Infectious Agents and Cancer

REVIEW

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Prophylactic vaccines against HPV-caused cervical cancer: novel vaccines are still demanded

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Abstract

Several high-risk types of human papillomaviruses (HPVs) are associated with cervical cancer and other malignancies. Despite the tremendous success of marketed prophylactic HPV vaccines for the past 18 years, cervical cancer remains a significant global challenge. A nearly 10% increase in new cervical cancer cases worldwide from 2020 to 2022 underscores the urgent need for enhanced vaccination efforts. Current HPV vaccines, including Cervarix®, Gardasil®, Gardasil®9, Cecolin®, and Walrinvax® utilize VLP (virus-like particle) structures and have demonstrated significant efficacy. However, challenges such as type-limited coverage, cold-chain requirements, and affordability emphasize the critical need for further research and development of novel HPV vaccines. Some investigational vaccines, for instance, those using VLPs to carry protective antigens with broader coverage across different viral types, show promise for the future of cervical cancer prevention. Realizing this hope and making further progress still depend on the dedication and innovation of the scientists and authorities involved. This review focuses on both approved and investigational preventive vaccines, including also those designed for simultaneous prevention and therapy. Clinical trials are briefly reviewed, and potential strategies to advance vaccination against HPV-induced cervical cancer are summarized. This review emphasizes approaches that require further investigation in the future.

Keywords HPV, Human papillomavirus, Cervical cancer, Cancer vaccines, Vaccine immunogenicity

Introduction

Cervical cancer (CxCa) ranked as the fourth most common cancer among women worldwide, with 661,021 newly diagnosed cases reported in 2022 [1]. It is a leading cause of cancer-related mortality in women, causing nearly 350,000 deaths globally in the same year [2]. Furthermore, CxCa is a significant contributor to maternal orphanhood, accounting for approximately 20% of cases worldwide, which leads to substantial social and economic implications that must be addressed [3]. Therefore, the control and elimination of CxCa is a public health priority.

Carcinogenic human papillomavirus (HPV) types are identified as the principal risk factor for CxCa in over 99% of cases. HPV Infections are more prevalent than

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any other sexually transmitted infection globally [4]. Additionally, CxCa shows a significant correlation with HIV infection, with studies showing that HIV infection contributes to a nearly six-fold increase in the incidence of CxCa. The highest incidence and approximately 94% of deaths due to CxCa occur in low- to middle-income countries, particularly sub-Saharan Africa, Southeast Asia, and Central America [5], where access to HPV vaccination and screening programs is inadequate. This situation highlights crucial disparities in socioeconomic factors that influence healthcare standards [2], especially since no specific genetic predisposition for CxCa has been documented [6].

The significant relationship between CxCa and persistent infection with HPV has fostered optimism regarding the potential impact of HPV vaccination on reducing the CxCa burden. Vaccination efforts can focus either on prevention or treatment. Currently, all marketed HPV vaccines are prophylactic, although research into therapeutic vaccines is ongoing.

Prophylactic vaccines are designed to generate immune protection by producing neutralizing antibodies (nAbs), primarily of the IgG type, while therapeutic vaccination aims to induce cell-mediated immunity, specifically targeting $CD8^+$ T cells against HPV [7]. Since the introduction of Gardasil®, the first approved HPV vaccine in 2006, prophylactic HPV vaccines have effectively prevented HPV infections and significantly reduced the burden of CxCa in some countries [8]. Current prophylactic HPV vaccines are based on virus-like particles (VLPs) [9], which primarily prevent HPV invasion by stimulating humoral immunity and generating nAbs that target the main HPV capsid protein, L1 [10].

Despite significant advances in vaccination and the development of novel HPV vaccines, recent statistics from Globocan indicate that the incidence and mortality of CxCa are still on the rise globally (Fig. 1). Even in low-income regions of the United States, a recent reversal of the declining trend of CxCa has been observed [11]. Furthermore, the World Health Organization (WHO) predicts a 16.9% increase in CxCa incidence and a 21.1% rise in the mortality rate by 2030 worldwide. These statistics and projections underscore the urgent need for developing novel approaches and strategies to control HPV infection through vaccination [2].

This paper aims to address this issue by first reviewing the structure and pathogenesis of HPV. It then summarizes the available and investigational prophylactic HPV vaccines, highlighting their platforms, efficacy, and outcomes in preventing CxCa. Additionally, the review succinctly discusses the types of vaccines that could bolster global CxCa prevention efforts in the future.

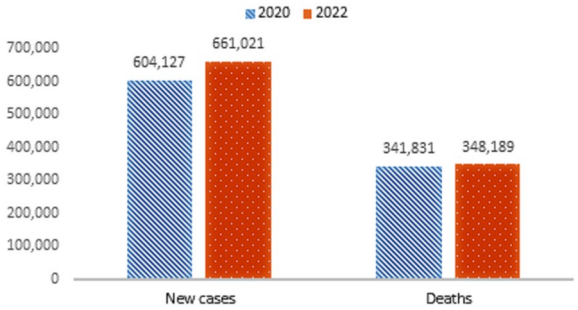

Fig. 1 Worldwide incidence and mortality caused by cervical cancer in 2020 and 2022, according to Global Cancer Statistics. New cases rose by approximately 9.42% in two years. The number of deaths slightly increased (1.86%). Although the greater number of cases of all cancers might explain the higher number of cases, the percentages of cases associated with cervix uteri incidence and mortality (versus all cancers) also slightly increased from 2022 to 2020 (3.3% vs. 3.1% and 3.6% vs. 3.4%, respectively [1] [176])

Human papillomavirus (HPV)

Structure

HPVs are nonenveloped icosahedral viruses containing circular double-stranded DNA. Their genome encodes six early proteins (E1, E2, E4, E5, E6, and E7), which play regulatory roles in replication and carcinogenesis, as well as two late proteins (L1 and L2) that form the capsid [12]. Specifically, E1 and E2 aid in replication, E4 facilitates virus release, and E5, E6, and E7 promote host cell proliferation [13]. Major (72 L1 pentamers) and minor (12–72 L2) proteins self-assemble and create the capsid [10]. The infection process begins with the attachment of the virions to basal cell heparan sulfate proteoglycans (HSPGs). Subsequently, during virus integration, L1 and L2 are deleted. Consequently, vaccines targeting these proteins will be ineffective if HPV-related diseases have already developed [14].

HPV diversity

HPVs are categorized into five main phylogenetic genera (α, β, γ, μ, and ν) according to the nucleotide sequence of the open reading frame (ORF) encoding the L1 protein. HPVs from different genera present less than 60% similarity in the L1 gene. In a genus, HPVs are grouped into species with 60 to 70% similarity. The International Human Papillomavirus Reference Center has identified 52 species and 228 subtypes of HPV, although only a limited number of these viruses are associated with health issues. Certain types of HPV can result in conditions such as genital warts, while others may lead to cancers of the cervix, vulva, vagina, oropharynx, or anus [15]. To date, more than 200 HPV genotypes have been identified and classified into three groups based on their carcinogenic

potency: high-risk (HR-HPV), potentially high-risk (pHR-HPV), and low-risk (LR-HPV). Fourteen types (HPV-16/18/31/33/35/39/45/51/52/56/58/59/66/68) are recognized as HR-HPVs for CxCa with varying probabilities of risk occurrence. LR-HPVs (HPV-6/11/40/42/43/44) are considered noncarcinogenic but can cause anogenital warts, such as condyloma acuminatum [4, 16]. Notably, HPV-16 and −18 are the most common carcinogenic HR-HPVs and are involved in approximately 50 and 20% of CXCas, respectively [17]. Moreover, five α–papillomaviruses (53/66/70/73/82) are recognized as pHR-HPVs due to limited data regarding their carcinogenicity [18].

Viral life cycle and pathogenesis

HPV is responsible for the majority of sexually transmitted infections worldwide. The natural clearance of HPV typically occurs within the first two years following infection. However, if the infection persists, a variety of diseases, including genital warts and cancer, may arise [19].

HPVs are viruses that infect the epithelial basal cells of the cervix and various other organs. The viral entry process begins when the virus binds to heparan sulfate proteoglycans (HSPGs) on the basement membrane [20]. Following this binding, conformational changes occur in the virus, exposing the N-terminus of its L2 protein [17]. This exposure facilitates the virus entry into keratinocytes. If the viral infection is not cleared, the virus then travels to endosomal compartments. After uncoating, it releases its genome into the cell nucleus through an L2-dependent mechanism. The viral genome can persist in an episomal state in basal cells—besides the host DNA—potentially leading to benign or precancerous lesions [14, 20].

In some cases, HPV can integrate into host DNA, disrupting host gene expression and producing oncoproteins, particularly E6 and E7. These oncoproteins interfere with key cell cycle regulatory proteins, promoting uncontrolled cell growth and contributing to malignant transformation [21]. Following integration, other early genes—namely E1, E2, E4, and E5—as well as the late genes—L1 and L2—are deleted [14]. This process can lead to cervical intraepithelial neoplasia (CIN) and, ultimately, cervical carcinoma [21].

Prophylactic vaccines for cervical cancer (CxCa)

Prophylactic vaccines targeting the L1 and L2 proteins of HPV, can inhibit the infectious cycle by promoting the production of antibodies against these targeted proteins. Upon viral entry into the cells, the antibodies produced following vaccination can recognize and neutralize the virus, leading to its subsequent elimination from the body. While L2-based vaccines are still in the investigational stages, several approved L1-based vaccines are already available on the market.

L1-based vaccines

L1 is the major capsid protein in HPV, with a molecular weight (MW) of 55 kDa, representing 80% of the viral capsid proteins [22]. The initial strategy for preventing HPV-induced CxCa involved the development of prophylactic vaccines containing the L1 protein. Currently, three L1-based HPV vaccines are licensed by the US FDA: Cervarix®, Gardasil®, and Gardasil®9, all of which have demonstrated excellent clinical efficacy [23, 24]. Moreover, three new prophylactic vaccines—Cecolin®, Walrinvax®, and Cervavac®— have recently been licensed in China and India. These three new biosimilar vaccines are expected to broaden access to HPV vaccination due to their considerably lower costs than the originators. The cost of each Cecolin® dose is about 55% to 65% lower than that of Gardasil® or Cervarix® in the Gavi program. Gavi Vaccine Alliance is an international organization that supports vaccination in low-income and low to middle-income countries [25]. WHO has already prequalified Cecolin® and Walrinvax® [25]. An *E. coli*-produced 9-valent vaccine is also under development in China by the manufacturer of Cecolin®. This vaccine has been reported to be non-inferior to Gardasil®9 in a head-to-head comparative immunogenicity study involving women aged 18–26 years in China [26].

All marketed L1-based vaccines (listed in Table 1) are produced using recombinant DNA technology in various hosts, allowing the L1 proteins to self-assemble into empty shells or VLPs. These particles typically vary in size and exhibit spherical or ellipsoidal shapes. Due to the three-dimensional structure of their VLPs, these vaccines should be stored at a refrigeration temperature of 2–8 °C [27].

Cervarix®

Cervarix®, marketed by GlaxoSmithKline in 2010, is a bivalent vaccine that contains HPV-16/18 VLPs (Table 1) [17]. These two genotypes are the most oncogenic types of HPV, accounting for approximately 70% of CxCas and nearly 90% of anal cancers [36, 37]. This recombinant vaccine is produced in baculovirus (insect) cells and includes an adjuvant system, known as AS04, which consists of aluminum hydroxide and 3-O-desacyl-4' monophosphoryl lipid A (MPL). MPL is a detoxified derivative of lipopolysaccharide (LPS) derived from the Gram-negative *Salmonella minnesota* R595 strain [38, 39].

Gardasil®

Gardasil®, also known as Silgard®, is a quadrivalent vaccine marketed by Merck Sharp & Dohme that contains

Table 1 Approved HPV vaccines

	Vaccine name	Company	Type and amount of L1 VLP	Year approved/ approving authority	Adjuvant	Expression system	Vaccination schedule [28]*	Reference
1	Cervarix®	GlaxoSmithKline (GSK)	20 µg HPV-16, 20 µg HPV-18	2009/ US-FDA	AS 04: 500 µg aluminum hydroxide and 50 µg 3-O-desacyl-4′ monophosphoryl lipid A (MPL)	Baculovirus (insect) cells	9–14 years: 2 doses with a minimum of 5-month interval 15 years and above: 3 doses administered at 0, 1–2, 6 months	[29]
2	Gardasil®	Merck Sharp & Dohme (MSD)	20 µg HPV-6, 40 µg HPV-11, 40 µg HPV-16, 20 µg HPV-18	2006/ US-FDA	225 µg amorphous aluminum hydroxy phosphate sulfate	*Saccharomyces cerevisiae*	9–13 years: 2 doses with a minimum of 5-month interval 14 years and above: 3 doses administered at 0, 1–2, 6 months	[30]
3	Gardasil®9	Merck Sharp & Dohme (MSD)	30 µg HPV-6, 40 µg HPV-11, 60 µg HPV-16, 40 µg HPV-18, 20 µg HPV-31, 20 µg HPV-33, 20 µg HPV-45, 20 µg HPV-52, 20 µg HPV-58	2014/ US-FDA	500 µg amorphous aluminum hydroxy phosphate sulfate	*S. cerevisiae*	9–14 years: 2 doses with a minimum of 5-month interval 15 years and above: 3 doses administered at 0, 1–2, 4–6 months	[31]
4	Cecolin®	Xiamen Innovax Biotech	40 µg HPV-16, 20 µg HPV-18	2019/ China-FDA	208 µg aluminum hydroxide	*Escherichia coli*	9–14 years: 2-dose schedule at months 0, 6 15 years and above 3-dose schedule at 0, 1–2, 5–8 months	[32] [33]
5	Walrinvax®	Walvax Yuxi Zerun Biotechnology Co	40 µg HPV-16, 20 µg HPV-18	2022/ China-FDA	225 µg aluminum phosphate	*Pichia pastoris*	9–14 years: 2-dose schedule at 0, 6 months From age 15: 3-dose schedule at 0, 2–3, 6–7 months	[34]
6	Cervavac®	Serum Institute of India	20 µg HPV-6, 40 µg HPV-11, 40 µg HPV-16, 20 µg HPV-18	2022/ Indian FDA	1.25 µg Al^{3+}	*Hansenula polymorpha*	9–26 years (girls and boys) 9–14 years: 2 doses at months 0, 6 15–26 years: 3-dose schedule at 0, 2, 6 months	[35]

* Off-label single-dose vaccination is already recommended by WHO (as of December 2022). The single-dose schedule for Cecolin® was announced by WHO in October 2024

The dosage of all vaccines is 0.5 ml/dose. They are all administered via intramuscular (IM) injection in the deltoid region. All these vaccines should be kept at a refrigeration temperature of 2–8 °C

VLPs of HPV-6/11/16/18 (Table 1) [17]. Compared with Cervarix®, the additional genotypes in Gardasil® are responsible for approximately 90% of genital warts [4, 16]. Approved in 2006, Gardasil® is produced using *Saccharomyces cerevisiae* as the recombinant expression system, and it contains an adjuvant composed of 225 µg of amorphous aluminum hydroxyphosphate sulfate [38]. Studies have shown that Gardasil® significantly reduces HPV infection in the anus, vulva, penis, and even the oral cavity [40].

Gardasil®9

Gardasil®9 is a VLP-based nonavalent vaccine developed by Merck Sharp & Dohme, containing HPV-6/11/16/18/31/33/45/52/58. The additional genotypes included in Gardasil®9 account for another 20% of CxCa cases, indicating the vaccine's potential to prevent nearly 90% of CxCa occurrence [41]. It was approved in 2014 for both females and males (Table 1) [32, 42].

Cecolin®

Cecolin®, developed by Xiamen Innovax Biotech in China, contains 40 μg of HPV-16 and 20 μg of HPV-18 recombinant L1 VLPs. *Escherichia coli* is the expression system used for its production. This vaccine contains an aluminum hydroxide vaccine adjuvant. Cecolin® successfully completed a Phase 3 clinical trial (NCT01735006), demonstrating high efficacy and tolerability [32], and it was approved by China's National Medical Products Administration in 2019. In October 2021, this vaccine received prequalification from the WHO. In March 2023, Cecolin® was licensed in multiple low- to middle-income countries such as Bangladesh, Morocco, Nepal, Thailand, the Democratic Republic of Congo, and Cambodia [26].

Walrinvax®

Walrinvax® (Table 1) is a bivalent VLP-based L1 vaccine, targeting HPV-16 and −18, produced in *Pichia pastoris*. Walrinvax® consists of 40 μg of HPV-16 and 20 μg of HPV-18 L1 protein VLPs adsorbed to 225 μg of aluminum phosphate and suspended in 0.5 ml of buffered saline (0.32 M sodium chloride, 10 mM L-histamine, 0.025 μg polysorbate 80) [43]. Walrinvax® is designed for intramuscular (IM) administration in women of 9–30 years. Developed by Shanghai Zerun Biotech Co., China, clinical trials for this vaccine are conducted in this country [34]. In August 2024, Walrinvax® received prequalification from the WHO, making it the fifth HPV vaccine available.

Cervavac®

Cervavac® is a quadrivalent vaccine (HPV-6/11/16/18) developed by the Serum Institute of India (Table 1). It is approved for both females and males aged 9–26 years in India [35]. This VLP vaccine uses Al^{3+} as an adjuvant and can increase the IgG geometric mean titer to more than 1000 times the baseline value [44].

Dosing and age of vaccination

Generally, the best time for vaccination to achieve maximum protection is before HPV exposure, since these preventive vaccines may not be effective against an existing infection. Thus, vaccination is usually recommended before sexual exposure commences [45]. The optimal starting age is 11–12 years. The approved vaccination age range for Gardasil® and Cervarix® is between 9 and 26 years and is 9–45 years for Gardasil®. However, the vaccination schedule varies depending on the vaccine's first administration [28]. Usually, if the first dose is administered at age 15, a two-dose schedule is recommended, with a minimum of a 5-month interval between the two doses (the optimal time is 6–12 months after the first dose). If the second dose is given within the first 5 months, a third dose is needed 4 months after the second dose. A three-dose vaccination plan is recommended to ensure efficacy if the initial vaccination age is over 15 years. If the schedule is interrupted, there is no need to restart, and vaccinations can continue as scheduled [28, 36, 46]. Additionally, the three-dose schedule is also recommended for individuals with a weakened immune system aged 9–26 years [47, 48]. Notably, as of December 2022, WHO has recommended a one- or two-dose schedule for girls aged 9–14 and girls and women aged 15–20 years [49, 50]. WHO updated its recommendations for the HPV vaccination schedule in early October 2024 to introduce a single-dose schedule of Cecolin® as an alternative that demonstrated efficacy and protection durability comparable to the previous two-dose schedule [25], following the previously announced off-label alternative schedule for using one single dose of HPV vaccines. The single-dose regimen was proposed as an off-label schedule. Single-dose vaccination against HPV has been gaining popularity; its usage increased from 20% in 2022 to 27% in 2023, with adoption by 23 countries in 2023.

All three US FDA-approved vaccines (Cervarix®, Gardasil®, and Gardasil®9) are extremely safe and well-tolerated at all ages [51]. The most common side effects of Cervarix® and Gardasil® are injection site reactions, including pain and swelling. However, Cervarix® may cause several systemic adverse effects, including fever, dizziness, myalgia, vomiting, nausea, and diarrhea [52]. Moreover, considering their manufacturing process, Gardasil® and Gardasil®9 are not recommended for people with hypersensitivity to yeasts because of the risk of anaphylactic reactions [53]. Fortunately, no significant observations have been made regarding the relationship between HPV vaccination and the new onset of autoimmune diseases, and studies have shown no serious adverse effects in people with medical conditions or pregnant women. Nevertheless, due to the lack of enough evidence for vaccination during pregnancy, postponing vaccination after childbirth is recommended [30].

The recommended dosing methods for Cecolin®, Walrinvax®, and Cervavac® are summarized in Table 1. Notably, the minimum protective antibody titer is still unknown, although some evidence indicates that one

immunization dose might be protective enough [54, 55]. In 2023, a mathematical modeling study published by Bénard and colleagues recommended the one-dose HPV vaccine strategy for children aged 9–14 years based on a calculated protection time of more than 20–30 years using the nonavalent vaccine. The single-dose regimen will be especially beneficial in low- and middle-income countries, as it expands access to HPV vaccines by reducing costs and facilitating administration [56].

Advantages of current HPV vaccines

The VLP structure of approved HPV vaccines offers multiple benefits, including the dense and repetitive display of antigens on the particle surface [57], resembling the conformation of real virions. Additionally, VLPs are non-infectious and non-oncogenic because VLPs lack viral genomes. Thus, as a class of subunit vaccines, they are safer than attenuated vaccines [58, 59]. Moreover, reports suggest that HPV L1 VLPs stimulate the production of several polyclonal antibodies, including nAbs, in the host. This humoral response is 10 to 100 times stronger than that generated by natural infection [60, 61]. A comparison of the long-term efficacy of different HPV vaccines in a systematic review reported the longest period of sustained clinical effect for the quadrivalent Gardasil® vaccine, which has been 12 years in real-world data and 14 years of seropositivity in the FUTURE II trial. Notably, similar outcomes are expected from the nonavalent vaccine; however, owing to its more recent introduction, a longer evaluation report is not yet available [62, 63]. The high impact of vaccines on cancer prevention has been recorded in many countries. Notably, a recent publication from Scotland reported no cases of CxCa in women vaccinated at 12–13 years of age during 12 years of follow-up [64]. Although longer follow-up studies are still needed to fully assess the long-term benefit of these vaccines, their effectiveness in reducing cancer incidence is widely recognized.

Limitations of current HPV vaccines

While the current L1-VLP prophylactic vaccines are sufficiently effective, efforts are underway to develop improved vaccines due to some barriers to the uptake of these vaccines at the global level. Some of these barriers are related to specific countries and particularly their socioeconomic conditions, which are beyond the scope of this review. The major shortcomings of these vaccines, which contribute to their limited uptake, along with suggested approaches to address these issues, are summarized in Table 2. One disadvantage of L1 VLP vaccines is their type-restricted immunity leading to incomplete prevention in regards of other HPV genotypes. Protection against several non-vaccine HPV genotypes–notably HPV-31, –45, and HPV-33/52/58–is reported to be stronger by Cervarix® than Gardasil®, probably due to its adjuvant system. Though L1-based vaccines might provide limited cross-protection for heterologous HPV viruses in general, some other oncogenic HPV genotypes are not covered by vaccination [65]. Moreover, the waning of cross-protection is observed. Besides, there is a risk of the emergence of viral types not covered by the vaccines over time. A report from Spain documented a higher incidence of several HPV types not covered by Cervarix® and Gardasil® vaccines, including HPV-31, HPV-52, and HPV-45 [66]. The emergence of virus types uncovered by marketed vaccines could be a serious concern, especially in the long term. The partial type-specific immunity provided by current L1 vaccines also necessitates more extensive manufacturing processes, because each VLP type included in the vaccine must be produced separately and combined later to create the final vaccine formulation. The VLP manufacturing process, which requires creating stable particles free from contamination and impurities, imposes challenges and increases costs [67]. Thus, scientists have sought novel ways to achieve broader cross-protection [45]. Furthermore, studies indicate regional variations in HPV genotypes and vaccine efficacy, necessitating post-vaccination cervical screening programs. However, these screening programs can be costly and not all women may adhere to them in a timely manner [68, 69]. The high cost of these vaccines has significantly hindered vaccination progress, particularly in low- to moderate-income populations and developing countries [70]. In response to supply limitations and high costs of Gardasil®, Gardasil®9, and Cervarix®, countries such as China and India have initiated research and development of vaccines such as Cecolin®, Walrinwax®, and Cervavac® to support global CxCa elimination efforts. Moreover, L1 vaccines require constant refrigeration at the optimal temperature, and exposure to extreme temperatures during storage or transfer can reduce their efficacy. This also increases vaccination costs [71] and restricts access to these vaccines in remote areas. Given the higher rates of HPV cancers in some developing countries–particularly in parts of Africa–and the limited infrastructure in rural areas, the ease of transport, storage, and administration is crucial for successful vaccination programs [72, 73].

The limitations of current HPV vaccines have spurred the development of second-generation vaccines, such as L2-based vaccines. These vaccines aim to overcome the issue of type-specificity and could potentially work against a wider range of HPV genotypes, offering hope for future advancements in HPV vaccine development.

Table 2 Limitations of the current VLP prophylactic HPV vaccines and some suggested approaches to solve these issues (the limitations discussed here are related mainly to vaccine characteristics, not their social acceptance and similar aspects)

	Characteristic of current vaccines	Limitation	Approaches to solve the issue
1	Type-specificity (partial)	1. Lack of complete cross-protection against other HPV types that are not included in the vaccine 2. More complex and lengthy manufacturing processes due to the inclusion of multiple types of HPV, which should be produced separately 3. The increased cost of vaccines for adding more HPV genotypes 4. The need for cancer screening remains 5. Risk of emergence of some uncovered HPV types	Employing protective L2 proteins of HPV in the vaccine (either exclusively or in combination with L1) to enhance cross-protection due to the higher conservancy of L2
2	No therapeutic indication	Not indicated for patients who already have cervical cancer	Developing therapeutic vaccines targeting the E proteins of HPV
3	High costs of vaccine	Lower access to vaccines, especially for low-income populations	1. Development of vaccines with lower costs, for example, using simpler hosts (such as *E. coli*) (already done: Cecolin® and Walrinvax®) 2. Encouraging local production of HPV vaccines in low- and middle-income countries
4	Cold-chain requirement	1. Increasing manufacturing, storage, and transfer costs 2. Not easy handling	1. Lyophilized powder formulations 2. Developing vaccines with more stability at room temperature, such as peptide vaccines
5	Injectable formulation	Need for nurses or other professionals for injecting vaccines Not preferred by individuals with a fear of injection	Development of non-parental formulations such as intranasal or oral vaccines
6	Need for multiple dosing *	Lower compliance for getting the vaccine, especially in remote areas	1. Optimizing vaccination schedules 2. Reminder or call services
7	Effective only in the early stages of the viral life cycle	Age limitation (The greatest benefit of the vaccine occurs when vaccination is completed before age 15, particularly prior to the onset of sexual activity. However, individuals vaccinated after age 15 still gain some benefit, though it is lower than those who began the HPV vaccine injection before turning 15.)	Development of novel vaccines targeting other proteins that are present in later stages of the disease, such as the E proteins of HPV

* Single-dose vaccination is already recommended by WHO (off-label)

Given the prophylactic nature of current L1 vaccines and their lack of efficacy against existing infections, ir is crucial to develop a therapeutic vaccine or a combination of therapeutic and preventive vaccines. This approach could greatly enhance the effectiveness of HPV vaccines and is an active area of research and development [74, 75]. Post-translational proteins E6 and E7 can be used as antigenic candidates for therapeutic vaccines, as high concentrations of these proteins are consistently found in CxCa cases [74, 75]. Some studies have also included E5 in addition to E6 and E7 [76–78].

Research on therapeutic vaccines is ongoing, as discussed elsewhere [79–81]. Chimeric L1–L2 VLPs have also been explored as alternative preventive vaccines [82, 83].

Under development L1-based vaccines

To reduce the manufacturing costs of VLP L1-based HPV vaccines, simpler expression systems, such as *E. coli*, have been employed. The manufacturer of Cecolin® (Xiamen Innovax Biotech, China) is working on a nonavalent vaccine (HPV-6/11/16/18/31/33/45/52/58) produced in *E. coli*, which is currently in Phase 3 (NCT05056402). Other examples include the Quadri (HPV-6/11/16/18) and nonavalent (HPV-6/11/16/18/31/33/45/52/58) L1 VLP vaccines, both developed by Shanghai Bovax Biotechnology using *Hansenula polymorpha* (a methylotrophic yeast), which are currently in Phase 3 clinical trials (NCT04425291). Several other companies engaged in the development of L1 HPV vaccines, including a trivalent vaccine by the Health Guard, China (HPV-16/18/58), which is in the preclinical phase [84]; a bivalent vaccine by Shanghai Zerun Biotechnology (HPV-16/18); a tetravalent vaccine by the China National Biotech Group (HPV-16/18/52/58); and another tetravalent vaccine by the Serum Institute of India (HPV-6/11/16/18) [85, 86].

The most recent and innovative prophylactic HPV vaccine is likely the eleven-valent vaccine, developed by the National Vaccine and Serum Institute in China, which is currently undergoing a Phase 3 clinical trial

Table 3 L1-based vaccines in different Phase 2/3 clinical trials

	Vaccine	Company	Type of included HPV VLP	Clinical phase	Expression system	Reference
1	Bivalent vaccine	Xiamen Innovax Biotech	HPV-16/11	II	*Escherichia coli*	[88]
2	Quadrivalent vaccine	Shanghai Bovax Biotechnology	HPV-6/11/16/18	III	*Hansenula polymorpha*	[89]
3	Nonavalent vaccine	Shanghai Bovax Biotechnology	HPV-6/11/16/18/31/33/45/52/58	III	*H. polymorpha*	[89]
4	Nonavalent vaccine	Xiamen Innovax Biotech	HPV-6/11/16/18/31/33/45/52/58	III	*E. coli*	[90]
5	11-valent vaccine	National Vaccine and Serum Institute, China	HPV-6/11/16/18/31/33/45/52/58/59/68	III	*H. polymorpha*	[87]
6	EG-HPV	Eyegene Inc., Korea	HPV-16/18	I	Yeast	[91]

(NCT05262010) [87]. The mentioned vaccines are listed in Table 3.

Furthermore, several alternative approaches have demonstrated significant efficacy in preclinical phases. For instance, bacterial vectors, specifically, live attenuated *Shigella*, have been employed to deliver HPV-16/58 L1 proteins [92]. Additionally, L1 capsomers (particularly HPV-16) have shown promise. As subunits of capsids, capsomers are more cost-effective and can be more easily replicated in recombinant bacteria, such as *E. coli* [93, 94]. Their thermostability may address the challenges associated with the preservation of VLPs [95].

L2-based vaccines

Many studies have been conducted to develop second-generation HPV vaccines using L2 capsid proteins. While L1-based vaccines have been proven to be highly effective, L2-based vaccines are being investigated mainly to enhance cross-protection against different types of HPVs because the L2 protein is highly conserved across various HPV types [10]. Studies have shown that nAbs can recognize L2 as a broadly protective antigen [96]. Certain L2 residues are conserved among multiple HPV types. Research on the L2 protein has shown that its first 120 amino acids (aa) at the N-terminus constitute the only region exposed to the external environment throughout the viral life cycle, making it a potential target for vaccine design [17, 22, 97]. This region plays a crucial role in virion assembly and infection. Additionally, it contains several highly conserved protective epitopes, including aa 17–36 [98], 69–81 [99], and 108–120 [100, 101] of the HPV-16 L2 protein.

Given the linearity of L2 epitopes, they can be produced cost-effectively in *E. coli*. Additionally, L2 epitope peptides can be integrated into various scaffolds. They can also be fused to toll-like receptors (TLRs) as adjuvants in the design of multi-epitope polypeptide vaccines [102, 103]. Generally, peptide-based epitope vaccines offer several advantages, including enhanced stability (which allows for less temperature-strict conditions for storage and transport), improved safety, reduced risk of triggering harmful autoimmune responses, and ease of production [104, 105].

However, immune responses to L2 vaccines, whether administered alone or in conjunction with a potent adjuvant, are generally lower than those elicited by the currently approved L1-based VLP vaccines [106]. This discrepancy is primarily attributed to the linearity of L2 vaccines, which results in a deficiency in T-helper cell activity [107]. Several strategies can potentially enhance the immunogenicity of this category of vaccines, including the use of VLP platforms, multimeric L2 peptides, recombinant bacteria as carriers for L2, the fusion of immunostimulatory agents, and DNA/mRNA vaccine platforms. While some of these strategies have demonstrated acceptable levels of immunogenicity, the exceptionally high efficacy of the present L-1-based vaccines has set high expectations for future vaccine development [57]. Multi-epitope polypeptide vaccines remain less immunogenic than L1-VLPs due to their lack of complex conformational structures. However, given their broader protective spectrum, their lower immunogenicity may be considered acceptable if they can generate adequate serum antibody titers compared to current vaccines [102]. Consequently, research in this area is ongoing. Various approaches have been undertaken to develop effective L2-based vaccines, as discussed below. The L2-based HPV vaccines that are currently in the preclinical phases are listed in Table 4, and one vaccine that has progressed to clinical trial is presented in Table 5.

VLP-based second-generation HPV vaccines

VLPs generated from the capsids of different viruses can be used as carriers to present L2 epitopes [142].

Papillomavirus VLPs Using L2 epitopes on L1 VLPs as scaffolds is a promising approach for achieving broader efficacy against diverse HPV types. Numerous studies have focused on this strategy [83, 99, 115, 116].

Table 4 Preclinical studies of L2-based HPV vaccines

	Vaccine platform	Used adjuvant	Employed antigens	Expression system	Type of study	Type of immune response	Year	Reference
1	HPV-16 L1 VLP	Alum-MPL	RG1 epitope (HPV-16 L2 aa 17–36	Insect cells	In vitro (rabbit)	Passive transfer of sera-protected mice against PsVs of HPV-16/18/45/31/33/52/58/35/39/51/59/68/56/73/26/53/66/34 and LR-HPV-6/43/44 nAbs against HR-HPV- 16/18/45/37/33/52/58/35/39/51/59/68/73/26/69/34/70, LR-HPV-6/11/32/40, and cutaneous HPV-2/27/3/76 in PBNA	2013	[108]
2	HPV-16 L1 VLP	Alum/MPL	RG1 epitope	Sf9 insect cells	In mice (RG1-VLP + HPV-18 L1-VLP) In rabbits	Cross-protection against vaginal challenge with HR-HPV-58 Cross-neutralization titers (50–1000) against HR-HPV-18/31/33/45/52/58/26/70	2019	[109]
3	HPV-16 L1 VLP	Alum-MPL	HPV-31 L2 (aa.17–38)	Bac-to-Bac baculovirus	In vivo (mice)	Cross-nAbs against HPV-2/5/6/11/16/18/27/31/33/35/39/52/57/58/59/68	2018	[110]
4	HPV-16 L1 VLP	Freund adjuvant	HPV-16 L2 aa108-120 aa 56–81 aa 17–36 BPV-1 L2 aa 1–88	Insect cells	In vitro In vivo (mice)	Most vaccinated mice could neutralize HPV-16 PsVs nAbs against HPV-16 nAbs against HPV-16 No nAbs	2013	[111]
5	HPV-16 L1 VLP	Alum-MPL	HPV-58 L2 aa.16–37	Baculovirus	In vivo (rabbits and mice)	nAbs against HPV-2/5/6/11/16/18/27/31/33/35/39/45/52/57/58/59/68	2017	[112]
6	HPV-16 L1 VLP	Alum with BECC470 (Bacterial enzymatic combinatorial chemistry)	HPV-16-L2 17–36 a.a. (RG1 epitope)	*Yersinia pestis*	In vitro and in vivo	nAbs against HPV-16/18/39	2021	[113]
7	HPV-16 L1 VLP	-	HPV-16 L2	*Pichia pastoris*	In vitro	Positive reaction against L1-HPV-16 antibody and L2 -HPV-16 antibody	2018	[114]
8	HPV-18 L1 VLP	AS04	HPV-16 L1, HPV-18 L1, and recombinant L2 fragment consisting of HPV-33 and HPV-58 (HPV-33 L2 (aa 17–36) + HPV-58 L2 (aa 56–75))	pAcSG2 baculovirus	In vivo (mouse and rabbit)	Protection against HPV-6/11/16/31/35/39/45/58/59 PsVs or quasivirions	2016	[115]
9	HPV-18 L1 VLP	Alum-MPL	HPV-45 L2 (aa 16–35)	Sf9 insect cells	In vitro In vivo (rabbits)	nAbs against HPV-18/39/45/68/70 Passive transfer of sera-protected vaginal challenge of mice with PsVs of HPV-18/39/45/68	2015	[116]
10	HPV-16 L1 HPV-5 L1 HPV-1 L1 HPV-16/18 L1	Alum-MPL	HPV-17 L2 RG1 HPV-17 L2 RG1 HPV-4 L2 RG1 HPV-5 L2 aa53-72	*Spodoptera frugiperda* (Sf9) insect cell	In vitro	PsVs neutralized in L2-based PBNA: HPV-5/8/20/23/24/36/49/80 HPV-20/24/36/92 HPV-4 No nAb in L2-based PBNA	2017	[82]

Table 4 (continued)

	Vaccine platform	Used adjuvant	Employed antigens	Expression system	Type of study	Type of immune response	Year	Reference
11	HPV-16 L1 VLP	-	HPV-16 L2 aa 108–120 aa 65–81 aa 56–81 aa 17–36	*Agrobacterium tumefaciens*	In vitro	PsVs neutralized in PBNA: HPV-16/58 HPV-11/16/18 HPV-18 No nAb	2019	[117]
12	MS2 bacteriophage VLP	Incomplete Freund's adjuvant	HPV-16 L2 aa 17–31 aa 20–29 aa 14–40 aa 14–65	C41 cells (Lucigen)	In vivo (mice)	Protection from vaginal challenge against HPV-5/6/16/31/33/35/39/45/51/53/58 Not tested Not tested Not tested	2012	[118]
13	MS2 bacteriophage VLP	Alum hydroxide	HPV-16 L2 aa 17–31 + HPV-31 L2 aa 20–31 + aa 69–86, 108–122 from a consensus L2 sequence	C41 cells *Escherichia coli*	In vivo (mice)	Protection of mice against cervicovaginal infection with HPV-16/18/31/33/45/58 PsVs	2017	[119]
14	MS2 Bacteriophage VLP	With and without alum hydroxide	HPV-16 L2 aa 17–31	*E. coli* (C41)	In vivo (mice)	Spray-dried VLPs were highly immunogenic and protected mice in the genital challenge with HPV-16 PsVs	2015	[120]
15	MS2 Bacteriophage VLP PP7 Bacteriophage VLP	Incomplete Freund's adjuvant (IFA)	L2 aa 17–31 of HPV-16/31 L2 aa 17–31 of HPV-16/18	C41 cells (Lucigen)	In vitro In vivo (mice)	Neutralized PsVs of HPV-16/18/31/45/58 Protected mice against vaginal challenge with HPV-6 PsVs	2014	[121]
16	MS2 Bacteriophage VLP	Cholera toxin and MPL	1) HPV-31 L2 aa 20–31 + HPV-16 L2 aa 17–31 2) Consensus aa 69–86	C41 *E. coli*	In vivo (mice)	Mixed VLPs contained both antigens to protect mice from PsVs; HPV-11/16/35/39/52/53/56/58	2019	[122]
17	MS2 Bacteriophage VLP	Alum	1) HPV-31 L2 aa 20–31 + HPV-16 L2 aa 17–31 2) Consensus aa 69–86	C41 *E. coli*	In vivo (mice)	Mixed VLPs protected against genital infection with PsVs of HPV-5/6/51	2021	[123]

Table 4 (continued)

	Vaccine platform	Used adjuvant	Employed antigens	Expression system	Type of study	Type of immune response	Year	Reference
18	Qβ bacteriophage VLP PP7 bacteriophage VLP	Incomplete Freund's adjuvant	HPV-16 L2 aa 34–52 aa 49–72 aa 108–120 aa 65–85 Consensus HPV-16 and −18 L2 aa 65–85 HPV-16 L2 aa 17–31 aa 51–65 aa 35–50	C41 *E. coli* (Lucigen)	In vitro In vivo *(mice)*	Protection against PsVs; Poor protection against HPV-16 Moderate protection against HPV-16 Strong protection against HPV-16 (in vivo) HPV-6/18/31/45/58 (in vivo) Strong protection against HPV-16 HPV-16 and −18 (in vivo) HPV-16/18/31/45/58(in vitro) Strong protection against HPV-16 Moderate protection against HPV-16 Poor protection against HPV-16	2014	[124]
19	Adeno-associated virus 2 VLP	Montanide ISA 51	HPV-16 and HPV-31 L2 aa 17–36	HEK 293T cells	In vitro and in vivo	nAbs against HPV-16/18/31/45/52/58 PsVs in mice and rabbits Passive transfer of sera-protected mice against vaginal challenge with HPV-16 PsVs	2012	[125]
20	Adenovirus type 5 VLP	Alum and MPL	HPV-16 L2 aa 12–41	HEK 293T cells	In vitro and in vivo	Protection against vaginal challenge with HPV-16 nAbs against HPV-16/73	2015	[126]
21	Adenovirus type 35 VLP	-	L2 RG1 epitope from HPV-6/11/16/18/31/33/45/52/58	PER C6 cells	In vitro	nAbs against HPV-16/18/31/59	2018	[127]
22	Hepatitis B core (HBc) VLP	Alum	aa 14–122 of HPV-16 L2	*Nicotiana benthamiana*	In vitro	nAbs against HPV-16	2019	[128]
23	*Pyrococcus furiosus* thioredoxin (trivalent-PfTrx)	Aluminum hydroxide- MPL	L2 aa 20–38 of HPV-16, HPV-31, and HPV-51	*Trichoplusia ni* (TN) High Five cells	In vitro (mice and guinea pigs) In vivo	Cross-nAbs against HPV-16/18/31/33/35/39/45/51//52/58/5968 Cross-protection against vaginal challenge with HPV-16/18/31/33/51	2015	[129]
24	*Pf*Trx–8-mer–OVX313	Alum-MPL or glycopyranosyl lipid A or Army liposome formulation-alum	L2 aa 20–31 of HPV-6/16/18/31/33/3551/59 fused to PfTrx and OVX313	*E. coli* BL21 cells	In vitro (mice and guinea pigs) In vivo	Cross-nAbs in PBNA test against HPV-6/11/16/18/31/33/35/39/45/51/52/56/58/59/68/73 Passive transfer of sera-protected mice against HPV-6/11/16/18/31/33/35/39/45/56/58	2018	[130] [131]
25	*P. furiosus* thioredoxin fused to ferritin nanoparticles (Pf FeTrx8mer)	AddaVax	HPV-16 L2 aa 20–38 of HPV-6/16/18/31/33/35/51/59	Baculovirus-infected insect cells	In vitro In vivo	nAbs titers against HPV-6/11/16/18/31/33/35/39/45/51/52/56/58/59/68/73 Passive transfer of sera-protected mice against HPV-6/11/16/18/33/35/39/45/51/56	2020	[132]

Table 4 (continued)

	Vaccine platform	Used adjuvant	Employed antigens	Expression system	Type of study	Type of immune response	Year	Reference
26	Lipidated triple-repeat HPV-16 RG-1 epitope and a hFccRI-specific single-chain antibody fragment (H22scFv) (LpE3H22)	MF59 and poly I:C	aa 17–36 of HPV-16 L2	*E. coli* (protein) 293T cells (PsVs)	In vivo	nAbs against HPV-2/5/6/11/16/18/31/33/35/39/45/52/58/59	2016	[133]
27	TLR5 ligand bacterial flagellin (Fla)	-	L2 aa 17–38 of HPV-6/18/31/39/52 added to HPV-16 L2 11–200 or 11–88 fused to flagellin	*E. coli*	In vitro In vivo	nAbs against HPV-6/16/18/31/45 Passive transfer of sera-protected rabbit against HPV-6/16/18/31/45/58	2017	[134]
28	L2 concatemer fused to flagellin (A cL2 epitope introduced at the C-terminus of flagellin.) (Fla-5PcL2)	Without adjuvant or alum or alum-MPL	L2 RG-1 of HPV-18/33/58/59 epitopes and HPV-16 L2 aa 11–88	*E. coli*	In vitro/in vivo	nAbs against HPV-16/18/31/33/58 Protection of mice against vaginal challenge with HPV-5/39/58	2019	[135]
29	Multimeric protein	Alum	L2 11–88 aa of HPV-6/16/18/31/39 (11–88 × 5) 88 × 8 (HPV-6/16/18/31/39/51/56/73	*E. coli* BL21	In vivo	Protection of mice against HPV-6/16/26/31/33/35/45/51/56/58/59 challenge	2013	[136]
30	*Lactobacillus casei*	-	HPV-16 L2 aa 1–224 fused to poly-γ-glutamic acid synthetase A	293T cell line	In vitro and in vivo	Oral administration protected mice against HPV-16/18/45/58	2012	[137]
31	DNA vaccine	-	Fused L1–L2 genes	HEK-293 cell line	In vitro In vivo (mice)	The high expression rate of recombinant L1-L2 HPV-16 DNA Protection against C3 tumor cells	2019	[138]
32	L2-based recombinant vaccine	Self-assembled peptide (OVX313) and thioredoxin	HPV-16 L2	*E. coli* BL21	In vivo (mice)	The larger antigen size correlates with enhanced B-cell induction and increased plasma half-life	2017	[74]
33	RG2-VLP	Aluminum hydroxide	RG2	Cottontail rabbit papillomavirus (CRPV) quasivirus	In vivo (mice)	Robust L2-specific antibody titers and protection against β-type HPV5 Similar HPV16- and HPV18-specific neutralizing Ab responses between RG2-VLP- and Gardasil®9 groups	2022	[139]
34	MS2-L2 VLPs	-	MS2-31L2/16L2 VLPs and MS2-consL2 (69–86)	*E. coli*	In vivo (mice)	Constant antibody titer against HPV16 L2 (aa 17–31) and HPV31 L2 (aa 17–31) peptides	2021	[123]

Table 4 (continued)

	Vaccine platform	Used adjuvant	Employed antigens	Expression system	Type of study	Type of immune response	Year	Reference
35	RG1-VLP	Aluminum hydroxide or poly[di (carboxylatoethylphenoxy) phosphazene] (PCEP)	HPV16-L1 L2 RG1	293-TTF cells	In vivo (mice)	Cross-neutralization of pseudovirion types HPV18 and HPV39 Sufficient level of Ab titer after two doses of vaccine with PCEP	2021	[140]
36	HPV16L2-N200 (first 200 aa of L2) HPV16L2-N88 as fused or mixed with Tat-protein transduction domain (PTD) as a CPP	Freund's adjuvant	HPV16-L2	*E. coli*	In vivo (mice)	Increased titer of anti-HPV16L2 total antibodies and neutralizing antibodies for HPV16 and other HR-HPVs (HPV16, 18, 31, 45, and 58) following vaccination with fused PTD-L2-N200	2016	[141]

HPV: Human papillomavirus; HR-HPV: high-risk HPV; LR-HPV: low-risk HPV; CPP: cell-penetrating peptide; MPL: Monophosphoryl Lipid A; nAbs: neutralizing antibodies; PBNA: pseudovirion-based neutralization assay; PsVs: Pseudovirions

Table 5 Clinical trials of L2-based vaccines

Vaccine	Class	Phase	Title of trial	Target disease/ HPV type	Administration route	Outcome measures	NCT	Sponsor	Status
Adeno-associated VLPs (AAVLP)	VLP-based vaccine	I	A study of safety, tolerability, and immunogenicity of HPV-L2 vaccine in healthy adult male and female subjects	Antibodies against HPV-6/11/16/18	Intramuscular (IM)	1. Percentage of local/ general symptoms 2. Percentage of adverse events 3. Vital signs	NCT03929172	2A Pharma AB	Completed/ Last updated in 2020

Inserting aa 17–36 of HPV-16 L2 (a frequently reported epitope on L2, also called the RG1 epitope) into the DE-surface loop of the HPV-16-VLP resulted in developing a potent chimeric HPV vaccine. Schellenbacher and colleagues reported that RG1-VLP vaccination with alum-MPL (aluminum hydroxide plus 3-O-desacyl-4′-monophosphoryl lipid A) elicited robust immune responses against L2 and provided protection against multiple types of cutaneous and mucosal HPV infections. They observed protection against mucosal HR-HPV types 16/18/45/37/33/52/58/35/39/51/59/68/73/26/69/34/70, LR-HPV types 6/11/32/40, and cutaneous HPV types 2/3/27/76 in a pseudovirion-based neutralization assay (PBNA). Furthermore, nAbs against mucosal HR-HPV-16/18/45/31/33/52/58/35/39/51/59/68/56/73/26/53/66/34 and LR-HPVs 6/43/44 were induced in rabbits and mice. Furthermore, the vaccine also stimulated robust cytotoxic T-cell responses with protection lasting one year. RG1-VLPs were produced under Current Good Manufacturing Practice (CGMP) to initiate a Phase 1 clinical trial, as noted in their 2013 publication [108]. However, no further updates regarding this project have been reported. Notably, chimeric VLPs displaying L2 epitopes can be generated with minor modifications to the existing approved HPV vaccines [106].

Bacteriophage VLPs Bacteriophages can serve as efficient platforms for presenting various antigens, such as L2 epitopes, to enhance the immune response. They can be produced in a bacterial system, such as *E. coli*, at a low cost and with a straightforward purification process. This platform may also act as a natural adjuvant, as encapsidated bacteriophage single-stranded (ss) RNAs can activate TLR-7 and −8 [106]. Pseudomonas phage 7 (PP7) [143, 144] and *Emesvirus zinderi* (MS2) [118] bacteriophages were used to display L2 epitopes. PP7 VLPs displaying the aa 17–31 of L2 induced cross-protection against HPV-16 and the heterologous HPV pseudovirion type HPV-45 following IM injection in mice [143].

Inserting the aa 17–31 L2 epitope at the N-terminus of the MS2 coat protein VLPs could induce significant protection against several heterologous HPV types in vivo. Cross-protection was observed following intravaginal challenge with mucosal HR-HPV-16/31/33/35/39/45/51/53/58 and LR-HPV-6 or intradermal challenge with beta-HPV5 in mouse models [118, 120, 144]. This candidate vaccine demonstrated high immunogenicity in mice, both with and without the alum adjuvant; and a single dose of the MS2-16 L2 VLPs vaccine elicited a robust immune response lasting more than 18 months. This study highlighted that in addition to the previously mentioned advantages of bacteriophage VLP display, spray drying technology allows VLPs to maintain their immunogenicity and stability for over one month at room temperature or 37 °C [120]. Importantly, Peabody's experiments indicated that spray-dried MS2-16 L2 VLPs exhibited thermostability for 34 months at room temperature and 14 months at 37 °C, allowing the vaccine to be immunogenic and effective in mice [71]. However, Agilvax® has ceased the development of the MS2 VLP-16 L2 vaccine technology following cGMP development [74].

Adeno-associated VLPs (AAVLPs) Adeno-associated viruses (AAV) are nonpathogenic ss DNA viruses that can be used for vaccine delivery. They exhibit stability across a wide range of pH and temperatures. Their capsid comprises 60 protein subunits, including virus protein 1 (VP1), VP2, and VP3, which can serve as peptide scaffolds in vaccine development. Adeno-associated VLPs (AAVLPs) were created by the double insertion of the RG1 epitope (aa 17–36) of HPV-16 and HPV-31 into the VP3 of AAV2 capsid. The vaccine demonstrated stability at various pH values and temperatures. AAVLP (HPV-16 and −31 L2) used with montanide adjuvant induced cross-nAbs against HPV-16/18/31/45/52/58 and bovine papillomavirus type 1 in mice. Furthermore, the immunogenicity of the lyophilized particles of AAVLP (HPV-16 and −31 L2) was maintained. Lyophilization can effectively address cold-chain limitations [125].

Jagu et al. investigated AAVLP (containing HPV-16 and −31 L2) vaccines with three different adjuvant systems: alum only, alum combined with MPL, and RIBI adjuvants. The administration of this candidate vaccine to mice without any adjuvants did not elicit an immune response against L2. In contrast, the alum adjuvant provided strong and durable immune protection that lasted over three months. The concurrent use of MPL and RIBI enhanced antibody titers. Furthermore, AAVLP (HPV-16/31 L2) alone, with alum ± MPL or RIBI adjuvants in rabbits, demonstrated potential effectiveness against HPV-16/31/35/39/45/58/59, with protective effects detectable 6–12 months post-immunization [145]. 2A Pharma AB has initiated a Phase 1 clinical trial for this candidate vaccine (NCT03929172, last updated in 2020).

Adenovirus VLPs Adenovirus 5 (ad5) has a major antigenic capsid protein, hexon, which comprises nine hypervariable regions (HVR). The insertion or substitution of aa 12–41 of L2 HPV-16 within HVR1 or HVR5 resulted in the creation of recombinant ad5. The specific L2 response to the recombinant ad5 was initially weak; however, the incorporation of alum and MPL adjuvants augmented antibody titers. NAb titers and protection against HPV-16 and −73 were observed in

mice but not against HPV-56 [126]. To broaden vaccine coverage, concatemers of the L2 RG1 epitopes from HPV-6/11/16/18/31/33/45/52 and 58 were inserted into the C-terminus of protein IX of adenovirus 35. The HAdV35 pIX-L2 (human adenovirus 35 protein IX) without an adjuvant elicited robust immune responses and nAbs against HPV-16/18/31/59 [126, 127].

Tobacco mosaic virus display Tobacco mosaic virus (TMV) was employed as a scaffold to present L2 epitopes [146, 147]. Palmer et al. used recombinant TMV as a vector to display aa 94–122 of the L2 of rabbit oral papillomavirus (ROPV) and Cottontail rabbit papillomavirus (CRPV) with the RIBI adjuvant. The CRPV rTMVs alone or with ROPV demonstrated strong immunogenicity in rabbits against the CRPV challenge; however, ROPV L2 rTMVs elicited a weak immune response against the CRPV challenge. The advantages of these scaffolds include ease of production and low purification costs [147].

Others The hepatitis B virus core (HBc) was utilized as a VLP carrying aa 14–122 of the HPV-16 L2. HBc VLPs resulted in elevated titers of L2 antibodies and nAbs against HPV-16 in mice [128]. Other studies have used recombinant potato virus VLPs [148] or grapevine fanleaf virus (GFLV) VLPs [149] to deliver L2 epitopes.

Peptide vaccines/recombinant L2 peptides

The intranasal administration of L2 aa 108–120, which contains cross-neutralizing epitopes without adjuvants, induced the mucosal (mainly IgA) and systemic (mostly IgG) antibodies in mice. A high dose of this candidate vaccine was tolerable in humans in a Phase 1 clinical trial. Neutralizing activities against HPV-16 and −52 were found in the sera of four patients (out of five). Based on the outcomes, L2 could be bound to immunostimulant agents or be designed as a concatemer of peptides to enhance immune responses [100, 150].

Vaccines consisting of epitope peptides fused to immunostimulants L2 peptides fused to different immunostimulatory peptides, such as thioredoxin, and some TLRs (including TLR-2, TLR-4, and TLR-5 agonists) have been investigated in several studies. Thioredoxin is an immunostimulatory agent obtained from different bacteria, which serves as a peptide scaffold. Incorporating HPV-16 L2 aa 20–38 into bacterial thioredoxin provoked immune responses in mice by inducing cross-nAbs against HPV-16/18/31/45/58 [151]. The incorporation of aa 20–38 L2 of HPV-16/31/51 into *Pyrococcus furiosus* thioredoxin (PfTrx) adjuvanted with aluminum hydroxide and MPL induced cross-nAbs against 12 of the 13 oncogenic HPV types (HPV-16/18/45/31/33/52/58/35/59/51/39/68) in mice and guinea pigs. Notably, the trivalent PfTrx L2 formulation demonstrated thermostability, which could mitigate cold storage challenges and also accelerate purification processes [129].

To enhance the immunogenicity of this strategy, Spagnoli et al. developed single peptides containing eight distinct epitopes of L2 (aa 20–38) bound to a bacterial thioredoxin carrier. To further enhance immunogenicity, these thioredoxin L2 polytopes were fused to OVX313 (a heptamerization domain). OVX313, a synthetic self-assembling polypeptide, is an IMX313 derivative. Both of these molecules were developed by the Osivax® Company from C4-binding protein (C4bp), a complement inhibitor [131]. C4bp naturally inhibits the classical and lectin pathways of the complement system. OVX313, which differs from human or murine C4bp due to some modifications, was shown to increase B-cell and T-cell responses; though the mechanism is not well understood [130]. PfTrx-OVX313 nanoparticles could be introduced as third-generation HPV vaccines, and this L2 scaffold was highly immunogenic. It could evoke nAbs against 10 HPV types in mice [131]. In another study by Pouyanfard et al., PfTrx–11-mer–OVX313, which contained aa 20–38 L2 of 11 HPV types, neutralized 14 types of oncogenic HPVs, HPV-6 and −11 (which are LR-HPVs), and some cutaneous HPVs [130]. The PANHPVAX (Trx-L2m8mer-OVX313 antigen) candidate vaccine, formulated with the cyclic di-adenosine monophosphate (cdA) adjuvant, has entered a Phase 1 clinical trial as of 2022 (NCT05208710) [152].

Another approach to enhance L2 epitope immunogenicity is to fuse it with a TLR agonist as an immunostimulant. An HPV lipopeptide vaccine was made from the RG1 epitope of L2 HPV-16 in combination with a T-helper epitope (P25) and the TLR2 ligand dipalmitoyl-S-glyceryl cysteine (P2C). The administration of P25-P2C-HPV lipopeptides in mice, either intranasally or subcutaneously, induced serum-nAbs that neutralized HPV-16 pseudovirions in addition to oncogenic cutaneous (HPV-5 and BPV1 [bovine papillomavirus 1]) and genital (HPV-18 and −45) types [153].

Several studies have used flagellin, a TLR5 ligand, as a vaccine adjuvant. Klannin and colleagues reported that the flagellin-L2 multimer, even without additional adjuvants, demonstrated promising results because it could act as a self-adjuvant antigen. Various studies have been conducted on multimer and monomer L2 residues. Consequently, adding aa 17–38 L2 of HPV-6/18/31/39/52 to aa 11–88 or aa 11–200 on L2 HPV-16 fused to flagellin caused robust and extensive protection lasting over one year [134, 154]. Another recombinant protein was designed by Zhang et al. and contains the HPV-18/33/58/59 RG1 epitopes, the HPV-16 aa 11–88, and

the L2 epitope (aa 65–85) fused to flagellin to provoke the immune system. Both subcutaneous and intranasal administration in mice elicited cross-nAbs against HPV-16/18/31/33/58 in mucosal secretions and protection against vaginal challenge by HPV-39/58/5 [135]. A multiepitope peptide vaccine designed in silico by our team, which incorporated flagellin and a synthetic TLR4 agonist [45], also induced several cytokines in mice, which could lead to humoral and cellular immune responses with a more Th-1 favored pattern [155].

An alternative approach was investigated in a study by Zeng et al. using a cell-penetrating peptide (CPP) to facilitate the intracellular delivery of the vaccine [156]. The protein transduction domain (PTD) of the Tat protein of human immunodeficiency virus (HIV) was used as a CPP, which was either mixed or fused with two peptides from the N-terminal of HPV16 L2. The fusion form of the vaccine showed increased humoral responses and cross-protection [157].

Alternative approaches focusing on nanovaccines Different vaccine development research has shown the great potential of nanovaccines and related delivery systems. The resemblance of nanovaccines to pathogens regarding their size is the main reason for their significant advantages as vaccines [9]. VLPs and some other well-known platforms could also fall into this category. However, there are other approaches not employed for HPV vaccines that may deserve exploring.

Exosomes, as natural nanovesicles responsible for the extracellular transfer of various materials (such as proteins and nucleic acids) within the body [158], could be used for vaccine delivery [9]. Exosomes have been investigated for a therapeutic HPV vaccine before [159], but not for prophylaxis. Alternative platforms such as self-assembled peptide nanoparticles, liposomes, and other structures found elsewhere [142], could also be investigated for HPV.

Multimer or synthetic peptides The use of concatemeric (multimeric) peptides has broadened protection against diverse HPVs. Generally, due to the low immunogenicity of multimeric peptides, they should be used in conjunction with potent adjuvants. Jagu et al. performed a series of studies on concatenated multitype L2 peptides [136, 160–162]. Bravovax® is currently developing the most effective structure identified in these experiments. They designed a concatemeric peptide by fusing L2 aa 11–88 of HPV-6/16/18/31/39 (11–88×5) and 11–88×8, adding HPV-51/56/73 to the previous types along with alum adjuvant. Antibodies elicited by this candidate vaccine protected mice against HPV-6/16/26/31/33/35/45/51/56/58/59 challenges. Importantly, since concatemeric peptides can be produced in bacteria, their production costs are lower than those of licensed vaccines, such as Gardasil®. Additionally, these concatemeric vaccines produced long-lasting (lasting more than one year) and broader immunity [136].

Recombinant bacteria

Recombinant bacteria can also be used to present the L2 protein. Yoon et al. used *Lactobacillus casei* to display HPV-16 L2 aa 1–224 fused to poly-γ-glutamic acid synthetase A on bacterial surfaces. Oral administration of this vaccine in mice resulted in the production of nAbs against HPV-16/18/45/58. *L. casei*, a member of the human gut microbiota, is considered safe. Additionally, the low cost of mass production is an advantage of this platform. The oral route could be convenient and especially beneficial in remote areas in countries with limited access to healthcare services, such as some African countries [137].

DNA and mRNA vaccines

DNA vaccines are appealing platforms due to their safety, stability, and low production cost. They also effectively elicit cytotoxic T cells and humoral responses. One study produced an L2 DNA vaccine by cloning L2 HPV-16 into a pTH vector. While L2-specific antibodies and cell-mediated responses were detected in mice, antibody titers were low, and the antibodies did not show neutralizing activity [163]. Namvar et al. reported that recombinant L1-L2 HPV-16 DNA expression in human embryonic kidney cells (HEK-293) was greater than in the L1 DNA construct because L2 facilitates DNA delivery and transfer across the cell barrier [164]. Furthermore, L1–L2 HPV-16 DNA constructs without adjuvants elicited effective and protective immune responses. Protection against C3 tumor cells has also been observed in mice [138]. Additionally, mRNA vaccines initially developed for COVID-19 and now under investigation as novel platforms for many diseases, could serve as safer alternatives. mRNA vaccines have been investigated as preventive HPV vaccines; however, to our knowledge, they have not yet been studied for preventive purposes. Figure 2 summarizes all the investigated prophylactic HPV vaccine platforms and their consequent immune responses.

Routes of administration and dosage forms

HPV vaccines are administered via IM injections, similar to many other vaccines. They are relatively easy to apply, create a reservoir of immunogenic substances, and have been shown to be sufficiently safe [7]. However, the demand for more convenient routes remains undeniable.

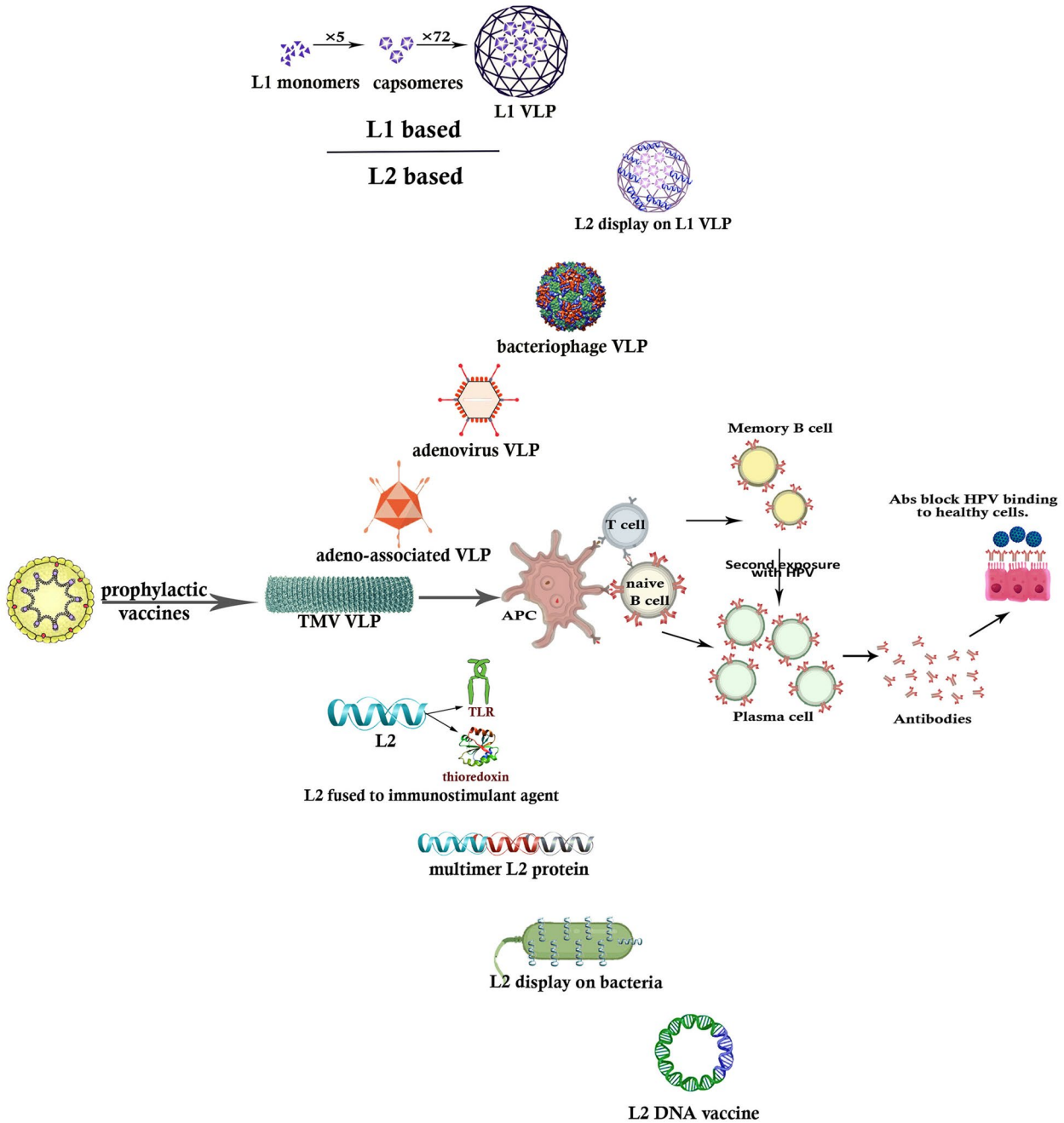

Fig. 2 Schematic representation of all investigated prophylactic HPV vaccine platforms for prophylaxis and their produced immune response

Electroporation can increase the injection efficiency. Electric pulses induce the breakdown of the keratinocyte structure in the skin. It facilitates the transport of immunogenic substances through the cell membrane, allowing a higher number of antigens to encounter immune compartments. For example, a DNA-based therapeutic vaccine (MEDI0457) targeting E6 and E7 of HPV-16 and −18, was investigated in a Phase 1 clinical trial using an electroporation device, called CELLECTRA®. After each vaccine dose, electroporation with CELLECTRA® was performed. Although no comparison was made between the use and nonuse of CELLECTRA®, the regimen was deemed safe and well-tolerated [165]. The transdermal route is another newly suggested route for HPV vaccine administration. Due to the abundance of immune cells, including

Langerhans cells, T cells, and dermal dendritic cells, transdermal delivery, similar to intradermal delivery, effectively facilitates the interaction between immunogenic substances and immune cells. For example, microparticles containing HPV16 L1 + L2 VLPs were prepared and loaded onto an AdminPatch®. The administration of this patch to BALB/c mice induced a robust immune response, as evidenced by elevated IgG titers and immune cell populations (CD4+, CD8+, CD27+, CD45R+, and CD62L+cells). This approach may offer a promising alternative for less invasive, efficient, and pain-free vaccine administration [166]. The intranasal and oral routes are also being evaluated for different vaccines due to their convenient usage and potential to induce mucosal immunity. These formulations could be specifically promising for first-line protection against HPV infection. Oral vaccines are of particular interest, especially in the form of particulate nanostructures. These particulate systems can protect antigen integrity under enzymatic and acidic conditions in the stomach and intestine. Moreover, nanoparticles can cross-present antigens to induce T-cell responses and usually do not require further adjuvants.

Current vaccines mostly employ adjuvants as essential formulation ingredients. For example, the Cervarix® formulation contains both Al2O3 and ASO4 (3-O-desacyl-4-monophosphoryl lipid A) as adjuvants, which help induce humoral immunity. However, the main immunogenicity of VLP vaccines, including Cervarix®, is attributed to the repetitive structure of epitopes on VLPs, which facilitates cross-linking of B cell receptors, inducing B cell responses, leading to the induction of nAbs following vaccination. Moreover, the uptake of VLPs by antigen-presenting cells (APCs) initiates the activation of adaptive immunity [167]. VLP structures could also be considered nanostructures [142]. When a vaccine is administered orally, antigens or antigen-containing particles are phagocytosed by M cells in Peyer's patches in the small intestine (aggregations of lymphoid tissue in the lowest portion of the small intestine) [168]. These cells deliver antigens to lymphoid tissues and resident macrophages and serve as APCs. This can induce mucosal immune responses [167]. Fraillery et al. examined the oral administration of recombinant *Salmonella enterica* expressing the L1 protein of HPV-16 and −18, which evoked a strong immune response [169].

The intranasal route represents a promising method for administering HPV vaccines, as it can induce a proper mucosal immune response. Mucosal immune activation is particularly significant, given that HPV infect and persists in epithelial cells and mucosa. A recombinant adeno-associated virus (AAV) expressing the HPV-16 L1 antigen was designed and intranasally administered to mice and rhesus macaques. Robust immune induction was observed without the use of adjuvants. AAVs can release antigens in the nasal mucosa for an extended duration. Although it supports the induction of a long-lasting immune response (remaining anti-L1 antibody titer 60 days after intranasal administration in mice), it may also cause T-cell exhaustion. Moreover, AAVs exhibit physical stability during lyophilization [170, 171]. Finally, a combined prophylactic and therapeutic intranasal vaccine was designed using recombinant AAV-5 and −9 expressing the L1 and E7 proteins of HPV-16. This intranasal vaccine produced a stronger humoral and cellular response than HPV-16 L1/E7 VLPs. The potential of lyophilization as an alternative to reduce added costs and simplify storage and distribution is promising for the future of vaccine administration [172, 173].

Access expansion and other strategies to further benefit from current HPV vaccines

The promising outcomes of vaccination programs in certain countries demonstrate that CxCa is preventable, highlighting the need for global initiatives to implement more robust strategies aimed at eliminating this cancer and other HPV-related tumors. Thus, various plans that support the acceleration of vaccination among different populations are critical. This could include improving national immunization programs in some countries, adding HPV vaccines to national childhood immunization programs [174], or even combining them with another essential vaccine and gender-neutral vaccination, which could prevent infections in women and men through herd immunity [175]. These approaches have been discussed elsewhere [174, 175]. Nevertheless, developing HPV prophylactic vaccines with broader coverage, improved accessibility, and reduced costs represents a significant advancement that could raise the bar in this field.

Conclusion and perspectives

Given the confirmed role of HPV in CxCa, immunization against HPV is recommended as a key preventive strategy. While the effectiveness of current prophylactic L1-based VLP vaccines against HPV has been demonstrated over nearly two decades, there is still a demand for novel vaccines with broader coverage, improved affordability, and easier transportation and storage conditions. Therefore, research on other platforms is ongoing, primarily summarized in this review. Though the vast volume of studies on HPV prophylactic vaccines over many years is a barrier to mentioning all the published works, we tried to cover the main approaches and steps undertaken to date.

Since the success of marketed vaccines has raised expectations for newcomers, no other studied approach has outperformed in clinical trials yet. In parallel with research on alternative vaccine platforms, the production of more affordable L1 VLP vaccines in countries such as China and India—specifically Cecolin®, Walrinvax®, and Cervavac®—is actively addressing issues related to supply and affordability. WHO has already prequalified Cecolin® and Walrinvax®. The recombinant production of Cecolin®, a vaccine with comparable efficacy to Cervarix®, in the *E. coli* host represents significant progress in HPV vaccine production. However, similar to previous L1 protein-based vaccines, it provides limited cross-protection against different HPV types and requires cold-chain maintenance. Overall, the recent biosimilar HPV vaccines produced in China and India have characteristics nearly identical to the original products regarding dosage, route of administration, and storage conditions. Their primary advantage lies in their lower prices, which can significantly enhance HPV vaccination rates by reducing financial barriers, especially in developing countries. While these biosimilars are beneficial, developing alternative novel vaccine platforms and formulations may offer a more comprehensive solution in the long run.

Vaccines containing L2 peptides seem promising candidates due to their broader coverage across different virus types and easier and lower-cost production. However, their linear structure results in decreased immunogenicity, highlighting the role of adjuvants or carrier systems in vaccine formulations. Despite extensive research on L2-based vaccines at preclinical stages (Table 3), only one vaccine containing AAVLPs has progressed to clinical trials (Table 4).

Consequently, linear L2-based protein vaccines seem far from achieving optimal efficacy unless combined with other immune-enhancing strategies. Employing scaffolds, carriers, and nanotechnological approaches, as well as combining several mentioned approaches may improve outcomes in the future. Using L2 antigens as mRNA or DNA vaccine platforms represents a potential strategy to enhance antigenicity while reducing type-specificity, warranting further investigation.

On the other hand, alternative routes of administration, such as oral or intranasal vaccines, could facilitate administration and improve access, particularly in remote areas and underdeveloped countries, thereby enhancing global vaccination coverage.

Combining L2 with E6, E7, or E5 viral proteins to confer both prophylactic and therapeutic capabilities can benefit more people worldwide. Although these vaccines are not yet sufficiently efficient, such approaches deserve further investigation and may open new avenues for future research.Overall, the fight against CxCa continues. Developing novel HPV vaccines, along with improved vaccination programs at the global level, is essential to achieve better control of HPV-induced CxCa and other malignancies.

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S.A., Sh.R., SMI.M., N.S., SA.F., and Sh.N. performed the searches and wrote the manuscript. SMI.M. coordinated the project and helped revise and edit the manuscript. A.B. helped write and revise the manuscript. M.N. conceived the idea, designed and supervised the project, and revised and edited the manuscript. All the authors reviewed the manuscript.

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