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## HPV Diagnosis in Vaccination Era

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Fátima Galán-Sánchez and  
Manuel Rodríguez-Iglesias

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<http://dx.doi.org/10.5772/55818>

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### 1. Introduction

Diagnostic methods for the detection of HPV should increase standardization and reproducibility due to two significant events in the future: the epidemiological monitoring changes in a vaccinated population and the involvement of HPV in tumors of other anatomical sites.

It will be necessary to diagnose HPV in processes other than cervical cancer, and we refer not only to their involvement in tumors with a demonstrated relationship, as in the anal region, but also to other locations in which their participation is suggested, as in the oropharyngeal tumors. Standardization in the collection and sample processing will be crucial in order to achieve accurate results involving an aid in the diagnosis and prognosis of these processes and their possible prevention.

However, the future challenge will be presented with the changes caused by the vaccination campaigns and their influence on the prevalence of cytological abnormalities and screening programs that will need to be designed according to the new status. There are already published results showing a decrease in the incidence of HPV in young women vaccinated Australia and confirm the estimates of predictive models [1].

Vaccination impact monitoring requires a multidisciplinary collaboration with technical and information support adequate to provide significant evidence to justify changes in health measures. However, screening should remain as an important strategy in cervical cancer control in both vaccinated and unvaccinated people. The reduction in incidence in young women after mass vaccination of adolescents together with greater range in controls and improved sensitivity of HPV testing draws a picture with attractive screening programs more efficient and cost effective in disease control.

The availability of sensitive diagnostic assays for HPV, reproducible and cost effectively will be critical in the monitoring of current vaccines and the design and implementation of vaccination programs. The HPV vaccine offers a unique opportunity to fight against cancer and diseases associated with HPV infection.

## 2. HPV epidemiology: The changes coming

Although the risk of cervical cancer was long known to be associated with risky sexual behavior in both the woman and her partner, it was not until around 3 decades ago that HPV was identified the likely culprit by Dr. Zur Hausen, whose work in cervical cancer won him the Nobel Prize. The development of superior molecular techniques advanced the field rapidly cementing the association between HPV and cervical cancer. This finding quickly led scientists to examine HPV's association with other anogenital cancers in women including vulvar, vaginal and anal cancers and in men, penile and anal cancers [2]. Most recently, oropharyngeal cancers have joined the list of HPV-associated cancers. Although HPV was found to be associated with these other anogenital cancers, most of the work continues to focus on cervical cancers since it remains 20 times more common than any of the other HPV-associated cancers. This phenomenon is likely due to the vulnerability of the cervix to HPV. Although the mechanisms are not well understood, the transformation zone (TZ) is likely one of the most vulnerable epithelia to HPV's carcinogenic powers [3]. The TZ change of columnar epithelium to stratified epithelium Mullerian urogenital area in the ectocervix. In the puberty columnar epithelium differentiates into stratified squamous epithelium. Metaplastic tissue is more vulnerable to HPV because its thickness to the basal layer is smaller and the virus requires cells in differentiation state for their survival, which is accelerated in metaplastic tissues [4]. It is likely that this metaplastic tissue supports viral persistence which is essential for cancer development. Interestingly metaplastic tissue is also found in the anal and tonsillar tissue.

According to a recent meta-analysis that included data from more than 1 million women in 59 countries, the prevalence of genital HPV infection among those with normal cytology ranges from 1.6% to 41.9% [5]. The estimated average global prevalence of HPV in this particular study was 11.7%. This study, along with others [6], reported an interesting trend in the female age-specific distribution of HPV whereby there is a first peak at younger ages (< 25 years) in all regions; and in the Americas, Africa and Europe, a clear second peak among individuals 45 years or older. The first peak, which comes shortly after sexual debut for most women, is generally attributed to higher levels of sexual activity with multiple partners and low viral immunity. After the first peak, a consistent age-related decline in HPV prevalence has been documented in numerous epidemiological studies. Although the reason for the smaller second peak at middle age still remains unclear, possible explanations include immunosenescence, hormonal changes prior to menopause, changes in male/female sexual behavior, cohort effects, or perhaps higher rates of HPV persistence at older ages [7]. However, new sexual exposures and latent reactivation are often invoked as explanation for the second peak of HPV prevalence observed in older women, particularly in Latin America [6]. Studies in humans are unable to directly demonstrate establishment of latency and induction of reactivation from the latent

state. However, animal models of papillomavirus infection have provided sufficient experimental evidence for papillomavirus latency [8]. HPV infects basal epithelial cells at sites of microtrauma, likely a normal consequence of sexual intercourse. Infected basal cells induced to differentiate to fill the wound will result in active papillomavirus infection. A few infected basal stem cells will retain HPV, but do not differentiate, and these infected cells are unlikely to be sampled using standard exfoliative techniques employed in most epidemiologic studies, which sample only the surface epithelium. Thus, HPV in a basal stem cell may remain undetectable until triggered to differentiate by undetermined stimuli such as wound repair and hormonal regulation [9]. With high cellular turnover in the cervical epithelium, one may expect relatively constant detection of HPV in this model. However, it is likely that a reactivated infection subsequent to latently infected stem cell differentiation is immediately recognized and brought under control by a functional memory immune response resulting in very short duration of active infection. This model would be consistent with the increase in new HPV DNA detection immediately following memory T cell depletion in acute HIV and in sexually abstinent women with chronic HIV infection [10, 11]. The relatively lower rates of recurrent HPV DNA detection in healthy populations suggests that the normal immune response is able to retain recurrence, but that background reactivation is common and likely associated with short duration of detectable viral DNA [12].

Among sexually active males, genital HPV infection is also very common; however, prevalence varies widely depending on geographic region, risk group, anatomical site, sampling method, and HPV testing methodology [13, 14]. In a recent systematic review, Smith et al [15] estimated HPV prevalence to be between 1% and 84% in low-risk sexually active men, and between 2% and 93% in high risk men. HPV infection declines substantially after about 30 years of age, The prevalence of HPV infection in males generally remains constant or declines only slightly with age after peak prevalence. One possible explanation for this is that men experience a higher rate of reinfection compared to women. Anal cancer is very rare in the general population, but much more common in well defined, high-risk populations, including women with a previous cervical precancer, men having sex with men (MSM), and immune-compromised individuals. Infection with carcinogenic human papillomavirus (HPV) has been increasingly recognised to cause anal cancer and Machalek et al [16] recorded a prevalence of high-risk anal HPV in HIV-positive MSM of 73.5%.

In 1995, the International Agency for Research on Cancer (IARC) first classified HPV types 16 and 18 as carcinogenic to humans, but based on more recent evidence, the list of carcinogenic HPV types has been expanded to include a total of 13 mucosotropic anogenital HPV types as being definite or probable carcinogens (grade 1 or 2a) based on their frequent association with invasive cervical cancer (ICC) and cervical intraepithelial neoplasia (CIN) [17]. The oncogenic types (mostly HPV 16) are also causally implicated in other cancers, including penile, anal, vulvar and vaginal cancers [18, 19]. The remaining genital types (e.g., HPV types 6, 11, 42, 43, 44 and some rarer types) are considered to be of low or no oncogenic risk [20]. However, these types may cause subclinical and clinically visible benign lesions known as flat and acuminated condylomata, respectively.

In descending order, the most common HPV types implicated in cervical cancer globally are: 16, 18, 58, 33, 45, 31, 52, 35, 59, 39, 51 and 56 [21]. HPV types 16 and 18 are the most dominant types implicated in cervical cancer in all continents, being responsible for ~ 70% of ICC cases globally. In many studies, estimating the fraction of cervical cancer cases attributable to the different HPV types is difficult due to the high prevalence of multiple type infections. For example, a recent meta-analysis estimated the prevalence of co-infection ( $\geq 2$  HPV types) in tumor specimens at 15.7% [21]. Other recent meta-analyses and cross sectional studies evaluating the worldwide distribution of HPV infections consistently reveal the same HPV prevalence patterns [5, 22]. This widespread circulation of HR-HPV types strengthens the potential for a phenomenon known as type-replacement, i.e., an increase in other non-vaccine genotypes following HPV vaccination. However, based on evidence that HPVs evolve very slowly and that HPV types do not normally compete with one another during natural infection [23-25], it is still unlikely that some other HPV type(s) will evolve to fill the niche currently occupied by vaccine target types. Furthermore, phase III trials evaluating both bivalent and quadrivalent vaccines indicate partial cross-type protection (cross-immunity) against many phylogenetically related HPV types [26-28], suggesting that the benefit from vaccination may be even greater than expected.

Infection of the oral cavity with high risk HPV is also now recognized as an important cause of oral and oropharyngeal cancers [29]. However, unlike cervical cancer in which 100% of cases are attributable to infection with HPV, only 25–35% of these cancers are attributable to HPV [2, 30]; the major risk factors being alcohol and tobacco use. Among cases of oral/oropharyngeal cancer linked to HPV infection, HPV 16 is by far the most common type detected in tumor specimens [31, 32]. Based on evaluation of risk factor profiles for cancers of the head and neck, comparing HPV 16-positive and HPV 16-negative cases, some researchers have decided that these should actually be considered distinct cancers [33]. In their study, sexual behavior (but not alcohol or tobacco use) was an important predictor of head and neck cancers among HPV 16-positive subjects, meanwhile the opposite was observed for HPV 16-negative subjects.

HPV 16 is the most common in all the HPV associated anogenital and oropharyngeal cancers therefore the HPV vaccines currently on the market are likely to make a significant impact on all these cancers in women and in men. In contrast, the role of HPV in penile cancers is less understood. Trauma and inflammation appear to be important risks suggesting that tissue damage and/or repair processes play a role in cancer development [34]. The natural history of HPV in the penis is also not well described. Although there is a histologic equivalent of the precancerous CIN 3 found in men, referred to as penile intraepithelial neoplasia (PIN), nothing is known about the natural history of these lesions and whether they have the same potential as CIN 3 (~ 12%) to progress to invasive cancer. One intriguing observation is that the penile cancer has a much lower prevalence than cervical cancer and yet the prevalence of HPV is much higher in the genitals of men than what is observed in women. Across all ages, HPV is found 2–4 times more common in the genital area of men than seen in women and the decline observed with age in women is not seen in men with similar prevalence across all ages. In contrast, the natural history of HPV in anogenital area in men behaves similar to HPV in women [35] in that HPV appears to be rapidly cleared so by 6–8 months, most infections are

undetected. These observations and the fact that antibodies to HPV are less common in men than in women suggest that most infections in men are relatively superficial and clearance is likely directed by local innate immune responses, but not by memory immune responses [36]. Since men do not appear to develop adequate HPV specific immune responses, they are not protected from re-infection. This is another argument for HPV vaccination in men. It may be that sexually active males would benefit from vaccination. Benefits would include prevention of penile, anal and oropharyngeal cancers and prevention of infections would, in theory, also benefit women [37].

After breast and colorectal cancer, cervical cancer is the 3rd leading cancer site worldwide irrespective of gender and second among women. In 2008, there were an estimated 530,000 cases and 270,000 deaths attributed to cervical cancer, with 86% of cases and 88% of deaths occurring in developing countries [38]. In these developing countries, the age-standardized incidence rate (ASIR) and age-standardized mortality rate (ASMR) were 18 and 10 per 100,000 women, respectively; whereas in more developed countries, the ASIR and ASMR were 9 and 3 per 100,000 women, respectively. Globally, incidence of ICC ranges from < 3 to > 50 cases per 100,000 women for low- and high-burden countries, respectively. These differences between countries are believed to reflect protection from screening, and variance in exposure to HPV and other cofactors like smoking and oral contraceptive use, and other sexually transmitted infections such as human immunodeficiency virus [38].

The global burden of other HPV related cancers is also substantial. Worldwide, approximately 97,215 cases of non-cervical cancers for which HPV infection may be an etiologic factor are diagnosed annually; roughly 50,780 in men (520 penile, 26,775 oropharyngeal and 13,485 anal cancers) and 46,435 in women (25,600 vaginal/vulvar, 6048 oropharyngeal and 14,787 anal cancers) [39]. However, it is important to recognize that not all of these cases are attributable to HPV and that these estimates represent the upper limit for the annual burden of cancers caused by HPV. only a quarter of oropharyngeal cancers are attributable to HPV; meanwhile approximately 90% of anal cancers, and 40% of penile, vaginal or vulvar cancers are attributable to the virus. Although there is some evidence implicating HPV with several other cancers (e.g., lung, colon, ovary, breast, prostate, urinary bladder, esophageal and nasal/sinonasal cancers), current molecular and epidemiological data are sparse and do not yet support a causal role for HPV in the etiology of these cancers [30, 40-42]. The role of HPV in the causation of esophageal squamous cell carcinoma is unclear.

Globally, HPV accounts for roughly 5.2% of the total cancer burden — the highest among all infectious agents. However, as may be expected, the distribution varies considerably according to country development status, where HPV accounts for approximately 7.7% and 2.2% of all cancer cases in developing and developed countries, respectively [42].

### **3. Screening methods for detect HPV infection**

HPV cannot be cultured in conventional cell cultures. Other classical direct virological diagnostic techniques, such as immunohistochemistry, lack the sensitivity as well as specificity

for the routine detection of HPV. Serological assays for the detection of anti-HPV antibodies have only limited analytical accuracy and their possible clinical utility is currently unresolved. Consequently, all HPV tests currently in diagnostic use rely, on the detection of HPV nucleic acids in clinical specimens.

Having relied on cervical cytology effectively for several decades, primary human papillomavirus (HPV) testing is increasingly and widely recognised as the means to deliver effective cervical screening and the prevention of cervical cancer. Despite differences in the interventions amongst the main primary screening studies [43-46], the key message seems to be clear and consistent: a primary HPV test will increase the sensitivity for detecting *cervical intraepithelial neoplasia, grade 2+ (CIN2+)*, compared with cytology, and will allow the screening interval to be extended with fewer life-time tests for women. Conventional cytology-based screening is very effective in reducing cervical cancer when delivered in well-managed programmes with high population coverage and robust quality assurance, but is costly in terms of workforce, finance and infrastructure. These programmes will be more difficult to sustain with lower detection rates of abnormalities in HPV-immunised populations. Yet cervical screening needs to sit alongside HPV immunisation to optimise cancer prevention. Screening programmes will differ between different resource settings: e.g. introducing basic screening where no previous programme has existed in low- or medium-resource settings, or providing more clinically effective prevention in highly resourced countries with organised or opportunistic screening. In protocols of prevention and treatment of cervical cancer involved, in addition to cytology and HPV detection, other diagnostic tools such as colposcopy, histological categorization and use of new biomarkers, with the correct risk stratification parameter that must be used to choose the appropriate procedures in the different phases of the clinical process: screening, triage, diagnosis and treatment [47].

Screening, defined as the preventive activity that can diagnose the disease in healthy population a priori, should have as target detecting cervical intraepithelial neoplasia grade 3 (CIN3), or being even stricter CIN2, histological stages of the disease are even preventable. Screening by cervical cytology detects only 50-60% of cases of CIN3, including cytological revisions very close in time. The analytical sensitivity of the detection of HPV DNA in exfoliated cells is much higher, above 90%, but the clinical specificity is low because of virological diagnostic predicts non persistence, necessary to cause the development of cancer. This limitation is accentuated in young women with a good chance of getting an HPV infection self-limited. However, the negative predictive value of HPV testing is very high and would, in patients free of viruses could increase the periods between two revisions in years. Using both techniques simultaneously (cytology + HPV), as has been done in some trial cohort (Northern California Kaiser Permanent), increases the control intervals to three years with good results. Implementation of HPV DNA testing in cervical screening leads to earlier detection of clinically relevant CIN grade 2 or worse, which when adequately treated, improves protection against CIN grade 3 or worse and cervical cancer. Early detection of high-grade cervical lesions caused by HPV16 was a major component of this benefit [48].

The costs are reduced in the triage by using a technique followed by another in accordance with the results of the first. The cytology followed by HPV testing has been used but as already

discussed the problems of the low sensitivity of cytology and uncertain cytology triage (ASCUS) or low-risk lesions (LSIL) can lead to positive HPV tests with unknown clinical significance. However, the benefits of the HPV test result prior cytology have been demonstrated [49]. Currently there are proposals to make the detection of HPV followed by cytology, which has the best results of clinical sensitivity, reducing costs to distance the negative control women but also may increase if performed in populations with a high incidence of infection, as in young women [50]. Hence, several protocols established older than 30-35 years for a suitable cost-effectiveness. It remains to determine the usefulness of combining HPV screening combined with a new biomarkers triage as detection of HPV mRNA, p16-INK, and other histological progression markers (Ki-67, MCM2 and TOP2A).

This scenario has increased the range of methods and technologies for the diagnosis of HPV in clinical samples, whether exfoliated cells or biopsy, even paraffin. Extraction procedures of nucleic acids, methods of viral genome detection and automation necessary for a determination which is becoming routine laboratories have acquired high levels of complexity for the most suitable choice [51]. The commercially available tests can be systematized in five groups [52]: 1) screening assays based on the detection of HPV DNA of high risk, which includes hybrid capture as the reference method because of the enormous experience gathered in previous studies, amplification using probes invader and PCR procedures, 2) similar screening tests for the detection of DNA of high-risk HPV concurrent determinations of genotypes or reflex HPV-16 and HPV-18 genotypes whose infection is a risk of developing cancer than other genotypes too, 3) genotyping assays based on HPV PCR using various technologies: reverse hybridization, microarray, array and RFLP suspension, which joins the pyrosequencing and other methods of mass sequencing, 4) based screening assays for the quantification of mRNA expression of E6/E7 high-risk HPVs, with the added value of its utility as a marker of progression, and 5) in situ hybridization, available but with limitations because of lower sensitivity.

#### **4. HPV-DNA screening methods**

High-risk HPV-DNA-based screening assays represent a group of qualitative or semi-quantitative multiplex assays in which the DNA of the targeted HPV types is detected using mixtures of probes (probe cocktails) for several HPV types with similar clinical characteristics. None of the assays from this group allow the exact determination of HPV type(s) present in a clinical specimen, but rather express the results of the tested group of HPV types as positive or negative. Until recently, such an approach has been widely accepted by the HPV community as the best way to present the results of hr-HPV testing to clinicians involved in primary screening for cervical carcinoma and management of patients with cervical intraepithelial neoplasia (CIN). The Hybrid Capture 2 (HC2) HPV DNA Test, originally developed by Digene Corporation (Gaithersburg, MD, USA) in 1997 and currently marketed by Qiagen, has been the most important HPV diagnostic assay for the last decade and is still the most frequently used diagnostic HPV test worldwide [53]. The main problems of the current version of HC2 are: analytical inaccuracy due to the cross-reactivity of its probe cocktails with untargeted HPV



types; and lack of internal control to evaluate specimen adequacy or the presence of potentially interfering substances. In order to resolve the current problems of analytical inaccuracy and to improve HC2 throughput, a next-generation diagnostic system has been developed [54]. Significant improvements have been engineered into the next-generation hybrid-capture system, which builds on the current advantages of the FDA-approved HPV screening technology, HC2. Central to the improved chemistry in the NextGen assay on the QIAensemble automated system is the new collection medium, DCM [54].

The Amplicor HPV Test (Amplicor; Roche Molecular Systems, Branchburg, NJ, USA), launched on the European market in 2004, is a qualitative PCR-based test designed to detect the same 13 HPV types as HC2: HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59 and HPV-68. Similarly to HC2, Amplicor expresses the results of the tested group of hr-HPV types as positive or negative. Amplicor is based on standard PCR amplification and detection of PCR products on microwell plates [55]. The Cervista HPV HR Test (Cervista; Hologic) is another FDA-approved signal amplification-based qualitative test for the routine detection of 14 HPVs. In March 2009, the FDA approved Cervista for two indications: to screen patients with ASC-US cervical cytology results to determine the need for referral to colposcopy; and to be used adjunctively with cervical cytology to screen women 30 years of age and older to assess the presence or absence of hr-HPV types [56]. In contrast to HC2, Cervista is based on the Invader chemistry, a signal amplification method for detecting specific nucleic acid sequences. With support from the Bill and Melinda Gates Foundation, a careHPV Test (Qiagen), based on simplified HC2 technology, has been recently developed to detect the 13 HPV types included in the original HC2 plus HPV-66, in approximately 3 h. Such rapid, simple and affordable HPV tests, whereby results can be given to a patient within the same visit, are anticipated to have the greatest impact in countries in which cervical cancer screening programs do not exist or in countries in which substantial loss to follow-up impairs the effectiveness of cervical cancer screening programs [57].

## 5. HPV-DNA screening methods with concurrent or reflex HPV-16 & HPV-18 genotyping

Reflex HR HPV DNA testing is now recognized as a cost-effective strategy to refer women with an ASC-US cytology for colposcopy [58, 59]. High-risk HPV-DNA-based screening assays with individual or pooled HPV-16 and HPV-18 genotyping are a group of novel HPV assays in which qualitative detection of 13–14 HPV types is combined with concurrent or reflex HPV-16 and HPV-18 genotyping. The Abbott RealTime High Risk HPV test (RealTime; Abbott Molecular, Des Plaines, IL, USA) is a real-time PCR assay based on concurrent individual genotyping for HPV-16 and HPV-18 and pooled detection of 12 other HPVs: HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, HPV-66 and HPV-68. Amplification of human  $\beta$ -globin is used as an internal control. The assay was launched on the European market in January 2009. RealTime is performed on the *m2000rt* real-time PCR instrument (Abbott Molecular) using a modified GP5+/GP6+ primer mix consisting of three forward and two reverse primers [60]. Analytical sensitivity of RealTime was comparable to

that of HC2 (97.6% vs 95.1%), whereas RealTime demonstrated significantly higher analytical specificity compared with HC2 [61, 62].

The cobas 4800 HPV Test (Roche Molecular Diagnostics, Pleasanton, CA, USA) is a real-time PCR assay based on concurrent individual genotyping for HPV-16 and HPV-18 and pooled detection of 12 other HPVs: HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, HPV-66 and HPV-68 [63-65]. An excellent agreement (>93%) was obtained between cobas 4800 HPV and HC2 or Linear Array (LA) for the detection of HR HPV DNA in CIN [66]. The cobas 4800 HPV test will refer fewer women to colposcopy than automatic referral of all women with ASC-US. It will also distinguish women infected with HPV16 or HPV18 from those infected with other HR HPV genotypes, although this feature is not yet included in clinical guidelines for the management of women with ASC-US. When the cobas 4800 HPV was utilized by a diagnostic laboratory, its performance was equivalent to that of HC2 and show comparable levels of performance including results for women >30 years old with ASCUS cytology [67]. Gage et al found agreement between Cobas and LA to be very good, better than that between Cobas and HC2 [68].

The Cervista HPV 16/18 Test (Hologic) is a signal amplification qualitative test based on the Invader chemistry specifically designed to detect HPV-16 and HPV-18 [69].

## 6. HPV-DNA genotyping methods

HPV DNA-based genotyping assays, which allow exact determination of several alpha-HPV types present in a clinical sample, are the largest group of currently available HPV commercial assays. In contrast to the previously described two groups of commercial HPV assays, the clinical value of HPV DNA-based genotyping assays has still not been finally determined [70-72]. Currently, HPV genotyping methods are indispensable as research tools for the study of the natural history, transmission, pathogenesis and prevention of HPV infection. However, it is highly likely that genotyping methods will also have a role in clinical management in the near future [70, 73, 74]. As the use of prophylactic HPV vaccines becomes more widespread, surveillance for population-level effectiveness will become an increasingly important activity, which will require the use of a HPV genotyping method. If HPV genotyping is to be used for diagnostic applications and not just as a research tool, it will require standardized and validated methods for the specific detection and identification of a defined spectrum of HPV types.

Although DNA sequencing is still considered to be the 'gold standard' for HPV genotyping, it is costly, time-consuming and difficult to apply in routine diagnostic settings. Thus, in daily practice, the majority of laboratories use nonsequencing based methods for HPV genotyping. However the next generation sequencing can be implemented in the HPV detection [75]. This methodology also provides a tumor genomic copy number karyogram, and in the samples analyzed here, a lower level of chromosome instability was detected in HPV-positive tumors compared to HPV-negative tumors, as observed in previous studies. Thus, the use of next-generation sequencing for the detection of HPV provides a multiplicity of data with clinical significance in a single test.

The most frequently used HPV genotyping assays today utilize the principle of reverse line-blot (RLB) hybridization. In these assays a fragment of the HPV genome is first PCR-amplified using biotinylated HPV-specific primers and the resulting amplicons are then denatured and hybridized with HPV-specific oligonucleotide probes immobilized as parallel lines on nylon or nitrocellulose membrane strip. After hybridization, streptavidin-conjugated alkaline phosphatase or horseradish peroxidase is added, which binds to any biotinylated hybrid previously formed. Incubation with chromogenic substrates (e.g., BCIP/NBT for alkaline phosphatase) yields a colored precipitate at the probe positions where hybridization occurs. The genotyping strip is then read and interpreted visually by comparing the pattern of HPV-positive probes to the test reference guide for each of the targeted HPV types. In addition to the in-house GP5+/GP6+ RLB Genotyping Assay which has been used in several important HPV trials [76, 77]. INNO-LiPA HPV Genotyping is one of the most widely used HPV genotyping tests. Several versions of this assay have been developed. In all versions, amplification of a 65 bp region of the HPV *L1* gene using biotinylated SPF10 primers is followed by hybridization of the resulting amplicons with 17–28 (depending on the assay version) HPV-specific oligonucleotide probes immobilized on a nitrocellulose strip [78]. An evaluation of INNO-LiPA *Extra*, performed on 70 HC2-positive samples, showed comparable genotyping results to the *digene* HPV Genotyping RH Test RUO (*digene* RH Test; Qiagen) and a significantly higher sensitivity of INNO-LiPA *Extra* for the detection of multiple infections [79]. Recently is described this method with realtime PCR [80]. The Linear Array HPV Genotyping Test (Linear Array) is one of the most commonly used HPV genotyping assays, which combines PCR amplification and reverse line-blot hybridization for the identification of 36 alpha-HPV types. Linear Array is based on the co-amplification of a 450 bp region of the HPV *L1* gene and a 268 bp region of the human  $\beta$ -globin gene, using biotinylated primer sets PGM09/PGMY11 and PC04/GH20 [81]. Steinau et al [82] compared the performance of three line blot assays (LBAs), the Linear Array HPV genotyping assay (LA) (Roche Diagnostics), INNO-LiPA HPV Genotyping Extra (LiPA) (Innogenetics), and the reverse hybridization assay (RH) (Qiagen). Although the assays had good concordance in the clinical samples, the greater accuracy and specificity in the plasmid panel suggest that LA has an advantage for internationally comparable genotyping studies.

Similar to reverse line-blot assays, microarray-based HPV genotyping assays also employ the principle of reverse hybridization. Following PCR amplification of a fragment of a viral genome with HPV-specific primers, the resulting amplicons are denatured and hybridized with a number of HPV-specific oligonucleotide probes attached on the surface of an insoluble supporter or DNA chip (also known as microchip, biochip and gene chip). DNA chips can consist of one to several DNA microarrays, thus enabling simultaneous analysis of multiple samples in a single experiment. After hybridization, fluorescence light from the bound PCR amplicon is detected by excitation with monochromatic light. Currently, laser scanners are used for the highly sensitive fluorescence microarray readout systems. The fluorescence label of the hybridizing amplicons can be introduced during PCR and/or during the hybridization step. Some of the microarray-based HPV genotyping tests utilize the principle of chromogenic precipitation instead of fluorescence detection. The PapilloCheck HPV-Screening Test (PapilloCheck; Greiner Bio-One GmbH, Frickenhausen, Germany) is currently one of the two most frequently used PCR-microarray-based assays. The assay allows identification of 24 alpha-HPV types. [83]. Clart HPV 2 – papillomavirus clinical arrays (Clart HPV 2; Genomica, Coslada, Spain) combine PCR amplification and an oligonucleotide microarray-based detection system for the identification

of 35 alpha-HPVs. The assay consists of pre-aliquoted, ready to use amplification mix tubes and allows amplification of a 450 bp region of the HPV *L1* gene together with an internal control template and the human *CFTR* gene using three different sets of biotinylated primers [84].

The suspension array that uses bead-based xMAP or Luminex technology is based on the use of polystyrene beads with a diameter of 5.6  $\mu\text{m}$ , which are internally dyed with various ratios of two spectrally distinct fluorophores (red and infrared). Different bead sets with specific absorption spectra can be mixed and used for the multiplexed detection of different analytes; currently, an array of 100 bead sets (bead mix) can be generated. For HPV genotyping purposes, each bead set in the bead mix is usually coupled to a single oligonucleotide probe specific for one HPV type. HPV genotyping is done by reverse hybridization technique using biotinylated PCR amplicons. After denaturation and hybridization of target HPV sequences to the bead-bound probes, labeling of the hybridized biotinylated amplicons is done using R-phycoerythrin-labeled streptavidin, serving as a reporter fluorophore. The bead sets are then read and analyzed on a Luminex analyzer; the HPV types are discerned according to the unique bead signature, whereas the presence of specific PCR amplicons is determined by R-phycoerythrin fluorescence. The Luminex readouts are expressed as the median fluorescent intensity of the reporter fluorescence for each HPV type. Positivity for the relevant types is calculated from the median fluorescent intensity over a defined threshold level, and can provide a semi-quantitative numerical output. Several in-house genotyping protocols based on xMAP technology have been developed in the last 5 years [85-91]; some of them are considered to be the most sensitive HPV detection methods currently available [85]. In addition, at least two commercial assays based on this technology are available at present.

Gel electrophoresis-based HPV genotyping assays utilize general primer-mediated PCR, type-specific or group-specific PCR, to screen for a broad spectrum of HPVs, followed by agarose gel electrophoresis. Restriction fragment length polymorphism (RFLP) is consequently applied to identify HPV type-specific restriction patterns. In addition to several in-house genotyping protocols based on RFLP, one commercial assay based on this technology is currently available.

Yi et al [92] report the development of a highly sensitive, highly automated assay based on the MALDI-TOF-MS platform for the detection and individual genotyping of 14 different HR-HPV types. The use of the MassARRAY technique and combination of automated DNA extraction/PCR pipetting procedures increased the detection throughput, which consequently decreased the cost per case of the assay. Now we can deal with 3,000 samples within 2 working days, and the current total cost per sample is about \$2 (US). The MS HPV genotyping assay is potentially suitable for routine HPV detection, especially in large-scale cervical cancer screening programs owing to its high throughput and low cost per case. Proper population-based clinical validation is needed to establish the clinical relevance of this highly analytically sensitive assay.

## 7. HPV E6/E7 mRNA screening methods

Several recent studies have clearly shown that testing for HPV mRNA instead of HPV DNA can be clinically useful [70, 93, 94]. The most relevant transcripts for diagnostic purposes are those encoding viral oncoproteins E6 and E7. The detection of viral mRNA can be done by reverse

transcriptase real time PCR [95] or by nucleic acid sequence-based amplification (NASBA). For the latter, three commercially available assays that detect E6/E7 transcripts are currently available. PreTect HPV-Proofer (HPV-Proofer; NorChip, Klokkearstua, Norway) is an assay based on NASBA technology, which allows qualitative determination of E6/E7 mRNA transcripts of the five most frequently identified hr-HPV types in cervical cancer worldwide: HPV-16, HPV-18, HPV-31, HPV-33 and HPV-45 [96]. The NucliSENS EasyQ HPV V1 assay (NucliSENS; bioMérieux) was launched in 2007 and is based on the original HPV-Proofer assay, except for the proprietary hardware platform and the software for NASBA measurements and data analysis [97]. APTIMA HPV Assay (APTIMA; Gen-Probe, San Diego, CA, USA) is a transcription-mediated amplification-based assay, which allows the detection of E6/E7 mRNA transcripts of 14 HPV types: HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, HPV-66 and HPV-68. The assay generates a qualitative result for the presence/absence of 14 targeted HPVs and does not allow the exact determination of HPV type(s) present in a clinical specimen [98]. APTIMA yielded similar sensitivity for CIN2+ compared with hc2, Amplicor and Linear Array (95.2 vs 99.6%, 98.9% and 98.2%, respectively), but a significantly higher specificity (42.2 vs 28.4%, 21.7% and 32.8%, respectively) [55]. In a comparative evaluation of APTIMA and hc2 on PreservCyt specimens collected from 800 women referred to colposcopy, APTIMA showed comparable sensitivity to hc2 for the detection of CIN2+ (91 vs 95%), as well as CIN3+ (98 vs 99%), but had higher clinical specificity (>55 vs 47% for CIN2+; 53 vs 44% for CIN3+) [99]. APTIMA had the best sensitivity/specificity balance measured by AUC (area under ROC curve) comparison test (significant for CIN2+), and the colposcopy referral rate (9.2%) comparable to that of liquid cytology (8.7%) [100].

## 8. In situ hybridization methods

*In situ* hybridization (ISH) is the only molecular method allowing reliable detection and identification of HPVs in topographical relation to their pathological lesions. Unlike other molecular methods, in ISH the whole HPV detection procedure occurs within the nuclei of infected cells and not on solid supports or in solutions. The result of the hybridization reaction is evaluated microscopically and the appearance of an appropriate precipitate within the nuclei of epithelial cells is indicative of the presence of HPVs in the specimen being tested. In addition, the physical state of the virus can be evaluated by the presence of punctuate signals for integrated virus and diffuse signals for episomal virus. Although commercially available HPV assays based on ISH have been validated technically, they are insufficiently clinically validated. In addition, current ISH-based assays are considered by many experts in the field to be too laborious and to have insufficient clinical sensitivity to be used in routine screening [52].

## 9. Progression markers

### 9.1. HPV viral load

HPV viral load is a product of the number of cells infected and number of viruses per infected cell and is therefore influenced by two main factors: (i) the extent of an HPV infection on the

cervical surface and (ii) the level of viral production in the area of infection. Viral load has been suggested to be a potential biomarker for cervical intraepithelial neoplasia grade 2 (CIN2) or greater, but currently there is no consistent evidence that a one-time measurement of viral load is a useful marker of prevalent disease or disease progression [101]. A widespread productive infection might be associated with high viral load, while a small incipient CIN3 with low-level virus production might be associated with low viral load. Furthermore, viral load in a cytological sample is subject to sampling variation in which there are varying proportions of lesional cells, normal epithelial cells, inflammatory exudate, and blood. A further complication in using viral load to predict neoplasia of CIN2 or greater is the high prevalence of multiple carcinogenic HPV infections detected in cervical samples. The current paradigm is that cervical lesions clonally expand following infection with one specific genotype (one virus-one lesion concept). On the cervical surface, multiple independent infections or lesions may occur that are caused by different genotypes. Without specific genotyping conducted *in situ*, assigning a causal HPV genotype to a specific lesion can only be based on assumptions [102]. HPV16 is the only genotype for which there is some indication that viral load may predict viral persistence and progression to precancer [103-105].

Quantitating HPV viral load seems to be a rational strategy of identifying women at risk for persistent HPV infection and progression to high-grade dysplasia. Accordingly, a high viral load could represent many cells with few virions each or a few cells containing many virions. An inaccurate description of the viral biology and the possible implications for the host could result from this discrepancy. Recently, studies have focused on longitudinal observations of viral load to predict viral clearance or lesion progression [106, 107]. Initial data indicate that repeated measurements can improve prediction of persistence or clearance, but these data are, so far, limited to HPV16 only. Although signal intensities from HC2 or various endpoint PCR-based assays have been proposed and partly used as surrogates for viral load, these approaches have limitations [108, 109]. HC2 only gives aggregate signal strength for a pool of 13 carcinogenic types, and commercial genotyping assays, such as the Roche Linear Array (LA) or Innogenetics InnoLiPA (line probe assay), do not formally report quantitative results. Overestimation of the presence of oncogenic HPV may result. Despite these caveats, the development of HPV viral load assays that may reliably be used as an adjunct screening tool to identify women at increased risk of progression to CIN 2+ and cervical cancer remains a promising tool in cervical cancer screening. Recently, Wentzensen et al [110] measuring signal intensities on LA HPV genotyping strips provides quantitative information comparable to viral load measurements based on Q-PCR. This approach offers the potential for viral load assessment for 37 types in parallel, simplifying conducting repeated measurements of viral load in epidemiologic studies and addressing the problems of multiple HPV genotype infections in studies of HPV load.

Screening for HPV integration into the host genome is a subcategory of HPV diagnostics. HPV integration is a key molecular event in the transition from an innocuous HPV infection to one that has oncogenic potential. Human papilloma virus integration results in increased expression of the viral E6 and E7 proteins. Increased expression of these proteins ultimately results in the disruption of host cell proteins, p53 and retinoblastoma protein [111]. Tests that detect

the integration of HPV into the host cell and corresponding risk of CIN 2+ or cancer are in development, and may provide a useful way of screening women at risk for cervical cancer. Studies have shown that viral integrants are detected in 100% of HPV-18-positive and 70–80% of HPV-16-positive cases of cervical carcinoma [112, 113]. A smaller subset of HSIL (15%) and 0% of LSIL contain transcriptionally active viral integrants [111].

Detection of p16(INK4a) correlates tightly with viral integration. In a normal cell, p16 blocks cyclin-dependent kinases (CDK) 4/6. Increased expression of the E6 and E7 oncogenes disrupt cell-cycle regulation, resulting in cell-cycle progression. In the normal cell, cell-cycle progression is activated by CDK 4/6 and in part regulated by p16. Because in HPV-transformed cells, cell-cycle activation is caused by E7 and not by CDK 4/6, p16 has no effect on the cell-cycle activation. Increased expression of p16 in cells driven by viral oncogene-mediated cell-cycle dysregulation can be detected through cellular immunostaining [114].

Because the correlation between HPV mRNA and high-grade dysplasia is a biologically plausible biomarker of risk, HPV mRNA detection may improve the specificity in the evaluation of women with ASCUS and LSIL Pap smears [115]. Many women have lesions that will not progress to CIN3 or invasive cancer, and these women currently present a treatment dilemma. No reliable methods can identify those lesions that are likely to regress. As a result, these women are monitored with serial colposcopic examinations at great expense to patients and the healthcare community. Detection and quantification of mRNA transcripts in these women may further refine current broad-spectrum, high-risk HPV DNA typing by allowing clinicians to know whether or not the virus is actively replicating E6 and E7 oncogenes. Messenger RNA transcript assays show great promise for being able to stratify the risk of progression to high-grade dysplasia in women with abnormal cytology.

The E6 strip test is also a biomarker that indicates viral integration. Schweizer et al. [116] evaluated the correlation of the HPV E6 test (Arbor Vita Corporation, Fremont, CA), which takes an hour to carry out and detects the HPV-E6 oncoprotein of HPV types 16,18 and 45, with detection of oncogenic HPV DNA in cytologic samples.

## 9.2. Screening methods identifying epigenetic changes

Many genes are currently being evaluated as potential methylation biomarkers for cervical cancer, but assay reliability for these methylation markers is highly variable. Within the human genome, methylation of cytosines in the CpG dinucleotides (also known as CpG sites) clustered into islands associated with transcriptional promoters is an important cellular mechanism to regulate gene expression. Methylation of HPV DNA by infected cells may alter the expression patterns of viral genes that are relevant for infection and transformation [117]. Increased methylation of CpG sites within the HPV16 genome before diagnosis and at the time of diagnosis was associated with cervical precancer [118]. Some promising candidate genes include DAPK1, CADM1, and RARB [119].

Another area of biomarker research is in the use of telomerase RNA component (TERC) identification by fluorescence in-situ hybridisation. Most cervical cancers have an extra copy of the long arm of chromosome 3, and consequently show amplification of TERC (present on

chromosome band 3q26), which seems to play a key role in progression from low-grade dysplasia to cancer [120]. Many studies indicate that TERC identification may become a useful screening tool for cervical cancer. A prospective study by Andersson *et al.* [121] found a correlation between increasing TERC detection in cytology specimens and higher grade of dysplasia. They showed that progression to cervical cancer is never seen without TERC amplification and that, conversely, specimens without extra copies of TERC were likely to undergo spontaneous regression of HPV infection.

Other biomarkers under early evaluation for cervical cancer screening include CDC6 and MCM5. These proteins are present in normal cells only during the activation of the cell cycle and help form prereplicative DNA complexes during the G1 phase. They are absent from the cell during quiescence and differentiation. Dysplastic cells have unregulated cell cycles and, as a result, CDC6 and MCM5 reflect cell proliferation [122]. Studies indicate that CDC6 may be a biomarker of high-grade and invasive lesions of the cervix, with limited use in low-grade dysplasia. MCM5 seems to be a biomarker that is expressed independent of high-risk HPV infection, and may in the future serve as a useful marker for both HPV dependent and HPV-independent cervical dysplasia [122].

General markers of cell proliferation like MIB-1, MCMs or ProEx C, and surrogate markers of high risk HPV infection like p16 INK4A have shown promising results. Other potential candidates need to be tested until we find an ideal combination. Following the example of the p16 INK4A /MIB-1 dual staining combination and the MCM2/TOP2A combination (ProEx C), combinations are often superior to any single marker and should be tested. A critical determinant for the success of future investigations will be the standardization of sample preparation and interpretation. Furthermore, before reaching the point of routine application, this ideal 'biomolecular' or 'immune-enhanced Papanicolaou test' needs to be evaluated in large prospective clinical trials with appropriate colposcopic, histological, and clinical endpoints as well as adequate follow-up [123].

### 9.3. Screening methods for detect HPV infection

In order to facilitate the acceptance of novel HPV assays, mainly for cervical screening purposes, several recommendations have recently been published [70, 124, 125]. Meijer *et al.* proposed that before a new HPV assay can be used for cervical screening purposes, it should demonstrate at least similar if not better clinical characteristics (sensitivity, specificity, reproducibility, and so on) for the detection of CIN2+ as hc2 [125]. Other experts believe that large-scale clinical trials, with an assessment of prospective disease outcomes, are required to validate any proposed HPV screening test and that cross-sectional comparisons of new HPV assay to HC2 using several hundred specimens are not an acceptable form of assay validation [70]. Stoler *et al.* recently proposed that any novel HPV assay aiming to be used for cervical screening should have a clinical sensitivity of  $92\% \pm 3\%$  for CIN3+ to render a high NPV or the capacity to predict the future detection of a CIN3+ outcome that might occur during a recommended screening interval [124]. The HPV assay aiming to be used for cervical screening should also have a clinical specificity of at least 85% to achieve an adequate PPV for CIN3. The common idea behind all proposed recommendations is that a clinically useful HPV assay



should achieve an optimal balance between clinical sensitivity and clinical specificity for detection of CIN2+/CIN3+ in order to minimize redundant or excessive follow-up procedures for hr-HPV-positive women with transient hr-HPV infections and/or without cervical lesions. Thus, as an example, a HPV assay with very high analytical sensitivity can yield a large number of clinically insignificant positive results, which will cause unnecessary clinical follow-up, unnecessary diagnostic procedures and unnecessary treatment of healthy women [126].

In forthcoming years, self-sampling may become increasingly important in cervical screening since self-collection for HPV testing (HPV self-sampling) has shown to persuade a subset of non-attendees to participate [127-129]. Targeting non-attendees is important, because they are at higher risk of developing cervical cancer. Additionally, self-sampling may make cervical screening accessible to women in developing regions [130].

An accurate and internationally comparable HPV DNA detection and genotyping methodology is an essential component both in the evaluation of HPV vaccines and in the effective implementation and monitoring of HPV vaccination programs. Genotyping assays used today differ in their analytical performance with regard to type-specific sensitivity and specificity [131]. The evaluation of assay performance needs to be performed in a standardized manner, where different assay performances can be evaluated and results can be compared against a known and accepted standard over time.

In 2008, the WHO HPV LabNet conducted a proficiency study based on HPV DNA plasmids containing the genomes from 14 oncogenic HPV types and 2 benign HPV types and open for participation to laboratories worldwide [132]. This study demonstrated that it is possible to perform global proficiency studies with unitage traceable to ISs based on plasmid DNA and that such studies can provide an overview of the status of the HPV detection and typing methodology worldwide. More recently, based on a proficiency panel composed of the same HPV DNA plasmid material used in 2008, with the amount of DNA titrated in amounts traceable to the IS. The use of the same panel material allowed a reproducible, standardized evaluation of assay sensitivity over time. Specificity was defined as absence of incorrect typing. The sample preprocessing was evaluated with extraction controls of cervical cancer cell lines. The panel was distributed to 105 laboratories worldwide and analyzed using a range of HPV DNA typing assays in a blinded manner [133]. Among laboratories that used the same assay in both years, 27% were proficient in 2008, whereas 30% were proficient in 2010. They also saw a strong trend toward increased sensitivity of assays. For example, among the laboratories using the same assay in 2008 and 2010, 50 IU of HPV-16 could be detected by all (100%) laboratories in 2010, whereas 86% of laboratories could detect 50 IU of HPV-16 in 2008. However, for several laboratories, the increased sensitivity was accompanied by increased amounts of false-positive results, resulting in nonproficiency, suggesting that recommendations for HPV laboratory testing include an increased emphasis on the use of negative controls in the assays.

The demands on sensitivity of HPV typing assays vary depending on the purpose of the testing. The WHO HPV LabNet proficiency panels are designed to evaluate the performance of HPV typing tests used in HPV vaccinology and HPV surveillance. In vaccinology, high analytical sensitivity is needed, as failure to detect prevalent infections at trial entry may result

in false vaccine failures in vaccination trials. It should be noted that the HPV tests used in cervical cancer screening programs have different requirements for evaluation, since for that purpose, only HPV infections associated with high-grade cervical intraepithelial neoplasia or cancer and not those transient HPV infections that do not give rise to clinically meaningful disease are relevant. Since the latter are characterized by low viral loads, HPV screening assays do not have demands on analytical sensitivity that are as high [125].

## 10. Measuring the immune response to vaccine

HPV serology is an essential technology for both HPV vaccinology and HPV epidemiology. Definitions of HPV-naïve subjects eligible for HPV vaccination trials include seronegativity for HPV. Immunogenicity of HPV vaccines has been used to bridge results from efficacy trials in adolescents to children and to evaluate different batches of HPV vaccines. Antibody measurements are also important in vaccinology research, e.g. for characterizing the immune response with respect to studies of seroconversion and antibody increases, cross-reactions, immune memory and immune persistence as well as kinetics of antibody responses and establishment of correlates of protection. Finally, HPV seroepidemiology is also useful for understanding the epidemiology of HPV infections in populations to be targeted by HPV vaccination programs. The lack of a standardized assay to measure HPV antibody levels has hindered both epidemiological studies of HPV infection and comparison of results from different HPV vaccine trials [134]. WHO Guidelines for HPV vaccines suggest that “initial assessment of immune responses to HPV VLP vaccines should be based on measurement of neutralizing antibodies in serum”. The available data [134-136] suggest that neutralizing and ELISA antibody titres are usually highly correlated when the ELISA antigen target is conformationally intact VLPs. Due to the complexity and labour-intensiveness of neutralization assays, VLP-based ELISAs have been preferred in large epidemiological studies. E.g., a study of HPV seroprevalences was conducted by measuring HPV 16 antibodies with an HPV16 L1 VLP-based ELISA to estimate the public-health impact of HPV vaccination strategies [137].

WHO has been coordinating work to develop standard assays that will help in assessing vaccine quality and monitoring impact after vaccination [138]. In 2006, WHO established a global HPV laboratory network (LabNet) with a main focus being the harmonization and standardization of laboratory testing procedures to support consistent laboratory evaluation of regional disease burden and monitoring of the performance of HPV vaccines. At a WHO consultation in January 2008, a group of experts recommended that the HPV LabNet should develop or identify standardized assays for general use and that efforts towards standardization on VLP-ELISA should be a high priority of the WHO HPV LabNet [139]. Following the recommendation, the WHO HPV LabNet launched a serology standardization program encompassing: (i) an international collaborative study to evaluate and refine a direct HPV 16 VLP-ELISA suggested Standard Operating Procedure (SOP), (ii) an international request for donations of VLPs to be used as international reference reagents for serology, followed by characterization and selection of optimal reagents, and (iii) an international collaborative

proficiency study on HPV 16 serology, where participating laboratories used the same standardized SOP and the same VLP reference reagent [140].

Serologic assays for the evaluation of HPV vaccine responses are currently limited to an enzyme-linked immunosorbent assay (ELISA) [141], three multiplex assay systems [142-144], and a pseudovirus neutralization assay [145], and emerging data suggest that each system has some utility for characterizing HPV vaccine antibody specificity [136, 146]. Protection against vaccine types is thought to be mediated by neutralizing antibodies [147], and while the mechanism of vaccine-induced cross-protection is uncertain, the measurement of antibodies against nonvaccine types may be useful as a potential correlate or surrogate of cross-protection [148, 149]. Recently, Bissett *et al* [150] obtain a sera panel. These plasma pools could be useful as reference reagents. They are currently available as 250- $\mu$ l aliquots of liquid plasma archived at  $-80^{\circ}\text{C}$  and can be obtained from the National Institute for Biological Standards and Control.

Type-specific L1 VLP-antibodies reach maximum titres at month 7, i.e. 1 month after administration of the third dose. Titres decline until month 24 and remain rather stable thereafter [151, 152]. At 3 years, antibody titres remain two- to 20-fold higher than in placebo controls [152]. Complete protection against HPV16 associated CIN lesions was observed over the whole follow-up duration of two Phase IIb trials: 6 years for the monovalent HPV16 vaccine, 5.5 years for the bivalent HPV16/18 vaccine [153, 154] and 4 years for the quadrivalent vaccine. Follow-up is continuing, and continued protection against HPV 16/18-associated disease end-points has been shown for the entire available observation time, even when specific antibody titres fall.

HPV infection is the most prevalent soon after sexual debut of a girl [155]. The current HPV vaccines, being prophylactic in nature, should be used before exposure of girls to HPV infection. National mass vaccination programs targeting adolescent girls between 12 and 17 years of age are available in most of the developed countries. The vaccine coverage varies greatly between countries, but school-based schemes have thus far demonstrated the highest coverage rate of 80% or higher [1, 156-158]. Australia is the first country to implement a school-based mass vaccination program with Gardasil in April 2007 and, within 3 years, has already witnessed a reduced incidence of high-grade abnormalities (HGAs) among women below 18 years of age [1].

High coverage rates of adolescent vaccination with rubella and hepatitis B suggest that, with appropriate parental and public education, adequate HPV vaccine uptake rate of 50% or more is achievable. This coverage rate is critical for mass vaccination to attain cost-effectiveness as a public measure in controlling cervical cancer burden [159]. It will also remove the imbalance in accessing screening and effectively diminish the unequal distribution of cervical cancer burden among women in the metropolitan and rural areas, among women of different ethnic groups, and among women of various socio-economic status in developed countries. Adolescent HPV infection protects women from the first exposure to HPV infection at sexual debut and abolishes the opportunity for latent infection to occur with a resultant reduction of cervical cancer in old age. The current HPV vaccines induce very high levels of anti-HPV-16 and anti-HPV-18 antibody levels to sustain protection from infection by these aggressive oncogenic HPV subtypes for at least 20 years to cover the average age period of sexual debut [160].

A measure to address the concern of vaccination efficacy for older women is to consider vaccinating women at middle ages. Undoubtedly, vaccinating mid-adult women misses the most at-risk period of a woman's life for contracting HPV infection at sexual debut. It is, however, shown that one must not dismiss lightly a mid-adult woman who wants to take the vaccination to prevent new or repeat HPV infection [161]. The vaccines have been shown to be immunogenic in adult women and significant prevention of persistent HPV-16 and HPV-18 infection has been demonstrated in a randomized control trial [162]. The effectiveness of the vaccines among these women in reducing the incidence of HGAs is being awaited with great interest. The shifting burden of cervical cancer to the young and the old in the screened population should disappear in the HPV vaccination era [163].

Immunization of boys with VLPs elicits a serum immune response similar to that in girls. Because genital HPV infection is sexually transmitted, immunization of men may help to prevent infection of women. Modeling studies on herd immunity, i.e. indirect protection of those who remain susceptible, owing to a reduced prevalence of infections in the risk group for disease, have been published [164]. The utility of immunization of males depends upon the assumed population coverage of vaccination, with successively smaller additional benefits seen in scenarios with high population coverage [165]. Modeling of programs with high population coverage (90%) have found that addition of male vaccination gives a more rapid infection control and have suggested that both sex vaccination programs may be required to achieve an ultimate eradication of the infection [137].

Limitations of the current HPV vaccines include the need for multiple parenteral doses, the lack of protection against some HPV types that cause cervical cancer, and a relatively high cost. The opportunity to overcome 1 or more of these limitations provides a rationale for developing candidate second-generation vaccines. Low-cost second-generation vaccines that could induce long-term protective immunity with fewer doses would be especially attractive for the developing world. Vaccines with activity against a broader range of the HPV types that cause cervical cancer could increase their effectiveness throughout the world, and Merck has indicated that a nonavalent (9 HPV targets) VLP vaccine is currently in clinical trials [166]. If successful, such a vaccine might reduce the frequency of potentially oncogenic infections to a degree that would permit a drastic reduction in cervical cancer screening, which accounts for most of the cost of HPV-associated disease in developed countries [167].

## 11. Future prospects and conclusions

HPV differs from most other vaccine-preventable diseases in that the major diseases to be prevented occur many decades after infection. Whereas clinical trials have documented prevention of infection and intermediate disease end-points (condylomas and precancers), surveillance following vaccine implementation will be required to document the expected gains in cancer prevention if there is appropriate population coverage. Surveillance will also provide data to indicate if type replacement or escape mutants occur. Other important tasks for the HPV surveillance include monitoring of the duration of protection, long-term safety

and actual effects on health-care cost consumption. Monitoring the impact of vaccination on type-specific infection could be important as it is the earliest change that could be anticipated, and failure to detect protection from infection will indicate failure to impact cancer in the decades that follow and allow appropriate changes in strategy to be introduced. As countries differ in their health-care priorities and infrastructure as well as in their incidence and prevalence of various HPV infections, their HPV vaccination strategies are also likely to differ [168]. As has been mentioned, the waning in the levels of HPV antibodies post-vaccination appears to plateau after 5 years. It is not known whether waning of HPV antibody levels in the longer term will require a vaccine booster. In addition, antibody correlates of protection have not been defined because there have so far been almost no cases of vaccination failure. If a reliable immunological correlate of protection can be identified, this will help in assessing the requirement for booster vaccinations and greatly facilitate the evaluation of second-generation vaccines.

As the type-specific prevalence of HPV infection is very high in young sexually active populations, the effect of a successful HPV vaccination programme should be detected quite rapidly by sentinel surveillance in these populations. The specific design of these sentinel studies will vary, but selecting clinics offering sexual counselling may be more efficient than school-based sampling. Reduction in the prevalence of types targeted by the vaccines as well as no increase in the prevalence of non-vaccine types are important end-points. Baseline data are needed to establish prevaccine prevalence as well as to determine the sample size required to observe impact beyond confidence intervals of sampling and testing errors. It is imperative that all HPV DNA prevalence surveys are performed using testing methodology that has been subjected to an international quality assurance, as comparability of data between countries or even before *versus* after will otherwise not be possible.

Screening remains an important strategy in cervical cancer control in the HPV vaccination era. Women who have received HPV vaccination should continue regular screening, as cases of HGAs and invasive cervical cancer among vaccinated women have been reported [169]. There is also a concern whether the protective effect of adolescent vaccination has an impact on the incidence of cervical cancer when the vaccinated cohort reaches older age. It is reassuring to see evidence that, in well informed communities, there appears to be no change in women's attitude toward continual screening [170]. Furthermore, screening remains the only effective method for cervical cancer prevention for women who opt out of vaccination. However, reduction in the incidence of HGAs in young women following adolescent mass HPV vaccination supports a strategy to delay screening to a later age than the current practice of starting screening once the girl becomes sexually active.

Evidence from meta-analysis has overwhelmingly demonstrated the superiority in sensitivity of HPV DNA test over cytology in cervical cancer screening. Indeed, retrospective analysis of invasive cervical cancer tissues in well screened populations showed that more than 80% of cases were related to HPV-16 or HPV-18 [171]. With elimination of HPV-16 and HPV-18 with mass vaccination, the remaining oncogenic subtypes of HPV, which have low neoplastic transformation rates, should significantly lower the incidence of HGAs. The sensitivity and positive predictive value of cytologic screening will be further compromised [172]. The

recently available HPV-16-specific and HPV-18-specific screening technology further enhances the technical advances that makes the HPV DNA screening test even more attractive in the vaccination era than before. The high sensitivity of the HPV DNA test should allow the screening interval to be increased to a longer period [173].

The reduction in background risk of cervical cancer by elimination of the most important HPV types will affect cost-effectiveness of screening programs and may, in the long term, allow increasing screening intervals. Co-ordinated quality assurance/monitoring of HPV vaccination and cervical screening is advisable for finding the most efficient strategies for cervical cancer control. Data on vaccination coverage will be essential for every country performing HPV vaccinations. HPV vaccination registries are preferable, but sales statistics and serosurveys may be alternatives.

For rapid assessment of vaccine program efficacy, the continuous monitoring of which HPV types are spreading in the population will become necessary for early monitoring of 'type replacement' phenomena, inappropriate vaccination strategies or other reasons for vaccination failure. Surveys in sexually active teenagers and/or in younger participants of cervical screening programs should be contemplated.

## Author details

Fátima Galán-Sánchez and Manuel Rodríguez-Iglesias

Department of Microbiology, School of Medicine and Clinical Microbiology Laboratory, Puerta del Mar Univ. Hosp. University of Cádiz, Spain

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