CHAPTER 12

General discussion and conclusions
In the last two decades, numerous in-house and commercial tests have been developed for the detection of human papillomavirus (HPV) DNA in clinical specimens, many of which involve PCR-based technologies. HPV tests can be defined on the basis of their analytical sensitivity or on their clinical sensitivity.

### 12.1 ANALYTICAL AND CLINICAL SENSITIVITY

Analytical sensitivity is defined as the detection of all HPV infections including transient HPV infections. Clinically validated HPV tests are defined by the pooled detection of hrHPV infections mainly associated with clinically meaningful disease, i.e., CIN2+ lesions. The range of HPV types targeted by these assays is usually limited to those 13 or 14 types associated with a high risk for the development of cervical cancer (so-called hrHPVs), i.e., HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 and sometimes 66. By this definition, the specificity for CIN2+ is high and prevents in a clinical setting over-referral for (transient) HPV infections not associated with CIN2+ (1). The read-out of analytically and clinically relevant HPV tests can differ according to their capacity to provide identification of individual genotypes (genotyping), or to detect a group of HPVs simultaneously (pooled or grouped detection), or a combination of both (partial genotyping).

### 12.2 USE OF HPV TESTS

In a research setting, HPV DNA tests with high analytical sensitivity usually have full genotyping capability for a broad range of HPVs. HPV tests that detect a group of HPVs simultaneously (e.g., SPF10-PCR-DEIA) are uncommon and only used as part of a genotyping test algorithm. Analytically sensitive HPV tests can be used to 1) determine HPV vaccine efficacy against several surrogate endpoints, from persistent HPV infection to high-grade CIN lesions (CIN2+) associated with vaccine-targeted HPVs (section 12.3.1) 2) monitor the effects of nation-wide vaccination on prevalence of HPV genotypes (and possibly type replacement) within an immunized cohort (section 12.3.2) 3) investigate the attribution of individual HPVs to (pre)malignant diseases (section 12.3.3).

In a clinical setting, clinically validated HPV tests that target a group of high-risk (hr)HPVs simultaneously (also known as hrHPV tests) have three possible clinical applications: 1) triage of women with equivocal cytological abnormalities, 2) follow-up after treatment of high-grade disease, and 3) for primary cervical cancer screening (section 12.4.1).

Clinically validated hrHPV tests with full genotyping capacity might allow 1) detection or exclusion of new incident CIN2+ after treatment for high-grade disease, and 2) monitoring persistence of infection by a specific genotype when hrHPV is present (section 12.4.2).

A recent generation of clinically validated hrHPV tests offering grouped detection of hrHPV genotypes has also implemented the option of concurrent partial genotyping for HPV16 and HPV18 only. Assays with partial genotyping are useful for 1) clinical management of women positive for HPV16/18, who have the highest risk of developing high-grade CIN, and 2) monitoring women treated for high-grade CIN, since women who are positive for HPV16 have the highest risk of recurrent CIN2+ (section 12.4.3). In a clinical setting HPV genotyping should only be done following or integrated in a clinically validated hrHPV test.

The current chapter provides a general discussion on considerations for the use of HPV genotyping tests, including the two different PCR-based technologies, i.e., SPF10 and GP5+/6+, described in this thesis. We review findings from this thesis in the context of other studies to illustrate that the design and validation of an HPV test should be in accordance with its intended application. In Chapter 2-5 we have already discussed technical aspects and characteristics of SPF10 and GP5+/6+–based genotyping techniques. Therefore, only technical aspects will be discussed that have not been highlighted before (section 12.6.2).
12.3 ANALYTICAL HPV TESTS INTENDED FOR VACCINOLOGY, SURVEILLANCE AND DISEASE ASSOCIATION

12.3.1 Vaccinology

An HPV DNA test with a high analytical accuracy and full genotyping capability is essential for investigations of (cross-)protective effects by licensed or candidate HPV vaccines. HPV genotyping assays are used to determine efficacy and protection against persistent vaccine-associated HPV infections and CIN2 associated with these HPV genotypes.

Different HPV genotyping technologies have been used in clinical trials with the licensed prophylactic bivalent HPV16/18 (Cervarix) and quadrivalent HPV6/11/16/18 (Gardasil) vaccines. As discussed in Chapter 1, quantitative type-specific (TS) PCRs (internally developed at Merck Research Laboratories) were used in all Phase III trials, e.g., the FUTURE I and II, of the quadrivalent vaccine. In the two largest trials of the bivalent vaccine, i.e., PATRICIA and the Costa Rican Vaccine Trial (CVT), a combined algorithm was used, comprising broad-spectrum PCR by SPF10 LiPA25 followed by TS PCR for vaccine types HPV16 and 18. The TS PCRs for these vaccine-targeted HPVs were added to the test algorithm, since SPF10 LiPA25 is based on broad-spectrum PCR and has therefore a suboptimal analytical sensitivity for multiple HPV infections (2) (Chapter 1).

In the PATRICIA trial, a technical limitation of the SPF10 LiPA25 and TS16/18 PCR test algorithm in determining a cross-protective effect of the bivalent vaccine against non-vaccine HPV types was reported (3). Despite cross-protective efficacy of the bivalent vaccine against non-vaccine type HPV33, 31, 45 and 51, a negative vaccine efficacy was noted for persistent HPV52 and 58 infections (3). This could be attributable to the reduced analytical sensitivity for non-vaccine types in multiple infections, as the combined algorithm of SPF10 LiPA25: broad-spectrum PCR and TS16/18 PCR did not comprise TS PCRs for non-vaccine HPVs.

Two TS PCR-based assays encompassing a broader range of HPVs were recently developed, i.e., MPTS12 RHA (4, 5) and MPTS123 Luminex (5) (Chapter 2). The analytical sensitivity in multiple infections increases when using SPF10 LiPA25 with these TS PCR assays in a combined algorithm, as opposed to utilizing each of them separately (5). A post-hoc HPV analysis of the specimens in the PATRICIA trial was performed using SPF10 LiPA25 with MPTS12 RHA, which demonstrated that the per protocol testing algorithm (i.e., SPF10 LiPA25 with TS16/18) had initially underestimated the vaccine efficacy against some non-vaccine hrHPV types (6). Thus, type-specific PCRs targeting non-vaccine types (i.e., other than HPV16/18) should be added to SPF10 LiPA25 test algorithm for more accurate evaluation of the cross-protective effects of HPV vaccines.

Key messages:

- HPV genotyping assays based on broad-spectrum PCR or type-specific PCRs with high analytical sensitivity are suitable for assessing HPV vaccine efficacy, HPV prevalence- and HPV disease association studies.
- Assays based on broad-spectrum PCR, e.g., the SPF10 LiPA25 algorithm, can slightly underestimate actual genotype prevalence due to PCR primer competition in mixed infections, which could impact vaccine efficacy trials that use virological endpoints.
- A combined test algorithm of broad-spectrum PCR with a range of type-specific PCRs increases the analytical accuracy for vaccine efficacy studies.
12.3.2 HPV genotype surveillance
The bivalent and quadrivalent vaccines have been implemented in vaccination programs in many countries. Several aspects should be monitored to evaluate the effects of an HPV vaccination program, i.e., 1) the occurrence of vaccine-preventable disease, 2) the concentration of naturally occurring HPV type-specific antibodies and the prevalence of HPV vaccine-associated genotypes, 3) the titers of HPV vaccine-type-associated antibody response, 4) vaccine uptake, and 5) vaccine safety. Knowledge on type-specific HPV infections pre- and post-vaccination is needed for evaluation of the (cross-)protective effects of the HPV vaccines on genotype diversity and possible type replacement. For example, in Suriname, a Caribbean country on the South American continent, the quadrivalent HPV vaccine was introduced in 2013. Before introduction of the vaccine, data on pre-vaccination prevalence of genital hrHPV genotypes among Surinamese women was limited. Therefore we determined the prevalence of HPV genotypes in a selected multi-ethnic population in Paramaribo using the SPF10 LiPA25 algorithm (Chapter 6). The prevalence of genital carcinogenic HPV genotypes was high among women in Suriname and comparable to other Caribbean populations. These baseline data enable future studies to evaluate changes in HPV genotype prevalence over time following HPV vaccination.

Key message:
- Pre- and post-vaccination data of HPV DNA genotyping assays on cervical specimens is needed for evaluation of the (cross)protective effects of the HPV vaccines on genotype diversity and possibly type replacement.

12.3.3 Disease association by established and probably/possibly carcinogenic HPVs
Studies towards epidemiology of HPV types and their association with anogenital malignancies, including cervical cancer, require HPV genotyping assays with high analytical sensitivity. Particularly when clinical specimens with poorly preserved DNA, e.g., formalin-fixed paraffin-embedded biopsies, are used, HPV tests using short PCR fragments (<100 bp), e.g., the SPF10 LiPA25 system, reach the highest analytical sensitivity.

In Chapter 7, HPV genotype attribution was estimated in the largest global collection of archival invasive cervical carcinoma (ICC) specimens to date (n=10,575) using a single algorithm for case selection and HPV analysis by SPF10 LiPA25. The combined relative attribution to ICC of vaccine-targeted HPV types 16 and 18 was estimated at 71%, ranging from 68% in Central/South America to 79% in North America. The combined attribution of the eight most common types, i.e., HPV16, 18, 31, 33, 35, 45, 52, and 58, was 91%. Regional differences in genotype prevalence were observed, most notably HPV33 ranking third in Europe and HPV58 and 52 ranking fourth and fifth (after HPV45), respectively, in Asia.

In addition to those genotypes identified by SPF10 LiPA25, other, infrequent HPVs were identified as single infections in ICC using a novel Sanger sequence methodology (Chapter 8). Besides HPV85 and 97, all genotypes in α-species 5, 6, 7, 9 and 11 were found as single infections in cervical cancers. These species together constitute a single evolutionary clade (7), comprising the 13 established and probably carcinogenic HPV types (Class 1 and 2A) and 12 possibly carcinogenic (Class 2B) HPV types (8). The classification of Class 2B HPVs is only based on a close phylogenetic relation to established carcinogenic HPVs, for which both mechanistic and epidemiologic evidence of an etiologic involvement in cervical carcinogenesis has been found (e.g., a specific pattern of expression of the viral E6 and E7 genes observed in carcinoma tissues; the immortalization of human keratinocytes by HPVs that express the E6 and E7 genes; the interaction of E6
and E7 proteins with various cellular proteins, particularly with pRb and p53; the direct demonstration that E6 and E7 proteins are responsible for the malignant phenotype of cervical carcinoma cells; the presence of HPV DNA in cervical carcinoma cell lines; and large-scale epidemiological studies that identified hrHPV types as the major risk factor for cervical cancer). In Chapter 8, the thirteen Class 1/2A HPVs contributed to 97.5% of the single HPVs detected in ICC, as opposed to only 2.3% by Class 2B HPVs.

The WHO IARC has undertaken a different approach to estimate the global HPV genotype distribution among ICC, i.e., meta-analyses based on multiple case-control studies in different geographical parts of the world (9, 10). The most recent meta-analysis was performed by Li et al on a selection of 30,848 ICC specimens from previous, smaller case studies (11). The global contribution of HPV16/18 was estimated at 73%, and their prevalence also differed per region, with the largest difference observed between Eastern Asia (68%) and Western/Central Asia (82%). HPV58 (8%) and 52 (5%) were the third and fourth most common types in Eastern Asia, which is in accordance with our finding that HPV 58 (4%) and 52 (4%) are the predominant types attributing to ICC in this region besides HPV16 and 18. Li et al found that HPV33 was also highly frequent in Europe (4%), but preceded by HPV31, a notable difference to our findings. In Africa, the third most common HPV according to our findings and to the study by Li was HPV45. The low frequency of Class 2B HPVs that we found in Chapter 8 was confirmed in the meta-analysis by Li et al. Most of these Class 2B HPVs were identified in less than 0.5% of ICC.

It should be noted that a bias could have arisen in our study, in terms of overestimation of the prevalence of a particular genotype since we cannot exclude that the participating pathology laboratories have performed selection of cases which were negative for HPV16 or 18 when contributing specimens (as discussed in Chapter 7). In addition, Li et al performed a meta-analysis involving studies that used less sensitive techniques for HPV analysis than in our study, which may have led to underestimation of the attribution of (rare) types in ICC.

Chapter 7 and other studies provide important estimates of the combined, global contribution of HPV16, 18, 31, 33, 35, 45, 52, and 58 to ICC, ranging from 81% (12), 91% (Chapter 7), to 95% (11). These eight HPVs are the main types that should be considered when studying (cross-)protective effects of licensed and candidate, next-generation vaccines, e.g., the nonavalent V503 (13, 14). HPVs classified as possibly carcinogenic had only a marginal contribution to ICC (individually ranging from 0.07% to 0.52%; cumulative contribution 2.3%) (Chapter 8). Individual prevalence of some Class 2B HPV types was even lower than that of HPV6. Prevalence of HPV6 is high in benign lesions (genital warts; estimated at 62%) (15) but extremely low in ICC (0.4%) (11). Therefore, prevalence of HPV6 in ICC has been taken as the arbitrary epidemiologic cut-off for non-carcinogenic HPV types (8). Contemporary hrHPV tests intended for cervical cancer screening target all hrHPVs from Class 1/2A and none of the Class 2B HPVs (except for HPV66). Even if stronger evidence for involvement in cervical carcinogenesis is provided in the future, inclusion of the very low prevalent, possibly carcogenic types in HPV screening assays holds the severe risk of yielding only small gains in clinical sensitivity at the cost of a substantial loss in clinical specificity leading to many unnecessary colposcopy referrals (16, 17).
Key messages:

- Virtually all HPV genotypes belonging to the same evolutionary clade of α-species 5, 6, 7, 9 and 11 are found as single infections in ICC.
- The possibly carcinogenic HPVs (Class 2B, according to the WHO classification) have only a marginal contribution to ICC (ranging 0.07% to 0.52%).
- These types should not be included in hrHPV screening tests, since the gain in sensitivity obtained by adding these rarely occurring Class 2B HPV types to HPV tests used for cervical screening does not outweigh the loss in specificity, leading to considerable increase in costs.

12.3.4 Dynamics of infection by HPV16 variant lineages

Prevalence of variant lineages of HPV16, the most carcinogenic HPV type, was investigated in Chapter 9 by a specifically designed HPV16 variant Reverse Hybridization Assay (RHA). The HPV16 genotype consistently detected in follow-up samples usually involves a persistent infection with the same variant. We found that multiple HPV16 variants in one woman are rarely observed as opposed to presence of multiple genotypes. The prevalence of mixed HPV16 variant infections in our study was estimated at 8.5%. This corroborates with the frequency of 4.8% reported recently by Mirabello et al, which was determined using a novel whole-genome next-generation sequence (NGS) technology (IPV Seattle 2014).

The HPV16 RHA is an easy-to-use and rapid tool for the recognition of the main HPV16 variant lineages (as well as some sublineages) in natural history and epidemiologic studies using a limited number of diagnostic SNPs. However, the HPV16 RHA does not have sufficient resolution for detailed genome-wide analysis of specific nucleotides or combination of nucleotides that might be associated with specific pathological consequences. NGS technology is becoming more affordable and can be used for viral genome-wide association studies (VWAS) to elucidate the genetics of HPV pathogenesis (18). The underlying genetic mechanisms causative for the large difference in cervical cancer risk between HPV16 and other hrHPV’s are poorly understood (19). Even within the HPV16 genome, evidence exists that genetic variants of the non-European lineage, particularly those of the D lineage (AA), have a stronger association with high-grade cervical neoplasia and cancer than the European lineage (20-22).

12.4 HPV TESTS INTENDED FOR CLINICAL PRACTICE (CLINICALLY VALIDATED HRHPV TESTS)

12.4.1 Clinical validation of hrHPV tests for pooled detection

Clinically validated HPV tests are defined by the pooled detection of hrHPV infections (usually 13 or 14 hrHPVs) mainly associated with clinically meaningful disease, i.e., CIN2+ lesions. As a result, the specificity for CIN2+ is high and prevents over-referral for (transient) HPV infections not associated with CIN2+.

Therefore, HPV DNA tests intended for screening require clinical validation. Evidence to support the introduction of a clinically validated HPV test must come from large, longitudinal randomized controlled clinical trials, or from showing equivalence to HPV tests that have already been validated in clinical trials, i.e., Hybrid Capture 2 HPV DNA Test (HC2, Qiagen , Hilden, Germany) and the GP5+/6+ PCR-based EIA (GP5+/6+ EIA, Diassay, Rijswijk, The Netherlands). The candidate HPV tests must, among other things, demonstrate that its clinical sensitivity and specificity for histologically confirmed CIN2+ are non-inferior to those of the validated comparator assay according to defined internationally agreed criteria (1).

A clinically validated test can be applied as a primary cervical cancer screening tool (alone or in
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conjunction with cytology), for triage of women with abnormal cytology, and/or as a test-of-cure after treatment for high-grade cervical disease. The HPV DNA tests that have been validated for one or more of these applications usually detect 13 or 14 hrHPV types as a group, and are listed in Chapter 1. Based on findings in Chapter 10, the LMNX Genotyping Kit HPV GP (GP5+/6+ LMNX; previously marketed as digene HPV Genotyping LQ Test) can be used for cervical cancer screening when 14 hrHPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) are detected as a group. GP5+/6+ LMNX and several other validated assays have partial or full genotyping capability. The potential role of genotyping in a clinical setting will be discussed in more detail in section 12.4.2 (full genotyping) and 12.4.3 (partial genotyping).

12.4.2 Clinical utility of hrHPV tests with full genotyping: measuring persistence

In a clinical setting, full genotyping of 14 hrHPVs, either following or integrated in a clinically validated HPV test, is currently not routinely performed. However, HPV genotyping allows monitoring persistence of a specific HPV genotype infection, which might be useful for two clinical applications.

Firstly, a clinically validated test with full hrHPV genotyping could be used to measure persistence of a specific hrHPV type infection after treatment for high-grade cervical disease or detect a new incident hrHPV genotype infection. Post-treatment persistence of a given hrHPV type could be indicative of disease recurrence (23). Recent studies have shown that post-treatment hrHPV testing is more sensitive for the detection of post-treatment CIN2+ than cytology (24-27). However, these studies generally apply hrHPV tests offering pooled detection of 13-14 hrHPVs as a proxy for type-specific persistence, i.e., non-type-specific persistence (24, 25) and cannot differentiate residual high-grade lesions from new, incident CIN2+. For accurate measurement of type-specific persistence, full hrHPV genotyping either following or integrated in a clinically validated HPV test should be used as opposed to repeated measurements with a pooled hrHPV test.

Secondly, monitoring persistence of a hrHPV genotype through an assay that offers full genotyping might also be of use in follow-up of women who tested positive by a clinically validated hrHPV test in cervical cancer screening. Women with a persistent hrHPV infection are associated with a high absolute risk of progression to high-grade cervical lesions (28). However, implementation of full hrHPV genotyping for the evaluation of persistence of certain HPV genotypes in cervical cancer screening is complicated and will be challenging. Direct risk-based management by partial HPV genotyping (HPV 16 and 18) seems more feasible and cost-effective for clinical practice.

12.4.3 Clinical utility of hrHPV tests with partial genotyping: direct risk management

Partial genotyping for HPV16 and 18 has been suggested as a strategy for direct risk management of women testing hrHPV-positive in screening. This strategy is based on 1) observational, longitudinal studies, showing that women testing positive for HPV16 and to a lesser extent for HPV18 have a higher risk of CIN3 and cancer compared to the women who tested positive for non-HPV16 hrHPVs (29-32), and 2) on the observation that, in contrast to other HPVs, HPV16 and 18 show a proportional increase in prevalence in women with normal cytology, precancer, and ICC (33, 34).

Thus, a recent generation of clinically validated hrHPV tests provides partial genotyping for HPV16 and 18 in addition to the pooled detection of the other (non-HPV16/18) hrHPVs (35). The utility of triaging HPV16/18-positive women to immediate colposcopy referral was evaluated in a Dutch screening population and in a US screening cohort (36, 37). Triage by HPV16/18 presence identifies women at increased risk, but women who are positive for the other hrHPVs also
have an unacceptably high risk and therefore still require an additional repeat test, e.g., cytology.

Partial genotyping could also be of use for risk management of women treated for high grade CIN. Women who are positive for HPV16 after treatment appear to have a higher risk for recurrent CIN than those positive for any of the other hrHPVs (25, 38).

12.5 HRHPV TESTS AND SELF-SAMPLING

12.5.1 Self-sampling devices and hrHPV testing

Screening coverage can be improved by offering HPV-self-sampling to women not responding to the program invitation (i.e., non-responders) or to women in low-resource areas lacking medical services (39-41). Cervicovaginal cell material is collected by women themselves in a non-clinical setting (e.g., at home) and sent to the laboratory for hrHPV testing. Self-collection methods can be brush-, swab-, tampon- or lavage-based devices and material can be stored in liquid-based medium, on solid carriers, or just dry on the brush. Self-collected specimens require analysis by molecular techniques, e.g., hrHPV testing, and are not suitable for cytology, due to the insufficient collection of intact ectocervical and/or endocervical cells from the transformation zone (42, 43). In general, hrHPV testing on self-samples seems an acceptable and clinically accurate alternative for HPV- and/or cytology-based analysis of conventional physician-collected cervical scrapes (i.e., physician-sampling), but the combination of self-sampling method and hrHPV test should be validated for reaching clinical equivalence in terms of detecting CIN2+ (extensively reviewed by Snijders et al (39) and Arbyn et al (44)).

In Chapter 11, we evaluated a novel self-sampling method, consisting of a Viba brush for self-collection of cervicovaginal material that is subsequently applied and stored on an FTA solid carrier cartridge, for hrHPV testing using the SPF10 or GP5+/6+ hrHPV assay. Self-collected specimens tested by the GP5+/6+ PCR assay demonstrated lower clinical accuracy compared to physician-collected liquid based specimens. However, the suboptimal self-collection method could be compensated by using the analytically more sensitive SPF10-LiPA25 algorithm for hrHPV detection. These observations confirmed that significant changes in the variables of the diagnostic process, e.g., collector (either physician or women herself), collection device and medium, specimen processing, and a predefined hrHPV test, should be validated in conjunction with the current gold standard in relation to a histologically confirmed endpoint of CIN2+/CIN3+. The current gold standard comprises physician-collected cervical scrapes stored in liquid-based medium and analyzed by a clinically validated methodology for processing and HPV testing.

12.5.2 Triage of hrHPV-positive women by self-sampling

hrHPV testing on most self-samplers is at least as sensitive but less specific than cytology on physician-collected specimens (39, 44). Thus, women testing hrHPV-positive via self-sampling require triage to prevent unnecessary referral for colposcopy. In most studies, triage is done by cytology, which would require a visit to a physician for collection of a new, conventional liquid-based specimen, bearing the risk of women being lost to follow-up. Another way already discussed previously is by partial HPV genotyping on self-collected material. Although HPV 16/18 positive women have an increased risk of CIN3+ that warrants direct referral for colposcopy, the risk for non-HPV16/18 positive women is still too high and therefore they require close surveillance in the next year.

Therefore, other new molecular triage markers should be developed that can be directly performed on the same (processed) self-collected specimen. Such a new development is the triage of women who tested hrHPV-positive by self-sampling using methylation marker panels directly on self-collected cervicovaginal samples. Tumour suppressor genes involved in cervical cancer are inactivated by hypermethylation of their promoter region. Technologies for analysis of
methylations patterns tend to preferably detect cervical cancers and more advanced high-grade precursor lesions (45). Recently, Verhoef et al showed in an RCT that triage by methylation marker panel MAL-miR124-2 directly on self-collected cervicovaginal samples is as sensitive as triage by cytology on a new physician-taken smear, although at the cost of a higher referral rate. This opens the way to full molecular screening on cervicovaginal material taken by women themselves (46).

Key message:

A careful clinical validation of the combination of collection device, DNA processing method and a predefined hrHPV test is required prior to implementation of self-sampling in a clinical setting.

12.6 QUALITY CONTROL IN HRHPV TESTING

12.6.1 Proficiency panels
Currently, standardization in HPV genotyping assays for clinical use is lacking. The WHO HPV laboratory network (HPV LabNet) attempted to introduce standardization in HPV genotyping through the annual distribution of a proficiency panel of purified, recombinant HPV DNA plasmids. This panel provides a standardized evaluation of the analytical performance of genotyping assays in different laboratories and consistency of testing over time (47). However, no clinical specimens were included in the panel to evaluate other aspects of the laboratory process, such as sample processing. In addition, the proficiency panel was intended for performance evaluation of genotyping tests used in HPV vaccinology and surveillance, but not for those used for HPV-based cervical cancer screening. Proficiency panels specifically designed for HPV (genotyping) tests used in HPV-based screening are currently unavailable.

12.6.2 Internal control
Although not mentioned among the criteria for HPV tests in a clinical setting (1), several PCR-based HPV tests have implemented an endogenous internal control strategy to aid identification of false-negative HPV results. COBAS, Abbott, PapilloCheck and GP5+/6+ LMNX have primers that can amplify a (conserved) fragment of the human genome extracted from cells present in the collected cervical specimen. If a specimen does not allow amplification of the target HPV DNA and the internal control, this could indicate inhibition of amplification or failure of DNA extraction. In addition, a positive endogenous human target indicates presence of human cells, and is therefore often interpreted as an accurately collected cervical specimen.

However, we should be careful in relying on this endogenous internal control for the following reasons:

1. An internal control for a human target can be positive due to presence of any type of human cells (e.g., human skin cells), which may then provide a false reassurance that cervical epithelial cells from the squamo-columnar junction have been accurately sampled.

2. In addition, there is no correlation between the HPV viral load and the amount of cells in cervical swabs. A threshold for the number of cells that constitutes an adequate sample for HPV testing is therefore difficult to determine.

3. Another concern when using an endogenous human DNA target as an internal control, is that the number of human gene copies may be much higher than the HPV copy number. The internal control may have an amplification advantage and thus not accurately test for inhibition of HPV amplification (48). Therefore, amplification reagents in e.g. the PapilloCheck and Aptima tests have been spiked with an exogenous internal control template at a fixed and relatively low concentration to monitor inhibition. Notably, inhibition of amplification and/or extraction failure in cervical swab samples has become a rare event with contemporary DNA extraction methods.
In conclusion, the endogenous internal control (IC) for human DNA implemented in some PCR-based hrHPV tests is of limited use in identifying potential false-negative hrHPV results. The non-detection of both viral and human DNA in (self-collected) cervical specimens points towards inadequate sampling. However, presence of human DNA in the absence of viral DNA is no guarantee for correct sampling for hrHPV testing. For monitoring sample processing, a spiked exogenous internal control (as well as parallel extraction and PCR controls) is more appropriate.

**Key message:**
- The endogenous internal control (IC) for human DNA implemented in some PCR-based HPV tests is useful to identify potential false-negative HPV results but care should be taken against over-interpretation.

### 12.7 FUTURE PERSPECTIVES

This section provides considerations, new developments and possible avenues of research involving HPV detection/identification technologies.

**Vaccinology**

Future trials of licensed and next-generation vaccines towards their (cross-)protective efficacy against high-grade lesions associated with vaccine-targeted HPV genotypes require highly sensitive HPV DNA genotyping algorithms. Type-specific (TS) PCR-based technology has been used in efficacy studies of the quadrivalent and bivalent vaccines, as a stand-alone assay or used in a combined testing algorithm with a sensitive broad-spectrum PCR, respectively. A higher analytical sensitivity was achieved by using the combined algorithm than by using TS PCR and broad-spectrum PCR separately, bringing together the advantages of both systems (5). A direct analytical comparison of the quantitative TS PCR assays used for the quadrivalent vaccine versus the combined algorithm of TS and broad-spectrum PCR used for the bivalent vaccine has not yet been performed. A thorough insight into their type-specific analytical characteristics, particularly in multiple infections, would be informative for the choice as to which HPV tests should be used in future vaccine trials.

**HPV surveillance**

Knowledge on type-specific HPV prevalence pre- and post-vaccination is required for surveillance of effects on genotype diversity and possibly type replacement in nations that have implemented a vaccination program. Broad-spectrum PCR-based genotyping tests with high analytical sensitivity should be used for short-term monitoring of reduction in types and possibly type replacement in vaccinated young women, in addition to the surveillance of CIN2+ prevalence associated with vaccine-targeted HPV’s. It is important to realize that a part of the effect of vaccination could be masked by a lower sensitivity in multiple infections, as already discussed for the vaccine trials (section 12.3.1).

Once vaccinated cohorts reach the age of the first screening round, the effect of vaccination on HPV genotype prevalence can also be monitored ‘real-time’ within organized screening programs. This can only be performed in countries that will implement primary HPV-based screening, such as The Netherlands, Australia and parts of Italy. Preferably, the clinically validated HPV test used should have either a partial or full genotyping component.

**Disease association**

Whole-genome ultra-deep sequencing or next generation sequencing (NGS) is a novel technology that could be suitable for elucidating the genotype-phenotype relationships between HPV genotypes or variants and clinical outcomes, such as cervical cancer. The identification of specific nucleotides or combination of nucleotides associated with disease could provide insight into the molecular basis of HPV-
associated malignancies (18). Elucidating the genetic basis of pathogenesis and of virus-host interactions might help understand the differences in carcinogenic potential between closely related HPV genotypes and intratypic variant lineages within alpha species 5, 6, 7, 9 and 11.

**HPV-based screening**

The Netherlands will be the first country to adopt hrHPV testing for primary cancer screening. This change is expected to be implemented in 2016. The choice of the hrHPV test(s) that will be used is currently not known. Around 10 commercial assays have been clinically validated, most of them by showing non-inferiority to HC2 or GP5+/6+ EIA. Other important factors contributing to the choice of an HPV test in screening are, among other things, price per test, degree of automation and hands-on time, turn-around time, and clinical performance on self-collection devices. The incorporation of an endogenous internal control is only marginally beneficial. As genotyping will not be recommended for triage of hrHPV-positive women in The Netherlands, assays do not necessarily require a (partial) HPV genotyping component. But using HPV tests with (partial) genotyping capability could be beneficial for ‘real-time’ monitoring of post-vaccination changes in genotype prevalence, as discussed previously.

**Post-vaccination screening**

As an additional means of cervical cancer prevention, a national immunization program was started in 2009 in The Netherlands using the bivalent prophylactic HPV vaccine. Since the vaccine-associated HPV types comprise around 70% of all cervical cancers, HPV-based screening will be needed for many years to come. As vaccinated young women reach the age of screening, a gradual decrease in cervical lesion prevalence will adversely impact HPV test performance (49). Evaluation of the impact of prophylactic HPV vaccination on cervical precursor lesions (CIN2+) will show whether the starting age of screening can be increased and whether the screening intervals can be extended. These changes might help retain cost-effectiveness of HPV-based screening of vaccinated populations (50, 51), but the optimal strategy remains to be determined.

In theory, around 70% of all cervical cancer cases could be prevented by the licensed bivalent and quadrivalent vaccines. This proportion will probably be around 76% due to partial cross-protection. If protection against HPV vaccine types is assumed, the remaining cases will be related to HPV types for which the vaccines do not offer cross-protection. Some of these types are not included in current HPV tests used in cervical screening. Thus, the relative importance of rarer cancer-related HPVs might increase. Whether the prevalence of these less frequent HPV types should be monitored in the future by HPV genotyping for these rare types is a matter of costs and requires further investigation.

**Full molecular-based screening**

In the envisioned hrHPV-based primary screening program in the Netherlands, cytology at baseline and repeat cytology after 6 months will be used for triage of hrHPV-positive women. However, as opposed to cytology, methylation markers have the capacity to specifically detect invasive cancers and advanced transforming CIN with a high short-term risk of progression, and reduce unnecessary referrals (45). As discussed earlier, methylation markers will also be useful for triage of women testing hrHPV-positive directly on self-collected cervicovaginal samples, as these are not suitable for cytology (46). Such an ‘objective’, full molecular-based screening strategy of hrHPV testing and methylation triage could encompass the next improvement in cervical cancer screening programs.
REFERENCES


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