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Increased human papillomavirus viral load is correlated to higher severity of cervical disease and poorer clinical outcome: A systematic review

Seth-Frerich Fobian[1,2,3,4] | Xionge Mei[1,2,3] | Johannes Crezee[1,3] | Barbara C. Snoek[1,2,3] | Renske D. M. Steenbergen[5] | Jiafen Hu[6] | Timo L. M. ten Hagen[4] | Louis Vermeulen[2,3,7] | Lukas J. A. Stalpers[1,2,3] | Arlene L. Oei[1,2,3]

[1]Department of Radiation Oncology, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands

[2]Center for Experimental and Molecular Medicine (CEMM), Laboratory for Experimental Oncology and Radiobiology (LEXOR), Amsterdam, The Netherlands

[3]Cancer Center Amsterdam, Cancer Biology and Immunology, Amsterdam, The Netherlands

[4]Precision Medicine in Oncology (PrMiO), Department of Pathology, Erasmus MC Cancer Institute, Rotterdam, The Netherlands

[5]Department of Pathology, Amsterdam UMC, Vrije University, Amsterdam, The Netherlands

[6]Jake Gittlen Laboratories of Cancer Research, Department of Pathology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania, USA

[7]Oncode Institute, Amsterdam, The Netherlands

Correspondence
Arlene L. Oei, Department of Radiation Oncology, Center for Experiment and Molecular Meidcine, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands.
Email: a.l.oei@amsterdamumc.nl

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Abstract

Cervical cancer is the fourth most common cancer in women worldwide and is caused by persistent infection with high-risk types of human papillomavirus (HPV). HPV viral load, the amount of HPV DNA in a sample, has been suggested to correlate with cervical disease severity, and with clinical outcome of cervical cancer. In this systematic review, we searched three databases (EMBASE, PubMed, Web of Science) to examine the current evidence on the association between HPV viral load in cervical samples and disease severity, as well as clinical outcome. After exclusion of articles not on HPV, cervical cancer, or containing clinical outcomes, 85 original studies involving 173 746 women were included. The vast majority (73/85 = 85.9%) reported that a higher viral load was correlated with higher disease severity or worse clinical outcome. Several studies reported either no correlation (3/85 = 3.5%), or the opposite correlation (9/85 = 10.6%); possible reasons being different categorization of HPV viral load levels, or the use of specific sampling methods. Despite variations in study design and populations, the above findings suggest that HPV viral load is

Work performed at Amsterdam UMC (location University of Amsterdam).

Seth-Frerich Fobian and Xionge Mei contributed equally.

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correlated to clinical outcome, and may become an important biomarker for treatment selection and response monitoring for cervical cancer.

KEYWORDS
cervical cancer, clinical outcome, disease severity, HPV, HPV viral load, human papillomavirus

1 | INTRODUCTION

Around 5% of all cancers worldwide are caused by infection with human papillomavirus (HPV; Family *Papillomaviridae*, genus *Alpha-papillomaviruses*, species 4–10).[1,2] HPV is a sexually transmitted virus infecting only differentiating epithelial cells (keratinocytes), especially though small wounded areas.[3] More than 200 types of HPV have been identified,[4] of which a number have been linked to cancer development.[5] Within the mucosal group of HPV types, a subdivision exists between low risk (lr) and high risk (hr). Low-risk human papillomavirus (lrHPV) types, including 6, 11, 42, 43, and 44, are known to cause benign genital warts and are much less frequently associated with cervical cancer.[5,6] High-risk human papillomavirus (hrHPV) types 16 and 18 are foremost associated with cervical cancers (~70%), followed by 45, 31, 33, 52, 58, and 35.[7] hrHPV is linked to the development of cancer of various origins, including the cervix (100%), anus (88%), vagina (78%), vulva (24.9%), penis (50%), and oropharynx (head and neck) (30.8%).[8] Though all these cancers have been studied in relation to HPV infection, this systematic review only focuses on cervical cancer.

Cervical cancer is the fourth most common cause of cancer-related mortality in women worldwide.[9] While approximately 80% of sexually active people get infected with HPV at some point during their life,[10,11] a vast majority (80%–90%) of infections are cleared by the host's immune system within 2 years[11,12] (Figure 1A). Briefly, viral clearance is mediated by $CD8^+$ cytotoxic T lymphocytes, primed by antigen presenting cells (APCs) which take up antigens for processing and presentation to T cells.[13,14] $CD8^+$ T cells bind to viral peptides presented on the surfaces of infected epithelial cells, which initiates the production of granzymes and perforins, causing apoptosis and membrane permeabilization of the targeted cell.[15]

However, in the remaining subset of persistent infections, this interaction is largely prevented by reduced immune surveillance within the layers of infected keratinocytes, as well as very low expression of viral proteins on infected cells.[14] Furthermore, while many viruses' replication cycles involve host cell lysis, that of HPV does not need to, given the short lifespan of keratinocytes.[16] This removes an opportunity for release, uptake, and processing of virions by APCs,[13] and also prevents an inflammatory immune response,[16] which would normally release danger signals as a chemoattractant for other immune cells. HPV-infected cells also show reduced interferon secretion, removing a key antiviral, immunostimulatory mechanism.[16] Such conditions create an environment in which development of cervical intraepithelial neoplasia (CIN) may occur.[3]

CIN lesions are graded 1–3 based on the degree of dysplastic cells. CIN 1 is characterized by mild dysplasia within one-third of the epithelium; CIN 2 by dysplasia up to two thirds of the epithelium, and CIN 3 by dysplasia from two-thirds up to the full thickness of the epithelium.

CIN 1 is a benign state of cervical dysplasia associated with viral replication and conservative treatment recommendations, as it is expected that 70%–90% of CIN1 lesions will regress within 2–3 years.[17] CIN 2 and CIN 3 are associated with transforming HPV infections and these lesions may progress to cancer when left untreated. The 30-year progression risk of CIN 3 is 30% when left untreated.[18] These are true pre-invasive precursor lesions warranting surgical intervention.[19] Progression from CIN 2/3 to cervical cancer is a process that can take 10–30 years.[20] Alongside these histological classifications, standardized cytological classifications have been developed known as the Bethesda System for reporting cervical cytology.[21] Each category has defined clinical implications,[22] with the following classifications: NILM (negative for intraepithelial lesion or malignancy), ASC-US (atypical squamous cells of undetermined significance), ASC-H (atypical squamous cells cannot exclude HSIL), LSIL (low grade squamous intraepithelial lesion), HSIL (high grade squamous intraepithelial lesion), and SCC (squamous cell carcinoma). The correlations between the CIN grades and Bethesda classification systems are shown in Figure 1A.[21]

The 5-year relative survival rate for patients with cervical cancer is approximately 67%,[23] but is typically worse in low–middle income countries,[24] indicating a dire clinical need for more cost-effective treatment and improved screening programs. To maximize therapeutic benefit, predictive biomarkers can help to stratify cancer patients for improved treatment responses. One such potentially predictive biomarker is measurement of the HPV viral load in cervical samples. Viral load is the average amount of HPV DNA present within a patient's cervical epithelium, which can be expressed by copies per unit or volume. The viral load has been suggested as a marker in relation to CIN grade, and progression and clinical outcome of cervical cancer, such as survival.[7,25] Although the determination of HPV viral load is currently not a standard protocol, it could potentially be of great value in improving treatment strategies and thereby possibly clinical outcome. A better understanding of the clinical value of HPV viral load in cancer patients is therefore important for better treatment strategies and subsequently a reduction in mortality.

In this systematic review, we first give a general overview of the association between HPV and CIN or cervical cancer, followed by an in-depth look at the methods and techniques of measurement of

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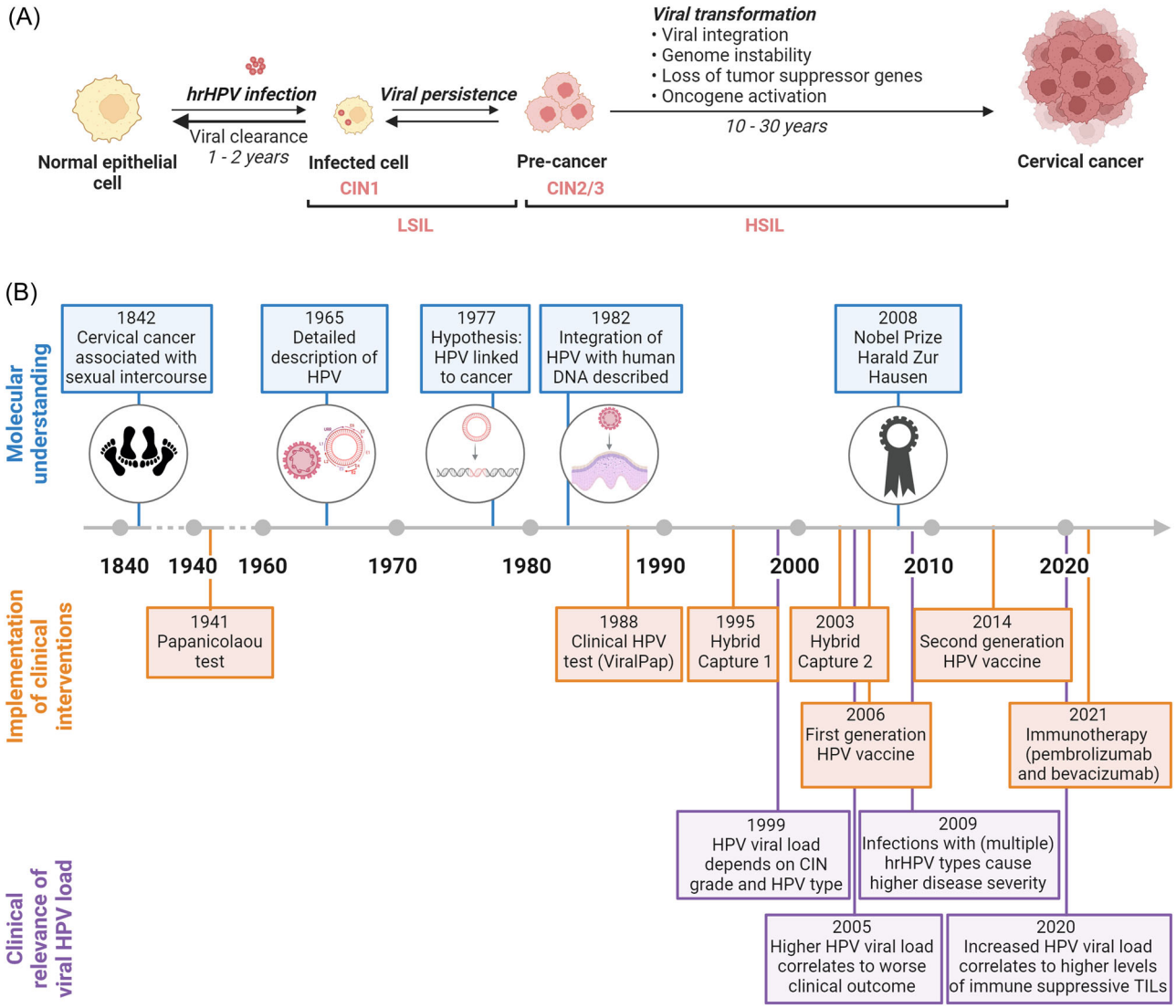

FIGURE 1 (A) Pathogenesis from normal cells to CIN to cervical cancer. (B) Historical milestones in the understanding of cervical cancer and HPV. CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion.

HPV viral load in relation to both precursor and cancer samples, as well as the reporting and potential clinical relevance thereof.

1.1 | History of HPV

In 1842, Rigoni-Stern described his observation that cervical cancer was mainly noted in married woman, widows and prostitutes, and rare in virgins and nuns.[26] This led to the conclusion that the development of cervical cancer had to be related to sexual contacts (Figure 1B). It was only a decade after Watson and Crick discovered the molecular structure of DNA,[27] that the double-stranded circular DNA structure of HPV was described.[28,29] HPV was first linked to cervical cancer in the 1970s,[26,30] and the integration of viral DNA in the human genome was confirmed by Harald Zur Hausen in 1982.[31,32] He received the Nobel Prize in 2008 for his important discovery that HPV causes cervical cancer.

1.2 | Pathogenesis of CIN and cervical cancer

HPV contains a small, non-enveloped circular double-stranded (ds) DNA genome of approximately 7900 base pairs and consists of several genomic regions. The early region encodes proteins necessary for viral replication (E1-2, E4-7), and the late region encodes the major and minor viral capsid proteins (L1 and L2, respectively) necessary for viral assembly, and an upstream regulatory region which is important for viral gene transcription.[33] Viral gene expression is regulated by multiple promotors, enabling the expression of different proteins at distinct stages of the viral life cycle.[33]

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A productive infection with HPV starts when virus particles enter the basal cells of epithelium via microabrasions. The viral genome enters the cell nucleus of the infected basal keratinocytes where the genome is replicated in conjunction with host DNA and maintained as stable episomes at low copy numbers (50–100 copies per cell).[34] Productive HPV infections may give rise to mild or moderate cellular abnormalities (CIN 1–2 lesions).[20]

A minority of productive HPV infections are able to persist and lead to the development of high-grade premalignant lesions (CIN 3+), These lesions are associated with so-called transformative HPV infections, in which the normal viral life cycle is aborted and the viral early genes E6 and E7 function as oncogenes. A transformative HPV infection is frequently characterized by integration of the virus in the host genome,[20] leading to increased expression of E6 and E7. In the dividing epithelial cells, oncoproteins E6 and E7 function by interacting with tumor suppressor p53 and retinoblastoma proteins (pRb), respectively.[35,36] E6 binds to E6-associated protein (E6AP), an E3 ubiquitin ligase, which results in ubiquitin-mediated proteasome degradation of p53. Consequently, p53-induced apoptosis, DNA repair and cell cycle arrest are inhibited, resulting in cell cycle progression. The E7 oncoprotein degrades pRb which results in entry into the S-phase of the cell cycle, thereby promoting uncontrolled cell proliferation. The combined action of E6 and E7 on cell cycle control and evasion of apoptosis results in genetic and epigenetic host cell changes that ultimately lead to genomic instabilities and the activation of oncogenes and inactivation of tumor suppressor genes. These events are crucial for progression from high-grade premalignant lesions to cervical cancer.[37–41]

1.3 | Clinical management of HPV

Detection of abnormal cells within the cervical area and the ability to distinguish them from normal healthy cells became possible with the discovery of the Papanicolaou (Pap) smear in 1928. Since 1941 it has been used as a screening method for early detection of cervical cancer.[42] The first test to clinically measure HPV was Food and Drug Administration (FDA)-approved in 1988.[43] Since the 1990s multiple clinically validated HPV tests have reached the market, and in several countries HPV testing is either an addition to testing with Pap smears, or it has completely replaced Pap smears.[44]

Next to screening methods for cervical cancer, in the last 20 years, HPV vaccines were introduced to the global market.[45,46] These provide protection before exposure to specific hr- and lrHPV-types. The first quadrivalent vaccine, approved by the FDA in 2006, prevents infections of both lr and hrHPV-types 6, 11, 16, and 18. Eight years later, the second generation HPV vaccine got FDA approved, adding protection against hrHPV-types 31, 33, 45, 52, and 58. However, the World Health Organization does not expect HPV vaccination to significantly reduce cervical cancer mortality before 2030,[47] because of a long latent period between initial exposure, infection and onset of cancer, as well as low uptake due to vaccine resistance, and low vaccine availability in low income countries.[48]

1.4 | Clinical relevance of HPV viral load

The first evidence of the link between an increased viral load of HPV and higher CIN grade was reported by Swan et al.[7] in 1999 (Figure 1B). Interestingly, contrary to the way in which the correlation was stated shortly thereafter (as an association,[49] or causation[50]), Swan et al.[7] stated that the HPV viral load is dependent on the grade of cervical disease. The question remains as to how the differences in HPV viral load occur or develop amongst patients.

Subsequently, studies have been carried out to investigate the link between increased HPV viral load and higher CIN grade, and clinical outcomes of cervical cancer.[51] HPV co-infections by multiple types has, often alongside viral load, been suggested as a factor affecting progression, citing a lower rate of clearance of the virus in multiple infections, leading to HPV persistence and CIN 2/3 development.[52,53] While some have shown evidence of this, the key factor seems to be co-infection of HPV16 with other types.[54] This has shown significant correlations to disease increased severity, more so than multiple infections, as such.[55–57] A combination of viral load, HPV viral co-infection status, and HPV genotype, may be needed to identify patients for better personalized cervical cancer treatment.[58]

The mechanisms by which viral load affects CIN development or cervical cancer progression are not well understood; however, Cao et al.[59] recently published a study in which they measured immune biomarkers, including suppressive $FOXP3^+$ tumor infiltrating lymphocytes (TILs), as well as $CD8^+/FOXP3^+$ T cell ratios, as a measure of effector T cell activity. A marked difference was found between high and low viral load groups of patients, with higher viral load patients experiencing shorter survival and decreased immune surveillance in the tumor microenvironment. Thus, the poorer outcome of women carrying a higher viral load may be explained either by possibly more aggressive HPV types (i.e., genotypes with reduced immunological clearance of the virus), or by a reduced local or systemic patient immune response.

1.5 | Objectives of study

In this systematic review, we have investigated the importance of viral load measurement in cytology samples taken for cervical cancer screening/referral, or tissue samples taken for diagnostic purposes, as well as the relation thereof to disease severity (CIN grade or stages of cervical cancer) and clinical outcomes (survival and recurrence). This was done in two groups of studies: those studying this effect in cytology samples, and those studying the same in tissue biopsies. We focus on the perspectives of whether the correlation between HPV viral load and disease severity, and clinical outcome, improve diagnosis and triage, lead to more personalized treatment strategies, potentially avoiding unnecessary radical surgeries and interventions. Alongside these, we have sought to identify trends in sampling techniques and methods for measurement of viral load, as differences in these

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and other features of the included studies can greatly impact the results thereof.

2 | MATERIALS AND METHODS

In this study, the Preferred Reporting Items for Systematic Reviews and Meta-Analyzes (PRISMA) guidelines were followed to enable thorough, transparent, and unbiased reporting of the findings made regarding the correlation between viral load and clinical outcomes and progression of cervical cancer.[60] The protocol carried out was not pre-registered. Exact queries and search strings can be viewed in Table S1.

2.1 | Search strategy and selection process

Three online databases were searched for the articles included in this systematic review; namely, Pubmed (NCBI), Embase (Elsevier), and Web of Science (Clarivate). Within these databases the following search terms were used: Medical Subject Heading (MeSH) or "/exp" (Emtree explosion) terms and words in title, abstract or keywords including "Cervical cancer" or "Cervical Intraepithelial Neoplasia" and "Human Papillomavirus" and "Viral Load" and Possible clinical outcome measurements. All digitally available publications were surveyed up until December 1st, 2023. One retracted paper and duplicates from databases were excluded. The remaining articles were assessed for eligibility according to the criteria detailed below. Literature screening was performed by three investigators independently (SFF, XM, ALO), and disagreements were resolved by consensus.

2.2 | Inclusion and exclusion criteria

Inclusion criteria were full-text, original data articles, written in English, and published in international, peer-reviewed journals. All studies meeting these criteria were considered. These articles were screened for relevance, and excluded if they were not on cervical cancer or CIN and HPV (including studies on co-infections with viruses such as human immunodeficiency virus, commonly reported alongside HPV). If studies only reported results on vaccination or epidemiology studies, or if they were only method-based (focusing on method evaluation, performance, optimization, validation, verification, or assay comparison), or made use of non-standard sampling approaches (such as cell-free DNA or plasma detection), or did not report a correlation between the viral load of HPV and clinical outcome, they were also excluded from the final analysis, as clinical outcomes were required to draw conclusions for this paper. There were no geographical inclusion or exclusion criteria; leaving this aspect unbiased. Distributions of included studies and counts thereof are shown in Figure S2.

2.3 | Participants and study outcomes

Women with a variety of cervical abnormalities were included based on different cytological and histological characteristics, ranging from atypical squamous cells of undetermined significance (ASC-US) to HSIL,[61] as well as CIN 1–3 and cervical cancer of multiple histotypes (squamous cell carcinoma, adenosquamous carcinoma, adenocarcinoma, and neuroendocrine carcinoma).[62] These cases were considered both with or without active HPV infection of various types, based on the exclusion criteria of individual studies. Outcomes sought included survival of any kind (including overall, disease-free, and progression-free survival), cancer recurrence, tumor or neoplasia progression, or other pathological features such as the presence of positive lymph nodes.

2.4 | Data extraction

The following data were extracted from each article: year of publication, detection method to measure viral load, definition of viral load with its unit of measurement, HPV types which were detected, cohort characteristics, including sample type, disease stage (if any) and potential exclusion of non-infected patients; number of subjects and possible clinical outcome with a measure of statistical significance related to the viral load and clinical outcome. The same three investigators carried out this task independently. Given the nature of the extracted data, no further meta-analyzes of any kind could be carried out.

2.5 | Study quality assessment

All included articles were assessed for quality and risk of bias by three investigators independently (SFF, XM, ALO). Where applicable, this was done with the guidelines outlined in the Newcastle-Ottawa Scale (NOS).[63] The NOS assessment scale can be viewed in Figure S1, and results of this assessment in Table S2.

3 | RESULTS

The PRISMA flow chart showing study identification, selection, and exclusion, is shown in Figure 2. A total of 2331 articles were found across the three surveyed databases, of which 1143 were duplicates. After elimination thereof, 1188 unique full-text English articles were found of which 113 were included after initial screening steps. A total of 28 full-text articles were then excluded for the following reasons: 12 described eligibility or optimization of methods to measure viral load, 9 articles did not measure viral load, and 7 articles did not relate to any clinical outcome. Finally, 85 articles were eligible for data extraction, involving a total of 173 746 (median: 361; range: 13–47 120) women. in the results of this literature search are

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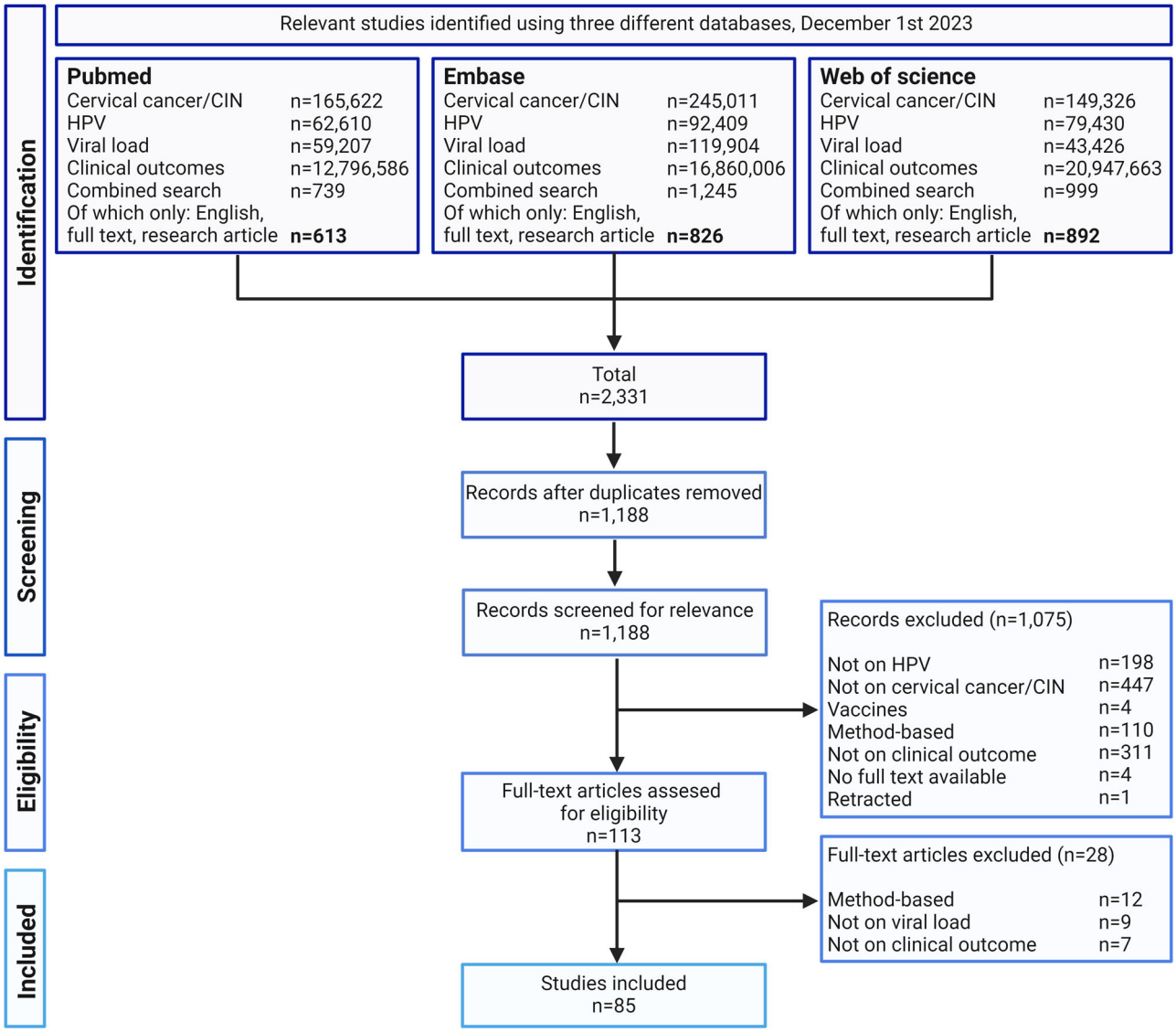

FIGURE 2 PRISMA flow chart. A systematic review of the viral load of HPV in cervical lesions and cancers, and its correlation to clinical outcome. HPV, human papillomavirus; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyzes.

reported in Table 1. The earliest included study dates back to 1999,[7] while the most recent was published in 2022.[64]

3.1 | Detection and quantitation of HPV viral load

Our First observation is that there is a large variation in methodology to measure HPV viral load. Common techniques used are (quantitative) polymerase chain reaction ((q)PCR), hybrid capture 2 (HC2), and in situ hybridization (ISH). Moreover, there is also little consensus on how to quantify and report viral load, neither within nor between the different techniques. Some qPCR users report copies per cell, per volume, or per host genome, while others using HC2 report results as relative light units per cut off (RLU/CO), and the unit integrated optical density is used more than once. Furthermore, there are no well-established cut-off values for categorizing a viral load value as "low," "medium," or "high." It is therefore not possible to compare all results amongst each other. Interestingly, the categorization of "low" viral load using RLU/CO values generated with HC2 as being less than approximately 163 or in some cases, less than 1000, can lead to opposite and/or misleading results. Although HC2 is less commonly used in current practice, when using HC2 to define HPV viral load, as the majority of studies have done, we recommend the categorizing for "low" viral load to be <10, given that a positive value is considered >1. Furthermore, when cytology samples rather than tissue samples were used to study clinical outcomes of cancer patients, it may have resulted in similarly misleading results. Noticeably, some studies also excluded patients who were HPV negative. While in many cases rationales were given, these omissions may also influence the interpretation of data, as the ranges of viral load, as well as medians and grouping distributions would shift.[68,72,112]

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TABLE 1 A: Details on viral load measurement in cytology samples obtained for screening or referral purposes and correlations to disease severity and clinical outcome reported in literature.

Screening or referral samples (cytology)								
Detection method					Patient information	Endpoint		
DNA load assay	Units	Low	Med	High	Cancer type, FIGO stage, or cervical disease (*n* =) at outset of study	Clinical outcome	*p*-value/HR/OR/RR (respectively to outcomes)	Reference
HC2	Genome equivalent/ 1000 cells	<3.2 $\log_{10}$		≥3.2 $\log_{10}$	Unknown (790), normal (45), CIN 1 (50)	Higher HPV16 VL significantly increased risk for high-grade CIN lesions	0.0001	Baumann et al.[65]
HC2	pg/mL	1.0–9.9	10.0–99.9	≥100.0	Unknown (33 288, at follow up CIN 3 (490), cancer (26))	Higher HPV16 VL significantly increased risk of CIN 3 & cancer, and non-significantly for HPV18	HR CI min 1.1	Thomsen et al.[66]
HC2	RLU/CO	<1000	0.81–3966.10, Median = 1129.98	>1000	SCC (21), I-IVA	Higher VL non-significantly increased progression to invasive cancer, but decreased DFS significantly	both ways	Datta et al.[67]
HC2	RLU/CO	<163.13	Median = 163.13	>163.13	SCC (222), AC/ASCC (24), IB2-IVA	Higher VL: increased DFS and OS	0.003, 0.009	Huang et al.[68]
HC2	RLU/CO	<Med	Median = 385.8 (IQR: 21.6–1168)	>Med	AC (16), SCC (151), IBI-IVB	Higher VL: increased DFS	0.002	Kim et al.[69]
HC2	RLU/CO	≤1000		≥1000	SCC (21), I (2), II (12), III (6), IV (1)	Higher VL: increased DFS in radiotherapy patients	ns	Datta et al.[70]
HC2	RLU/CO	≤100		>100	AC (5), ASCC (4), SCC (24), other (1), IA1-IB2	Higher VL: decreased PFS	0.7756	Kim et al.[71]
HC2	RLU/CO	≤132.5	Median = 132.5	>132.5	SCC (305), AC/ASCC (37), IA2-IIIA, other (4)	Higher VL: lymphovascular & stromal invasion, decreased DFS	0.026, 0.024, 0.037	Deng et al.[72]
HC2	RLU/CO	≤387	Median = 387	>387	AC/ASCC (11), SCC (145), IB-IVB	Higher VL: increased RFS	0.001	Song et al.[73]
HC2	RLU/CO	≤Median	Median = 356.1 (RH) and 294.29 (CCRT)	>Median	SCC (520), IB1-IVA	Higher VL: increased PFS/OS	<0.001	Zuo et al.[74]
HC2	RLU/CO	1–<10	10–<100	>100	Unknown (258), CIN (1739)	Higher VL: progression of precancerous lesions	0.343	Zhao et al.[75]
HC2	RLU/CO	1–<100	100–<1000	>1000	Normal (125), CIN (120), cancer (20)	Higher VL: higher risk of high grade CIN and cervical cancer	0.001, 0.002	Liu et al.[76]
HC2	RLU/CO	1–10	11–100	>100	Normal (69), CIN (202), SCC (236)	VL significantly increased from CIN 1 to CIN 2/3	0.001	Huang and Huang[77]

(Continues)

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TABLE 1 (Continued)

Screening or referral samples (cytology)								
Detection method					**Patient information**	**Endpoint**		
DNA load assay	**Units**	**Low**	**Med**	**High**	**Cancer type, FIGO stage, or cervical disease (*n* =) at outset of study**	**Clinical outcome**	***p*-value/HR/OR/RR (respectively to outcomes)**	**Reference**
HC2	RLU/CO	1–10	11–100	>100	ASC-US (596), LSIL (372), HSIL (27)	HSIL was significantly associated with higher VL	0.011	Xu et al.[78]
HC2	RLU/CO	1–10	11–100	101–1000	ASC-US (388)	Higher VL: higher CIN grade, especially between normal & CIN 1	0.67	Jarboe et al.[79]
HC2	RLU/CO	1–10	11–100	>100	Normal (294), ASC-US (295), LSIL (205), ASC-H + HSIL (23)	Higher VL: CIN progression	0.0001	Kim et al.[80]
HC2	RLU/CO	1–10	11–100	101–1000; <1000	Normal (282), CIN 1 (268), CIN 2/3 (64)	Higher VL significantly correlated to CIN 2/3	0.0001	Origoni et al.[81]
HC2	RLU/CO	1–10	11–100	>100	Normal (282), CIN 1 (268), CIN 2/3 (64)	Higher VL: significant increase in rates of CIN 1-3, especially CIN 2/3	0.001, 0.0002	Origoni et al.[82]
HC2	RLU/CO	1–10	10–100	>100	Normal (68), CIN (262), cervicitis (139), cancer (541)	Higher VL: CIN progression	OR CI min 17.8	Wu et al.[83]
HC2	RLU/CO	Ranged, 0.12–3427.17			AC (45), ASCC (7), SCC (152), IB-IIA	Higher VL: lower RR	0.227	Kang et al.[84]
HC2	RLU/CO	Ranged: 1.0–3286.8, severity cutoff = 2385			Normal (174), ASC-US (101), LSIL (47), ASC-H (3), HSIL (14), cancer (4)	Higher VL correlated significantly to CIN severity	<0.05	Chang et al.[85]
HC2	RLU/CO	Ranged: 0.2–2632, mean (normal) = 6.7, (CIN 1) = 34.3, (CIN 2) = 683.5, (CIN 3+) = 62.5			Controls (175), CIN 1/2 (91)	Higher VL correlated significantly to CIN severity	0.0001	Tsai et al.[86]
HC2	RLU/CO	Ranged: 2–4880 (mean = 1514.54)			Normal (9), CIN 2 (18), CIN 3 (19)	VL significantly higher in CIN 2 and CIN 3 patients compared to controls	0.01/0.02	Bencomo-Alvarez et al.[87]
HC2	RLU/CO	Ranged: max mean + dev = 1385.39			ASC-US (361, of which cervicitis (166), CIN (119), SCC (76))	VL significantly correlated to cervicitis, CIN, and SCC; VL increased with lesion severity	<0.0001	Shen et al.[88]
HC2, Cobas, Geno-Array	RLU/CO for HC2, others qualitative	1–<10	10–<100	>100	Normal (1505), LSIL (721), HSIL (1188), HSIL+ (1233), cancer (45)	VL increased significantly in HSIL+	<0.0001 (trend)	Wang et al.[89]
HC2	RLU/positive control	<0.2	0.2–0.8	<0.8	Normal (80), LSIL (10), HSIL + (20)	Higher VL significantly increases SIL and cancer risk	0.01	Sun et al.[90]

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TABLE 1 (Continued)

Screening or referral samples (cytology)								
Detection method					**Patient information**	**Endpoint**		
DNA load assay	**Units**	**Low**	**Med**	**High**	**Cancer type, FIGO stage, or cervical disease (*n* =) at outset of study**	**Clinical outcome**	***p*-value/HR/OR/RR (respectively to outcomes)**	**Reference**
HC2	RLU/positive control	<0.6	0.6–10.0	<10.0	Normal (22), LSIL (19), HSIL (32)	Higher VL significantly increases risk of HSIL+ and larger lesions	0.0004, 0.008	Sun et al.[91]
HC2	RLU/positive control	0.01–1.0	1.01–2.0	2.0–3.6	Normal (182), CIN 1–3 (182)	Higher VL increased significantly with CIN grade	0.001	Hernandez-Hernandez et al.[92]
HC2	RLU/positive control	1–<10	10–<100	>100; >1000	Normal (2853), CIN 3+ (88)	Higher VL strongly increased risk of CIN 3 + , but did not predict risk predict risk thereof	ns	Lorincz et al.[93]
HC2	RLU/positive control	1–9	10–99	>100	Normal (652), unknown (8), ASC-US/LSIL (121)	Higher VL significantly correlated to CIN 2/3	<0.0001	Dalstein et al.[94]
HC2	RLU/positive control	N/A			Normal (236), ASC-US/ LSIL (308)	VL did not predict SIL progression. Medium VL increases incidence while high load decreases incidence	ns, both ways	Szoke et al.[95]
HC2	RLU/positive control	N/A			Normal (29), ASC-US (26), ASC-H (29), AGC (3), LSIL (98), HSIL (178)	HPV16 VL increased with CIN 1/3 lesions	ns	Briolat et al.[96]
ISH, linear array	Staining intensity	Observer values: low, intermediate, high			Normal (25 281), LSIL (19 946), HSIL (1893)	HPV16 VL increased with CIN 3 lesions	<0.0001	Adcock et al.[97]
ISH, qPCR	Dot blot oligonucleotide hybridization signal intensity	Ranged: scale of 1–5			CIN < 2—cancer	HPV16, as well as other types: VL higher in CIN 2+	HR CI min 4.4, 2.1	Gravitt et al.[98]
qPCR	10e-7 Copies/μL	Ranged: 0.38–7.99			ASC (61), AGC (18), LSIL (103), HSIL (14), SCC (7), AC (3)	Higher VL significantly associated with abnormal cytology	0.001	Al-Awadhi et al.[99]
qPCR	$2^{-\Delta Ct}$ values	N/A			Normal (3165), inflammation (3570), CIN 1 (215), CIN 2 (119), CIN 3 (209), ICC (75), other (394)	VL of HPV58/16/33 significantly correlated with the severity of cervical lesions	<0.001, 0.016, 0.026	Long et al.[100]
qPCR	Copies/μg of cellular genome	Ranged: 0.32–3700			Normal (25), CIN 1–3 (40), IA1-IVB (70, of which AC (8), ASCC (6), SCC (56))	High VL correlated to increased cancerous lesions	ns	Ho et al.[101]

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TABLE 1 (Continued)

Screening or referral samples (cytology)							
Detection method					**Patient information**	**Endpoint**	
DNA load assay	**Units**	**Low**	**Med**	**High**	**Cancer type, FIGO stage, or cervical disease (*n* =) at outset of study**	**Clinical outcome**	***p*-value/HR/OR/RR (respectively to outcomes)**	**Reference**
qPCR	Copies/μL	644–9e8, Median = 1 669 812			Normal (12), CIN 1 (12), CIN 2 (20), 27 had CIN 3 (27), SCC (19), AC (1)	HPV52 VL increases with CIN I progression to cancer	ns	Cheung et al.[102]
qPCR	Copies/μL	Ranged: max = 33 850 583 (HPV18) & ~100 000 (HPV16)			Normal (635), unknown (26), ASC-US (18), LSIL (68), HSIL (65), cancer (121),	HPV16/18 VL significantly associated with cytology grade ($p < 0.05$).	<0.05	Obeid et al.[103]
qPCR	Copies/μL or cell	Study duration dependent			CIS (621 cases, 621 controls), SCC (457 cases, 457 controls)	HPV16 VL increased continuously in HPV16-positive cancer cases 10 y prediagnosis	ns	Sundstrom et al.[104]
qPCR	Copies/1000 cells	Ranged: 1–337 398			ASC-US (2011)	Higher HPV16/18 VL associated with increased CIN risk	HR CI min 1.38, 1.25	Constandinou-Williams et al.[105]
qPCR	Copies/1000 cells	Ranged: max mean + dev = ~6			Normal (1341), LSIL(209), HSIL (392), SCC (520), AC (51)	HPV VL increases with histological grade from normal to SCC, and peaked in LSIL and HSIL	<0.01 (differences), <0.05 (association)	Wu et al.[106]
qPCR	Copies/10 000 cells	Ranged: ~1–~8			Of total (15 518), HPV-infected samples were CIN 1 (1481), CIN 2/3 (849), cancer (149)	HPV16 and similar types' VL significantly higher in CIN 2/3 and cancer vs. LSIL/CIN 1	0.001	Wang et al.[107]
qPCR	Copies/300 ng of total DNA	Significance threshold = 1.38e6			LSIL/CIN I (72), 94 HSIL (94); of which CIN 2/3 (83) & SCC (11)	VL significantly higher in HSIL than in LSIL	0.001	Cricca et al.[108]
qPCR	Copies/cell	<0.45		>0.45	Unknown (999), ASC-US (100), at follow-up CIN 1 (29), CIN 2 (27), CIN 3 (49)	Higher hrHPV VL: significant correlation to CIN 1/2, but especially in CIN 3+	<0.0001	Schmitt et al.[109]
qPCR	Copies/cell	<0.46		>0.46	Normal (913), ASC-US (136), LSIL (114), HSIL (110)	Higher hrHPV VL increased from normal to ASC-US, LSIL to HSIL	<0.05	Schmitt et al.[110]
qPCR	Copies/cell	<80th percentile of controls		>80th percentile of controls	Normal (129), HSIL (57), cancer (216)	VL of HPV 16/18/58 significantly correlated to HSIL+ lesions	<0.001	Kim et al.[111]
qPCR	Copies/cell	≤0	0–105	>105	Normal (114), CIN 1 (56), CIN 2+ (3)	Lesions more frequent in low VL HPV16 patients	OR CI min 1.16	Del Rio-Ospina et al.[112]

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TABLE 1 (Continued)

Screening or referral samples (cytology)								
Detection method					**Patient information**	**Endpoint**		
DNA load assay	**Units**	**Low**	**Med**	**High**	**Cancer type, FIGO stage, or cervical disease (*n* =) at outset of study**	**Clinical outcome**	***p*-value/HR/OR/RR (respectively to outcomes)**	**Reference**
qPCR	Copies/cell	≤1367.79	Median = 132.5	>1367.79	Negative (49), Inflammation (28), unknown (9), ASC-US (13), LSIL (31), HSIL (38), CIS (8)	Higher VL associated with significant risk of HSIL + , especially HPV16	<0.0001 and CI min 2.75	Alvarez-Paredes et al.[113]
qPCR	Copies/cell	1–10	11–100	101–1000; >1000	Normal (2081), LSIL/HSIL at follow-up	Higher HPV VL increased risk for SIL	RR 95% CI 1.9 for higher copy number	Schlecht et al.[114]
qPCR	Copies/cell	Ranged: 0.04–23.65			Normal (65), LSIL (127), HSIL (170)	VL associated with abnormal cytology	<0.001	Marongiu et al.[115]
qPCR	Copies/cell	Ranged: <0.01–5807.7-2	Significance threshold = 33rd percentile (0.2)		Normal (674), CIN 1 (37), CIN 2+ (125)	hrHPV VL significantly associated with CIN 3	0.001	Snijders et al.[116]
qPCR	Copies/cell	Ranged: 0.21–28.0; Median = 2.15			ASC-US (17 235)	Higher HPV16 VL has significant correlation to CIN 2+ lesions	0.001	Tao et al.[64]
qPCR	Copies/cell	Ranged: 1.27–74.61			Control (79), normal (39), CIN 1 (4), CIN 2 (5), CIN 3/cancer (31)	Higher VL increases non-significantly with cervical lesion progression	ns	Manawapat et al.[117]
qPCR	Copies/ng of cellular DNA	Ranged: 0.27–4.40			ASC-US (503), LSIL (318)	Higher VL increases CIN 3 risk	ns	Xi et al.[118]
qPCR	Copies/scrape	Ranged: 5–4e9	Significance thresholds = 2.4e4 (normal), 4.3e6 (CIN 2/3)		Group A = control (47), CIN 2/3 (12); Group B = control (25), CIN 2/3 (38)	HPV16 VL significantly higher in cancer cases and in CIN 2/3 vs. CIN ≤ 1	0.04	van Duin et al.[119]
qPCR	Ct value	>45.60		<39.62	Control (608), Carcinoma (478)	Higher HPV16 VL increases probability of developing carcinoma	ns	Ylitalo et al.[120]
qPCR	Ct value	Ranged: <39.9–50.0			Control (608), Carcinoma (478)	Higher HPV16 VL significantly increases probability of developing CIS	HR CI min 1.1 up to min 15.8	Josefsson et al.[25]
qPCR	HPV copies/ genomic DNA	Ranged (HPV16): 0.00060–96.56597; (HPV52): 0.00125–13.0894			Normal (19), ASC-US (5), LSIL (14), HSIL (10)	HPV 16/52 VL increases cervical lesion progression	0.001, 0.003	Hamaguchi et al.[121]
qPCR	HPV genomes/ 10 000 cells	<Median	Median = 1307	>Median	Normal (125), ASC-US (105), LSIL (200), HSIL (152), cancer (15)	HPV16/31 VL increases significantly with cytology grade	<0.05	Broccolo et al.[56]

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TABLE 1 (Continued)

Screening or referral samples (cytology)								
Detection method					**Patient information**	**Endpoint**		
DNA load assay	**Units**	**Low**	**Med**	**High**	**Cancer type, FIGO stage, or cervical disease (*n* =) at outset of study**	**Clinical outcome**	***p*-value/HR/OR/RR (respectively to outcomes)**	**Reference**
qPCR	HPV genomes/ 10 000 cells	log ≤3	log 3–5	log >5	Unknown (294), at follow up CIN (114), cancer (3)	HPV16 VL is significantly associated with higher grade cervical lesions	<0.0001	Oyervides-Munoz et al.[122]
qPCR	HPV genomes/cell	Ranged: 0.019–4194			Normal (47), CIN 1 (11), CIN 2 (44), CIN 3 (85), other & unknown (9 & 41)	HPV16 VL is associated with higher grade cervical lesions	0.0624	Fiander et al.[123]
qPCR	HPV genomes/ human genome equivalent	0–1	1–10	>10	Controls (1049), SCC (201)	HPV16 VL (and others to a less significant degree) correlated with invasive cancer presence	0.0001	Moberg et al.[124]
qPCR	HPV genomes/ human genome equivalent	25th percentile	Mean	75th percentile	Controls (552), CIS (457)	Higher VL for most of the HPV types studied increased risk of cancer, especially HPV16	0.0001	Moberg et al.[57]
qPCR	Log copies E6	Median (HPV16) = 4.15, (18) = 3.76, (52) = 4.92			CIN 2–3 (97), cancer (81)	HPV 16/18/52 significantly correlated to invasive cervical cancer development compared to CIN 2–3	0.022, 0.003, 0.001	Ho et al.[125]
qPCR	Log copies HPV18/ ng cellular DNA	Ranged: 1.12–6.62			Normal (60), ASC-US (82), LSIL (127), HSIL (34)	HPV18 VL correlated significantly to increasing severity of cervical cytology	0.001	Xi et al.[126]
qPCR	log copies/μg DNA	N/A			Normal (270), CIN 1 (176), CIN 2–3 (149)	HPV16 VL increased with epithelial abnormality	ns	Swan et al.[7]
qPCR	Log copies/μL (or cell equivalent)	0.01–9.0e5; Median = 56			CIN 1—cancer	Higher VL was seen in CIN 1/2, but controls and CIN 3 patents overlapped	ns, both ways	Chan et al.[127]
qPCR	Log copies/10,000 cells	Ranged: 1.7–6.5			CIN 1 (2821), CIN 2/3 (785), cancer (122)	HP16/18/31/33/51/52/53/58 VL increases with cervical pathological grade	<0.001	Li et al.[128]
qPCR	Log copies/cell	Logscale categories from <0.01 to >1000			Normal (73), CIN 1–3 (395), cancer (164)	HPV16/18/31 VL increases with precancerous lesions	OR CI min 1.11	Malagon et al.[129]

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TABLE 1 (Continued)

Screening or referral samples (cytology)								
Detection method					**Patient information**	**Endpoint**		
DNA load assay	**Units**	**Low**	**Med**	**High**	**Cancer type, FIGO stage, or cervical disease (*n* =) at outset of study**	**Clinical outcome**	***p*-value/HR/OR/RR (respectively to outcomes)**	**Reference**
qPCR	log copies/ng cellular DNA)	Ranged: max mean + dev = 5.32			Normal (845), ASC-US (692), LSIL (701), HSL (217), unknown (447)	HPV16 and similar VLs are associated with risk of development of CIN 2/3	HR CI min 1.14	Fu Xi et al.[55]
qPCR	Log E6 signal	Ranged: 3.1–7.2			Normal (9), CIN 1 (19), CIN 2/3/CIS (25), I–II (39), III–IV (29)	HPV16 VL predicts tumor progression for normal vs. CIN 2/3/CIS/FIGOI/II, and normal vs. FIGO III/IV	0.002, 0.001	Wanram et al.[130]
B: Details on viral load measurement in tissue samples obtained for diagnostic purposes and correlations to disease severity and clinical outcome reported in literature								
Biopsy samples (tissue)								
Detection method					**Patient information**	**Endpoint**		
DNA load assay	**Units**	**Low**	**Med**	**High**	**Cancer type, FIGO stage, or cervical disease (*n*=) at outset of study**	**Clinical outcome**	***p*-value/HR/OR/RR (respectively to outcomes)**	**Reference**
HC2	RLU/CO	Ranged: 0.84–2701.47, Medians (Normal) = 42.68, (CIN I) = 146.45, (CIN II) = 156.43			ASC-US of which: Normal (162), CIN 1 (135), CIN 2+ (52)	VL correlated to CIN presence but not severity	0.001	Lee et al.[131]
PCR	Band size/control	Observer values: low & high			AC (5), SCC (39), I-III	Higher VL: decreased PFS/OS	0.04, 0.2	Kahla et al.[132]
qPCR	$2^{-\Delta Ct}$ values	2.63152 ± 0.68173e-05 (LSIL), 3.08763 ± 0.59262e-05 (HSIL), and 3.35171 ± 0.57651e-05 (SCC)			Normal (20), LSIL (52), HSIL (46), SCC (29)	VL significantly correlated to epithelial dysplasia and non-significantly to lesion progression (LSIL-SCC)	<0.05 (dysplasia), >0.05 (trend)	Wu et al.[133]
qPCR	Copies/cell	<5	–	>5	NECA (21), IB-IIA	Higher VL: increased DFS	0.028	Siriaunkgul et al.[134]
qPCR	Copies/cell	Ranged: 0.002–36.9			AC (5), ASCC (5), SCC (14), IB1–IIA	Longer (5 years) surviving patients did not have a difference in HPV 16 or 18 VL compared to those surviving <2 years	ns	Dahlgren et al.[135]
qPCR	Copies/cell	Ranged: 12–1800; Median = 450			SCC (15), IB–IIA	Higher VL: increased LN	0.016	Chan et al.[51]
qPCR	Log copies per cell	Ranged: Approx. log(−2.0)–log(5.0)			CIN 1–3	Higher VL: increase from CIN 1 to CIN 2	ns	Andersson et al.[38]
qPCR	Log copies/ng	4.4	–	11.3	AC (3), ASCC (1), SCC (9), IB-IIIB	High VL in positive lymph nodes	0.005	Lillo et al.[136]

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TABLE 1 (Continued)

B: Details on viral load measurement in tissue samples obtained for diagnostic purposes and correlations to disease severity and clinical outcome reported in literature

Biopsy samples (tissue)

Detection method					Patient information	Endpoint		
DNA load assay	Units	Low	Med	High	Cancer type, FIGO stage, or cervical disease (*n*=) at outset of study	Clinical outcome	*p*-value/HR/OR/RR (respectively to outcomes)	Reference
qPCR	Log E6 signal	N/A			Normal (20), CIN 1 (27), CIN 2 (28), CIN 3 (33) SCC (29)	No significant differences in VL observed across the disease categories	0.15 (HPV16), 0.74 (other types)	Guo et al.[137]
qPCR	Log viral DNA/ 100 ng genomic DNA	Ranged: 3.736–22.764, Medians (cases) = 17.21, (controls) = 9.86.			Controls (87), cancer (152)	VL significantly higher in cases vs. controls	0.001	Das et al.[50]
qPCR	Viral/genomic DNA ratio	Ranged: 0.00–1.93e6			Controls (26), wart controls (29), cancer (85)	VL in cancer samples higher than that in controls; also increased with progression from FIGO I to II	0.011	Chang et al.[138]
ISH	IOD	<50%	–	>50%	IIB–IIIB	Higher VL: decreased OS	0.001	Cao et al.[59]
ISH	IOD	Ranged: 2293.9–5734.75			SCC (116), IIB–IIIB	Higher VL: decreased OS	0.001	Cao et al.[139]

Note: Cervical cancers are staged according to the International Federation of Gynecology and Obstetrics (FIGO) 2018 guidelines, based on pathological and microscopic characteristics including size, invasion, location, and metastasis[59]; Arrows "→" denote "correlated to".

Abbreviations: AC, adenocarcinoma; AGC, atypical glandular cells; ASCC, adenosquamous cell carcinoma; ASC-H, atypical squamous cells, cannot exclude a high-grade squamous intraepithelial lesion; ASC-US, atypical squamous cells of undetermined significance; CCRT, concurrent chemoradiotherapy; CI, confidence interval; CIN, cervical intraepithelial neoplasia; CO, cutoff; DFS, disease-free survival; HC2, hybrid capture 2; HPV, human papillomavirus; HR, hazard ratio; HSIL, high-grade squamous intraepithelial lesions (ASC-US, ASC-H, AGC, and L/H-SIL are ratings of abnormal cytology using the Bethesda system[58]); IOD, integrated optical density; ISH, in situ hybridization; LBC, liquid-based cytology; LSIL, low-grade squamous intraepithelial lesions; NECA, neuroendocrine carcinoma; OR, odds ratio; OS, overall survival; PC, positive control; PFS, progression-free survival; (q)PCR, (quantitative) polymerase chain reaction; RH, radical hysterectomy; RLU, relative light units; ROC, receiver operating characteristic; SCC, squamous cell carcinoma; VL, viral load.

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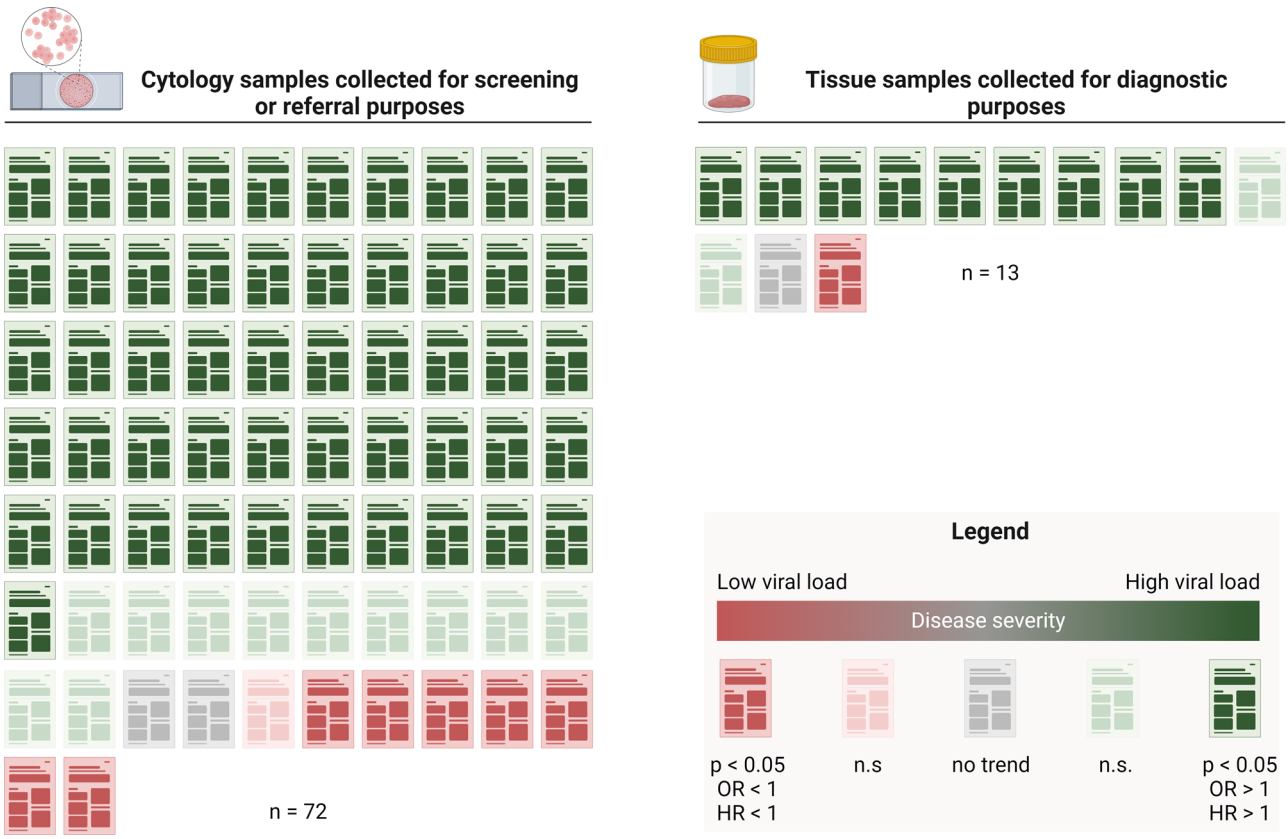

FIGURE 3 Illustration of data collection and the correlation between the viral load of HPV and clinical outcomes. HPV, human papillomavirus; HR, hazard ratio, n.s., nonsignificant; OR, odds ratio.

3.2 | Higher viral load correlates to more severe disease and worse clinical outcome

The majority of studies (72/85 = 84.7%) used cytology samples collected for screening/referral purposes, from 172 227 women enrolled. The remaining studies (13/85 = 15.3%) used tissue samples taken for diagnostic purposes, from 1519 women. The differences between the sample types, and their association with disease severity and/or clinical outcomes are discussed in the next sections.

In general, there is substantial disparity in the sampling purposes, testing methods, and viral load quantification ("high," "medium," and "low") as a prognostic marker in cervical cancer. However, despite these differences, the vast majority (87.1%; 74/85) of studies found a trend towards poor prognoses with higher viral loads, which includes CIN grades, or higher stages (disease severity) of cancer, worse overall survival, disease-free survival, progression-free survival, or higher recurrence rates (Figure 3). No differences in correlation were observed between both larger and smaller cohorts, and older and more recent studies, which implies that even with smaller populations and improvements in technology, trends have remained similar. However, the screening of a wider range of HPV types, using both HC2 and qPCR methods, will likely improve reporting of such information as is contained in this review, especially in terms of cutoff values and methodology. Notably, some studies also resolved to exclude non-HPV-infected samples from their analyzes. While this is a logical choice for viral load analysis, a study carried out in a larger cohort should be reported alongside healthy controls, to prevent bias. This also increases the discordance between results. Last, it was found that several groups studying biopsies made efforts to exclude non-neoplastic tissue from analyzes.[59,134] The same would be impossible for cytology samples, affecting findings, and therefore it is strongly discouraged to compare results between these two types of sampling methods.

3.3 | Cytology samples: Higher viral load correlates to disease severity

Of the 72 articles listed which studied cytology samples taken for screening/referral purposes, 62 (86.1%) noted that a higher viral load was linked to (a higher) CIN grade or cervical cancer stage. These were used to determine the difference between normal (healthy) cells and abnormal cells, CIN 1–3, or cancer. Moreover, a clear majority used approximately the same cut off RLU/CO values for grouping viral load levels, namely for "low" 1–10, "medium" 11–100, and "high" 101–1000. Of these 62 articles

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that concluded that a higher viral load was linked to a higher disease severity, 30 studies found that a higher viral load was only correlated to a higher CIN grade, but not more severe stages of cancer, and 29 studies reported a higher viral load being linked to a higher CIN grade and/or cervical cancer. In two of these studies, no correlation between viral load and CIN grade or cancer was found, and one reported the opposite correlation, noting an increase in presence of lesions among that population with decreased presence of HPV16.[112] This is, in fact, particularly unusual given that 27/72 (37.5%) articles specifically cite that especially HPV16 is primary driver of the observed increase in severity.[65,66,105,120]

In 12.5% (9/72) of the above articles, cytology samples were used to investigate a potential link between viral load and clinical outcomes, including disease-free survival and recurrence rate. A trend towards a less severe clinical outcome was linked to a higher viral load in 7 out of these 9 studies, a result strikingly opposite to the majority. A possible explanation for such a discrepancy could be that this sampling method (cytology) should not be used to evaluate clinical outcomes such as survival of cancer patients. Moreover, it was evident that these studies, all of which measuring DNA via HC2, had categorized their "low" HPV viral load group as <100 (or even 10-fold higher), whereas many other studies have a cut off at <10. This once again emphasizes the need for consistency between studies.

3.4 | Diagnostic tissue samples: Higher viral load correlates to worse clinical outcome

In 13 studies, with a total of 1519 women enrolled, biopsies were collected from patients for diagnostic purposes to determine the cancer stage and type to decide what treatment plan they should receive. Most of these studies used qPCR-based methods to quantify and demonstrate the detected range for the viral load, and actively excluded non-cancer cells in their analyzes. In the majority (11/13 = 84.6%) of included articles, an increased viral load correlated to higher stages of cancer (median: 128 patients/study, range: 13–349 patients), and to a worse clinical outcome. The disease outcomes included significantly worse overall survival ($p = 0.02$; $p = 0.001$; $p = 0.001$), progression-free survival ($p = 0.04$), progression of cervical cancer ($p = 0.011$), and presence of positive lymph nodes ($p = 0.016$; $p = 0.005$).

Only in one study (Siriaunkgul et al.[134]) was a lower viral load correlated to a worse treatment outcome (shorter disease free survival, $p = 0.028$). However, the cohort in this study only consisted of 21 neuroendocrine cervical cancer patients. This is a rare and aggressive type of cervical cancer. All patients were HPV18 positive, and only a few patients had co-infections with HPV16, which is notable because it is more likely for the positive correlation between a high viral load and worse clinical outcome to be observed in cases with HPV16 infections,[33,126,134,140] which could explain the opposite correlation observed in this study.

4 | DISCUSSION

The majority of published studies show that a higher viral load is associated with more severe disease or poorer patient outcomes, such as worse survival. Even though there was a large variety in different sample types, methodology, definition of HPV viral loads. Moreover several studies, in which patients biopsies were collected for diagnosis of cancer, efforts were made to actively exclude non-neoplastic tissue for analyzes of HPV viral load. This was not possible for cytology samples, possibly explaining the discrepancy in survival when correlating HPV viral load with samples obtained for screening/referral purposes (mainly cytology samples) or diagnosis of cancer (mainly biopsy samples).

CIN and cervical cancer were taken together in this study following the same practice having been followed and published in several of the included papers relating viral load to severity of disease in those studies that used cytology samples. Specifically, long-term follow-up[66,125,129,130] and retrospective/prospective[76,89,98] studies, where progression to higher grades of CIN (2+) and cervical cancer was reported to be significantly linked to higher HPV viral load as a risk factor. Others reported the same trend, albeit not statistically significant.[117] Several non-longitudinal, observational, or cross-sectional studies followed the same inclusion of disease states from CIN 1 through to invasive cervical cancer using both cytology[77,78,83,85,99,101–103,106–108,128] and tissue samples.[133,137] This approach yielded a body of evidence upon which sufficient conclusions could be made. To study viral load in relation to clinical outcome of cancer patients both studies using cytology and tissue samples were available, all but one of which reporting a tend for high HPV viral load increasing risk of progression to higher grades of CIN or cancer.

The mechanism by which the above takes place is of interest, and may inform the way patients are managed and stratified. This, along with multiple infections and the integration status of the virus (episomal or integrated),[108,141] are being investigated widely as diagnostic and treatment outcome biomarkers in cervical lesions.[97] Higher incidence of HPV has been found in patients with primary and secondary immune deficiencies, supporting the crucial role of the immune system in the control and clearance of HPV infections,[142–144] while HPV-mediated immunosuppression also creates a tumor microenvironment in which the viral load in these patients may increase.[145] Higher proportions of regulatory T cells, HPV oncoprotein-mediated interference with adaptive immunity pathways at multiple levels, and a T-helper (Th)1/2 and cytokine imbalance, all potentiate this phenomenon.[146,147] Upon this basis, the finding that a higher viral load makes for a more suppressive tumor microenvironment is key in understanding the mechanisms behind the trend reported in this review and should certainly be described in more detail in future studies.[59]

The findings presented in this study may suggest that higher viral load-bearing tumors could respond differently to treatment than those with an overall lower viral load, and are most in need of

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improved therapies. This is especially important with the recent FDA-approval of immunotherapies, including pembrolizumab for use in PD-L1$^+$ cervical cancer (Figure 1B).[148] Tumors with a higher viral load could potentially be sensitive to immunotherapy, as PD-L1 has been shown to be overexpressed in cervical cancer tissue compared to normal cervical tissue,[149,150] and increases with cervical cancer stage.[150] Taken alongside the findings presented in this systematic review, of increased viral load present in more advanced disease, it is plausible that these two parameters may be directly or indirectly linked, warranting further investigation. Accordingly, reports have indicated that higher levels of CD8$^+$ TILs are present in patients with higher HPV16 viral load.[151] Furthermore, it has been widely reported that patients in low CD8$^+$TIL and high PD-L1 expression groups had significantly worse clinical outcome and worse survival.[150] Other studies have found the same in HPV-related[152] and other cancers.[153] Therefore, the prognostic impact and stratification potential of HPV status, viral load, and CD8$^+$ TILs, and PD-L1 expression as well as combinations of these, are not to be overlooked, especially when considering (immuno)therapeutic outlooks.

Due to the dynamic nature of PD-L1 expression on a variety of tumors, and the responses thereof to a variety of stimuli,[154–156] one may also be interested in the expression products of HPV at a protein or messenger RNA (mRNA) level. However, viral load is not closely correlated to the expression of HPV genes.[157] de Boer et al.[158] found that the copy number of HPV DNA was not predictive of overall survival, but the expression (mRNA level) of HPV E6/E7 was correlated to poor prognosis in cervical cancer patients. On mRNA level, many epigenetic factors influence the expression of HPV genes,[20] including methylation,[115,159] microRNA expression,[160] and others, all of which have been found to impact clinical outcomes. Wu et al.[161] demonstrated that HPV16 E6 mRNA was significantly increased in invasive cervical cancer versus HSIL lesions. However, some studies have shown no significant correlation between viral load and E6/E7 mRNA expression level.[162] Thus, at this time, little is known about how HPV viral load and E6/E7 gene expression may change during the progression of the disease, and during the course of treatment. Multiple cervical sampling during the course of disease is technically simple, but may cause a considerable burden to the patient. Viral load of the initial biopsy, as reviewed in the present study, may be sufficiently predictive to evaluate and improve new treatment strategies. The clinical situation, due to papillomavirus species specificity, and vast differences in infectivity or various organs, symptoms, and other implications for papillomaviruses infecting animals,[163] is difficult and complex to accurately recapitulate. Several naturally occurring animal papillomavirus models have been applied to study viral-host interaction for HPV pathogenesis and played a pivotal role in better understanding of the mechanisms underlying tumor progression to cancer.[164–167] Therefore, a novel mouse papillomavirus model that representatively mimics HPV-associated infections and disease progression in the lower genital tract could be useful to determine these correlations.[168] Last, as a further potentially useful DNA-level measurement, tumor mutational burden (TMB) has been studied in cervical cancer. It is currently unclear which particular genes contribute most to the TMB,[169,170] as the integration site of HPV within the host genome is not universally conserved.[171] There is evidence that high TMB cervical cancer patients exhibit more severe disease, along with increased stage and lymph nodes.[169] An increased TMB motivates for the use of immunotherapies such as checkpoint inhibitors, in high TMB patients, due to an increased likelihood of chemo/radiotherapy resistance.[169,172] Specifically, pembrolizumab was approved in a tissue and site-agnostic manner based on the TMB of enrolled patients.[173] The same has been confirmed by other groups, who found that TMB indeed not only holds prognostic value, but is also related to the infiltration level of immune cells within the tumor microenvironment, which directly links to the success of checkpoint inhibitors.[170] Furthermore, HPV-positive cervical cancers have been shown to have higher TMB,[174] leaving the question as to whether this may be linked to viral load open to further research.

4.1 | Limitations of the study

This systematic study on the clinical role of viral load in cervical cancer and its precursors assessed a wide range of published studies. Although a vast amount of data was found in 85 useful studies, the comparability between studies was limited by a large variety of laboratory methods and clinical outcomes. The possibility of including a meta-analysis was explored, but was found not to be feasible due to differences in methodology, measurement techniques, units reported, and patient selection. Therefore, much of the value of the present systematic review is to help future researchers improve standardization and comparability, particularly to overcome differences in the measurement of viral load levels, extraction of patient material, and documentation of study outcomes. In Table 1 we listed technical procedures which may benefit from standardization. Practical suggestions and recommendations for more standardized reporting and communication of results are also provided based on the findings, such as use of similar methodologies (where available), reporting similar units within similar thresholds, as the categorization of "low," "medium," and "high" HPV load is extremely important for interpretation of data. Furthermore, classifications of disease severity were inconsistent throughout the studies assessed; thus, what was reported had to be conserved to maintain accuracy. While this makes use of both cytological and histological cervical lesion classifications, as well as FIGO staging for cancerous samples, the overall trend for all sample types is clear and useful for other groups embarking on similar scientific questions in the future.

5 | CONCLUSION

This systematic review on HPV viral load in cervical cancer and its precursors confirms a strong association between viral load and severity and outcome of disease (overall survival and disease-free

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survival). Measurement of viral load is not a standard procedure, nor is there consensus on the methodology or quantitative values. Despite these differences, there is a clear convergence of results from both cytology and tissue samples collected for screening/referral or diagnostic purposes, that a viral load is an independent prognostic factor, next to HPV genotype, HPV co-infection, genome integration status, and other clinical factors. There is increasing evidence that viral load also is a predictive marker for response to a range of treatments potentially including immunotherapies, and may therefore be used as future guidance for personalized treatment selection. Future studies on viral load need more standardization and alignment of techniques and result reporting, to improve comparability and for practical use as a prognostic and predictive marker of cervical cancer and its precursors.

AUTHOR CONTRIBUTIONS

Seth-Frerich Fobian*: Study design, writing of manuscript, performing searches, extracting data, editing. **Xionge Mei***: Study design, performing searches, writing of original manuscript. **Barbara C. Snoek, Renske D. M. Steenbergen, Timo L. M. ten Hagen, Louis Vermeulen, and Jiafen Hu**: Expert review and editing of manuscript. **Johannes Crezee and Lukas J. A. Stalpers**: Expert review, advice, and manuscript editing. **Arlene L. Oei**: Study conceptualization, design, writing of manuscript, performing searches, editing, and co-ordination of all authors (***equal contributors**).

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CONFLICT OF INTEREST STATEMENT

Louis Vermeulen is currently an employee of Genentech Inc. Renske D.M. Steenbergen is a minority shareholder of Self-screen BV.

DATA AVAILABILITY STATEMENT

The exact database search queries and data extracted from articles contained herein are available from the authors upon reasonable request. Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ORCID

Seth-Frerich Fobian http://orcid.org/0000-0001-6550-4304
Xionge Mei http://orcid.org/0000-0001-8697-2721
Johannes Crezee http://orcid.org/0000-0002-7474-0533
Barbara C. Snoek http://orcid.org/0000-0002-3558-2165
Renske D. M. Steenbergen http://orcid.org/0000-0002-2327-9839
Jiafen Hu http://orcid.org/0000-0001-8700-9937
Timo L. M. ten Hagen http://orcid.org/0000-0002-0955-7740
Louis Vermeulen http://orcid.org/0000-0002-6066-789X
Lukas J. A. Stalpers http://orcid.org/0000-0001-9574-0295
Arlene L. Oei http://orcid.org/0000-0002-6936-1934

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