M	Oropharynx	T2N1	I	No	RT-CHT	Positive
HU012	54	M	Oropharynx	T1N1	I	No	RT-CHT	Positive
HU018	67	M	Oropharynx	T4N0	III	No	RT-CHT	Positive
HU024	54	M	Oropharynx	T2N1	I	Ex, 10P/Y	RT-CHT	Positive
HU025	61	F	Oropharynx	T1N1	I	Ex, 3P/Y	RT	Positive
HU026	49	M	Oropharynx	T1N1	I	Ex, 2P/Y	RT-CHT	Positive
HU027	49	M	Oropharynx	T3N2	III	No	RT-CHT	Positive
HU036	77	M	Oropharynx	T2N2	II	No	RT-CHT	Positive
HU039	52	F	Oropharynx	T1N1	I	No	RT-CHT	Positive
HU040	79	M	Oropharynx	T4N1	III	EX, UNK	RT-CHT	Positive
HU041	41	F	Oropharynx	T2N1	I	YES, 15P/Y	RT-CHT	Positive
HU043	69	M	Oropharynx	T4N1	III	EX, 50 P/Y	RT-CHT	Positive
HU045	67	M	Oropharynx	T4N1	III	NO	RT-CHT	Positive
HU046	59	F	Oropharynx	T2N1	I	NO	RT-CHT	Positive
HU048	64	M	Oropharynx	T2N1	I	NO	RT-CHT	Positive
INT002	51	M	Oropharynx	T1N1	I	NO	RT-CHT	Positive
INT003	70	M	Oropharynx	T4N1	III	EX, 10P/Y	RT-CHT	Positive
INT006	59	M	Oropharynx	T3N1	II	YES, 15P/Y	RT-CHT	Positive
INT010	51	M	Oropharynx	T3N1	II	NO	RT-CHT	Positive
INT012	55	M	Oropharynx	T1N1	I	NO	RT-CHT	Positive
INT013	65	M	Oropharynx	T4N1	III	YES, 11P/Y	RT-CHT	Positive
INT017	67	M	Oropharynx	T4N1	III	EX, 5P/Y	RT-CHT	Positive
CA002	76	M	Oropharynx	NA	NA	No	Surgery	Positive
CA013	NA	NA	Oropharynx	NA	NA	NA	RT-CHT	Positive
BO012	65	M	Oropharynx	T4N2	III	YES, 75P/Y	RT-CHT	Positive
MB005	64	M	Oropharynx	T4N2M0	III	NA	RT	Positive
MB007	62	M	Oropharynx	T4N1	III	Ex	CHT + RT	Positive
BS014	57	F	Larynx	T4N3	IV	Ex, 48P/Y	Surgery	Negative
BS025	52	M	Oropharynx	T4N2	IV	No	RT-CHT	Negative
BS026	67	M	Oropharynx	T2N2	IV	Ex, 60P/Y	RT-CHT	Negative
BS038	61	M	Hypopharynx	T4N2	IV	Yes, 50P/Y	RT-CHT	Negative
BS052	60	M	Hypopharynx	T3N0	III	Ex, 30P/Y	RT-CHT	Negative

BS067	55	M	Oropharynx	T2N3	IV	Ex, 1,25P/Y	RT-CHT	Negative
MB002	66	M	Oral cavity	T2N1M0	III	NA	Surgery +RT	Negative
MB003	79	M	Larynx	T3N2M0	IV	NA	Surgery +RT	Negative
MB004	67	M	Larynx	T3N0M0	III	Si, Ex	Surgery RT	Negative
MB008	67	M	Larynx	T3N0	III	10 sig/die da 50 aa	Surgery + RT	Negative
MB009	55	M	Larynx	pT4aPN0	IV	NA	Surgery + RT	Negative
MB010	74	F	Oral cavity	T1N0	I	NA	Surgery	Negative
MB011	78	F	Oral cavity	T2N0	II	No	Surgery	Negative
MB012	88	F	Oral cavity	T1N0	I	No	Surgery	Negative
MB013	61	M	Oral cavity	T3N2B	IV	No	Surgery	Negative
HU020	78	M	Larynx	T2N0	II	Ex, 60P/Y	RT	Negative
HU022	74	F	Oropharynx	T4N2	IV	Ex, NA	RT-CHT	Negative
BS011	53	M	Oral cavity	T4N3	IV	Ex, 7,5P/Y	Surgery	Negative
BS019	59	M	Oropharynx	T1N3	IV	Yes, 30P/Y	RT-CHT	Negative
BS068	65	M	Hypopharynx	T2N1	III	Yes, 80P/Y	RT	Negative
HU021	46	M	Hypopharynx	T3N3	IV	Ex, 45P/Y	RT-CHT	Negative
HU037	59	M	Oropharynx	TXN1	I	NO	SURGER Y	unknown

p16-positive patients
 p16-negative patients
 Patient with unknown p16 status
 NA= not available

Table 15: A comprehensive description of patients' demographics

5.1.3 Disease Stage and Tumour Site Distribution

The 67 patients were categorised according to the site of the tumour and the stage of the disease. Among patients with oropharyngeal cancer (N=51), the majority were diagnosed at Stage I = 20 and Stage III = 18, while a smaller proportion presented with advanced disease (Stage IV = 5); all patients in this latter group were p16-negative. Patients with cancer of the oral cavity (N=6) showed a balanced distribution across all stages. Laryngeal (N=6) and hypopharyngeal (N=4) cancers were more frequently diagnosed at advanced stages (Stage IV = 3 and 2, respectively) (Figure 23).

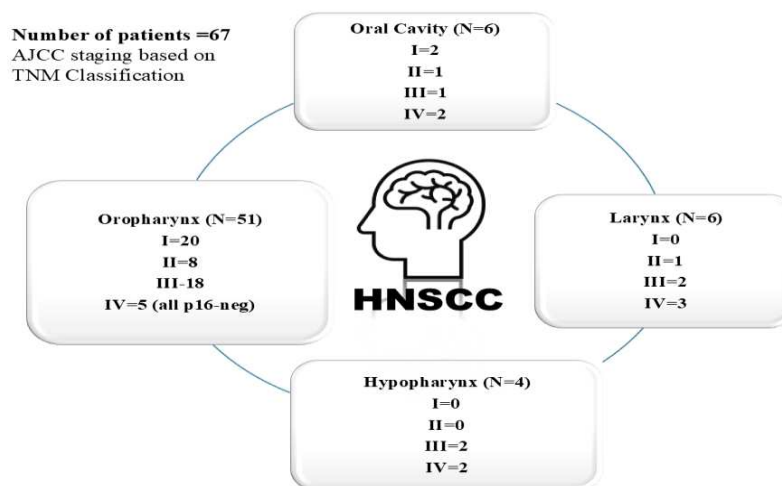


Figure 23: Patient cohort stratification based on disease stage and disease site.

5.2 Baseline (T0) HPV DNA Detection and Performance of the OncoPredict HPV QT Assay

5.2.1 HPV DNA Detection in Plasma Samples

A total of 62 patients had plasma samples at T0 out of the overall 67 patients. Out of these 62 blood samples, 41 samples had a positive p16 status, while 20 were p16-negative, and 1 had an unknown p16 status. The overall HPV positivity observed in the plasma samples at T0 was 59.7% (37/62; 95% CI= 47%-71%). In particular, ctHPVDNA was detected in 91.9% (34/37; 95% CI= 78% - 98%) of p16-positive patients, while only 8.1% (3/37; 95% CI= 2.1% - 22%) of p16-negative patients had detectable ctHPVDNA (Figure 24).

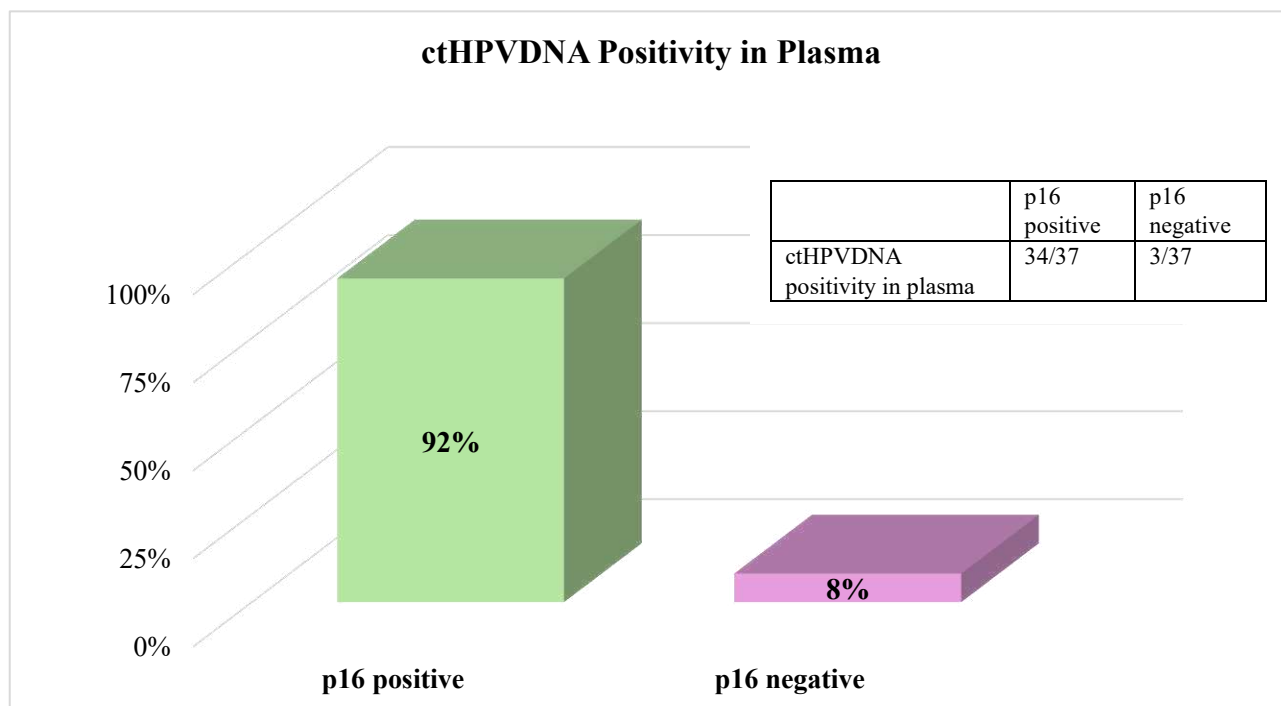


Figure 24: Percentage of HPV positivity in plasma

A concordance analysis using Cohen's kappa between the OncoPredict HPV QT assay in blood liquid biopsy and p16 immunohistochemistry in tissue biopsies, considering only samples with known p16 status. A good concordance with a kappa value of 0.65 (Table 16) was observed with a sensitivity and specificity of 82.9% and 85.0% respectively.

		p16 IHC			K=0.65
		Positive	Negative		
HPV OncoPredict QT assay	Positive	34	3	37	
	Negative	7	17	24	
		41	20	61	

Table 16: Cohen's Kappa for HPV OncoPredict QT assay positivity in blood vs p16IHC in tissue biopsy

5.2.2 HPV DNA Detection in Saliva Samples

A total of 61 patients had saliva samples at T0 out of the overall 67 patients. Out of these 61 saliva samples, 40 were p16-positive, 20 were p16-negative, and 1 had an unknown p16 status. Among the patients with saliva samples, the overall HPV positivity was approximately 49.2% (30/60; 95% CI= 37% - 61%). HPV DNA was detected in 96.7% (29/30; 95% CI= 82% -

100%) of p16-positive patients, while only 3.3% (1/30; 95% CI= 0.01% - 18%) of p16-negative patients had detectable HPV DNA (Figure 25).

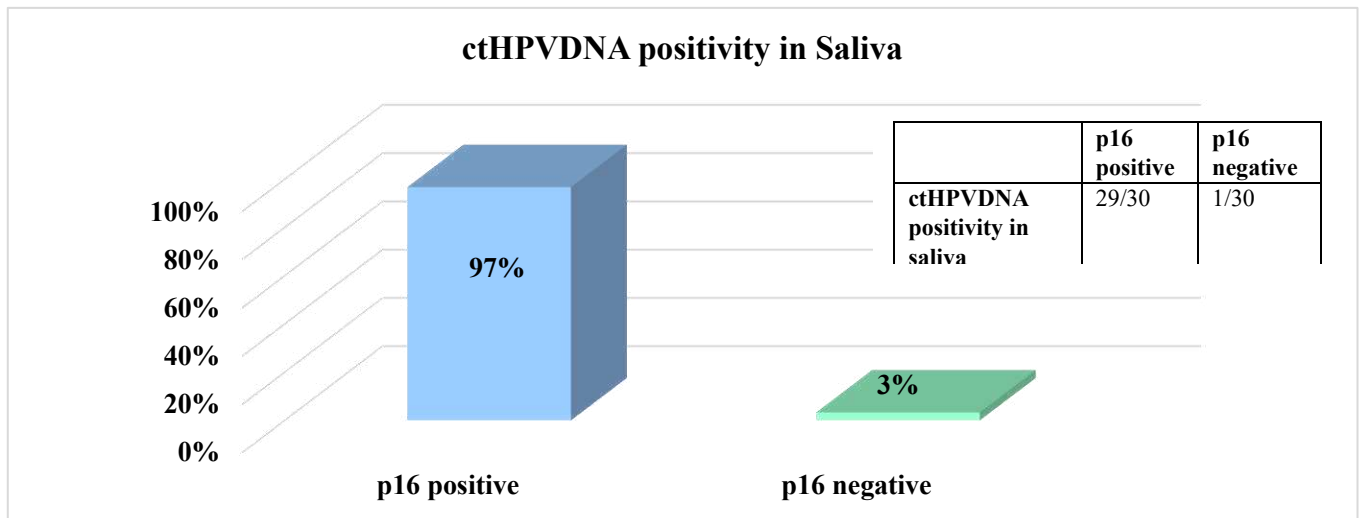


Figure 25: Percentage of HPV DNA positivity.

A concordance analysis using Cohen's kappa between the OncoPredict HPV QT assay in saliva samples and p16 immunohistochemistry, for those with known p16 status. A moderate concordance with a kappa value of 0.60 (Table 17) was observed with a sensitivity and specificity of 72.5% and 95.0%, respectively.

		p16 IHC		
		Positive	Negative	
HPV OncoPredict QT assay	Positive	29	1	30
	Negative	11	19	30
		40	20	60

K=0.60

Table 17: Cohen's Kappa for OncoPredict HPV QT assay in saliva vs p16IHC in tissue biopsy

5.2.3 Concordance of HPV DNA Detection in Paired Plasma and Saliva Samples

Focusing on the 55 (36 p16-positive and 19 p16-negative) patients with paired plasma and saliva samples collected at baseline (T0), the overall HPV DNA positivity rate increased to 61.8% (34/55; 95% CI= 49% - 74%) when compared to only plasma (59.7%) or saliva (49.2%). Among the p16+ patients, HPV DNA was detected in 94.1% (32/34; 95% CI = 80% - 99%) and in only 2/34 (5.9%; 95% CI=0.7% - 20%) of p16-negative patients. For the 34 HPV-positive patients, HPV genotypes were consistent across both plasma and saliva samples in the majority of double-positive cases (24/34). One exception was patient BO012, who tested positive for HPV 56 in plasma and for HPV 52 and HPV 31 in saliva. Multiple infections were observed only in saliva samples. In the p16-negative group, one patient showed HPV 16 positivity in both sample types.

The Cohen's kappa was calculated, and a good agreement of 0.77 was observed (Table 18). A sensitivity and specificity of 88.9% and 89.5% and a PPV of 94.1% (95% CI = 81.1% - 98.4%) and NPV of 81.9% (95% CI = 62.5% -91.6%) were observed.

		p16 IHC			
		Positive	Negative		
HPV OncoPredict QT assay	Positive	32	2	34	K= 0.77
	Negative	4	17	21	
		36	19	55	

Table 18: Cohen's Kappa for HPV OncoPredict QT assay vs p16 IHC in both plasma and saliva

5.2.4 Predominant HPV Genotypes and Viral Load

Plasma Samples

HPV 16 (31/37) was the most predominant HPV genotype detected in plasma, followed by HPV 33 (3/37) (Figure 26). The HPV 16 viral load varied widely, as seen in Figure 27a, ranging from as low as 7.77 copies/mL (BS019) to as high as 696,000 viral copies/mL (BS070). Among the patients positive for HPV genotypes other than HPV 16, the HPV viral load was between 25.1 to 26,300 viral copies/mL (Figure 27b).

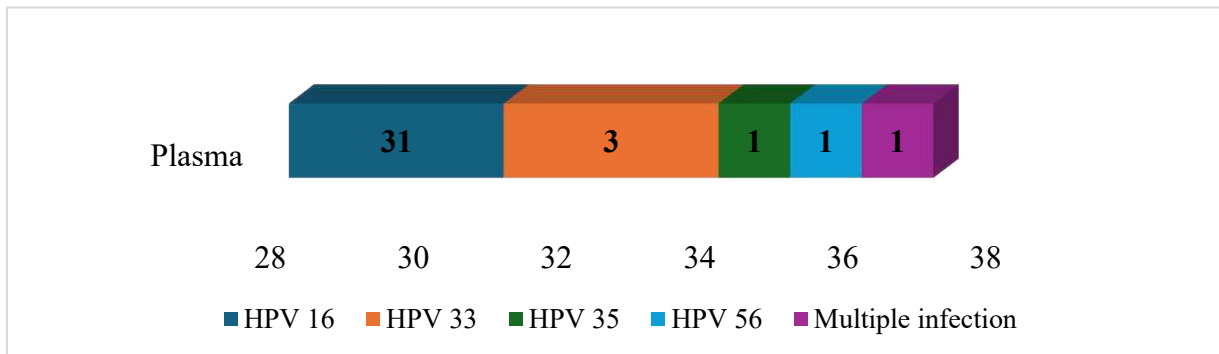


Figure 26: HPV genotypes detected in plasma

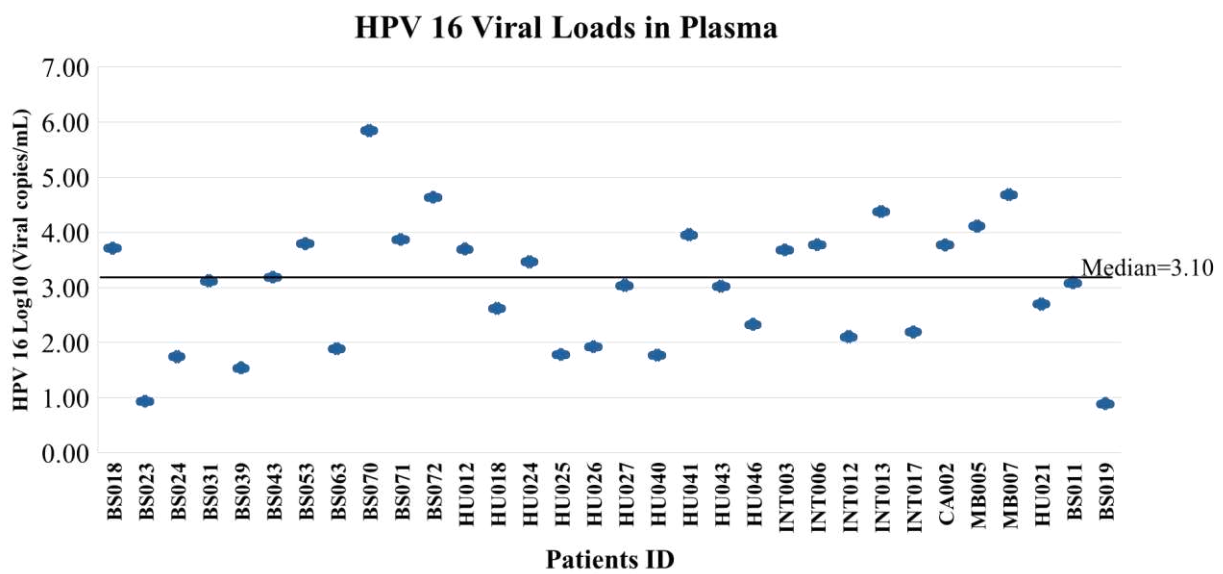


Figure 27a: HPV 16 viral loads expressed as Log10 (viral copies/ml) in plasma samples

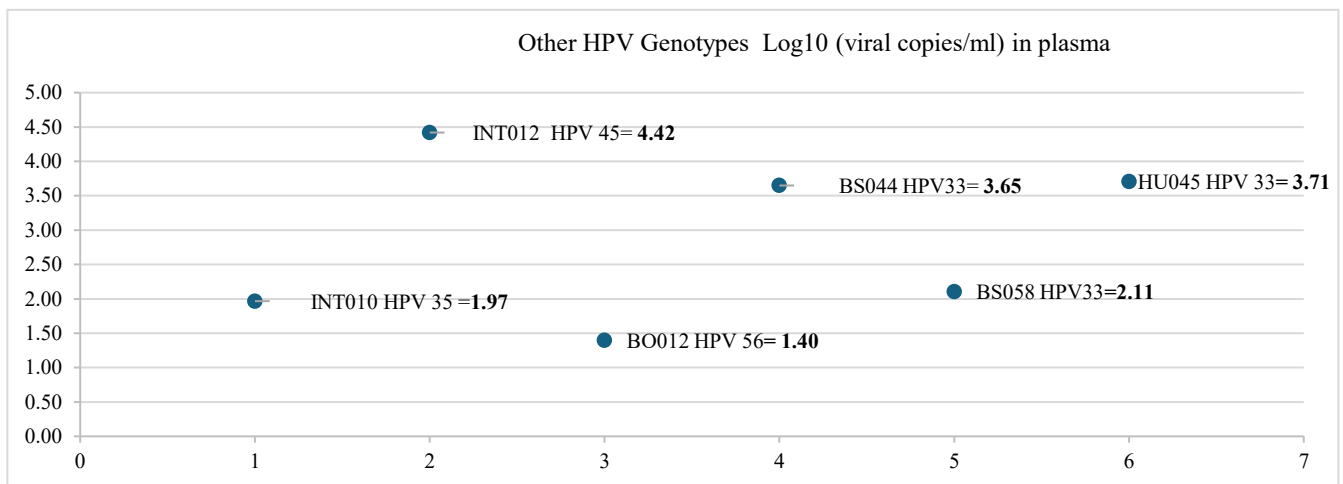


Figure 27b: Other HPV genotypes viral loads expressed as Log10 (viral copies/ml) in plasma samples

Saliva Samples

Among the saliva samples, the most prevalent HPV genotype was HPV 16 same as in plasma samples, followed by HPV 33 in one patient (Figure 28a). The HPV 16 viral load varied largely, ranging from as low as 0.012 viral copies/ 10^4 cells (BS039) to as high as 150,000 viral copies/ 10^4 cells (MB007) (Figure 28b). 6 patients had multiple infections, and HPV 16 and HPV 52 were the most common among them (Table 19).

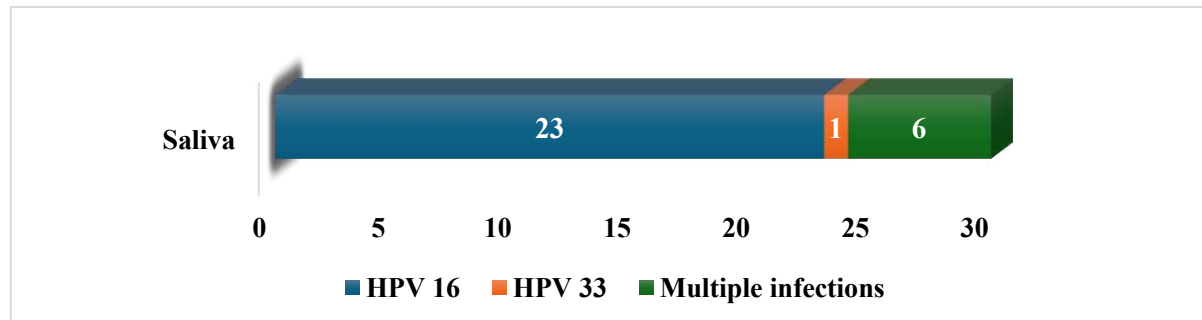


Figure 28a: HPV genotypes detected in saliva

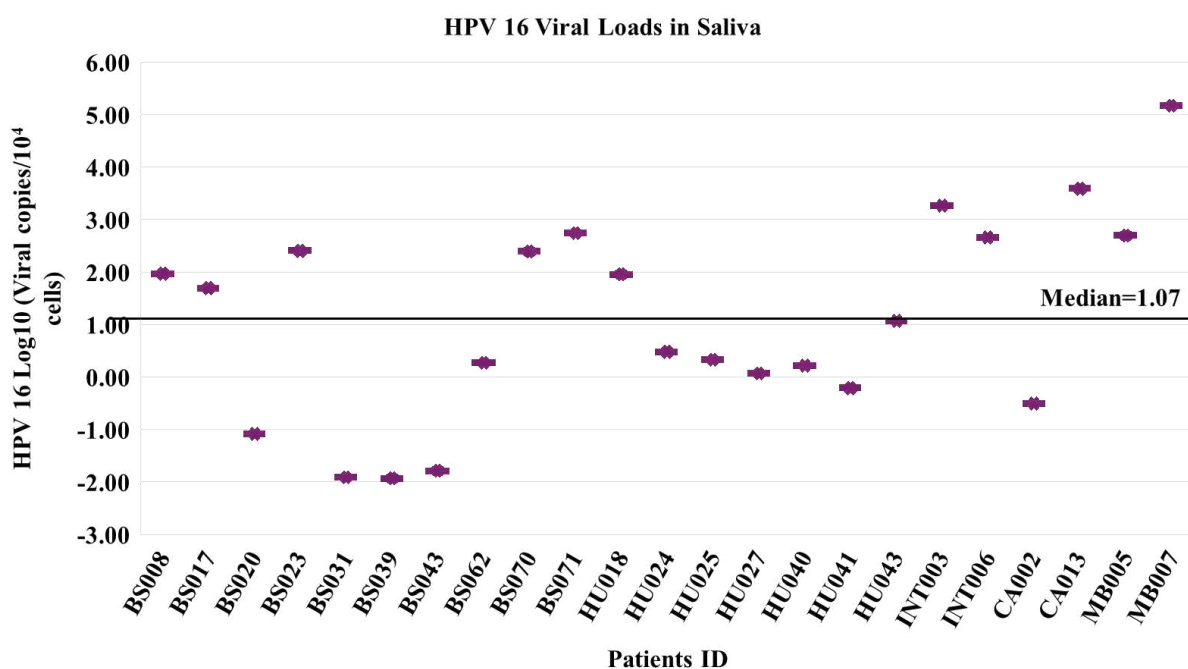


Figure 28b: HPV 16 viral loads expressed in Log10 (viral copies/ 10^4 cells) in saliva samples

Patients ID	Multiple Infections	
	HPV genotypes	Viral copies /10 ⁴ cells
BS013	HPV16	5.77E+03
	HPV45	1.12E+02
	HPV35	1.40E-01
BS053	HPV16	1.21E+02
	HPV58	3.05E-01
BS063	HPV16	4.07E-01
	HPV52	1.14E-01
HU026	HPV16	8.36E-01
	HPV52	2.33E-01
BO012	HPV52	4.07E-01
	HPV 31	1.10E+01
BS019*	HPV16	7.27E-02
	HPV51	2.79E-02

Table 19: Multiple HPV infections and their corresponding viral loads (copies/10⁴ cells) detected in p16-positive patients, along with one patient of p16-negative status. * = p16-negative patients

5.2.5 Comparison of OncoPredict HPV QT Assay with Droplet Digital PCR (ddPCR)

The viral load of HPV16 was assessed using both the OncoPredict HPV QT assay and droplet digital PCR (ddPCR) to evaluate the concordance between the two methods.

5.2.5a Comparison in Plasma Samples

The viral load of HPV16 was assessed in 34 plasma samples using both the OncoPredict HPV QT assay (5 µL input) and droplet digital PCR (ddPCR, 3 µL input). The results showed a high level of concordance between both methods, with most samples showing comparable HPV16 viral copy numbers (Figure 29).

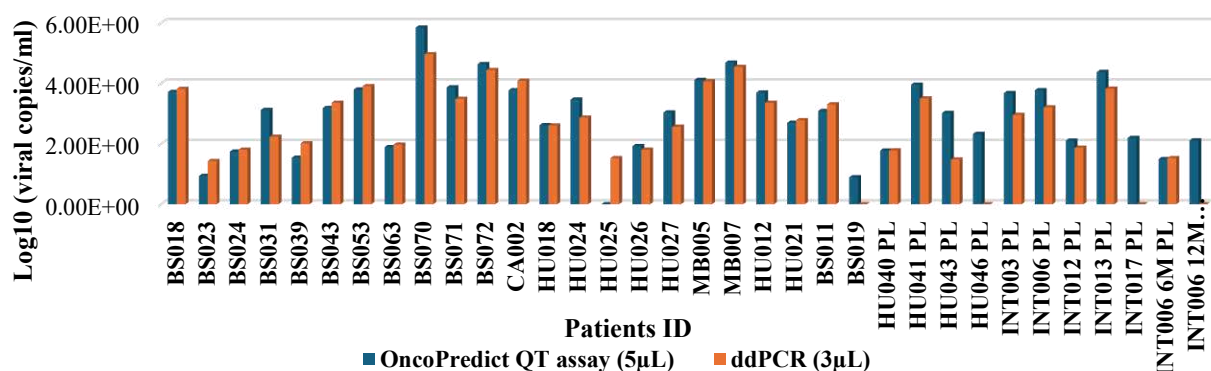


Figure 29: Comparison of HPV16 Viral Load Measured by OncoPredict HPV QT Assay and ddPCR in blood samples. The 2 methods use different volumes of extract for inoculation: the OncoPredict HPV QT assay = 5 µL and ddPCR = 3 µL

5.2.5b Comparison in Saliva Samples

The viral load of HPV16 was assessed in 29 saliva samples using both the OncoPredict HPV QT assay (5 µL input) and droplet digital PCR (ddPCR, 1 µL input). The results demonstrated a high level of concordance between both methods, with most samples showing comparable HPV16 viral copy numbers (Figure 30).

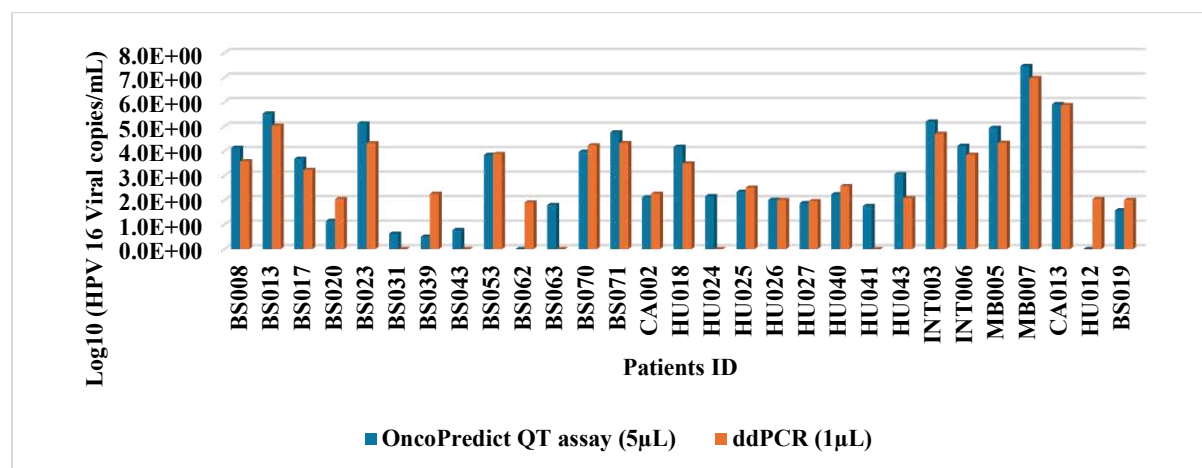


Figure 30: Comparison of HPV16 Viral Load Measured by OncoPredict HPV QT Assay and ddPCR in saliva samples. The 2 methods use different volumes of extract for inoculation: the OncoPredict QT assay = 5 µL and ddPCR = 1 µL

5.3 Association of HPV status with various demographic and clinical characteristics in plasma and saliva

The relationship between HPV DNA status determined by the OncoPredict HPV QT assay in both blood and saliva samples, and various demographic and clinical characteristics of the patient cohort at baseline, was determined.

5.3.1 HPV status associations with Sex and Age

In plasma samples, HPV positivity was more common among male patients (32/48) compared to females (5/13), although this difference did not reach statistical significance ($p = 0.1$). There was no significant association between HPV status and age group in blood samples. Similarly, in saliva samples, HPV positivity was more common among males (24/46) compared to females (4/13), but this difference was also not statistically significant ($p = 0.2$). No significant association was found between HPV status and age group in saliva samples (Table 20).

	HPV Status	Sex		<i>p</i> value*	Age		<i>p</i> value*
		Male	Female		< 60	>60	
Plasma	Positive (n)	32	5	0.1	18	19	0.4
	Negative (n)	16	8		9	15	
Saliva	Positive (n)	24	4	0.2	11	17	1.0
	Negative (n)	22	9		13	18	

Table 20: The distribution of HPV DNA detected by OncoPredict HPV QT assay in both plasma and blood samples in relation to patient sex, and age group (<60 vs. >60. *p-value**= Assessed using the two-sided Fisher's test and p -values < 0.05 were considered statistically significant.

5.3.2 HPV status association with Tumour Site

A statistically significant association was observed between HPV status and tumour site for both blood and saliva samples. In blood, HPV positivity was significantly higher in patients with oropharyngeal squamous cell carcinoma compared to non-OPSCC cases ($p = <0.001$). This trend was also significant in saliva samples, where HPV positivity was notably higher in OPSCC patients compared to non-OPSCC cases ($p = <0.001$) (Table 21).

	HPV Status	Site of disease		<i>p</i> value*
		OPSCC	Non-OPSCC	
Plasma	Positive (n)	22	4	<0.001
	Negative (n)	8	10	
Saliva	Positive (n)	22	1	<0.001
	Negative (n)	11	12	

Table 21: The distribution of HPV DNA detected by OncoPredict HPV QT assay, both in plasma and blood samples, in relation to patient sex, and age group (<60 vs. >60. *p-value**= Assessed using the two-sided Fisher's test and p-values < 0.05 were considered statistically significant.

5.4 Distribution of HPV viral loads across Disease stages

5.4.1 Distribution of HPV viral loads across disease stages in plasma

The distribution of HPV viral loads in plasma samples varied distinctly across the different disease stages (1, 2, 3) (Figure 31). Patients in stage 1 showed a wide range of viral copies (0.93 -5.84) with a median viral load of 2.33 viral copies/ml. In contrast, stage 2 had the highest median Log10 viral copies/ml (3.71) with a narrower range (1.97 – 3.86 viral copies/ml). For stage 3, the median viral load was observed to decrease to 3.03 with a broader range of 1.40 - 4.68 Log10 viral copies/ml. Given the observed non-linear pattern of viral load progression across stages, further statistical correlation analysis was not performed.

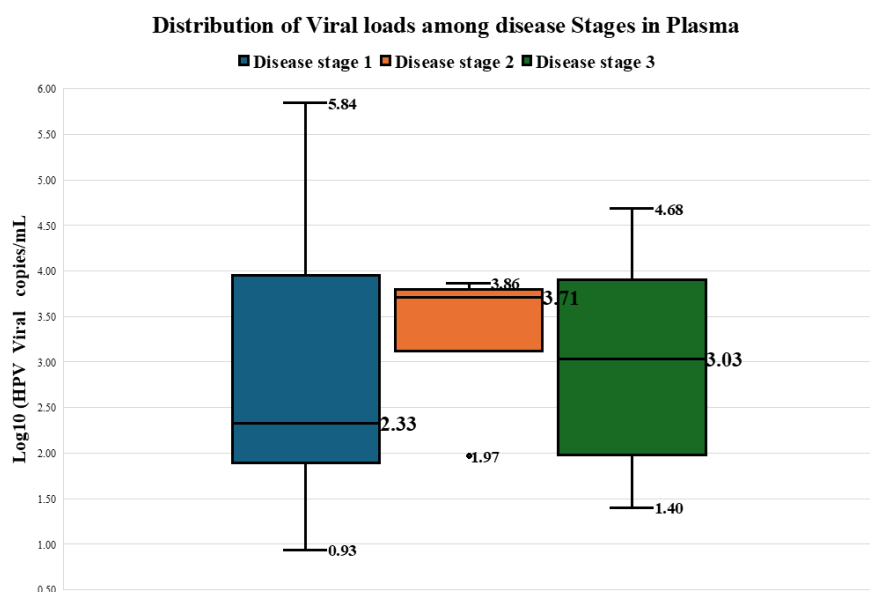


Figure 31: Distribution of HPV viral loads across disease stages in plasma. The viral loads are expressed in Log10 HPV viral copies/ml.

5.4.2 Distribution of HPV viral loads across disease stages in saliva

The distribution of HPV viral loads in saliva samples exhibited a considerable variability within and across the 3 disease stages (1, 2, 3) (Figure 32). Patients in stage 1 had the lowest median viral load of 0.13 viral copies/ 10^4 cells, ranging from -0.94 to 2.41 viral copies/ 10^4 cells. Stage 2 showed the highest median viral load (2.08 viral copies/ 10^4 cells) but within a broader range of -1.91– 2.75 viral copies/ 10^4 cells. A subsequent decrease in the median viral load to 1.05 viral copies/ 10^4 cells copies/cellularity was also observed in stage 3 patients with an even wider range of viral loads (-1.93– 5.18 viral copies/ 10^4 cells). Consistent with the plasma analysis, due to the non-linear progression observed in viral load across these stages, further statistical correlation analysis was not undertaken.

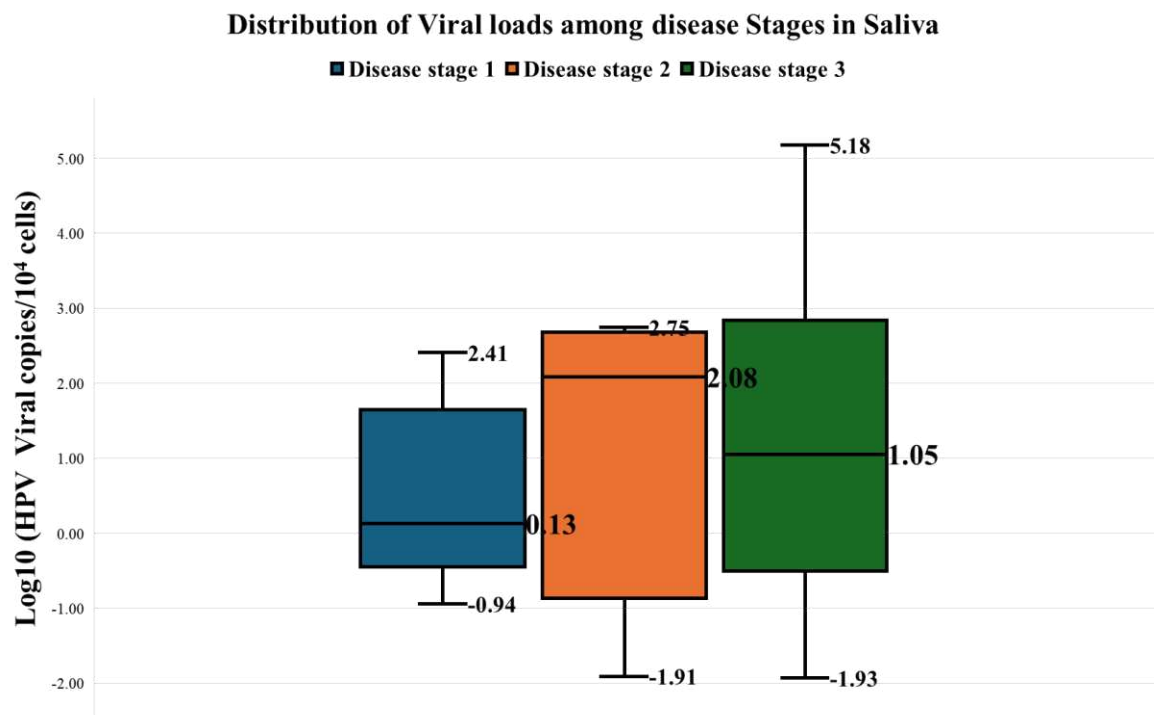


Figure 32: Distribution of HPV viral loads across disease stages in saliva. The viral loads are expressed in Log₁₀ HPV viral copies/ 10^4 cells.

5.5 HPV OncoPredict QT assay for Post-Treatment Longitudinal Monitoring

The capacity of the OncoPredict HPV QT assay in monitoring the changes of HPV detection in blood and saliva over various time-points post-treatment was analysed, considering patients with samples at baseline and at least one follow-up time-point.

A total of 47 patients fall within this criterion. Out of these 47, 35 patients are p16-positive, and 12 were p16-negative. They have a median age of 63 years (range 39-80). The majority of them were male and had the oropharynx as their tumour primary site. As noted with the whole data, not all patients have both sample types. We had 42 patients with plasma samples (31 samples p16-positive and 11 were p16-negative), and 39 patients with available saliva samples (28 p16-positive samples and 11 p16-negative).

5.5.1 Longitudinal HPV DNA Detection in Plasma Samples

HPV DNA was detected in 27/31 (95% CI = 17.793 - 39.284) p16-positive plasma samples at T0. Following treatment, a reduction in HPV DNA was observed at subsequent follow-up timepoints (Figure 33a). For p16-negative samples, 2 patients initially showed HPV DNA positivity at T0, which was cleared after treatments (Figure 32). HPV 16 (23/27) was the most predominant. Among these, 22 were single infections, while one patient (INT012) presented with a coinfection of HPV 16 and HPV 45 had a coinfection of HPV 16 and HPV 45. Other HPV genotypes detected were HPV 33 (2), HPV 35 (1), and HPV 56 (1) at baseline.

During follow-up, 3 patients showed HPV DNA positivity, one patient (INT006) exhibited a persistent HPV 16 infection with a gradually increasing viral load and this was also observed in a patient (BS034) that did not have a plasma sample at T0, but the samples collected at FU3M and FU6M showed persistent HPV 16 infection with increasing viral load as seen in figure 33b. Of the remaining 2 patients, one (CA002) had an HPV 16 infection at T0 and tested positive for HPV 56 at the 6-month follow-up. The other patient (BS062) was negative at baseline but positive for HPV 16 at the 12-month follow-up (Table 22).

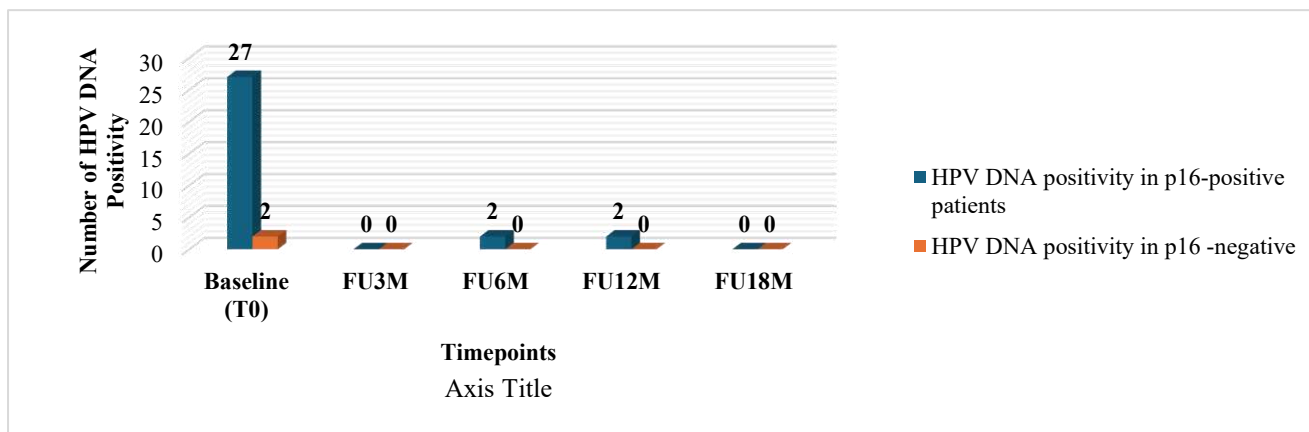


Figure 33a: Longitudinal HPV DNA Positivity in Plasma Samples of p16-Positive and p16-Negative Patients.

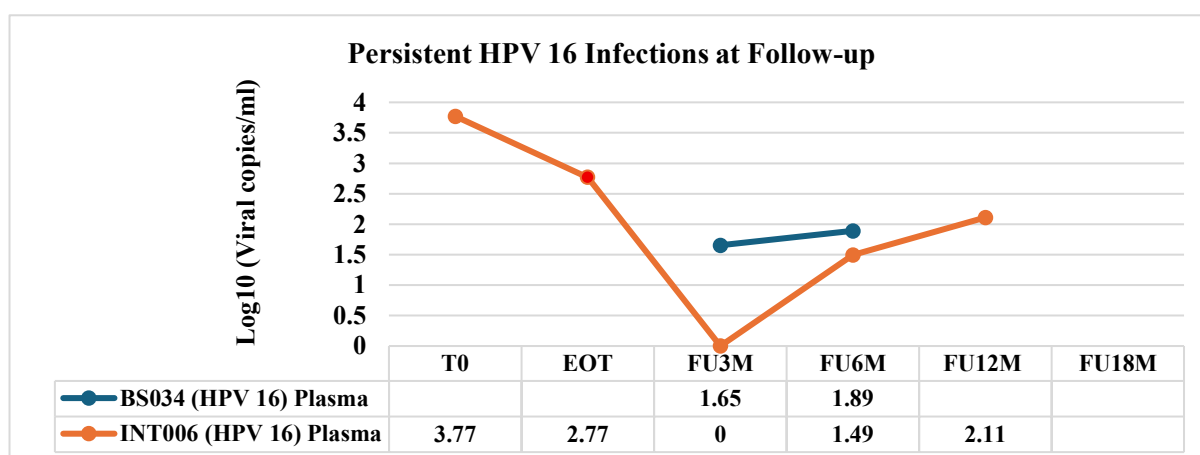


Figure 33b: Patients with persistent HPV 16 with increasing viral loads at different follow-up timepoints. Results are expressed in Log10(Viral copies/ml)

Patients ID				
	BS062	INT006	CA002	BS034*
Timepoints	HPV viral copies/ml			
Baseline (T0)	Neg	HPV 16: 5.92E+03	HPV 16: 5.88E+03	-
FU3M	Neg	Neg	Neg	HPV 16: 4.51E+01
FU6M	Neg	HPV 16: 3.11E+01	HPV 56: 2.54E+01	HPV 16: 7.78E+01
FU12M	HPV 16: 4.24E+02	HPV 16: 1.29E+02		
FU18M				

Table 22: An overview of the longitudinal HPV DNA detection in plasma samples at baseline and follow-up timepoints (3M, 6M, 12M, 18M). * = this patient plasma sample at T0 was missing.

5.5.2 Longitudinal HPV DNA Detection in Saliva Samples

Among the 39 patients with available saliva samples for testing (28 p16-positive and 11 p16-negative), HPV DNA was detected in 23 out of 28 (82.1%; 95% CI = 14.6% - 34.5%) p16-positive patients at T0 (Figure 34). Of these 23 HPV DNA-positive samples, HPV 16 was identified as a single infection in 17 cases, while multiple infections were detected in 5 samples. During the follow-up period, six patients had detectable HPV DNA at various timepoints. Notably, one of these six patients presented with a persistent HPV 52 infection, and another a persistent HPV 51 infection, associated with very low viral loads (Table 23).

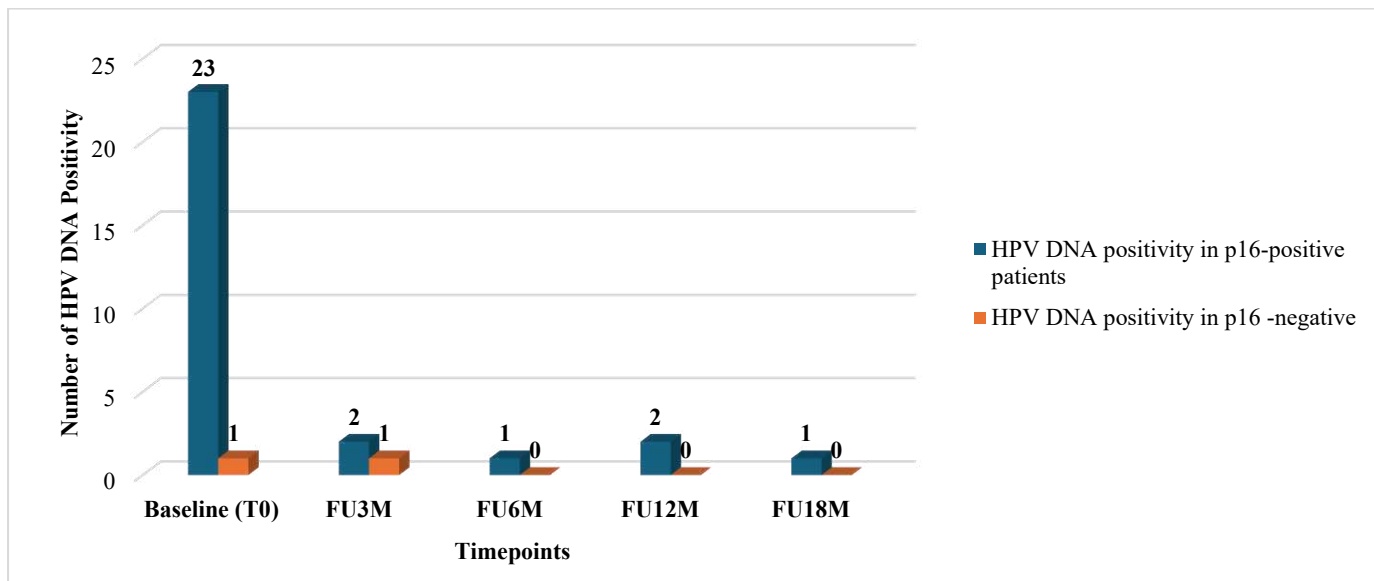


Figure 34: Longitudinal HPV DNA positivity in saliva samples of p16-positive and p16-negative patients.

	Patients ID					
	BS013	BS044	CA002	BS063	BS071	BS019
Timepoints	HPV Genotype: viral copies/10 ⁴ cells					
Baseline (T0)	HPV 16: 5.77E+03 HPV 45: 1.12E+02 HPV35: 1.40E-01	Neg	HPV 16: 3.14E-01	HPV 16: 4.07E-01 HPV 52: 1.14E-01	HPV 16: 5.56E+02	HPV 16: 7.27E-02 HPV 51: 2.79E-02
FU3M	-	HPV 35: 4.92E-02	-	-	-	HPV 51: 3.16E-01
FU6M	-	-	HPV 45: 1.37E+01	-	-	
FU12M	-	-	-	HPV 52: 9.71E-01	HPV18: 9.81E+02	
FU18M	HPV 58: 4.25E-01	-	-	-	-	

Table 232: An overview of the longitudinal HPV DNA detection in saliva samples at baseline and follow-up timepoints (3M, 6M, 12M, 18M).

CHAPTER 6: DISCUSSION

The rising global incidence of HPV-related head and neck squamous cell carcinoma (HNSCC) presents a significant clinical challenge due to its characteristically high mortality and low overall survival rates [2,11,42]. Despite this, there is still no validated screening program for the early detection of HPV-related HNSCC, which could substantially improve clinical outcomes. The poor prognosis currently associated with these cancers is often a consequence of late-stage diagnosis, leading to the need for aggressive treatment modalities. While these intensive therapeutic strategies aim to control disease progression, they are frequently associated with long-term toxicities, increased morbidity, and a reduced quality of life [62]. The development of non-invasive saliva- or plasma-based screening, treatment monitoring and post-treatment surveillance biomarkers for HPV-related HNSCC could therefore play a crucial role in the early detection of primary tumours, evaluating efficacy of treatment for potential de-escalation therapy, to minimise the burden of short- and long-term toxicities on patients, and in identifying recurrences before the onset of clinical symptoms.

This thesis is focused on the validation of the OncoPredict HPV Quantitative Typing assay (QT), assessing its performance for detecting and quantifying the 12 carcinogenic HPV type-specific DNA in plasma and saliva liquid biopsy samples from HNSCC patients enrolled in the Identify study. Among the 67 patients included in this analysis, 76.1% (51/67) presented with OPSCC, and 45 of the 51 were p16-positive. The study successfully demonstrates the OncoPredict HPV QT molecular assay's high accuracy and sensitivity in the analytical performance, its strong concordance with recognised biomarkers and its potential application in the clinical management of HPV-related HNSCC.

This validation study critically evaluated the agreement between the OncoPredict HPV QT assay agreement with p16 immunohistochemistry, which is a widely accepted surrogate marker for transcriptionally active HPV infection in HNSCC [112,113]. The study found a concordance ranging from moderate to strong concordance between the two methods. More precisely, plasma samples yielded a Cohen's kappa value of 0.65, indicating good agreement, while saliva samples showed moderate concordance ($k=0.60$). When considering combined plasma and saliva samples, an even stronger agreement was evident, with a Cohen's kappa of 0.77. This strong alignment with p16 IHC supports the OncoPredict HPV QT assay as a reliable indicator of HPV presence in HNSCC patients and a potential complementary tool to p16 IHC, which, although considered the gold standard, has well-recognised limitations. Previous studies

have reported that relying solely on p16 IHC for HPV status determination can lead to false positives in approximately 8–10% of p16-positive cases, as p16 overexpression can also be triggered by non-HPV-related cellular pathways [112–114]. Another limitation of p16 IHC is the potential for false negatives, which may arise from the collection of small tissue biopsies or from inconsistent staining thresholds. These factors can lead to an underestimation of p16 expression in HPV-related tumours. Although current guidelines recommend strong and diffuse nuclear and cytoplasmic staining in at least 70% of tumour cells as the criterion for positivity, this threshold has also been shown to produce a notable rate of false negatives [115,116].

The study evaluated the potential of the OncoPredict HPV QT assay in detecting HPV DNA plasma and saliva at baseline, offering a diverse yet complementary insight into HPV status.

The baseline HPV DNA positivity for all patients with plasma samples was 59.7%. Specifically, plasma ctHPVDNA was detected in 91.9% of p16-positive patients. The OncoPredict HPV QT assay demonstrated a sensitivity of 82.9% and a specificity of 85% in plasma. These findings are consistent with prior studies where they also calculated the sensitivity and specificity assuming p16-positive samples as diseased and p16-negative as non-diseased, demonstrating the use of plasma ctHPVDNA in diagnosing and monitoring HPV-related OPSCC [117–119]. Plasma ctHPVDNA is widely recognised for its clinical utility in HPV-related HNSCC due to its correlation with tumour burden and its capacity to reflect systemic disease [65,91]. For instance, the sensitivity of HPV DNA detection in plasma using ddPCR has been reported to vary widely from as high as 98.4% in the study by Siravegna et al. (for HPV types 16, 18, 33, 35, and 45) to as low as 43% in the study by Rettig et al., for the absolute quantification of HPV16 [120,121]. The results of this study have shown a similar performance of the commercially available real-time quantitative PCR assay, OncoPredict HPV QT, to that of ddPCR. This represents an important advantage in the clinical laboratory setting, offering the possibility to implement reliable testing of plasma for the detection and quantification of circulating HPV DNA using a commercially available, standardised, and CE-marked test, reflecting tumour activity and prognosis.

Saliva also proved to be a valuable biofluid for HPV DNA detection using the OncoPredict HPV QT assay, demonstrating an HPV DNA positivity rate of 49.2% in the overall patient cohort at baseline. In p16-positive patients, the OncoPredict HPV QT assay detected HPV DNA in 96.7% of cases from saliva. The assay yielded a sensitivity of 72.5% and a specificity

of 95% in saliva. These results align with literature indicating that salivary HPV DNA is a promising biomarker in minimally invasive samples for HPV-related HNSCC [19,122]. Salivary HPV DNA offers the advantage of being non-invasive and can directly reflect the local tumour microenvironment, especially for oral cavity and oropharyngeal cancers [123]. When comparing results from different studies, it must be taken into account that the different methods used for saliva collection may influence HPV DNA detection. Oral rinse or gargle samples using sterile saline or alcohol-based mouthwash and spitting are the most commonly used sampling methods in studies involving the oral cavity and oropharynx [124,125]. While these methods offer advantages in terms of ease of collection, transport, and cost-effectiveness, they also present specific challenges. Samples generally require immediate processing, often within 30 minutes of collection, due to the absence of stabilising preservatives [125]. Furthermore, in patients with HNSCC, reduced salivary production post-treatment can make sampling by spitting quite difficult. An alternative approach involves swabbing the oral cavity, for which commercial collection devices incorporating liquid preservatives are available [84,124]. This improves the stability of the oral sample for downstream molecular work. A meta-analysis by Jakobsen et al. focusing on the diagnostic accuracy of HPV detection in OPSCC reported a pooled sensitivity of 77.64% and a specificity of 72.191% for oral sampling methods when compared to p16 tumour tissue markers [126]. They also observed considerable variability in sensitivity and specificity (ranging from 60.9% to 86.1% and 50% to 91% respectively, in some studies) among various oral sampling techniques, further highlighting these challenges [126]. This variability could also be attributed to the type of HPV detection genotyping assay used, because HPV viral load in oral samples is lower than that in genital sites; as such, testing oral specimens requires the use of HPV assays with higher analytical sensitivity [124].

Detection of HPV DNA in saliva has been shown to inform locoregional disease status in advanced HPV-related OPSCC [127] and can serve as a non-invasive tool for early diagnosis, prognosis, and monitoring treatment response [19].

In this study, the HPV DNA detection rates were analysed in paired plasma and saliva samples from OPSCC patients. Among a cohort of 55 patients with paired plasma and saliva samples (including 36 p16-positive and 19 p16-negative cases), the total HPV DNA positivity was 61.8% (34/55; 95% CI= 49% - 74%). A higher HPV DNA positivity was also seen among the p16-positive patients. The OncoPredict HPV QT assay detected HPV DNA in 94.1% (32/34;

95% CI = 80% - 99%) of these cases. A stronger concordance of the paired HPV positivity in both sample types (Cohen's Kappa=0.77) was demonstrated than what was observed in evaluating only plasma (Cohen's Kappa=0.65) or saliva samples (Cohen's Kappa=0.60) alone. A sensitivity of 88.9% and specificity of 89.55% were observed, and this was higher than what was reported by a previous study, where the combined HPV DNA positivity in plasma and saliva samples had a sensitivity of 65% and a specificity of 87% [128]. Another study showed an even lower sensitivity (76%) but a higher specificity (100%) [129]. The results obtained in this study confirmed the better predictive values indicated in previous studies for combined plasma and saliva samples HPV DNA detection using the OncoPredict HPV QT assay. A PPV of 94.1% (95% CI = 81.1% - 98.4%) was achieved, alongside a NPV of 81.9% (95% CI = 62.5% - 91.6%). These metrics underscore the assay's capacity to accurately identify both HPV-positive and HPV-negative cases, suggesting its potential in clinical decision-making.

This further supports that the combination of multiple samples can be a useful tool for the diagnosis and management of HPV-related OPSCC. This was also recently highlighted by a study by Lewis et al., who underlined improved sensitivity by combining HPV serology with ctHPVDNA detection using ddPCR [130].

Interestingly, a small proportion (5.9%; 2/34; 95% CI=0.7% - 20%) of p16-negative patients also presented with detectable HPV DNA. This observation highlights the inherent complexity of HPV detection and suggests the potential for liquid biopsy to identify occult or early disease that might be missed by tissue-based methods alone. Moreover, it could indicate false-negative p16 IHC results [131,132]. The detection of HPV DNA in p16-negative cases suggests the possibility of false-negative p16 IHC results or the presence of transcriptionally active HPV DNA without corresponding p16 overexpression. This can lead to the misclassification of HPV-driven tumours, which is crucial because HPV status has a substantial influence on the survival probability of patients and is used to stratify them for treatment escalation or de-escalation [133,134]. Studies have consistently demonstrated discordance between p16 status and HPV DNA or RNA status [53]. For instance, a multicentre analysis by Mehanna et al. quantified this discordance and its prognostic implications, indicating that p16 alone is often insufficient for accurately inferring HPV status [53]. Similarly, von Buchwald et al. found that double HPV DNA/p16 status is superior to p16 alone in predicting outcome, emphasising that the detection of HPV significantly impacts survival probability even in p16-negative tumours [31]. While p16 IHC is valuable as an initial screen due to its cost-effectiveness, its utility as a

standalone biomarker is limited by the clinical specificity and sensitivity of this HPV surrogate biomarker, with the potential for false positive and false negative results, as well as requiring more invasive tissue biopsy samples [132]. Therefore, a multimodal diagnostic strategy is crucial; for example, combining p16 IHC with an assay like OncoPredict HPV QT, which is a standardised, commercially available multiplex real-time PCR-based assay, would improve specificity and sensitivity, thereby reducing false-positives and false-negatives. For this reason, future studies should evaluate the use of the OncoPredict HPV QT assay on tissue biopsies in combination with p16 IHC, in addition to liquid biopsies, for a comprehensive and accurate diagnosis of HPV-related OPSCC.

The OncoPredict HPV QT assay's ability to detect the 12 HR-HPV genotypes and quantify the genotype-specific viral loads provides a valuable insight into the biology of HPV-related OPSCC. Among our cohorts, HPV 16 was the most prevalent both in plasma (83.8%) and saliva (76.8%), this is followed by HPV 33 in both sample types. Other HPV genotypes were also detected (HPV 31, HPV 35, HPV 51, HPV 52 and HPV 58); most of these genotypes were detected as co-infections in saliva. The clearly higher prevalence of HPV 16 in OPSCC is consistent with extensive epidemiological data recognising HPV 16 as the primary oncogenic driver in HPV-related HNSCC globally [112,135]. Nevertheless, the results of this study have also shown an association with other HR-HPV genotypes, which is in keeping with the recent update of the HNSCC guidelines on HPV testing by the College of American Pathologists, which indicate that 12% of HPV-related OPSCC were associated with non-HPV 16/18 types. This study confirms that the use of HR-HPV-specific tests allowing only HPV 16 detection should not be used, as they would miss a significant number of HPV-related OPSCC cases, reducing the clinical sensitivity of the testing. Thereby, the multiple detection of HR-HPV PCR should include at least HR-HPV types 16,18, 26, 33, 35, and 58 [136]. The OncoPredict HPV QT assay includes all HPV types besides HPV 26 genotype, which is classified as a probable HR-HPV type. Moreover, the OncoPredict HPV QT assay allows for genotype-specific viral load determination with a high analytical sensitivity, offering a great advantage in monitoring the efficacy of treatment and in the early detection of disease recurrences.

HPV16 viral load in plasma ranged from 7.77 viral copies/mL - 696,000 viral copies/mL (median=1,210 viral copies/ mL), reflecting wide variability consistent with findings from Warlow et al. (1–30,380 viral copies/mL, median 279 viral copies/mL) and Cao et al. (0–5,500

viral copies/mL, median 222 viral copies/mL) [117,137]. Similar results were observed by Chera et al., who reported a median of 419 viral copies/mL (range, 8–22,579) [90]. A broad viral load range was also observed for other HPV genotypes (25.1 to 26,300 viral copies/mL). This quantitative capability of the OncoPredict HPV QT assay represents a major advantage, as HPV DNA levels have been shown to correlate with tumour burden, treatment response, and patient outcomes [138,139]. High ctHPVDNA viral loads at diagnosis have been linked to more advanced disease stages and less favourable prognostic indicators, reinforcing the importance of precise quantitative measurements [139]. Among the saliva samples, a broader ctHPV16DNA viral load range was detected with a median value of 11.7 viral copies/10⁴ cells (range = 0.012-150,00 viral copies/10⁴ cells). An additional advantage of the OncoPredict HPV QT assay is that it allows normalisation of the viral load according to sample cellularity in saliva samples for improved standardisation of the results.

The study also explored associations between HPV status (as determined by the OncoPredict HPV QT assay) and various demographic and clinical parameters. No statistically significant associations were found for sex or age in either plasma or saliva samples, consistent with findings by Galati et al [118]. Though it has been indicated that HPV prevalence can vary by gender and age in certain populations [112]. However, a statistically significant correlation was observed with tumour site for both blood and saliva samples. HPV positivity was significantly higher in patients with oropharyngeal squamous cell carcinoma compared to non-OPSCC cases ($p < 0.001$ for both blood and saliva). This finding is consistent with the well-established epidemiology of HPV-HNSCC, where the oropharynx is the primary site for HPV-driven tumours [42,112,118].

To further establish the analytical validity and precision of the OncoPredict HPV QT assay, its performance in quantifying HPV 16 viral loads was rigorously compared with droplet digital PCR (ddPCR). ddPCR is widely regarded as a highly sensitive and precise method for nucleic acid quantification, often considered a gold standard due to its absolute quantification capabilities and improved sensitivity at low template concentrations [140].

The study found a high level of concordance between the OncoPredict HPV QT assay and ddPCR for HPV 16 viral load assessment. In 34 plasma samples, as well as in 29 saliva samples, the majority of samples showed comparable viral copy numbers between the two methods, despite differences in input volumes (OncoPredict QT assay = 5 μ L; ddPCR for plasma = 3 μ L; ddPCR for saliva = 1 μ L). This strong agreement between the two methods validates the

OncoPredict HPV QT assays' analytical robustness and reliability. Although ddPCR has the advantage of high sensitivity, especially for targets with low copy numbers, previous studies as shown a correlation between qPCR-based assays and ddPCR for HPV DNA quantification [141,142].

The OncoPredict HPV QT assay, being qPCR-based, offers several advantages over ddPCR for routine clinical practice. It offers a more cost-effective solution with a lower turnaround time of approximately 2 hours, allows for the detection of all 12 HR-HPV genotypes, and operates with a standard input sample volume. These characteristics address some of the known limitations of ddPCR, such as its higher cost, longer turnaround time, limited probe multiplexing, and challenges with sample quality and quantity [140]. The OncoPredict HPV QT assay thus offers a standardised and potentially more clinically accessible tool for quantitative viral load assessment. This will make it easy to be used in routine diagnostic practice and ultimately broaden access to advanced HPV testing for HNSCC patients.

The reliability of the *C-C Motif Chemokine Receptor 5 (CCR5)* gene, used as a sample adequacy control in the OncoPredict HPV QT assay, was evaluated. This control is added as an independent Quality Control (QC) reaction, and it quantifies the single-copy human CCR5 gene. This quantification is carried out by the normalisation of the HPV viral loads based on the number of human cells present in the sample, thereby improving the viral load determination. This is particularly important for sample collection involving mucosal surfaces, such as cervical, vaginal and oral cavity samples, where there may be a great variability in sample cellularity, which will influence viral load determination and require normalisation. To validate its performance, CCR5-based cellular quantification results were compared with two conventional methods: the microscopy-based Thoma cell-counting chamber and Fluorescence-Activated Cell Sorting (FACS). The cellularity values obtained were consistent across all three approaches, confirming CCR5 as a robust molecular marker for reliable cellular quantification. These findings, which have been published, support the use of CCR5 as an effective sample adequacy control in the OncoPredict assay, minimising the risk of false-negative results and enhancing overall assay reliability [143].

This study initiated an evaluation of the OncoPredict HPV QT assay's capacity for post-treatment longitudinal monitoring. Preliminary analysis in patients with samples at baseline

and at least one follow-up time-point post-treatment assessed changes in HPV DNA detection in blood and saliva. The longitudinal analysis revealed a significant drop in HPV DNA positivity following treatment in p16-positive patients, and this was in line with what has been reported [89,90]. Notably, in our cohort, two patients with persistent HPV 16 DNA exhibited gradually increasing viral loads, both of whom subsequently had image-proven relapse. One of the patients (INT006) was positive for HPV16 at baseline, who had detectable ctHPV16DNA at the end of treatment (slightly lower than baseline), which cleared at 3 months post-treatment. However, it became detectable again during the 6-month and 12-month follow-up with increasing viral loads, and a month later, disease relapse was confirmed by imaging. These findings are consistent with existing literature, where longitudinal monitoring of ctHPV DNA has emerged as a sensitive and specific tool for detecting disease recurrences or relapse in patients with HPV-related OPSCC [89,144]. The monitoring of ctHPV DNA has been shown to predict treatment failures weeks to months before conventional imaging methods, and the persistence or increasing HPV cfDNA is a strong indicator of disease recurrence or relapses [89,145,146]. The quantitative nature of the OncoPredict HPV QT assay positions it well for integration into these surveillance strategies, potentially allowing for earlier intervention and improved patient outcomes.

Despite the promising findings, this study has several limitations. The sample size for some specific analyses, while providing valuable initial insights, could benefit from expansion in future studies to further enhance statistical power and generalizability. Notably, the distribution of HPV viral loads across disease stages in both plasma and saliva exhibited considerable variability, and a non-linear pattern of viral load progression was observed across stages. Due to this non-linear progression, further statistical correlation analysis between viral load and disease stages was not performed. This highlights the complexity of viral load kinetics in a heterogeneous patient population. Another limitation was the lack of tissue biopsy data to confirm the genotypes detected in the liquid biopsy, which would further strengthen the correlation. While promising, the evaluation of the assay's utility for detecting recurrence in longitudinal monitoring was initiated with a limited number of follow-up samples, necessitating larger prospective studies to fully establish its role in clinical surveillance.

In conclusion, the validation of the OncoPredict HPV QT assay demonstrates its reliability and potential as a valuable tool for HPV DNA detection and quantification in HPV-related OPSCC patients. Its strong concordance with p16 IHC, robust predictive values, and high agreement

with ddPCR affirm its analytical performance, consistent with the evolving landscape of HPV diagnostics. The assay's ability to identify predominant genotypes and quantify viral load holds significant promise for disease stratification, monitoring treatment response, and assessing tumour burden[139]. Future studies should prioritise expanding cohort sizes, integrating longitudinal data with definitive clinical outcomes (e.g., recurrence-free survival, overall survival).

The Implementation of a Standard Operating Procedure (SOP) for the IDENTIFY study, including the preanalytical procedure starting from sample (blood and saliva), collection and processing up to the validation of the analytical procedure, involving the OncoPredict HPV QT assay, offers a robust framework for standardising liquid biopsy workflows in HPV-related HNSCC. In particular, this standardisation encompasses meticulous sample collection, transport and processing, using Streck Cell-Free DNA BCT tubes for blood and FLOQSwabs® in eNAT® for saliva, specifically designed for optimal nucleic acid preservation. It further extends through automated nucleic acid isolation and purification, the inclusion of an exogenous extraction control to verify the efficiency of extraction, an exogenous amplification control to verify the presence of inhibitors, and a CCR5-based sample adequacy control within the OncoPredict HPV QT assay, confirming adequate sample cellularity and ensuring accurate viral load quantification. The subsequent real-time PCR analysis and dedicated software for results interpretation establish a highly defined and reproducible pipeline. This comprehensive, end-to-end approach is instrumental in reducing analytical inconsistencies and enhancing the reliability and comparability of HPV detection results from the use of liquid biopsies.

The successful integration of this validated, standardised, commercially available CE-marked assay into a comprehensive liquid biopsy strategy has the potential to significantly advance the early detection, personalised management, and continuous surveillance of this challenging disease, ultimately improving patient care.

As part of my industrial PhD program (PON “Ricerca e Innovazione”), I conducted some part of my PhD activities at Hiantis Srl, Brescia. During the first 6 months of the program, I was involved in the analytical and clinical validation of the novel in-vitro diagnostic devices (IVDs) (OncoPredict HPV assays) developed by Hiantis Srl as part of a Horizon 2020 SME Instrument Phase 2 Project (<https://cordis.europa.eu/project/id/806551>). The OncoPredict HPV assays are

made up of two modules: the OncoPredict HPV SCReening assay (OncoPredict HPV SCR assay) and the OncoPredict HPV Quantitative Typing assay (OncoPredict HPV QT assay). A novel feature is that both assays introduce a Quality Control (QC) module/well; this QC reaction well evaluates the sample's cellularity as a measure of sample adequacy, efficiency of nucleic acid extraction, and amplification control as an indicator of PCR inhibition.

During this time in the company, I participated in the analytical validation of the OncoPredict HPV assays by evaluating and defining their analytical sensitivity and specificity, stability, reproducibility, the Limit of Detection (LoD)/Quantification (upper and lower limits) according to the In Vitro Diagnostic Regulation EU 2017/746. The data obtained from the experiments were included in the IVDs' technical dossier (the technical dossier is a document required for the CE marking of new IVDs, which describes the analytical performance of the in-vitro diagnostic kits intended to demonstrate compliance with regulations, including information about the intended use, claims, and analytical/clinical performances).

Furthermore, during this PhD period, I contributed to the experimental phase required for the initial clinical validation of the HPV OncoPredict SCR and QT assay for their intended use in cervical cancer screening programs according to international criteria using the VALGENT Framework [98,147]. I actively contributed to performing laboratory testing of clinical samples collected as part of the clinical validation of the HPV OncoPredict SCR and QT IVDs on self-collected samples collected as part of the longitudinal prospective observational EUROPEAN VALHUDES Study (ClinicalTrials.gov ID: NCT04312737- EU-VALHUDES) [103] and on cervical samples collected to evaluate the HPV OncoPredict QT assay for the molecular triage of HPV screen-positive women as part of the VALTRIHP Study (ClinicalTrials.gov ID: NCT05193669- VALHTRIP) for the clinical validation process of the OncoPredict HPV QT assay.

These research activities resulted in the publication of the following articles:

- Evaluation of the clinical performance of OncoPredict HPV® SCR assay within the VALGENT-2 framework- DOI: 10.1002/jmv.28417.
- Clinical performance of a novel full-genotyping OncoPredict HPV® Quantitative Typing (QT) assay using the VALGENT framework- DOI: 10.1002/ijc.34754.
- Analytical and clinical performance of OncoPredict HPV Screening (SCR) assay on self-collected vaginal and urine specimens within the VALHUDES Framework- <https://pmc.ncbi.nlm.nih.gov/articles/PMC11590042/>

- Accuracy of OncoPredict HPV Quantitative Typing (QT) Assay on Self-Samples- <https://doi.org/10.1016/j.jcv.2024.105737>
- Evaluation of C-C Motif Chemokine Receptor 5 (CCR5) as a Sample Adequacy Control in HPV molecular diagnostics- <https://doi.org/10.3390/diagnostics14192194> <https://www.mdpi.com/2075-4418/14/19/2194>).

During the final months of my program, I was actively involved in the production process of the OncoPredict HPV assays at Hiantis Srl, working within the Quality Control Department. My role included performing comprehensive quality control analyses on all raw materials and on the newly produced assay batches to ensure that each product met the required safety, performance, and quality standards. In addition, I conducted routine environmental monitoring to verify laboratory sterility and prevent potential contamination. All procedures were carried out in strict compliance with Good Manufacturing Practices (GMP) [148].

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Overall list of the Doctoral candidates' publications:

- Evaluation of the clinical performance of OncoPredict HPV® SCR assay within the VALGENT-2 framework- DOI: 10.1002/jmv.28417.
- Clinical performance of a novel full-genotyping OncoPredict HPV® Quantitative Typing (QT) assay using the VALGENT framework- DOI: 10.1002/ijc.34754.
- Evaluation of two alternative non-alcohol-based media for the suspension of self-collected vaginal swabs for HPV testing in cervical cancer screening - doi.org/10.1016/j.heliyon.2024.e31032.
- Evaluation of C-C Motif Chemokine Receptor 5 (CCR5) as a Sample Adequacy Control in HPV molecular diagnostics- <https://doi.org/10.3390/diagnostics14192194> <https://www.mdpi.com/2075-4418/14/19/2194>).
- Analytical and clinical performance of OncoPredict HPV Screening (SCR) assay on self-collected vaginal and urine specimens within the VALHUDES Framework- <https://pmc.ncbi.nlm.nih.gov/articles/PMC11590042/>
- Accuracy of OncoPredict HPV Quantitative Typing (Qt) Assay on Self Samples- <https://doi.org/10.1016/j.jcv.2024.105737>
- Quality Assurance in Cervical Cancer Screening: Evaluation of Sample Adequacy in HPV DNA Testing- doi: 10.1002/jmv.70482
- Molecular Detection of Human Papillomavirus (HPV) and Other Sexually Transmitted Pathogens in Cervical and Self-Collected Specimens- doi: 10.3390/ijms26031296



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Article

Evaluation of C-C Motif Chemokine Receptor 5 (CCR5) as a Sample Adequacy Control in HPV Molecular Diagnostics

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Abstract: Background: Reliable Human Papillomavirus (HPV) testing and genotyping are essential for quality assurance in HPV-based primary screening, disease management and for monitoring the impact of HPV vaccination. The clinical validation of HPV molecular diagnostic assays has significantly contributed to these objectives; however, little emphasis has been placed on assuring sample quality. This study aimed to evaluate the accuracy of sample cellularity assessment using the C-C Motif Chemokine Receptor 5 (CCR5) gene target as a marker of sample adequacy in molecular diagnostics. **Methods:** Jurkat cell line samples were counted using both a Thoma cell-counting chamber and Fluorescence-Activated Cell Sorting (FACS). Jurkat cell line samples at three different concentrations were subsequently evaluated using the OncoPredict HPV Quality Control (QC) real-time PCR assay, employing CCR5 for molecular cellularity quantification. **Results:** The cellularity values obtained were comparable across the three different methods for all dilutions of the cell line tested. **Conclusions:** The results obtained from this study show that CCR5 represents a promising molecular marker for the accurate quantification of sample cellularity, confirming its use as a reliable sample adequacy control, thus reducing the risk of “false-negative” results.

Keywords: C-C Motif Chemokine Receptor 5 (CCR5); Human Papillomavirus (HPV); sample adequacy control (SAC); OncoPredict HPV Quality Control (QC) assay



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1. Introduction

The implementation of molecular Human Papilloma Virus (HPV) assays has accelerated programs based on HPV primary screening for the prevention of HPV-related Cervical Cancer (CC). HPV primary screening is a new screening algorithm for women over the age of 30 that involves the use of molecular HPV assays. If the HPV test result is positive, the patient is then referred for cytological testing. The improved clinical sensitivity of molecular HPV assays based on nucleic acid amplification test (NAAT) methods over cervical cytology in screening programs has helped to reduce the incidence and mortality arising from this cancer [1–5]. Moreover, the introduction of molecular HPV assays has allowed the possible transition to the use of HPV primary screening on self-collected samples (vaginal or urine samples autonomously collected by the patient), thereby broadening women's participation in cervical cancer screening programs.

Self-collected sampling has been widely accepted as a method of cervical screening, especially in low-income and middle-income countries [6–10]. Its acceptance is largely due to its convenience and flexibility in implementation, as it can be performed in various settings such as health care facilities, homes or workplaces facilitated by community health outreach teams. Additionally, HPV molecular assays can be used on self-collected samples

as point-of-care cervical cancer screening [7,11–22]. Validation of HPV assays on self-samples is therefore becoming increasingly important and essential to provide assurance for their use in routine clinical settings.

The internationally recognized criteria for the validation of HPV tests to be used in cervical cancer screening programs have focused on the importance of assuring the clinical sensitivity and specificity of the assays for the detection of women with precancerous lesions, but less attention has been placed on sample adequacy assessment [5].

Currently, there are several commercially available HPV kits in the global market, but not all of them have been validated according to the international validation criteria, and a recent review by Poljak et al. showed that only about 21% of these kits meet the validation criteria [23]. Furthermore, a systemic review by Arbyn et al. reported that not all validated HPV assays contain an internal control (IC) [24]. In the majority of assays which include an IC, this is associated with the qualitative detection of a *Beta-globin* gene target, used to assess both the presence of human cells in the sample as well as any potential PCR inhibition in the same reaction well used for the detection of HPV DNA targets.

As previously reported, the inclusion of a sample adequacy control (SAC) in a separate reaction well from the molecular diagnostic targets allows a more reliable assessment of sample cellularity, avoiding potential competition for the reaction reagents [25–27]. Moreover, a SAC would represent an important quality assurance tool in HPV molecular testing, boosting confidence in negative results [25,28,29]. In particular, the inclusion of a reliable sample adequacy assessment would help to reduce the potential risk of false-negative results due to inadequate sample collection, particularly in cervical screening programs based on self-collected samples, as clearly pointed out in a recent report [5,26].

The importance of evaluating cell counts in other diagnostic laboratory investigations by means of quantitative tools has previously been established and adopted for diagnostic and prognostic analysis in illness such as leukemia and lymphoma [30,31].

The *C-C Motif Chemokine Receptor 5* (*CCR5*) gene has been employed in the OncoPredict HPV assays (Hiantis Srl, Milan, Italy) as an independent Quality Control (QC) reaction to assess sample adequacy through the quantitative determination of the single-copy human *CCR5* gene, also allowing a more reliable HPV viral load determination by normalization according to sample cellularity [32,33]. Similarly, the use of the *CCR5* gene target has also been previously described for the normalization of HIV-1 pro-viral loads in clinical samples [34,35].

This study aimed to evaluate the performance of the molecular target *CCR5* included in the OncoPredict HPV QC module for the reliable quantitation of cell numbers by comparing the results with a cellularity assessment based on a Thoma cell-counting chamber and Fluorescence-activated Cell Sorting (FACS).

2. Materials and Methods

Human Jurkat cell lines were used to evaluate the accuracy of molecular quantitative cellularity assessment using *CCR5*, a single-copy human gene located on chromosome 3, included as an independent quantitative Real Time PCR (qRT-PCR) reaction of the OncoPredict HPV kit (Hiantis Srl Milan, Italy), by comparing the results with those obtained using two other cell-counting methods based on microscopy cell-counting using a Thoma chamber and Fluorescence-activated Cell Sorting (FACS), as described in the workflow reported in Figure 1.

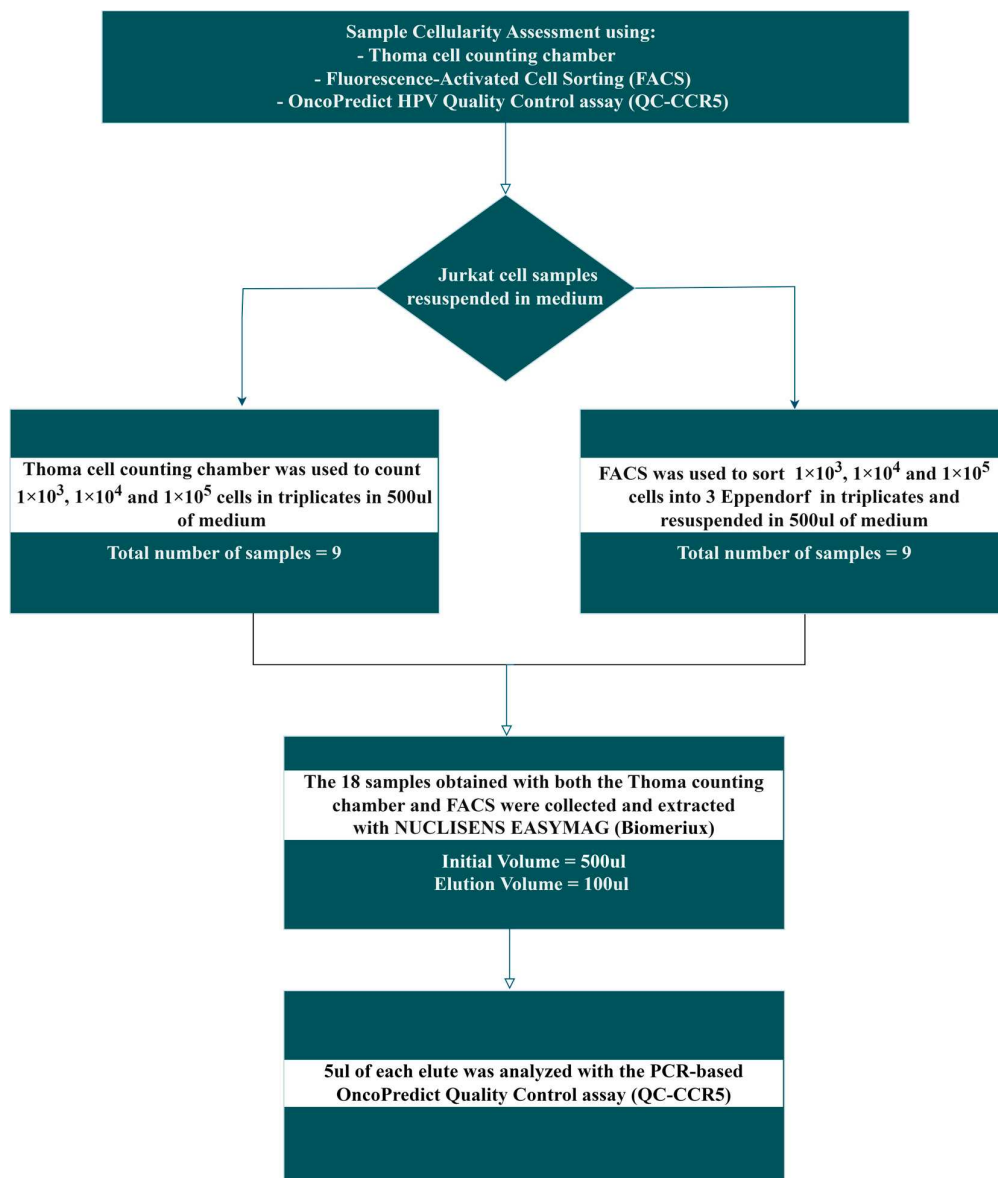


Figure 1. Study workflow.

The frequency of copy number variants and structural variants present in the human *CCR5* gene were also investigated as part of this study to ensure that this gene target could represent a reliable marker in cellularity assessment.

2.1. Analysis of the Frequency of Copy Number Variants and Structural Variants in the Human *CCR5* Gene

It has been shown that presence of copy number variants (CNVs) or structural variants (SVs) can affect the results of PCR analysis [36]. We thus interrogated a catalog of genomic variants in the Jurkat cell line [37]. We confirmed that *CCR5* is a single-copy gene in these cells and that no copy number variants (CNVs) or structural variants (SVs) affect its coding sequence.

We also explored the frequency in the human population of CNVs and SVs that involve the *CCR5* gene. To this aim, we interrogated the Genome Aggregation Database (gnomAD, v4 release) [38]. GnomAD v4 includes data from ~800,000 individuals. Although the majority of such individuals are of European ancestry, the resource is representative of worldwide genetic diversity, with more than 150,000 samples from subjects with non-European origin (<https://gnomad.broadinstitute.org/> accessed on 25 September 2024).

2.2. Cell Line: Cell Count with Thoma Chamber

Human Jurkat cell line sample obtained from ATCC (Manassas, VA, USA) was cultured in RPMI-1640 medium supplemented with 10% FBS, 1% Pen/Strep, Fungizone and Glutamine (Complete medium; Euroclone, Milan, Italy) at 37 °C and 5% CO₂.

After harvesting the Jurkat cells were centrifuged at 1200 rpm for 5 min at room temperature and the pellet was resuspended in 5 mL of complete medium. The cells were then filtered with 40 µm cell strainer (Euroclone) to eliminate cell aggregates.

Cell counting was performed using Trypan Blue solution (Sigma-Aldrich, St. Louis, MO, USA) to exclude non-viable cells, with living cells counted in a double-grid Thoma chamber (Marienfeld Superior, Lauda-Königshofen, Germany). The Thoma chamber slide was observed using an inverted microscope (Olympus) with a 10× objective. Subsequently, the following cell suspensions of 1000, 10,000 and 100,000 cells were prepared in triplicate in 500 µL (total number = 9 Eppendorf tubes) of complete medium. The remaining cells were used for the cell-sorting procedure.

2.3. Cell Line: Fluorescence-Activated Cell Sorting (FACS)

The remaining Jurkat cell suspensions were sorted using a MoFlo Astrios Cell Sorter (Beckman Coulter, Brea, CA, USA). During the Set Sort Decision phase, the following criteria were used: “Purify and 1-2 Drop”. Using the “Limit Event” function, 1,000, 10,000 and 100,000 cells were sorted in triplicate (Total number = 9 Eppendorf tubes) according to the gate strategy shown in Figure 2. An averaged sort rate of 200–2000 events per second and a sorting pressure of 25 PSI with a 100 µm nozzle were maintained throughout the process. Kaluza software version 1.2 was used for .fcs file analysis.

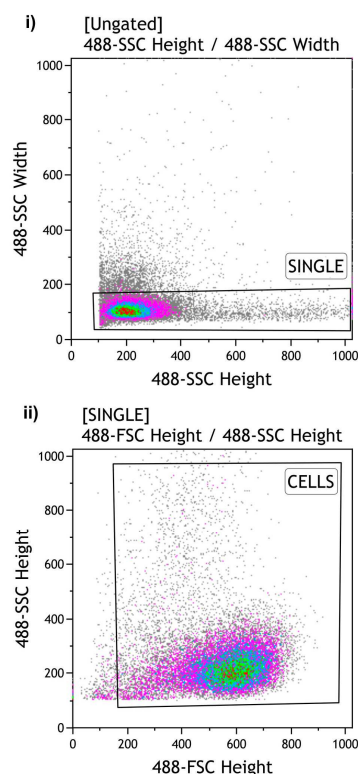


Figure 2. Gating strategy of Flow Cytometry: The gating strategy of Flow Cytometry for the sorting of Jurkat cells was: (i) “SINGLE” cells to exclude aggregates (ii) “CELLS” to sort the desired cell number. SSC: Side Scatter. FSC: Forward Scatter.

2.4. Extraction of Human Jurkat Cell Line Samples

A total of 18 Eppendorf tubes containing the human Jurkat cell line samples obtained following both cell-counting by Thoma chamber and sorting by FACS in a volume of about

500 µL underwent nucleic acid extraction using the automated NucliSENS® easyMag® platform (bioMérieux, Marcy-l'Étoile, France) which exploits BOOM technology with magnetic silica particles diluted 1:2. Prior to extraction, 10 µL of the exogenous spike-in control gene target (synthetic gene-firefly luciferase DNA) included in the OncoPredict HPV Quantitative Typing (QT) kit, used to assess efficiency of nucleic acid extraction as part of the assay's QC [36], was added to the Jurkat cell line samples. The extraction protocol "Specific B Protocol 2.0.1", characterized by an elution temperature of 70 °C, was selected and performed according to manufacturer's instructions using a final elution volume of 100 µL.

2.5. Testing of the Jurkat Cell Line Samples with OncoPredict HPV QC Module

The real-time assay of the Quality Control (QC) module of OncoPredict HPV Quantitative Typing (QT) kit (Hiantis SRL, Milan, Italy) was performed on the CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using 5 µL of extracted sample in a total reaction volume of 15 µL. The QC module includes an accurate evaluation of the number of human cells present in the sample through the quantitative determination of CCR5 gene; the efficiency of nucleic acid extraction through the recovery of an exogenous control gene target added to the sample before preanalytical processing; and the potential PCR inhibition through the amplification of a control target (synthetic custom-designed gene/not human DNA) included in the OncoPredict HPV QT. Standard curves for the quantification of the CCR5 gene were constructed based on the cycle threshold (Ct) values of 4 quantitative calibrators run in triplicate.

2.6. Statistical Analysis

The cellularity was extrapolated from the CCR5 standard curve and a general estimate of the cellularity value for samples was calculated using the equation below. The *p*-value was calculated using *t*-test for independent samples.

The cellularity value for samples was calculated using Equation (1).

$$= \text{Number of CCR5 (cells/reactions)} * 20 \left(\frac{100 \mu\text{L (elution volume)}}{5 \mu\text{L (innoculated volume)}} \right) \quad (1)$$

Equation used to determine the sample cellularity value.

3. Results

3.1. Frequency of CNVs and SVs in the Human CCR5 Gene

Upon interrogation of the Genome Aggregation Database (gnomAD, v4 release) [38], we detected six CNVs and nine SVs that involve the human CCR5 gene. However, the reported frequency of individual CNVs and SVs was found to be extremely low (the most common one having a frequency of 0.0033), suggesting that they do not pose a problem in the use of this gene as a sample adequacy control.

3.2. Performance of CCR5

After extracting and performing real-time PCR analysis on the 18 Jurkat cell line samples obtained from both the Thoma counting chamber and FACS, the Ct values obtained from each tested sample were used to calculate the number of cells from the CCR5 standard curve (Figure 3). These cell numbers were reported as CCR5 cellularity (cells/sample) and Log10 CCR5 cellularity (cells/sample).

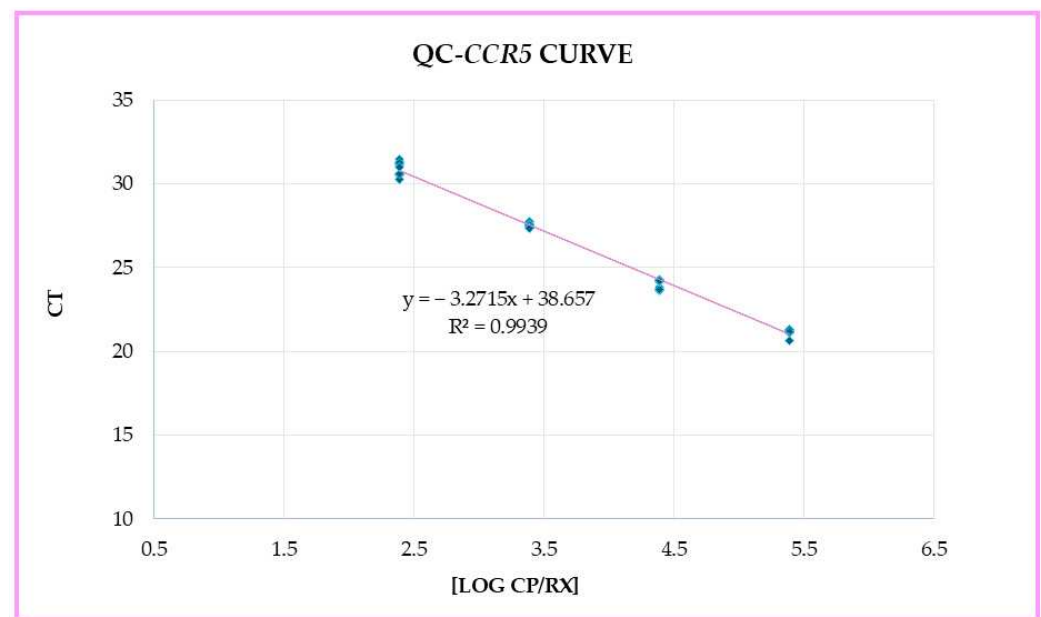


Figure 3. CCR5 standard curve plot.

From Table 1 and Figure 4, we observed that the number of cells obtained using the QC-CCR5 assay correlates to the actual number of cells counted using the Thoma chamber and FACS.

Table 1. Cellularity values obtained from the analysis of QC CCR5 assay across three different initial cell numbers (10^3 , 10^4 and 10^5). Each initial cell number had three replicates (a, b, c). * (Initial cell numbers indicate the absolute cell count (1,000; 10,000; 100,000) detected through both the Thoma chamber and FACS methods.)

Initial Cell Number *	Triplicate Samples Counted Using Thoma Chamber	Triplicate Samples Counted Using FACS
	CCR5 Molecular Cellularity Determination (Cells/Samples)	
10^3 a	2.54×10^3	1.94×10^3
10^3 b	2.35×10^3	1.70×10^3
10^3 c	1.89×10^3	2.11×10^3
10^4 a	2.20×10^4	2.31×10^4
10^4 b	2.11×10^4	2.50×10^4
10^4 c	2.63×10^4	2.25×10^4
10^5 a	1.21×10^5	1.27×10^5
10^5 b	1.26×10^5	1.44×10^5
10^5 c	1.40×10^5	1.14×10^5

Finally, no significant difference was observed between samples obtained from the Thoma chamber and FACS analysis ($p = 0.96$) using a *t*-test for independent samples.

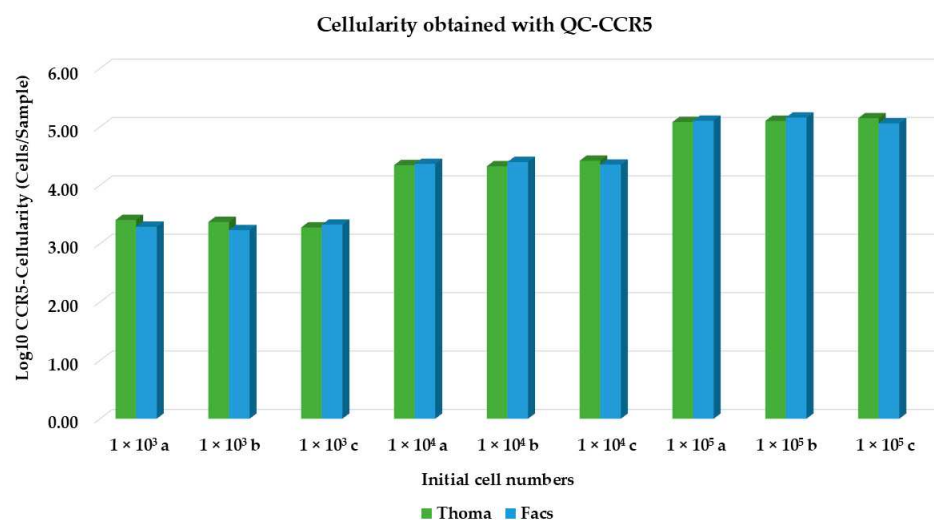


Figure 4. Graphic representation of the QC CCR5 cellularity value. Cellularity values (Log10 CCR5 cellularity, cells/sample) are presented for three replicates (a, b, c) across initial cell numbers (10^3 , 10^4 and 10^5).

4. Discussion

The diagnostic accuracy of HPV molecular assays has received an important focus in the scientific community, which has led to a consensus on international validation guidelines, known as the “Meijer criteria”, which establish minimum requirements for novel HPV tests in terms of clinical sensitivity, specificity and reproducibility [39]. Additionally, two complementary international initiatives, VALGENT (VALidation of HPV GENotyping Tests) and VALHUDES (VALidation of HUMAN papillomavirus assays and collection DEvices for HPV testing on Self-samples and urine samples), have been introduced to evaluate the clinical performance validation of HPV assays on cervical and self-collected samples [40,41]. Notwithstanding these validation criteria, only a limited number of studies have focused on evaluating the importance of sample adequacy assessment in molecular HPV-based primary testing in cervical cancer screening [5,25,42]. Moreover, the causes of “invalid results” in molecular HPV testing, which could result from multiple pre-analytical and analytical issues, such as inadequate sampling, sample processing and/or PCR inhibition, have not been fully investigated. Furthermore, presently commercially available HPV assays which include an internal human gene control often apply arbitrarily chosen IC cut-offs, in terms of cycle threshold (Ct) values, by the assay manufacturers, which have not undergone more stringent validation, such as those that are presently required for determining HPV cut-offs in terms of clinical sensitivity and specificity. To our knowledge there are no previously reported studies evaluating what should be considered an appropriate number of human cells in relation to sample adequacy for molecular HPV testing.

The results of our study, comparing the performance of molecular CCR5 detection to microscopy and FACS cell-counting, showed that the cellularity assessment was comparable between the different methods.

Our study results for using CCR5 to assess cell counts align with the study conducted by Malnati and his group. In their study, CCR5 as a single-copy human gene was used to determine the cellularity of human cells from clinical samples to achieve the absolute quantification of proviral HIV-1 DNA [34]. Similarly, a study carried out by Hong et al. used CCR5 copy numbers to calculate HIV-1 DNA copies per 1 million cells [35]. Additionally, Cocuzza et al. reported the use of CCR5 to determine the number of human cells present in clinical samples, thereby enabling the normalization of viral copy numbers per cell for the accurate quantification of HPV viral loads. This highlights an additional advantage of using CCR5 [33].

The presence of a sample adequacy control (SAC) in HPV assays is also expected to ensure the reliability of negative results by confirming the quality of the clinical sample to be tested and of the sample-collection procedure. In cervical cancer screening molecular HPV testing, a “fixed” volume of resuspended cervical or self-sample is used to perform the analysis based on manufacturers indications, irrespective of the abundance of sample collection. The latter is influenced by the fact that both clinician-collected cervical samples and self-collected vaginal samples originate from the brushing or swabbing of a mucosal surface which is influenced by the sampling procedure and by the person who is performing the collection, as also previously reported for the mucosal sampling used for SARS-CoV-2 molecular testing [43]. Moreover, negative results from molecular tests may not only be due to the absence of cells but also to errors in extraction or amplification processes [26]. The OncoPredict HPV QC test addresses these issues by including an exogenous control for both nucleic acid extraction and PCR amplification, which enable the separate monitoring of both the extraction efficiency and the potential PCR inhibition for each sample.

A SAC might be external, as in the case of the OncoPredict HPV assay, where it is amplified in a separate reaction well, or as an internal control (IC), where it is co-amplified with HPV nucleic acid [25]. Nevertheless, an appropriate IC molecular assay cut-off needs to be established in order to appropriately assess the sample adequacy.

Although no cost-benefit analysis has been performed, the additional detection of a reliable SAC, especially if it is performed in a separated reaction, is of course associated with an increased cost of the test which can however be justified by more reliable and robust results.

The reliability of negative results in HPV screening is critical for patient health, as it can influence clinical decisions. HPV testing, with its strong negative predictive value, has allowed the extension of screening intervals from approximately three years with cytology-based primary-screening protocols to five years for those who test negative [29]. This could mean that an undetected infection or early-stage precancerous lesions could progress into more advanced disease, potentially resulting in cervical cancer due to untimely follow-up care.

The risk of inadequate samples being collected is expected to increase with the recent move promoted by the World Health Organization (WHO) towards the use of self-collection for cervical cancer prevention, which includes vaginal samples being collected by the woman herself. Additionally, inadequate samples may also represent a problem in clinician-collected cervical samples, particularly in relation to women’s age. It has been shown, for example, that in post-menopausal women the transformation zone retreats further into the cervical canal, making it more difficult to obtain adequate samples. All these factors can impact the quality assurance of molecular HPV testing of clinical samples [44–46].

One of the major drawbacks of the use of molecular HPV assays is the lack of standardized criteria regarding the minimum cellularity required to define sample adequacy. The use of molecular HPV assay across various clinical settings requires the implementation of the quality assurance parameters stated by Cuschieri and colleagues [5].

On the contrary, parameters have been well-defined for both traditional Pap smears and for cervical liquid-based cytology, with a required minimum cellularity count of 8000 cells and 5000 cells, respectively, according to the Bethesda system [47]. A better understanding and further studies are needed to assess quality assurance in the performance of the more recently introduced HPV-based primary cervical cancer screening programs. In particular the assessment of the minimum cellularity required to define molecular cut-offs for sample adequacy it is necessary by the use of appropriate human gene target with a reliable number of copies per cell.

To the best of our knowledge, this is the first study comparing the performance of *CCR5*, a single-copy human gene target, to other laboratory reference cell-counting methods for the accurate molecular assessment of human cell numbers. The limitations of this study include the small number of cell-line samples tested and that these were not derived from true clinical samples, nor human vaginal or cervical cells. Moreover, this study did not

include a direct comparison with other human genes such as *beta-globin (HBB)*, *ARHGEF11*, *DNA topoisomerase III (TOP3)* and other available internal controls already included as SAC in presently available commercial HPV assays. Future studies will need to include a larger number of clinical samples allowing to better evaluate the range of cellularity required for quality assurance in molecular HPV testing and a direct comparison of *CCR5* gene to other available internal controls [42].

5. Conclusions

The results obtained from this study show that *CCR5* represents a promising molecular marker to assess sample cellularity in molecular diagnostics, with particular reference to molecular HPV testing used in cervical cancer screening. It will be important in future to define specific cellularity cut-off values for specific sample types and for the quality assurance of molecular HPV-based primary screening through the use of samples collected from human mucosal surfaces.

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RESEARCH ARTICLE OPEN ACCESS

Quality Assurance in Cervical Cancer Screening: Evaluation of Sample Adequacy in HPV DNA Testing

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Keywords: cervical cancer screening | HPV-DNA test quality assurance (QA) | sample adequacy | sample cellularity

ABSTRACT

In HPV-primary screening, sample quality significantly influences test accuracy. Unlike cytology-based screening, no consensus guidelines presently exist for sample quality assessment in HPV testing. This study aims to evaluate the impact of sample cellularity on HPV testing. A total of 37 592 liquid-based cytology (LBC) samples from women undergoing HPV-primary screening (aged 30–64, median 48; IQR: 40–56 years) were analyzed using Cobas 4800 HPV Test (Roche). Sample adequacy was assessed by the assay's β -globin internal control and by an independent quantitative cellularity assessment (OncoPredict HPV, Hiantis). HPV positivity rates (PR) were stratified according to β -globin Ct values. Among the analyzed samples, 50.0%, 47.1%, 2.3%, and 0.6% had β -globin Ct values of ≤ 28 , > 28 to ≤ 32 , > 32 to ≤ 34 , and > 34 , respectively. Overall HPV-PR was 7.7% (2891/37 592). PR reached 9.7% in samples with β -globin ≤ 28 Ct (1820/18 801), decreasing markedly to 1.4% for β -globin > 34 Ct (3/214), ($p < 0.001$). Quantitative analysis showed that Cobas 4800 β -globin Ct = 34 corresponds to approximately 1.5×10^3 nucleated cells/reaction. A subset of 195 HPV-negative samples with β -globin Ct ≥ 34 was evaluated by liquid based cytology (LBC): 19% had inadequate cellularity according to LBC guidelines, 8% were $\geq$ ASC-US and 73% NILMs. 65% of adequate LBC showed cellular atrophy. These findings emphasize the importance of assessing cellularity in HPV-screening to avoid potentially false-negative results due to inadequate samples. Future research should focus on establishing standardized cellularity thresholds to improve screening accuracy.

1 | Background

Cervical cancer (CC) is determined by persistent infection with high-risk (HR) human papillomavirus (HPV) genotypes. The aim of HPV-primary screening is the prevention of CC in asymptomatic women using evidence-based protocols [1–3], balancing benefits and harms on the basis of the clinical

sensitivity and specificity of validated tests. HPV-primary screening algorithms [1, 2] are presently recommended by European Guidelines, as they have been shown to markedly improve the effectiveness of CC prevention. Despite the improved clinical sensitivity of HPV screening (92.6%) versus Pap-test (62.8%) [3], 5.5%–16.9% of CCs are still reported as HPV-negative, especially in women over the age of 50 years [4–7].

Abbreviations: ASC-H, atypical squamous cells-high-grade cannot be excluded; ASC-US, atypical squamous cells of undetermined significance; CC, cervical cancer; CCR5, *C-C chemokine receptor type 5*; CCS, cervical cancer screening; CEL, Local Ethic Committee; CI, confidence interval; CIN, cervical intraepithelial neoplasia; Ct, cycles threshold; EU, European Union; GPSC, Good Performance Screening Centers; HPV, human papillomavirus; HR, high risk; H-SIL, high-grade squamous intraepithelial lesion; IC, internal control; IFU, instruction for use; LBC, liquid-based cytology; L-SIL, low-grade squamous intraepithelial lesion; MPSC, Medium Performance Screening Centers; NILM, negative for intraepithelial lesion or malignancy; OR, odds ratio; PCR, polymerase chain reaction; PPSC, Poor Performance Screening Centers; PR, positive rate; QC, quality control; RXN, reaction; SC, Screening Clinic; TPSC, Top Performance Screening Centers.

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Recent studies have shown that HR-HPV negative LBC (Liquid Based Cytology) and Formalin-Fixed Paraffin-Embedded tissue samples represent 5%–7.8% of all histologically confirmed CCs [8], therefore only few rare CCs are truly HPV-negative [8–13] even after testing with highly sensitive assays such as Next Generation Sequencing (NGS) [4, 8].

Clinical validation studies of new HPV assays [14, 15] have focused on the clinical sensitivity and specificity of HPV targets' cut-offs for the detection of CIN2+ lesions [16] but less attention has been placed on the assessment of sample adequacy.

Sample quality is well defined for LBC by the Bethesda System which classifies samples as “satisfactory” based on the assessment of samples' cellularity and the presence of transformation zone components [17–20]. In cervical cytology, a minimum of 5000–8000 squamous epithelial cells per slide is recommended [18, 19, 21], as inadequate cellularity can lead to false negative results.

Most commercially available HPV assays are qualitative and arbitrarily estimate the amount of collected cells by applying a Cycle threshold (Ct) cut-off for the amplification of a human internal control (IC) target, as a measure of sample adequacy. Although sample adequacy is essential for the quality assurance of HPV DNA molecular testing in CC prevention, this parameter has not been strictly evaluated, nor a minimum required number of cells has been defined to improve confidence in “HPV-negative” results, as in the case of cervical cytology. Low-quality samples can in fact result in HPV-DNA false negative results due to low viral loads or other pre-analytical issues [4, 8, 9].

The inclusion of an appropriate Sample Adequacy Control is critical in the quality assurance of PCR testing, ensuring the presence of sufficient, good quality human nucleic acids in the reaction [22, 23]. The absence of IC in some HPV assays [24–27] may increase false negatives, as issues in sample collection or pre-analytical processing can compromise results [8, 28]. Currently, a negative IC result indicates an invalid test outcome [28, 29] in acellular samples, however, detectable IC amplification at high PCR Ct values, within the indicated assays' manufacturer's cut-offs, does not always guarantee the presence of sufficient biological material. Quantitative cellularity assessment or appropriately “selected” Ct values can strengthen confidence in HPV negative results [22, 23].

The aim of this study was to determine whether the cellularity of clinical samples can influence HPV positivity rates in cervical cancer screening (CCS) programs.

2 | Materials and Methods

2.1 | Sample Collection and Study Population

In Apulia (Italy) the CCS program transitioned to primary HPV-based algorithm at the end of 2022. This study analyzed 37 592 samples collected from women living in Bari metropolitan area, aged 30 to 64 years (median age 48 years; IQR: 40–56) and attending the first round of HPV-based primary CCS, from January to December 2023. Cervical samples were collected using Cervex-Brush (Rovers) and 20 mL PreservCyt Collection Medium

(Hologic), according to manufacturer's instructions, in 45 different public CC screening clinics (SCs) by professionally trained midwives. All samples were subsequently centralized and analyzed in a laboratory performing both molecular HPV testing and cytological triage, within 1 week from sample collection.

2.2 | HPV-DNA Assay

Samples were analyzed using the Cobas 4800 HPV Test (Roche): this test targets the L1 gene [29] and reports individually HPV16, HPV18 and a pool of 12 additional HR-HPV types. Human β -globin gene is amplified as IC, confirming the qualitative presence of human nucleated cells in the sample and evaluating any potential PCR inhibition.

Testing was performed strictly following the assay's “European Instructions for Use” (EU IFU), by loading the primary collection tube onto the Cobas®x480 immediately after vortexing and decapping. From 400 μ L starting volume, DNA was extracted and purified in 150 μ L of elution buffer from which 25 μ L was used for PCR and HPV detection.

The clinical cut-off values for each channel (Ct < 40.5, 40, and 40 for HPV16, HPV18, and HPV “other” HR, respectively) were previously established by Rao et al. [29] to achieve the required clinical sensitivity and specificity for the detection of CIN2+ lesions. The manufacturer's IC cut-off value for β -globin is set at Ct 40.

2.3 | Liquid-Based Cytology

LBC was performed using the ThinPrep 2000 Processor (Hologic), following the manufacturer's instructions. Cytology results were reported according to the Bethesda System guidelines. LBC results were classified as “negative” if cytology was Negative for Intraepithelial Lesions or Malignancy, (NILM); as “inadequate” or “non-diagnostic” in cases such as low cellularity (< 5000 cells/slide), excessive blood, mucus, or inflammatory cells, presence of noncellular amorphous material or “amorphous debris”; any other result was considered positive. Among NILM samples, those showing atrophy were annotated [17].

As part of the local primary HPV-based screening algorithm, LBC was performed for the triage of HPV-positive samples. For the present study, LBC was also performed on a subset of 195 HPV-negative samples with β -globin Ct $\geq$ 34 following which HPV-negative women with ASC-US+ or inadequate cytology were recalled for screening.

2.4 | Testing of Random HPV-Negative Samples With OncoPredict HPV QC Module

The Quality Control (QC) module of the OncoPredict HPV Quantitative Typing (QT) kit (Hiantis SRL, Milan, Italy) was used on the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using 5 μ L of the same DNA eluate of 125 random HPV-negative samples extracted and tested by the Cobas x480 instrument (24 < β -globin Ct < 40). The QC module accurately quantifies the number of human cells in the sample

through quantitative CCR5 gene Real-time PCR reaction and assesses potential PCR inhibition through the separate amplification of a synthetic control target [30, 31]. Standard curves for CCR5 quantification were constructed based on the Ct values of four quantitative standards provided by the kit which were run in triplicate.

2.5 | Evaluation of PreservCyt Volume Required for LBC Setup

The volume of 110 ThinPrep containers with IC amplified at varying Ct values was assessed, before and after cytology preparation.

2.6 | Statistical Analysis

Data were generated by Cobas 4800 Software v2.2.0. The data archived were imported into Cobas 4800 ArchiveViewer3.0 Software. Selected runs were exported into Microsoft Excel files, and data were consolidated into a single Excel file with barcode, HPV HR Ct, HPV-16 Ct, HPV18 Ct and β -globin Ct. Age and data of women screened at different participating SCs associated with barcodes were exported from the Laboratory Information System. A merged pseudonymized data file was generated by a Visual Basic for Applications (VBA) code. Data were evaluated for the distribution of β -globin Ct values, age ranges and the performance of SCs in relation to HPV results. Descriptive statistics were computed and Chi-squared (χ^2) tests were used. A logistic regression analysis was performed to identify factors associated with the presence of HPV. Both univariate and multivariate models were applied. The dependent variable was “HPV presence” (positive/negative) and independent variables included β -globin Ct values, the performance of SCs and women’s age intervals.

β -globin Ct values were grouped into four categories: ≤ 28 (reference), $28 < \beta$ -globin ≤ 32 , $32 < \beta$ -globin ≤ 34 and $34 < \beta$ -globin ≤ 40 . Performances of SCs were categorized into four groups based on β -globin ranges as indicator of sample collection quality: Top Performance SCs (TPSC), Good Performance SCs (GPSC), Medium Performance SCs (MPSC), Poor Performance SC (PPSC), (Table SI). Women’s age intervals were arbitrarily divided into four groups: 30–39, 40–49, and 50–59, 60–64 years.

Univariate logistic regression was first conducted to evaluate the association of each independent variable with HPV-result and odds ratios (ORs) with 95% confidence intervals (CIs) were calculated. Variables with a p -value < 0.05 in the univariate analysis were subsequently included in the multivariate model to adjust for potential confounders. Statistical significance was set at $p < 0.05$. All analyses were performed using STATA Statistical Software version 17.0.

3 | Results

3.1 | HPV Positivity Rates Related to β -globin Ct

The results from the first round of HPV-based primary CCS of women living in the Bari metropolitan area, showed an

overall HPV positive rate (PR) of 7.7% (2891/37 592). Among the HPV positive samples, 2203 samples (76.2%) were identified as positive for “other” HR-HPV types, 419 samples (14.5%) were positive only for HPV16, 123 (4.2%) only for HPV18, 106 (3.7%) for HPV16 and “other” HR types, 34 (1.2%) for HPV18 and “other” HR types, 2 samples (0.06%) were co-positive for HPV16 and HPV18, and 4 (0.14%) samples showed positivity for a combination of HPV16, HPV18, and “other” HR types.

As part of the laboratory quality assurance assessment, HPV-PR were analysed in association with β -globin Ct values. Among the 37 592 analyzed samples, 50% (18 801), 47.1% (17 704), 2.3% (837) and 0.6% (214) had a β -globin Ct value respectively ≤ 28 , $28 < \beta$ -globin ≤ 32 , $32 < \beta$ -globin ≤ 34 , and > 34 . Data showed a marked decrease in HR-HPV PR as the β -globin Ct values increased. Specifically, PR were highest, at 9.7%, for β -globin ≤ 28 Ct, decreasing to 5.8% for the range $28 < \beta$ -globin ≤ 32 , further dropping to 1.4% in the β -globin Ct range > 34 (Table 1). From logistic regression β -globin Ct values showed a strong inverse association with HPV PR ($\chi^2 = 219.8$, $p < 0.001$, Table 2). In both univariate and multivariate analyses, samples with a range $28 < \beta$ -globin ≤ 32 had significantly lower odds of HPV PR compared to the reference group (β -globin ≤ 28), with ORs of 0.58 (95% CI: 0.53–0.63, $p < 0.001$) and 0.63 (95% CI: 0.58–0.68, $p < 0.001$), respectively. The association was even stronger for samples with $32 < \beta$ -globin ≤ 34 , which had ORs of 0.38 (95% CI: 0.27–0.57, $p < 0.001$) and 0.42 (95% CI: 0.30–0.60, $p < 0.001$). Samples with $34 < \beta$ -globin ≤ 40 range had the lowest odds of HPV-PR, with ORs of 0.13 (95% CI: 0.04–0.41, $p < 0.001$) in the univariate model and 0.14 (95% CI: 0.05–0.45, $p = 0.001$) in the multivariate model.

3.2 | Age-Related Sample Cellularity and HPV-PRH

DNA yields and HPV-PR were influenced by patients’ age. Table 3 shows slight but significant differences in median age across the β -globin Ct groups (unpaired t -test p value < 0.0001). Generally, lower Ct values (indicating higher DNA quantity) were associated with women’s younger median ages (46, IQR: 38–53). This trend may suggest that sample cellularity, as reflected by β -globin Ct, could vary with age. However, the differences are not substantial, suggesting that age may not be the only factor influencing it. The association between age ranges and HPV-PR was statistically significant ($\chi^2 = 273.0$, $p < 0.001$) (Table 4), confirming previously reported data [32, 33]. In the present study, age was a significant factor in HPV-PR: 11.5% of samples were HPV-positive in the 30–39 yrs age group, compared to 4.8% in the 60–64 yrs age group. The 30–39 years age group had the highest odds of HPV-PR, with ORs of 2.55 (95% CI: 2.20–2.96, $p < 0.001$) in the univariate model and 2.18 (95% CI: 1.87–2.53, $p < 0.001$) in the multivariate model. The 40–49 years group had ORs of 1.70 (95% CI: 1.46–1.97, $p < 0.001$) and 1.50 (95% CI: 1.28–1.74, $p < 0.001$) in univariate and multivariate analyses, respectively. The 50–59 years age group had lower but still significant odds, with ORs of 1.26 (95% CI: 1.08–1.47, $p = 0.003$) and 1.19

TABLE 1 | HPV test results sorted by β -globin Ct-value.

HPV	β -globin (Ct values)				Total N (%)	Chi squared χ^2 (p-value)
	β -globin ≤ 28 N (%)	$28 < \beta$ -globin ≤ 32 N (%)	$32 < \beta$ -globin ≤ 34 N (%)	$34 < \beta$ -globin ≤ 40 N (%)		
Negative	16 981 (90.3)	16 670 (94.2)	839 (96.1)	211 (98.6)	34 701 (92.3)	219.9 < 0.001
Positive	1820 (9.7)	1034 (5.8)	34 (3.9)	3 (1.4)	2891 (7.7)	
Total	18 801 (100)	17 704 (100)	873 (100)	214 (100)	37 592 (100)	

Note: Numbers (N) and percentages (%) of positive and negative HPV cases for each Ct group of β -globin amplification.

TABLE 2 | Association between HPV Positivity Rate (PR) and respectively β -globin Ct values, age range and screening out-patients' clinics (SC) grouped by estimated performance.

Variables	Univariate model (OR [IC 95%]; p-value)	Multivariate model (OR [IC 95%]; p-value)
β -globin (Ct values)		
β -globin ≤ 28	Reference	Reference
$28 < \beta$ -globin ≤ 32	0.58 [0.53–0.63]; < 0.001	0.63 [0.58–0.68]; < 0.001
$32 < \beta$ -globin ≤ 34	0.38 [0.27–0.53]; < 0.001	0.42 [0.30–0.60]; < 0.001
$34 < \beta$ -globin ≤ 40	0.13 [0.04–0.41]; < 0.001	0.14 [0.05–0.45]; 0.001
Age range		
30–39 years	2.55 [2.20–2.96]; < 0.001	2.18 [1.87–2.53]; < 0.001
40–49 years	1.70 [1.46–1.97]; < 0.001	1.50 [1.28–1.74]; < 0.001
50–59 years	1.26 [1.08–1.47]; 0.003	1.19 [1.02–1.38]; 0.03
60–64 years	Reference	Reference
Sampling Centres (SC)		
TPSC	Reference	Reference
GPSC	0.77 [0.64–0.93]; 0.006	0.81 [0.67–0.98]; 0.028
MPSC	0.69 [0.58–0.82]; < 0.001	0.76 [0.63–0.91]; 0.003
PPSC	0.79 [0.66–0.95]; 0.011	0.92 [0.77–1.11]; 0.42

Note: Results of logistic regression analyses (univariate and multivariate) are reported as Odds Ratio and IC95%. Top (TPSC), Good (GPSC), Medium (MPSC), Poor Performance SC (PPSC).

(95% CI: 1.02–1.38, $p = 0.025$) in the univariate and multivariate models, respectively, (Table 2).

3.3 | Sample Collection Variability Across Screening Clinics

The study also evaluated the variation in sample cellularity across 45 different SCs in the metropolitan area of Bari, where samples were collected for HPV testing. β -globin Ct distributions were assessed in samples collected from different SCs, and it was observed that in some clinics the samples collected showed a significantly higher cellularity compared to others as indicated by the distribution of β -globin Ct values. Given that patient age did not significantly differ across centers (patient median age in clinics = 48, IQR: 41–56), these variations are likely to reflect differences in performing sample collection by the health professionals operating in the different SCs.

Therefore, we sorted SCs into four groups (Table SI). As shown in Table 5, some SCs had a higher percentage of samples in the β -globin ≤ 28 Ct range as compared to others which had a greater proportion of samples with higher intervals of β -globin Ct values ($28 < \beta$ -globin ≤ 32 , $32 < \beta$ -globin ≤ 34 and $34 < \beta$ -globin ≤ 40), (Table SII).

The correlation between performance of SCs and HPV PR was assessed with a regression model (Table 2). GPSC showed reduced odds of HPV-PR compared to TPSC in both univariate (OR = 0.77, 95% CI: 0.64–0.93, $p = 0.006$) and multivariate analyses (OR = 0.81, 95% CI: 0.67–0.98, $p = 0.028$). MPSC had ORs of 0.69 (95% CI: 0.58–0.82, $p < 0.001$) and 0.76 (95% CI: 0.63–0.91, $p = 0.003$) in univariate and multivariate models, respectively. PPSC were significantly associated with reduced odds of HPV PR only in the univariate analysis (OR = 0.79, 95% CI: 0.66–0.95, $p = 0.011$), but not in the multivariate model (OR = 0.92, 95% CI: 0.77–1.11, $p = 0.402$).

3.4 | Correlation Between Sample Cellularity and β -Globin Ct Values

The study further evaluated the relationship between β -globin Ct values and a quantitative sample cellularity assessment. The results of testing 125 HPV-negative random samples with OncoPredict HPV QC module [30, 31] underlined that higher β -globin Ct values are due to a lower initial amount of human DNA or cells in the sample. Moreover, the PCR inhibition control included in the OncoPredict HPV QC module did not show any evidence of inhibition of the reaction among tested

TABLE 3 | Median age and interquartile range (IQR) in each β -globin Ct range.

Cellularity	Age (Median)	IQR	N	%
β -globin ≤ 28 ct				
Positive	43.0	36–51	1820	50
Negative	46.0	39–53	16 981	
Total	46.0	38–53	18 801	
$28 < \beta$ -globin ≤ 32				
Positive	47.0	39–56	1034	47.1
Negative	51.0	43–58	16 670	
Total	51.0	43–57	17 704	
$32 < \beta$ -globin ≤ 34				
Positive	52.5	42–58	34	2.3
Negative	55.0	47–59	839	
Total	55.0	47–59	873	
$34 < \beta$ -globin ≤ 40				
Positive	42.0	31–60	3	0.6
Negative	54.0	47–59	211	
Total	54.0	47–59	214	
Total				
Positive	45.0	37–53	2891	100
Negative	49.0	41–56	34 701	
Grand Total	48.0	40–56	37 592	

Note: N = sample count in each group. Age unpaired T-Test p value < 0.0001 vs. β -globin ≤ 28 ct group.

samples, which could account for the higher β -globin Ct values. This is consistent with the hypothesis that β -globin late amplification using Cobas 4800 correlates with samples with a lower cellular content, rather than resulting from PCR inhibition issues.

Graph in Figure 1 plots the number of cells per mL, as a function of β -globin Ct values, calculated by CCR5 standard curve, in 125 samples with β -globin Ct ranging from 25 to 40.

Quantification showed cellularity in the LBC sample and in Cobas 4800 PCR reaction mix respectively of about 2×10^5 cells/mL and 1.5×10^4 cells/reaction (rxn) at a β -globin Ct of 28, 6×10^4 cells/mL and 4×10^3 cells/rxn at a β -globin Ct of 32, 2.5×10^4 cells/mL and 1.5×10^3 cells/rxn at a β -globin Ct of 34, showing a significant drop in nucleated cells with increasing Ct. At a Ct of 39, the cellularity reached the lowest measurable levels, close to 10 cells/mL and 1 equivalent genome/rxn, indicating that very few nucleated cells are present in the tested sample, with HPV DNA test results for these samples being reported by Cobas 4800 software as “valid” and “HPV-negative”. Moreover, IC Ct value reflects the overall number of nucleated cells in the sample, including inflammatory cells and not just the squamous epithelial cells (the primary target for HPV infection [34]), which means that Ct values represent total nucleated cell content rather than solely the target cell type for HPV.

3.5 | Cytology and Adequacy of Samples With Late β -globin Amplification

In a subset of 195 samples with β -globin ≥ 34 Ct, LBC slides were prepared to assess sample adequacy. Cytology revealed that 19% (37/195) of these samples exhibited inadequate cellularity, with less than 5000 cells per slide, 8% were positive for abnormal cytology (16/195: 7 ASC-US, 7 L-SIL, 1 ASC-H, 1 H-SIL), and 65% showed normal cytology with marked atrophy, suggesting that atrophic changes in the cervical epithelium may contribute to the reduced cellular yield. The remaining samples (8%) were NILMs but not atrophic.

Within this subset of samples, those found to be HPV-negative with inadequate cytology or positive for $\geq$ ASC-US were reported as “inadequate”, and women were invited to repeat the

TABLE 4 | HPV test results sorted by age range.

HPV	Age range				Total N (%)	Chi squared χ^2 (p-value)
	30–39 N (%)	40–49 N (%)	50–59 N (%)	60–64 N (%)		
Negative	7459 (88.54)	10 996 (92.08)	11 790 (93.99)	4456 (95.17)	34 701 (92.3)	273.0 < 0.001
Positive	965 (11.5)	946 (7.9)	754 (6.01)	226 (4.8)	2891 (7.7)	
Total	17 701 (100)	18 728 (100)	931 (100)	232 (100)	37 592 (100)	

Note: Numbers (N) and percentages (%) of positive and negative cases for each age range; the p-value from the χ^2 test for each group is compared to the total group.

TABLE 5 | DNA yields grouped by β -globin Ct ranges sorted by the performance of screening clinics (SC) involved in the study.

β -globin (Ct values)	Performance of sampling centres (SC)				Total N (%)	Chi squared χ^2 (p-value)
	TPSC N (%)	GPSC N (%)	MPSC N (%)	PPSC N (%)		
β -globin ≤ 28	857 (57.4)	4036 (55.4)	9241 (51.9)	4667 (42.4)	18 801 (50)	881.6 < 0.001
$28 < \beta$ -globin ≤ 32	609 (40.8)	3221 (44.2)	8192 (46)	5682 (51.6)	17 704 (47.1)	
$32 < \beta$ -globin ≤ 34	20 (1.8)	23 (0.4)	293 (1.6)	537 (4.8)	873 (2.3)	
$34 < \beta$ -globin < 40	7 (0.5)	0 (0.0)	74 (0.4)	133 (1.2)	214 (0.6)	
Total	1493 (100)	7280 (100)	17 800 (100)	11 019 (100)	37 592 (100)	

Note: The p -value from the χ^2 test for each group is compared to the total group. Screening Clinics (SCs) were sorted in four groups on the basis of cellularity of the samples collected: Top (TPSC), Good (GPSC), Medium (MPSC), Poor Performance SC (PPSC).

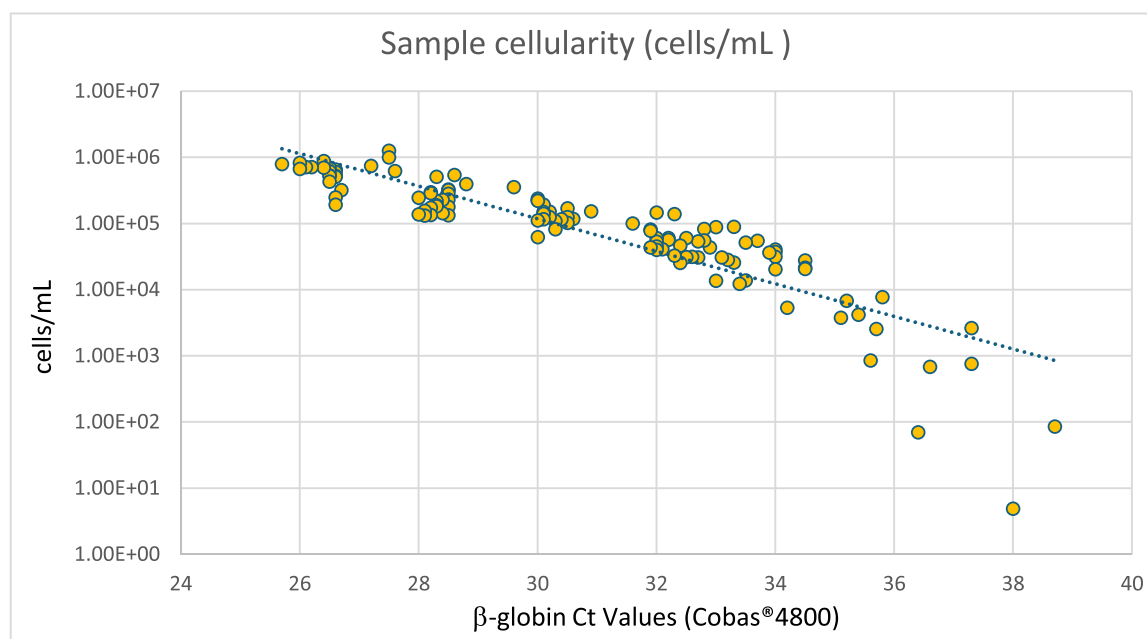


FIGURE 1 | Sample cellularity/mL vs β -globin Ct values assessed by CCR5-qPCR assay.

test. Among 16 samples with positive cytology, 4 were HPV positive at the recall visit, 4 missed the recall, 8 were HPV negative (2 with β -globin > 32 Ct). Among inadequate samples ($N = 37$), 12 women missed the recall, 3 were inadequate on repeated testing and 22 were HPV-negative (14 with β -globin > 32 Ct). These further emphasize the need for robust sample collection techniques to avoid missing clinically significant infections and need for recalling systems, [of note that 16/53 (30.2%) missed the recall visit].

Among the 2891 HPV-positive samples, only four women (0.13%) had inadequate LBC results, due both to insufficient epithelial cervical squamous cells and excessive blood contamination. The β -globin Ct values in these samples ranged from 28.2 to 30.6 (mean Ct value 29.7). These IC Ct values, all below

34, could potentially be explained by an increased number of white blood cells (WBCs) in the sample, as the β -globin gene is present in all human nucleated cells.

Moreover, we observed that among samples with β -globin late amplification, very little PreservCyt remained in the ThinPrep vial after LBC preparation. To investigate this further and to assess potential differences in residual volume, we measured the amount of PreservCyt in 110 ThinPrep vials, with samples at varying β -globin Ct values, before and after cytology preparation. As shown in Figure 2, there is a strong correlation between the volume aspirated for cytology preparation and the β -globin Ct. This confirms that when the IC Ct is higher, indicating a lower concentration of cells/mL in the sample, the instrument requires to aspirate more liquid to create an adequate LBC slide.

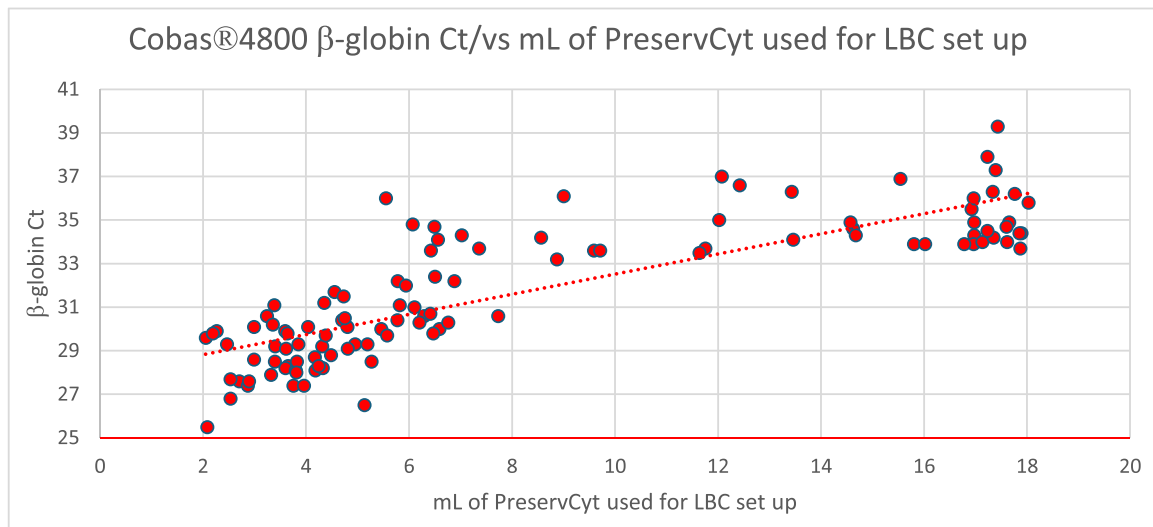


FIGURE 2 | PreservCyt (mL) aspirated for LBC setup by ThinPrep 2000 vs PCR β -globin Ct.

4 | Discussion

HPV DNA tests validated for CCS, adhering to Meijer [14] and VALGENT [15] criteria, focus on achieving appropriate clinical sensitivity and specificity for the detection of CIN2+ lesions, as assessed by HR-HPV assays' specific cut-offs. This approach allows to discriminate clinically relevant from transient HR-HPV infections, the latter usually associated with low viral loads, so avoiding potentially overtreatment. However, HPV DNA target cut-offs can also be influenced by sample cellularity, affected by the intrinsic variability associated with collection from a mucosal surface, the use of different devices, volumes and collection media as well as being operator-dependant.

This cross-sectional study based on a screening population of 37 592 women investigated the association of HR-HPV positivity with β -globin Ct values. Interestingly, decreasing HR-HPV PR were observed in samples with increasing β -globin values, dropping from 9.7% in samples with β -globin Ct ≤ 28 to 1.4% in samples with β -globin Ct > 34 ($p < 0.001$). Further investigations suggested that cervical samples containing not less than 2.5×10^4 cells/mL (β -globin ≤ 34 Ct) could be considered acceptable for screening purpose. The preliminary results of this study therefore underline the potential influence of sample adequacy in the performance of HPV-primary screening programs.

While our results emphasize the importance of assessing sample cellularity in HPV molecular testing, it is difficult to establish a common Ct value cut-off for the different molecular assays as they are based on different human gene targets. Further studies are needed to define standardized cellularity and Ct thresholds for the human targets of specific HPV assays. Moreover, as different HPV assays require different starting volumes for DNA extraction, elution and amplification, a specific sample quality assessment study is required to determine the acceptable cellular content required for each assay. Commercial HPV assays need to be assessed using the different pre-analytic and analytic protocols used in the clinical validation studies, which can influence the IC Ct values [16, 21–23, 29].

In the present study only 50% of all cervical samples achieved β -globin amplification within a Ct of 28, which is in contrast with the results reported from another HPV-primary screening program in Norway [35] where all samples run on Cobas 4800 had an IC Ct value ≤ 28 . This difference could be explained by the different pre-analytic procedure used in Norway, which involved vortexing and immediate aliquoting of 1.25 mL of sample into analysis tubes, whilst in our screening laboratory primary sample vials (20 mL) were loaded directly into the instrument, following preliminary vortexing and decapping as indicated in Roche European IFUs. The higher sample volume and longer time interval, from vortexing to sample pipetting and analysis, may also account for the observed differences in the IC Ct values.

Although HPV-based CCS has been shown to be highly effective, this study underlines the importance of implementing quality assurance measures in both the preanalytical and analytical steps.

Cellularity in cervical samples could be influenced by several factors, such as:

1. Non-adherence to Standard Operating Procedures for cervical sample collection [36–38]: such as improper rotation of the brush, which can result in insufficient cellular material being transferred to the sample resuspension volume [38]. In this context it is worth investigating whether sample collection performance may depend on the type of collection device, brush, or swab, used.
2. Pre-analytical Processing Issues:
 - If the brush, used for sample collection, is not adequately and quickly rinsed in the solution through proper bristle opening and mixing [36, 37], many cells may remain on the device, leading to lower sample's cellularity.
 - Inadequate vortexing may cause cells to remain clumped or settle at the bottom of the tube, rather than being evenly suspended.

- A delay between vortexing and sample processing can exacerbate this problem, as cells may precipitate before the test system aspirates the required volume from the meniscus of the primary vial after loading the sample on the assay's liquid handler.
3. Test IC target: different assays have different target genes and/or sequences within the gene target, which may be present in multiple and/or variable number of copies within human cells, influencing sample adequacy assessment and cellular thresholds.

It is possible to improve CCS by adequate sample collection and by improved reliability of HPV-primary testing by addressing factors such as improved training of health operators on the procedure of sample collection and on performing pre-analytical and analytical quality controls during sample processing and testing.

Moreover, among the causes of poor sample cellularity, patient's age and hormonal status must be taken in consideration. In older or postmenopausal and postpartum patients, the cervix may exhibit mucosal atrophic changes. Atrophy often leads to a reduced cellular yield and the formation of cellular clumps, which can negatively impact on the quality and quantity of biological material available for analysis. The literature highlights an age-related decline in test sensitivity due to sampling challenges with reports pointing to an elevated risk of CIN2+ in older women with ASCUS but HPV-negative [39, 40]. Although CC is more frequently diagnosed in younger women, due to routine screening, the median age at diagnosis remains 50 years. Women over 65 years account for over 20% of new CC cases and 37% of CC deaths in the United States [40], emphasizing the need for improved quality assurance measures to enhance screening effectiveness in older populations.

Despite these challenges, LBC systems, like ThinPrep 2000, partially mitigate these issues by aspirating larger volumes of medium (ranging from 2 to 18 mL depending on cellular concentration) and transferring cells onto a slide for optimal microscopic evaluation. This flexibility ensures the presence of a sufficient number of cells for cytological examination. Additionally, LBC protocols include established quality assurance benchmarks based on the number of cells, which provide a reference for sample adequacy and enhance reliability in cytological assessment as previously discussed [17–21]. In contrast, HPV testing currently lacks standardized quality assurance benchmarks for cellularity. These findings do not define specific cellularity thresholds applicable to all settings, and additional research is required to determine optimal cut-off values. Moreover, standard workflows use a fixed starting sample volume, typically 0.2–0.4 mL of the resuspended cervical sample, used for DNA extraction, irrespective of the starting sample cellularity. The possibility to concentrate samples should be investigated although less feasible in high throughput screening laboratories. Another possible solution could be to report for HPV-negative results, an indication of sample cellularity, categorizing cellular quantity as good, fair, or poor. Moreover, providing midwives feedback on their performance in sample collection could serve as an incentive for improvement.

In screening programs based on self-collected samples, ensuring adequate sample cellularity is crucial for quality assurance and should be taken into consideration. Self-sampling studies have shown that some women prefer clinician-collected samples due to concerns about their ability to perform self-collection correctly [41], highlighting the need for confidence-building measures and quality assurance strategies.

In conclusion, future clinical studies are necessary to establish shared guidelines for minimum sample cellularity in HPV molecular testing. Such research would help define optimal IC cut-off values across different diagnostic systems and sample collection methods, improving confidence in HPV-negative results in cervical cancer screening programs.

Author Contributions

Conceptualization, writing, data curation, and editing: Morena d'Avenia, Clementina E. Cocuzza. Molecular biology experiments: Morena d'Avenia, Njoku Chinyere Ruth. Cytology evaluation: Loredana Santomauro, Michela Iacobellis. Data collection: Morena d'Avenia, Njoku Chinyere R. Statistical Analysis, Tables and Figures: Filippo Dell'Anno, Morena d'Avenia. Writing review: Clementina Cocuzza, Morena d'Avenia, Marianna Martinelli, L.S. Arroyo Mühr, Filippo Dell'Anno. Supervision and funding acquisition: Michela Iacobellis, Clementina Cocuzza. All authors have read and agreed to the published version of the manuscript.

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Ethics Statement

The study received ethical approval from the Local Ethics Committee (Ref. 7438/CEL/2023, addendum 1809/CEL/2024).

Consent

Women involved in the observational study gave written consent.

Conflicts of Interest

C. E. C. institution has received research grants and/or gratis consumables from Beckton Dickinson, Copan Italia, Seegene, Novosanis and Fujirebio. C. E. C. has received speaker honoraria and/or travel funds from Seegene, Beckton Dickinson, Copan Italia. C. E. C. is a minority shareholder of Hiantis Srl.

Data Availability Statement

The data that support the findings of this study 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.

Evaluation of the clinical performance of OncoPredict HPV[®] SCR assay within the VALGENT-2 framework

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Abstract

Human papillomavirus (HPV) assays used in cervical cancer screening should be clinically validated according to international criteria. OncoPredict HPV[®] Screening (SCR) is a partial genotyping multiplex real-time PCR assay targeting E6/E7 genes of 13 high-risk (hr) HPVs. OncoPredict HPV[®] SCR (index assay) identifies HPV-16 and HPV-18 separately, 11 other hrHPV in aggregate and includes quality controls for sample adequacy, DNA extraction efficiency and PCR inhibition. 1300 VALGENT-2 study samples (from women aged 20–60 attending the Scottish cervical cancer screening program) were tested with the index assay and the GP5+/6+ PCR enzyme immunoassay (standard comparator assay). Non-inferior accuracy detecting cervical intraepithelial neoplasia of grade 2 or worse (CIN2+) of the index versus comparator was verified. Intra- and interlaboratory reproducibility of the index was evaluated by overall concordance and Cohen's kappa, using a sub-population ($n = 526$). Relative sensitivity and specificity for CIN2+ of the index versus comparator were 1.01 (95% confidence interval [CI]: 0.99–1.03) and 1.02 (95% CI: 1.0–1.04), respectively. Noninferiority p values were all ≤ 0.05 , except for CIN3+ in patients ≥ 30 years. Excellent intra- and interlaboratory reproducibility was shown with concordance $>98\%$ and kappas >0.95 . OncoPredict HPV[®] SCR fulfills the three international validation criteria for hrHPV DNA tests in cervical cancer screening.

KEYWORDS

cervical cancer, HPV genotyping, human papillomavirus, OncoPredict HPV[®], test validation, VALGENT

1 | INTRODUCTION

Human papillomavirus (HPV) infection is the most common sexually transmitted infection globally. It is estimated that more than 85% of individuals will acquire any HPV infection once in their lifetime.¹

Whilst most HPV infections resolve spontaneously without any clinical manifestation within two years, 10% of infections persist and may progress to cervical precancerous lesions.² HPVs are classified according to their oncogenic potential, with 12 genotypes considered carcinogenic (Group 1) by the International Agency for Research on

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Cancer (IARC) and referred to as high-risk (hr) HPV types (HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58 and HPV59).^{3,4}

These 12 hrHPV types are together responsible for more than 90% of all cervical cancer cases; with HPV 16 possessing the highest oncogenic potential followed by HPV 18, causing together more than 70% of cervical cancers worldwide.^{5,6} As cervical cancer develops slowly, over approximately 20 years from HPV infection to cancer, screening is an important tool for the early detection and treatment of pre-cancerous lesions, avoiding progression to invasive disease. Cervical cancer is one of the most successfully preventable neoplasia emphasizing the importance of effective screening.^{7,8} Strong evidence from large randomized clinical trials has demonstrated a reduction of incidence and mortality from cervical cancer when hrHPV-based screening is used as compared to screening with conventional cytology.^{9–11} As a result, many countries have replaced cytology with HPV testing as a primary screening tool.^{12–16} This paradigm shift to hrHPV-based primary screening has been accompanied by the influx and rapid increase of the number of HPV assays on the global market.^{17,18} Several new HPV molecular assays allowing point of care, manual or high-throughput testing have been developed, based on various technologies, ranging from immunoassays, PCR and CRISPR-Cas12a-based platforms. One of the first HPV assays introduced in cervical cancer screening programs is based on enzyme immunoassay (EIA) technology that detects PCR amplification products. However the majority of current HPV DNA molecular assays are based on real-time PCR, which is less labor-intensive and time-consuming than EIA. More recently droplet digital PCR (ddPCR) has been explored, allowing not only HPV detection but also the accurate and sensitive determination of HPV viral load.¹⁹ Isothermal amplification and CRISPR-Cas12a technologies have also been evaluated as rapid HPV detection methods, which may prove useful in resources-constrained settings.^{20,21} HPV assays selected for cervical cancer screening need to be properly validated in terms of clinical accuracy and reproducibility, according to recognized international guidelines for their use in cervical cancer screening.²²

International criteria for the validation of a new HPV assay require assessment of non-inferior sensitivity and specificity of the assay compared to a standard comparator test, in addition to achieving high intra and inter-laboratory reproducibility using well preserved samples that are representative of a screening population.²³

The VALidation of HPV GENotyping Tests (VALGENT) is a collaborative consortium for the comparison and validation of HPV assays that are intended for screening according to the international validation guidelines.²⁴ Established first in 2012, there have been four iterations of the VALGENT framework to date from different countries in Europe. The VALGENT framework allows evaluation of new HPV assays by providing samples that are representative of a screening population. In this study, we aim to evaluate the clinical performance and reproducibility of the OncoPredict® SCR assay using samples that are nested within an organized screening program from the second installment of the VALGENT framework (VALGENT-2).

2 | MATERIALS AND METHODS

2.1 | Study population and VALGENT-2 panel

The VALGENT-2¹⁸ iteration was conducted in Scotland. Scotland has a national organized cervical screening program with high uptake of around 70%. At the time of sample collection in 2012, women were then screened by cytology at three-year intervals from the age of 20 up to 60 years old. The study population for VALGENT-2 included 1300 samples of which 1000 were from consecutive samples collated from the routinely screened population (screening population) and 300 samples were cytologically abnormal samples (enrichment population). All samples for the VALGENT-2 panel were collated at one of the National Health Service (NHS) cytopathology laboratories that supported the cervical screening program in the storage medium PreservCyt liquid (Hologic). The panel was collated in August 2012. The median age of the women contributing the specimens was 38 years (range 19–68 years).

2.2 | Testing of samples with index assay

OncoPredict HPV® SCR assay (Hiantis) is a partial genotyping qualitative real-time PCR assay targeting E6 and E7 DNA sequences of 13 hrHPV types. It allows separate genotyping for HPV-16 and HPV-18 and detection of 11 other hrHPV types (HPV -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, and -68) in aggregate. The multiplex real-time PCR assay includes two reaction wells. The first well is the sample's quality control tube (QC) which permits evaluation of: (i) sample's cellularity by a *C-C Motif Chemokine Receptor 5* (CCR5) gene through a quantitative assay; (ii) an exogenous control gene target which is added to the sample prior to nucleic acid extraction, to assess DNA recovery; (iii) an amplification control target included in the real-time PCR mix to determine potential PCR inhibition. The second well allows screening for the presence or absence of HPV-16, HPV-18 and the 11 hrHPV types. This tube also includes the amplification control to verify for PCR inhibition. The CCR5 DNA control dilutions required to prepare the standard curve for target quantification are included in the OncoPredict HPV® kit.

Nucleic acid extraction and HPV testing with OncoPredict HPV® SCR assay were performed at the University of Milano-Bicocca from May to June 2021, using a Fluent 480 (Tecan) automated workstation. DNA extraction was performed by means of *Quick-DNA/ RNA MagBead* (Zymo) from 400 µl of sample starting volume. Ten microliters of exogenous control was added to the sample before nucleic acids extraction to evaluate the efficiency of the extraction procedure. The extracted DNA was eluted in a final 100 µl volume into a clean 96-well plate. OncoPredict HPV® SCR was performed according to the manufacturer's protocol. Briefly, the automated liquid handler dispensed 10 µl of mastermix QC and SCR, respectively into two reaction wells, and subsequently 5 µl of sample's DNA extract added to each well, resulting in a 15 µl total PCR reaction

volume. Negative and positive controls provided by the manufacturer were included in each PCR run. PCR was carried out using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad). Samples were considered adequate for the analysis if (i) containing at least 400 cells/reaction; (ii) extraction efficiency $\geq 10\%$; (iii) amplification control cycle threshold (Ct) ≤ 30 . Samples were defined as HPV positive if the Ct was ≤ 40 for the HPV channels.

2.3 | Testing of samples with standard comparator test

GP5+/6+ PCR enzyme immunoassay (hereinafter referred to as GP-EIA [Diassay]²⁵ was the standard comparator test used for the VALGENT-2 samples. GP-EIA is an enzyme immunoassay-based method able to detect HPV DNA, after PCR amplification of the targeted DNA sequences. It detects an aggregated presence of 14 hrHPV types (HPV -16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68) and an aggregated presence of six low-risk HPV types (HPV -6, -11, -40, -42, -43, and -44). The GP-EIA, together with the Hybrid Capture 2 (HC2)²⁶ assay, is one of the clinically validated assays currently recommended as standard comparator test, given substantial evidence of their clinical performance coming from large randomized controlled trials.^{5,27} GP-EIA was the comparator assay against which the index assay OncoPredict HPV[®] SCR was evaluated. As the GP-EIA has no genotyping capability, the LMNX genotyping kit HPV GP hr (GP5+/6+LMNX Diassay) (hereinafter referred to as "LMNX Diassay" [Diassay BV—previous version marketed as the *digene* HPV Genotyping LQ Test by Qiagen])²³ was used to compare type-specific HPV genotyping. The LMNX Diassay includes individual resolution of 18 HPV types, including the same 14 hrHPV types as in GP-EIA in addition to four additional probable/possible high-risk (pHR)-HPV types (IARC Group 2 A/B carcinogens) (HPV -26, -53, -73, and -82) and an internal control for a human DNA target to evaluate the quality of the specimen.²² Testing of samples with the GP-EIA and LMNX Diassay was performed starting from 500 μ l of the original samples at DDL Diagnostic Laboratory during April to May 2013 and April to September 2013.²³ All samples have since been stored at -80°C in the Scottish HPV Archive, a biobank to support HPV research at the University of Edinburgh, Scotland (<https://shine.ed.ac.uk/research-updates/scottish-hpv-archive>). They were retrieved from -80°C and dispatched with a dedicated courier for cold storage to University of Milano-Bicocca for testing with the index assay.

2.4 | Reproducibility

To assess the intra- and interlaboratory reproducibility of the OncoPredict HPV[®] SCR, a subset of 526 samples were randomly selected by the Unit of Cancer Epidemiology, Sciensano from the initial VALGENT-2 panel, which included 30% hrHPV positive samples as foreseen by the validation guidelines.¹⁶ In particular the

reproducibility panel contained 157 hrHPV positive samples and 369 hrHPV negative samples, based on the results of VALGENT-2 standard comparator test GP-EIA. Residual DNA extracts following initial testing for the selected 526 samples were retested twice at the University of Milano-Bicocca (Laboratory 1) to assess intra-laboratory reproducibility and subsequently tested once at PTP (Parco Tecnologico Padano) (Laboratory 2) to assess inter-laboratory reproducibility. A flowchart depicting the sample flow and testing procedures conducted in this study is displayed in Figure 1.

2.5 | Clinical outcomes

Classification of cytological and histological outcomes was based on the reporting guidelines by the British Society for Clinical Cytopathology.²⁴ According to the screening program guidance in Scotland, women with abnormal cytological results were referred to colposcopy and biopsies were performed where clinically indicated. For this study, outcomes for clinical sensitivity were determined when patients received a histologically confirmed diagnosis of cervical intraepithelial neoplasia grade 2 or worse (CIN2+). Outcomes for clinical specificity or the nondiseased group estimates were determined when women had two repeated negative cytology samples across two screening rounds. In this case, women who received two negative cytology results enrolled in the Scottish screening program over the span of 3 years were considered the non-diseased group.

2.6 | Statistical analyses

The absolute clinical sensitivity for CIN2+ and specificity for $\leq$ CIN1 of the OncoPredict HPV[®] SCR were calculated with 95% CI in all women and women aged 30 and older.¹⁶ Non-inferior accuracy statistics compared to the standard comparator test (GP-EIA) as proposed by Tang et al.²⁸ were assessed applying the benchmarks of 0.90 for relative sensitivity and 0.98 for relative specificity. McNemar (McN) test was applied to determine any differences between matched proportions.

Intra-reproducibility of the OncoPredict HPV[®] SCR testing was assessed by the University of Milano-Bicocca laboratory, whereas the inter-reproducibility assessment included the initial testing at the University of Milano-Bicocca and subsequent testing at the PTP laboratory (Parco Tecnologico Padano). The reproducibility was expressed as the overall percentage agreement which is the ratio of the number of concordant results (positive on both assays + negative on both assays) overall test results. The reproducibility validation criterion was considered as fulfilled when the left 95% confidence interval (CI) bound for hrHPV concordance exceeded 87% and the kappa > 0.5 .¹⁶

Additionally, genotype-specific concordance was assessed between the OncoPredict HPV[®] SCR and LMNX Diassay. The concordance between OncoPredict HPV[®] SCR and LMNX Diassay was assessed for HPV-16 and HPV-18 and aggregate

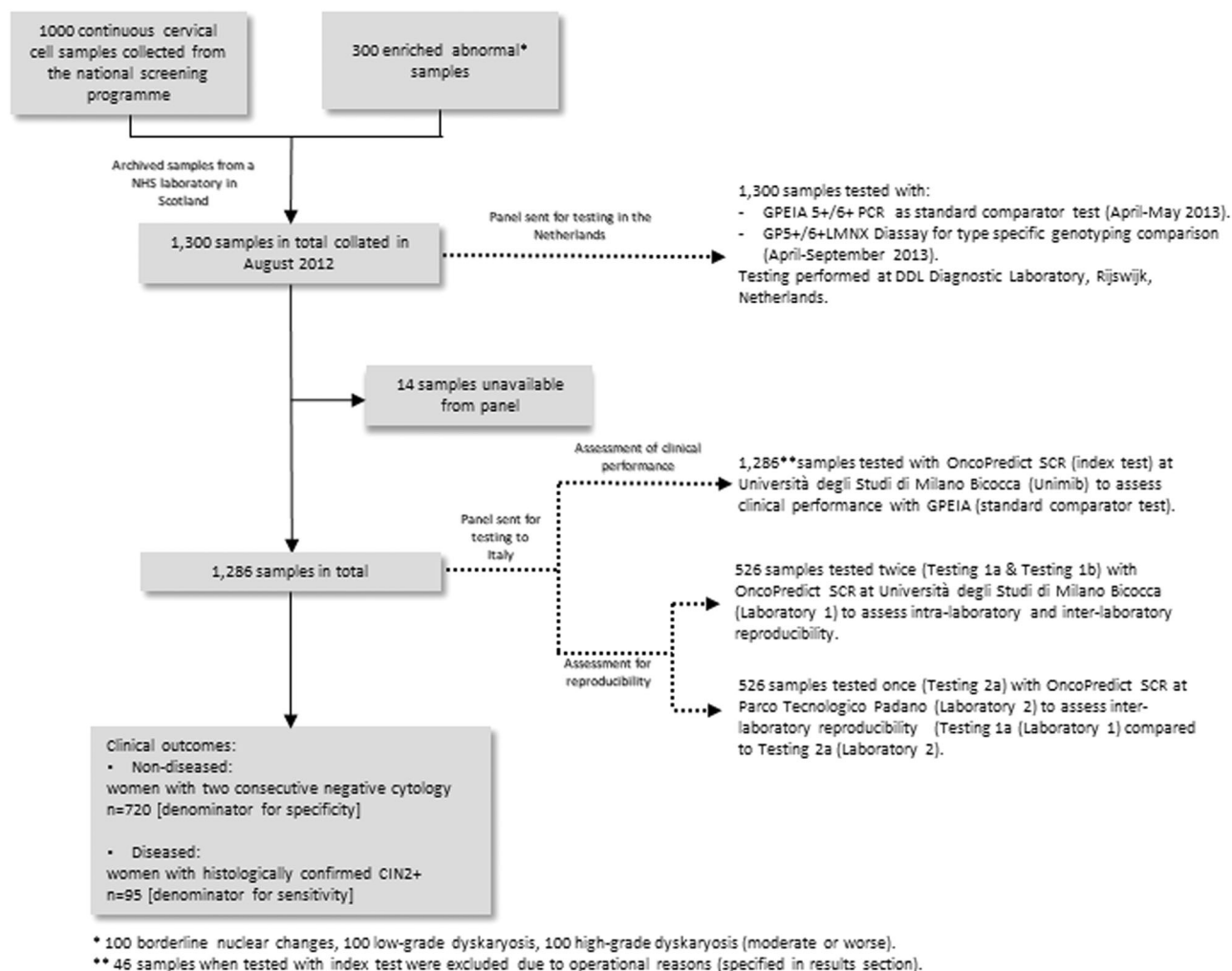


FIGURE 1 Flowchart of samples and testing of the OncoPredict HPV® SCR assay using the VALGENT-2 panel

hrHPVs using Kappa statistics. Cohen's kappa (as defined by Fleiss²⁹) were categorized as following levels of agreement between the two assays: ($1.00 \geq K > 0.80$): excellent; ($0.80 \geq K > 0.60$): good; ($0.60 \geq K > 0.40$): moderate; ($0.40 \geq K > 0.20$): fair; ($0.20 \geq K > 0.00$): poor.

Ninety-five percent exact CIs were calculated (95% CI) for all proportions. The CI around relative sensitivity and specificity took the paired design of comparisons into account. The level of statistical significance was set at 0.05. Statistical analyses were performed with STATA version 16.

2.7 | Ethical approval statement

Ethical approval was obtained for the study. As the cervical samples are anonymized residual samples surplus to diagnostic requirements which have been donated to the Scottish HPV Archive which comes under the governance of the National Research Scotland Lothian Bioresource (East of Scotland Research Ethics Service Ref¹⁵-ES-0094 and 20/ES/0061). Samples were provided for this study following

approval from the Scottish HPV Archive steering committee (HPV Archive application Ref-0060).

3 | RESULTS

3.1 | Study population

The demographics and cytopathological results of the VALGENT-2 population have been described previously.¹⁸ Of the 1300 samples from VALGENT-2, only 1286 residual aliquots were available for the present study. Of these, a further 46 (25 from the screening and 21 from the enrichment population) were excluded, 17 due to an insufficient starting volume ($<400 \mu\text{l}$, required for testing with OncoPredict HPV® SCR) and 29 (2.3%) of invalid samples due to either low sample cellularity (six samples with <400 cells/reaction) or reduced nucleic acid extraction efficiency (23 with nucleic acid recovery $<10\%$). In total, there were 1240 samples with valid OncoPredict HPV® SCR results. Initial testing of the 1300 samples with GP-EIA excluded two samples due to operational issues.

Therefore, the final number of matched samples between GP-EIA and OncoPredict HPV® SCR was $n = 1239$. From the screening population, 720 women had two consecutive negative cytology results and were used as the nondiseased group whereas 95 patients had a diagnosis of CIN2+ and were used as the diseased group. From the diseased group, 50 patients had a diagnosis of CIN3+. The hrHPV prevalence in the Scottish screening population was 16.2% (95% CI = 13.9%–18.7%) when assessed with OncoPredict HPV® SCR and 17.3% (95% CI = 14.9%–19.8%) when assessed with GP-EIA (Table 1).

3.2 | Clinical performance of OncoPredict HPV® SCR assay

In the total study population, OncoPredict HPV® SCR detected 90/95 of CIN2+ and 49/50 of CIN3+ cases corresponding to a sensitivity of 94.7% (95% CI = 93.2%–96.3%) and 98.0% (95% CI = 97.0–99.0), respectively. In the nondiseased group, 659/720 samples were OncoPredict HPV® SCR negative resulting in a specificity for $\leq$ CIN1 of 91.5% (95% CI = 89.6%–93.5%). In patients aged 30 years or older, the absolute sensitivity for CIN2+ and CIN3+ was 92.9% (95% CI = 90.9%–94.8%) and 95.5% (95%

CI = 93.8%–97.1%), respectively. Specificity for $\leq$ CIN1 of OncoPredict HPV® SCR was slightly higher in women aged 30 years and older compared to the total study population and was 93.3% (95% CI = 91.4–95.2). The accuracy estimates of OncoPredict HPV® SCR and GP-EIA 5+/6+ are shown in Table 2.

Relative sensitivities for CIN2+ and CIN3+ and relative specificity for $\leq$ CIN1 of OncoPredict HPV® SCR versus GP-EIA 5+/6+ are shown in Table 3. The relative sensitivity for CIN2+ in the total study population was 1.01 (95% CI = 0.99–1.03) and 1.00 (95% CI = 0.95–1.05) in patients aged 30 years and older. The relative specificity for $\leq$ CIN1 was 1.02 (95% CI = 1.00–1.04) and 1.01 (95% CI = 0.99–1.03) in all women and in women aged 30 years or older, respectively. OncoPredict HPV® SCR demonstrated noninferior sensitivity for CIN2+ and CIN3+ and noninferior specificity for $\leq$ CIN1 compared to GP-EIA 5+/6+ ($p_{ni} = 0.001$ and $p_{ni} = 0.0004$, respectively) in the total study population and also in the population of patients of 30 years and older (p_{ni} always >0.05 , except for the sensitivity for CIN3+ among patients aged 30 years and older). However, the relative sensitivity for CIN3+ in this latter population was the same (21/22 for OncoPredict HPV® SCR and 21/22 for GP-EIA) and the p_{McN} was 1.00.

3.3 | Intra- and interlaboratory reproducibility of the OncoPredict HPV® SCR assay

Of the 526 samples that were selected for the reproducibility assessment, three samples were excluded due to low sample cellularity (<400 cells/reaction). Results for the intralaboratory assessment showed 136 and 381 samples that were concordant for hrHPV positivity and negativity, whereas six sample pairs were discordant leaving an excellent overall agreement of 98.9% (95% CI = 97.5%–99.6%) based on a kappa value of 0.971 (95%

TABLE 1 hrHPV positivity within the VALGENT-2 screening population assessed with OncoPredict HPV® SCR assay and GP5+/6+-PCR-EIA

	Number screened	Number hrHPV+	% hrHPV+
OncoPredict HPV SCR	968	157	16.2%
GP5+/6+-PCR-EIA	968	167	17.3%

Abbreviations: HPV, human papillomavirus; hrHPV, high-risk HPV.

TABLE 2 Absolute sensitivity and specificity of the OncoPredict HPV® SCR compared to GP-EIA 5+/6+ in the total study population and women aged ≥ 30 years

Parameter	Outcome	n/N^a	OncoPredict HPV® SCR, % (95% CI)	n/N^a	GP-EIA 5+/6+, % (95% CI)
<i>Total study population</i>					
Sensitivity	CIN2+	90/95	94.7 (93.2–96.3)	89/95	93.7 (92.0–95.4)
Sensitivity	CIN3+	49/50	98.0 (97.0–99.0)	49/50	98.0 (97.0–99.0)
Specificity	$\leq$ CIN1	659/720	91.5 (89.6–93.5)	649/720	90.1 (88.1–92.2)
<i>Women ≥ 30 years</i>					
Sensitivity	CIN2+	39/42	92.9 (90.9–94.8)	39/42	92.9 (90.9–94.8)
Sensitivity	CIN3+	21/22	95.5 (93.8–97.1)	21/22	95.5 (93.8–97.1)
Specificity	$\leq$ CIN1	569/610	93.3 (91.4–95.2)	563/610	92.3 (90.3–94.3)

Note: For more details, see Supporting Information: Table 1.

Abbreviation: HPV, human papillomavirus.

^aFor sensitivity, n is number of hrHPV+ among N women with disease (CIN2+ or CIN3+). For specificity, n is number of hrHPV- among N women with two consecutive negative cytology results (i.e., non-diseased group [$\leq$ CIN1]).

Comparison	Outcome	Relative accuracy (95% CI)	p_{McN}	$p_{n.inf}$
<i>Total study population</i>				
Relative sensitivity	CIN2+	1.01 (0.99–1.03)	1.000	<0.0005
Relative sensitivity	CIN3+	1.00 (0.96–1.03)	1.000	0.0098
Relative specificity	≤CIN1	1.02 (1.00–1.04)	0.164	0.0004
<i>Women ≥ 30 years</i>				
Relative sensitivity	CIN2+	1.00 (0.95–1.05)	1.000	0.0187
Relative sensitivity	CIN3+	1.00 (0.92–1.09)	1.000	0.0633
Relative specificity	≤CIN1	1.01 (0.99–1.03)	0.377	0.0021

Abbreviation: HPV, human papillomavirus.

TABLE 4 Intralaboratory and interlaboratory reproducibility of OncoPredict HPV® SCR

First analysis in Unimib			
<i>Intralaboratory analysis^a</i>			
Second analysis in Unimib	Positive	Negative	Total
Positive	136	1	137
Negative	5	381	376
Total	141	382	523
<i>Interlaboratory analysis^b</i>			
Second analysis in PTP	Positive	Negative	Total
Positive	132	1	133
Negative	9	381	390
Total	141	832	523

Abbreviation: HPV, human papillomavirus.

^aOverall HPV test agreement: 98.9 (95% CI 97.5%–99.6%); Kappa: 0.971 (95% CI 0.947–0.994)—indicating an excellent intralaboratory agreement.

^bOverall HPV test agreement: 98.1 (95% CI 96.5%–99.1%); Kappa: 0.951 (95% CI 0.920–0.981)—indicating an excellent inter-laboratory agreement.

CI = 0.947–0.994). The interlaboratory analysis showed 132 concordantly hrHPV positive and 381 concordantly hrHPV negative results whereas 10 sample pairs were discordant: excellent overall agreement of 98.1% (95% CI = 96.5%–99.1%) based on a kappa value of 0.951 (95% CI = 0.920–0.981). Results of the reproducibility analyses are presented in Table 4.

3.4 | Type-specific HPV prevalence of OncoPredict HPV® SCR and GP5+/6+ LMNX

Genotyping agreement when compared to the GP5+/6+ LMNX in the total study population was excellent for HPV16 genotyping (general agreement of 99.1% [95% CI: 98%–100%], a kappa value of 0.94), good for HPV 18 genotyping (general agreement of 98.8% [95% CI: 98%–99%], a kappa value of 0.77), and excellent for the pooled identification of other hrHPV types (general

TABLE 3 Relative sensitivity and specificity of the OncoPredict HPV® SCR compared to GP 5+/6+ PCR-EIA

agreement of 95.6% [95% CI: 94%–97%], a kappa value of 0.87) (Table 5).

4 | DISCUSSION

In this study, the clinical performance of OncoPredict HPV® SCR was assessed, in combination with an automated preanalytical workflow for nucleic acid extraction and PCR plate preparation, using VALGENT-2 framework samples collected as part of a routine cervical screening program, according to international clinical validation criteria. hrHPV DNA testing with OncoPredict HPV® SCR assay has shown non-inferior accuracy for the detection of cervical precancerous lesions and a level of intra- and inter-laboratory reproducibility that meets the international validation criteria for cervical cancer screening.

The strong evidence of the effectiveness of HPV-based screening in the last decade has seen a sudden increase in the number of HPV assays in the market. A recent inventory estimated that over 250 distinct HPV assays are currently available in the market but over 90% have not been clinically validated according to international validation guidelines for use in cervical cancer screening.¹³ It is essential that new HPV assays will be clinically validated using samples that are representative of the screening context. OncoPredict HPV® SCR is a novel HPV assay with partial genotyping capability that enables the detection of 13 hrHPV types in a single reaction well, with separate identification of HPV-16, HPV-18, and 11 other pooled hrHPVs. The OncoPredict HPV® SCR assay also includes a unique quality control assessment of both sample adequacy and sample processing in a second reaction well. This feature enables an evaluation of the quality of sample collection and technical issues that may occur during the end to end process of testing. In particular, cellularity is evaluated by the quantification of the human CCR5 gene, present in a constant copy number/cell, allowing to control for the quality of sample collection. The amplification of the human target is evaluated in a separate reaction well from that set up for the detection of HPV positivity, so avoiding any potential interference due to competition of the amplification reactions. Moreover spiking the specimen with an exogenous control target before DNA extraction provides insight into yield and recovery of DNA in this preanalytical phase, allowing the

TABLE 5 hrHPV genotyping agreement in the total study population between OncoPredict HPV® SCR and GP5+/6+ PCR-LMNX

HPV type	-/- ^a	+/+ ^a	-/+ ^a	+/- ^a	General agreement (95% CI)	Kappa (95% CI)
HPV 16	1135	93	1	10	99% (98%–100%)	0.94 (0.90–0.98)
HPV 18	1198	26	2	13	99% (98%–99%)	0.77 (0.66–0.88)
Other hrHPV ^b	948	236	37	18	96% (94%–97%)	0.87 (0.83–0.90)

Note: Color legend (adapted from Landis & Koch³¹) for the levels of agreement: dark green ($1.00 \geq K > 0.80$): excellent; light green ($0.80 \geq K > 0.60$): good; yellow ($0.60 \geq K > 0.40$): moderate; orange ($0.40 \geq K > 0.20$): fair; red ($0.20 \geq K > 0.00$): poor.

Abbreviations: HPV, human papillomavirus; hrHPV, high-risk HPV.

^a-/- both tests are concordantly negative; +/+ both tests are concordantly positive; -/+ OncoPredict HPV® SCR negative, GP5+/6+ LMNX positive; +/- OncoPredict HPV® SCR positive, GP5+/6+ LMNX negative.

^bOther hrHPV includes the aggregate of HPV types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 for OncoPredict HPV® SCR whilst GP5+/6+ LMNX also includes the additional HPV types: -66, -26, -53, -73, and -82.

possibility for laboratories to use OncoPredict HPV® SCR assay on their own extraction systems according to local available infrastructure/liquid handling platforms. Furthermore, potential PCR inhibition is assessed by means of an amplification control target included in both QC and SCR mastermixes. This extended quality control assessment of OncoPredict HPV® SCR assay allows a reduction of potential “false negative” results due to inadequate sample collection, particularly important in the case of self-collected samples, and can highlight technical issues related to analytical (PCR) steps. This allows for more accurate troubleshooting and mitigation.

Although the VALGENT-2 panel was collated back in 2012, this study shows that the performance of OncoPredict HPV® SCR was not affected by specimen storage over approximately 10 years. This study showed only 0.5% (6/1286) of samples invalid due to inadequate cellularity, possibly pointing to changes in the number of cells usable for HPV testing. While further exploration of a sample's storage life and its effects on the quality of HPV testing is justifiable, it is notable that valid samples that were tested with OncoPredict HPV® SCR did not show any reduction of sensitivity and specificity when compared to the GP-EIA. A linkage of baseline data with the Scottish Pathology registry beyond the VALGENT2 follow-up period is planned when all aliquots will be exhausted. This will identify cases with CIN2+ not yet discovered within the VALGENT observation period.

As HPV-16 and HPV-18 are linked to over 70% of cervical cancer cases worldwide,^{5,6} limited genotyping assays which delineate HPV-16 and HPV-18 are often used to triage positive hrHPV samples. The use of assays with limited genotyping offers high-throughput and quick turnover time when used in a screening context.

In conclusion, OncoPredict HPV® SCR shows non-inferior clinical accuracy to that of the standard comparator test and sufficient interand intralaboratory reproducibility using the stringent international validation criteria evaluated through the VALGENT-2 framework. This means that OncoPredict HPV® SCR may be added to the list of hrHPV assays considered as validated for screening.³⁰

AUTHOR CONTRIBUTIONS

Sharonjit K. Dhillon and Marc Arbyn have contributed to the study design and were involved in the practical coordination of the study.

Clementina E. Cocuzza, Marianna Martinelli, Chiara Giubbi, Ruth C. Njoku, Ramya Bhatia, and Kate Cuschieri were involved in data collection and/or samples testing. Sharonjit K. Dhillon and Marc Arbyn have analyzed the data. Sharonjit K. Dhillon, Clementina E. Cocuzza, Pui Yan Jenny Chung, and Marc Arbyn have written the manuscript. All authors reviewed and/or edited the manuscript. All co-authors approved the final manuscript and its submission to this journal.

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CONFLICT OF INTEREST

C. E. C. is a minority shareholder of Hiantis Srl and received research grants and/or gratis consumables from Beckton Dickinson, Copan Italia, Seegene, Novosanis and Fujirebio. C. E. C. has received speaker honoraria and/or travel funds from Seegene, Beckton Dickinson, Copan Italia. C. E. C. is a minority shareholder of Hiantis Srl. K. C. and R. B.'s institution has received research funding or gratis consumables to support research from the following commercial entities in the last 3 years: Cepheid, Roche, Abbott, Euroimmun, GeneFirst, SelfScreen, Hiantis, Seegene and Hologic.

DATA AVAILABILITY STATEMENT

No data were deposited in a repository.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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RESEARCH ARTICLE

Infectious Causes of Cancer

Clinical performance of the novel full-genotyping OncoPredict HPV Quantitative Typing assay using the VALGENT framework

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Abstract

Clinical validation of human papillomavirus (HPV) assays according to international criteria is prerequisite for their implementation in cervical cancer screening. OncoPredict HPV Quantitative Typing (QT) assay (Hiantis Srl, Milan, Italy) is a novel full-genotyping multiplex real-time PCR quantitative assay targeting E6/E7 genes, allowing individual viral load determination of 12 high-risk (HR) HPV types. Quality controls for sample adequacy, efficiency of nucleic acid extraction and PCR inhibition are included in the assay. Clinical performance of OncoPredict HPV QT test was assessed as part of the “Validation of HPV Genotyping Tests” (VALGENT-2) framework, consisting of 1300 cervical liquid-based cytology (LBC) samples of women aged between 20 and 60 years who had originally attended for routine cervical screening in Scotland. The clinical accuracy of the OncoPredict HPV QT (index test) for the detection of CIN2+ was assessed relative to the GP5+/6+ Enzyme Immuno-Assay (GP5+/6+ EIA) (comparator test), using noninferiority criteria. Intra- and interlaboratory reproducibility of the assay was assessed on a subpopulation, comprising 526 samples. The relative sensitivity and specificity for OncoPredict HPV QT vs GP5+/6+-PCR-EIA were 1.01 (95% CI: 0.99-1.03) and 1.03 (95% CI: 1.0-1.06) respectively. The *P*-values for noninferiority were ≤ 0.001 . The intra- and inter-laboratory reproducibility demonstrated a high concordance (>98.7%) with kappas for individual types ranging from 0.66 to 1.00. OncoPredict HPV QT fulfills the international validation criteria for the use of HPV tests in cervical cancer screening.

Abbreviations: BSCC, British Society for Clinical Cytopathology; CCR5, C-C motif chemokine receptor 5; CI, confidence interval; CIN, cervical intraepithelial neoplasia; CIN1, cervical intraepithelial neoplasia grade 1; CIN2, cervical intraepithelial neoplasia grade 2; CIN3, cervical intraepithelial neoplasia grade 3; Ct, cycle threshold; GPEIA, GP5+/6+ Enzyme ImmunoAssay; HC2, Hybrid capture 2; HPV, human papillomavirus; HR, high-risk; HR-HPV, high-risk human papillomavirus; LBC, liquid-based cytology; NHS, National Health Service; PTP, Parco Tecnologico Padano; QC, quality control; QT, quantitative typing; UniMiB, University of Milano-Bicocca; VALGENT, Validation of HPV Genotyping Tests; VIA, visual inspection with acetic acid.

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KEYWORDS

cervical cancer prevention, clinical performance study, full-genotyping HPV viral load, OncoPredict HPV quantitative typing (QT), VALGENT framework

What's new?

Testing for high-risk HPV infection is an effective screening tool for the prevention of invasive cervical cancer. Many HPV assays are commercially available, but few have been fully validated according to international guidelines. Here, the authors evaluated the clinical accuracy and reproducibility of OncoPredict HPV Quantitative Typing assay, a novel full-genotyping assay targeting the 12 oncogenic HPV types. They showed that OncoPredict HPV QT performed with comparable accuracy to the well-established GP5+/GP6+ Enzyme ImmunoAssay and had good reproducibility within and between labs. Its ability to determine the genotype-specific viral load makes the OncoPredict HPV QT also useful for the risk stratification and follow-up monitoring of HPV-positive women.

1 | INTRODUCTION

Cervical cancer is the second most common cause of death in women of reproductive age worldwide.¹ Persistent infection with high-risk HPV (HR-HPV) is now known to be associated with progression to precancer and ultimately invasive cancer.² Several large, randomized trials have shown that HR-HPV testing offers better protection in the prevention of invasive cervical cancer as compared to cytology or visual inspection with acetic acid (VIA), allowing also for longer screening intervals.³⁻⁵ Moreover, some of the HR-HPV tests recently introduced on the market have extended or full genotyping capability, which in clinical practice may be useful in the risk-stratification and triage of HPV-positive women.⁶⁻⁸ Consequently, many countries worldwide have already, or are now shifting to HR-HPV primary screening, using HPV tests that have undergone clinical validation according to international criteria.⁹ In recent years many HPV assays have become commercially available but only a relatively small number have been fully validated according to the international guidelines.^{10,11} The European VALidation of HPV GENotyping Tests (VALGENT) collaborative framework was designed to support robust evaluation and validation of HPV tests including those with genotyping capacity. It involves the application of HPV tests to samples derived from a screening population and a disease-enriched population, where clinical outcomes are known.¹² Our study aimed to evaluate the clinical accuracy and intra- and interlaboratory reproducibility of the novel quantitative full-genotyping assay, OncoPredict HPV QT, which allows normalized genotype-specific viral load determination. Evaluation of clinical performance and reproducibility was performed through the VALGENT-2 iteration.

2 | MATERIALS AND METHODS

2.1 | Study population and VALGENT-2 panel

The VALGENT-2 framework/collection is based on samples collected from the organized screening program in Scotland in 2012. Dimensions of the sample are described in detail elsewhere.¹³ However, in brief, the population included 1300 samples comprising of 1000

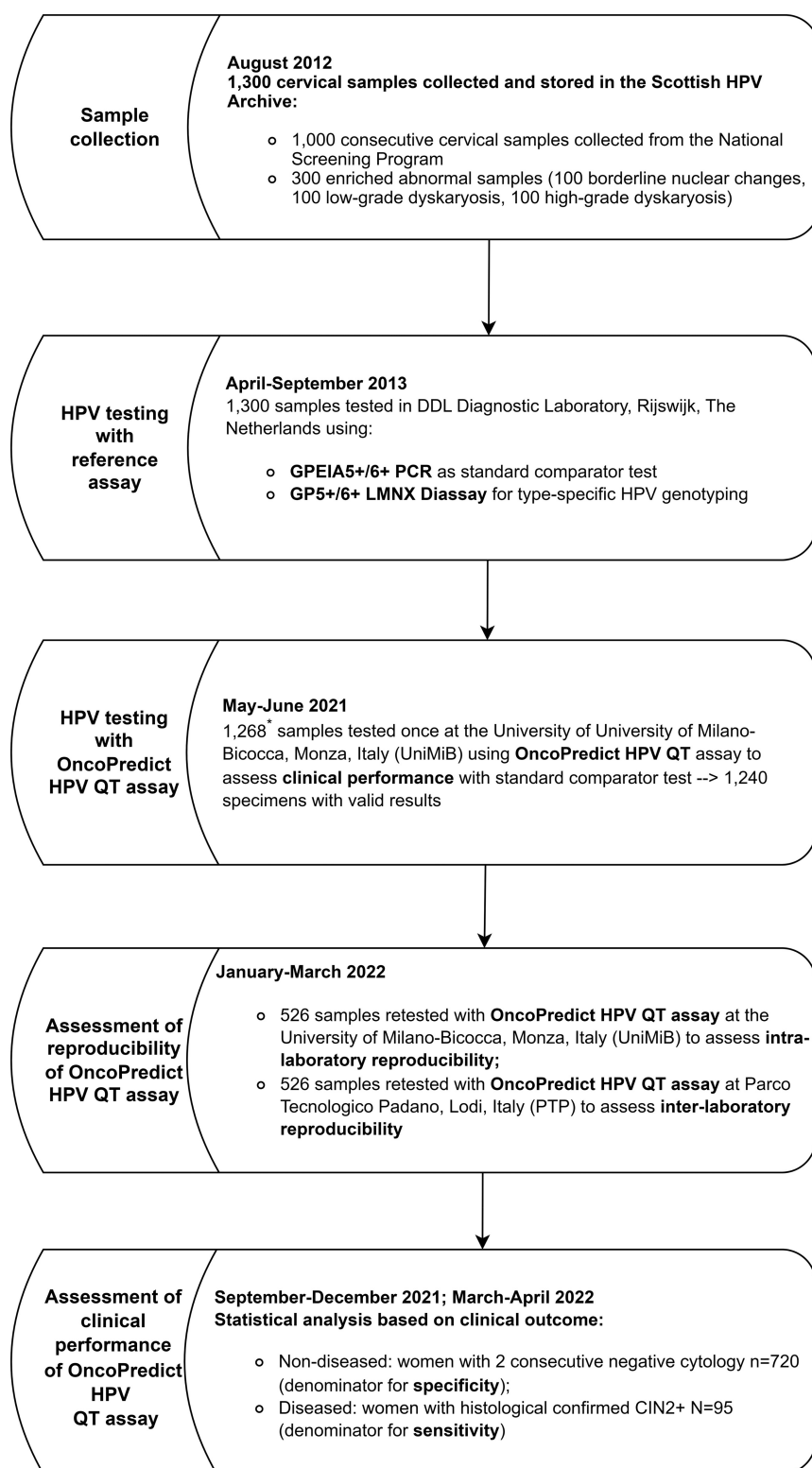
consecutive samples collected from the routinely screened population (screening population) enriched with 300 cytologically abnormal samples (disease-enriched population). All liquid-based cytology (LBC) samples resuspended in PreservCyt liquid medium (Hologic, Bedford, MA) were aliquoted and stored at -80°C in the Scottish HPV Archive. A separate aliquot of the original LBC specimen was defrosted only once before its use for the validation of an HPV DNA assay as part of the VALGENT-2 Framework. When considering the total population (screening and disease-enriched) women's median age was 38 years (ranging from 19 to 68 years).

Figure 1 describes the complete flowchart of sample collection and testing procedure used in this validation study.

2.2 | Testing of samples with OncoPredict HPV QT

OncoPredict HPV QT (Hiantis Srl, Milan, Italy) is a CE-marked full-genotyping quantitative assay, based on multiplex real-time PCR technology, targeting specific E6 and E7 DNA sequences for the detection of 12 HR-HPV types: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59. The E6/E7 amplicons detected by the OncoPredict HPV QT test vary according to the high-risk HPV genotypes detected, ranging from 60 to 128 bp. The assay is composed of five separate reaction wells (as shown in Figure 2): four of them (QT1 to QT4) allow the independent identification of three separate HR-HPV types and of an external amplification control primed to detect an exogenous target (synthetic-custom designed gene/not human DNA); the fifth serves as a quality control (QC) reaction to reflect sample adequacy and end-to-end sample processing. The QC assessment includes an accurate evaluation of: the number of human cells present in the sample, by the quantitative determination of the single-copy human gene *C-C Motif Chemokine Receptor 5* (CCR5); the efficiency of nucleic acid extraction, by the recovery of an exogenous control gene target (synthetic gene—firefly luciferase DNA) added to the sample before pre-analytical processing; and the evaluation of potential PCR inhibition, by the amplification of a control target (synthetic-custom designed gene/not human DNA) included in the OncoPredict HPV QT.

FIGURE 1 Flowchart of samples tested for the evaluation of clinical accuracy and reproducibility of OncoPredict HPV QT within the VALGENT-2 framework.

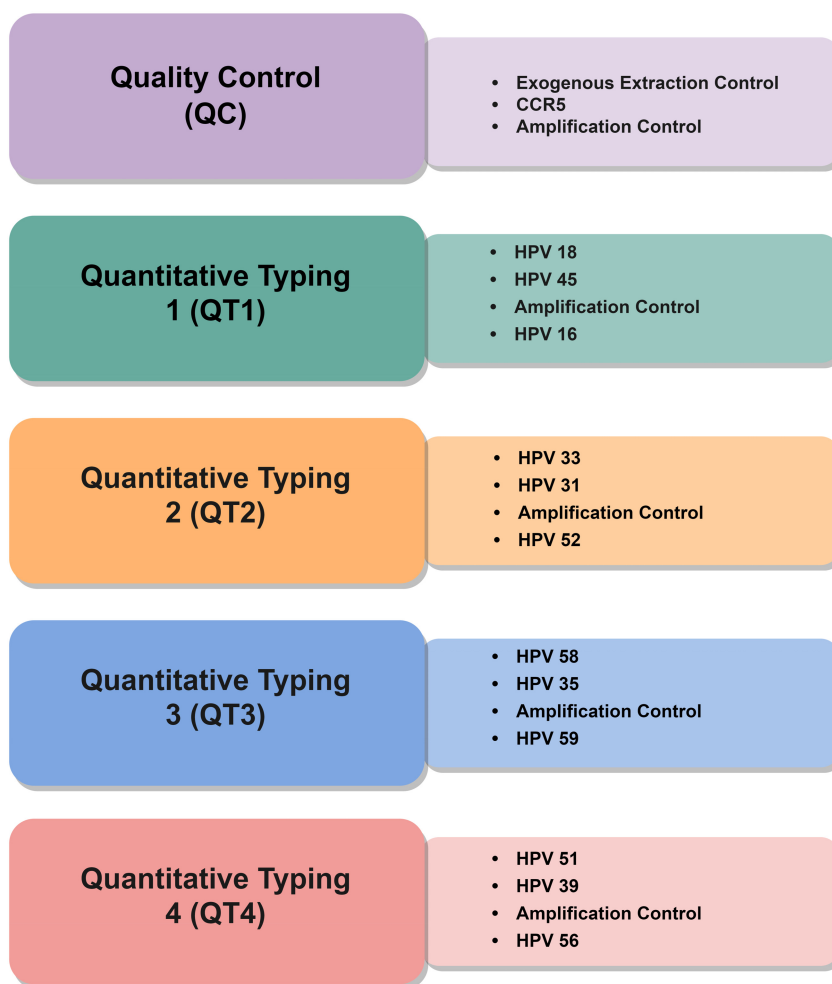


* 14 samples not available for HPV testing with OncoPredict HPV QT assay

Fully automated nucleic acid extraction and real-time PCR plate preparation, using OncoPredict HPV QT assay, were performed at the University of Milano-Bicocca (Monza, Italy) from May to June 2021, by means of a Fluent 480 (Tecan, Switzerland) liquid-handler workstation. DNA extraction was performed using Quick-DNA/RNA MagBead kit (Zymo, Irvine, CA), starting from a 400 μ L of sample volume; the

extracted nucleic acids were eluted in a final volume of 100 μ L and transferred into a clean 96-well plate. OncoPredict HPV QT testing was performed according to the manufacturer's protocol. Briefly, the automated liquid handler dispensed the PCR master mix containing 10 μ L of QC reagents and 10 μ L for each of the four additional mixes (QT1 to QT4) per sample well. Subsequently, a 5 μ L volume of DNA template

FIGURE 2 Set-up of OncoPredict HPV QT assay composed by quality control (QC) for the evaluation of sample adequacy and 4 Quantitative modules (QT1 to QT4) for the assessment of type-specific viral loads.



was added to each sample well, resulting in a 15 μ L total reaction volume. Negative and positive controls, provided by the manufacturer, were included in each PCR run. Standard curves were constructed based on the cycle threshold (Ct) values of quantitative gene targets calibrators, included in the OncoPredict HPV QT kit, allowing for the determination of the normalized viral load of each of the 12 HR-HPV types (viral genomic units/ 10^4 cells). PCR was carried out using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Samples were considered adequate for the HR-HPV analysis if at least 400 cells/reaction were detected, if nucleic acid extraction efficiency was $\geq 10\%$ and if the amplification control cycle threshold (Ct) was ≤ 30 . Samples were then considered to be HR-HPV positive if a viral load ≥ 50 copies/ 10^4 cells was detected. In the case of multiple HR-HPV infections, the HPV type with the highest viral load or predominant HR-HPV type was used for assessing positivity.

2.3 | Testing of samples with standard comparator test

In VALGENT-2, the GP5+/6+ PCR enzyme immunoassay (GPEIA)¹⁴ was used as the standard comparator test to assess noninferior clinical

accuracy of HR-HPV testing with OncoPredict HPV QT. LMNX Genotyping Kit GP HR (LMNX; Diassay BV, Rijswijk, the Netherlands), subsequently referred to as “LMNX Diassay,”¹⁵ was used to evaluate the genotyping capability of OncoPredict HPV QT. The overall HR-HPV prevalence in both the screening and disease-enriched populations according to baseline cytology and age was determined using OncoPredict HPV QT and LMNX Diassay. Type-specific agreement was assessed using the kappa statistics for the 12 HR-HPV types resolved by the OncoPredict HPV QT assay.

Testing of VALGENT-2 samples with the GPEIA and LMNX Diassay was performed at DDL Diagnostic Laboratory, Rijswijk, The Netherlands from April to September 2013¹⁶ (Figure 1).

2.4 | Reproducibility assessment

The intra- and interlaboratory reproducibility of the OncoPredict HPV QT was assessed on a subset of 526 samples, randomly selected from the original VALGENT-2 panel imposing 30% HR-HPV GPEIA positives samples, as indicated by the validation guidelines.⁹ The reproducibility panel included 157 HR-HPV positive and 369 HR-HPV negative samples, as indicated in Figure 1.

The remaining extracted DNA following initial testing was frozen and subsequently thawed to perform reproducibility analysis. Intralaboratory reproducibility of the OncoPredict HPV QT testing was assessed by repeated testing at UniMiB, whereas the interlaboratory reproducibility assessment was performed by repeating the testing at Parco Tecnologico Padano (PTP) Laboratory, Lodi, Italy.

2.5 | Clinical outcomes

The British Society for Clinical Cytopathology (BSCC) terminology and the cervical intraepithelial neoplasia (CIN) nomenclature were used for reporting cytology and histology results, respectively.^{16,17} Women with abnormal cervical cytology were managed according to the Scottish guideline criteria. In particular, women with abnormal cytological results were referred to colposcopy and colposcopy-directed biopsies were taken when clinically indicated according to local protocols. HPV test results did not influence patients' management.

The clinical sensitivity of OncoPredict HPV QT assay was based on test performance for the detection of histologically confirmed diagnosis of cervical intraepithelial neoplasia (CIN) grade 2 or worse (CIN2+) within 18 months of sample collection. The clinical specificity was determined using samples from women who had two consecutive negative cytology samples across two screening rounds (control group). Clinical performance of the index test was assessed for women irrespective of age as well as separately for women ≥ 30 years of age.

2.6 | Statistical analyses

The absolute clinical sensitivity for CIN2+ and specificity for $\leq$ CIN1 of the OncoPredict HPV QT were calculated with 95% CI for women irrespective of age and in women aged 30 years and older. Noninferior clinical accuracy of the index test compared to the standard comparator test was assessed as proposed by Tang¹⁸ applying the benchmarks of 0.90 for relative sensitivity and 0.98 for relative specificity.

Differences in accuracy between index and comparator tests were also assessed by 95% confidence intervals (95% CIs) around the relative sensitivity and specificity taking the paired design into account and by McNemar (McN) tests.

The reproducibility was expressed as the overall percentage agreement, which is the proportion of the number of concordant results (positive on both assays + negative on both assays) overall test results. The reproducibility validation criterion was considered as fulfilled when the left 95% CI bound for HR-HPV concordance exceeded 87% and the kappa > 0.5 .⁹

The analytical concordance between OncoPredict HPV QT and LMNX Diassay was assessed using Kappa statistics¹⁹ for the overall HR-HPV positivity and individually for the 12 HR-HPV types detected by the OncoPredict HPV QT assay. Kappas were categorized as proposed by Landis²⁰: ($1.00 \geq K > 0.80$): excellent; ($0.80 \geq K > 0.60$): good; ($0.60 \geq K > 0.40$): moderate; ($0.40 \geq K > 0.20$): fair; ($0.20 \geq K > 0.00$): poor.

The level of statistical significance was set at 0.05. Statistical analyses were performed with STATA version 16 (College Station, TX).

3 | RESULTS

3.1 | Cytopathological findings, adequacy of specimens for HPV testing

The cytopathological findings in the screening population were as follows: 89.8% were cytology negative; 5.4% had borderline nuclear changes; 3.8% low-grade dyskaryosis; 1% moderate to severe (high-grade) dyskaryosis. The disease-enriched population was composed by design¹³ of 100 samples with borderline nuclear changes, 100 with low-grade dyskaryosis and 100 with high-grade dyskaryosis.

Out of the total 1300 samples comprising the VALGENT-2 panel, 1286 were available for the purpose of this clinical performance study. Out of the 1286 available samples, a further 46 samples (25 from the screening and 21 from the disease-enriched population) had to be excluded from the analysis due to insufficient starting volume (17 samples' aliquots contained < 400 μ L volume required for nucleic acid extraction and testing according to OncoPredict HPV QT protocol) or to "invalid result" due to low sample cellularity or reduced nucleic acid extraction efficiency (6 samples with < 400 cells/reaction; 23 with nucleic acid recovery $< 10\%$). Testing of the VALGENT-2 panel with GPEIA at DDL Diagnostic Laboratory yielded two samples that were previously excluded due to operational issues. The final number of matched samples where valid results were available for both GPEIA and OncoPredict HPV QT were $n = 1239$.

Overall, there were a total of 95 women with CIN2+ (denominator for clinical sensitivity); of these 50 women had CIN3+ or worse. A total of 720 women had two consecutive cytology negative results (denominator for clinical specificity).

3.2 | HR-HPV prevalence

The prevalence of HR-HPV for OncoPredict HPV QT assay was 14.3% (95% CI: 12.1%-16.6%) (138/968) and 67.5% (95% CI: 61.6%-73.1%) (183/271) in the screening and the disease enriched population respectively. Comparatively, the prevalence of HR-HPV when tested with the comparator assay (GPEIA) was 17.3% (95% CI: 14.9%-19.8%) (167/968) in the screening population and 71.6% (95% CI: 65.8%-76.9%) (194/271) in the diseased enriched population. The prevalence of HR-HPV by cytological findings can be found in Tables S1 (single and multiple HR-HPV infections) and S2 (only single HR-HPV infections).

3.3 | Clinical accuracy of OncoPredict HPV QT

Table 1 provides a summary of the data on clinical sensitivity and specificity for both assays as well as relative sensitivity and specificity for women of all ages and for women > 30 years of age.

In the total study population, OncoPredict HPV QT showed a relative sensitivity for CIN2+ of 1.01 (95% CI: 0.99-1.03) and a relative specificity for $\leq$ CIN1 of 1.03 (95% CI: 1.00-1.06) compared to GPEIA. The sensitivity for CIN2+ and specificity for $\leq$ CIN1 of OncoPredict HPV QT was noninferior to GPEIA ($P = < 0.0001$ for both). Similar results were found when restricting the analysis to women aged 30 years and older.

TABLE 1 (a) Absolute and (b) relative sensitivity for CIN2+ and CIN3+ and specificity for $\leq$ CIN1 of OncoPredict HPV QT compared to GPEIA.^a

(a) Age group and outcome	OncoPredict HPV QT				GPEIA			
	Sensitivity % (95% CI)	n/N	Specificity % (95% CI)	n/N	Sensitivity % (95% CI)	n/N	Specificity % (95% CI)	n/N
<i>Total study population</i>								
CIN2+	94.7 (93.2-96.3)	90/95			93.7 (92.0-95.4)	89/95		
CIN3+	98.0 (97.0-99.0)	49/50			98.0 (97.0-99.0)	49/50		
$\leq$ CIN1 ^b			93.6 (91.9-95.4)	674/720			90.1 (88.1-92.2)	649/720
<i>Women $\geq$ 30 years</i>								
CIN2+	92.9 (90.9-94.8)	39/42			92.9 (90.9-94.8)	39/42		
CIN3+	95.5 (93.8-97.1)	21/22			95.5 (93.8-97.1)	21/22		
$\leq$ CIN1 ^b			94.9 (93.2-96.6)	579/610			92.3 (90.3-94.3)	563/610
(b)								
		Relative accuracy of OncoPredict HPV QT vs GPEIA (95% CI)						
	Outcome	Relative sensitivity		Relative specificity		McNemar, P^c		Noninferiority P^d
Total study population	CIN2+	1.01 (0.99-1.03)				1.000		<.0001
	CIN3+	1.00 (1.00-1.06)				1.000		.009
	$\leq$ CIN1 ^b			1.03 (1.00-1.06)		.0001		<.0001
Women $\geq$ 30 years	CIN2+	1.00 (0.95-1.05)				1.000		.0187
	CIN3+	1.00 (0.92-1.09)				1.000		.06
	$\leq$ CIN1 ^b			1.02 (1.01-1.05)		.005		<.0001

^aPositivity for the detection of 12 HR-HPV types.^bWomen with two consecutive negative cytology results.^c P for the McNemar test for a difference between matched proportions; P values of $>.05$ indicate that the sensitivity or specificity of the OncoPredict HPV QT assay is not significantly different from that of GPEIA.^d P for the test of noninferiority; P values of $<.05$ indicate that the sensitivity or specificity of the OncoPredict HPV QT assay is not significantly lower than that of GPEIA.**TABLE 2** Intra- (a) and inter- (b) laboratory reproducibility of HR-HPV testing with the OncoPredict HPV QT assay evaluated by the laboratories of UniMiB and PTP.

(a)	Second testing at UniMiB			
		Positive	Negative	Total
First testing at UniMiB	Positive	122	2	124
	Negative	2	397	399
	Total	124	399	523
(b)	Second testing at PTP			
		Positive	Negative	Total
First testing at UniMiB	Positive	121	3	124
	Negative	4	395	399
	Total	125	398	523

3.4 | Intra- and interlaboratory reproducibility of OncoPredict HPV QT assay

The intra- and interlaboratory reproducibility results are reported in Table 2. Among the subset of 526 samples, included in the reproducibility panel, 3 samples were excluded because they were invalid on at

least of one retesting. Out of the 523 valid samples, 519 were concordant over the two intralaboratory runs, while 516 concordant results were obtained on repeating testing at PTP. The intra- and interlaboratory reproducibility was 99.2% (95% CI: 98.1-99.8; $k = 0.98$) and 98.7% (95% CI: 97.3-99.5; $k = 0.96$), respectively.

Reproducibility was also high at type-specific level with general concordance $>99\%$ and kappa values ranging from 0.66 to 1.00, as reported in Table 3.

3.5 | HPV genotyping prevalence of OncoPredict HPV QT assay and GP5+/6+ LMNX

Overall, 321/1239 (25.9%; 95% CI: 23.5-28.4) and 338/1239 (27.2%; 95% CI: 24.8-29.9) women were found to be HR-HPV positive using OncoPredict HPV QT and LMNX Diassay, respectively. HR-HPV type-specific agreement between OncoPredict HPV QT and LMNX Diassay ranged from good to excellent with kappa values ranging from 0.64 for HPV 58 to >0.90 for HPV types 16, 31, 33, 39 and 52. Full details of the analytical concordance for the detection of the 12 HR-HPV types detected by both assays in the total population are illustrated in Table 4.

Values of genotype-specific viral load (copies/ 10^4 cells) are reported in the Table S3.

TABLE 3 Intra- and interlaboratory reproducibility of HR-HPV and type-specific HPV testing with OncoPredict HPV QT evaluated by the laboratories of UniMiB and PTP.

HPV type	Intralaboratory reproducibility		Interlaboratory reproducibility	
	Concordance, % (95% CI)	K value (95% CI)	Concordance, % (95% CI)	K value (95% CI)
HR-HPV	99.2 (98.1-99.8)	0.98 (0.96-1.00)	98.7 (97.3-99.5)	0.96 (0.94-0.99)
HPV 16	99.6 (98.6-100.0)	0.95 (0.89-1.00)	99.8 (98.9-100.0)	0.98 (0.93-1.00)
HPV 18	99.8 (98.9-100.0)	0.92 (0.77-1.00)	99.0 (97.8-99.7)	0.66 (0.38-0.94)
HPV 31	99.8 (98.9-100.0)	0.96 (0.90-1.00)	99.8 (98.9-100.0)	0.96 (0.895-1.00)
HPV 33	100.0 (99.3-100.0)	1.00 (1.00-1.00)	100.0 (99.3-100.0)	1.00 (1.00-1.00)
HPV 35	100.0 (99.3-100.0)	1.00 (1.00-1.00)	100.0 (99.3-100.0)	1.00 (1.00-1.00)
HPV 39	100.0 (99.3-100.0)	1.00 (1.00-1.00)	100.0 (99.3-100.0)	1.00 (1.00-1.00)
HPV 45	100.0 (99.3-100.0)	1.00 (1.00-1.00)	100.0 (99.3-100.0)	1.00 (1.00-1.00)
HPV 51	99.6 (98.6-100.0)	0.95 (0.89-1.00)	99.2 (98.1-99.8)	0.91 (0.81-1.00)
HPV 52	99.6 (98.6-100.0)	0.95 (0.87-1.00)	99.4 (98.3-99.9)	0.92 (0.82-1.00)
HPV 56	100.0 (99.3-100.0)	1.00 (1.00-1.00)	100.0 (99.3-100.0)	1.00 (1.00-1.00)
HPV 58	99.6 (98.6-100.0)	0.92 (0.80-1.00)	99.8 (98.9-100.0)	0.96 (0.88-1.00)
HPV 59	100.0 (99.3-100.0)	1.00 (1.00-1.00)	99.8 (98.9-100.0)	0.95 (0.86-1.00)

Note: Color legend (adapted from Landis and Koch)²⁰ for the concordance: dark green ($1.00 \geq K > 0.80$): excellent; light green ($0.80 \geq K > 0.60$): good; yellow ($0.60 \geq K > 0.40$): moderate; orange ($0.40 \geq K > 0.20$): fair; red ($0.20 \geq K > 0.00$): poor.

TABLE 4 Concordance of OncoPredict HPV QT and LMNX Diassay results in the total population for types individually resolved by both assays.

HPV type	Negative in both assays (n)	Positive in both assays (n)	Positive in OncoPredict HPV QT only (n)	Positive in LMNX Diassay assay only (n)	Kappa (95% CI)
HPV 16	1133	89	3	14	0.91 (0.86, 0.95)
HPV 18	1200	20	0	19	0.67 (0.53, 0.81)
HPV 31	1187	45	2	5	0.92 (0.87, 0.98)
HPV 33	1203	30	0	6	0.91 (0.83, 0.98)
HPV 35	1220	15	3	1	0.88 (0.76, 1.00)
HPV 39	1196	36	6	1	0.91 (0.84, 0.98)
HPV 45	1209	16	0	14	0.69 (0.54, 0.84)
HPV 51	1191	34	13	1	0.82 (0.73, 0.91)
HPV 52	1200	35	4	0	0.94 (0.89, 1.00)
HPV 56	1192	33	6	8	0.82 (0.73, 0.91)
HPV 58	1201	18	18	2	0.64 (0.49, 0.78)
HPV 59	1204	22	7	6	0.77 (0.64, 0.89)

Note: Color legend (adapted from Landis and Koch)²⁰ for the concordance: dark green ($1.00 \geq K > 0.80$): excellent; light green ($0.80 \geq K > 0.60$): good; yellow ($0.60 \geq K > 0.40$): moderate; orange ($0.40 \geq K > 0.20$): fair; red ($0.20 \geq K > 0.00$): poor.

4 | DISCUSSION

Worldwide cervical cancer screening programs are transitioning from cytology to primary HPV testing resulting in molecular HPV assays becoming increasingly available commercially; however only a relatively small number of them have been assessed according to international criteria designed to validate HPV DNA tests for use in cervical cancer screening.^{10,21}

This clinical performance study demonstrated the noninferior accuracy of OncoPredict HPV QT compared to GPEIA for the

detection of cervical precancer. The assay also showed excellent intra- and interlaboratory reproducibility of qualitative HPV detection. Furthermore, OncoPredict HPV QT was evaluated as part of the 2019 HPV Labnet international proficiency study, which reported it to be 100% proficient in the detection of the targeted HR-HPV types.²²

OncoPredict HPV QT assay is a novel quantitative full-genotyping assay targeting E6/E7 viral genes, allowing normalized genotype-specific viral load determination of 12 HR-HPVs defined as 1A carcinogens by the International Agency for Research on Cancer.²³ A key and unique feature of this assay is the extent of quality controls

included to assess sample adequacy, efficiency of nucleic acid extraction and amplification. Sample adequacy is evaluated through the quantitative assessment of a single-copy human gene, CCR5, located on chromosome 3, allowing the number of human cells present in the clinical sample to be determined as well as allowing the accurate normalization of viral loads, as previously described.^{24,25} In particular, CCR5 is not a pseudogene, as in the case of several housekeeping genes used as internal controls in nucleic acid amplification tests (NAATs) which have variable copy numbers in the human genome. Moreover, the detection of this single-copy human gene in a reaction-well separate from those dedicated to the amplification of HR-HPV targets prevents “amplification competition,” as previously reported in samples with high viral loads,²⁶ which could compromise the accuracy of viral load determination. The efficiency of nucleic acid recovery, through an external control introduced in the sample before nucleic acid extraction, provides information on the quality of this important preanalytical step. It also supports evaluation, validation and comparison of the assay with different extraction methods/protocols. Finally, an external amplification control is included in each of the five separate real-time PCR reaction wells, part of the analytical step, allowing potential PCR inhibition and/or reagent failures to be discerned.

The OncoPredict HPV QT assay has been CE marked for its use in full automation, combining high-throughput automated DNA extraction and amplification/detection by real-time PCR, as well as for a manual preanalytical and analytical set-up, thus allowing the use of the test both in large scale screening and in smaller molecular diagnostics laboratories.

The additional characteristics of the OncoPredict HPV QT assay, such as the possibility to determine the full-genotype specific normalized viral load (viral copies/no. of human cells), support its application not only in screening programs for cervical cancer prevention but also in the risk-stratification and follow-up of HPV-positive women.^{27–30} Studies are ongoing to evaluate the potential value of OncoPredict HPV QT in the molecular triage of HPV-positive women following primary HPV-based screening. Moreover, HR-HPV genotype-specific viral load is expected to be a useful marker in the post-treatment management of women with high-grade dysplasia (test-of-cure). In the present study the genotyping performance of OncoPredict HPV QT assay has been compared to that of GP5+/6+ PCR EIA, according to the current validation guidelines.⁹ The latter has been replaced operationally by newer assays in HPV-based screening programmes²¹ and as a result guidelines are in the process of being updated to include alternative potential comparators in addition to HC2 and GP5+/6+ PCR EIA. In case of multiple infections, the HPV genotype with the highest viral load was defined as the predominant HPV-type and was used to assess OncoPredict HPV QT positivity, as previously reported for RIATOL qPCR assay.^{26,31} Moreover, validation of RIATOL qPCR assay showed similar results in terms of sensitivity and specificity to detect CIN2+ when considering either the cumulative hrHPV viral loads or the genotype with the highest viral load.³¹

Meijer Guidelines⁹ suggest that clinical validation of new candidate HPV tests for their use in cervical cancer screening should be performed on a selected panel of CIN2+ cases and <CIN2 controls

from women of at least 30 years of age. However, in the current validation study, conducted as part of the VALGENT-2 framework,¹³ cervical samples were collected in 2012 in Scotland, when at the time, routine screening was offered to women from 20 to 60 years of age; representing a “real-life” screening population. We also note that samples collected in VALGENT-1 and VALGENT-4 framework came from an HPV-primary screening setting, while those collected during VALGENT-2 and VALGENT-3 from a cytology-based program; however, a multivariate analysis confirmed that primary screening modality did not affect validation outcomes; this aids in extrapolating the findings to contemporary settings.²¹

While the present study focused on clinician-taken samples, there is an increasing global practice and appetite to apply HPV tests to self-taken samples. In the future, there will be a greater onus on demonstrating tests are validated for both clinician- and self-collected specimens.³² To this end, accuracy of type-specific viral load determination with OncoPredict HPV QT on self-collected as compared to clinician-collected specimens is currently being addressed in the VAL-HUDES framework.³³

In conclusion, OncoPredict HPV QT is a novel full-genotyping HR-HPV test with the unique features of allowing genotype-specific normalized viral load determination as well as independent quality controls to assess sample adequacy and preanalytical/analytical processing. The present study demonstrated noninferior accuracy to detect cervical precancer compared to GPEIA in addition to robust intra- and interlaboratory reproducibility. OncoPredict HPV QT, therefore, fulfills all three international validation criteria for HPV tests suitable for primary cervical cancer screening.

AUTHOR CONTRIBUTIONS

Clementina Elvezia Cocuzza: Conceptualization; Methodology, validation and/or data curation; Formal analysis and writing – review & editing. **Sharonjit Kaur Dhillon:** Conceptualization; Formal analysis and writing – review & editing. **Marianna Martinelli:** Methodology, validation and/or data curation. **Chiara Giubbi:** Methodology, validation and/or data curation. **Ruth Chinyere Njoku:** Methodology, validation and/or data curation. **Ramya Bhatia:** Methodology, validation and/or data curation. **Kate Cuschieri:** Conceptualization; Methodology, validation and/or data curation; Formal analysis and writing – review & editing. **Marc Arbyn:** Conceptualization; Formal analysis and writing – review & editing. The work reported in the article has been performed by the authors, unless clearly specified in the text.

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CONFLICT OF INTEREST STATEMENT

CEC received research funding or gratis consumables to support research from the following commercial entities in the last 3 years: BD, Seegene, Copan, Novosanis and Hiantis; CEC is a minority shareholder of Hiantis Srl. SKD and MA's institution has received support from VALGENT, as explained in the VALGENT protocol (Arbyn 2016 J Clin Virol). MA and SKD were supported by the Horizon 2020 Framework Programme for Research and Innovation of the European Commission, through the RISCC Network (Grant No. 847845) and by the European Commission funded SME Instrument Phase 2 Project (Grant agreement ID: 806551). KC's institution has received research funding or gratis consumables to support research from the following commercial entities in the last 3 years: Cepheid, Roche, Vaccitech, Euroimmun, GeneFirst, SelfScreen, Hiantis, Seegene and Hologic. The work was carried out when RB was employed within NHS Lothian and University of Edinburgh, while at the time of publication, RB is employed by QIAGEN as a full-time employee. Marianna Martinelli, Chiara Giubbi and Ruth Chinyere Njoku have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

Data will be provided upon reasonable request to the corresponding author and in line with due process of governance.

ETHICS STATEMENT

The VALGENT-2 samples were obtained from the Scottish HPV Archive which comes under the guardianship and approval of the National Research Scotland Lothian Bioresource (East of Scotland Research Ethics Service Ref 20/ES/0061). Samples were provided for the present study following approval from the Scottish HPV Archive Steering Committee (HPV Archive application Ref-0060).

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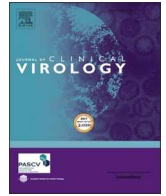
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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Clinical accuracy of OncoPredict HPV Quantitative Typing (QT) assay on self-samples

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ABSTRACT

Background: The VALHUDES initiative was established to assess the clinical accuracy of HPV assays to detect cervical precancers using urine and vaginal self-samples compared to cervical clinician-collected samples. Here, the clinical performance of OncoPredict HPV Quantitative Typing (QT) assay (OncoPredict QT) was evaluated. **Methods:** 490 women referred to colposcopy self-collected a urine and a vaginal specimen using Colli-Pee and FLOQSwab, respectively. Subsequently, a colposcopy was performed, and a cervical sample was collected with Cervex-Brush, followed by biopsy if clinically indicated. Vaginal samples were transported dry and resuspended in 5 mL of eNAT medium, whilst cervical brushings were immediately transferred in 20 mL ThinPrep.

Results: The clinical sensitivity of OncoPredict HPV QT testing for CIN2+ in urine and vaginal self-samples was similar to cervical samples (ratios of 0.99 [95 % CI 0.94–1.05] and 1.00 [95 % CI 0.96–1.04]), respectively, when manufacturer's cut-offs were applied. The specificity for <CIN2 on both self-samples was lower than on cervical samples (urine/cervical ratio = 0.91 [95 % CI 0.84–0.98]; vaginal/cervical ratio = 0.90 [95 % CI 0.84–0.98]). Cut-off optimisation improved specificity without compromising sensitivity. Median viral load values adjusted for cellularity were significantly higher in cervical samples compared to urine or vaginal self-samples, in general for all 12 high-risk HPV and in particular for HPV16, 18, 31, 33, 35, 45, 51, 58 ($p < 0.05$). No difference was observed in median viral loads between urine and vaginal samples.

Conclusion: Following cut-off optimisation OncoPredict HPV QT assay demonstrated similar accuracy on self-collected versus cervical samples.

Introduction

High-risk (hr) HPV testing for detection of high-grade cervical intraepithelial neoplasia (CIN) has been successfully used as a primary cervical cancer screening tool [1]. In addition, HPV testing on self-samples offers opportunities to increase screening coverage in

under-screened women [2,3]. Since self-samples are often well accepted among women and show similar sensitivity for the detection of cervical precancer, many countries are implementing self-sampling strategies within screening programmes routinely [4].

Meta-analyses have shown that polymerase chain reaction (PCR)-based HPV DNA tests, validated on clinician-taken samples are similarly

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accurate to detect high-grade CIN as testing on vaginal or urine self-samples [2,3,5]. These meta-analyses provided initial robust evidence demonstrating a high level of acceptance of self-sampling among women. Nevertheless, optimisation of pre- and post-analytical workflows is still lacking [6].

HPV testing using cervical samples for cervical cancer screening is well established. Performance criteria relevant for suitable HPV assays are incorporated within the Meijer 2009 or VALGENT criteria [7,8]. More than a dozen of HPV tests presently fulfil these international criteria and are considered clinically validated for cervical cancer screening [9]. Nevertheless, even if a test has been validated for cervical cancer screening using clinician-collected cervical samples, it does not automatically imply that this test can be applied to self-samples. Therefore, the VALHUDES framework was established to provide additional evidence of the HPV test accuracy on self-samples [10]. Several HPV tests were evaluated within the Belgian VALHUDES framework and have shown similar clinical accuracy on both first-void urine (FVU) and vaginal samples compared to clinician-taken samples using the manufacturer cut-off or after cut-off optimisation [11–16].

The second VALHUDES initiative was established with the support of the European Union (Grant No. 806551), bringing together the collaborative efforts of Italy, Ireland, and Scotland, however certain elements of the VALHUDES study protocols differed. In the first VALHUDES study, vaginal samples were collected with Evalyn Brush or Qvintip and resuspended in 20 mL ThinPrep, whereas in the European VALHUDES initiative a FLOQSwab was used in combination with 5 mL eNAT, an alcohol-free liquid-based medium which inactivates viruses and bacteria, preserving nucleic acids at room temperature. In the current VALHUDES report, we aimed to evaluate the accuracy of OncoPredict HPV Quantitative Typing (QT) assay to detect cervical precancer on FVU and vaginal self-samples collected with Colli-Pee and FLOQSwab devices, respectively. In addition, normalized type-specific HPV viral load was compared across specimen types. OncoPredict HPV QT assay is a quantitative full-genotyping assay targeting 12 hr HPV types and yielding normalized genotype-specific viral load. The assay has been validated within the VALGENT framework and can be used in cervical cancer screening based on clinician-collected samples [17].

Materials and methods

Study design

Matched cervical, vaginal and urine samples were collected from 500 patients recruited for the study within the European VALHUDES framework according to the STARD guidelines [18]. FVU was collected first using the Colli-Pee FV5000 collection device (Novosanis, Wijnegem, Belgium) followed by a vaginal self-collected sample with FLOQSwabs (Copan Italia Spa, Brescia, Italy), which was kept dry following collection. Thereafter, a cervical sample was taken by a gynaecologist using Cervex-Brush (Rovers Medical Devices, Oss, The Netherlands) prior to colposcopy. Colposcopy was performed as recommended by the European guidelines and a biopsy was taken if clinically indicated. Cervical samples were immediately transferred into 20 mL PreservCyt solution (Hologic, Marlborough, MA). All samples were labelled and sent to the laboratory connected with the colposcopy unit. After arrival in the laboratory, the dry vaginal samples were resuspended into 5 mL of eNAT medium (Copan Italia Spa, Brescia, Italy). The FVU samples were sent to the laboratory in the Colli-Pee container without further manipulation. At the laboratory, cervical and urine samples were divided into 1.5 mL aliquots, whereas vaginal samples were divided into 0.4 mL aliquots. All sample aliquots were properly labelled and stored at -20°C until further dispatching to the MIRRI-IT Biobank of the University of Milano-Bicocca for storage at -80°C . Study recruitment details are described in the supplementary methods.

HPV testing

HPV testing was performed using the OncoPredict HPV QT assay (Hiantis Srl, Milan, Italy), which is a quantitative full genotyping real-time PCR assay. The assay can detect and differentiate 12 hrHPV genotypes in four separate reaction wells (QT1: HPV 16/18/45; QT2: HPV 31/33/52; QT3: HPV 35/58/59; QT4: HPV 39/51/56). It targets *E6* and *E7* oncogenes using genotype-specific primers and probes. A fifth reaction well is used to quantitatively assess sample cellularity as well as the preanalytical nucleic acid extraction efficiency. Sample cellularity is calculated targeting the human *C-C chemokine receptor type 5 (CCR5)* gene. Both, sample cellularity and HPV viral loads are calculated using specific standard calibration curves. HPV type-specific viral loads are expressed as normalized viral genomic units/ 10^4 human cells based on the number of cells present in the sample determined by the quantitative CCR5 result. To assess DNA extraction efficiency, an external calibrator gene target is added to the sample prior to nucleic acid extraction and extraction efficiency is evaluated in terms of percentage recovery. Each reaction well also contains an exogenous amplification control, to independently assess PCR inhibition in each reaction mix. DNA extraction and quality control steps are described in the supplementary methods [17].

HPV positivity was defined using normalised genotype specific viral load expressed in viral copies/ 10^4 cells. Samples were considered HPV positive if ≥ 50 viral copies/ 10^4 cells were detected in cervical specimens and ≥ 10 copies/ 10^4 cells in vaginal and urine specimens, as recommended by the manufacturer. Cut-off optimization was subsequently performed based on the study results in order to achieve a balance between clinical specificity and sensitivity. Revised cut-offs were established at ≥ 15 HPV copies/ 10^4 cells for all HPV types on urine and vaginal samples. Additionally, normalised and non-normalised metrics of HPV16 positive samples were compared for their ability to identify CIN2+ cases. Normalised viral load per 10,000 cells of HPV16 was plotted against HPV16 Ct-value, as an approximation of “non-normalised viral load” per reaction, along with the clinical outcome. As the defined assay’s cut-offs were 50 and 15 copies/ 10^4 cells for cervical and self-samples, respectively, we identified Ct-value cut-offs alternative to non-normalised 50 and 15 copies per reaction using standard calibration curves.

Statistical analysis

Given the matched design applied in VALHUDES studies, McNemar test was used to assess differences between index and comparator tests. McNemar standard errors were used to compute 95 % confidence intervals of the VALHUDES study. Statistical significance was accepted if p -values < 0.05 or when the 95 % confidence intervals around relative accuracy excluded unity.

Overall and type-specific HPV test agreement among self- and cervical samples was evaluated using Cohen’s kappa values, categorized as: poor (0.00–0.19), fair (0.20–0.39), moderate (0.40–0.59), good (0.60–0.79), and excellent (0.80–1.00). Concordance was assessed for the entire study population and according to disease outcome. Differences in median signal levels between samples were assessed with the matched Mann-Whitney test for paired comparisons, whereas for non-paired comparisons, the Wilcoxon signed-rank test was used. The natural logarithm of the viral load and cellularity was used in comparisons. The Spearman’s correlation coefficient was used to assess the correlation between log (normalised viral load) of different sample types with application of the Benjamini–Hochberg (BH) false discovery correction [19]. All statistical analyses were conducted using Stata 16.1 (College Station, Texas, USA) and Jupyter Notebook 6.5.4.

Ethical approval

European VALHUDES study (NCT04312737) was approved by the

Ethics Committee of the Coordinating Centre, ASST degli Spedali Civili di Brescia, Brescia, Italy (Ethics approval number: NP 3879- Studio WP6-HPVONC) and subsequently by the local Ethics Committees of the other participating centres. The study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all study participants prior to enrolment.

Results

Study population

In total, 500 women were recruited (median age = 37 years; IQR: 31–47 years; range 25–64) from whom triplet samples were collected for the study. Ten patients were excluded due to major protocol violations as shown in Fig. 1. Twenty-seven cervical (27/490, 5.5 %), 13 vaginal (13/490, 2.7 %) and 14 urine (14/490, 2.9 %) samples were considered invalid. After excluding 27 participants due to invalid cervical samples, the dataset contained 463 participants: 452 matched cervical and vaginal and 450 matched cervical and urine samples were included in the evaluation of relative accuracy. Colposcopy was performed for 462 participants: 123 (123/462, 26.6 %) had negative colposcopy findings, 231 (231/462, 50.0 %) had minor colposcopy findings, 103 (103/462, 22.3 %) had major colposcopy findings and six (5/462, 1.1 %) had suspicion of cancer. Median age was higher in women without CIN (median age = 38; IQR 31–48) compared to CIN2 (median age = 34; IQR 30–41) or CIN3+ (median age = 33; IQR 30–40) ($P < 0.001$). Significantly more CIN2+ and CIN3+ cases were recruited in Milan than in the three other participating colposcopy clinics ($P < 0.001$). Characteristics of the study population are shown in Table 1.

Clinical accuracy of OncoPredict HPV QT assay

HPV testing with OncoPredict HPV QT assay was similarly sensitive for CIN2+ and CIN3+ on both urine and vaginal samples compared to cervical samples when manufacturer's cut-off were applied. Relative sensitivity was 0.99 [95 % CI 0.94–1.05] and 0.98 [95 % CI 0.91–1.06] for CIN2+ and CIN3+, respectively, on urine versus cervical samples. The relative sensitivity for CIN2+ on vaginal versus cervical samples was 1.00 [95 % CI 0.96–1.04] and for CIN3+ was 1.00 [95 % CI NA]. However, the specificity for <CIN2 was significantly lower on both urine

(ratio = 0.91 [95 % CI 0.84–0.98], $P = 0.02$ and vaginal (ratio = 0.90 [95 % CI 0.84–0.98], $P = 0.01$) self-samples relative to cervical specimens (Table 2).

Following a posterior cut-off optimisation specificity improved to 0.94 [95 % CI 0.87–1.01] for urine and 0.94 [95 % CI 0.87–1.01] for vaginal samples, while relative sensitivity remained the same for vaginal samples (ratio = 1.00 [95 % CI 0.96–1.04]) and decreased by 0.02 for urine samples (ratio = 0.97 [95 % CI 0.90–1.03]) (Table 2). Absolute accuracy for the whole study population and for women 30 years and older is reported in Supplementary Tables S1 and S2. Relative accuracy for the study group of 30 years and older women resembled the accuracy of the whole study population (Supplementary Table S3).

Sample cellularity and HPV viral load

Median number of cells was higher in cervical samples (median = 3576 cells/reaction) compared to urine (median = 2540 cells/reaction), but lower than in vaginal samples (median = 41,347 cells/reaction) ($P < 0.001$) (Fig. 2).

Since, the amount of targeted DNA is inversely correlated with Ct-values, as expected, median Ct-values for overall hrHPV types and for HPV16, 18, 33, 35, 45, and CCR5 gene were lower in cervical compared to urine samples (Fig. 3 and Supplementary Table S4). However, median Ct-values in vaginal samples were lower than in cervical samples considering all hrHPV types and individually HPV31, 51, 56 and CCR5 gene, indicating higher amounts of DNA in vaginal samples (Fig. 3 and Supplementary Table S5). Comparison between urine and vaginal samples showed lower median Ct-values in vaginal samples for all hrHPV genotypes and for CCR5 gene (Fig. 3 and Supplementary Table S6).

On the contrary, from the analysis of normalized viral load (viral copies/ 10^4 cells), median viral loads for overall hrHPV types and for HPV16, 18, 31, 33, 35, 45, 51, 52 and 58 were significantly higher in cervical samples compared to urine (Fig. 4 and Supplementary Table S7). Moreover, the median viral loads of overall hrHPV and HPV16, 18, 31, 33, 35, 39, 45, 51, 56 and 58 were also significantly higher in cervical compared to vaginal samples (Fig. 4 and Supplementary Table S8). In general, no differences (except for HPV35) were observed in median viral loads between urine and vaginal samples (Supplementary Table S9).

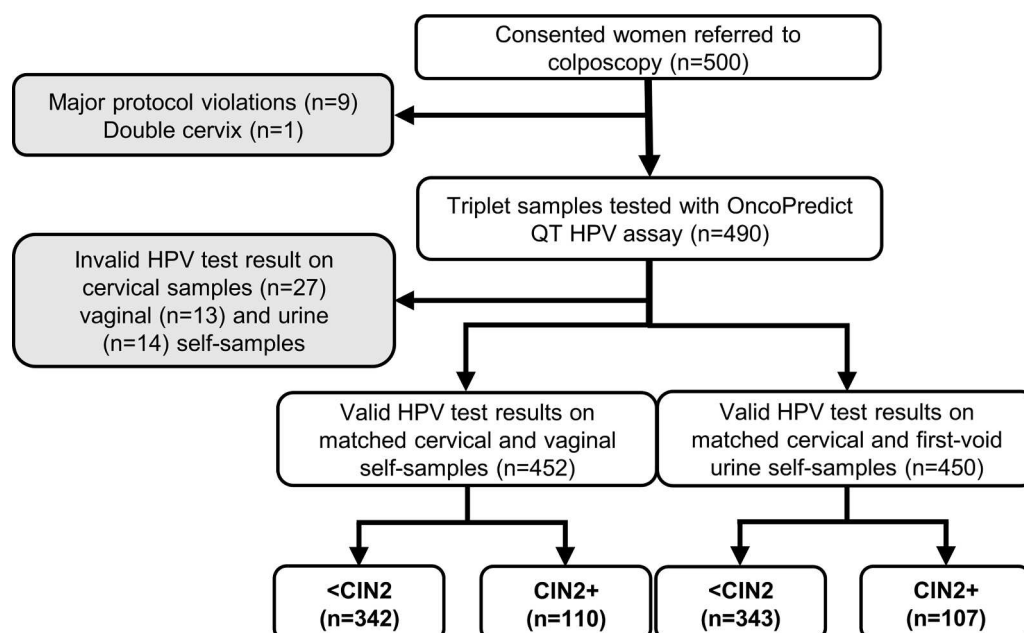


Fig. 1. Flow chart of samples included in the European VALHUDES trial tested with the OncoPredict HPV QT assay. Grey boxes represent excluded samples.

Table 1
Study population characteristics.

Age category (years)	Participants N (%)	Cervical hrHPV	Vaginal hrHPV	Urine hrHPV	Disease outcome		
		Pos N (%)	Pos N (%)	Pos N (%)	≤CIN1 N (%)	≥CIN2 N (%)	≥CIN3 N (%)
<30	91 (19.7)	66 (25.2)	69 (24.8)	68 (25.0)	68 (19.3)	23 (20.9)	14 (20.3)
30–39	174 (37.6)	106 (40.5)	109 (39.2)	102 (37.5)	118 (33.4)	56 (50.9)	37 (53.6)
40–49	115 (24.8)	54 (20.6)	60 (21.6)	63 (23.2)	93 (26.4)	22 (20.0)	14 (20.3)
50–59	69 (14.9)	31 (11.8)	34 (12.2)	34 (12.5)	61 (17.3)	8 (7.3)	3 (4.4)
60+	14 (3.0)	5 (1.91)	6 (2.2)	5 (1.8)	13 (3.7)	1 (0.9)	1 (1.5)
Total	463 (100.0)	262 (100.0)	278 (100.0)	272 (100.0)	353 (100.0)	112 (100.0)	69 (100.0)
Colposcopy centre	Participants N (%)	Pos N (%)	Pos N (%)	Pos N (%)	≤CIN1 N (%)	≥CIN2 N (%)	≥CIN3 N (%)
Edinburgh	186 (40.2)	102 (38.9)	105 (37.8)	101 (37.1)	149 (42.2)	37 (33.6)	27 (39.1)
Brescia	47 (10.2)	19 (7.3)	23 (8.3)	23 (8.5)	42 (11.9)	5 (4.6)	0 (0.0)
Milan	137 (29.6)	98 (37.4)	102 (36.7)	101 (37.1)	76 (21.5)	61 (55.5)	40 (58.0)
Sassari	93 (20.1)	43 (16.4)	48 (17.3)	47 (17.3)	86 (24.6)	7 (6.4)	2 (2.9)

hrHPV, high-risk HPV; CIN, cervical intraepithelial neoplasia, Pos, HPV positivity.
Supplementary Fig. 1 shows number of overlapping HPV positive cases stratified by age category.

Table 2
Relative accuracy of OncoPredict HPV QT assay on cervical versus self-samples (urine or vaginal), observed in the whole study population.

	CIN2+sensitivity [95 %CI]	CIN3+sensitivity [95 %CI]	<CIN2 specificity [95 %CI]
<i>Manufacturer cut-offs¹</i>			
Urine	0.99 [0.94–1.05]	0.98 [0.91–1.06]	0.91 [0.84–0.98]
Vaginal	1.00 [0.96–1.04]	1.00 [NA]	0.90 [0.84–0.98]
<i>New cut-offs²</i>			
Urine	0.97 [0.90–1.03]	0.97 [0.89–1.05]	0.94 [0.87–1.01]
Vaginal	1.00 [0.96–1.04]	1.00 [NA]	0.94 [0.87–1.01]

CI, confidence interval; CIN, cervical intraepithelial neoplasia;
¹ Manufacturer’s HPV positivity threshold for cervical samples: hrHPV ≥ 50 copies/10⁴ cells; for urine or vaginal self-samples: hrHPV ≥ 10 copies/10⁴ cells.
² New a posteriori defined cut-offs for vaginal and urine samples at ≥15 copy/10⁴ cells for all types.
Relative sensitivity and specificity for women ≥ 30 years old are shown in Supplementary Table S3.

HPV genotype-specific concordance and normalized viral load correlation between specimens

Good and excellent concordance with Kappa values between 0.68 and 0.93 was observed between both self- and cervical specimens

(Supplementary Tables S10 and S11).
Supplementary Fig. S2 shows a correlation matrix of 630 total unique possible associations of the 12 individual hrHPV genotypes across three specimen types using normalised viral load. A significant positive normalised viral load correlation was observed across all three specimen types (cervical, urine and vaginal) for the 21 pairs of seven genotypes HPV16, 18, 31, 33, 45, 51 and 56, with correlation coefficients ranging from 0.49 to 0.92 ($P < 0.05$). For the remaining 5 individual genotypes (15 pairs) correlation was significant only for seven pairs. Of the remaining 594 unique possible associations across different HPV genotypes, 25 significantly positive and 18 inverse correlations were identified after BH false discovery correction (Supplementary Fig. S2, Supplementary Tables S12 and S13) ($P < 0.05$). For instance, HPV16 infections were positively correlated with HPV18 and HPV45, but inversely correlated with HPV39, HPV58 and HPV59 ($P < 0.05$).

Discussion

In this study, the performance of OncoPredict HPV QT genotyping assay, which incorporates normalized genotype-specific quantitative detection of 12 hrHPV, was evaluated on vaginal and urine self-samples. This is the first study to evaluate the clinical performance of OncoPredict HPV QT test on self-samples and the first, we believe, to detail performance of an assay which detects viral copy number in the context of self-

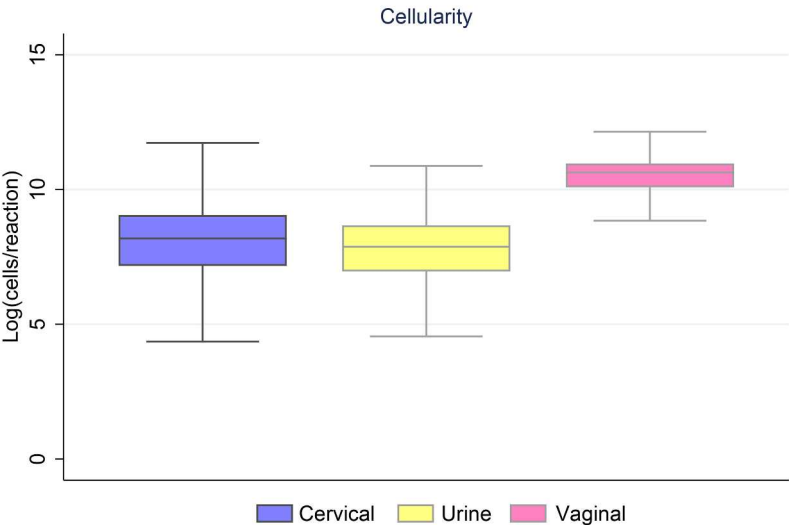


Fig. 2. Median log (cells/reaction) for all sample types. Boxplots indicate median log (cells/reaction) interquartile ranges, and extreme values (whiskers).

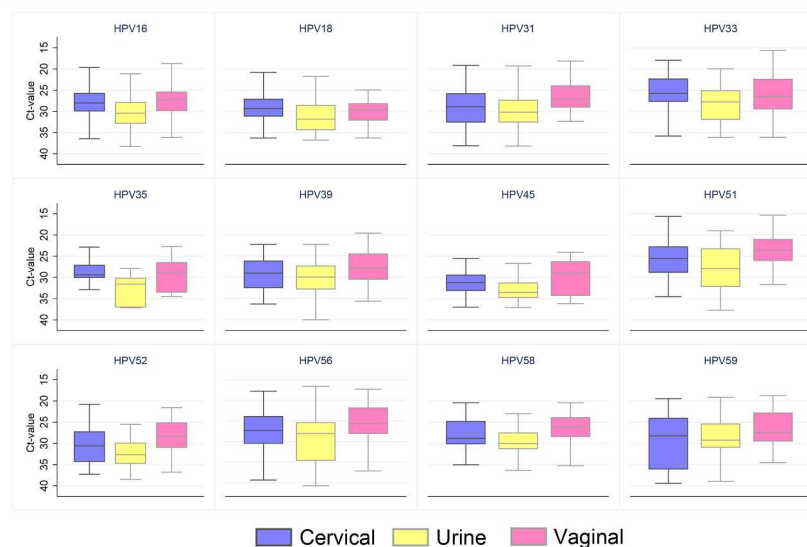


Fig. 3. Individual HPV median Ct-values for all sample types. In case of multiple infections, the highest viral load was considered. Boxplots indicate median Ct-values, interquartile ranges, and extreme values (whiskers). Note: Y-axis has been reversed so that lowest Ct values (corresponding with the highest amount of amplified HPV) are on top.

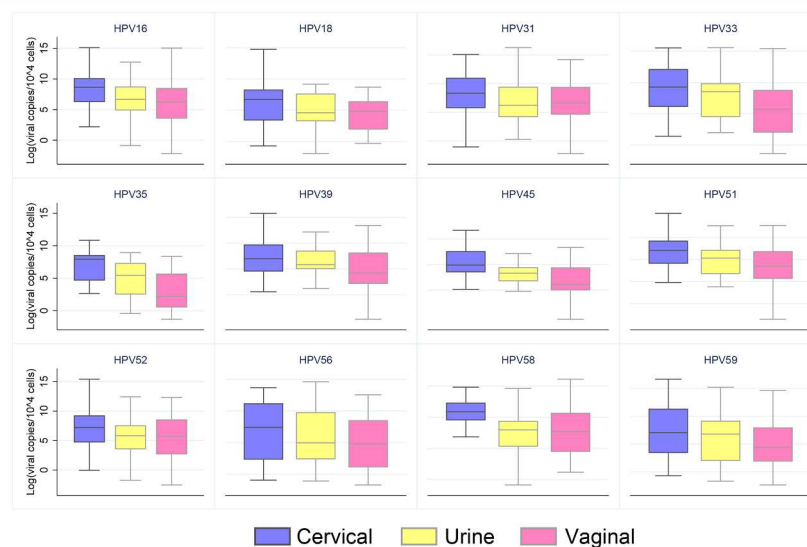


Fig. 4. Individual HPV median log (viral copies/ 10^4 cells) for all sample types. In case of multiple infections, the highest viral load was considered. Boxplots indicate median log (viral copies/ 10^4 cells), interquartile ranges, and extreme values (whiskers).

taken samples. In addition, the OncoPredict HPV QT assay is unique as compared to other more established HPV assays used for cervical screening in that it targets only the twelve HPV genotypes considered by IARC as carcinogenic.

The OncoPredict HPV QT test demonstrated similar sensitivity for precancerous lesions on both urine and vaginal samples compared to cervical samples, whereas specificity was lower in both types of self-collected samples, using manufacturer's cut-offs. Following cut-off optimisation specificity improved without compromising sensitivity on both types of self-collected specimens. Lower specificity on vaginal samples may be explained by the resuspension of the dry vaginal device in a small volume (5 mL) [6]. Consistent with our findings, previous studies have reported lower specificity when dry devices were placed in

smaller media volumes [6,12,20]. In the Belgian VALHUDES project, the Alinity HPV assay was evaluated using Abbott multi-Collect swab resuspended in 2.5 mL of Abbott Cerci-Collect buffer [12]. In another validation study, BD HPV test was evaluated in combination with FLOQSwab resuspended in 3 mL of BD HPV self-collection diluent [20]. In both studies, sensitivity of the HPV assay on self-samples was similar to that on the cervical sample, whereas specificity was lower. The lower resuspension volumes may generate a more concentrated sample, which could lead to increased HPV detection and, therefore, lower specificity. Moreover, reduced specificity in both the urine and vaginal self-samples may also result from the higher rates of hrHPV infections in the lower vaginal tract not associated with CIN2+ as compared to the cervix [21, 22]. Nevertheless, an optimal balance between specificity and

sensitivity has been made possible through cut-off optimisation in both the present and previous reports [12,20].

To date four other HPV assays have been validated within the VALHUDES framework (Alinity m HR HPV, BD Onclarity HPV, RealTime High Risk HPV and Xpert HPV); of these Alinity m HR HPV and RealTime High Risk HPV also required cut-off optimisation [11–16]. A posterior cut-off optimisation is often required specifically when evaluating assays on self-samples even if those assays have been previously validated and used on cervical samples. Self-samples are collected from different gynaecological niches and often require different laboratory workflows as compared to clinician-collected samples. Given these differences, manufacturers of HPV assays should adapt their assays' cut-offs for self-samples based on the results of the relative validation studies. Or, for new products, consider and optimise specifications with self-samples in mind from the outset.

In the European VALHUDES project, the medium used for the resuspension of vaginal swabs was eNAT, whereas in the Belgian VALHUDES ThinPrep was used. eNAT is an alcohol-free medium, characterized by its capacity to preserve nucleic acids and inactivate microbial agents by cell lysis, which represents an important cost-effective alternative for self-samples resuspension in low-resource settings [6,23–25].

In general, this study showed a wide range of cellularity within the different sample types collected from participating women, pointing to potential differences in performing sample collection. The quantitative evaluation of sample cellularity by a single copy human gene as well as the inclusion of nucleic acids extraction and PCR amplification controls represent a unique feature of the OncoPredict HPV QT assay, providing a level of increased diagnostic confidence in reporting negative results. This is particularly important when evaluating sample adequacy on testing self-collected samples [26]. Presently, a major limitation of most commercially available HPV tests is either the lack of an internal quality control or the use of human controls that do not accurately assess sample cellularity [27,28]. The extensive quality control of the OncoPredict HPV assay has probably resulted in higher invalidity rates, which could be challenging in a real-world cervical cancer screening scenario as a significant number of women may need to be recalled to the clinic to collect another self-sample. Nevertheless, this strict quality control ensures that the valid samples are truly negative, reducing the risk of missing an underlying lesion.

In the present study, higher median viral loads were observed in cervical compared to both self-sample types when viral load was normalised by cellularity. However, Ct-value comparison revealed that median HPV Ct-values were highest in urine, followed by cervical and lowest in vaginal samples. As the Ct-value is inversely correlated with DNA concentration, this suggests a higher amount of viral DNA in vaginal samples before normalization compared to urine or cervical samples. Such differences could be expected, especially given the tenfold difference in cellularity observed in vaginal versus urine or cervical specimens, due in part to the different resuspension volumes used for vaginal and cervical samples. Although Ct-values reflect the initial quantity of viral DNA template in the reaction, it is important to underline that the measurement of viral load is uncertain without a sample's reference mass/volume unit. This is particularly the case for samples collected from mucosal surfaces where an intrinsic variability in cellularity can occur due to differences in the operator's collection technique or the patient's tolerance during the sampling process [29,30]. Moreover, viral load normalization based on sample cellularity can also account for preanalytical variables such as sample-collection, resuspension volumes as well as different nucleic acid extraction and amplification systems, particularly important for vaginal samples. Normalisation by cellularity could help mitigate these differences and provide more consistent results across various sample types. Furthermore, the normalization process in self-samples may also play a role in detecting clinically relevant infections. In order to demonstrate the potential advantages of viral load normalisation, normalised viral load and non-normalised Ct-values of HPV16 positive samples were compared to

identify CIN2+ cases. On applying a threshold of 50 copies/ 10^4 cells normalised viral load for HPV16 positivity on cervical samples versus HPV16 positivity with Ct-value threshold of 32.95, which corresponds to 50 copies per reaction non-normalised viral load, three additional CIN2+ cases were identified (Supplementary Fig. S3). Similarly, on self-samples, 15 viral copies/ 10^4 cells threshold for normalised viral load resulted in detection of four additional CIN2+ cases versus 34.51 Ct-value cut-off, corresponding to non-normalised 15 copies per reaction. Differences in the amount of DNA quantities have been previously reported with the highest viral signals in the upper genital tract decreasing towards the lower genital tract [21]. Moreover, cervical samples are collected by a physician from the transformation zone, where HPV infection and replication occur, while vaginal samples are collected from the lower vaginal environment by women themselves. In terms of urine collection, it contains cervical exfoliated epithelial cells, and free viral particles, accumulated between two urinations and washed out with the first urine stream. Therefore, it is not unexpected to observe these differences in normalised and non-normalised viral signals across different specimen types.

Additionally, we explored the correlation between individual HPV types within urine, vaginal and cervical specimens, as well as across different HPV types. Given that HPV concordance is estimated based on qualitative data, we aimed to explore alternative metrics using quantitative normalised viral load. Spearman's correlation was applied to evaluate these associations which could be considered as an indicator of viral load concordance [31]. For example, the kappa concordance between cervical and vaginal samples for HPV16 was 0.90, indicating high agreement, while the correlation coefficient for normalised viral load between these specimens was 0.60. On the other hand, inverse and forward correlation was identified between different genotypes suggesting competition or co-occurrence. For instance, inverse HPV16 and HPV58 correlation was present in our study, which was previously reported by Mejlhede *et al.*, who employed a more complex model with OR as an indicator of co-occurrence or competition [32]. Such correlation studies could be useful in identifying HPV interactions in vaccinated and non-vaccinated populations. However, it is important to acknowledge that our study did not account for multiple infections, which could potentially introduce bias into our results.

In conclusion, this VALHUDES study demonstrated that HPV testing with OncoPredict HPV QT assay on self-vaginal and first-void urine samples, collected with FLOQSwab and Colli-Pee respectively, is as sensitive but less specific for precancerous lesions compared to cervical samples. Post-hoc cut-off optimisation enhanced specificity on self-samples without compromising sensitivity.

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CRediT authorship contribution statement

Ardashel Latsuzbaia: Writing – review & editing, Writing – original draft, Visualization, Formal analysis. **Marianna Martinelli:** Writing – review & editing, Methodology, Data curation. **Chiara Giubbi:** Writing – review & editing, Methodology. **Kate Cuschieri:** Writing – review & editing. **Hana Elasifer:** Writing – review & editing. **Anna D. Iacobone:** Writing – review & editing, Methodology, Data curation. **Fabio Bottari:** Writing – review & editing, Methodology. **Andrea F. Piana:** Writing – review & editing, Methodology. **Roberto Pietri:** Writing – review & editing, Methodology. **Giancarlo Tisi:** Writing – review & editing. **Franco Odicino:** Writing – review & editing. **Clementina E. Cocuzza:** Writing – review & editing, Methodology, Resources, Supervision,

Project administration, Funding acquisition, Conceptualization. **Marc Arbyn**: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

The European VALHUDES is an investigator initiated study, coordinated by University of Milano-Bicocca (Milan, Italy), Sciensano (Bruxelles, Belgium), Istituto Europeo di Oncologia (Milan, Italy), University of Sassari (Sassari, Italy), U.O. Coordinamento Consultori Familiari, ASSL Sassari – ATS Sardegna (Sassari, Italy), NHS Lothian, University of Edinburgh (Edinburgh, Scotland), Trinity College Dublin (Dublin, Ireland).

Manufacturers of HPV assays (GeneFirst, Oxford, UK and Hiantis, Milan, Italy) and devices (Copan Italia Spa, Brescia, Italy and Novosanis, Belgium) participated in the European VALHUDES framework contributing equipment for laboratory testing under the condition of accepting independent publication of results. Funding received from the companies are managed by the directors of the respective collaborating institutions. Sciensano authors do not have any personal or material conflict of interest.

The study group received free self-sample collection devices from Copan Italia Spa (Brescia, Italy) and Novosanis (Belgium) and free OncoPredict HPV assay from (Hiantis, Milan, Italy).

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Copan Italia Spa (Brescia, Italy) has provided for free FLOQSwab devices and eNAT solution. Novosanis (Wijnegem, Belgium) has provided free Colli-Pee devices. Hiantis (Milan, Italy) has provided free kits and reagents for HPV testing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2024.105737.

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RESEARCH ARTICLE OPEN ACCESS

Clinical Performance of OncoPredict HPV Screening Assay on Self-Collected Vaginal and Urine Specimens Within the VALHUDES Framework

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ABSTRACT

The introduction of self-sampling in cervical cancer screening has raised the importance of HPV test validation on self-collected samples. This study aimed to evaluate the clinical accuracy of the OncoPredict HPV Screening (SCR) assay on self-collected vaginal and first-void urine (FVU) samples, relative to cervical specimens, using the VALHUDES Framework. FVU and vaginal self-samples followed by a clinician-collected cervical brushing were collected from 500 women referred to colposcopy and tested using OncoPredict HPV SCR assay. The assay demonstrated clinical sensitivity to detect cervical intraepithelial neoplasia grade 2 or worse ($\geq$ CIN2) similar to cervical samples in FVU (ratio: 0.95, [95% CI: 0.88–1.02]) and vaginal self-samples (ratio: 0.96 [95% CI: 0.90–1.02]). The clinical specificity for $<$ CIN2 was lower in vaginal (ratio: 0.90 [95% CI: 0.84–0.96]) but not in FVU samples (ratio: 1.03 [95% CI: 0.96–1.12]) when compared to cervical samples. However, the relative specificity improved following cut-off optimization (ratio: 0.94, 95% CI: [0.88–1.01]). Moderate to excellent agreement in HPV detection between self-collected and cervical samples was demonstrated (Kappa values: 0.53–1.00). To conclude, OncoPredict HPV SCR assay demonstrated similar accuracy on FVU and cervical samples. On vaginal compared to cervical samples sensitivity was similar with a lower specificity, which improved with cut-off optimization.

1 | Introduction

Women who do not participate to cervical cancer screening programs have the highest risk of developing cervical cancer.

For this reason a key target of the World Health Organization (WHO) Call to Action is to achieve the participation of at least 70% of eligible women to cervical cancer screening using a high-performance test by the age of 35, and again by the age of 45, in

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support of the elimination of cervical cancer by 2030 [1]. Human Papillomavirus (HPV) testing has been demonstrated to be more effective than cytology for the secondary prevention of cervical cancer [2, 3] and is therefore recommended as a primary screening tool in current screening algorithms [4]. Additionally, meta-analyses have shown that the clinical accuracy of PCR-based HPV tests on self-samples can be similar to that on cervical samples. Furthermore, offering self-samples could increase participation of women to cervical cancer screening [5, 6].

In 2021, 48 countries worldwide recommended primary HPV-based screening and 17 introduced self-sampling in their national programs or guidelines [7] as response to the WHO Call to Action [1].

While several HPV tests are currently validated for use in cervical cancer screening [8] according to the VALGENT Framework and Meijer Guidelines [9, 10], only a few are formally validated for their use on self-collected specimens. The VALidation of HUman papillomavirus assays and collection Devices for Self-samples and urine samples (VALHUDES) Framework has defined a standardized protocol to assess the clinical performance of HPV tests in combination with self-collection devices [11], to evaluate HPV tests previously validated on clinician-collected cervical samples. Results of a first installment of VALHUDES demonstrated similar accuracy of first void urine (FVU) and vaginal self-collected specimens compared to clinician-collected cervical samples of several PCR-based HPV assays in combination with different sample collection devices [12–17]. This study reports on a second iteration of VALHUDES undertaken in a different geographic setting and utilizing a different approach to vaginal sampling.

The OncoPredict HPV Screening (SCR) assay, previously validated for its use in cervical cancer screening on clinician-taken cervical samples [18], is a partial genotyping assay able to detect HPV-16, and HPV-18 individually and 11 “other” hrHPV genotypes (HPV-31, -33, -35, -39, -45, -51, -52, -56, -58, -59, and -68) as a pool. Moreover, an innovative feature of this assay is that it includes an accurate sample adequacy control, particularly important in the quality assurance of testing samples which have been self-collected. The present study aimed to evaluate the clinical performance of the OncoPredict HPV SCR assay on vaginal self-samples collected using FLOQSwab, transported dry and subsequently resuspended in 5 mL eNAT, and on FVU, collected using the Colli-Pee device, as compared to clinician-collected cervical scrapes to detect high-grade cervical lesions. Secondly, we investigated the analytical performance of the assay and evaluated the adequacy of self-collected samples.

2 | Materials and Methods

2.1 | Study Design

The European VALHUDES study was performed in compliance with ISO 20916:2019 (In vitro diagnostic medical devices—Clinical performance studies using specimens from human subjects—Good study practice) and reported in ClinicalTrials.gov Register (NCT04312737).

Within the European VALHUDES Framework, 600 women, referred to colposcopy following a previous cervical abnormality or HPV positivity, were enrolled between July 2020 and February 2022 in four colposcopy centers (NHS Lothian, Edinburgh; ASST degli Spedali Civili di Brescia, Brescia, Italy; European Institute of Oncology IRCCS, Milan, Italy; U.O. Coordinamento Consultori Familiari, ASSL Sassari - ATS Sardegna, Sassari, Italy). Exclusion criteria have already been described [19].

All women were asked to self-collect a urine sample followed by a vaginal specimen. FVU was collected using Colli-Pee FV5000 (Novosanis, Wijnegem, Belgium). The device captures approximately 13 mL of FVU that are mixed with 7 mL nucleic acid preservative included within the collection device. Vaginal self-collection was performed using a FLOQSwab (Copan Italia Spa, Brescia, Italy). During gynecological examination, a cervical specimen was collected by a clinician with Cervex-Brush (Rovers Medical Devices, Oss, The Netherlands) and immediately transferred in 20 mL PreservCyt (Hologic Inc., Bedford, Massachusetts, USA).

All women underwent colposcopy and biopsy was performed only if clinically required, according to the local protocols. The histological result of the biopsy was used to determine the disease outcome: lesions were classified as Cervical Intraepithelial Neoplasia grade 0 (CIN0), CIN1, CIN2, CIN3 and Cervical Cancer [20]. Cervical intraepithelial neoplasia grade 2 or worse ($\geq$ CIN2) were considered as high-grade lesions, while lesions $<$ CIN2 were considered low-grade lesions.

Self-collected vaginal samples were transported dry to the laboratory together with the 20 mL PreservCyt vial containing cervical samples and the Colli-Pee tube containing FVU. All specimens were transported to the laboratories affiliated with the enrolling colposcopy centers. After arrival in the laboratories, cervical and FVU specimens were shaken for 30 s and divided into 1.5 mL aliquots. The dry vaginal swabs were resuspended in 5 mL of PreservCyt (Hologic Inc., Bedford, Massachusetts, USA) for the first 100 women enrolled in the study; subsequently vaginal samples collected by the remaining 500 women were resuspended in 5 mL eNAT (Copan Italia Spa, Brescia, Italy). Vaginal samples were further aliquoted into 0.4 mL volumes. All aliquots were stored at -20°C until transferred to MIRRI-IT Biobank of the University of Milano-Bicocca where they were stored at -80°C . Results reported in this manuscript are referred to those 500 women whose vaginal swabs were resuspended in 5 mL eNAT.

2.2 | HPV Testing

Testing of all specimens was performed at the Laboratory Clinical Microbiology and Virology, School of Medicine and Surgery, University of Milano-Bicocca (Monza, Italy). Nucleic acid extraction was performed using a Fluent 480 (Tecan, Männedorf, Switzerland) automated platform with Quick-DNA/RNA MagBead (Zymo, USA) starting from 400 μL of

sample. Fluent 480 workstation was also used to set-up the real-time PCR plate of OncoPredict HPV SCR assay (Hiantis, Milan, Italy) according to manufacturer instructions using 10 μ L of mastermix and 5 μ L of sample's DNA extract. The OncoPredict HPV SCR assay, previously validated for testing on cervical scrapes in a screening setting [18], is a partial genotyping assay targeting E6 and E7 DNA sequences of 13 high-risk human papillomavirus (hrHPV) types (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, and -68). The test is composed of two separate real-time PCR reactions. A quality control reaction (QC) well which allows the assessment of nucleic acid extraction recovery, by means of an exogenous control gene target added to the sample before preanalytical processing, as well as adequacy of sample collection by the determination of human cellularity using *C-C Motif Chemokine Receptor 5* (CCR5) gene [21]. The second reaction well is used to assess the presence of HPV16, HPV18 individually and 11 "other" hrHPV types as a pool. Both wells also include an exogenous amplification control to evaluate potential PCR inhibition. PCR was carried out using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, USA). All results were considered valid if HPV positivity signal was reported. In case of HPV negative result(s) samples were defined as inadequate if (i) the extraction efficiency was below 10%; (ii) less than 400 cells/reaction in cervical samples [18] or 150 cells/reaction in urine and vaginal samples were detected and (iii) there was PCR inhibition in any of the two reaction wells.

PCR cycle threshold (Ct) interpretation was performed with the support of Hiantis' Reader, an Artificial Intelligence-based reading software (CE Marked), developed for Hiantis by Hi-Future, Teoresi Group Company (Italy).

Manufacturer's cut-off for all hrHPV types in cervical samples and vaginal self-samples was $Ct \leq 40$, in FVU $Ct \leq 44$. New a posteriori hrHPV positivity cut-offs for vaginal self-samples were $Ct \leq 39$ for HPV16, $Ct \leq 37$ for HPV18 and $Ct \leq 38$ for "other" hrHPV.

2.3 | Statistical Analysis

Clinical sensitivity was estimated for cervical intraepithelial neoplasia grade 2 or worse ($\geq$ CIN2) and for cervical intraepithelial neoplasia grade 3 or worse ($\geq$ CIN3). Specificity was estimated for < CIN2 outcome or by accepting negative colposcopy as clinical endpoint when biopsy was not taken based on the colposcopist's clinical judgment. McNemar tests was used to evaluate the accuracy differences between index and comparator tests with statistical significance accepted if $p < 0.05$ or when the 95% confidence intervals excluded 1. Cohen's kappa was employed to assess HPV test concordance between self- and clinician-taken samples for the entire study population and according to disease status among specimens, categorized as: poor (0.00–0.19), fair (0.20–0.39), moderate (0.40–0.59), good (0.60–0.79), and excellent (0.80–1.00). Mann-Whitney test was used to evaluate differences in median Ct-values and median number of cells/reaction. All statistical analyses were conducted using Stata 16.1 (Statacorp, College Station, TX, USA).

2.4 | Ethical Approval

The European VALHUDES study (ClinicalTrail.gov: NCT04312737) was conducted in accordance with the Declaration of Helsinki and approved by the central Ethics Committee of the Coordinating Centre, ASST degli Spedali Civili di Brescia, Brescia, Italy (Ethics approval number: NP 3879- Studio WP6-HPVONC) on the 16th of July 2020, and subsequently by the local Ethics Committees of the other participating centers. All women signed a written informed consent form before enrollment.

Consorzio Italiano per la Ricerca in Medicina (C.I.R.M.), Milano, Italy, performed on site and remote monitoring of the study conduction, as previously described [19].

3 | Results

3.1 | Study Population

490 out of the 500 women were included in the study as reported in Figure 1. The median age of the women included in the study was 37 years (IQR: 31–47 years, range: 25–64 years). Median age of women with $\geq$ CIN2 lesions was significantly lower than those with < CIN2, as previously described [19]. 489 women had colposcopy with the following outcomes: 134 (27.4%) negative, 245 (50.1%) minor colposcopy findings, 104 (21.3%) major colposcopy and 6 (1.2%) suspicion of cancer. 55% (271/490) of women underwent biopsy and diagnosis of $\geq$ CIN2 was confirmed in 41.3% (112/271) of cases. Table 1 reports the characteristics of the study population by age group and colposcopy center. 28 cervical specimens, 16 vaginal swabs and 19 FVU samples were excluded from the analysis because they were inadequate and HPV-negative (Figure 1). Finally, the number of matched cervical and vaginal samples available from the same woman was 449, while the number of matched cervical specimens and urine from the same woman was 447 as reported in Figure 1.

3.2 | Sample's Adequacy

All hrHPV-positive samples were considered valid. 5.7% (28/490) cervical, 3.3% (16/490) vaginal and 3.9% (19/490) FVU hrHPV-negative specimens were inadequate. Most invalid cervical (23/28) and FVU (12/19) samples resulted from a low cellularity, while only 3 vaginal samples showed cellularity below the cut-off. Invalidity in this group of samples was mainly related to extraction efficiency (13/16). As shown in Table 2, the cellularity of vaginal self-collected specimens (resuspended into 5 mL of eNAT) was demonstrated to be more than 10-fold higher than that of cervical (resuspended into 20 mL PreservCyt) and FVU samples.

3.3 | Clinical Accuracy of OncoPredict HPV SCR Assay

Clinical sensitivity for the detection of $\geq$ CIN2 and $\geq$ CIN3 and specificity for the detection of < CIN2 of OncoPredict HPV SCR assay on self-collected samples relative to cervical scrapes are reported in Table 3. Using manufacturer's cut-offs, the absolute

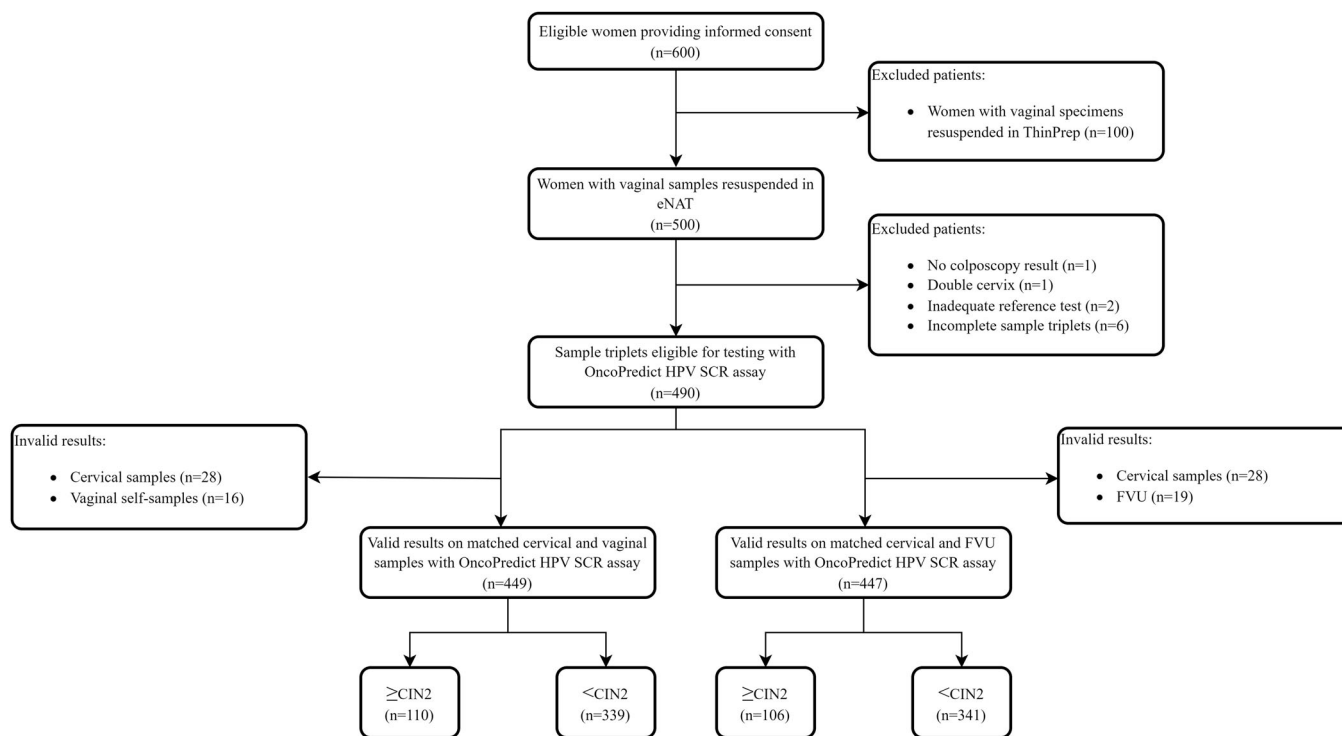


FIGURE 1 | Flowchart of samples included in the analysis for the accuracy of OncoPredict HPV SCR assay within the VALHUDES Framework.

TABLE 1 | Disease outcomes by biopsy and/or colposcopy findings divided by age group and colposcopy center.

Age group (years)	Participants (n (%))	Disease outcome		
		≥ CIN2 (n (%))	≥ CIN3 (n (%))	< CIN2 (n (%))*
< 30	93 (19.0)	23 (20.5)	14 (20.0)	70 (18.5)
≥ 30	397 (81.0)	89 (79.5)	56 (80.0)	308 (81.5)
Total	490 (100.0)	112 (100.0)	70 (100.0)	378 (100%)

Colposcopy center	Participants (n (%))	Disease outcome		
		≥ CIN2 (n (%))	≥ CIN3 (n (%))	< CIN2 (n (%))*
Edinburgh	191 (39.0)	37 (33.0)	27 (38.6)	154 (40.8)
Brescia	49 (10.0)	5 (4.5)	0 (0.0)	44 (11.6)
Milan	150 (30.6)	63 (56.2)	41 (58.6)	87 (23.0)
Sassari	100 (20.4)	7 (6.3)	2 (2.8)	93 (24.6)
Total	490 (100.0)	112 (100.0)	70 (100.0)	378 (100%)

Abbreviation: CIN, cervical intraepithelial neoplasia.

*217 cases were classified as < CIN2 based on colposcopy findings without biopsy.

sensitivity for the detection of $\geq$ CIN2 lesions was 87% (95% CI: 80%–93%) on cervical samples, 82% (95% CI: 74%–89%) on vaginal self-samples and 81% (95% CI: 72%–88%) on FVU. Absolute specificity for < CIN2 detection were respectively 54% (95% CI: 49%–60%), 50% (95% CI: 44%–55%) and 57% (95% CI: 52%–62%). Absolute clinical sensitivity and specificity results are reported in Supporting Information S1: Table 1. Clinical sensitivity of OncoPredict HPV SCR assay for $\geq$ CIN2 on matched FVU and vaginal samples was not different to that of cervical specimens (relative sensitivity FVU/cervical sample = 0.95 [95% CI: 0.88–1.02]) (relative sensitivity vaginal self-sample/cervical specimen = 0.96 [95% CI: 0.90–1.02]). Specificity for < CIN2 on FVU was similar to cervical scrapes (ratio = 1.03 [95% CI: 0.96–1.12]), whereas specificity on vaginal samples was slightly lower (ratio = 0.90 [95% CI: 0.84–0.96]).

After cut-off adjustment, the relative specificity on vaginal samples improved (ratio = 0.94 [95% CI: 0.88–1.01]). Similar clinical accuracy estimates were observed when restricting the analysis for women of 30 years or older as reported in Supporting Information S1: Table 2.

3.4 | hrHPV Positivity and Concordance

Out of 449 women with matched cervical and vaginal specimens, 256 (57.0%) cervical and 270 (60.1%) vaginal specimens were hrHPV-positive. Out of 447 matched cervical and FVU samples, 250 (55.9%) cervical swabs and 256 (57.3%) FVU tested hrHPV-positive.

TABLE 2 | Median values of cellularity (cells/reaction) across different types of samples.

	Matched cervical and vaginal specimens (<i>n</i> = 449)*		Matched cervical and FVU specimens (<i>n</i> = 447)*	
	Clinician- collected cervical samples	Vaginal self-collected samples	Clinician-collected cervical samples	FVU self-collected samples
Median cellularity (IQR)	3875 (1469–8956)	42049 (25 823–59 300)	3925 (1549–8902)	2879 (1220–6130)
				<i>p</i> value
				< 0.001
				< 0.001

*Invalid samples have been excluded from the analysis.

Moderate to excellent agreement with Kappa values ranging from 0.53 to 1.00 between both vaginal and FVU self-collected samples and cervical scrapes was demonstrated using manufacturer's cut-offs (Tables 4 and 5). In general, vaginal samples showed higher test agreement with cervical specimens than FVU. Similar concordance rates were observed between self-collected vaginal and clinician-collected cervical samples using new cut-offs as shown in Supporting Information S1: Table 3.

In matched cervical and vaginal hrHPV-positive samples, median Ct values were significantly lower in vaginal samples compared to cervical for 11 “other” hrHPV, but not for HPV16 or HPV18 (Supporting Information S1: Figure 1). In matched hrHPV-positive cervical and FVU samples, median Ct values were higher in FVU as compared to cervical samples. However, the difference was not significant for HPV18 (Supporting Information S1: Figure 2).

4 | Discussion

The introduction of self-sampling in cervical cancer screening programs, further enhanced by the COVID19 pandemics [22], is an important instrument to reach 70% screening coverage as proposed in by the WHO Call to Action [1]. A similar clinical accuracy of PCR-based HPV tests on self-samples and clinician-collected cervical scrapes has been demonstrated in previous validation studies for other assays based on the VALHUDES protocol [12–17].

The present study demonstrated that the use of OncoPredict HPV SCR assay on self-collected vaginal specimens using FLOQSwabs, resuspended in 5 mL of eNAT, and FVU collected using Colli-Pee FV5000 has a similar clinical accuracy to detect $\geq$ CIN2+ and $\geq$ CIN3 lesions as compared to testing clinician-collected cervical samples. Clinical sensitivity of OncoPredict HPV SCR assay on FVU and vaginal samples was similar to that on cervical specimens, however specificity on vaginal samples was lower when applying manufacturer cut-off values. Cut-off optimization on vaginal self-collected samples resulted in an improvement in specificity without compromising sensitivity. A lower specificity for the detection of < CIN2 as compared to cervical specimen was also reported for the validation of BD Onclarity HPV test on FLOQSwabs resuspended in 3 mL of BD HPV self-collection diluent [23]. On the other hand, in the Belgian VALHUDES, where vaginal samples were resuspended in 20 mL of PreservCyt, a posteriori cut-offs adjustment was necessary to improve the clinical sensitivity of some of the evaluated HPV assays, but was not required for specificity [13, 14].

Different preanalytical workflows of self-collected vaginal samples may affect the clinical accuracy of the test. Therefore, optimization and standardization of the procedures for handling and testing self-samples are fundamental to ensure an optimal performance of the assay [24]. Presently, the VALHUDES protocol has been developed to assess the performance of HPV tests in combination with self-collection devices [11]. In the European VALHUDES, vaginal swabs have been collected using FLOQSwabs resuspended in 5 mL of eNAT medium, while FVU were collected using a 20 mL Colli-Pee device. eNAT is a

TABLE 3 | Relative accuracy of OncoPredict HPV SCR assay on vaginal and FVU self-samples versus cervical specimens.

	Relative sensitivity [95% CI] for $\geq$ CIN2 detection	Relative sensitivity [95% CI] for $\geq$ CIN3 detection	Relative specificity [95% CI] for $<$ CIN2 detection
Manufacturer cut-offs^a			
Vaginal self-sample	0.96 [0.90–1.02]	0.95 [0.87–1.04]	0.90 [0.84–0.96]
FVU	0.95 [0.88–1.02]	0.93 [0.85–1.03]	1.03 [0.96–1.12]
New cut-offs^b			
Vaginal self-sample	0.95 [0.90–1.00]	0.93 [0.86–1.01]	0.94 [0.88–1.01]

Abbreviations: CI, confidence interval; CIN, cervical intraepithelial neoplasia.

^aManufacturer's positivity threshold for all hrHPV types in cervical samples and vaginal self-samples: Ct $\leq$ 40; in FVU: Ct $\leq$ 44.

^bNew a posteriori cut-offs vaginal self-sample: HPV16 Ct $\leq$ 39, HPV18 Ct $\leq$ 37, other hrHPV Ct $\leq$ 38.

TABLE 4 | Concordance between self-collected vaginal and clinician-collected cervical samples using manufacturer's cut-offs.

	HPV type	+/+	+/-	-/+	-/-	Agreement [%]	Kappa [95% CI]
Total population (n = 449)	hrHPV	241	15	29	164	90.2	0.798 (0.742–0.855)
	HPV16	60	7	10	372	96.2	0.854 (0.786–0.921)
	HPV18	13	2	4	430	98.7	0.806 (0.654–0.957)
	Other hrHPV	185	13	38	213	88.6	0.773 (0.714–0.831)
$\geq$ CIN2 (n = 110)	hrHPV	90	6	2	12	92.7	0.708 (0.519–0.897)
	HPV16	34	4	5	67	91.8	0.820 (0.708–0.933)
	HPV18	3	1	0	106	99.1	0.853 (0.568–1.000)
	Other hrHPV	64	4	5	37	91.8	0.826 (0.717–0.935)
$<$ CIN2 (n = 339)	hrHPV	151	9	27	152	89.4	0.788 (0.723–0.853)
	HPV16	26	3	5	305	97.7	0.854 (0.754–0.953)
	HPV18	10	1	4	324	98.5	0.792 (0.616–0.969)
	Other hrHPV	121	9	33	176	87.6	0.747 (0.677–0.818)

Note: Color legend: for the concordance: dark green ($1.00 \geq K > 0.80$): excellent; light green ($0.80 \geq K > 0.60$): good; yellow ($0.60 \geq K > 0.40$): moderate; orange ($0.40 \geq K > 0.20$): fair; red ($0.20 \geq K > 0.00$): poor. Manufacturer's positivity threshold for all hrHPV types in cervical samples and vaginal self-samples Ct $\leq$ 40. Abbreviations: 95% CI, 95% confidence interval; CIN, cervical intraepithelial neoplasia; N, number.

TABLE 5 | Concordance between FVU and clinician-collected cervical samples using manufacturer's cut-offs.

	HPV type	+/+	+/-	-/+	-/-	Agreement [%]	Kappa [95% CI]
Total population (n = 447)	hrHPV	212	38	27	170	85.5	0.707 (0.641–0.773)
	HPV16	56	10	6	375	96.4	0.854 (0.784–0.924)
	HPV18	11	3	3	430	98.7	0.779 (0.607–0.951)
	Other hrHPV	156	38	35	218	83.6	0.667 (0.597–0.737)
$\geq$ CIN2 (n = 106)	hrHPV	84	8	3	11	89.6	0.607 (0.398–0.816)
	HPV16	31	6	5	64	89.6	0.770 (0.642–0.898)
	HPV18	2	1	1	102	98.1	0.657 (0.212–1.000)
	Other hrHPV	56	10	5	35	85.9	0.706 (0.569–0.843)
$<$ CIN2 (n = 341)	hrHPV	128	30	24	159	84.2	0.681 (0.603–0.759)
	HPV16	25	4	1	311	98.5	0.901 (0.816–0.987)
	HPV18	9	2	2	328	98.8	0.812 (0.632–0.992)
	Other hrHPV	100	28	30	183	83.0	0.638 (0.554–0.723)

Note: Color legend: for the concordance: dark green ($1.00 \geq K > 0.80$): excellent; light green ($0.80 \geq K > 0.60$): good; yellow ($0.60 \geq K > 0.40$): moderate; orange ($0.40 \geq K > 0.20$): fair; red ($0.20 \geq K > 0.00$): poor. Manufacturer's positivity threshold for all hrHPV types in cervical samples: Ct $\leq$ 40; FVU: Ct $\leq$ 44. +/+ positive on FVU and cervical samples, +/- positive only on cervical samples, -/+ positive only on FVU samples, -/- negative on both sample types. Abbreviations: 95% CI, 95% confidence interval; CIN, cervical intraepithelial neoplasia; N, number.

transport medium that allows the preservation of nucleic acids, denaturation of proteins and inactivation of microbial agents. It has been previously used in combination with HPV molecular assays [25, 26]; two previous studies demonstrated a good analytical performance of FLOQSwabs self-collected vaginal samples resuspended in 5 mL eNAT as compared to cervical samples [27, 28]. Moderate to excellent agreement in the concordance of HPV test results between vaginal and cervical specimens was also demonstrated in the present study.

Both urine and vaginal self-collected samples are well accepted by women [29], in particular this study confirmed that FVU is a noninvasive collection method with a clinical accuracy for the detection of $\geq$ CIN2 lesions comparable to cervical specimens, as also previously reported for other HPV assays [16, 17].

Ensuring sample adequacy is crucial in terms of quality assurance in HPV-based primary cervical cancer screening, particularly important when testing self-collected samples, allowing to reduce potential false-negative results [21, 30–32]. One of the main advantages of OncoPredict HPV SCR assay is the inclusion of a thorough quality assessment, both for the preanalytical and analytical phases. The assay allows to assess the efficiency of nucleic acid extraction and potential PCR inhibition through the use of external calibrators, as well as evaluating the adequacy of sample collection through a quantitative cellularity assessment. In general, most of the clinically validated molecular assays include an internal housekeeping gene which is used for both sample adequacy and amplification assessment. Recent studies have underlined the importance of quality controls in molecular diagnostics, such as in HPV-based primary screening, including the possibility of a quantitative sample adequacy control in a separate reaction well [30, 31]. In the present study, no invalid result related to PCR inhibition was detected, underlying the good performance of the analytical process. The invalidity due to a low extraction efficiency could be attributed to errors in specific nucleic acid extraction runs that may be resolved by retesting the sample following repeated nucleic acids extraction. On the contrary, in case of low cellularity samples, in absence of other invalidity reasons, sample collection should be repeated [30]. In general, in this study the invalidity rate was higher in cervical samples than in self-collected samples. This could be related to different limits of acceptable cellularity for cervical and self-collected samples. Moreover, as previously discussed, in the present study vaginal samples were resuspended in 5 mL of eNAT while cervical swabs in 20 mL of PreservCyt which may have resulted in lower sample cellularity. In conclusion, the inclusion of the QC module in OncoPredict HPV SCR assay, allowing assessment of sample adequacy, may improve confidence in the reporting of negative HPV results in cervical cancer screening.

OncoPredict HPV SCR assay is a limited genotyping assay, identifying HPV16, HPV18 and/or 11 “other” hrHPV genotypes, whereas OncoPredict HPV Quantitative Typing (QT) is a full genotyping assay that can distinguish all the 12 hrHPV types separately, based on the same preanalytical workflow. Both assays have been independently validated on cervical and self-samples within VALGENT and VALHUDES Frameworks [18, 19, 33], respectively. In future strategies for the molecular triage of HPV-positive self-collected samples, OncoPredict HPV

SCR screen-positive samples may benefit from reflex testing using the quantitative, full-genotyping OncoPredict HPV QT assay, on the same sample's nucleic acid extract.

In conclusion, following a posteriori cut-offs adjustment the OncoPredict HPV SCR assay demonstrated similar clinical accuracy for $\geq$ CIN2 lesions on self-collected vaginal and FVU samples compared to testing on clinician-collected cervical samples.

Author Contributions

Principal investigator and conceptualization: C.E.C., M.A. Protocol development: M.A., C.E.C. Funding acquisition: C.E.C., M.A. Project administration: C.E.C., M.A. Enrollment of patients: A.D.I., R.P. Data curation and formal analysis: A.L., M.A., M.M. Sample collection, handling and Methodology: A.D.I., R.P., M.M., C.G., F.B., A.F.P. Drafting original manuscript: C.G. Critical review and editing of manuscript: A.L., A.D.I., A.F.P., C.E.C., C.G., F.B., F.O., G.T., H.E., K.C., M.A., M.M., R.P.

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Ethics Statement

The European VALHUDES study (ClinicalTrials.gov: NCT04312737) was conducted under the Declaration of Helsinki. The study was approved

by the central Ethics Committee of the Coordinating Centre, ASST degli Spedali Civili di Brescia, Brescia, Italy (Ethics approval number: NP 3879-Studio WP6-HPVONC) and subsequently by the local Ethics Committees of the other participating centers. All women signed a written informed consent form before to enrollment.

Conflicts of Interest

The European VALHUDES is a researcher-induced study, coordinated by the University of Milano-Bicocca (Milan, Italy), Sciensano (Bruxelles, Belgium), Istituto Europeo di Oncologia (Milan, Italy), University of Sassari (Sassari, Italy), U.O. Coordinamento Consultori Familiari, ASSL Sassari-ATS Sardegna (Sassari, Italy), NHS Lothian, University of Edinburgh (Edinburgh, Scotland), Trinity College Dublin (Dublin, Ireland).

Manufacturers of HPV assays (GeneFirst, Oxford, UK and Hiantis, Milan, Italy) and devices (Copan Italia Spa, Brescia, Italy and Novosanis, Belgium) participated in the European VALHUDES framework under the condition of accepting independent publication of results. Funding received from the companies are managed by the directors of the respective collaborating institutions. The study group received free self-sample collection devices from Copan Italia Spa (Brescia, Italy) and Novosanis (Belgium) and free OncoPredict HPV assay from (Hiantis, Milan, Italy).

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Sciensano authors do not have any personal or material conflict of interest. A.D.I., A.F.P., F.B., F.O., G.T., H.E., K.C., M.M., C.G., R.P. declare no conflict of interest.

Data Availability Statement

Final study data sets generated by the study will be stored locally and securely at Sciensano. Anonymized data will be available by request to the corresponding author on a case-by-case basis pending approval from the information security coordinator at Sciensano.

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Supporting Information

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



Research article

Evaluation of two alternative non-alcohol-based media for the suspension of self-collected vaginal swabs for HPV testing in cervical cancer screening

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ABSTRACT

The introduction of Human Papillomavirus (HPV) testing in cervical cancer screening enhanced the opportunity to introduce self-collection as an innovative approach to improve coverage rates. Validation and standardization of the pre-analytical and analytical procedures are crucial for the quality assurance of HPV tests on self-collected samples.

This study evaluated the analytical performance and the stability of self-collected vaginal samples resuspended in 5 mL of two non-alcohol-based media, eNat® and MSwab® compared to a professionally collected cervical sample, resuspended in 20 mL ThinPrep®, for the detection of high-risk HPV (hrHPV). The impact of the suspension volumes on analytical performance was also evaluated (2 and 5 mL).

A good analytical concordance in hrHPV detection in cervical and vaginal self-collected swabs suspended in 5 mL of both non-alcohol-based media was demonstrated (eNat®: 91.2 %, $k = 0.821$; MSwab®: 91.4 %, $k = 0.798$). A similar analytical performance was found for samples resuspended in 2 mL (eNat®: 92.9 %, $k = 0.811$; MSwab®: 92.9 %, $k = 0.811$) compared to cervical samples.

Good nucleic acid stability was demonstrated for vaginal samples stored at 20–25 °C and 37 °C for up to 4 weeks.

Results of this preliminary study support the introduction of these media for vaginal self-sampling-based prevention programs. Nevertheless, further research is necessary to evaluate clinical accuracy in larger settings.

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Abbreviations	
HPV	Human Papillomavirus
hrHPV	high-risk Human Papillomavirus
WHO	World Health Organization
NILM	negative for intraepithelial lesion or malignancy
ASCUS	Atypical squamous cells of undetermined significance
LSIL	Low-grade squamous intraepithelial lesion
ASCH	Atypical squamous cells suspicious for HSIL
HSIL	High-grade squamous intraepithelial lesion
AGC	Atypical glandular cells
CIN1	cervical intraepithelial neoplasia grade 1
CIN2	cervical intraepithelial neoplasia grade 2
CIN3	cervical intraepithelial neoplasia grade 3

1. Introduction

Persistent infection of the cervical tract with high-risk Human Papillomavirus (hrHPV) has been recognized to be the major cause of cervical cancer. Cervical cancer is largely preventable through both HPV vaccination and screening for precursor lesions [1].

HPV nucleic acid testing of physician-collected cervical scrapes has demonstrated superior efficacy in reducing the incidence of cervical cancer compared to cervical cytology and its implementation in screening programs has been advocated by European guidelines [2]. Despite these advances in secondary prevention, there are still approximately 500,000 new cases of cervical cancer every year worldwide [3], occurring principally in developing countries, where women do not have access to screening programs, but also in industrialized countries in under- or never-screened women.

A promising strategy to implement cervical cancer screening programs in rural or low-resource settings as well as improving the participation rate of non-responders has been the implementation of self-collection-based screening [4–6]. The validation and standardization of both pre-analytical and analytical procedures of HPV testing on self-collected samples is however of fundamental importance in the quality assurance of HPV primary screening programs.

A protocol for the validation of human papillomavirus assays in combination with collections devices for HPV testing on self-samples has been published [7] allowing the evaluation of HPV test accuracy on self-samples as compared to physician-collected cervical scrapes. These studies allow to assess not only different sampling approaches using commercially available collection devices but also to extend the validation to different volumes and compositions of non-alcohol-based storage media for the resuspension of vaginal self-collected samples. Previously published validation studies of HPV tests on self-collected vaginal samples, using different vaginal collection devices, have shown great variability in the resuspension procedure used for vaginal samples, in terms of time from collection to sample processing, resuspension volume and type of liquid media being used [8–10].

Clinician-collected cervical samples are generally resuspended in 20 mL of ThinPrep® or PreservCyt® (Hologic, USA), an alcohol-based medium, which allows both HPV DNA molecular testing and liquid-based cytology for the triage of HPV-positive women from the same initial sample. ThinPrep® is however presently available only in 20 mL vials containing 60 % methanol, making it flammable and toxic (UN1993 Dangerous Good), with specialised handling and packaging requirements for air transportation. These aspects, together with the higher costs of alcohol-based media, represent important constraints, particularly in low-middle-income countries, making it unsuitable for vaginal sample resuspension, particularly as the latter is not an appropriate sample type for its use in cytology triage.

eNat® (Copan Italia Spa, Brescia, Italy) is a transport medium for the preservation and stabilization of nucleic acids compatible with numerous molecular assays and previously used for HPV molecular detection [11,12]. It also has the advantage of inactivating microbial agents and denaturing proteins, allowing safer sample handling in the laboratory. MSwab® medium (Copan Italia Spa, Brescia, Italy) is compatible with both bacterial and viral culture as well as nucleic acids detection. Moreover, it allows rapid nucleic acid heat extraction, thus reducing the time and costs of HPV molecular testing.

This pilot study aimed to evaluate the concordance of HPV testing of self-collected vaginal samples suspended in two alternative non-alcohol-based media, eNat® and MSwab®, using two different suspension volumes, 5 mL vs 2 mL, compared to the reference clinician-collected cervical samples suspended in 20 mL of ThinPrep®. Furthermore, viral nucleic acid stability of resuspended vaginal samples in both eNat® and MSwab® was evaluated following storage at both room temperature (RT) and 37 °C, over a timeframe of 4 weeks.

2. Materials and methods

2.1. Study design and samples collection

The study was conducted according to documentation approved by the Ethics Committee of the University of Milano-Bicocca (Protocol n. 0037320/2017 and subsequent update 0086409/2018).

A clinician-taken cervical sample and 2 self-collected vaginal swabs were obtained from 100 women referred to colposcopy at Fondazione IRCCS San Gerardo dei Tintori (Monza, Italy) following a recent abnormal cervical cytology.

Women who agreed to participate in the study by the signature of an informed consent at the time of the colposcopy visit were instructed to collect 2 vaginal swabs using FLOQSwab® 552.80 (Copan Italia Spa, Brescia, Italy), prior to gynaecological examination, while cervical swabs were taken by the physician before performing colposcopy, using L-shaped Endo/Esocervical FLOQSwab® (Copan Italia Spa, Brescia, Italy) and resuspended immediately into 20 ml of ThinPrep® (Hologic, USA). All samples, including dry vaginal swabs, were transported within 24 h from collection to the Clinical Microbiology Laboratory of the University of Milano-Bicocca, Italy.

Women underwent biopsy or conization based on the outcome of the colposcopy and on the clinical judgment. Cytological lesions were classified according to the Bethesda system [13], whilst outcomes of histological analysis according to the World Health Organization (WHO) histological classification of tumours [14].

2.2. Pre-analytical samples processing and HPV testing

On arrival at the laboratory, cervical samples were vortexed for 30 s and aliquots of 1.5 ml were dispensed in sterile cryotubes and stored at -20°C until testing.

Vaginal self-samples arrived dry at the laboratory where they were suspended in the different suspension media (ThinPrep®, eNat® and MSwab®) as described in Fig. 1. Arbitrarily, the first and second self-collected vaginal swabs were suspended respectively in the alternative non-alcohol based medium and ThinPrep® in the first two sets of 36 women. For the evaluation of the two different volumes of the alternative media, the first vaginal swab was suspended in 5 mL and the second self-sample in the 2 mL volume, respectively.

200 μl of all sample types were used to perform nucleic acid extraction using STARMag 96 $\times$ 4 Universal Cartridge Kit (Seegene, Korea) on the Nimbus platform. The automated workstation also allowed the preparation of the real-time PCR assay Anyplex™II HR HPV (Seegene, Korea). The kit was able to individually detect 14 different genotypes of hrHPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and a cellular gene target by melting curves analysis. Real-time assays were performed on the CFX96 (Bio-Rad, Hercules, USA) with 5 μl of template DNA in a total volume of 20 μl . As reported by the manufacturer, if sample positivity is detected before the first cyclic Catcher Melting Temperature Analysis (cyclic-CMTA) after the first 30 cycles of amplification, the result is indicated as “+++”; if positivity is detected between the first and the second cyclic-CMTA ($30 < \text{Amplification Cycles} < 40$) as “++”; if between the second and the third cyclic-CMTA ($40 < \text{Amplification Cycles} < 50$) as “+”. Samples with an invalid result (no detection of the cellular gene or cellular gene detected with just “+”) were retested and excluded from the analysis if the invalidity was confirmed.

2.3. Evaluation of nucleic acid stability

In order to evaluate nucleic acid stability of 10 vaginal self-samples suspended in 5 ml of MSwab® and eNat®, aliquots of 0.4 mL of self-collected vaginal samples were dispensed in sterile cryotubes and stored at either RT ($20\text{--}25^{\circ}\text{C}$) and 37°C until further testing.

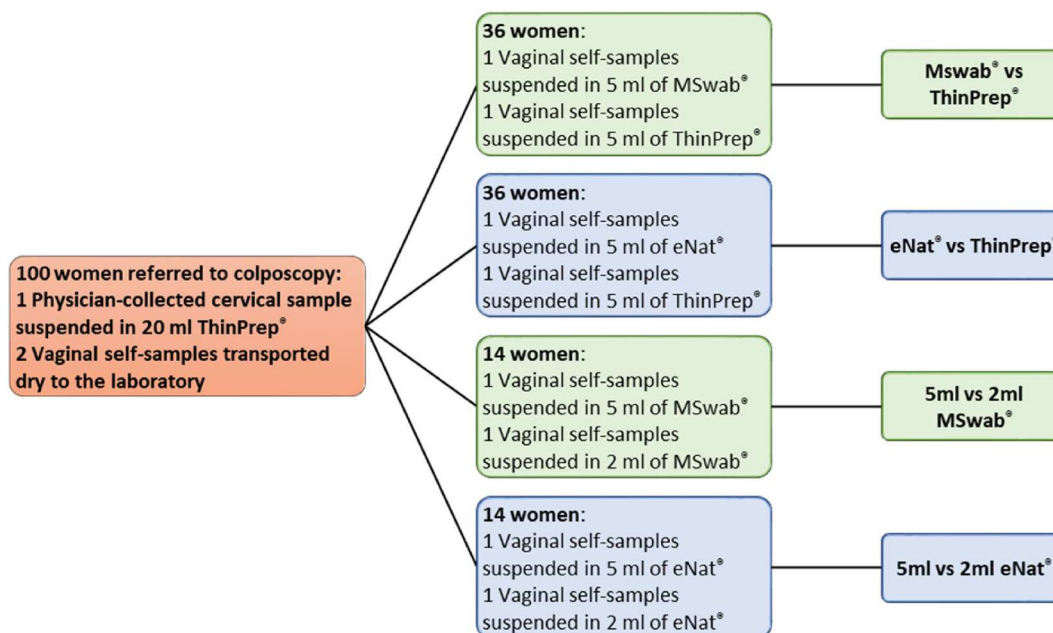


Fig. 1. Study design.

Nucleic acids were re-extracted starting from a new sample aliquot and hrHPV testing repeated at the time points and storage conditions described in Fig. 2.

2.4. Statistical analysis

Agreement between results of HPV testing of different types of samples was evaluated with Cohen's kappa (κ) statistics and defined as slight ($0.00 < \kappa < 0.20$), fair ($0.20 < \kappa < 0.40$), moderate ($0.41 < \kappa < 0.60$), substantial ($0.61 < \kappa < 0.80$) and almost perfect ($0.81 < \kappa < 1.00$).

3. Results

3.1. Study population

100 women were enrolled between January 2020 and February 2022 as part of this pilot study. Three patients were excluded from the analysis: 2 of them had a reduced number of cells in the professionally collected cervical sample, resulting in an invalid HPV test result, while the other invalid result was obtained in a vaginal swab suspended in MSwab®. In total, the analysis of the results was performed on samples from 97 women (49 with one of the vaginal swabs suspended in 5 ml of MSwab® and 48 with one vaginal specimen suspended in 5 ml of eNat®).

Overall, forty-one women had a positive colposcopy examination, while in 56 no abnormalities were found. Table 1 reports the clinical data of the 97 women evaluated in this pilot study.

3.2. hrHPV positivity and genotypes distribution: evaluation of alternative media

Among women whose self-collected vaginal samples were suspended in 5 ml of ThinPrep® and MSwab®, 23/35 (71.4 %) cervical samples and 26/35 (74.3 %) vaginal swabs suspended in each of the two media were found to be hrHPV-positive. Among cervical samples, 8/23 (34.8 %) women had single infections and 15/23 (65.2 %) had multiple infections. 8/26 (30.8 %) vaginal specimens suspended in ThinPrep® showed single infections and 18/26 (69.2 %) multiple infections, while those suspended in MSwab® showed 7 single infections and 19 multiple infections.

Among those with vaginal self-specimens suspended in 5 ml of ThinPrep® and eNat®, the hrHPV positivity rate in cervical samples was 52.9 % (18/34), while in vaginal self-samples suspended in each of the two media was 61.8 % (21/34). The distribution of co-infections was slightly different among the three sample types: 13 single and 5 multiple infections were detected in cervical specimens, 13 single and 8 multiple infections in vaginal specimens suspended in ThinPrep® and 11 single and 10 multiple infections in vaginal swabs suspended in eNat®.

HPV16 and HPV31 were the genotypes most frequently detected. Fig. 3 and Supplementary Table 1 show the complete hrHPV genotypes distribution in cervical and vaginal self-samples in both groups of women. Among vaginal self-samples resuspended in MSwab®, 20/35 (57.1 %) samples showed the presence of the same hr-HPV types found in both the self-collected vaginal sample

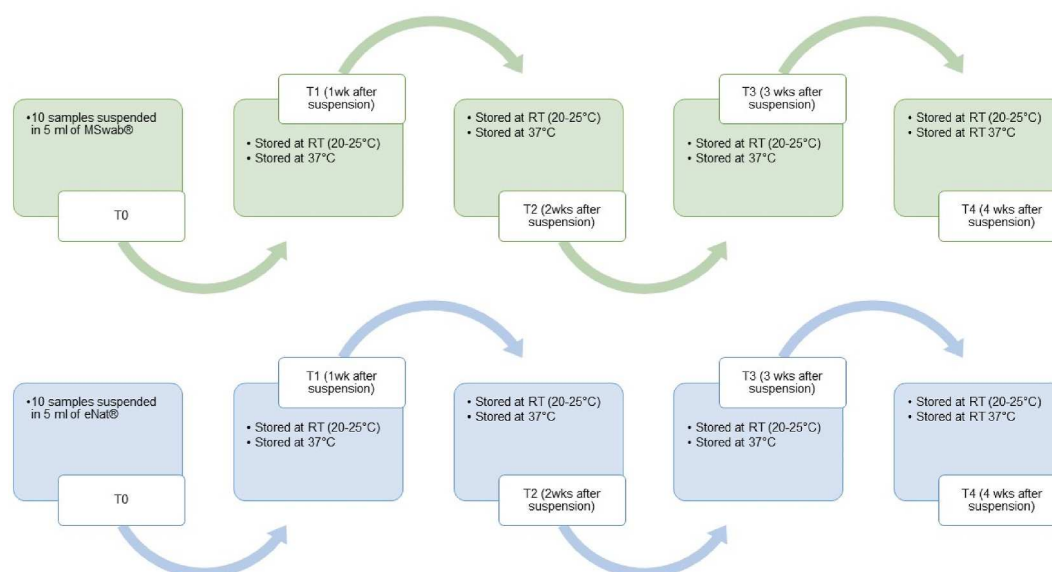


Fig. 2. Time points and storage conditions of vaginal self-samples suspended in MSwab® and eNat® prior to testing.

Table 1
Clinical data of patients enrolled in the study.

	n (%)
Cytology	Tot. 97
HSIL	14 (14.4)
ASCH	3 (3.1)
LSIL	36 (37.1)
ASCUS	19 (19.6)
AGC	2 (2.1)
NILM	23 (23.7)
Biopsy/Conisation	Tot. 18
Cervical cancer	1 (5.6)
CIN3	9 (50.0)
CIN2	2 (11.1)
CIN1	5 (27.7)
Negative	1 (5.6)

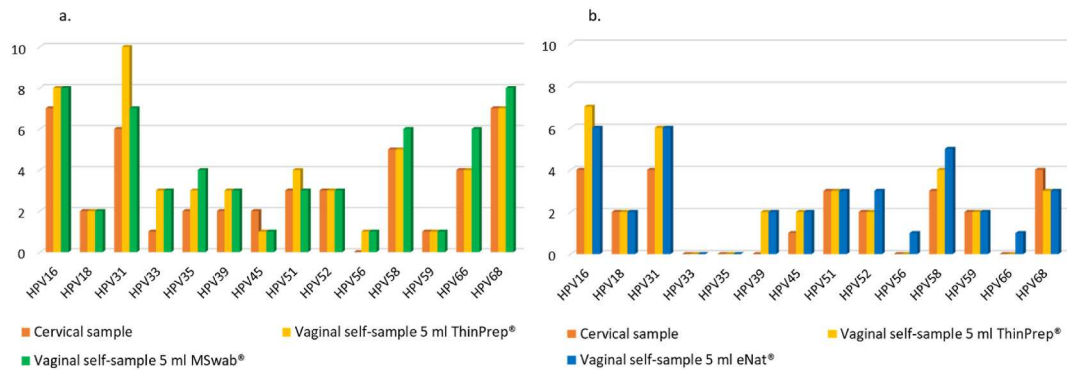


Fig. 3. hrHPV genotypes distribution in cervical and vaginal self-samples suspended in 5 ml of ThinPrep® and 5 ml MSwab® (a) and 5 ml ThinPrep® and 5 ml of eNat® (b).

resuspended in ThinPrep® and in the cervical sample. A concordance for at least one hrHPV genotype among the three different types of samples was observed in 11/35 (31.4 %). On the contrary, among the 35 triplets of samples, 4 (11.4 %) have shown a discordant positive/negative result at least in one of the sample types. In 3 of these cases, the positivity found was related to just one ‘+’, meaning that this positivity is associated with a very high Ct value ranging from 40 to 50 (Supplementary Table 1). Regarding the vaginal self-collected samples resuspended in eNat®, 24/34 (70.6 %) triplets of samples showed the presence of the same hrHPV types and 7/34 (20.6 %) demonstrated a concordance for at least one hrHPV genotype. Three triplets of samples (8.8 %) have shown a discordant result (positive/negative) and in one case the positivity found was associated with just one ‘+’.

3.3. Analytical agreement between cervical and vaginal samples: evaluation of alternative media

As shown in Table 2, the concordance of hrHPV detection of cervical samples and vaginal self-collected swabs suspended in 5 ml of MSwab® and the same volume of ThinPrep® was demonstrated to be good (with k ranging from $k = 0.798$ to $k = 0.821$) irrespective of the medium used.

Table 2
Agreement in hrHPV detection in vaginal self-samples suspended in 5 ml of ThinPrep® and 5 ml of MSwab® (a) and 5 ml of ThinPrep® and 5 ml of eNat® (b) as compared to the reference professionally collected cervical sample.

a.	Vaginal self-sample 5 ml ThinPrep®		Vaginal self-sample 5 ml MSwab®	
	Overall concordance n (%)	k (95 % c.i.)	Overall concordance n (%)	k (95 % c.i.)
	Cervical sample	31 (91.2)	0.821 (0.631–1.000)	31 (91.2)
b.	Vaginal self-sample 5 ml ThinPrep®		Vaginal self-sample 5 ml eNat®	
	Overall concordance n (%)	k (95 % c.i.)	Overall concordance n (%)	k (95 % c.i.)
	Cervical sample	32 (91.4)	0.798 (0.583–1.000)	32 (91.4)

When comparing the self-collected swabs, almost no differences were obtained between samples suspended in ThinPrep® and in the alternative media (5 mL MSwab® vs 5 mL ThinPrep®: 94.3 % [33/35]; k [95 % c.i.]: 0.850 [0.650–1.000]; 5 mL eNat® vs 5 mL ThinPrep®: 100.0 % [34/34]; k [95 % c.i.]: 1.000 [1.000–1.000]).

3.4. hrHPV positivity and genotypes distribution: evaluation of alternative volumes

When considering the group of women whose vaginal specimens were suspended in both 2 and 5 ml of MSwab®, 10/14 (71.4 %) cervical samples and 11/14 (78.6 %) vaginal swabs suspended in the volumes of MSwab® were hrHPV-positive. Cervical specimens showed 8 single infections and 2 co-infections, while vaginal specimens suspended in 2 and 5 ml of MSwab® showed 7 single and 4 multiple infections.

In women with vaginal self-samples placed in 2 and 5 ml of eNat®, 10/14 cervical samples, 9/14 vaginal specimens suspended in 5 ml of eNat® and 11/14 vaginal swabs suspended in 2 ml of eNat® were hrHPV-positive. In the case of the two additional women whose vaginal specimen was hrHPV positive if suspended in 2 ml, but not in 5 ml of eNat®, the cervical samples were positive for HPV31 with “+” or “++” and the relative vaginal swabs suspended in 2 ml were HPV31 positive with “+”. 8 single infections and 2 multiple hrHPV infections were detected in cervical samples; 7 single infections and 4 co-infections on vaginal swabs suspended in 2 ml of eNat® and 4 single and 4 multiple infections on vaginal swabs suspended in 5 ml of eNat®.

hrHPV genotypes distribution in the two groups of enrolled patients is shown in Fig. 4 and Supplementary Table 2.

3.5. Analytical agreement between cervical and vaginal samples: evaluation of alternative volumes

In women with vaginal specimens suspended in 2 and 5 ml of MSwab®, an almost perfect concordance was demonstrated ($k = 0.811$) between the cervical sample and the vaginal swabs suspended in the two different volumes.

In the group of patients with double self-collection performed in the two volumes of eNat®, the agreement was higher when 2 ml instead of 5 ml of medium was used (92.9 %, $k = 0.811$ vs 78.6 %, $k = 0.512$), probably related to low hrHPV copies number as previously described.

Data are reported in Table 3.

3.6. Stability

Good stability for up to 4 weeks was demonstrated for vaginal samples suspended in MSwab® and eNat® at room temperature (20–25 °C) and 37 °C. The results of HPV testing at the different time points and storage conditions are shown in Table 4. As reported in Table 4, the discrepancies are mainly related to a low number of viral copies with hrHPV detection with “+” using Anyplex™II HR HPV.

4. Discussion

In 2021, 17 countries recommended the use of HPV tests in combination with self-sampling devices in organised screening programs, while 10 countries were conducting pilot studies on self-collected samples [15]. The introduction of self-sampling-based HPV screening programs is an opportunity to increase coverage rate by reaching non-attendant women in countries with ongoing organised screening programs, but most of all to set up new procedures in low-middle income countries where resources are limited.

In order to evaluate the impact of different suspension media and volumes on HPV test conducted on self-collected samples, we investigated the performance of two non-alcohol-based media, eNat® and MSwab®, and two suspension volumes, 5 mL vs 2 mL of each alternative medium by assessing the analytical agreement with the clinician-collected cervical sample suspended in 20 mL of ThinPrep® from the same woman.

In this study, vaginal self-collected samples were transported dry to the laboratory where they were resuspended in the evaluated

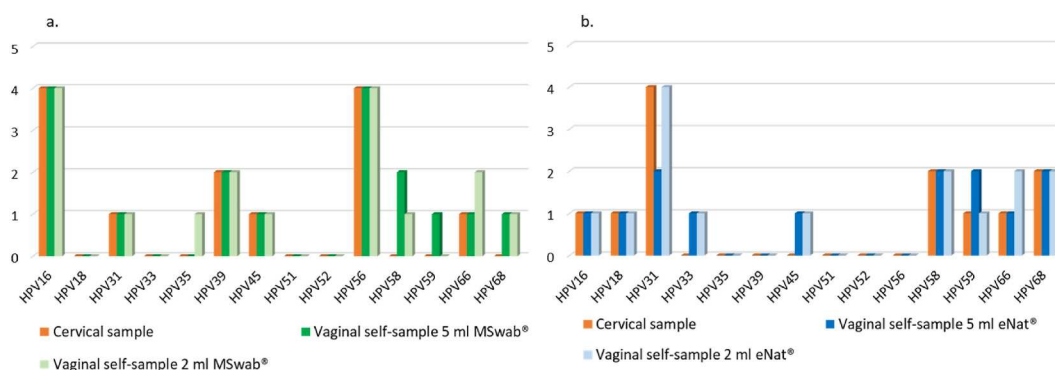


Fig. 4. hrHPV genotypes distribution in cervical and vaginal self-samples suspended in 5 ml and 2 ml of MSwab® and 5 ml 2 ml of eNat®.

Table 3
Agreement in hrHPV detection in vaginal self-samples suspended in 5 ml and 2 ml of MSwab® (a) and 5 ml 2 ml of eNat® (b).

a.	Vaginal self-sample 2 ml MSwab®		Vaginal self-sample 5 ml MSwab®	
	Overall concordance n (%)	k (95 % c.i.)	Overall concordance n (%)	k (95 % c.i.)
Cervical sample	13 (92.9)	0.811 (0.460–1.000)	13 (92.9)	0.811 (0.460–1.000)
b.	Vaginal self-sample 2 ml eNat®		Vaginal self-sample 5 ml eNat®	
	Overall concordance n (%)	k (95 % c.i.)	Overall concordance n (%)	k (95 % c.i.)
Cervical sample	13 (92.9)	0.811 (0.460–1.000)	11 (78.6)	0.512 (0.037–0.987)

media. The use of dry swabs is a suitable solution to reduce costs and avoid the risk of leakage during transport. Costs related to vaginal specimens' management are further reduced on using non-alcohol-based media for the resuspension of the vaginal swabs and by reducing the resuspension volume compared to that used for cervical samples.

Specific protocols to evaluate the performance of HPV tests in combination with self-collection devices have been introduced [7]. Optimal sample management is a crucial step to assure sample adequacy, particularly in the case of self-collection. Technical analysis to evaluate the best workflow procedure is required, as recently underlined by Arbyn and colleagues [16]. Up to now, self-collection procedures, volumes and types of suspension media have varied across different settings and just a few studies have been conducted to evaluate the impact of pre-analytical workflow on the HPV test result on clinical samples.

The different chemical compositions of the suspension media may influence the results of the test. Some previous studies have evaluated the impact of different suspension media and volumes on HPV test results [17,18] using artificially HPV-spiked samples. Badman and colleagues tested serial dilutions of HPV-positive cell lines with 5 different suspension media and suggested that MSwab® could be considered a valid alternative to ThinPrep® [17]. The results of our preliminary analysis confirm the good analytical performance of MSwab® in HPV detection also on clinical samples and further suggest that eNat® could also be considered as a good alternative to ThinPrep®. Furthermore, in a previous published study from our group conducted on clinical specimens, the analytical performance for HPV detection on vaginal samples collected with FLOQSwab® resuspended in 5 ml eNat® was demonstrated to be comparable to that of FLOQSwab® in 5 ml ThinPrep® [19].

The potential impact of the suspension volume on the clinical performance of vaginal specimens for the detection of high-grade cervical lesions has been discussed by Inturrisi and colleagues [20] and further confirmed by Arbyn and colleagues [21]. In a more recent study, FLOQSwab® and Evalyn®Brush were suspended in different volumes of ThinPrep® spiked with various concentrations of HPV16-positive cell lines. The resuspension in a volume of 5 ml or less was demonstrated to maximize HPV detection [18]. In other studies, conducted in a colposcopy setting, paired cervical and vaginal samples were collected and suspended in different types and volumes of media underlining the effect of these factors in terms of clinical accuracy [22,23]. Furthermore, a recent study conducted by our group demonstrated a similar accuracy in HPV detection of a vaginal sample collected using FLOQSwab® suspended in 5 ml of ThinPrep® as compared to clinician-collected scrape in a group of women referred to colposcopy [24]. In the current study, a concordance of over 91 % was found between vaginal swabs suspended in 5 ml of the three tested media (ThinPrep®, MSwab® and eNat®) and cervical specimens.

Looking at the comparison of the two suspension volumes of MSwab® and eNat®, no differences were detected in the analytical performance of MSwab®, while the use of 2 ml of eNat® seemed to improve the analytical sensitivity of the method as compared to the higher 5 mL volume; however, the small sample size of women evaluated in this pilot study does not allow to make definitive conclusions. A previously published paper already evaluated the performance of 2 ml of eNat® in a screening setting [25]. Furthermore, in the present study, the discordances were detected in two women in case of a low number of copies with HPV31 detected with “+” in vaginal swabs suspended in 2 ml of the medium and no HPV detected in those suspended in 5 ml. One of the two women in whom discordances were detected had an ASCUS cytology and a negative colposcopy outcome, the other a positive colposcopy outcome and an ASCH cytology. One of the two discordances could be considered as not clinically relevant. Since the comparison was performed in a small group of women, future studies on a larger number of participants will be necessary to further investigate the potential impact of suspension volume on the analytical and clinical performance of vaginal swabs. Moreover, the data need to be better correlated with the clinical outcomes of the patients to eventually establish different cut-off values to define HPV positivity in self-collected samples as compared to cervical swabs.

Some previous studies on clinician-collected cervical samples have investigated the stability of nucleic acids in SurePath® [26] and PreservCyt® [27]. Most of the studies conducted on vaginal samples, on the contrary, focused on demonstrating the stability of dry samples to highlight the possibility of transporting samples without any suspension media to reduce costs and improve safety [28,29]. Our preliminary results on real clinical samples, using clinician-collected cervical samples as reference, indicate that MSwab® and eNat® may offer two valid alternatives for the resuspension of self-collected samples because they allow for adequate preservation of nucleic acids for up to 4 weeks at room temperature (20–25 °C) and 37 °C. The small differences across the time points are mainly related to samples with low copy numbers (detected as “+” by the assay) which are likely to result from transient infections, not significant for the development of high-grade cervical lesions. Interestingly, no invalid results were obtained at the different time points underlining the good preservation of nucleic acids in MSwab® and eNat®. Future studies using quantitative real-time PCR assay may allow a more precise evaluation of nucleic acids' stability at the different storage conditions.

Table 4

hrHPV positivity in cervical samples and vaginal self-specimens at the different time points and storage conditions suspended in MSwab® (a) and eNat® (b).

(a)														
MSwab®	Clinical Data			Results at T0	Results after 1 week of storage		Results after 2 weeks of storage		Results after 3 weeks of storage		Results after 4 weeks of storage			
Sample ID	Colposcopy	Cytology	Histology	Vaginal self-sample	20–25 °C	37 °C	20–25 °C	37 °C	20–25 °C	37 °C	20–25 °C	37 °C		
MO211	NEG	LSIL	–	33+	33+	33++	16+	33++	16+	33++	16+	33++		
				59++	59++	59++	33+	59++	33+	59++	33 + 59++	59++		
							59++		59++					
MO213	POS	HSIL	CIN 3	16++	16+++	16+++		16++		16++	16++	16++		
MO214	NEG	LSIL	–	16+	68+	68+	68+	NEG	16++	NEG	NEG	16++	68+	68+
				68+										
MO216	NEG	LSIL	–	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
MO14 T24	NEG	NILM	–	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
MO218	NEG	NILM	–	16+++	16+++	16+++	16+++	16+++	16+++	16+++	16+++	16+++	51+++	16+++
				51+++	51+++	51+++	51+++	51+++	51+++	51+++	51+++	51+++	51+++	
MO219	NEG	ASCUS	–	18++	18++	18++	18++	18++	18++	18++	18++	18++	33++	18++
				33++	33++	33++	33++	33++	33++	33++	33++	33++	33++	33++
MO220	POS	ASCUS	–	31++	31++	31++	31++	31++	31++	31++	31++	31++	31+++	31+++
				66+	68+	68+	66+	66+	68+	68+	68+	68+	68+	68+
				68+			68+	68+						
MO221	NEG	ASCUS	–	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
MO222	NEG	NILM	–	31+	31+	68++	31+	31+	31+	31+	31+	31+	31++ 68++	31++
				68+++	68++		68++	68++	68++	68++	68++	68++	68+++	

(b)														
eNat®	Clinical Data			Results at T0	Results after 1 week of storage		Results after 2 weeks of storage		Results after 3 weeks of storage		Results after 4 weeks of storage			
Sample ID	Colposcopy	Cytology	Histology	Vaginal self-sample	20–25 °C	37 °C	20–25 °C	37 °C	20–25 °C	37 °C	20–25 °C	37 °C		
MO235 T6	NEG	LSIL	–	16+++	16+++	16+++	16++	16+++	16+++	16+++	16++	16+++		
					18+			18+				18+		
MO269	POS	ASCUS	–	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
MO270	NEG	ASCUS	–	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
MO230 T12	NEG	NILM	–	31+	31+	31+	52++	31+	31+	31+	52++	52++		
				52++	52++	52++	52++	52++	52++	52++	52++	52++		
MO222 T12	NEG	NILM	–	52+	68+++	52+	68++	68++	68+++	68+++	68+	68+++		
				68+++		68+++								
MO271	POS	ASCUS	–	59+	59+	59+	NEG	59+	59+	59+	59+	NEG	59+	
MO272	POS	LSIL	–	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
MO239 T6	NEG	NILM	–	NEG	NEG	52+	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
MO273	POS	HSIL	CIN 3	16++	16+++	16+++	16++	16+++	16+++	16+++	16++	16+++	16++	16+++
														68+

5. Conclusions

These preliminary results demonstrated the good performance of MSwab® and eNat® as alternative media to ThinPrep® for the suspension of vaginal specimens for HPV detection.

The main limitation of this work is the reduced sample size. Since the preliminary results are promising, future analysis will be performed to evaluate the best combination of medium/volume for suspension of vaginal samples in order to achieve an accurate and cost-effective strategy for the implementation of self-sampling in cervical cancer screening programs.

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Ethics statement

The study was conducted according to documentation approved by the Ethics Committee of the University of Milano-Bicocca (Protocol n. 0037320/2017 and subsequent update 0086409/2018). Women agreed to participate to the study by the signature of a written informed consent.

Data availability statement

Final study datasets generated by the study are stored locally and securely at the University of Milano-Bicocca. Anonymized data will be available by request to the corresponding author on a case-by-case basis pending approval by the University of Milano-Bicocca.

CRediT authorship contribution statement

Chiara Giubbi: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Marianna Martinelli:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Maria Letizia Di Meo:** Writing – review & editing, Data curation. **Ruth Chinyere Njoku:** Writing – review & editing, Methodology, Data curation. **Federica Perdoni:** Writing – review & editing, Methodology. **Robert Fruscio:** Writing – review & editing. **Fabio Landoni:** Writing – review & editing. **Clementina Elvezia Cocuzza:** Writing – review & editing, Supervision, Project administration, Investigation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Clementina Elvezia Cocuzza received research funding or gratis consumables to support research from the following commercial entities in the last 3 years: BD, Seegene, Copan, Novosanis and Hiantis.

Other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.heliyon.2024.e31032>.

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Article

Molecular Detection of Human Papillomavirus (HPV) and Other Sexually Transmitted Pathogens in Cervical and Self-Collected Specimens

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Abstract: This study investigated the detection of high-risk Human Papillomavirus (hrHPV) and seven other pathogens associated with sexually transmitted infections (STIs) in matched clinician-collected cervical samples and self-taken vaginal and urine specimens collected from 342 asymptomatic women referred to colposcopy to evaluate (i) the concordance in the molecular detection of investigated pathogen in three different sample types; (ii) the analytical sensitivity and specificity of STIs detection on self-samples; and (iii) the distribution of STIs in hrHPV-positive and hrHPV-negative women. Pathogens detection was performed using AnyplexTMII HR and AnyplexTMII STI-7e, respectively. Good/substantial agreement was observed between cervical and self-taken samples in detecting hrHPV ($\kappa = 0.870$ and $\kappa = 0.773$ for vaginal and urine). The agreement between cervical and self-taken samples for detecting STIs was found to be significant ($\kappa = 0.779$ and $\kappa = 0.738$ for vaginal and urine), with almost perfect agreement between urine and vaginal specimens ($\kappa = 0.899$). The positivity rate for all investigated STIs was found to be higher in hrHPV-positive compared to hrHPV-negative women. In conclusion, self-sampling proved to be a valid alternative to cervical samples to detect hrHPV and STIs, but further studies are required to evaluate the role of STI coinfections in cervical lesions development and progression.

Keywords: high-risk human papillomavirus (hrHPV); sexually transmitted infections (STIs); self-sampling



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1. Introduction

Cervical cancer represents a serious threat to women's health globally, with an age-standardized incidence rate of 14.1 per 100,000 women. It is the fourth most common cancer among women, with 661,000 new cases and 348,000 deaths reported in 2022, according to GLOBOCAN data [1]. Persistent infection with high-risk Human Papillomavirus (hrHPV) is widely acknowledged as the primary cause of cervical cancer and associated with a large percentage of anal cancers and cancers of the vulva, vagina, penis and oropharynx [2].

To date, nearly 200 different HPV types have been identified, including 12 classified by the International Agency for Research on Cancer (IARC) as oncogenic or "high-risk"

HPV types belonging to the Group 1 (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59), with HPV16 and HPV18 being the two genotypes more frequently associated with cervical cancer development [3].

Presently, molecular testing for the detection of HPV nucleic acids is gradually replacing cytology in cervical cancer screening and more than 200 HPV tests are commercially available, using different technologies and targeting different HPV genotypes. Some of them are full genotyping assays detecting each HPV type individually; others are partial, extended or without genotyping [4]. Only few tests are validated for the use in cervical cancer prevention according to internationally recognized criteria [4].

The use of self-samples is currently being promoted in cervical cancer prevention worldwide to improve the participation of women in screening programs [5,6]. Self-collection, a non-invasive and easy-to-perform procedure, may attenuate cultural and/or socio-economic barriers, increasing the participation in prevention programs of underserved cohorts [7–9].

The use of PCR-based HPV assays on self-collected samples has been described as having similar accuracy to that of clinician-collected cervical specimens [10,11], as also reported in a previous study by our group [12]. Recently, the VALHUDES (Validation of Human Papillomavirus Assays and Collection Devices for Self-samples and Urine Samples) protocol has been developed to evaluate the clinical accuracy of HPV tests in combination with self-collection devices [13]. Self-collection may represent a convenient way to screen women for other sexually transmitted infections (STIs), increasing the uptake of testing services compared to samples collected by healthcare professionals [14].

The increased prevalence of STIs reported by the European Centre for Disease Prevention and Control (ECDC) [15], urges the scientific community to consider new strategies to implement STI screening, diagnosis and treatment. In particular, in women, STIs are frequently asymptomatic, which, if untreated, can result in the persistence and/or spread of infections through sexual contacts [16]. The delayed diagnosis and treatment of STIs can also increase the risk of long-term health complications, including pelvic inflammatory disease and infertility in women [14]. The diagnosis of STIs usually requires screening and/or diagnostic procedures, which may be difficult to implement due to the social stigma associated with these infections and the limited access to healthcare, particularly in low–middle-income countries [9,16].

Interactions between HPV and other microorganisms sharing similar anatomical sites may be associated with the persistence of HPV infection and an increased risk of disease progression. However, the role of coinfections as a risk factor for the development of cervical cancer requires further investigation [17–19]. Some studies reported that *Chlamydia trachomatis* (CT) persistent infection may be associated with DNA damage due to reactive oxygen species increasing the risk of carcinogenesis related to hrHPV [18,20,21]; others indicated that CT may increase the risk of acquiring hrHPV infection by the disruption of cadherin–catenin junctions in cervical epithelial cells [22]. Smith and colleagues reported an association between CT infection and squamous cells intraepithelial cervical cancer [23], while Castle and colleagues did not find any association with the severity of cervical neoplasia [24]. The chronic inflammation caused by *Ureaplasma* spp. and *Mycoplasma* spp. seems to permit the entry of other pathogens or induce genetic alterations that might lead to the carcinogenesis of epithelial cells [25–27].

The present study aimed at evaluating the accuracy in terms of analytical sensitivity, specificity and agreement of self-collected vaginal and urine specimens, compared to clinician-collected cervical samples, for the detection of 14 hrHPV genotypes (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and seven other sexually transmitted microorganisms (*Ureaplasma parvum* (UP), *Ureaplasma urealyticum* (UU), *Mycoplasma genitalium* (MG),

Mycoplasma hominis (MH), *Chlamydia trachomatis* (CT), *Neisseria gonorrhoeae* (NG), and *Trichomonas vaginalis* (TV)). In addition, we aimed to investigate the distribution of STIs among hrHPV-positive and hrHPV-negative women to evaluate the possible association of hrHPV-STI coinfections with the severity of cervical dysplasia.

2. Results

2.1. Study Population

Three hundred and forty-five women with a prior abnormal Pap smear were recruited at the first colposcopy referral visit. Their median (interquartile range, IQR) age was 37 (29–46) years. In total, 25.2% of women were younger than 30 years, 31.3% were aged 30–40, 28.7% were aged 41–50, 12.5% were aged 51–60 and 2.3% were older than 60 years. The cytology results show that 47.5% of women had low-grade squamous intraepithelial lesion (LSIL), followed by those with atypical squamous cells of undetermined significance (ASCUS) (24.9%) and those with high-grade squamous intraepithelial lesion (HSIL) (13.6%). The colposcopy examination showed positive findings in 127 patients, while 218 patients had negative findings. Patients underwent cervical biopsy and/or treatment with conization according to clinical judgment and local clinical protocols. The histological results showed four cases of cervical cancer: two cases of squamous cell carcinomas, one of adenocarcinoma “in situ” and one cervical carcinoma with dual histological components (Table 1).

Table 1. Clinical data of the study group.

Cytology (n = 345)	N	%
HSIL	47	13.6%
ASCH	26	7.5%
LSIL	164	47.5%
ASCUS	86	24.9%
AGC	14	4.1%
NILM	8	2.3%
Colposcopy (n = 345)		
Positive	127	36.8%
Negative	218	63.2%
Histological Outcome (n = 84)		
Negative	11	13.1%
CIN 1	13	15.5%
CIN 2	12	14.3%
CIN 3	44	52.4%
Cervical cancer	4	4.8%

HSIL (high-grade squamous intraepithelial lesion); ASCH (atypical squamous cells—cannot exclude HSIL); LSIL (low-grade squamous intraepithelial lesion); ASCUS (atypical squamous cells of undetermined significance); AGC (atypical glandular cell); NILM (negative for intraepithelial lesion or malignancy); CIN 1 (cervical intraepithelial neoplasia grade 1); CIN 2 (cervical intraepithelial neoplasia grade 2); CIN 3 (cervical intraepithelial neoplasia grade 3).

2.2. HPV Detection and Genotyping

Among the enrolled study population (n = 345), three women were excluded from the analysis because one of their matched samples tested invalid twice. As a result, 342 women were included in the study.

The prevalence of hrHPV, in this cohort of women referred to colposcopy for abnormal cervical cytology, was 67.0% (229/342), 71.3% (244/342), and 68.1% (233/342) in cervical, vaginal, and urine samples, respectively, with HPV16 and HPV31 being the most common types, followed by HPV58 and HPV66 (Figure 1).

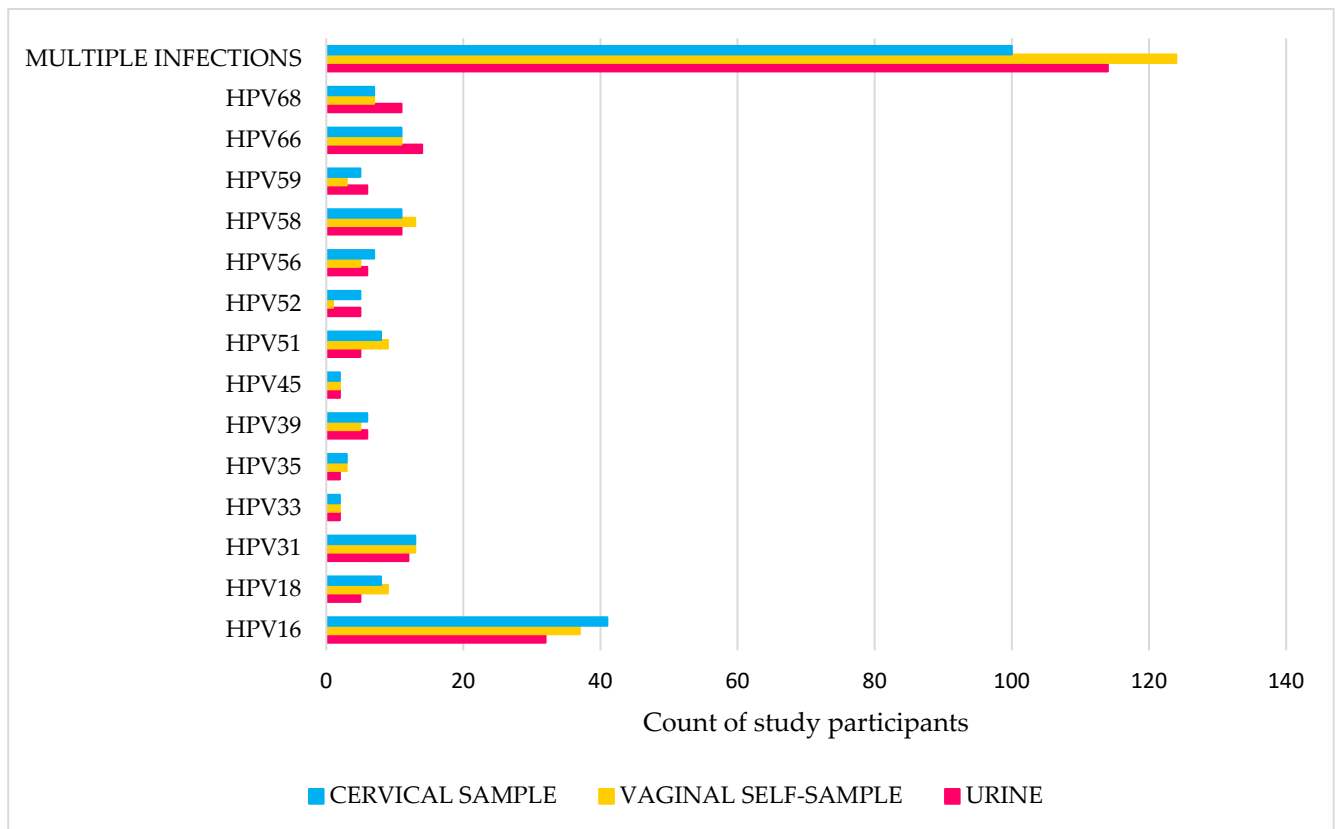


Figure 1. Prevalence of hrHPV genotypes in different samples.

In total, 56.3% (129/229), 49.2% (120/244) and 51.1% (119/233) of the women showed a single infection with only one hrHPV type in the cervical swab, self-collected vaginal and urine samples, respectively, whereas multiple hrHPV infections were detected in 43.7% (100/229), 50.8% (124/244) and 48.9% (114/233) of patients in the cervical samples, vaginal samples and urine samples, respectively (Supplementary Table S1). No statistical difference was found in single and multiple infections rates between sample types (Supplementary Table S2).

2.3. Analytical Sensitivity, Specificity and Type-Specific Agreement of Self-Collected Samples for hrHPV Detection

The analytical sensitivity and specificity for the detection of any hrHPV in vaginal self-collected specimens relative to the results on cervical samples were 99.1% and 85.0%, respectively, while those in urine were 93.4% and 83.5%. Good/substantial agreement was observed between cervical and self-taken samples in detecting hrHPV (agreement 94.4% with $\kappa = 0.870$ and agreement 90.1% with $\kappa = 0.773$ for vaginal and urine specimens, respectively) (Figure 2 and Supplementary Table S3; Figure 3 and Supplementary Table S4).

2.4. STI Prevalence

In total, 47.9% (164/342), 57.9% (198/342), and 56.4% (193/342) of investigated women resulted positive for at least one of the seven STI-related pathogens under investigation in their cervical, vaginal and urine samples, respectively.

Ureaplasma parvum was the most prevalent pathogen in all samples, followed by *UUU* and *MH*. No women showed infection with *NG*. In general, for most of the sexually transmitted pathogens, the detection rate was lower in cervical swabs (Table 2 and Supplementary Figure S1). Still, statistically significant differences using the X^2 test with Yates correction were only found for *UP* positivity rates in vaginal ($p < 0.01$) and urine ($p < 0.05$) specimens compared to cervical samples (Supplementary Tables S5 and S6).



Figure 2. Type-specific detection and test concordance between cervical and vaginal self-samples.

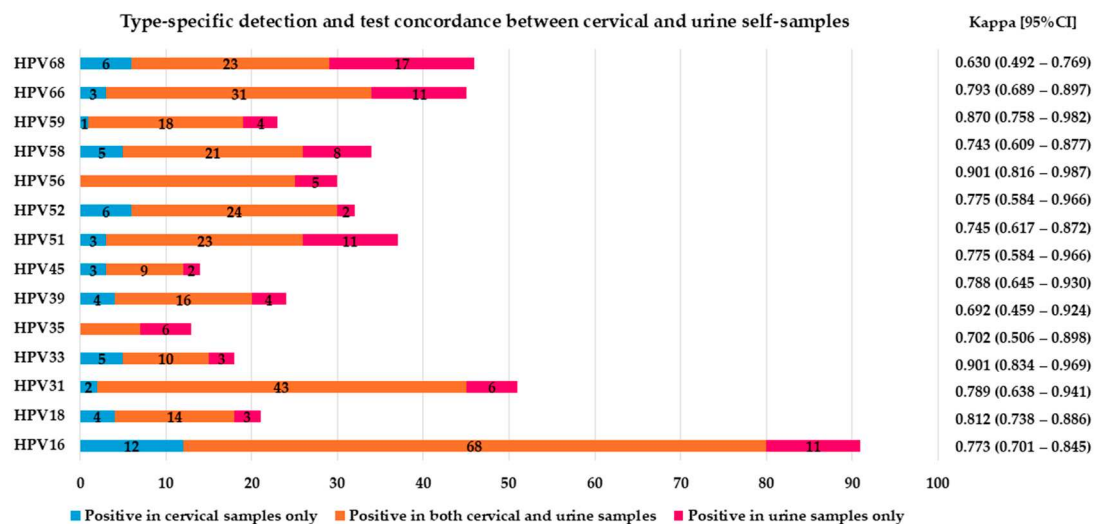


Figure 3. Type-specific detection and test concordance between cervical and urine self-samples.

Table 2. Distribution of STIs in the three sample types.

	UP n (%)	UU n (%)	MH n (%)	MG n (%)	CT n (%)	NG n (%)	TV n (%)
Cervical sample (n = 342)	129 (37.7%)	34 (9.9%)	31 (9.1%)	8 (2.3%)	11 (3.2%)	0 (0%)	4 (1.2%)
Vaginal swab (n = 342)	165 (48.2%)	40 (11.7%)	40 (11.7%)	11 (3.2%)	13 (3.8%)	0 (0%)	3 (0.9%)
Urine (n = 342)	158 (46.2%)	38 (11.1%)	39 (11.4%)	10 (2.9%)	11 (3.2%)	0 (0%)	3 (0.9%)

UP (*Ureaplasma parvum*), UU (*Ureaplasma urealyticum*), MH (*Mycoplasma hominis*), MG (*Mycoplasma genitalium*), CT (*Chlamydia trachomatis*), NG (*Neisseria gonorrhoeae*), TV (*Trichomonas vaginalis*).

The majority of women with positivity for the STIs molecular panel were infected by a single microorganism in all sample types (cervical samples: 74.4%; vaginal self-collected samples: 72.2%; and urine: 73.1%) (Supplementary Tables S7 and S8).

2.5. Analytical Sensitivity, Specificity and Agreement of Self-Collected Samples for STIs Detection

Considering the results of the clinician-collected cervical samples as reference, the analytical sensitivity and specificity for the detection of any of the STIs of vaginal self-collected specimens were 98.8% and 79.8%, respectively, while those of urine were 95.1% and 79.2%.

The agreement between cervical and self-taken samples for detecting STIs was found to be significant (agreement 88.9% with $\kappa = 0.779$ and agreement 86.8% with $\kappa = 0.738$

for vaginal and urine specimens, respectively), with almost perfect agreement between urine and vaginal swab specimens (agreement 95% with $\kappa = 0.899$) (Figures 4 and 5, and Supplementary Tables S9 and S10).

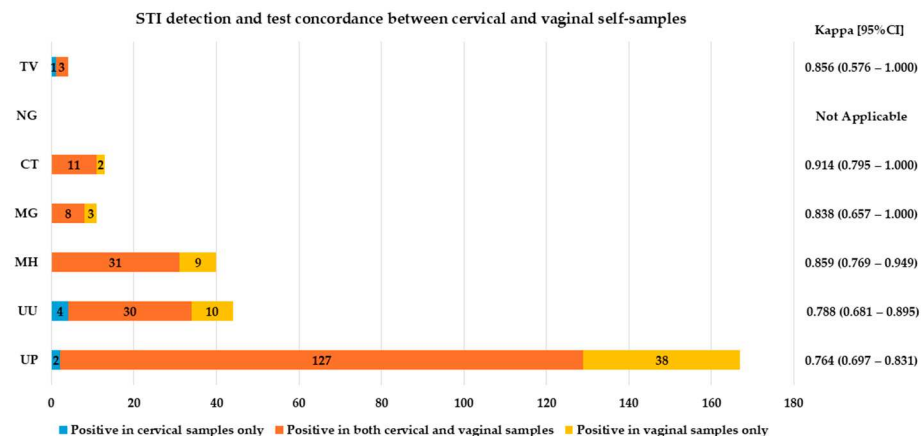


Figure 4. STIs detection and test concordance between cervical and vaginal self-samples. CT (*Chlamydia trachomatis*), NG (*Neisseria gonorrhoeae*), TV (*Trichomonas vaginalis*), MH (*Mycoplasma hominis*), MG (*Mycoplasma genitalium*), UU (*Ureaplasma urealyticum*) and UP (*Ureaplasma parvum*); CI (confidence interval); Kappa concordance between the self- and clinician-collected cervical samples is presented as follows: slight ($0.00 < \kappa < 0.20$), fair ($0.21 < \kappa < 0.40$), moderate ($0.41 < \kappa < 0.60$), substantial ($0.61 < \kappa < 0.80$) and almost perfect ($0.81 < \kappa < 1.00$).

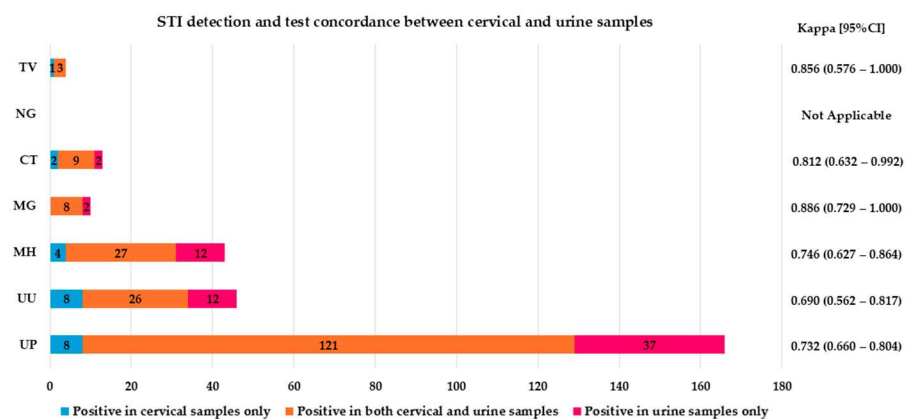


Figure 5. STI detection and test concordance between cervical and urine self-samples. CT (*Chlamydia trachomatis*), NG (*Neisseria gonorrhoeae*), TV (*Trichomonas vaginalis*), MH (*Mycoplasma hominis*), MG (*Mycoplasma genitalium*), UU (*Ureaplasma urealyticum*) and UP (*Ureaplasma parvum*); CI (confidence interval); Kappa concordance between the self- and clinician-collected cervical samples is presented as follows: slight ($0.00 < \kappa < 0.20$), fair ($0.21 < \kappa < 0.40$), moderate ($0.41 < \kappa < 0.60$), substantial ($0.61 < \kappa < 0.80$) and almost perfect ($0.81 < \kappa < 1.00$).

2.6. hrHPV and STI Prevalence in the Different Age Groups

Figure 6 shows a comparison between hrHPV and STI prevalence in cervical, vaginal and urine samples across the different age groups of women referred to colposcopy for abnormal cervical cytology. hrHPV prevalence was higher in the younger age groups in all sample types. In cervical samples, the highest hrHPV prevalence (75.5%) was observed in the 30–40 age group of women, whilst hrHPV prevalence was slightly higher in self-collected samples compared to cervical samples among women younger than 30.

Younger women had also a higher prevalence of STIs. In the <30 years of age group, 55.2% of cervical samples, 71.3% of vaginal self-samples and 69.0% of urine were STI-positive. The STIs rates decreased with age, with the lowest prevalence in the >60 years of group, where all sample types showed a prevalence of 25%.

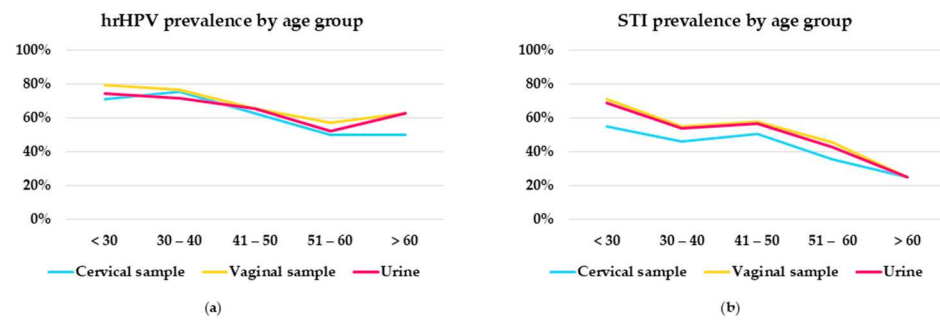


Figure 6. Comparison of hrHPV (a) and STI (b) prevalence by age group.

2.7. hrHPV and STI Co-Infections

Table 3 shows a comparison between STI prevalence in hrHPV-positive and hrHPV-negative women. For almost all the STIs investigated, the positivity rate among hrHPV-positive women was higher compared to the hrHPV-negative group in all sample types. The difference was statistically significant for *UP* and *MH* in all sample types and for *MG* in vaginal swabs. Details on the statistical analysis conducted are reported in Supplementary Table S11.

Table 3. Distribution of STIs in hrHPV-positive and -negative women in the three sample types.

		<i>UP</i>		<i>UU</i>		<i>MH</i>		<i>MG</i>		<i>CT</i>		<i>NG</i>		<i>TV</i>	
		<i>n</i> (%)	<i>p</i>	<i>n</i> (%)	<i>p</i>	<i>n</i> (%)	<i>p</i>	<i>n</i> (%)	<i>p</i>	<i>n</i> (%)	<i>p</i>	<i>n</i> (%)	<i>p</i>	<i>n</i> (%)	<i>p</i>
Cervical sample	hrHPV-positive (<i>n</i> = 229)	102 (44.5%)	0.0003	27 (11.8%)	0.15	29 (12.7%)	0.0005	8 (3.5%)	0.06	10 (4.4%)	0.11	0 (0%)	1	3 (1.3%)	1
	hrHPV-negative (<i>n</i> = 113)	27 (23.9%)		7 (6.2%)		2 (1.8%)		0 (0%)		1 (0.9%)		0 (0%)		1 (0.9%)	
Vaginal swab	hrHPV-positive (<i>n</i> = 244)	137 (56.1%)	0.000007	32 (13.1%)	0.27	37 (15.2%)	0.001	11 (4.5%)	0.04	12 (4.9%)	0.12	0 (0%)	1	2 (0.8%)	1
	hrHPV-negative (<i>n</i> = 98)	28 (28.6%)		8 (8.2%)		3 (3.1%)		0 (0%)		1 (1.0%)		0 (0%)		1 (1.0%)	
Urine	hrHPV-positive (<i>n</i> = 233)	125 (53.6%)	0.00009	30 (12.9%)	0.18	34 (14.6%)	0.006	9 (3.9%)	0.18	10 (4.3%)	0.18	0 (0%)	1	2 (0.9%)	1
	hrHPV-negative (<i>n</i> = 109)	33 (30.3%)		8 (7.3%)		5 (4.6%)		1 (0.9%)		1 (0.9%)		0 (0%)		1 (0.9%)	

hrHPV (high-risk Human Papillomavirus), *UP* (*Ureaplasma parvum*), *UU* (*Ureaplasma urealyticum*), *MH* (*Mycoplasma hominis*), *MG* (*Mycoplasma genitalium*), *CT* (*Chlamydia trachomatis*), *NG* (*Neisseria gonorrhoeae*), *TV* (*Trichomonas vaginalis*).

The highest prevalence of co-infections with hrHPV and other sexually transmitted pathogens was found in women under 30 years (46.0%), the lowest in women over 60 years (25.0%). Among women with positive and negative colposcopy outcomes, 45.6% and 33.6% of women showed co-infections with hrHPV and STI pathogens, respectively. Rates of co-infections with hrHPV and sexually transmitted pathogens among women with low-grade cervical lesions ranged from 21.4% to 43.8%, while among those with high-grade cervical lesions, the rates ranged from 34.0% to 50.0%. In the group of women with histologically confirmed $\geq$ CIN 2 lesions, 96.6% of women were hrHPV-positive. However, no difference was observed between those with or without concomitant coinfections with other sexually transmitted microorganisms (48.3% vs. 48.3%), as illustrated in Table 4.

Table 4. Distribution of hrHPV and STI co-infection in cervical samples according to clinical data, including age, cytology, colposcopy and histology results of the study population.

	hrHPV+/STI+	hrHPV+/STI−	hrHPV−/STI+	hrHPV−/STI−	Total
Total Population	130 (38.0%)	99 (28.9%)	34 (9.9%)	79 (23.1%)	342
Age in Years (<i>n</i> = 342)					
<30	40 (46.0%)	22 (25.3%)	8 (9.2%)	17 (19.5%)	87

Table 4. Cont.

	hrHPV+/STI+	hrHPV+/STI−	hrHPV−/STI+	hrHPV−/STI−	Total
30–40	40 (37.7%)	40 (37.7%)	9 (8.5%)	17 (16.1%)	106
41–50	34 (34.3%)	27 (27.3%)	16 (16.2%)	22 (22.2%)	99
51–60	13 (31.0%)	8 (19.0%)	2 (4.8%)	19 (45.2%)	42
>60	2 (25.0%)	2 (25.0%)	0 (0%)	4 (50.0%)	8
Cytology (n = 342)					
NILM	0 (0%)	1 (12.5%)	1 (12.5%)	6 (75.0%)	8
ASCUS	26 (30.6%)	26 (30.6%)	10 (11.8%)	23 (27.0%)	85
AGC	3 (21.4%)	2 (14.3%)	1 (7.1%)	8 (57.1%)	14
LSIL	71 (43.8%)	41 (25.3%)	15 (9.3%)	35 (21.6%)	162
ASCH	13 (50.0%)	8 (30.8%)	0 (0%)	5 (19.2%)	26
HSIL	16 (34.0%)	22 (46.8%)	7 (14.9%)	2 (4.3%)	47
Colposcopy (n = 342)					
Negative	73 (33.6%)	53 (24.4%)	28 (12.9%)	63 (29.1%)	217
Positive	57 (45.6%)	46 (36.8%)	6 (4.8%)	16 (12.8%)	125
Histology (n = 84)					
<CIN 2	10 (41.7%)	6 (25.0%)	2 (8.3%)	6 (25.0%)	24
≥CIN 2	29 (48.3%)	29 (48.3%)	2 (3.4%)	0 (0%)	60

hrHPV (high-risk Human Papillomavirus), STI (sexually transmitted infection), HSIL (high-grade squamous intraepithelial lesion); ASCUS (atypical squamous cells—cannot exclude HSIL); LSIL (low-grade squamous intraepithelial lesion); ASCUS (atypical squamous cells of undetermined significance); AGC (atypical glandular cell); NILM (negative for intraepithelial lesion or malignancy); <CIN 2 (cervical intraepithelial neoplasia inferior to grade 2); ≥CIN 2 (cervical intraepithelial neoplasia grade 2 or worse).

3. Discussion

In the present study, good analytical agreement was observed between cervical samples and self-collected vaginal and urine samples for the detection of hrHPV, in accordance with data reported by our group in a similar study that investigated the detection of both low- and high-risk HPV genotypes among women referred to colposcopy [12]. The concordance rate of cervical and vaginal specimens was higher than that of cervical and urine samples, consistent with previously reported data [12,28]. Cervical samples confirmed a higher prevalence of single hrHPV infections compared to self-collected samples [12]. These discrepancies may be related to the different anatomical sites of sample collection.

Self-collected specimens have been reported as a valid non-invasive alternative for the detection of hrHPV, contributing to improving women's participation in HPV screening, and promoted by the World Health Organization (WHO) as a key strategy to accelerate the global fight against cervical cancer [6]. Furthermore, self-collection has shown good acceptability in low-resource settings, improving success in cervical cancer elimination [29,30] by facilitating women's access to screening, reducing public health costs and alleviating feelings of shame and discomfort by offering privacy to women [29].

The acceptability of self-collection in cervical cancer prevention depends on socio-cultural aspects and women's perceptions, but it can also be influenced by the attitude of providers who may express doubts about patients' ability to adequately perform self-collection [31]. Currently, countries such as New Zealand, Australia, the Netherlands, France and Sweden have included self-sampling in their national cervical cancer screening programs [5,11].

The present study has also demonstrated a substantial agreement between self-collected and cervical samples for the detection of STIs in asymptomatic women referred to colposcopy, with the only exception of *UIP*, which showed higher positivity rates in self-collected samples. *UIP* is commonly found in the genital tract of healthy people, so its pathogenic role can be difficult to prove [32]. Low *MG*, *CT*, *NG* and *TV* positivity rates were detected in this study, probably due to the fact that participants were asymptomatic and recruited when referred to colposcopy for an abnormal Pap smear.

The good analytical sensitivity and specificity for STIs in self-collected samples compared to clinician-collected samples should, however, be further confirmed using diagnostic test accuracy studies to compare the clinical accuracy of different sample types, as recom-

mended for the validation of the hrHPV test by the VALHUDES protocol [33]. The correct assessment of the clinical performance of STI tests, associated with potential false positive and/or false negative results, is essential to understand the cost-benefit of introducing self-sampling as a potential STIs screening method for the general population. Moreover, it is fundamental to understand the clinical relevance of each microorganism and the eventual need for treatment depending on the woman's clinical presentation. This is particularly true in the case of samples collected from the lower genital tract where more infections from the *Mycoplasmataceae* family have been reported. The improved detection and treatment of STIs may reduce the transmission of these infections, but their over-treatment may be responsible for increased antimicrobial resistance and anxiety in patients receiving a positive result.

In the present study, STIs were more frequently detected among hrHPV-positive than hrHPV-negative women. While persistent hrHPV infections have been demonstrated to be the necessary cause of the onset of cervical precancer and cancer, the potential role of other STIs as cofactors in the development of cervical lesions is still controversial. Some studies have suggested an association between the presence of STIs and a higher risk of developing high-grade cervical lesions [18,19,23,34,35], whilst others have reported no association [24,36].

It is well documented that productive HPV infection is favoured at the ectocervix and that lesion formation begins from the infection of an epithelial stem cell at the transformation zone or endocervix [37]. Changes in vaginal microbiota and infections with other sexually transmitted pathogens have been proposed to act as cofactors in determining HPV-related disease, potentially facilitating viral entry and persistence through chronic cervical inflammation and the ulceration of the upper layers of the cervical epithelium, as well as through a reduction in host cell-mediated immunity [38]. The increased probability of viral entry at the basal layer makes it easier for the virus to persist inside the cells and to advance viral genome integration in the human host cell. Viral integration activates the overproduction of E6 and E7 oncoproteins that are associated with different carcinogenesis pathways [39].

Persistent cervicitis might also enhance the progress of undetected precancerous cervical lesions [25,40]. In fact, previously published reports indicate that cervical carcinogenesis is associated with inflammation [25,40], driven by the hormonal milieu, regulatory cytokines and chemokines, as well as multiple cervicovaginal microorganisms [41]. Inflammation may disrupt the homeostasis of the genital tract, facilitating the entry of virions. The chronic inflammation caused by *Ureaplasma* spp. infections might favour the entry of other microorganisms or induce chromosomal alterations that might lead to the carcinogenesis of epithelial cells [42]. In an earlier study, Lukic et al. postulated that *UUU* is related to HPV persistence and early cervical cytological changes [43] through several inflammatory responses, involving the production of reactive oxidative metabolites, the increased expression of cytokines, chemokines and angiogenic factors, decreased cell-mediated immunity and the generation of free radicals [26,35]. Moreover, it is well known that the immunological status of a patient may influence the persistence of HPV infection. The cross-sectional design of the study cannot assess the potential role of persistent STIs and hrHPV infections in cervical lesion progression.

In this study, statistically significant differences were found in the distribution of *UP* and *MH* between hrHPV-positive and hrHPV-negative women in all sample types and of *MG* in vaginal self-samples. Similar results have been previously reported in cervical samples with a significantly higher *UP* infection rate in the HPV-positive group of women compared to the HPV-negative group [27]. In a previous study, Parthenis et al. recruited 345 asymptomatic women participating in routine cervical cancer screening and

reported *Ureaplasma* spp. as the most frequently isolated microorganism detected in 30.2% of hrHPV-positive women [44]. Verteramo et al. also showed an increased infection rate of *UU* in HPV-positive women [38]. *Mycoplasma* infections have been linked to “in vitro” chromosomal changes and cell transformation [45,46]. The association between *CT* and cervical cancer has also been widely investigated [23,26,38,47,48]. *CT* might increase susceptibility to HPV causing micro-abrasions or cervical epithelial cells and molecular alterations, facilitating the entry of virions [23,38]. However, in this study, no significant difference was found between *CT*, *TV* and *UU* distribution in hrHPV-positive women and hrHPV-negative women, probably because of the low frequency of these infections in our population.

The results of the present study further confirm the high prevalence of STIs in young women, as previously described in the literature [49,50]. The risk of acquiring sexually transmitted infections decreases with the decrease in number of sexual encounters, usually associated with an older age. Since this study was conducted in a colposcopy setting, as expected, the prevalence of hrHPV infections was very high as well as the number of women with confirmed cervical lesions. As a result, the high hrHPV positivity rate found in older women may not reflect the trend of hrHPV infections distribution in the general population. The choice of enrolling women in a colposcopy setting was determined by the possibility to investigate the performance of the molecular assays on cervical and self-collected samples on a smaller study population with expected higher positivity rates for hrHPV and STIs; however this may also be considered as a limitation of the present work as it does not provide data on the prevalence of hrHPV and STIs in the general population. Moreover, the limited number of participants and the cross-sectional nature of the study do not allow to completely understand the potential role of hrHPV-STI co-infections in affecting the progression of cervical lesions. Future longitudinal studies, including a larger number of women, will allow to better investigate the possible role of coinfections in cervical lesion development.

In conclusion, the present study confirms that self-taken specimens may be a good alternative for the diagnosis and screening of both hrHPV and other STIs. Due to the increase in the STI rate recently reported, associated with asymptomatic and complicated infections [15], it is important to consider the possibility to include molecular screening for STIs on self-samples collected from women who participate in cervical cancer screening programs. At the same time, improving sexual education, especially among young people, may help to control the transmission of these pathogens and move towards the elimination of STIs and cervical cancer.

4. Materials and Methods

4.1. Study Design and Sample Collection

For this cross-sectional study, 345 consecutive women from 18 to 69 years (median age 39 years, IQR: 29–46 years) were enrolled at the Colposcopy Clinics of Fondazione IRCCS San Gerardo dei Tintori (Monza, Italy) from May 2017 to September 2024, following the signing of informed consent. Women were referred to colposcopy because of a recent abnormal cervical cytology result. All women were asymptomatic. The following exclusion criteria were applied: immunocompromising or the presence of HIV infection, the state of pregnancy, presumed or ascertained, the presence of immune and autoimmune system diseases, a diagnosis of malignant tumour pathology, and chemotherapy in progress or completed in the 6 months prior to the study.

Prior to the gynaecological examination, all women were asked to autonomously collect a first-void urine sample using a Colli-Pee® device (Novosanis, Belgium) and a vaginal swab using FLOQSwab® 552.80 (Copan Italia Spa, Brescia, Italy).

Before performing the colposcopy, the physician collected a cervical specimen from each woman using an L-shaped Endo/Esocervical FLOQSwab® (Copan Italia Spa, Brescia, Italy) that was immediately resuspended into 20 mL of ThinPrep® PreservCyt® Solution (HOLOGIC™, Marlborough, MA, USA).

Based on the colposcopy findings and clinical judgment, women underwent biopsy and/or treatment with conisation.

The classification of cytological lesions was conducted according to the Bethesda system [51], whilst the histological outcomes were classified according to the WHO histological classification of tumours [52]. Histological lesions worse than cervical intraepithelial neoplasia grade 2 ($\geq$ CIN2) were considered high-grade lesions.

This study was conducted following the approval of the Ethics Committee of the University of Milano-Bicocca (Protocol n. 0037320/2017 and update n. 0086409/2018).

4.2. Pre-Analytical Sample Processing and Nucleic Acid Extraction

All samples were processed at the Laboratory of Clinical Microbiology of the University of Milano-Bicocca, Monza, Italy. On their arrival at the laboratory, cervical samples were vortexed for 30 s and aliquots of 1.5 mL were dispensed into sterile cryotubes and stored at -20°C until testing.

First-void urine collected using Colli-Pee® was also shaken on the vortex for 30 s and aliquots of 1.5 mL were stored at -20°C in sterile cryotubes until testing.

Vaginal self-samples were transported dry at the laboratory where they were suspended in 5.5 mL of ThinPrep® PreservCyt® Solution. Moreover, 1 mL was then dispensed into sterile cryotubes and stored at -20°C until testing.

In total, 200 μL of all sample types were used to perform nucleic acid extraction using the STARMag 96 $\times$ 4 Universal Cartridge Kit (Seegene, Seoul, Republic of Korea) on the MicroLab Nimbus workstation (Hamilton, Reno, NV, USA) with a final elution volume of 100 μL .

4.3. HPV and STIs Detection

The Nimbus platform allows the real-time PCR plate preparation of Anyplex™ II HR HPV (Seegene, Seoul, Republic of Korea) and Anyplex™ II STI-7e (Seegene, Seoul, Republic of Korea) assays.

Anyplex™ II HR HPV is a full genotyping HR-HPV assay that individually detects 14 different genotypes of hrHPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and a cellular gene target by melting curve analysis. The analysis is performed on the CFX96 (Bio-Rad, Hercules, CA, USA) with 5 μL of template DNA in a total volume of 20 μL , as indicated in the manufacturer's instructions.

Anyplex™ II STI-7e allows the detection of 7 sexually transmitted pathogens, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, *Mycoplasma hominis*, *Mycoplasma genitalium*, *Ureaplasma urealyticum* and *Ureaplasma parvum*. According to the manufacturer's instructions, the real-time PCR analysis is performed on the CFX96 using 5 μL of template DNA in a total volume of 20 μL .

Data interpretation of the results obtained with both assays was conducted using the Seegene Viewer software (V3 version) according to the manufacturer's instructions.

Samples that were invalid according to the software interpretation were retested. After two invalid results, samples were excluded from the analysis.

4.4. Statistical Analysis

Qualitative and quantitative variables were summarized using absolute and relative (percentage) frequencies and medians (interquartile ranges, IQR), respectively. Concordance between the results of cervical and self-collected specimens with the two assays

was evaluated using Cohen's kappa (κ) statistics and defined as follows: slight ($0.00 < \kappa < 0.20$), fair ($0.21 < \kappa < 0.40$), moderate ($0.41 < \kappa < 0.60$), substantial ($0.61 < \kappa < 0.80$) and almost perfect ($0.81 < \kappa < 1.00$). Statistical analyses were performed with R software (R Core Team 2021 v. 4.4.0). Statistical significance between positivity rates was calculated using Pearson's Chi-squared (χ^2) test with Yates' correction or Fisher's exact test, as appropriate (Supplementary Tables S2, S5, S6, S8 and S11).

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms26031296/s1>.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Final study datasets generated by the study are stored locally and securely at the University of Milano-Bicocca. Anonymized data will be available by request to the corresponding author on a case-by-case basis pending approval by the University of Milano-Bicocca.

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Conflicts of Interest: The Clinical Microbiology Research Group of the University of Milano-Bicocca received free cervical and vaginal self-sample collection devices from Copan Italia Spa (Brescia, Italy), free Colli-pee® (20 mL) from Novosanis (Belgium), and STARMag 96 × 4 Universal Cartridge Kit, Anyplex™ II HR HPV and Anyplex™ II STI-7e from Arrows Diagnostics (Italy). The University of Milano-Bicocca also received research grants from BD Diagnostics, Seegene, Arrows Diagnostics, Copan, GeneFirst, Hiantis and VITRO. C.E.C. is a minority shareholder of Hiantis. C.G., M.M., M.R., M.L.D.M., R.C.N., F.P., G.M., R.M., R.F., and F.L. declare no conflicts of interest.

Abbreviations

AGC	Atypical glandular cell
ASCH	Atypical squamous cells—cannot exclude HSIL
ASCUS	Atypical squamous cells of undetermined significance
CI	Confidence interval
CIN 1	Cervical intraepithelial neoplasia grade 1
CIN 2	Cervical intraepithelial neoplasia grade 2
CIN 3	Cervical intraepithelial neoplasia grade 3
CT	<i>Chlamydia trachomatis</i>
ECDC	European Centre for Disease Prevention and Control
hrHPV	High-risk Human Papillomavirus
HPV	Human Papillomavirus

HSIL	High-grade squamous intraepithelial lesion
IARC	International Agency for Research on Cancer
κ	Cohen's kappa
IQR	Interquartile ranges
LSIL	Low-grade squamous intraepithelial lesion
MG	<i>Mycoplasma genitalium</i>
MH	<i>Mycoplasma hominis</i>
NG	<i>Neisseria gonorrhoeae</i>
NILM	Negative for intraepithelial lesion or malignancy
STI	Sexually transmitted infection
TV	<i>Trichomonas vaginalis</i>
UP	<i>Ureaplasma parvum</i>
UU	<i>Ureaplasma urealyticum</i>
VALHUDES	Validation of Human Papillomavirus Assays and Collection Devices for Self-samples and Urine Samples
WHO	World Health Organization

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