Persistent high-risk human papillomavirus (HPV) infections as surrogate endpoints of progressive cervical disease. Potential new endpoint for efficacy studies with new-generation (non-HPV16/18) prophylactic HPV vaccines

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Summary

Recent data indicate that persistent HR-HPV infections represent a significantly increased risk of developing incident high-grade CIN and cervical cancer. Accordingly, 6-month (6M+) or 12-month (12M+) type-specific persistence of HR-HPV have been proposed as powerful surrogates of progressive disease. Because of substantial practical impact in future HPV vaccine trials using non-HPV16/18 vaccines, studies on HR-HPV persistence as a surrogate endpoint of progressive CIN have been subject to a comprehensive meta-analyses recently. The present communication was solicited to bring this important and timely topic to the awareness of the readers, in a format consisting of a review of the recent literature, supplemented with the author's own experience from different studies. Based on a large number of relevant studies, there remains little doubt that persistence of HR-HPV for 6+ or 12+ months is associated with a significantly increased risk of developing incident high-grade CIN. However, some data also disclosed several important issues that need to be carefully considered and/or adequately resolved before adopting 6M+ or 12M+ HR-HPV persistence as a surrogate of progressive disease. These include i) definitions of HPV persistence, ii) HPV detection techniques and iii) testing intervals and iv) length of follow-up, as well as v) diagnosis of the surrogate endpoints, and vi) other study characteristics, including vii) the type of reference category used in calculating the risk estimates. All these issues are critically discussed in the present communication. Of major impact seems to be the reference category used to calculate these risk estimates, as evident from the NIS-LAMS cohort. Taken together, it is suggested that in all future studies using the 6M+ or 12M+ HR-HPV persistence as a surrogate endpoint of progressive disease, a "gold standard" should be used in calculating the risk estimates. In addition to deciding, 1) whether to use 6M+ or 12M+ persistence criteria, and 2) cytological, histological or combined surrogate endpoints (SIL, CIN1, CIN2, CIN/SIL), one should 3) use exclusively the HPV negative reference group in calculating the risk estimates for viral persistence endpoints. This is supported by the data from the recent meta-analysis as well as from the author's combined NIS-LAMS cohort, both implicating that the most consistent association to progressive disease is obtained when women with persistent HR-HPV are compared with HPV-negative women. It is the conviction of this author that the two other reference categories (HPV transient and HPV mixed outcome) are far too heterogeneous and subject to potential misclassifications to give consistent and reproducible risk estimates for HR-HPV persistence as a surrogate endpoint of progressive CIN.

Key words: Human papillomavirus; High-risk types; Persistent infection; 6-mo and 12-mo persistence; Surrogate endpoint; Progressive disease; HPV vaccines; Efficacy trials; Study power; NIS cohort; LAMS study.

Introduction

Since the recognition of human papillomavirus (HPV) as the causal agent of cervical cancer (CC) and its precursor lesions (CIN), epidemiological data from different countries confirmed that the peak prevalence of cervical HPV infections occurs between 22-24 years of age, with constant decline with progressing age [1-7]. More recent studies on the natural history of HPV infections have further refined their dynamics in different populations. Accordingly, incident HR-HPV infections are clearly age-dependent, the 3-year cumulative incidence exceeding 50% among young women following the onset of their sexual activity [8-11].

On the other hand, clearance of the virus does not show such strict age-dependence [12], but continues at a rather constant rate from 30 years onwards when the clearance rates exceed the acquisition rates resulting in declining age-specific prevalence rates [13-15]. However, not all HPV infections will undergo spontaneous clearance; some of the acquired infections remain persistent [6, 8, 14, 15]. These persistent infections of the high-risk (HR) HPV types are

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considered as prerequisite for developing a progressive disease intensely studied, e.g., for the necessary cofactors of HPV [12, 16-21].

Persistent infections and CIN are established from fewer than 10% of all new infections [8, 18, 19]. There is some evidence that HPV16 persists longer than the other HR-HPV types [18, 19]. Furthermore, prevalent infections persist longer in older women than in younger women [22], most probably because of increased probability of virus integration over time [23]. Time to progression from HPV infection to CIN2+ among HPV carriers is variable, but a majority of cases occur within the first three years [22-25]. Since persistent HR-HPV infection plays a key role in the development of CC, the detection of persistent HR-HPV infection represents a specific marker of an increased risk [19-25].

Indeed, several studies have demonstrated very high relative and absolute risks of CIN2+ and CC ascribable to type-specific persistent HR-HPV infections [18, 26-29]. This is particularly true with the women who acquire persistent HPV16 or HPV18 infection, but also applies to other HR-HPV types. The emerging data implicate, however, that HPV16 and HPV18 infections progress more rapidly than the other HR-HPV types [28-30]. Clearly, persistent HR-HPV infections represent a sign of increased risk of CC, and as such, 6-month (6M+) or 12-month (12M+) type-specific persistence of HR-HPV have been proposed as powerful surrogates of progressive disease, e.g., in ongoing HPV vaccine trials [19, 31, 32]. Recently, persistent HR-HPV infections have also been implicated as potential intermediate endpoints of high-grade CIN in CC screening [33, 34].

As shown by the published and ongoing vaccine trials [24], a relatively short-term vaccine efficacy study will have sufficient power to evaluate histological (CIN2+) endpoints associated with HPV16 and HPV18, but this is unlikely to be the case for the other HR-HPV types [19, 31, 32]. Indeed, a similarly powered study to evaluate CIN2+ endpoints for non-HPV16/18 types would need a significantly larger sample size (> 100,000 women) and significantly longer follow-up (up to 10 years), which would make such a study not feasible [19]. Thus, search for other (non-histological) endpoints as potential surrogate markers of disease progression is urgent. Indeed, persistent HR-HPV infection might be a potential candidate for being such a surrogate marker [35].

Because of its substantial practical impact related to the conduct of all future HPV vaccine trials using non-HPV16/18 vaccines, the data on HR-HPV persistence as a surrogate endpoint of progressive cervical disease were subject to comprehensive meta-analysis recently [35]. This exhaustive analysis disclosed several important issues that need to be carefully considered and/or adequately solved before implementation of 6M+ or 12M+ HR-HPV persistence as a surrogate of progressive disease in these future trials.

Unfortunately, no unanimous agreement has been reached as yet how to define persistent HR-HPV infection [35]. In some studies, HPV persistence is defined as two or more HPV DNA-positive tests [36, 37], while in some others, HPV persistence has been assessed using the time to clearance (i.e., duration of infection) [38-40], and yet in some others, as a proportion of HPV-positive visits [41, 42]. This issue is further complicated by differences in HPV detection methods, intervals in HPV testing, as well as whether a type-specific or non-type-specific HPV persistence is measured, and whether the analysis is restricted to HR-HPV types in general or to individual HR-HPV genotypes [35]. There is an urgent need to validate those multiple variables affecting the risk estimates of these virological endpoints [35-42]. It is also essential to reach a standardised definition of HPV persistence, to be uniformly applied in the future studies using HPV persistence as surrogate of disease progression.

Prompted by the rapid arousal of this topic into a sharp focus due to its major practical importance, we decided to explore the issue in our combined NIS and LAMS study cohort of over 15,000 women (to be described later), and recently analysed several potential (viral and other) surrogate markers of disease progression in two separate studies [43, 44]. The first one [43] was completed before the meta-analysis of Koshiol *et al.:* [35] was published, while the second one [44] was designed to elucidate several of the key open issues that were raised in that meta-analysis.

The present communication was solicited to bring this important and timely topic to the awareness of the readers, in a format consisting of discussion of the core literature [35], supplemented with the experience of this author based on two multi-centre screening trials (NIS and LAMS studies) [43, 44]. We start with an address of the key information from a recent meta-analysis [35], followed by the description of the two approaches recently made by the author's research group to tackle the issues that remain unsolved in the published literature. At the end, these key issues related to the acceptance of persistent HR-HPV as a surrogate endpoint in the future efficacy trials with new-generation non-HPV16/18 vaccines will be discussed. Recommendations are given how these assessments should be conducted to obtain (for 6M+ and/or 12M+ persistent HR-HPV infection) the most consistent risk estimates for the surrogate endpoint markers (CIN, SIL) of a progressive cervical disease.

Data on recent meta-analysis

In this communication, the purpose is by no means to reiterate the comprehensive meta-analysis recently published by Koshiol *et al.:* [35], which the reader is referred to for further details. For introduction to the topic, however, it is appropriate to identify the key studies included in this meta-analysis as well as to summarise the core data extracted from these studies in a highly synthetic form.

Altogether, the authors went through a substantial amount of literature and identified 41 eligible studies that covered > 22,500 women analysed (in different designs) for the association between HPV persistence and cervical neoplasia.

All abstracted data were reviewed twice by independent readers to ensure data accuracy. In many studies, several relative risks (RR) were given, but for the meta-analysis, these were selected through decision rules for analyses of the association of HPV persistence and CIN2-3/HSIL+ endpoint, to maintain the independence of observations [35]. Sensitivity analyses suggested that the results of this meta-analysis were robust to reasonable changes in the decision rules, and funnel plot asymmetry analyses showed little evidence of publication bias.

In this meta-analysis [35], the data extracted from these studies were presented as several figures and tables, summarising the key indicators of these studies [16, 18, 26, 42, 45-62]. For the purpose of the present communication, the key observations can be summarised in one table, synthesising the data most relevant to the present discussion (Table 1). There are several important observations that deserve to be addressed in some more detail here.

First of all, there remains little doubt that HPV persistence is strongly and consistently associated with incident CIN2-3/HSIL+ in practically all these studies [16, 18, 26, 42, 45-62]. This led the authors to emphasise the value of HPV persistence as a clinical marker and as an endpoint in clinical vaccine trials and also to suggest that sequential HPV DNA testing may be useful in the screening programs by identifying women who are at high risk of CC [35]. Any further discussion of this last subject, however, falls outside the scope of the present communication.

In the published studies included in this meta-analysis [35], several different surrogate endpoints of progressive disease were used, ranging from ASCUS Pap smear to biopsy-confirmed CIN2/3 [16, 18, 26, 42, 45-62]. As evident from Table 1, the strength of the association between HPV persistence and cervical neoplasia increased with increas-

Table 1. — *Risk estimates for HR-HPV persistence as a predictor of surrogate endpoints (ASCUS, LSIL, CIN1+, CIN2-3, HSIL) using optional reference categories*.*

Author (Reference No.]	Relative Risk	95% Confidence Interval	
		Lower bound	Upper bound
HPV-NEGATIVE REFERENCE			
ASCUS surrogate endpoint			
Kjaer *et al*. [26]	4.9	0.9	26.7
Liaw *et al*. [45]	13.2	6.5	27.0
LSIL/CIN1 surrogate endpoint			
Cuzick *et al*. [46]	4.1	0.93	17.9
Kjaer *et al*. [26]	117.7	45.2	417.7
Liaw *et al*. [45]	100.6	37.7	268.4
HSIL/CIN2-3 surrogate endpoint			
Bais *et al*. [47]	27.5	1.6	474.6
Moberg *et al*. [48]	33.3	17.7	62.7
Cuzick *et al*. [46]	14.0	0.83	237.5
Dalstein *et al*. [16]	239.9	14.8	3893.5
Kjaer *et al*. [26]	813.0	168.2	3229.2
Schlecht *et al*. [49]	12.3	2.6	57.5
Liaw *et al*. [45]	497.1	29.8	8290.2
Wallin *et al*. [50]	213.4	18.1	1600.0
Koutsky *et al*. [51]	26.0	6.5	112.0
HPV MIXED OUTCOME REFERENCE			
ASCUS surrogate endpoint			
Kjaer *et al*. [26]	4.0	1.4	11.5
Liaw *et al*. [45]	5.6	3.3	9.7
LSIL/CIN1 surrogate endpoint			
Cuzick *et al*. [46]	4.4	1.4	13.3
Kjaer *et al*. [26]	7.4	4.7	11.7
Liaw *et al*. [45]	4.7	1.8	12.2
HSIL/CIN2-3 surrogate endpoint			
Bais *et al*. [47]	36.9	2.1	638.2
Schiffman *et al*. [18]	94.6	53.4	167.7
Dannecker *et al*. [52]	5.7	2.9	11.3
Cuzick *et al*. [46]	29.8	1.8	507.3
Dalstein *et al*. [16]	260.6	16.1	4229.3
Kjaer *et al*. [26]	46.6	25.8	83.8
Paraskevaidis *et al*. [42]	21.3	10.3	44.3
Elfgren *et al*. [53]	27.3	1.4	452.0
Ylitalo *et al*. [54]	25.9	9.3	72.1
Liaw *et al*. [45]	26.8	12.4	57.6
Wallin *et al*. [50]	36.1	4.8	271.6
HPV TRANSIENT REFERENCE			
ASCUS surrogate endpoint			
Cuschieri *et al*. [55]	8.8	0.38	208.0
Bory *et al*. [56]	11.4	4.1	31.8
Kjaer *et al*. [26]	2.7	0.49	14.6
Liaw *et al*. [45]	4.7	1.8	12.2
LSIL/CIN1 surrogate endpoint			
Cuschieri *et al*. [55]	16.3	2.1	125.6
Harris *et al*. [57]	3.7	2.2	6.1
Cuzick *et al*. [46]	4.3	0.97	18.8
Bory *et al*. [56]	6.2	2.7	13.9
Kjaer *et al*. [26]	27.3	6.3	119.3
Liaw *et al*. [45]	6.9	2.9	16.4
ter Harmsel *et al*. [58]	0.7	0.15	3.2
Moscicki *et al*. [59]	1.6	0.68	4.0
Saito *et al*. [60]	42.0	2.5	712.9
HSIL/CIN2-3 surrogate endpoint			
Bais *et al*. [47]	9.4	0.56	157.2
Cuschieri *et al*. [55]	22.9	1.3	408.1
Peto *et al*. [61]	37.1	2.2	620.5
Harris *et al*. [57]	5.4	3.2	9.2
Cuzick *et al*. [46]	14.7	0.87	249.6
Dalstein *et al*. [16]	88.2	5.5	1427.9
Bory *et al*. [56]	119.1	7.4	1926.2
Kjaer *et al*. [26]	18.2	6.1	54.3
Beskow *et al*. [62]	79.0	10.4	597.3
Liaw *et al*. [45]	8.6	2.5	30.0
ter Harmsel *et al*. [58]	9.4	1.3	68.4
Wallin *et al*. [50]	13.5	0.98	185.5
Moscicki *et al*. [59]	14.1	2.3	84.5

*Meta-analysis by Koshiol *et al*. [35].

ing grade of cervical lesions. This is consistent with the view that CIN1 lesions (and their surrogates LSIL and/or ASCUS) in most of the cases represent only transient HPV infections, being very common among young sexually active women, and possess a high tendency for spontaneous regression accompanied by virus clearance [1, 2, 8, 14, 15, 17]. In contrast, long-term persistence of HR-HPV positivity is clearly associated with neoplastic transformation and thus clinically relevant as a surrogate endpoint of progressive disease [1, 2, 5-8, 11-17, 35].

Another observation of major importance is that the strength of association of HPV persistence and incident CIN2-3/HSIL+ varied widely and was partially dependent on the HPV referent group (Table 1) [35]. In settings like this [16, 18, 26, 42, 45-62], three different reference groups can be used in testing the strength of persistent HPV as predictor of progressive disease: 1) HPV-negative women, 2) women with mixed HPV outcome, and 3) women with transient HPV infections. These three referent categories will be discussed in more detail later. While viewing the data in Table 1, it is obvious that those studies comparing women with persistent HPV infections with those who were HPV-negative produced the highest and most consistent relative risks [16, 26, 45-51]. This is feasible because it is commonly agreed that the risk of developing high-grade CIN is very low among HPV-negative women [1, 2, 8-10]. On the other hand, comparing women with persistent HPV infections to those with transient infections produced the weakest relative risks [16, 26, 45-47, 50, 55-62]. This could implicate that the risk of high-grade CIN among women with HPV infections of shorter duration (transient) is by no means negligible. As the authors correctly point out, one cannot exclude the possibility that a substantial proportion of these women classified as transient HPV infections in these studies (i.e., duration < 6 months) do, in fact, represent persistent infections with an onset well before the baseline visit, and upon clearance soon after, were actually misclassified [35].

In overall evaluation, the second most consistent risk estimates were obtained in studies where HPV persistence is compared with mixed HPV outcome [16, 18, 26, 42, 45-47, 50, 52-54]. As the name implies, this reference category consists of women with mixed patterns of HPV outcomes, including short-term persistors with clearance, followed by reactivation etc, i.e., a pattern known as fluctuation [1, 2, 8]. As shown in long-term follow-up studies [2,8], the majority of these fluctuators eventually turn out to clear their infections, which seems to make this reference category more close to a HPV-negative referent group as to the obtained risk estimates for HPV persistence as predictors of high-grade disease (Table 1).

An additional point is associated with the duration of HPV persistence which is closely linked with the HPV testing interval [35]. It was noted that the associations between HPV persistence and cervical neoplasia appeared stronger in studies with longer duration of HPV infection (> 12 months) and longer HPV testing intervals (> 6 months or > 12 months) [16, 18, 26, 42, 45-62]. This is not unexpected, because these variables could reflect a longer exposure to oncogenic HPV conferring a higher risk for developing high-grade CIN and CC. Similarly, testing HR-HPV+ at longer testing intervals also decreases the potential misclassification (of both exposure and outcome), because the majority of HPV infections associated with low-grade lesions will regress during the testing interval [2, 8, 11, 14, 15, 35].

Finally, the meta-analysis under discussion [35] clearly disclosed a major gap in our knowledge, i.e., the lack of studies providing data on type-specific persistence and its association with disease progression. Astonishing as it might sound, only few studies provide such data for HPV16 and HPV18. Although the associations between HPV16 and/or HPV18 persistence and high-grade CIN were consistently positive, there was a major heterogeneity in the risk estimates varying from 4.5 (95% CI, 0.24-85.1) (not significant) to 279.7 (95% CI, 16.0-4,894.5) [35]. Knowing that even a single detection of HPV16/18 appears to increase the risk of developing CIN3 and CC [2, 3, 10, 12, 28, 30, 63], it is of utmost importance in the future studies to focus on these associations at the HPV genotype level.

Based on a large number of relevant studies [16, 18, 26, 42, 45-62], it is easy to agree with the authors of this meta-analysis [35] stating that these data demonstrate that two HPV-positive visits are associated with increased risk of high-grade CIN. This comprehensive meta-analysis clearly confirmed that repeated HPV detection is associated with an increased risk of invasive CC, and its precursors, despite the differences in i) definitions of HPV persistence, ii) HPV detection techniques and iii) testing intervals, iv) diagnosis of surrogate endpoints, and v) other study characteristics, including the type of reference category [35]. Needless to say, several of these differences need further assessment and standardization, before consistent and reproducible risk estimates can be provided for the association of persistent HPV infection and development of cervical neoplasia.

Experience from the combined NIS and LAMS study cohort

Prompted by the rapidly increasing interest in this topic, we decided (in 2008) to explore this issue in our combined NIS and LAMS study cohort, comprising over 15,000 women, of whom almost 2,000 have been prospectively followed-up for detection of incident cervical disease. We recently analysed several potential (viral and other) surrogate markers of disease progression in two separate studies [43, 44]. The first one [43] was completed before the meta-analysis of Koshiol *et al.:* [35] was published, while the second one [44] was designed to elucidate several of the key open issues that were raised in that meta-analysis.

Aims

In the first of our two studies [43], we found the 6M+ and 12M+ persistence of HR-HPV was a powerful surrogate of developing an incident CIN, but second only to persistent HSIL Pap smear. It also became obvious that these risk estimates are critically dependent on which endpoints (SIL, CIN1+, CIN2+) are used, and particularly how the reference category is defined [43]. Our second analysis was the first study to directly compare the impact of the three optional reference categories; i) HPV-negative, ii) transient HPV, and iii) mixed HPV outcome on the strength of the association between 6M+ and 12M+ HR-HPV persistence and disease progression [44]. Instead of a single surrogate endpoint, we used four: i) SIL (cytology), ii) CIN1+ (biopsy), iii) CIN2+ (biopsy) and iv) combined CIN/SIL (cytology/biopsy) surrogate endpoints of progressive disease. In both studies, the combined cohort consisted of 1,865 women, prospectively followed-up in the NIS study [64] and in the LAMS study [65], derived from the total cohort of 15,301 women [43, 44].

Study design

Our two recent analyses [43, 44] are based on a combined cohort of the NIS and the LAMS studies, previously described in a series of original reports [64-69]. Both studies are international multi-centre trials testing optional screening tools in low-resource settings of three NIS (New Independent States of the Former Soviet Union) countries (Russia, Belarus and Latvia) [64] as well as in two Latin American countries (Brazil and Argentina), respectively [65]. The design and baseline data of both cohort studies have been previously detailed [64, 65], and described here only in brief so as to give the necessary background to the data discussed next.

The NIS Cohort

The material of the NIS study cohort comprises 3,187 consecutive women attending six different outpatient clinics in the three NIS countries between 1998-2002. These women derived from three different groups: i) women participating in cervical cancer screening (= SCR patients); ii) gynaecological outpatients (= GYN patients), and iii) patients attending STD clinics (= STD patients). The mean age of these women at enrolment was 32.6 (± 10.7 SD) years (median 30.6, range 15-85 years) [64]. The study design, baseline data and interim results have been detailed in a series of reports already cited [11, 14, 15, 17, 64]. All eligible women had Pap smears taken and were tested for HR-HPV using HC2 assay, and also with PCR (n = 1,500) and confirmative hybridisation. Patients with ASC-US or higher Pap had biopsy confirmation at baseline [64].

The LAMS study

The LAMS study is a combination of a population-based, cross-sectional study and a longitudinal cohort study of women enrolled in regions with different (low, intermediate, high) incidence of CC in Brazil and Argentina, as described in detail recently [65]. In the first phase the four clinics examined a total of 12,114 women between February 2002 and June 2003, enrolled in the original LAMS Study cohort. The mean age of these women at enrolment was 37.9 years (range 14-67; median 37.7 yrs). In this screening trial, eight different diagnostic tests were compared: cervical cytology (conventional and liquid-based cytology) was compared with 1) four optional screening tools suggested for low-resource settings: a) visual inspection with acetic acid (VIA), b) visual inspection with Lugol iodine (VILI), c) cervicography, d) screening colposcopy); and 2) with the new molecular diagnostic tools (HPV testing by HC2 assay), performed a) in samples collected by physicians, and b) in those taken by self-sampling devices [65-69]. Women testing positive with any of these techniques were examined by colposcopy at the second visit. In addition, a 5% random sample of all test-negative (PAP, VIA, VILI, HC2) women was referred for colposcopy to assess false negative exams, and 20% of baseline HC2-negative women were referred for new HC2 to assess incident HPV infections.

Prospective follow-up

In both studies, prospective follow-up (FU) is an essential component of the design [11, 14, 15, 17, 64-69]. In the NIS cohort, all women presenting with biopsy-confirmed low-grade lesions were assigned for prospective FU, while high-grade lesions were treated. Altogether, FU data are available from 887 women, divided into four sub-cohorts according to their baseline HPV/Pap smear status [11, 14, 15, 17, 64]. Altogether, 33 women with baseline CIN3 were excluded from the analysis, leaving 854 women in the final FU-cohort of the NIS study. FU visits were scheduled at 6-mo intervals, planned to cover 24 months. The mean FU time reached in this trial was 17.2 mo (SD, 11.6 mo; median, 16.6 mo; range 1-43 mo) [64].

In the LAMS study the same criteria were used to allocate the women into the FU and treatment groups [65]. A total of 1,011 women completed at least one FU visit including examination by Pap smear, VIA/VILI, colposcopy and biopsy, whenever abnormalities were detected. Also in the LAMS study, FU visits were scheduled at 6-mo intervals,

planned to cover 36 months. The mean FU time reached in this study was 21.7 mo (SD, 8.1 mo; median, 24.2 mo; range 1-54 mo). All high-grade lesions were promptly treated and followed-up for the same period, using repeated Pap test and colposcopy at 6-month intervals, and HC2 assay at 12-month intervals [65-69].

Outcomes and endpoints of cervical lesions and HR-HPV infections

For both recent analyses [43, 44], the data of the 854 women from the NIS Cohort and 1,011 women from the LAMS study were merged into the same file, and the combined cohort of 1,865 women was analysed for the four surrogate endpoints of disease progression, based on cytology or histology or both: 1) progression to SIL; 2) progression to CIN1+, 3) progression to CIN2+, and 4) progression to CIN/SIL. Progression to SIL (any degree) was based on detection of either LSIL or HSIL in any of the Pap smears taken during the FU of a baseline Pap-negative woman. Being an endpoint of progressive disease, women who subsequently cleared their incident SIL (the last visit status) were excluded from this sub-group [43, 44]. Similarly, baseline biopsy-negative women who developed CIN1+ in any of the consecutive biopsies taken during the FU were defined as progression to CIN1+. As a progression to CIN2+ was defined any case where biopsy-confirmed progression from baseline negative-, NCIN- or CIN1 biopsy was established in the subsequent FU-visits. Finally, a fourth category was built up, consisting of women in whom the progression was defined by both biopsy and cytology, i.e., progression to LSIL/HSIL or to CIN1+/CIN2+. Times to progression into SIL, CIN1+, CIN2+, and CIN/SIL were calculated from the baseline visit to the respective FU-visit when the progression event was first detected. Progression rates were calculated dividing the numbers of progression events by woman months at risk (WMR), and expressed per 1,000 WMR [43, 44].

Methods

Because have been detailed in a series of recent reports [11, 14, 15, 17, 64-69], the methods used in the NIS cohort and in the LAMS study are described here only as far as pertinent to elaborating the data discussed in this communication.

Detection of HPV DNA by Hybrid Capture 2 assay

In both studies, HPV testing was performed by Hybrid Capture 2 (HC2) assay using cervical swabs (collected by a physician) and self-sampling devices (tampons, in LAMS study only), as described previously [64-69]. The samples were analysed only for the presence of HR-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. The usual limit of 1 pg/ml of HPV16 DNA was used as the positive control (CO), i.e., samples were classified as HR-HPV positive with the RLU/CO ≥ 1.0 pg/ml cut-off.

Assessment of HPV persistence

Statistical analyses were performed using the SPSS and STATA software packages (PASW for Windows, Version 17.0.2., SPSS Inc., Chicago, USA and STATA/SE 11.0. Stata Corp., TX, USA). The incidence rates (of SIL, CIN1+, CIN2+, CIN/SIL) were expressed as cases/1,000 WMR, and their 95% confidence intervals (95%CI). Incidence rates in the NIS and LAMS cohorts were compared by calculating RR (rate ratio) statistics (with 95%CI).

In both studies [43, 44] the power of two viral endpoints was estimated: i) more than 6-mo persistent (6M+) HR-HPV infection, and ii) more than 12-mo persistent (12M+) infection, as predictors of progressive cervical disease, defined by intermediate surrogate endpoints, i.e., progression to SIL, CIN1, CIN2. In the second study, also CIN/SIL was included among these intermediate endpoints of progressive disease [44]. These two viral surrogates (6M+ and 12M+) are based on detection of HR-HPV infection in two and three, respectively, subsequent samples taken at > 6 and > 12 months apart during the FU. Because of the longitudinal nature of the combined NIS-LAMS cohort, the data file was transformed to a panel data suitable for analysis by a generalized estimating equation (GEE) model, clustered by women-ID (subject variable) and FU visit (within-subject variable) [70, 71]. GEE adjusts for the serial correlation within subjects by modeling the covariance structure within subjects. Because all dependent variables are binomial (presence/absence of SIL, CIN1, CIN2, CIN/SIL), the logit link function was used. The exchangeable working correlation structure with a robust variance estimator to account for within-subject correlation was selected as the best-fit covariance pattern, using the Quasi-likelihood Information Criterion (QIC) [70, 71]. Because HR-HPV persistence clearly depends on time since the previous sample, therefore, a time variable was included as a covariate in these GEE models.

In the longitudinal assessment of HR-HPV persistence, women who were HR-HPV-positive at a specific (baseline or FU) visit *(t)* were considered 1) 6M+ persistent, if their subsequent assessment (t+1) was also HR-HPV positive, and 2) 12M+ persistent, if also the next visit (t+2) sample was HR-HPV positive. However, also the exact sampling intervals were calculated for each individual woman, and the strict 6.0-month and 12.0-month cut-offs were applied to adjust the above defined 6M+ and 12M+ persistence categories at the level of individual women.

In the first of these two analyses [43], no attention was paid to the reference category used in the above calculations, any women not fulfilling the 6M+ or 12M+ persistence criteria were used as a reference. In the second analysis [44], however, we were particularly interested in assessing the impact of the optional referent groups on the strength of the association between 6M+/12M+ HR-HPV persistence and the surrogate endpoints (SIL, CIN1, CIN2, CIN/SIL). Three optional reference categories exist: 1) women who cleared their HR-HPV after 6M+ or 12M+ persistence (= HPV transient reference); 2) women who remained HR-HPV negative throughout the whole FU period (= HPV negative reference); and 3) women whose HR-HPV infection run a fluctuating (mixed) course, with HR-HPV positive test followed by temporary clearance, subsequent activation, etc. (= HPV mixed outcome reference). This last category excluded women whose HR-HPV was cleared at the last FU visit. In the GEE file, six new panels were created comparing the i) 6M+ and ii) 12M+ persistence criteria (= 1) with each of the three referent groups (= 0) as follows; 1) +/+ or +/+/+ (= 1) compared with +/- or +/-/- (= 0) at their (t+1,2,… jth) visits; 2) +/+ or +/+/+ (= 1) compared with -/-/-/- (= 0) at all their (t+1,2,… jth) visits; and 3) +/+ or +/+/+ (= 1) compared with -/+ or -/+/- or -/+/-/+ (= 0) at their (t+1,2,… jth) visits. Relative risks (RR; 95% CI) were calculated for the associations of 6M+ and 12M+ persistence with the four intermediate surrogates (SIL, CIN1, CIN2, CIN/SIL) separately using the three reference categories. All statistical tests performed were two-sided and declared significant at a p value < 0.05 level [43, 44].

HPV persistence as a surrogate of progressive disease in the combined NIS-LAMS cohort

Combined reference category

In the first analysis [43], we calculated the risk estimates (relative risk, RR) for the potential surrogates as predictors of disease progression using three intermediate outcome events: SIL, CIN1+ and CIN2+ (Table 2). A wide variety of potential endpoints were tested, including those assessed at the baseline visit, those available at the 6-month and 12-month FU-visits, as well as several of those based on persistent viral events (HR-HPV assay) and persistent clinical abnormalities (Pap smear cut-offs). In this study, no attempt was made to distinguish between the different reference categories in these calculations. HPV genotype-specific data are scanty and available from the NIS cohort only [43, 64].

Of the predictors available at baseline, testing HR-HPV positive with HC2 assay is the single most powerful risk factor for subsequent progression to SIL, CIN1+ and CIN2+, with the highest RR = 8.69 obtained for CIN2+ endpoint. This far exceeds the power of baseline ASCUS, LSIL and HSIL cytology, and these cytological endpoints are of no value in predicting CIN1+. This is in sharp contrast to these cytological endpoints assessed at the 6-month FU-visit, of which HSIL is by far the single most powerful predictor of progression to CIN2+, with RR=47.14 (95% CI 17.29-128.66), and less powerful for CIN1+ (RR = 9.67). ASCUS and LSIL are all significantly associated with the development of CIN1+ and CIN2+, with RR varying between 2.5 and 4.6. Importantly, ASCUS at the 6-month FU visit is a powerful predictor of SIL outcome, with RR = 17.98, which is among the highest of all these associations (Table 2).

The strength of all these cytological endpoints is substantially diminished when assessed at the 12-month FU-visit as compared with the 6-month visit. However, HSIL at the 12-month FU-visit is still a significant risk factor for CIN2+ progression, with RR = 21.48, which is second only to that of HSIL at the 6-month visit. In contrast to the declining power of these cytological endpoints, the strength of the virological endpoints, i.e., testing HR-HPV+ at FU visits, is markedly increasing. Indeed, testing HR-HPV+ at the 12-month visit is associated with progression to CIN2+ with RR=10.72 (95% CI 3.16-36.37), and to CIN1+ and SIL outcomes with RR > 4.0.

Of the virological endpoints of interest in the present discussion, the most powerful seems to be the 6M+ persistent HR-HPV as a predictor of incident CIN1+; RR = 18.61 (95% CI 2.53-136.50), being among the highest in this panel (Table 2). No additional benefit is obtained using the 12M+ HR-HPV persistence criteria in predicting any of the three outcomes. The limited number of cases in each strata hampers the calculations of these data for individual HPV genotypes. In Table 2, non computable (NC) denotes situations where none of the < 6M+ or < 12M+ persistors progressed to the relevant outcome event while 6M+ and 12M+ persistors did, and NCC is for situations where no cases were in either of the outcome (progressed/not progressed) categories. Albeit formally not calculable in both situations, RR in the former can be considered to reach infinity.

Analogous to the 6M+ and 12M+ persistent HR-HPV, we made similar calculations for persistent Pap abnormalities, separately for ASCUS and SIL. As shown in Table 2, 6M+ persistent SIL is a powerful predictor of disease progression to CIN1+ (RR = 13.75) and CIN2+ (RR = 8.93), being superior in this respect to 6M+ persistent ASCUS. Interestingly, this power is practically lost when the 12M+ persistence criteria are used.

Specific Reference Categories

In the second study, prepared after the appearance of the above discussed meta-analysis [35], we conducted the same analysis using three different reference groups on women with 6M+ and 12M+ HR-HPV persistence [44]. Table 3 summarises the RRs for the 6M+ and 12M+ HR-HPV persistence to predict the four surrogate endpoints of progressive

Table 2. — *Risk of developing SIL, CIN1+ and CIN2+ associated with different intermediate endpoints in the combined NIS and LAMS follow-up cohorts*.*

Intermediate endpoint	Disease progressed to:					
	SIL (n = 131)		CIN1+ (n = 90)		CIN2+ (n = 39)	
	RR	95%CI	RR	95%CI	RR	95%CI
HR-HPV at baseline	**7.03**	**3.40-14.55**	**6.41**	**2.77-14.83**	**8.69**	**2.08-36.34**
ASCUS baseline	0.83	0.56-1.23	1.53	0.99-2.36	**2.56**	**1.35-4.84**
LSIL baseline	NA	NA	1.55	0.91-2.61	**2.27**	**1.11-4.61**
HSIL baseline	NA	NA	0.89	0.21-3.73	2.17	0.50-9.32
HR-HPV at 6-mo visit[1]	**6.01**	**2.34-15.41**	0.60	0.21-1.67	1.39	0.25-7.64
ASCUS at 6-mo visit	**17.98**	**11.13-29.06**	**2.49**	**1.53-4.06**	**4.15**	**2.00-8.61**
LSIL at 6-mo visit	NA	NA	**3.42**	**2.00-5.84**	**4.62**	**2.16-9.89**
HSIL at 6-mo visit	NA	NA	**9.67**	**3.56-26.24**	**47.14**	**17.29-128.66**
HR-HPV at 12-mo visit[1]	**4.06**	**2.48-6.63**	**4.93**	**2.64-9.21**	**10.72**	**3.16-36.37**
ASCUS at 12-mo visit	**9.49**	**6.03-14.93**	**2.83**	**1.60-5.00**	**4.48**	**2.02-9.94**
LSIL at 12-mo visit	NA	NA	**3.08**	**1.38-6.86**	**5.11**	**1.85-14.09**
HSIL at 12-mo visit	NA	NA	**7.58**	**1.85-30.97**	**21.48**	**5.08-90.79**
Incident HR-HPV[2]	**12.66**	**1.98-80.91**	1.02	1.00-1.03	1.003	0.99-1.01
Incident abnormal PAP[3]	**8.08**	**5.57-11.73**	**2.37**	**1.95-2.89**	**2.05**	**1.52-2.77**
6M+ persistent PAP-ASCUS	**2.77**	**1.47-5.24**	**5.47**	**1.86-16.05**	**5.87**	**1.32-26.04**
6M+ persistent PAP-SIL	NA	NA	**13.75**	**2.97-63.51**	**8.93**	**1.04-76.72**
12M+ persistent PAP-ASCUS	1.12	0.63-2.01	**2.54**	**1.20-5.35**	2.10	0.80-5.49
12M+ persistent PAP-SIL	NA	NA	2.87	0.97-8.47	1.39	0.25-7.61
6M+ persistent HR-HPV	**2.40**	**1.23-4.70**	**18.61**	**2.53-136.50**	NC	NC
HPV16	1.67	0.52-5.31	3.67	0.39-33.84	NC	NC
HPV18	2.89	0.31-27.05	NC	NC	NCC	NCC
HPV31	2.50	0.44-14.02	NC	NC	NC	NC
HPV33	1.55	0.39-6.04	NC	NC	NCC	NCC
HPV35	NC	NC	NCC	NCC	NCC	NCC
HPV39	2.75	0.24-30.51	NCC	NCC	NCC	NCC
MULT	1.44	0.32-6.52	NC	NC	NC	NC
12M+ persistent HR-HPV	**2.17**	**1.30-3.62**	**7.02**	**3.06-16.11**	**3.00**	**1.13-7.92**
HPV16	0.83	0.23-2.97	NC	NC	NC	NC
HPV18	NC	NC	NC	NC	NCC	NCC
HPV31	0.69	0.06-7.25	NCC	NCC	NCC	NCC
HPV33	0.94	0.15-5.63	NC	NC	NCC	NCC
HPV35	NC	NC	NCC	NCC	NCC	NCC
HPV39	1.66	0.10-25.43	NCC	NCC	NCC	NCC
MULT	0.32	0.03-3.04	NC	NC	NCC	NCC
HPV16 integration status	2.24	0.58-8.59	0.70	0.13-3.57	2.16	0.22-21.25
HPV16 integration load (hi/lo)[4]	**8.93**	**1.07-74.33**	0.47	0.04-5.35	0.47	0.04-5.35

*Syrjänen *et al*. 2009 [ref. 43]
RR, relative risk; [1]Single point detection; [2]Incident infection in baseline HR-HPV- woman; [3]New PAP abnormality (ASCUS+) in baseline PAP-negative women; [4]Integration load (log) high/low, median cut-off; NA, not applicable, LSIL and HSIL are outcomes here; NC, non-computable, none of the < 6M or < 12M cases progressed; NCC, non-computable due to the lack of any cases in one category (progressed/not progressed); significant results in bold.

disease (SIL, CIN1+, CIN2+, CIN/SIL) in the GEE model, run separately for the three referent groups. A series of cytological surrogates (i.e., HSIL at 6- and 12-mo FU visit, 6M+ and 12M+ persistent SIL and ASCUS) were similarly evaluated as predictors of progressive disease, just to make a comparison with the virological surrogates.

The results presented in Table 3 are markedly different from those in Table 2. This is because the strength of the association of both 6M+ and 12M+ HR-HPV persistence with all four surrogate endpoints is critically dependent on the reference category used in these calculations. With few exceptions, the highest RRs are obtained when the HPV-negative reference category is used, irrespective of whether the surrogate endpoint is SIL, CIN2+ or CIN/SIL. As to the CIN1+ surrogate endpoint, however, RR for 12M+ persistence is the highest (RR = 10.2) when the HPV transient reference is used, and RR for 6M+ persistence is highest (RR = 21.6) using the HPV mixed-outcome reference. The difference between the three reference categories is most marked when RRs of both 6M+ and 12M+ persistence are compared in the SIL surrogate endpoint, followed by CIN2+ surrogate and CIN/SIL surrogate groups. In fact, RRs are far above 10 in all associations of 6M+ and 12M+ persistence with SIL, CIN2+ and CIN/SIL surrogates when the HPV-negative reference group is used, whereas these associations almost lose their significance in many occasions when the HPV transient- and HPV mixed-outcome referent groups are being used. With the HPV-negative reference group, the

Table 3. — *Risk estimates for 6M+ and 12M+ HR-HPV persistence as surrogate endpoints of SIL, CIN1+, CIN2+ and CIN/SIL using optional reference categories in GEE models**

Endpoint/Reference category	Intermediate Surrogate Endpoint of Progression							
	SIL (n=131)		CIN1+ (n=90)		CIN2+ (n=39)		CIN/SIL (n=204)	
	RR	95%CI	RR	95%CI	RR	95%CI	RR	95%CI
HPV negative reference:								
6M+ Persistent HR-HPV	**20.87**	**6.46-67.43**	**6.62**	**2.76-15.87**	**18.84**	**2.50-141.60**	**11.71**	**5.78-23.70**
12M+ Persistent HR-HPV	**27.59**	**8.40-90.57**	**9.55**	**3.91-23.37**	**19.27**	**2.49-149.04**	**16.42**	**7.95-33.90**
HPV transient reference:								
6M+ Persistent HR-HPV	**3.36**	**1.01-11.14**	NC	NC	NC	NC	**5.76**	**1.75-18.92**
12M+ Persistent HR-HPV	**3.78**	**1.56-9.17**	**10.18**	**2.37-43.61**	6.80	0.87-53.04	**5.31**	**2.44-11.55**
HPV mixed outcome reference:								
6M+ Persistent HR-HPV	**2.62**	**1.31-5.25**	**21.56**	**2.93-158.66**	NC	NC	**4.13**	**2.15-7.92**
12M+ Persistent HR-HPV	**2.10**	**1.21-3.66**	**5.72**	**2.40-13.60**	2.04	0.75-5.54	**2.89**	**1.77-4.70**
Other surrogate endpoints:								
HSIL Pap at 6M FU-visit	NA	NA	**9.67**	**3.56-26.24**	**47.14**	**17.29-128.70**	**9.08**	**3.63-22.69**
HSIL Pap at 12M FU-visit	NA	NA	**7.58**	**1.85-30.97**	**21.48**	**5.08-90.79**	**7.67**	**2.03-28.89**
6M+ Persistent Pap (ASCUS)	**2.77**	**1.47-5.24**	**5.47**	**1.86-16.05**	**5.87**	**1.32-26.04**	**4.03**	**2.21-7.35**
6M+ Persistent Pap (SIL)	NA	NA	**13.75**	**2.97-63.51**	**8.93**	**1.04-76.72**	**6.16**	**2.44-15.53**
12M+ persistent Pap (ASCUS)	1.12	0.63-2.01	**2.54**	**1.20-5.35**	2.10	0.80-5.49	1.44	0.85-2.43
12M+ persistent Pap (SIL)	NA	NA	2.87	0.97-8.47	1.39	0.25-7.61	1.98	0.78-5.01

*Syrjänen *et al*. 2010 [ref 44]

RR, relative risk; NA, not applicable, LSIL and HSIL are outcomes here; NC, non-computable, none of the <6M or <12M cases progressed; NCC, non-computable due to the lack of any cases in one category (progressed/not progressed); significant results in bold.

strongest single association is obtained between 12M+ persistence and SIL surrogate (RR = 27.6), followed by the 6M+ persistence and SIL (RR = 20.9), 12M+ and CIN2+ (RR = 19.3) as well as 6M+ and CIN2+ (RR = 18.8). In contrast, when the HPV transient- and HPV mixed-utcomes are used as referent groups, RR=10 is exceeded in two associations only, i.e., 12M+ with CIN1+ and 6M+ with CIN1+, respectively (Table 3).

In addition to these virological endpoints, also most of the cytological markers seem to be significantly associated with the four surrogate endpoints (Table 3). Of all cytological predictors, HSIL at the 6-mo FU visit shows the strongest association with progression to CIN2+, with RR = 47.14 (95% CI 17.29-128.70), followed by HSIL and CIN2+ at 12-mo FU visit, with RR = 21.48 (95%CI 5.08-90.79). Of the 6M+ or 12M+ persistent Pap smear abnormalities, the single most powerful association is obtained between the 6M+ persistent SIL and CIN1+ (RR=13.75, 95%CI 2.97-63.51), while most of the others are not significant predictors of CIN1+, CIN2+ or CIN/SIL.

Considerations for the future

From the studies discussed above [43, 44] as well as from the recent meta-analysis [35], several important issues arise that deserve some discussion in this context. As mentioned above, these issues are related to definition of HPV persistence, definition of progressive disease, intervals of HPV testing, reference category used in calculations, as well as the HPV detection methods. Discussion of the latter falls outside the scope of this communication, however, the reader is being referred to comprehensive HPV textbooks addressing the technical aspects of HPV detection methods [1, 2]. Needless to emphasise that there is an urgent need to validate those multiple variables affecting the risk estimates of the discussed virological endpoints [35, 44], and it is also essential to reach a standardised definition of HPV persistence, to be uniformly applied in future studies using HPV persistence as a surrogate of disease progression.

Definition of HPV persistence

As recently pointed out [35, 44], no unanimous agreement has been reached as how to define persistent HR-HPV infection. In some studies, HPV persistence is defined as two or more HPV DNA-positive tests [36, 37], while in some others HPV persistence has been assessed using the time to clearance (i.e., duration of infection) [38-40], and yet in some others, as a proportion of HPV-positive visits [41, 42]. Certainly, this is among the first issues to be agreed on because of a major cause of misclassification (persistent or not) and as such a major source of biased estimates in all studies assessing the risk conferred by HR-HPV persistence for disease progression [16, 18, 26, 35, 42-44, 45-62].

Indeed, definition and predictors (determinants) of HPV persistence [17] are among the key issues in understanding the natural history of genital HPV infections, recognised already in the early prospective follow-up studies since the 1980s [8, 72-74]. When a prospective cohort of women with a cervical HPV lesion at baseline is prospectively followed-up for a prolonged period of time, at least six different disease outcomes can be distinguished, as recently

detailed in the textbook of this author [8]. These can be defines as follows: 1) early regression, 2) persistence, 3) fluctuation, 4) late regression, 5) progression, and 6) recurrent disease. As repeatedly emphasized [8, 75-77], the definition of these disease outcomes is based on clinical assessment only, i.e., colposcopy, Pap smear and biopsy.

The issue becomes more complicated when the dynamics of viral events are being assessed in a longitudinal setting, as done more recently when robust HPV detection techniques became generally available since the late 1990s [11, 14, 15, 17]. As to these viral outcomes, the three most obvious ones are: 1) acquisition of new infection, 2) persistent infection, and 3) clearance of the infection. However, referring back to long-term prospective cohort studies [72-74], at least two other outcomes are well established: 1) persistently HPV DNA negative, and 2) fluctuation. The former is straightforward, including women who remain persistently HPV DNA-negative in repeated HPV testing (e.g., at 6-mo intervals) for the entire follow-up time [11, 14, 15, 17]. The latter is far more complex, and not well understood even today, characterised by intermittent appearance, disappearance and reappearance of HPV DNA in repeated sampling of women in a longitudinal setting. Undoubtedly, many of the women included in the HPV mixed-outcome referent category in the recent studies on HPV persistence [16, 18, 26, 35, 42-44, 45-62] represent women with this type of fluctuating HPV outcome.

While considering HPV persistence, there are actually several different issues that need to be considered. Apart from the strict definition of persistence as an event (phenomenon), we need to consider whether 1) this persistence is type-specific or non-type-specific (= any HPV type), 2) whether the analysis is restricted to HR-HPV types collectively or to individual HR-HPV genotypes [35]. These considerations are closely linked with HPV detection techniques, and certainly responsible for the major differences in the results reported in the studies included in the above discussed meta-analysis [35]. Importantly, the data are completely insufficient at this stage to draw any firm conclusions on HPV persistence at the genotype level as a surrogate of progressive disease, albeit well established when HR-HPV types are counted collectively [35, 43, 44].

At least equally important as to provide genotype-specific data, is the jointly agreed definition of HPV persistence as the viral event itself. The optimal should be to record exact times of persistence for each individual woman in such analysis, e.g., at a 1-month accuracy level. Unfortunately, this can only be done when the sampling interval is short. As compared with the current situation, much improvement could be achieved if the sampling could be done at 3-month intervals. In most settings (large-scale multi-centre trials), this is not a realistic goal, however, but the sampling intervals are necessarily longer, ranging from six months to one year or even longer. It is the conviction of this author that the misclassification (persistent/not persistent) increases rapidly in settings where the sampling intervals exceed one year. This is simply because (due to the reasons not well understood) many of the key events affecting HPV persistence (acquisition, clearance) do seem to take place within the time frame of around 12 months or even shorter [appropriate literature cited in 11, 14, 15, 17]. With the sampling interval of six months, these events can be traced with reasonable accuracy, albeit not flawless to define the exact duration of infections in all cases.

To increase the complexity, the context at which HPV persistence is detected also needs to be considered. Referring back to the six outcome patterns established in the early follow-up studies cited above [8, 72-77], it is evident that viral persistence can be associated with all of them, and not only among women classified as persistors in this overall outcome assessment, who represent a minority of the cohort after ten years of follow-up [8, 72-77]. To fully exploit the entire follow-up cohort in any such analysis for HPV persistence, all six outcome patterns need to be considered and further stratified to identify every single woman with the recorded HPV detection data fulfilling the criteria of e.g., 6-month or 12-month persistence. This applies equally well to 1) early regressors (those cleared after > 6 months of duration), to 2) persistors, and 3) fluctuators (with persistent episodes lasting > 6 months), as well as to 4) late regressors, 5) progressors, and 6) recurrent disease category, the latter being closely associated with viral persistence [78]. Because of its inherent study design, the Finnish HPV Cohort Study run between 1981-1998 did not include women who were baseline negative for clinical HPV lesions. Thus, another outcome category needs to be added in the above list; women with incident disease, which usually follows incident HPV infection with two to three months of delay [11, 15].

Because of the fact that the aim of all these efforts [35] is to assess, as reliably as possible, the real value of HR-HPV persistence as a surrogate endpoint of progressive disease, it is essential to consider only those cases which represent true viral persistence. Referring to the above listed seven patterns (incident event included), caution must be exerted while classifying women as persistors from some of these categories. By definition, HPV infection is not persistent if it is transient in outcome. Of the above seven outcome categories, viral persistence is explicit only in three of them: persistors, progressors and recurrent outcome. In all others (early regression, late regression, fluctuation, incident disease), HPV infection can be potentially transient in nature. Strictly speaking, women from these outcome patterns should not be automatically included among the 6M+ or 12M+ HPV persistence group (even if otherwise eligible), without definite confirmation of their final outcome. Because there is no means to verify this "final outcome" beyond the termination of patient monitoring, the simple most consistent approach of doing this is to assess whether HPV infection persists or is cleared at the last follow-up visit. If persistent, the woman is eligible for the HPV persistence group, otherwise she should be included in the reference group (HPV-transient reference). Because these issues are closely related to the definitions of the referent groups, they will be discussed in more detail in that specific section.

Algorithm for HPV genotype-specific persistence

To translate the data on HPV persistence accumulated from our long-term cohort studies [8, 64, 65, 72-77] to the level of individual genotypes, an algorithm has recently been created to define HPV genotype-specific persistence [79]. Because pertinent to considerations as how to produce reproducible results, this new algorithm deserves some more treatise also in this context.

In longitudinal studies with repeated HPV testing results available using any of the genotyping techniques, the necessary first step is to define the genotype-specific outcome of HPV infection for each woman, by comparing the viral status at each FU visit to the baseline HPV status. As the first step, the six distinct main outcomes must be identified: 1) always HPV negative; 2) incident HPV infection; 3) genotype-specific persistence; 4) non-genotype-specific persistence; 5) fluctuation, and 6) virus clearance. Of these 1) and 2) are straightforward. 3) Genotype-specific persistence denotes for any case with two (or more) consecutive FU-samples positive for the same individual genotype (or genotypes in multiple-type infections). 4) Non-genotype specific persistence includes all cases with two (or more) consecutive samples being HPV positive, but not for the same individual genotype. 5) Fluctuation is a pattern where consecutive samples are intermittently HPV+ and HPV-, without any two consecutive samples positive for the same or different viral genotype. 6) In this primary categorization, virus clearance should include only the baseline HPV+ cases who have cleared their infection by or at the last FU visit.

As explained above, HPV infections in individual women can fall into more than one outcome category, and a secondary classification is necessary to maximally exploit the data of all outcomes. Accordingly, the patterns 2, 3, 4 and 5 listed above can be further stratified to the two persistent-outcome categories (type-specific and not type-specific) as follows. Among category 2 (incident infections), we can pick up all cases demonstrating genotype- and non-genotype-specific persistence of this incident infection. Similarly, the original categories 3 and 4 can be further stratified to genotype-specific and non-genotype specific persistence, whenever different or additional to these same categories in the first-line assessment. By definition, category 5 (fluctuation) does not include any viral persistence, but, importantly, the cases testing HPV-negative at the last FU visit should be classified as clearance, to indicate that these infections were transient in nature. The same might apply to category 2 as well, if the incident infection is cleared without persistence. Of the last category 6), the cases fulfilling the criteria of type-specific or non-type specific persistence to be included as such in this secondary classification can also be identified. As discussed later, in calculations comparing HPV persistence using the HPV transient outcome reference group, these women should be included in the latter [44].

As the final step, persistors from the first-line and second-line classifications must be combined to create a new variable (e.g., combined persistence), including both type-specific and non-type-specific persistors. Having completed this, the individual genotypes responsible for either type-specific or non-type-specific persistence at each FU visit are identified, called persisting genotype. Because of the multitude of individual genotypes and their combinations showing persistence, the last step would be the conversion of individual genotypes to HPV species, following the newly described phylogenetic classification of papillomaviruses [80]. By doing that, it can also be easily assessed whether or not the species that persists is the same as detected at the first HPV-positive visit (baseline species), which enables computing the species-specific persistence [79].

Intermediate endpoints of disease progression

The other variable with significant impact on the risk estimates for HPV persistence as a predictor of progressive disease is the intermediate (surrogate) endpoint used to define the progressive disease [35, 43, 44]. In most of the published studies, only CIN2/3 or CC has been used as an endpoint [26, 35, 45-50], whereas the lower grade endpoints (ASCUS, CIN1/LSIL) have been used more rarely [26, 45, 46]. One of the messages of the meta-analysis was that the strength of association between HPV persistence and cervical neoplasia seems to increase with the increasing grade of the endpoint [35].

This was confirmed only in part by our recent studies where several surrogate endpoints of progressive disease were tested, including both histological and cytological as well as their combination [43, 44]. As evident from Table 3, RRs are higher for the CIN2+ endpoint (RR = > 18) than for the CIN1+ endpoint (RR = 6.6-9.6), but this is only true with the HPV negative referent group, and even then, RRs for the SIL endpoint seem to be slightly higher (RR = > 20). The SIL endpoint in our study also includes all HSIL cases, however, contributing to the increased RR for this surrogate endpoint [44]. On the other hand, RRs with the combined CIN/SIL (RR = 11.71-16.42) endpoint are slightly downgraded by the inclusion of all CIN1 and LSIL cases among this surrogate [44].

As to the effect of the surrogate endpoints in referent groups other than the HPV negative group, there was no consistent grade-related trend (Table 3). The highest single RRs were obtained for the CIN1+ endpoint, but because of no events, RRs were not computable for CIN2+ (6M+ persistence). Noteworthy is the fact that both 6M+ and 12M+ viral persistence was significantly associated also with SIL and CIN/SIL endpoints, when either HPV transient or HPV mixed outcome referent groups were used. In these settings, RRs fall between 2 and 5.5, which seems to be in good agreement with the RRs (far below 10) in comparable studies included in the meta-analysis of Koshiol *et al*. [35].

HR-HPV cofactors for incident CIN1, CIN2 and CIN3 are different

One of the reasons for this obvious difference in the strength of association with HR-HPV persistence between the different surrogate endpoints (SIL, CIN1, CIN2, CIN3) might be true differences in their biological potential as surrogates of progressive disease [63]. Indeed, a novel approach to gain further insights in the genuine biological differences between CIN1, CIN2 and CIN3 is to assess whether the cofactors needed to promote the HR-HPV-driven disease progression differ at different stages of CIN. In other words, it is of interest to know if the HPV cofactors needed for progression from normal epithelium to i) CIN1 are different from those required for progression to ii) CIN2 and further iii) to CIN3, and whether any of these eventual differences in cofactors are related to the individual HPV genotypes [81-83]. We recently completed the first analysis of these cofactors in a prospective setting, using multinomial (polytomous) regression analysis for HR-HPV cofactors in increasing the risk of incident CIN1, CIN2 and CIN3 [63].

In the combined NIS-LAMS cohort of 1,865 women, 90 (4.8%), 39 (2.1%) and 14 (1.4%) cases progressed to CIN1, CIN2, and CIN3, respectively [63]. Baseline HR-HPV was the single most powerful predictor of incident CIN1, CIN2 and CIN3. When controlled for residual HPV confounding by analysing HR-HPV positive women only (n = 1,105), the risk profiles of incident CIN1, CIN2 and CIN3 were unique, i.e., completely different HPV cofactors were associated with progression to CIN1, CIN2 and CIN3 in univariate and multivariate analysis, irrespective of whether non-progression, CIN1 or CIN2 was used as the reference outcome. This study using polytomous logistic regression models in a prospective setting where residual confounding by HR-HPV was controlled, unequivocally demonstrates that different HPV cofactors are associated with incident CIN1, CIN2 and CIN3. These data substantiate the concept that each CIN grade represents a distinct biological entity, as also suggested by the extensive natural history data [8, 72-77]. This should have important implications in at least two fields: 1) lumping together of CIN2 and CIN3 in the histological classification of cervical cancer precursors should be revisited, and 2) one should reconsider using the combined CIN2/CIN3 endpoint in any studies assessing the risk factors of CIN/CC. The next urgent step is to assess whether these different cofactor profiles are linked with individual HR-HPV genotypes in this prospective cohort.

Extent of the follow-up time

Given the rarity of the incident CIN2+ and CIN3+, reliable incidence rates cannot be expected in studies where the follow-up times are too short, particularly if less than two years. This is well illustrated by our historical Kuopio cohort, where > 500 women with clinical HPV infection at baseline were followed-up for almost 20 years (1981-1998). Results from this study have been presented in several communications [72-77], and discussed in detail in our textbook [8]. Some of the lessons learned from this classical study deserve to be addressed here as well, because they are pertinent to the present discussion.

Soon after the onset of the Kuopio cohort in 1981, it became apparent that cervical HPV lesions in individual patients can adopt at least four outcomes: regression, persistence, progression, and recurrence [8]. It soon became evident also that the proportion of women classifiable into these four categories is clearly time-dependent and subject to continuous variation over time. During this long-term follow-up, two important patterns in the natural history of cervical HPV lesions emerged: 1) trends in regression, and 2) those in disease progression. Importantly, the effect of the follow-up time on these two outcomes is entirely different [8].

During the follow-up, it was observed that the rate of spontaneous regression increased in parallel with the follow-up time, from 24.8% at 25 months to 39.7% at 45 months, further to 54% at 57 months, and up to 63.8% after 83 months. Thereafter, the proportion of lesions undergoing regression increased very slowly, to 66.4% at 96 months, and reaching the 68.9% plateau after 123 months (i.e., 10 years) of the mean observation period. This is important while interpreting the different cohort studies, the majority of which have been run for a relatively short time only [84]. Importantly, a very long follow-up is needed to reach the plateau in the spontaneous regression rates, which approaches 70% in ten years [8, 72-77].

Even more important than spontaneous regression is, however, progression of HPV lesions to high-grade CIN. The Kuopio cohort was the first to demonstrate that disease progression follows a temporal pattern completely different from that of regression. In contrast to increasing regression rates over time, no such increase could be observed in progression rates [8, 72-77]. The hard fact seems to be that the progression rate levels off at around 14%-15% after the first 24 months of follow-up, and remains unchanged through the entire 10-year observation period [8]. In practical terms, this means that HPV lesions predestined to progression do so quite rapidly, almost invariably during the first two years of follow-up. It is important to remember, however, that all women in the Kuopio cohort were HPV-positive at baseline (i.e., clinical disease on Pap smear, colposcopy and biopsy), which certainly contributes to this relatively short time to progression. Certainly, progression to CIN among baseline HPV-negative women is 1) much more rare, and 2) takes a considerably longer time. Recent evidence also indicates that there are differences in progression times and progression rates between individual HR-HPV genotypes [28-34], and the same applies to the times of type-specific persistence, which seems to be subject to considerable variation as well [79, 85].

The most important implication of these historical data has been that practically all major cohort studies conducted for baseline HPV-positive women have started relying on this two-year observation period, during which practically all

progressing cases can be detected. The same is true with the NIS and LAMS cohorts as well. In practical terms, ten years are needed to detect all cases that will eventually regress, but only two years to disclose the progression of events among these baseline HPV-positive women.

Reference groups used in calculating the risk estimates

In the above, reference was already made to those several issues that remain to be clarified, before reproducible risk estimates can be provided for the association between persistent HPV infection and progression to CIN [35, 43, 44]. Most of these have been addressed in the preceding sections of this communication. The last but not least of these open issues is the way how the risk estimates are calculated for the association of HR-HPV persistence and incident CIN [35, 44]. In the above-mentioned meta-analysis [35] as well as in our recent approach [44], it became apparent that one of the variables with the highest impact on these risk estimates is the type of the reference category used in these calculations. In calculating the risk estimates for persistent HR-HPV to predict incident CIN endpoints, three different reference categories can be used: 1) HPV negative women; 2) women with transient HR-HPV; and 3) HPV mixed-outcome group [35, 44] to be discussed in brief next.

HPV mixed outcome reference group

As the name implies, this is the most heterogeneous of these three reference groups, consisting of women in whom HPV infections can run a highly divergent course. Due to this reason, also the risk estimates for 6M+ and 12M+ persistence obtained using this reference group are low compared with those calculated using the HPV-negative reference category, but not markedly different from those obtained with the HPV transient outcome category [35, 44].

As discussed in context with our classical cohort [8, 72-77], at least six different outcome patterns can be recognised for HPV infections during long-term follow-up. When always negative women and those showing transient infections (= virus clearance) are excluded, this leaves all the other outcomes eligible for this mixed outcome reference category. In a recent approach [44], included in this category were all women who did not meet the criteria of HPV negative- or HPV transient groups. Most notably, this group includes women whose HPV infections demonstrated a fluctuating course, with HPV-positive test(s) followed by negative test(s) and subsequently by another positive test, or alternatively -/+/-. Importantly, these women i) did not demonstrate 6M+ or 12M+ persistence, and ii) they did not have their HR-HPV infection cleared at the last visit, which make them distinct from the HPV transient group. Even today, this fluctuating course is still poorly understood [8], and it remains a potential source of several types of bias in these assessments. Indeed, we cannot exclude the possibility that the last-visit HPV+ sample represents the onset of persistence, which, however, remains undetected because the follow-up is terminated at this visit. In the same way, if a long HPV-negative phase extends over 2 to several visits in this process of fluctuation, there is a possibility of misclassifying these women as HPV-negative or even HPV transient, if this period overlaps the last follow-up visit.

On the other hand, some of the women with short-term fluctuation might eventually prove to be those with persistent infection in long-term (several years) follow-up [8]. In our analysis, this is indirectly supported by the fact that these women are at relatively high risk for incident CIN2+, as shown by the non-significant RR (2.04, 95% CI 0.75-5.54) for 12M+ persistence using this reference category (Table 3). On the other hand, the risk of developing CIN1+ seems low because 6M+ persistence, RR = 21.56 (95% CI 2.93-158.7), is higher than obtained in comparison with the HPV-negative referent group. The risk estimates for SIL and CIN/SIL endpoints are low (but significant), implicating that the risk of developing these endpoints is somewhat lower than among 6M+ and 12M+ HPV persistent women, but still substantial [44].

HPV transient reference group

In the discussed meta-analysis, studies where women with persistent HPV infections were compared to those with transient HPV infections usually reported the lowest RRs [35]. This was our experience as well [44]. Again, we need to pay some attention to the approach used to define these transient infections. In some studies, all infections that eventually regress during the follow-up are transient, whereas in some others, infections that cleared within less than six months (6M-) or 12 months (12M-) were defined as transient. In the former, such transient infections potentially estimate the effect of HPV persistence beyond short-term infections, whereas in the latter, they clearly denote short-term viral exposure. In our study [44], transient was defined as any HR-HPV infection that cleared during the prospective follow-up, i.e., tested HPV-negative at the last visit, irrespective the duration of infection. In so doing, we wanted to distinguish this group of transient infections from those women who have persistent (6M+ or 12M+) infection that did not clear during the follow-up. Using this strict definition (cleared/not cleared), we obtain RRs for 12M+ persistence as high as 10.18, whereas the RRs for 6M+ were either not computable (for CIN1+ and CIN2+ surrogate) or fall around 3 (for SIL) and 5 (for CIN/SIL) (Table 3).

Being substantially lower (but still statistically significant) than RRs obtained in comparison with the HPV negative reference group, these risk estimates in the persistent-transient comparison indicate that women in the latter represent a heterogeneous group, where: i) the risk of disease progression is far from zero in some women, but ii) very low in

some others. The former would include those who had their transient infection persisting but cleared at the end, whereas the latter represent-women with true short-term transient infections. Furthermore, the possibility cannot be completely excluded that some of the women classified as transient HPV infections actually had persistent infections prior to the baseline HPV testing, and as such were actually misclassified. The only way to control for that is to include only baseline HPV-negative women, which would significantly reduce the size of this reference group, however [44].

HPV negative reference group

As pointed out, of all influential variables, the reference group has the most dramatic impact on the risk estimates for the association between 6M+ and 12M+ viral persistence and disease progression [35, 44]. This was clearly shown in the analysis as well, where RRs were of different magnitude (RR = 10-27) as compared with the others (RR = 2-5), when the HPV negative referent group was used in calculating these estimates (Table 3). This applies to all other surrogates except CIN1+, in which the highest RRs were obtained using the two other reference categories. These data are fully consistent with the results of the studies included in the recent meta-analysis [35], where the use of HPV negative women as the reference group resulted in the highest RRs. This is feasibly explained by the fact that the risk of incident CIN2-3/HSIL+ approaches zero among HPV negative women [26, 35, 45-50, 85]. This seems to apply equally well to SIL and CIN/SIL endpoints [85], as shown by RRs up to 27 when women with 6M+/12M+ persistent HR-HPV are compared with HPV negative women in the NIS-LAMS cohort (Table 3) [44].

Conclusions

The present communication addressed the issues to be considered before adoption of viral endpoints (6M+ and/or 12M+ HR-HPV persistence) instead of the conventional histological endpoint (CIN2+) as new surrogates of progressive disease, e.g., in future efficacy trials with the new generation prophylactic non-HPV16/18 vaccines [19, 31, 32]. The data reviewed in this discussion implicate that persistent HR-HPV infections (6M+ and 12M+) are powerful predictors consistently associated with progressive cervical disease defined by surrogate intermediate endpoints SIL, CIN1+, CIN2+ and CIN/SIL. However, there seems to be substantial variation in the risk estimates of these associations, which seem to depend on several variables, as discussed in more detail above.

One of the questions is, whether the 6M+ or 12M+ HR-HPV persistence endpoint should be selected, i.e., are there major differences in the risk estimates between these two? Indeed, there seems to be some variation in these risk estimates, depending of the length of HR-HPV persistence [43, 44]. In our study, RRs calculated for 12M+ persistence were higher than those obtained for 6M+ persistence criteria, with few exceptions. This difference seems to persist, irrespective of which of the three reference categories was used. The only major exception was the substantially higher risk (RR = 21.6) for 6M+ persistence to associate with CIN1+ as compared with that (RR = 5.7) of 12M+ persistence, when HPV mixed outcome was used as the reference group [44]. Importantly, there were no exceptions to this rule when the data were calculated using the HPV negative reference category, where 12M+ persistence invariably gave higher RRs (Table 3). These observations are in full agreement with the data reported in the meta-analysis, where the associations between HPV persistence and CIN/CC appeared stronger in studies with longer duration of HPV infection (12M+) [35]. To this author, this observation sounds the only logical one considering the basic biology of HPV infections [8, 12, 16, 17], indicating that a longer duration of HR-HPV infections means longer exposure to viral oncogenes and increases the likelihood of developing progressive disease [85].

Taken together, it is suggested that in all future studies (whether vaccine efficacy trials or screening trials), using the 6M+ or 12M+ HR-HPV persistence as a surrogate endpoint of progressive disease, a “gold standard” should be used in calculating the risk estimates for this association. In addition to deciding, 1) whether to use 6M+ or 12M+ persistence criteria, and 2) cytological, histological or combined surrogate endpoints (SIL, CIN1, CIN2, CIN/SIL), one should 3) start using exclusively the HPV negative reference group in calculating the risk estimates for viral persistence endpoints. This is supported by the data from the recent meta-analysis [35] as well as from the author’s combined NIS-LAMS cohort [43, 44], both implicating that the most consistent association to progressive disease is obtained when women with persistent HR-HPV are compared with HPV-negative women. It is the conviction of this author that the two other reference categories (HPV transient and HPV mixed outcome) are far too heterogeneous and subject to potential misclassifications to give consistent and reproducible risk estimates for HR-HPV persistence as surrogate endpoint of progressive CIN.

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References

[1] Syrjänen K, Gissmann L, Koss LG. (eds.): "Papillomaviruses and Human Disease". Heidelberg, Springer Verlag, 1987, 1.
[2] Syrjänen K., Syrjänen S.: "Papillomavirus Infections in Human Pathology". New York, J. Wiley & Sons, 2000, 1.
[3] zur Hausen H.: "Papillomaviruses in the causation of human cancers- a brief historical account". *Virology,* 2009, *384*, 260.
[4] Syrjänen S., Syrjänen K.: "The history of papillomavirus research". *Cent. Eur. J. Public Health,* 2008, *16* (suppl.), S7.
[5] Burk R.D., Kelly P., Feldman J., Bromberg J., Vermund S.H., Dehovitz J.A. *et al.:* "Declining prevalence of cervicovaginal human papillomavirus infection with age is independent of other risk factors". *Sex Transm Dis.,* 1996, *23,* 333.
[6] Kjaer S.K., van den Brule A.J., Bock J.E., Poll P.A., Engholm G., Sherman M.E. *et al.:* "Determinants for genital human papillomavirus (HPV) infection in 1000 randomly chosen young Danish women with normal Pap smear: are there different risk profiles for oncogenic and nononcogenic HPV types?". *Cancer Epidemiol Biomarkers Prev.,* 1997, *6,* 799.
[7] Melkert P.W., Hopman E., van den Brule A.J., Risse E.K., van Diest P.J., Bleker O.P. *et al.::* "Prevalence of HPV in cytomorphologically normal cervical smears, as determined by the polymerase chain reaction, is age-dependent". *Int. J. Cancer,* 1993, *53*, 919.
[8] Syrjänen K.: "Natural history of cervical HPV infections and CIN". Chapter 6. In: Syrjänen K. and Syrjänen S. "Papillomavirus Infections in Human Pathology". New York, J. Wiley & Sons, 2000, 142.
[9] Herrero R., Hildesheim A., Bratti C., Sherman M.E., Hutchinson M., Morales J. *et al.:* "Population-based study of human papillomavirus infection and cervical neoplasia in rural Costa Rica". *J. Natl. Cancer Inst.,* 2000, *92,* 464.
[10] Winer R.L., Lee S.K., Hughes J.P., Adam D.E., Kiviat N.B., Koutsky L.A.: "Genital human papillomavirus infection: incidence and risk factors in a cohort of female university students". *Am. J. Epidemiol*., 2003, *157*, 218.
[11] Syrjänen S., Shabalova I.P., Petrovichev N., Kozachenko V.P., Zakharova T., Pajanidi A., Podistov J.I., Chemeris G., Sozaeva L.G. *et al.*: "Acquisition of incident high-risk human papillomavirus (HPV) DNA and PAP smear abnormalities in a cohort of women subjected to HPV screening in the New Independent States (NIS) of the Former Soviet Union". *J. Clin. Microbiol.,* 2004, *42,* 505.
[12] Brummer O., Hollwitz B., Bohmer G., Kuhnle H., Petry K.U.: "Human papillomavirus-type persistence patterns predict the clinical outcome of cervical intraepithelial neoplasia". *Gynecol. Oncol*., 2006, *102*, 517.
[13] Hildesheim A., Schiffman M.H., Gravitt P.E., Glass A.G., Greer C.E., Zhang T. *et al.:* "Persistence of type-specific human papillomavirus infection among cytologically normal women". *J. Infect. Dis.,* 1994, *169,* 235.
[14] Syrjänen S., Shabalova I.P., Petrovichev N., Kozachenko V.P., Zakharova T., Pajanidi A. *et al.*: "Clearance of high-risk human papillomavirus (HPV) DNA and PAP smear abnormalities in a cohort of women subjected to HPV screening in the New Independent States (NIS) of the Former Soviet Union". *Eur. J. Gynecol. Obstet. Reprod. Biol*., 2005, *119,* 219.
[15] Syrjänen S., Shabalova I.P., Petrovichev N., Kozachenko V.P., Zakharova T., Pajanidi A. *et al.*: "Age-specific incidence and clearance rates of high-risk Human Papillomavirus (HPV) infections in women subjected to HPV screening in the new independent states". *Int. J. STD & AIDS,* 2005, *16,* 217.
[16] Dalstein V., Riethmuller D., Pretet J.L., Le Bail C.K., Sautiere J.L., Carbillet J.P. *et al.:* "Persistence and load of high-risk HPV are predictors for development of high-grade cervical lesions: a longitudinal French cohort study". *Int. J. Cancer,* 2003, *106,* 396
[17] Syrjänen S., Shabalova I.P., Petrovichev N., Kozachenko V.P., Zakharova T., Pajanidi A. *et al.*: "Factors predicting persistence of high-risk human papillomavirus (HPV) infections in women prospectively followed-up in three New Independent States (NIS) of the Former Soviet Union". *Eur. J. Gynaecol. Oncol.,* 2005, *26,* 491.
[18] Schiffman M., Herrero R., DeSalle R., Hildesheim A., Wacholder S., Cecilia Rodriguez A. *et al.:* "The carcinogenicity of human papillomavirus types reflects viral evolution". *Virology,* 2005, *337,* 76.
[19] Schiffman M., Castle P.E., Jeronimo J., Rodriguez A.C., Wacholder S.: "Human papillomavirus and cervical cancer". *Lancet,* 2007, *370,* 890.
[20] Wang S.S., Zuna R.E., Wentzensen N., Dunn S.T., Sherman M.E., Gold M.A. *et al.:* "Human papillomavirus cofactors by disease progression and human papillomavirus types in the study to understand cervical cancer early endpoints and determinants". *Cancer Epidemiol. Biomarkers Prev*., 2009, *18*, 113.
[21] Belinson S., Smith J.S., Myers E., Olshan A., Belinson J., Pretorius R. *et al.:* "Descriptive evidence that risk profiles for cervical intraepithelial neoplasia 1, 2, and 3 are unique". *Cancer Epidemiol. Biomarkers Prev.,* 2008, *17,* 2350.
[22] Castle P.E., Schiffman M., Herrero R., Hildesheim A., Rodriguez A.C., Bratti M.C. *et al.:* "A prospective study of age trends in cervical human papillomavirus acquisition and persistence in Guanacaste, Costa Rica". *J. Infect. Dis*., 2005, *191,* 1808.
[23] Syrjänen K.J., Kulmala S.-M., Shabalova I.P., Petrovichev N., Kozachenko V.P., Zakharova T. *et al.*: "Epidemiological, clinical and viral determinants of the increased prevalence of high-risk human papillomavirus (HPV) infections in elderly women". *Eur. J. Gynecol. Oncol*., 2008, *29*, 114.
[24] Harper D.M., Franco E.L., Wheeler C.M., Moscicki A.B., Romanowski B., Roteli-Martins C.M. *et al.:* "Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial". *Lancet,* 2006, *367*, 1247.
[25] Plummer M., Schiffman M., Castle P.E., Maucort-Boulch D., Wheeler C.M.: "A 2-year prospective study of human papillomavirus persistence among women with a cytological diagnosis of atypical squamous cells of undetermined significance or low-grade squamous intraepithelial lesion". *J. Infect. Dis.,* 2007, *195,* 1582.
[26] Kjaer S.K., van den Brule A.J., Paull G., Svare E.I., Sherman M.E., Thomsen B.L. *et al.:* "Type specific persistence of high risk human papillomavirus (HPV) as indicator of high grade cervical squamous intraepithelial lesions in young women: population based prospective follow up study". *BMJ,* 2002, *325*, 572.
[27] Kjaer S., Hogdall E., Frederiksen K., Munk C., Van den B.A., Svare E. *et al.:* "The absolute risk of cervical abnormalities in high-risk human papillomavirus-positive, cytologically normal women over a 10-year period". *Cancer Res.,* 2006, *66,* 10630.
[28] Khan MJ, Castle PE, Lorincz AT, Wacholder S, Sherman M, Scott DR *et al.:* "The elevated 10-year risk of cervical precancer and cancer in women with human papillomavirus (HPV) type 16 or 18 and the possible utility of type-specific HPV testing in clinical practice". *J. Natl. Cancer Inst.,* 2005, *97,* 1072.
[29] Winer R.L., Kiviat N.B., Hughes J.P., Adam D.E., Lee S.K., Kuypers J.M. *et al.:* "Development and duration of human papillomavirus lesions, after initial infection". *J. Infect. Dis.,* 2005, *191,* 731.
[30] Castle P.E., Solomon D., Schiffman M., Wheeler C.M.: "Human papillomavirus type 16 infections and 2-year absolute risk of cervical precancer in women with equivocal or mild cytologic abnormalities". *J. Natl. Cancer Inst*., 2005, *97*, 1066.
[31] Harper D.M., Franco E.L., Wheeler C. *et al.:* "Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial". *Lancet*, 2004, *364*, 1757.
[32] Mao C., Koutsky L.A., Ault K.A. *et al.:* "Efficacy of human papillomavirus-16 vaccine to prevent cervical intraepithelial neoplasia: a randomized controlled trial". *Obstet. Gynecol*., 2006, *107*, 18.
[33] Kulasingam S.L., Hughes J.P., Kiviat N.B. *et al.:* "Evaluation of human papillomavirus testing in primary screening for cervical abnormalities: comparison of sensitivity, specificity, and frequency of referral". *JAMA*, 2002, *288*, 1749.

[34] Perrons C., Brink N., Jalal H. *et al*.: "The impact of high risk human papillomavirus testing in an inner London colposcopy clinic". *J. Med. Virol*., 2005, *76*, 576.
[35] Koshiol J., Lindsay L., Pimenta J.M., Poole C., Jenkins D., Smith J.S.: "Persistent human papillomavirus infection and cervical neoplasia: a systematic review and meta-analysis". *Am. J. Epidemiol*., 2008, *168*, 123.
[36] Schiffman M., Kjaer S.K.: "Chapter 2: Natural history of anogenital human papillomavirus infection and neoplasia". *J. Natl. Cancer Inst. Monogr*., 2003, *31*, 14.
[37] Woodman C.B., Collins S.: "A critique of cohort studies examining the role of human papillomavirus infection in cervical neoplasia". *BJOG*, 2002, *109*, 1311.
[38] Giuliano A.R., Harris R., Sedjo R.L., Baldwin S., Roe D., Papenfuss M.R. *et al*.: "Incidence, prevalence, and clearance of type-specific human papillomavirus infections: The Young Women's Health Study". *J. Infect. Dis*., 2002, *186*, 462.
[39] Molano M., Van den Brule A., Plummer M., Weiderpass E., Posso H., Arslan A. *et al*.: "Determinants of clearance of human papillomavirus infections in Colombian women with normal cytology: a population-based, 5-year follow-up study". *Am. J. Epidemiol*., 2003, *158*, 486.
[40] Moscicki A.B., Ellenberg J.H., Crowley-Nowick P., Darragh T.M., Xu J., Fahrat S.: "Risk of high-grade squamous intraepithelial lesion in HIV-infected adolescents". *J. Infect. Dis*., 2004, *190*, 1413.
[41] Ahdieh L., Klein R.S., Burk R., Cu-Uvin S., Schuman P., Duerr A. *et al*.: "Prevalence, incidence, and type-specific persistence of human papillomavirus in human immunodeficiency virus (HIV)-positive and HIV-negative women". *J. Infect. Dis*., 2001, *184*, 682.
[42] Paraskevaidis E., Kaponis A., Malamou-Mitsi V., Davidson E.J., Hirsch P.M., Koliopoulos G. *et al*.: "The natural history of HPV infection of the uterine cervix. Long-term observational and histological data". *Anticancer Res*., 2002, *22*, 1177.
[43] Syrjänen K., Shabalova I., Naud P., Kozachenko V., Derchain S., Zakharchenko S., Roteli-Martins C. *et al*.: "Persistent high-risk human papillomavirus (HPV) infections and other endpoint markers of progressive cervical disease among women prospectively followed-up in the NIS and LAMS cohorts". *Int. J. Gynecol. Cancer*, 2009, *19*, 934.
[44] Syrjänen K., Shabalova I., Naud P., Sarian L., Derchain S., Kozachenko V. *et al*.: "Risk estimates for persistent high-risk human papillomavirus (HPV) infections as surrogate endpoints of progressive cervical disease critically depend on reference category. Analysis of the combined prospective cohort of the NIS and LAMS studies". *Int. J. STD & AIDS*, 2010, in press.
[45] Liaw K.L., Glass A.G., Manos M.M., Greer C.E., Scott D.R., Sherman M. *et al*.: "Detection of human papillomavirus DNA in cytologically normal women and subsequent cervical squamous intraepithelial lesions". *J. Natl. Cancer Inst*., 1999, *91*, 954.
[46] Cuzick J., Szarewski A., Cubie H., Hulman G., Kitchener H., Luesley D. *et al*.: "Management of women who test positive for high-risk types of human papillomavirus: the HART study". *Lancet*, 2003, *362*, 1871.
[47] Bais A.G., Rebolj M., Snijders P.J., de Schipper F.A., van der Meulen D.A., Verheijen R.H. *et al*.: "Triage using HPV testing in persistent borderline and mildly dyskaryotic smears: proposal for new guidelines". *Int. J. Cancer*, 2005, *116*, 122.
[48] Moberg M., Gustavsson I., Gyllensten U.: "Type-specific associations of human papillomavirus load with risk of developing cervical carcinoma in situ". *Int. J. Cancer*, 2004, *112*, 854.
[49] Schlecht N.F., Kulaga S., Robitaille J., Ferreira S., Santos M., Miyamura R.A. *et al*.: "Persistent human papillomavirus infection as a predictor of cervical intraepithelial neoplasia". *JAMA*, 2001, *286*, 3106.
[50] Wallin K.L., Wiklund F., Angstrom T., Bergman F., Stendahl U., Wadell G. *et al*.: "Type-specific persistence of human papillomavirus DNA before the development of invasive cervical cancer". *N. Engl. J. Med*., 1999, *341*, 1633.
[51] Koutsky L.A., Holmes K.K., Critchlow C.W., Stevens C.E., Paavonen J., Beckmann A.M. *et al*.: "A cohort study of the risk of cervical intraepithelial neoplasia grade 2 or 3 in relation to papillomavirus infection". *N. Engl. J. Med*., 1992, *327*, 1272.
[52] Dannecker C., Siebert U., Thaler C.J., Kiermeir D., Hepp H., Hillemanns P.: "Primary cervical cancer screening by self-sampling of human papillomavirus DNA in internal medicine outpatient clinics". *Ann. Oncol*., 2004, *15*, 863.
[53] Elfgren K., Kalantari M., Moberger B., Hagmar B., Dillner J.: "A population-based five-year follow-up study of cervical human papillomavirus infection". *Am. J. Obstet. Gynecol*., 2000, *183*, 561.
[54] Ylitalo N., Josefsson A., Melbye M., Sörensen P., Frisch M., Andersen P.K. *et al*.: "A prospective study showing long-term infection with human papillomavirus 16 before the development of cervical carcinoma in situ". *Cancer Res*., 2000, *60*, 6027.
[55] Cuschieri K.S., Cubie H.A., Whitley M.W., Gilkison G., Arends M.J, Graham C. *et al*.: "Persistent high risk HPV infection associated with development of cervical neoplasia in a prospective population study". *J. Clin. Pathol*., 2005, *58*, 946.
[56] Bory J.P., Cucherousset J., Lorenzato M., Gabriel R., Quereux C., Birembaut P. *et al*.: "Recurrent human papillomavirus infection detected with the hybrid capture II assay selects women with normal cervical smears at risk for developing high grade cervical lesions: a longitudinal study of 3,091 women". *Int. J. Cancer*, 2002, *102*, 519.
[57] Harris T.G., Kulasingam S.L., Kiviat N.B., Mao C., Agoff S.N., Feng Q. *et al*.: "Cigarette smoking, oncogenic human papillomavirus, Ki-67 antigen, and cervical intraepithelial neoplasia". *Am. J. Epidemiol*., 2004, *159*, 834.
[58] ter Harmsel B., Smedts F., Kuijpers J., van Muyden R., Oosterhuis W., Quint W.: "Relationship between human papillomavirus type 16 in the cervix and intraepithelial neoplasia". *Obstet. Gynecol*., 1999, *93*, 46.
[59] Moscicki A.B., Shiboski S., Broering J., Powell K., Clayton L., Jay N. *et al*.: "The natural history of human papillomavirus infection as measured by repeated DNA testing in adolescent and young women". *J. Pediatr*., 1998, *132*, 277.
[60] Saito J., Sumiyoshi M., Nakatani H., Ikeda M., Hoshiar H., Noda K.: "Dysplasia and HPV infection initially detected by DNA analysis in cytomorphologically normal cervical smears". *Int. J. Gynaecol. Obstet*., 1995, *51*, 43.
[61] Peto J., Gilham C., Deacon J., Taylor C., Evans C., Binns W. *et al*.: "Cervical HPV infection and neoplasia in a large population-based prospective study: the Manchester cohort". *Br. J. Cancer*, 2004, *91*, 942.
[62] Beskow A.H., Josefsson A.M., Gyllensten U.B.: "HLA class II alleles associated with infection by HPV16 in cervical cancer in situ". *Int. J. Cancer*, 2001, *93*, 817.
[63] Syrjänen K., Shabalova I., Naud P., Sarian L., Derchain S., Kozachenko V. *et al*.: "Cofactors of high-risk human papillomavirus (HPV) infections display unique profiles in incident CIN1, CIN2 and CIN3. Analysis of the combined prospective cohort of the NIS* and LAMS studies". *Int. J. STD & AIDS 2010*, in press.
[64] Syrjänen S., Shabalova I.P., Petrovichev N., Kozachenko V.P., Zakharova T., Pajanidi A. *et al*.: "Human Papillomavirus testing and conventional PAP smear cytology as optional screening tools of women at different risk for cervical cancer in countries of former Soviet Union". *J. Lower Genital Tract. Dis*., 2002, *6*, 97.
[65] Syrjänen K., Naud P., Derchain S.M., Roteli-Martins C., Longatto-Filho A., Tatti S. *et al*.: "Comparing PAP smear cytology, aided visual inspection, screening colposcopy, cervicography and HPV testing as optional screening tools in Latin America. Study design and baseline data of the LAMS Study". *Anticancer Res*., 2005, *25*, 3469.
[66] Longatto-Filho A., Maeda M.Y.S., Eržen M., Branca M., Roteli-Martins C., Naud P. *et al*.: "Conventional PAP smear and liquid-based cytology as screening tools in low-resource settings of Latin America. Experience of the Latin American Screening Study". *Acta Cytol*., 2005, *49*, 500.

[67] Sarian L., Derchain S.M., Naud P., Roteli-Martins C., Longatto-Filho A., Tatti S. *et al*.: "Evaluation of visual inspection with acetic acid (VIA), Lugol's iodine (VILI) cervical cytology and HPV testing as cervical screening tools in Latin America. This report refers to partial results from the LAMS (Latin American Screening) study". *J. Med. Screen*, 2005, *12*, 142.
[68] Longatto-Filho A., Eržen M., Branca M., Roteli-Martins C., Naud P., Derchain S. *et al*.: "Human papillomavirus (HPV) testing as an optional screening tool in low-resource settings of Latin America. Experience from the LAMS Study". *Int. J. Gynecol. Cancer*, 2006, *16*, 955.
[69] Hammes L.S., Naud P., Passos E., Rivoire W., Matos J., Brouwers K., Syrjänen K.J.: "Value of the International Federation of Colposcopy and Cervical Pathology (IFCPC) terminology in predicting cervical pathology". *J. Lower Genital Tract. Dis*., 2007, *11*, 158-165.
[70] Diggle P.J., Liang K.-Y., Zeger S.L.: "Analysis of Longitudinal Data", 1st edition, Oxford, Oxford University Press, 1994.
[71] Hardin J., Hilbe J.M.: "Generalized estimating equations". Boca Raton, FL, Chapman & Hall/CRC, 2003.
[72] Syrjänen K., Väyrynen M., Saarikoski S., Mäntyjärvi R., Parkkinen S., Hippeläinen M., Castren O.: "Natural history of cervical Human papillomavirus (HPV) infections based on prospective follow-up". *Brit. J. Obstet. Gynaecol*., 1985, *92*, 1086.
[73] Syrjänen K., Mäntyjärvi R., Saarikoski S., Väyrynen M., Syrjänen S., Parkkinen S. *et al*.: "Factors associated with progression of cervical Human papillomavirus (HPV) infections into carcinoma in situ during a long-term prospective follow-up". *Brit. J. Obstet. Gynaecol*., 1988, *95*, 1096.
[74] Kataja V., Syrjänen S., Yliskoski M., Hippeläinen M., Väyrynen M., Saarikoski S. *et al*.: "Risk factors associated with cervical human papillomavirus (HPV) infections: A case-control study". *Am. J. Epidemiol*., 1993, *138*, 735.
[75] Syrjänen K.J.: "Spontaneous evolution of intraepithelial lesions according to the grade and type of the implicated HPV". *Eur. J. Obstet. Gynecol. Reprod. Biol*., 1996, *65*, 45.
[76] Syrjänen K.J.: "Biological behaviour of cervical intraepithelial neoplasia". In: Franco E., Monsonego J. (eds.): "New Developments in Cervical Cancer Screening and Prevention". *Oxford, Blackwell Science*, 1997, 93.
[77] Syrjänen K.J.: "Natural history of HPV infections: Recent developments". In: Monsonego J. (ed.). 5th International Multidisciplinary Congress EUROGIN 2003. Monduzi Editore, Milano 2003, 1.
[78] Costa S., Simone P.D., Venturoli S., Cricca M., Zerbini M.L., Musiani M. *et al*.: "Factors predicting Human papillomavirus (HPV) clearance in cervical intraepithelial neoplasia (CIN) lesions treated by conization". *Gynecol. Oncol*., 2003, *90*, 358.
[79] Louvanto K., Rintala M.A., Syrjänen K.J., Grénman S.E., Syrjänen S.M.: "Genotype-specific persistence of genital high- and low-risk human papillomavirus (HPV) infections in women followed-up for 6 years in the Finnish Family HPV Study". *J. Infect. Dis*., 2010, *202,* 436.
[80] De Villiers E.M., Fauquet C., Broker T.R., Bernard H.U., zur Hausen H.: "Classification of papillomaviruses". *Virology*, 2004, *324*, 17.
[81] Wang S.S., Zuna R.E., Wentzensen N., Dunn S.T., Sherman M.E., Gold M.A. *et al.:* "Human papillomavirus cofactors by disease progression and human papillomavirus types in the study to understand cervical cancer early endpoints and determinants". *Cancer Epidemiol. Biomarkers Prev*., 2009, *18*, 113.
[82] Belinson S., Smith J.S., Myers E., Olshan A., Belinson J., Pretorius R. *et al.:* "Descriptive evidence that risk profiles for cervical intraepithelial neoplasia 1, 2, and 3 are unique". *Cancer Epidemiol. Biomarkers Prev*., 2008, *17*, 2350.
[83] Moscicki A.B., Ma Y., Wibbelsman C., Powers A., Darragh T.M., Farhat S. *et al.:* "Risks for Cervical Intraepithelial Neoplasia 3 Among Adolescents and Young Women With Abnormal Cytology". *Obstet. Gynecol*., 2008, *112*, 1335.
[84] Östor A.G.: "Natural history of cervical intraepithelial neoplasia a critical review". *Int. J. Gynecol. Pathol*., 1993, *12*, 186.
[85] Louvanto K., Syrjänen K.J., Rintala M.A., Grénman S.E., Syrjänen S.M.: "Human papillomavirus (HPV) and other predictors of incident CIN among young mothers prospectively followed-up for 6 years in the Finnish Family HPV Study". *BMC Infect. Dis. Submitted.*

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