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REVIEW ARTICLE

Cell-Free HPV-DNA in Screening, Diagnosis, Prognosis, and Treatment Response Monitoring of Cervical Cancer

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Abstract

Persistent infection with high-risk human papillomavirus (HPV) is a significant factor in cervical cancer (CC) development. Although CC screening programs have reduced the incidence of this neoplasm, the number of deaths remains high, especially in developing countries: CC remains the fourth most common neoplasm in the female population globally. Currently, an HPV test has been replacing cytological analysis because it is a more sensitive screening method. However, the collection of gynecological material is still necessary, which can be a barrier to adherence to testing in the target population. Host cells presenting with a viral infection release fragments of their DNA into circulation, known as cell-free DNA (cfDNA); this allows detection through venous puncture, a routine procedure in clinical laboratories. Thus, the objective of this review was to evaluate the role of cfDNA of HPV (cfHPV-DNA) as an alternative tool for CC screening, diagnosis, prognosis, and treatment response monitoring. Furthermore, the development of sensitive methods, such as droplet digital PCR (ddPCR) and next-generation sequencing (NGS), have proven useful in identifying tumor markers for CC. The specificity of the primers, the size of the target DNA fragments, and variables such as sample type and volume, in addition to the cfDNA extraction kit used, can influence the results of cfHPV-DNA detection. Although the detection of cfHPV-DNA in plasma and serum of patients with CC is feasible, there were conflicting results regarding cfHPV-DNA detection in the blood circulation of patients with premalignant lesions. On the other hand, when CC is already established, the detection and quantification of cfHPV-DNA have shown potential as a biomarker for tumor staging, prognosis definition, and treatment response monitoring.

Key Points

cfHPV-DNA appears to be a promising biomarker for cervical cancer, being useful in the diagnosis, prognosis, and treatment monitoring of women with this neoplasm.

There are conflicting results regarding cfHPV-DNA detection in the blood circulation of patients with premalignant lesions.

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1 Introduction

Cervical cancer (CC) results from a continuous process, starting with the infection of the cervical epithelium by high-risk human papillomavirus (hr-HPV), development of premalignant lesions, and invasive carcinoma. However,

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in most cases, the process from HPV infection to cancer development occurs over a period of years and is naturally reversible, since the immune system can combat and eliminate the viral infection. When HPV persists and leads to the development of precancerous lesions, these can be successfully treated in most cases if diagnosed early [1, 2]. The introduction of HPV vaccines in many countries has shown positive effects on the incidence of pre-neoplastic lesions and invasive cancer, yet most women in the world are not vaccinated [3]. Thus, despite being a preventable outcome, incidence and mortality rates caused by CC remain high, especially in developing countries. CC remains the fourth most common type of neoplasm in the global female population, with an estimated 661,021 new cases and 348,189 deaths in 2022 [4].

The most common symptoms of CC are bleeding after sexual intercourse, intermenstrual bleeding, and vaginal discharge. However, these symptoms are common in young women with non-malignant conditions such as genital infections. Moreover, even though symptom overlap contributes to diagnostic delays, the late identification of the condition is also affected by factors such as insufficient screening and a low level of clinical suspicion during asymptomatic phases. Thus, more than 55% of patients are diagnosed with the disease at advanced stages (stage II–IV according to the International Federation of Gynecology and Obstetrics (FIGO)), with high rates of recurrence and mortality [5–8].

Once CC is established, clinical data such as cervical cytology, HPV status, tumor stage, as well as imaging tests are used for treatment evaluation and monitoring of recurrence. However, the prognostic predictability of existing biomarkers and their ability to discriminate against residual tumors remain limited. Measurement of tumor markers in blood, such as squamous cell carcinoma antigen (SCC), carcinoembryonic antigen (CEA), and CA125, are also used for disease monitoring and treatment response assessment. However, the clinical value of serum levels of these markers is limited, as they are expressed only by a small proportion of patients and may change during the course of the disease. Moreover, invasive procedures such as biopsy continue to be necessary [9–11]. On the other hand, liquid biopsy allows for identification of genetic information from body fluids such as blood, urine, or saliva, in a less invasive manner than tissue biopsies. Liquid biopsies can be easily obtained, thus facilitating monitoring throughout the course of the disease [12–14]. Cells release DNA fragments into the bloodstream, known as cell-free DNA (cfDNA), due to cell death or secretion processes. Thus, the objective of this review is to describe the role of cell-free DNA of HPV (cfHPV-DNA) as a biomarker of CC, considering its application in the screening of this neoplasm, as well as in evaluating the course of the disease.

2 HPV and Cervical Cancer

HPV is a non-enveloped virus approximately 55 nm in diameter. Its genome is a circular double-stranded DNA molecule of approximately 8000 bp divided into a control region (Long Control Region (LCR)) and eight open reading frames (ORF)—*E1, E2, E4, E5, E6, E7, L1*, and *L2* [2] (Fig. 1A). Expression of the viral non-structural genes (*E*) delays cell differentiation and promotes replication of infected cells, allowing for amplification of the viral genetic material. After expression of the structural genes (*L*), already in mature epithelial cells, the viral genome is packaged, and mature virions are released, which are capable of infecting other cells [2, 15–17]. Integration of the viral genome into the host DNA is likely one of the decisive steps for oncogenesis. With the interruption of the *E2* gene, overexpression of oncogenes *E6* and *E7* occurs, due to the loss of *E2*-mediated repression of the transcription of these genes (Fig. 1A). It occurs preferentially in transcriptionally active regions, near DNA sites that are weakened, and are observed in most invasive cancers. Additionally, in some low-grade lesions and most high-grade lesions, the HPV genome is integrated into the chromosomes [18, 19].

More than 200 types of HPV have been identified and classified phylogenetically, with some viral types classified epidemiologically according to their oncogenic risk [20]. hr-HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) are considered associated with CC, included in Category 1A of carcinogenicity by the International Agency for Research on Cancer (IARC). Among them, types 16 and 18 are the most prevalent, accounting for approximately 70% of CC cases [20–23].

The development of CC basically occurs through four stages: infection with hr-HPV, persistence of viral infection, development of pre-cancerous lesions, and establishment of invasive cancer [16, 24] (Fig. 1B). Although the entire anogenital epithelium can be infected by HPV, the transformation zone of the cervix, an area composed of metaplastic tissue located between the vaginal squamous epithelium and the glandular tissue of the endocervical canal, is especially susceptible to carcinogenesis. Approximately 75% of squamous cell carcinoma cases occur in the ectocervix, while adenocarcinomas more frequently affect the endocervical region [24, 25].

HPV replicates in the stratified squamous epithelium, using tissue differentiation to regulate its replication (Fig. 1B). Therefore, infection requires the availability of epidermis or mucosal epithelial cells that are still capable of proliferating, in addition to interaction of the virus with proteoglycans on the cell surface. Virus-infected basal layer cells divide, and the infection spreads laterally. Some

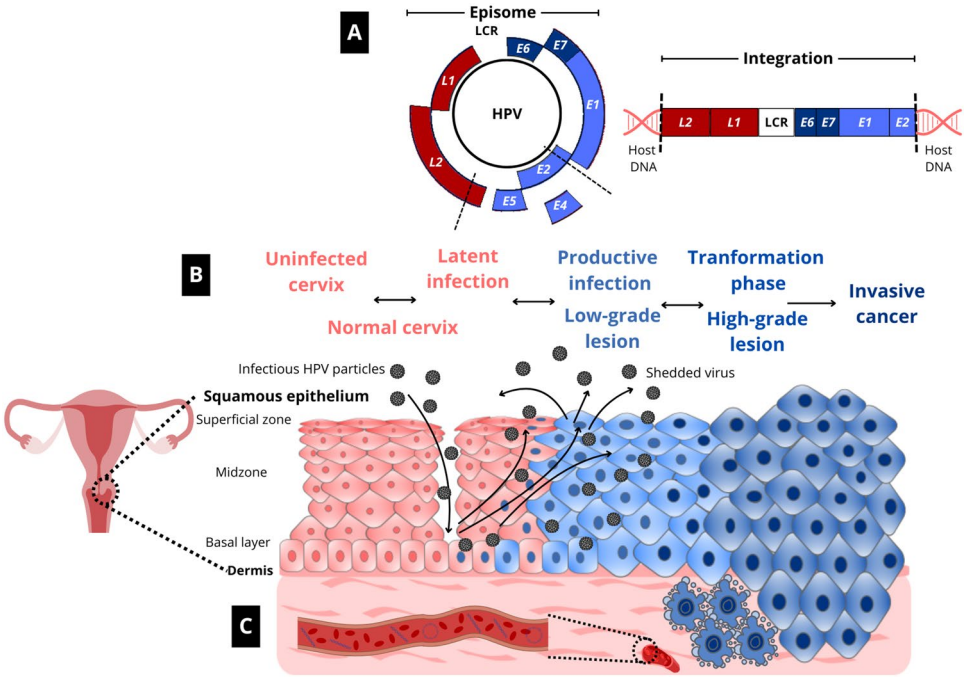

Fig. 1 HPV and cervical carcinogenesis. **A** The HPV genome is a circular double-stranded DNA molecule of approximately 8000 bp, divided into a control region (long control region (LCR)) and eight open reading frames (ORFs)—*E1, E2, E4, E5, E6, E7, L1*, and *L2*. When the viral DNA integrates into the host genome, the *E4/E5* genes, and part of the *E2* and *L2* genes are deleted. **B** The entire anogenital epithelium can be infected by HPV, but the transformation zone of the cervix is especially susceptible to carcinogenesis. HPV replicates in the stratified squamous epithelium, utilizing tissue differentiation to regulate its replication. Viral infection may regress or progress, leading to the appearance of cytopathological abnormalities. **C** Infected cells release HPV-DNA fragments into blood circulation, known as cell-free HPV-DNA (cfHPV-DNA), due to cell death or secretion processes. The cfHPV-DNA will contain specific viral (onco)genes

of the descendant cells migrate to the suprabasal cell layer, where viral genes are activated [26].

For most types of HPV infections, little or no local pathology and absence of measurable adaptive immune response is observed, with no seroconversion noted in more than 40% of infected women. Many infections are transient and resolve within 1 to 2 years (*latent infection*), and result in little or no local pathology (Fig. 1B). However, viral infection persists in some cases, resulting in the development of low-grade pre-neoplastic cervical lesions. Expression of the viral *E* genes delays cell differentiation and promotes replication of infected cells, allowing for amplification of the viral genetic material. After expression of the structural genes (*L*), already in mature epithelial cells, the viral genome is packaged, and mature virions are released, capable of infecting other cells (*productive infection*). The infection may persist and progress, leading to high-grade precancer, and the integration of the viral genome into the host DNA is likely one of the decisive steps for oncogenesis (*transformation phase*) (Fig. 1B) [16, 17, 27].

In the absence of treatment, the transition from dysplasia to invasive carcinoma can take years to occur in most women. However, in about 10% of cases, progression to cancer occurs in less than a year [25]. In addition to the immune response, other factors modulate the course of viral infection, the infecting viral type (hr-HPV tending towards more persistent infection), age (older individuals have a higher chance of infection persistence), nutritional status, and genetic variations [28–31].

3 Laboratory Evaluation of cfHPV-DNA

3.1 Cell-Free DNA

In the last decade, body fluids have been gaining attention for biomarker detection, as they are easy to collect and handle, and readily transferrable to clinical practice. The detection of cfDNA originating from tumor cells (circulation-tumor DNA (ctDNA)) has been used for diagnosis, molecular characterization, treatment monitoring, and prognosis assessment of various types of cancer. Since the half-life of cfDNA is very short (about 2 h), ctDNA provides current molecular status of tumor. CC differs from other neoplasms because HPV infection is a critical step in carcinogenesis. Infected cells release HPV-DNA fragments into blood circulation,

known as cell-free HPV-DNA (cfHPV-DNA), due to death of tumor cells by apoptosis, necrosis, pyroptosis, phagocytosis, cytoplasmic swelling (oncosis) (Fig. 1C) [32–34]. Therefore, the detection of the HPV DNA in the blood of these patients could be considered a liquid biopsy with diagnostic and prognostic potential (Fig. 2A) [7].

Studies indicate that cfHPV-DNA detection may be more sensitive than ctDNA detection of point mutations in genes associated with cancer development. This is attributed to the presence of multiple (one to several thousand) HPV DNA copies in each tumor cell, which are released into the bloodstream upon cell death, potentially increasing detection rates [32, 34].

We found studies using next-generation sequencing (NGS), droplet digital PCR (ddPCR), mass spectrometry, real-time PCR, nested PCR and conventional PCR, published from 2003 to 2025 (to date), for evaluation of cfHPV-DNA among women with CC or preneoplastic cervical abnormalities. Different methods and kits were used to extract cfDNA, from serum or plasma collected in EDTA or in a specific medium for conservation of the sample (Tables 1, 2, 3).

3.2 Blood Collection, cfDNA Extraction, and Methods for cfHPV-DNA Detection

Initially, the detection of cfHPV-DNA in the blood of women with cervical intraepithelial neoplasia (CIN) or CC was performed using the same techniques employed for HPV detection in cervical samples, such as conventional PCR, and later, real-time PCR (Fig. 2B). However, the rates of viral detection in the blood were highly variable due to the low clinical sensitivity of these methods against the low concentration of cfHPV-DNA. On the other hand, with the development of more sensitive techniques, such as ddPCR and NGS, the detection and quantification of cfHPV-DNA have been proving useful as a tumor marker for CC (Fig. 2A).

Both NGS and ddPCR have been demonstrated to be more accurate than real-time PCR to study cfHPV-DNA, as well as NGS being a high throughput technique. The use of endogenous control to ensure the presence of human DNA in the sample is essential to guarantee that samples negative for cfHPV-DNA are truly negative.

Some methodology-related variables include the type and volume of the analyzed sample, as well as the extraction kit used to extract cfDNA, which may influence the performance of cfHPV-DNA detection. According to Jeannot et al. [35], there was no qualitative difference in cfHPV-DNA detection by ddPCR in serum and plasma samples collected in EDTA from women with CC. However, quantitative assessment showed that the average levels of cfHPV-DNA in plasma were slightly higher (1360 ± 2003 copies/ml) if compared to quantification in serum (1101 ± 1881 copies/ml) ($p = 0.09$) [35]. In a meta-analysis performed by Gu and colleagues [36], it was observed that the sample source (serum vs. plasma) of cfHPV- DNA significantly influenced the heterogeneity of universal diagnostic value. Furthermore, there was lower sensitivity in the analysis of serum or plasma collected in EDTA for cfHPV-DNA detection if compared to studies conducted with blood samples collected in tubes with specific preservatives for cfDNA [37]. It was also observed that there was an increase in the limit of detection (LoD) of up to ten times when using a larger volume of blood and a greater fraction of purified DNA for ddPCR

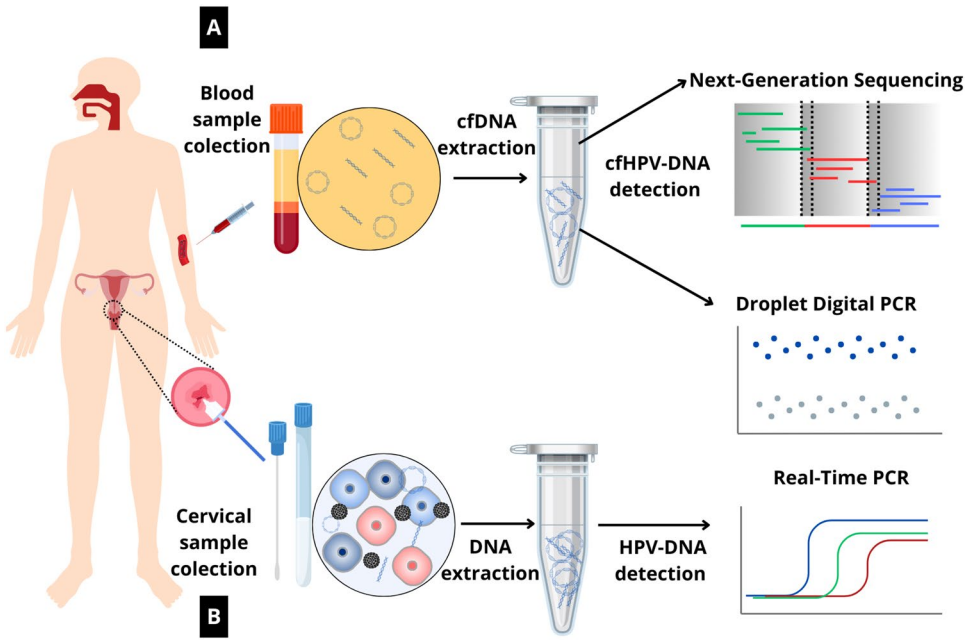

Fig. 2 Methods for laboratory detection of HPV. **A** cfHPV-DNA detection from the bloodstream: the blood sample is collected, and plasma/serum is separated from the leukocytes in the buffy coat and the red blood cells. Then, cfHPV-DNA can be extracted and is analyzed using next-generation sequencing (NGS) or droplet digital PCR (ddPCR), for example. **B** HPV-DNA detection from cervix: the cervical sample is collected, and the epithelial cell are stored in a buffer, such as PBS. Then, HPV-DNA can be extracted and analyzed using real-time PCR, for example

Table 1 Analytical performance techniques used to detect cfHPV-DNA

References	Technique	Sample (collection medium)	cfDNA purification kit	Analytical performance
Han et al. [37]	NGS or ddPCR[a]	Plasma (Cell-free DNA BCT tubes, Streck)	QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany)	LoD NGS: 0.023 copies/ml plasma LoD ddPCR: 0.18 copies/ml plasma
Bonlokee et al. [44]	NGS	Plasma (Cell-free DNA BCT tubes, Streck)	QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany)	LoD: 4 reads/test (LoD among negatives: 1 read/test)
Leung et al. [43]	NGS	Plasma (EDTA)	QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany)	LoD: <1 copies/mL plasma
Seo et al. [40]	ddPCR	Plasma (Cell-free DNA BCT tubes, Streck)	QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany)	LoD: 16 copies/mL plasma
Sivars et al. [58]	ddPCR	Plasma (Cell-free DNA BCT tubes, Streck)	QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany)	LoD: 3 molecules for all HPV types except for HPV31 (LoD 5.4 and HPV58 (LoD 6.0)
Bonlokee et al. [7]	ddPCR	Plasma (Cell-free DNA BCT tubes, Streck)	QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany)	LoD: >3 copies/ml plasma
Cabel et al. [32]	ddPCR	Serum or Plasma	QIAsymphonySP/AS workflow (Qiagen, Courtaboeuf, France)	LoD (log 2 scale): 0.05% for HPV35 and HPV52 0.1% for HPV31, HPV45 and HPV73 0.25% for HPV33 and HPV58
Rungkamoltip et al. [41]	ddPCR	Serum	Maxwell RSCVRccfDNA plasmakit (AS1480; Promega, Madison, WI, USA)	LoD HPV16: 80 copies/mL serum LoD HPV18: 86 copies/mL serum
Kang et al. [38]	ddPCR	Serum	Maxwell RSC Instrument (Promega, Madison, WI, USA)	LoD: ≤50 copies/mL serum
Shimada et al. [42]	Real-Time PCR	Plasma	QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany)	LoD: 50 copies/ml plasma
Wei et al. [84]	Nested PCR	Plasma (EDTA)	QIAmp blood kit (Qiagen, Hilden, Germany)	LoD: one copy/test (1.2×10^{-8} ng/test)

NGS next-generation sequencing, *PCR* polymerase chain reaction, *ddPCR* droplet digital PCR, *LoD* limit of detection

[a]Agreement of 92.8% between the two methods, with those not detected by ddPCR having low levels of cf-HPV-DNA (0.29–17.3 copies/mL)

analysis [38]. Additionally, Diefenbach and colleagues found differences in the performance of six commercially available kits for cfDNA extraction and purification when analyzing the pattern of DNA fragments obtained. In this study, the kits from Qiagen (Hilden, Germany) showed better results [39].

Table 1 presents the analytical performance for cfHPV-DNA detection, according to the technique, target, medium for blood collection, and cfDNA and purification kit. Considering the studies in which blood samples were collected in a specific preservation medium for cfDNA and that this was extracted with a specific kit, the analysis using NGS showed a lower LoD (0.023 copies/ml plasma) when compared to the analyses performed by ddPCR, the LoD of which ranged from 0.18 to 16 copies/ml plasma [37, 40]. On the other hand, when the analysis was performed on serum or plasma collected in EDTA and/or the extraction kit was not specific for cfDNA, the LoD for cfHPV-DNA detection with ddPCR increased to 50–86 copies/ml plasma or serum [38, 41], similar to the performance of real-time PCR (50 copies/ml plasma) [42] (Table 1). It is important to emphasize that the comparison of analytical performance of different studies, which used different ways to normalize the viral load quantification, must be interpreted with caution.

Jeannot and colleagues [35] observed an increase in the detection rate of HPV16 and HPV18 from 60 to 83% of serum samples from women with CC using real-time PCR and ddPCR, respectively. Additionally, with ddPCR, it was possible to detect cfHPV-DNA in two cases with microinvasive cervical carcinomas, with tumors only 1 mm in

Table 2 Clinical performance techniques used to detect cfHPV-DNA for CC diagnosis

References	Technique	Target	Sample (collection medium)	cfDNA purification kit	cfHPV-DNA positive/total (CC samples)	Clinical performance
Bonlokee et al. [44]	NGS	L1/E2/E6/E7 all HPVs	Plasma (Cell-free DNA BCT tubes, Streck)	QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany)	66/139	Sensitivity: 80.6% Specificity: 92.0% PPV: 93.1% NPV: 78.9%
Sastre-Garau et al. [57]	NGS	all HPVs	Plasma	In house method	60/62	Sensitivity: 96.8% Specificity: 100%
Seo et al. [40]	ddPCR	E6/E7 all HPVs	Plasma (Cell-free DNA BCT tubes, Streck)	QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany)	66 LACC	Sensitivity: 72.7%
Gupta et al. [63]	ddPCR	E7 HPV16 and 18	Plasma	Maxwell RSC® ccfDNA Plasma Kit (Promega, Madison, WI, USA)	HPV 16 positive samples: 29/35 HPV 18 positive samples: 16/35	Sensitivity: HPV 16 82.86% HPV 18 45.71%
Sivars et al. [58]	ddPCR	E7 hr-HPV	Plasma (Cell-free DNA BCT tubes, Streck)	QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany)	49/53 LACC[a]	Sensitivity: 92.4%
Rungkamoltip et al. [41]	ddPCR	E7 HPV16 and 18	Serum	Maxwell RSCVRccfDNA plasmakit (AS1480; Promega, Madison, WI, USA)	12/39 (14 FIGO I/II 25 FIGO III/IV)	Sensitivity: 30.77% Specificity: 100% AUC: 0.65
Cheung et al. [49]	ddPCR	E7/L1 HPV 16 and 18	Plasma	QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany)	HPV 16 and 18 positive samples: 85/138	Sensitivity: 61.6% Combining ddPCRtest results for E7 and L1
Han et al. [45]	ddPCR	E6/E7 all HPV	Plasma (EDTA)	QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany)	HPV positive samples: 19/19	Sensitivity: 100%
Galati et al. [59]	E7 type-specific multiplex genotyping assay[d]	E7 HPV 16	Plasma	QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany)	59/79	Sensitivity: 74.7% Specificity: 97.8%
Herbst et al. [60]	Mass Spectrometry	24 HPV types, including hr-HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68)	Plasma (EDTA)	QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany)	26/40	Sensitivity: 65.0% (95% CI = 50.0–80.0%).

LLAC local advanced cervical cancer, *NGS* next-generation sequencing, *PCR* polymerase chain reaction, *ddPCR* droplet digital PCR, *PPV* positive predictive value, *NPV* negative predictive value, *FIGO* Federation of Gynecology and Obstetrics

Table 3 cfHPV-DNA detection in pre-malignant lesions

References	Method	Target	Sample (collection medium)	cfDNA purification kit	Pre-malignant lesions (cfHPV-DNA positive/total)
Bonlokee et al. [44]	NGS	L1/E2/E6/E7 all HPVs	Plasma (Cell-free DNA BCT tubes, Streck)	QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany)	0/25 (CIN III)
Bonlokee et al. [7]	ddPCR	E7 HPV16 and 18	Plasma (Cell-free DNA BCT tubes, Streck)	QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany)	0/8 (CIN III)
Jeannot et al. [35]	ddPCR	E7 HPV16 and 18	Serum	QIAamp Min Elute Virus Spin Kit (Qiagen, Hilden, Germany)	0/16 (HSIL)
Rungkamoltip et al. [41]	ddPCR	E7 HPV16 and 18	Serum	Maxwell RSCVRc-cfDNA plasmakit (AS1480; Promega, Madison, WI, USA)	0/5 (1 CINI and 4 CINIII)
Sivars et al. [61]	ddPCR	E7 HPV16, 18 and 45	Plasma (Cell-free DNA BCT tubes, Streck)	QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany)	0/21 (HPV16, 18 or 45) (LSIL, HSIL, AIS)
Galati et al. [59]	E7 type-specific multiplex genotyping assay[a]	E7 HPV 16	Plasma	QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany)	0/18 (CIN III)
Herbst et al. [60]	Mass spectrometry	24 HPV types, including hr-HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68)			0/20 (HSIL, HPV positive)
Kay et al. [79]	Nested PCR	E6 HPV 16 and 18	Whole blood (EDTA)	DNA Blood Mini kit (Qiagen, Valencia, CA)	0/12 (HPV 16 and 18) (4 ASC-US, 3 LSIL, 5 HSIL)
Cocuzza et al. [46]	Real-Time PCR	E1 HPV 16, 18, 33, 45, 51, 52, and E2 HPV31	Plasma (Heparin)	NucliSENS easyMag® (bioMérieux)	33/94[b] (8/24 ASC/US 16/52 LSIL 9/18 HSIL)
Gnanamony et al. [62]	Real-Time PCR	E6/E7 HPV16 and HPV 18	Plasma	High Pure Viral Nucleic Acid kit (Roche Molecular Systems)	1/6 (CINIII and HPV16)
Ho et al. [78]	Real-Time PCR	HPV 16, 18 and 52	Blood	QIAmp blood kit (Qiagen, Hilden, Germany)	0/10 (CIN I) 0/10 (CINII) 0/20 (CINIII)
Shimada et al. [42]	Real-Time PCR	E6/E7 HPV16	Plasma	QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany)	0/21 (HSIL) 0/1 (LSIL) (HPV16)
Hsu et al. [80]	PCR	L1 all HPV	Serum	High Pure Viral Nucleic Acid kit (Boehringer-Mannheim Biochemicals, Indianapolis, IN),	0/10 carcinoma in situ HPV negative 0/10 carcinoma in situ HPV positive
Sathish et al. [81]	PCR	L1 all HPV	Plasma (EDTA)	QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany)	0/1 (CIN I) 0/1 (CINII) 0/8 (CINIII) (HPV positive)

Table 3 (continued)

References	Method	Target	Sample (collection medium)	cfDNA purification kit	Pre-malignant lesions (cfHPV-DNA positive/total)
Yang et al. [82]	Real-Time PCR	E6/E7 HPV16 and HPV 18	Plasma (Ficoll)	High Pure PCR Product purification kit (Roche Applied Science, Mannheim, Germany)	3/15 (LSIL) 6/18 (HSIL)

CIN cervical intraepithelial neoplasia, *ASC-US* atypical squamous cells of undetermined significance, *LSIL* low-grade squamous intraepithelial lesion, *HSIL* high-grade squamous intraepithelial lesion, *NGS* next-generation sequencing

[a]Performance comparable to ddPCR

[b]HSIL history 6 months ago

size, while the same did not occur with real-time PCR [35]. When comparing ddPCR with NGS, a concordance of 92.8% was observed between the two methods. Among the discordant samples, cfHPV-DNA was detected only by NGS, and the viral load was low (0.29–17.3 copies/mL plasma) [37]. Another study demonstrated a high correlation between NGS and ddPCR for the detection of cfHPV-DNA ($R^2 = 0.95$, $P = 1.9 \times 10^{-29}$). With NGS, it was possible to detect cfHPV-DNA at levels below 0.03 copies/mL of plasma in samples from post-treatment CC women that were negative for ddPCR [43]. Furthermore, it was possible to detect cfHPV-DNA in all women with CC showing positivity for viral DNA in cervical samples with NGS or ddPCR [37, 38, 44, 45]. Through an NGS technique, cfHPV-DNA was detectable even in the plasma of cervical HPV-negative women, reinforcing the potential of employing a broad NGS panel for accurate HPV genotyping, which could replace tumor tissue analysis [37].

3.3 Primer Sets

The specificity of the method used can also change the positive rate of viral detection. This occurs due to the integration of HPV DNA into the host's genetic material in the transformation phase of infection (Fig. 1A). Using ddPCR duplex for detection of HPV 16 and 18 with specific primers for viral *E7* gene, Rungkamoltip and colleagues [41] observed a low sensitivity of 30.77% for CC detection. The integration of HPV *E7* oncogene into the DNA of host cells, which targets *Rb* gene family, is considered an important hallmark for cell malignant transformation. Furthermore, the *E7* gene is highly conserved and allows more options of primer and probe design [41]. However, when assessing the presence of cfHPV-DNA using real-time PCR and primers for the *E1* gene of viral types 16, 18, 33, 45, 51, 52, and the *E2* gene of HPV 31, Cocuzza et al. [46] obtained higher positivity rates of cf-HPV-DNA detection in women with pre-neoplastic cervical abnormalities if compared with a positivity rate using real-time PCR with primers for *E6/E7* genes. This discrepancy may arise from the disruption of the *E2* gene during HPV genome integration [46]. Therefore, the detection of the *E2* gene may predominantly correspond to the episomal form of HPV, while the *E6/E7* genes can be found in both episomal and integrated forms. Thus, the development of CC detection tests based on cfHPV-DNA specific to the *E1/E2* and *E6/E7* regions of the HPV genome may increase the likelihood of detecting precancerous lesions and CC [41].

Cervical tumor cells can also contain only episomal HPV DNA [18, 19]. Carow et al. [47] evaluated the use of integration sites as molecular markers for disease monitoring or prognosis, using specific primers for viral-cellular junction sequences (vcj-PCR), which frequently arise during cervical carcinogenesis. These integration sites are unique and highly specific, being suitable as versatile tumor markers [47]. However, junction fragments were detectable in the serum of only five out of 21 patients with CC. On the other hand, a similar study detected cfHPV-DNA in the serum of 11 out of 16 women with CC above stage I, with sensitivity of 85% in cases of stage II–IV tumors [48].

The positivity of cfHPV-DNA was also influenced by HPV genotype, with cfHPV18-DNA positivity being lower than cfHPV16-DNA [32, 49]. This can be explained on the basis that the presence of viral DNA in the episomal state was observed in approximately 40% of tumors positive for HPV16, while the integrated state was observed in almost all tumors positive for HPV18 [50]. Since patients with episomal HPV have a higher viral load compared to those with integrated HPV, the integration of HPV-18 may lead to lower viral load, and, consequently, much lower detection of cfHPV18-DNA. Thus, the detection and quantification of cfHPV-DNA have also proven useful for assessing HPV DNA integration into the host genome. Tumor cells from patients with non-integrated HPV have a high viral load and present free and intact HPV genomes. It seems plausible that virus DNA could be released into the general circulation

with or without preceding cell death, thereby increasing the detection rate of cfHPV-DNA [7, 32, 35, 44, 49].

Another inherent issue in defining primers for cfHPV-DNA detection is the size of the fragment generated by amplification. Since the size of cfDNA fragments from apoptotic tumor cells is typically around 180 base pairs, primers targeting a shorter DNA fragment may identify more patients with detectable cfHPV-DNA [36, 51]. Thus, when using ddPCR with specific primers for the HPV 16 and 18 *E7* gene, which generated fragments of 183 bp and 93 bp, respectively, Cheung and colleagues [49] did not detect cfHPV-DNA in eight patients. However, when using primers targeting the *L1* gene, which generated smaller fragments (107 bp for HPV16 and 98 bp for HPV18), it was possible to detect cfHPV-DNA in these same samples. Consequently, there was an increase in sensitivity from 55.8% to 61.6% when two pairs of primers were used. Therefore, the use of more than one primer pair may offer an additional increase in the detection rate [49].

4 cfHPV-DNA Detection for Cervical Cancer Diagnosis, Prognosis, and Treatment Monitoring Response

The detection and quantification of cfHPV-DNA has clinical value for patients with neoplasms related to viral infection, including CC, as it can be used for disease staging and tumor size determination, detection of minimal residual disease (MRD), treatment assessment, and prognosis determination [14]. Furthermore, cfHPV-DNA appears to be a stable tumor marker over time, as the same HPV genes, fragments, and type identified in the primary tumor are also observed in metastatic tissue [47, 52].

4.1 Diagnosis

Once installed, CC prognosis and response to treatment are generally good when it is diagnosed at an early stage (stage IA–IB2, FIGO 2018), with a 5-year survival rate of at least 92%. On the other hand, women diagnosed at advanced stages (stage IB–IVB, FIGO 2018) present a poor prognosis, with higher recurrence rates and worse survival: when lymph nodes are affected (stage IIIC), the 5-year survival rate is 59%, and only 17% when metastatic disease is found (stage IVA–IVB, FIGO 2018). Moreover, it is estimated that at least 30% of patients in advanced stages will experience recurrences [14, 53]. Early stages of CC are typically treated with surgery, while locally advanced stages are treated with chemoradiotherapy. Metastatic disease is managed with palliative chemotherapy, often combined with radiation for symptom control such as bleeding or pelvic pain. Current guidelines recommend patient follow-up and therapy monitoring based on clinical examination and, when indicated, imaging tests [14, 54, 55].

The specificity of the method used can change the positive rate of cfHPV-DNA detection in women with CC. NGS-specific panels to simultaneously detect various types of HPV can be used to increase viral detection [14, 56]. We observed a sensitivity of 80.6–96.8% of cfHPV-DNA in samples of women with CC and using these panels (Table 2) [44, 57]. When using NGS to detect cfDNA from 13 types of hr-HPV, instead of a specific panel for HPV 16 and 18, Mittelstadt et al. [14] observed an increase in sensitivity from 61.5 to 84.6% in women with CC. This illustrates that a broader panel of HPV types can increase the detection rate and thus the value of cfHPV-DNA itself as a biomarker. Similarly, using primers for the detection of all HPV types using ddPCR, the sensitivity of CC detection ranged from 72.7 to 100% [40, 45]. Moreover, the sensitivity was 92.4% using primers specific to hr-HPV detection [58]. When the primers were HPV 16 and/or HPV 18 type specifics, the sensitivity was lower, ranging from 30.77 to 74.7% [41, 59] (Table 2). Furthermore, we found a study using mass spectrometry to detect cfHPV-DNA in women with CC, specific for 24 HPV types; the observed sensitivity was 65% (Table 2) [60].

In addition to the analytical specificity of the technique used, the positivity rate can also interfere with the observed positivity of cfHPV-DNA in the blood of woman with CC. It occurs because the detection and quantification of cfHPV-DNA were related to tumor size and disease stage, serving as a marker to determine tumor burden in patients with CC [7, 32, 35, 38, 41, 44, 45, 52, 60–62]. Thus, it has been observed that cfHPV-DNA levels are correlated with FIGO stage and reached a detection rate of 100% for FIGO IB3–IVB. Furthermore, the detection rate can be up to 100% for patients with metastatic CC [14, 38, 45]. Moreover, Gupta et al. [63] observed a significantly lower median cfDNA concentration among healthy controls than the levels observed in CC patients both before and after treatment Thus, the consistently elevated cfDNA levels in patients compared to healthy individuals reinforce the potential of cfDNA as a non-invasive biomarker for detecting and monitoring CC [63].

Some meta-analyses were carried out to evaluate the role of cfHPV-DNA detection in CC diagnosis. Elasifer et al. [64] found that the range of sensitivity was between 0.06 and 1.00 and the pooled sensitivity and specificity found in 16 studies were 0.36 (95% confidence interval (CI) 0.34–0.39) and 0.96 (95% CI 0.94–0.97), respectively. Similar results were obtained by Gu et al: the pooled sensitivity and specificity were 0.27 (95% CI 0.24–0.30) and 0.94 (95% CI 0.92–0.96), respectively [36]. According to these authors, although the studies included in this meta-analysis showed very high specificity, the sensitivity of cfHPV-DNA as a biomarker for CC detection was low. The pooled results indicated that

there was significant heterogeneity in sensitivity that could impact diagnostic accuracy [36]. This may have been due to differences in the experimental protocols, as discussed previously. Thus, a meta-analysis considering the data of seven studies published from 2016 to 2022, that used more sensitive detection methods to evaluate cfHPV-DNA as a diagnostic measure in CC, found a sensitivity of 0.63 (95% CI 0.58–0.68) and specificity of 0.97 (95% CI 0.95–0.99) [64]. Moreover, Balachandra and colleagues [65] showed that the association of circulating cfHPV16-DNA with CC was modest but significant (crude odds ratio [cOR], 15.7; 95% CI 3.4–72.6). However, the performance characteristics of circulating cfHPV18-DNA were weaker than those of cfHPV16-DNA for CC diagnostic [65].

Again, it is important to emphasize that the comparison of clinical performance techniques used by different groups under different conditions must be interpreted with caution.

4.2 Prognosis and Treatment Monitoring Response

Studies have shown that the presence of detectable cfHPV-DNA in women with CC, before treatment, is associated with an increased risk of disease recurrence and death, and that monitoring cfHPV-DNA can be correlated with treatment response [38, 45, 49, 58, 61]. Therefore, patients with higher viral load, measured by high rates of detectable cfHPV-DNA, at the end of chemoradiotherapy treatment had a greater risk of recurrence, which was associated with inferior progression-free survival (PFS). On the other hand, the decrease in cfHPV-DNA levels after treatment corresponded to remission [14, 37, 40, 45, 47, 52, 58, 64]. Leung and colleagues [43] found that detectable cfHPV-DNA at the end of treatment was associated with inferior PFS with 100% sensitivity and 67% specificity for recurrence. According to these authors, if this high sensitivity is confirmed in larger studies, patients with undetectable levels of cfHPV-DNA at the end of treatment could be candidates for observation and thus spared from adjuvant therapy or intensive surveillance. According to Sivars and colleagues [58], the PPV for predicting a relapse increased from 57% at the end of treatment to 86% and 88% during early follow-up and tumor evaluation, respectively, suggesting that waiting a few weeks after finished treatment before analyzing ctHPV DNA might be beneficial. Moreover, a meta-analysis showed that CC patients who have detectable cfHPV-DNA at the end of treatment or three months post-treatment may experience a shorter PFS compared to those in the negative group [66]. Also, cfHPV-DNA detection can predict the rate of relapse or recurrence after treatment, with post-treatment measurement being more effective than baseline assessment [34]. Therefore, quantification of cfHPV-DNA has proven to be an accurate test for evaluating patients at high risk of recurrence and with MRD. Thus, liquid biopsy could allow personalized use of adjuvant therapy by identifying patients who are cured after radical therapy and, therefore, would not benefit from additional treatment [43].

However, a positive result at the end of treatment may not guarantee subsequent recurrence and may require repeating tests or alternative surveillance procedures, such as imaging exams [43]. To ensure good performance of cfHPV-DNA detection, it may be preferable to repeat liquid biopsy in the weeks or months following the end of chemoradiotherapy treatment in patients with low levels of cfHPV-DNA, especially in the case of women who had high levels of cfHPV-DNA at the time of CC diagnosis. The ability to anticipate the presence of MRD up to 15 months before the diagnosis of recurrence has high clinical value. Early detection of persistent/recurrent disease is particularly important in irradiated tissues to reduce the risk of radical surgeries [32, 52].

Thus, Mittelstadt et al. [14] found that: (1) cfHPV-DNA levels are correlated with FIGO stage, with higher levels being detected in advanced tumors, thus, it can be correlated with an increased risk of recurrence; (2) the detection of high levels of cfHPV-DNA can quickly decrease to (almost) undetectable levels when a favorable therapeutic response is achieved; (3) despite the initial tumor stage, the decrease in cfHPV-DNA to nearly undetectable levels (without significant MRD) seems to correlate with a better prognosis; (4) high levels of detectable cfHPV-DNA post-treatment (significant MRD) may correlate with worst prognosis, indicating high risk of relapse, even without evidence of disease on histopathological examination; and (5) in the early stage, cfHPV-DNA rapid decrease can accompany reduction in tumor burden, but low levels of cfHPV-DNA may be associated with residual disease. Thus, quantification of cfHPV-DNA after therapy could help improve therapeutic algorithms [9]. Furthermore, studies have shown that monitoring the treatment of patients with CC can be performed by quantifying cfHPV-DNA during its course, not only at the end [14, 32, 61].

Kang et al. [38] also defined cfHPV-DNA quantification as a potential biomarker for anti-tumor activities of experimental therapies, by evaluating the effect of tumor-infiltrating lymphocytes (TIL) in women with metastatic CC. In this study, the authors observed a rapid peak of cfHPV-DNA 2–3 days after the initiation of TIL treatment, which could be interpreted as tumor DNA being released from degraded CC cells. Thus, cfHPV-DNA may serve as a novel type of pharmacodynamic biomarker for cancer patients undergoing experimental therapies and may have potential value in early clinical development of experimental cancer therapeutics [38].

5 cfHPV-DNA for Cervical Cancer Screening

Programs for CC screening that were based on primary cytology (Pap smear) are currently being replaced by primary HPV testing, which offers greater sensitivity than cytological analysis for the primary screening of women over 25 years of age [67–69]. Data from follow-up analysis of four large, randomized cohorts show that HPV-based cervical screening provides 60–70% greater protection against invasive cancer compared with cytology-based screening [70]. Furthermore, a meta-analysis observed that the pooled sensitivity estimates for HPV testing and Pap smear were 89.9% and 62.5%, respectively [71]. Additionally, patients diagnosed with high-grade pre-neoplastic lesions require careful lifelong monitoring for possible recurrence or development of CC. Molecular techniques have made it possible to research HPV DNA in cervical samples [25, 72, 73]. Real-time PCR is the most used technique, and many cervical HPV DNA tests are validated for CC screening and are commercially available, such as Cobas 4800 HPV Test (Roche Molecular Diagnostics, Pleasanton, CA, USA), BD Onclarity HPV Assay (BD Diagnostics, Sparks, MD, USA), and APTIMA HPV Assay (Hologic, Bedford, MA, USA). There is a growing interest in researching new techniques for HPV detection that present low cost and high analytical performance, enabling the inclusion of HPV testing in CC screening in developing countries [74–76].

According to guidelines, it is recommended that women whose test result is positive for HPV 16 or 18 should be referred for colposcopy. If the test result is positive for other hr-HPV, cervical cytological analysis should be performed, and if no abnormalities are found, the woman should repeat the HPV test in one year. Otherwise, if cytological analysis shows abnormalities, the patient should undergo colposcopy. On the other hand, patients with a negative HPV test should be retested after 5 years [77].

As the need for cervical samples may still represent an obstacle for satisfactory adherence to screening programs, the detection of viral DNA in blood samples may be an alternative tool in this scenario. Nevertheless, few studies have evaluated the detection of cfHPV-DNA in women with pre-neoplastic lesions, and the results found were unsatisfactory (Table 2).

Only one study using NGS [44] and five using ddPCR [7, 35, 41, 59, 61] evaluated the presence of cfHPV-DNA in women presenting CIN I, CIN III, low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), or adenocarcinoma in situ (AIS). It was not possible to detect the presence of viral DNA in plasma or serum samples in none of them, even when using specific extraction kits for cfDNA, when the technique was specific for all HPV types, and the cervical samples from the patients were known to be positive for the presence of the virus (Table 3). However, the sample size evaluated by these authors was small, ranging from 16 to 40 samples. Similar results were obtained in older studies with real-time PCR [42, 78], nested PCR [79] or conventional PCR [80, 81], once again, with small sample sizes (Table 2). Thus, these studies indicated that cfHPV-DNA constitutes a specific form of ctDNA, only being released into circulation in cases of invasive carcinomas caused by HPV. It could be used as a tumor marker for better diagnosis, prognosis, and treatment monitoring [7, 44, 57], but not in the screening of this neoplasm. Although the exact source of cfHPV-DNA is not clear, its levels may represent the lysis of circulating cancer cells or micrometastases released by the tumor [42].

On the other hand, Cocuzza and colleagues [46] detected cfHPV-DNA through real-time PCR specific for viral types 16, 18, 31, 33, 45, 51, and 52 (*E1* and *E2* genes) in 33 out of 94 plasma samples from HPV-positive women with a history of HSIL, who, at that time of the study, showed persistent lesions or regression to atypical squamous cells of undetermined significance (ASC-US) or LSIL (Table 2). Furthermore, these authors also detected cfHPV-DNA in eight of 26 women who showed complete regression of HSIL [46]. It was also observed that the rate of HPV detection in cervical and plasma samples increased with the advancement of the disease stage, ranging from 15.4% in women with lesion regression to 38.9% in women with persistent HSIL. Additionally, 25 women (20.8%) tested positive for HPV in both cervical and plasma samples, with 44% showing the same viral type in both samples. The discrepancy between plasma and cervical positivity for HPV observed in this study may be explained by the high frequency of transient cervical HPV infections, often associated with various types, as well as potential hr-HPV infection at sites other than the genital mucosa, such as the oropharyngeal cavity. Furthermore, these infections may independently result in viral persistence and/or replication in the bloodstream, which may also explain the fact that 16 women tested positive for cfHPV-DNA but negative for cervical HPV [46]. Further studies investigating the presence of virions in the bloodstream or evidence of viral replication through the expression of oncogenic transcripts will help clarify whether these cells may act solely as carriers for viral propagation or if they can also be permissive for HPV replication and persistence. This would have important implications for both non-sexual transmission and the development of tumors in anatomical tissues distant from the primary mucocutaneous sites of viral replication [46, 60].

A similar result was obtained by Yang and colleagues [82] when evaluating the presence of cfHPV-DNA in 96 women without cytological abnormalities (control group),

15 women with LSIL, and 18 with HSIL, where viral DNA was detected in the plasma of 14, three, and six patients, respectively. Also using real-time PCR, another study detected cfHPV-DNA in the plasma of one of six women with CIN III and positive for cervical HPV16 [62].

Thus, even using a less sensitive methodology (kits not specific for cfDNA extraction and real-time PCR), it was possible to detect cfHPV-DNA in samples from women with pre-neoplastic lesions. Evidence suggests that if cfHPV-DNA is indeed released into the circulation of patients with pre-malignant lesions, this is a rare event that may indicate invasive disease [61], potentially serving as an indicator of poor prognosis. On the other hand, studies with larger sample sizes, using NGS or ddPCR specific for all types of hr-HPV, flanking regions of both integrated and episomal viral genes, should be conducted to assess the real potential of cfHPV-DNA detection in CC screening. However, cfHPV-DNA is not specific to CC development, as hr-HPV infection has also been associated with the development of oropharyngeal and another anogenital cancer, as well as the occurrence of other extragenital tumors such as breast, lung, bladder, and colon cancer [83].

6 Perspectives

Liquid biopsy has been extensively evaluated as a sensitive, simple and feasible tool for the detection, prognosis, treatment assessment, and recurrence monitoring for various types of cancers. In this context, cfHPV-DNA emerges as a non-invasive, sensitive, and specific biomarker for CC [59]. However, the detection rates of cfHPV-DNA are variable, not only for CC, but concerning HPV-associated tumors in general. The possible explanations for inconclusive results among studies include: (1) the use of methodologies with different analytical sensitivity and specificity; (2) the number of patients included; and (3) the characteristics of patients, such as tumor size and disease stage [14]. Furthermore, there is significant variation in establishing a reliable cutoff for cfHPV-DNA positivity (Ct value or viral load, for example), and some studies did not include negative control samples [7, 36, 44]. Therefore, the need for new studies with standardized methodologies, validated clinical parameters, robust sample sizes, and representative subjects is evidenced.

High-sensitivity techniques are ideal for cfHPV-DNA analysis, and the technique employed must be carefully chosen. While ddPCR provides a quantitative measure of cfHPV-DNA based on a set of primers and probes specific to different sites of the HPV genome, a NGS panel for cfHPV-DNA detection can be specific to various regions of the HPV genome, ensuring that cfHPV-DNA can still be detected if genetic alterations or integration occur in one or more regions of the viral genome [14]. NGS-specific panels for a broad spectrum of hr-HPV types are shown to increase cfHPV-DNA detection sensitivity. When using ddPCR, besides detecting a wide range of hr-HPV types, primer design should be carefully evaluated, aiming at conserved HPV-genome sites that are not affected by viral integration [14, 49].

The exciting potential applications of cfHPV-DNA as a biomarker can be explored for personalized management of CC, particularly in monitoring real-time treatment response and viral load, as well as predicting recurrence and MRD, thereby guiding the clinical management of patients [38, 45, 49, 61]. It can also be considered a non-invasive, sensitive, and specific prognostic biomarker, especially for invasive and metastatic CC cases [14, 38, 45]. Additional research must be performed in order to better understand the precise source of cfHPV-DNA, as well as the biological mechanisms behind their release and their levels in the bloodstream across different pathological contexts [42].

7 Conclusion

The detection of cfHPV-DNA appears to be a promising biomarker for CC, being useful in the diagnosis, prognosis, and treatment monitoring of women with this neoplasm. On the other hand, there are conflicting results regarding cfHPV-DNA detection in the blood circulation of patients with pre-malignant lesions. Thus, studies with larger sample sizes, evaluating plasma collected in tubes specifically designed for ctDNA preservation, using specific extraction kits for cfDNA, and employing a more sensitive methodology for analysis, such as ddPCR or NGS, capable of detecting all types of hr-HPV, will be useful to assess whether cfHPV-DNA detection can be an alternative for CC screening.

Declarations

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