**Distinguished Expert Series**

# Covariates of high-risk human papillomavirus (HPV) infections are distinct for incident CIN1, CIN2 and CIN3 as disclosed by competing-risks regression models

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**NIS, New Independent States of the Former Soviet Union; **LAMS, Latin American Screening Study*

**Summary**

*Background:* In addition to the oncogenic human papillomavirus (HPV), several cofactors are needed in cervical carcinogenesis, but whether the HPV covariates associated with incident i) CIN1 are different from those of incident ii) CIN2 and iii) CIN3 needs further assessment. *Objectives:* To gain further insights into the true biological differences between CIN1, CIN2 and CIN3, we assessed HPV covariates associated with incident CIN1, CIN2, and CIN3. *Study Design and Methods:* HPV covariates associated with progression to CIN1, CIN2 and CIN3 were analysed in the combined cohort of the NIS (n = 3,187) and LAMS study (n = 12,114), using competing-risks regression models (in panel data) for baseline HR-HPV-positive women (n = 1,105), who represent a sub-cohort of all 1,865 women prospectively followed-up in these two studies. *Results:* Altogether, 90 (4.8%), 39 (2.1%) and 14 (1.4%) cases progressed to CIN1, CIN2, and CIN3, respectively. Among these baseline HR-HPV-positive women, the risk profiles of incident CIN1, CIN2 and CIN3 were unique in that completely different HPV covariates were associated with progression to CIN1, CIN2 and CIN3, irrespective which categories (non-progression, CIN1, CIN2, CIN3 or all) were used as competing-risks events in univariate and multivariate models. *Conclusions:* These data confirm our previous analysis based on multinomial regression models implicating that distinct covariates of HR-HPV are associated with progression to CIN1, CIN2 and CIN3. This emphasises true biological differences between the three grades of CIN, which revisits the concept of combining CIN2 with CIN3 or with CIN1 in histological classification or used as a common endpoint, e.g., in HPV vaccine trials.

*Key words:* CIN; HPV; Covariates; Progression; Competing-risks regression; Univariate; Multivariate; Prospective follow-up; NIS Cohort; LAMS Study.

## Introduction

Since the first evidence on human papillomavirus (HPV) as the causal agent of cervical cancer (CC) and its precursor (CIN) lesions [1-4], a substantial amount of data has accumulated on the potential risk factors of HPV infections [2, 3, 5-7]. Oncogenic HPV types are associated with CC and CIN in nearly 100% of the cases, but it is increasingly clear that several other cofactors are needed to complete the progression to high-grade CIN and invasive CC [2-4, 7-12]. Of these potential covariates of HPV, those associated with reproduction have attracted particular interest, including oral contraception (OC), parity, age at first intercourse, number of sexual partners, age at first full term delivery, age at menarche, and menopause [7, 13-17]. Cigarette smoking is suggested to increase the persistence of oncogenic HPV infections [18-20], and more recently, also drug addiction has been included in the list of potential risk factors [21].

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CC precursors have been classified by different approaches, including the 3-grade CIN terminology (CIN1-3) [22]. This concept was challenged by the Bethesda System (TBS), simplifying their classification into two categories (low-grade and high-grade) [23], abandoning the intermediate (CIN2) category. According to the leading European authorities, however, maintaining CIN2 in this classification can be based on solid i) morphological, ii) biological, and iii) clinical arguments [24, 25]. In addition, recent biomarker studies have disclosed some early molecular markers up-regulated in CIN1, and many more late markers being over-expressed only upon transition from CIN2 to CIN3 [26, 27], thus not advocating the clumping together of these two entities [23].

A novel approach to gain further insights in the genuine biological differences between CIN1, CIN2 and CIN3 is to assess whether the HPV covariates needed for progression from normal epithelium to i) CIN1 are different from those required for further progression to ii) CIN2 and iii) CIN3. Until today, four such studies (three for prevalent CIN and one for incident CIN) have been published [7, 28-30], all suggesting that CIN lesions are associated with HPV covariates that are unique for CIN1, CIN2 and CIN3.

All these studies used multinomial regression analysis [7, 28-30], which might not be the optimal technique for modelling particularly the longitudinal data on prospective settings [30]. Prompted by the recent advocates of marginal and mixed-effect models for analysis of HPV natural history data (based on repeated measures of individual women) instead of standard logistic regression models [31], we decided to use competing-risks regression models [32, 33] to analyse the panel data of our combined NIS-LAMS cohort [34] for HR-HPV covariates associated with incident CIN1, CIN2 and CIN3. To control for residual confounding by HPV, only the baseline HR-HPV-positive women (n = 1,105) were included, who represent a sub-cohort of all 1,865 women prospectively followed-up in these two studies [34].

## Material and Methods

### *The NIS and the LAMS cohort study*

The present analysis is based on the combined cohort of the NIS and the LAMS studies described in recent reports. Both studies are international multi-centre trials testing optional screening tools in three NIS (New Independent States of the Former Soviet Union) countries (Russia, Belarus and Latvia) [35] as well as in two Latin American countries (Brazil and Argentina) [36]. The design and baseline data of both cohorts have been previously detailed [35, 36].

### *Patients and study design*

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) cervical cancer screening (= SCR patients); ii) attendants of gynaecologic outpatient clinics (= GYN patients), and iii) patients examined at 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) [35]. The study design has been detailed in a series of reports [15-1]. All eligible women had PAP smear taken and were tested for HR-HPV using HC2 and the first 1,500 women also with PCR and hybridisation. Patients with ASC-US or higher PAP had biopsy confirmation at baseline [15-18, 35].

*The LAMS study* is a longitudinal cohort of women enrolled in regions with low, intermediate, and high incidence of CC in Brazil and Argentina [36]. A total of 12,114 women were enrolled by the four clinics. The mean age of these women at enrolment was 37.9 years (median 37.7, range 14-67). In this trial, eight different diagnostic tests were compared as follows: cervical cytology (conventional Pap and liquid based cytology, LBC) was compared with i) 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 ii) with the new molecular diagnostic tools (HPV testing by Hybrid Capture II; HC2), performed a) in samples collected by physicians, and b) in those collected by self-sampling devices [36-39]. Women testing positive with any of these techniques were examined by colposcopy.

### *Prospective follow-up*

Prospective follow-up (FU) is an essential component of both studies. In the NIS cohort, all women who presented with biopsy-confirmed low-grade lesions were assigned for FU, while high-grade lesions were treated. FU data are available for 887 women, of whom 33 patients with baseline CIN3 were excluded from this analysis, leaving 854 women in the final prospective NIS cohort. The mean FU time was 17.2 mo (SD, 11.6 mo; median, 16.6 mo; range 1-43 mo) [15-18].

In the LAMS study, the same criteria were used to allocate the women into the FU and treatment groups [36-39]. A total of 1,011 women completed at least one FU visit, scheduled at 6-month intervals. The mean FU time was 21.7 mo (SD, 8.09 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.

### *Outcomes and endpoints*

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 four outcomes: 1) no progression, 2) progression to CIN1; 3) progression

to CIN2, and 4) progression to CIN3, representing competing-risks events. Baseline biopsy-negative women who developed CIN1 at any time point during FU were defined as progression to CIN1. As progression to CIN2, we defined any case where biopsy-confirmed progression from baseline negative, NCIN, or CIN1 lesion was confirmed at any of the FU-visits. The same criteria were used to define cases that progressed to CIN3. Times to incident CIN1, CIN2 and CIN3 were calculated from the baseline visit to the respective FU-visit when the progression event was first confirmed. Progression rates (PR) were calculated dividing the progression events by woman months at risk (wmr), and expressed as events/1,000 wmr. Because the interest in the present analysis was on HPV cofactors associated with progression to CIN1, CIN2 and CIN3, all analyses were done in a sub-cohort of 1,105 women, who tested HR-HPV positive at baseline.

Table 1. — *Disease progression to CIN1, CIN2 and CIN3 endpoints in the NIS and LAMS cohorts.*

| Disease progression | Progression parameters | | | | | |
|---|---|---|---|---|---|---|
| | Progressed cases | | Progression times (mo) | | Progression rate (Events/1000 WMR) | |
| | No | Percent | Mean | Range | WMR | Rate/1000 WMR |
| CIN1 | | | | | | |
| NIS cohort | 14 | 1.6 | 16.8 | 11.7-21.8 | 14668 | 0.9 |
| LAMS cohort | 76 | 7.5 | 14.1 | 12.5-15.6 | 21969 | 3.4 |
| Combined | 90 | 4.8 | 14.5 | 13.0-16.0 | 36637 | 2.4 |
| | p = 0.0001 | | [1]p = 0.335 | | RR = 0.27; (95% CI 0.16-0.47) p = 0.0001 | |
| CIN2 | | | | | | |
| NIS cohort | 7 | 0.8 | 19.6 | 8.5-30.6 | 14668 | 0.5 |
| LAMS cohort | 32 | 3.2 | 15.4 | 12.8-18.0 | 21969 | 1.4 |
| Combined | 39 | 2.1 | 16.2 | 13.5-18.9 | 36637 | 1.0 |
| | p = 0.0001 | | [1]p = 0.535 | | RR = 0.32; (95% CI 0.15-0.71) p = 0.0018 | |
| CIN3 | | | | | | |
| NIS cohort | 0 | 0.0 | | | 14668 | 0.0 |
| LAMS cohort | 14 | 1.4 | 15.8 | 12.1-28.1 | 21969 | 0.6 |
| Combined | 14 | 1.4 | 15.8 | 12.1-28.1 | 36637 | 0.6 |
| | NC | | NC | | NC | |

WMR, woman months at risk; *chi-square, LR test; [1]Mann-Whitney; RR, rate ratio; NC, not computable.

### *Methods*

Because detailed in several reports [15-18, 36-39], the methods used in these two cohort studies are described here only as far as pertinent to elaborating the data used in the present analysis.

### *Epidemiological questionnaire*

In both studies, all women who gave their consent to participate filled in a detailed inquiry concerning the risk factors of HPV, CIN and CC. In combining the two databases, only the variables that were recorded in both cohorts were maintained to make the data consistent. The present analysis is based on the following variables recorded at baseline: age, marital status, years of education, race, age at first sexual intercourse, number of pregnancies, -live births, -abortions, number of life-time sexual partners, number of sexual partners during the past 12 months, partners' STD history, mode of contraception, years of hormonal contraception, history of STDs, previous Pap history, history of CIN, history of genital warts, smoking history [35, 36, 40].

### *Papanicolaou (Pap) smears*

In the NIS study, all women were examined using the conventional Pap smear [35], whereas in the LAMS study, three methods were used: conventional Pap and two different LBC techniques (DNA-Citoliq; Digene Brazil, Sao Paulo, and SurePath; TriPath, Durham, NC, USA) [37]. In the present analysis, only the results of the conventional Pap test were used (available from all patients).

### *Directed Punch Biopsy*

Directed punch biopsies (and cones) were fixed in formalin, embedded in paraffin, and processed into 5-µm-thick haematoxylin-eosin (HE)-stained sections for light microscopy, following the routine procedures. All biopsies were examined among the daily routine in the Pathology Departments of the partner institutions, and diagnosed using the commonly agreed CIN nomenclature [22, 24, 25]. Lesions presenting with morphological signs of HPV but not fulfilling the criteria of CIN were called HPV-NCIN (= flat HPV lesions without CIN). In statistical analysis, these lesions were treated as baseline-negative biopsies [35, 36].

### *Detection of HR-HPV DNA by Hybrid Capture 2 (HC2) assay*

In both studies, the principal HPV testing method was HC2 assay, performed using cervical swabs (collected by a physician) or self-sampling devices (tampons, in LAMS study only), as described previously [35, 36, 39]. HC2 assay (n = 3,084 baseline tests in the NIS and n = 4,694 in the LAMS) was performed using the automated HC2 test system according to the manufacturer's protocol. 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. Samples were classified as HR-HPV positive with the RLU/CO ≥ 1.0 pg/ml cut-off.

### *Statistical analyses*

All statistical analyses were performed using the SPSS 19.0.1. for Windows (IBM, NY, USA) and STATA/SE 12.0 software (STATA Corp., Texas, USA). Frequency tables for categorical variables were analysed using the chi-square test, with likelihood ratio (LR) or Fisher's exact test for significance. Differences in the means of continuous variables were analysed using non-parametric tests (Mann-Whitney, Kruskal-Wallis) or ANOVA. The incidence rates of CIN1, CIN2, CIN3 were expressed as events/1,000 wmr, and their 95% confidence intervals (95% CI). Incidence rates were compared by RR (rate ratio) statistics (with 95% CI).

This longitudinal data file was constructed into a panel data, clustered by women-ID and using the FU-visits as the time (repeated measures) variable. Competing-risks regression models [32, 33] were first used in univariate mode to estimate the risk, i.e., crude subhazard ratios (SHR and 95% CI) of different HPV covariates to associate with incident i) CIN1, ii) CIN2 and iii) CIN3 (among baseline HR-HPV+ women). Multivariate models were constructed to disclose independent HPV covariates, calculating SHRs (95%

Table 2. — *Covariates of HR-HPV associated with progression to CIN1, CIN2 and CIN3 in univariate competing-risks regression model (with non-progression and other CIN grades as competing events)@.*

| HPV Covariate | Progressed to CIN1 (vs non-progression, CIN2, CIN3) | | Progressed to CIN2 (vs non-progression, CIN1, CIN3) | | Progressed to CIN3 (vs non-progression, CIN1, CIN2) | |
|---|---|---|---|---|---|---|
| | SHR (95% CI) | *p* | SHR (95% CI) | *p* | SHR (95% CI) | *p* |
| Age | | | | | | |
| Above 35 years | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| Below 35 years | 0.98 (0.52-1.83) | 0.951 | 1.22 (0.48-3.09) | 0.674 | 0.32 (0.10-1.02) | 0.055 |
| Marital status: | | | | | | |
| Living with partner | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| Single | 1.12 (0.60-2.09) | 0.715 | 1.66 (0.71-3.87) | 0.234 | 0.48 (0.11-2.18) | 0.434 |
| Baseline Pap test ASCUS+ | | | | | | |
| PAP negative | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| PAP ASCUS+ | 3.00 (1.58-5.71) | 0.001 | 3.93 (1.48-10.45) | 0.006 | 35.47 (4.50-279.57) | 0.001 |
| Baseline Pap test LSIL+ | | | | | | |
| PAP < LSIL | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| PAP LSIL+ | 3.59 (1.79-717) | 0.0001 | 1.95 (0.55-6.84) | 0.297 | 19.78 (5.14-76.03) | 0.0001 |
| Baseline Pap test HSIL | | | | | | |
| PAP < HSIL | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| PAP HSIL+ | 3.47 (0.82-14.68) | 0.090 | 4.05 (0.53-30.43) | 0.174 | 146.23(39.25-544.76) | 0.0001 |
| Years of education | | | | | | |
| More than 11 years | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| Between 8-11 years | 1.53 (0.57-4.07) | 0.389 | 1.67 (0.35-7.98) | 0.515 | 0.48 (0.03-7.61) | 0.603 |
| Between 5-8 years | 1.06 (0.36-3.14) | 0.922 | 0.98 (0.17-5.81) | 0.988 | 3.30 (0.39-27.82) | 0.272 |
| Less than 5 years | 1.11 (0.37-3.30) | 0.845 | 1.39 (0.26-7.48) | 0.699 | 3.49 (0.41-29.50) | 0.249 |
| Race | | | | | | |
| White | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| Non-white (black, mixed, other) | 0.87 (0.37-2.04) | 0.756 | 1.23 (0.42-3.60)) | 0.704 | 1.10 (0.24-5.01) | 0.894 |
| Age at onset of sexual activity | | | | | | |
| At or above 20 years | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| Between 17 and 20 years | 1.43 (0.65-3.12) | 0.368 | 1.09 (0.35-3.72) | 0.879 | 0.34 (0.06-1.84) | 0.210 |
| Between 15 and 17 years | 1.49 (0.63-3.55) | 0.366 | 1.70 (0.54-5.32) | 0.356 | 1.52 (0.41-5.62) | 0.527 |
| Below 15 years | 2.10 (0.72-6.13) | 0.172 | 1.51 (0.36-3.29) | 0.884 | 0.95 (0.10-8.37) | 0.960 |
| Ever been pregnant | | | | | | |
| No | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| Yes | 0.73 (0.40-1.35) | 0.326 | 0.74 (0.31-1.75) | 0.492 | 0.84 (0.25-2.79) | 0.784 |
| Number of pregnancies | | | | | | |
| 0 | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| 1 | 1.07 (0.51-2.23) | 0.851 | 0.71 (0.21-2.35) | 0.580 | 0.36 (0.04-3.18) | 0.356 |
| 2 | 0.63 (0.25-1.59) | 0.333 | 0.84 (0.26-2.78) | 0.782 | 0.42 (0.05-3.75) | 0.440 |
| 3 | 0.43 (0.12-1.44) | 0.173 | 0.86 (0.23-3.18) | 0.816 | 2.28 (0.58-9.03) | 0.238 |
| 4 or more | 0.73 (0.30-1.75) | 0.489 | 0.63 (0.17-2.35) | 0.493 | 0.84 (0.16-4.56) | 0.841 |
| Number of live births | | | | | | |
| 0 | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| 1 | 0.69 (0.32-1.48) | 0.350 | 0.82 (0.31-2.13) | 0.685 | 0.44 (0.05-3.96) | 0.468 |
| 2 | 0.91 (0.39-2.09) | 0.826 | 0.22 (0.03-1.75) | 0.156 | 3.74 (1.01-13.82) | 0.047 |
| 3 or more | 1.06 (0.41-2.74) | 0.896 | 0.75 (0.17-3.29) | 0.706 | 2.45 (0.45-13.25) | 0.297 |
| Number of life-time sexual partners | | | | | | |
| 1 | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| 2-3 | 1.26 (0.57-2.76) | 0.559 | 0.67 (0.13-3.27) | 0.618 | 0.26 (0.05-1.36) | 0.112 |
| 4-5 | 1.03 (0.39-2.70) | 0.942 | 2.67 (0.68-10.50) | 0.157 | 0.80 (0.19-3.29) | 0.757 |
| 6 or more | 0.96 (0.30-3.02) | 0.950 | 2.18 (0.45-10.59) | 0.332 | 0.86 (0.17-4.37) | 0.864 |
| Number of partners during past 12 months | | | | | | |
| 0 | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| 1 | 0.93 (0.22-3.78) | 0.922 | 0.94 (0.12-76.89) | 0.946 | NC | NC |
| 2 or more | 1.03 (0.23-4.59) | 0.961 | 0.83 (0.09-7.28) | 0.868 | 0.37 (0.20-0.65) | 0.001 |
| Any sexual partner with diagnosed STD | | | | | | |
| No | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| Yes | 1.01 (0.40-2.51) | 0.986 | 0.78 (0.18-3.33) | 0.745 | 1.57 (0.34-7.13) | 0.555 |
| Mode of contraception | | | | | | |
| No contraception | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| Oral contraception | 2.38 (1.00-5.69) | 0.050 | 2.16 (0.76-6.16) | 0.149 | 3.43 (0.72-16.44) | 0.122 |
| Other contraception | 1.96 (0.83-4.61) | 0.123 | 0.87 (0.27-2.82) | 0.813 | 1.08 (0.18-6.45) | 0.930 |
| Oral contraception | | | | | | |
| Never | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| Ever (current and past) | 1.53 (0.84-2.79) | 0.163 | 2.34 (1.02-5.35) | 0.044 | 3.27 (1.04-10.26) | 0.041 |

Table 2. — *Covariates of HR-HPV associated with progression to CIN1, CIN2 and CIN3 in univariate competing-risks regression model (with non-progression and other CIN grades as competing events)@.*

| HPV Covariate | Progressed to CIN1 (vs non-progression, CIN2, CIN3) | | Progressed to CIN2 (vs non-progression, CIN1, CIN3) | | Progressed to CIN3 (vs non-progression, CIN1, CIN2) | |
|---|---|---|---|---|---|---|
| | SHR (95% CI) | *p* | SHR (95% CI) | *p* | SHR (95% CI) | *p* |
| History of STD | | | | | | |
| Never | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| Ever | 0.94 (0.40-2.20) | 0.895 | 0.94 (0.28-3.16) | 0.927 | 1.19 (0.26-5.42) | 0.815 |
| Previous Pap smear taken | | | | | | |
| Never | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| Ever | 5.65 (2.03-15.73) | 0.001 | 1.90 (0.70-5.14) | 0.207 | 6.52 (0.84-50.44) | 0.072 |
| Time since last Pap smear | | | | | | |
| More than 24 months | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| Between 12 and 24 months | 0.71 (0.28-1.78) | 0.466 | 4.40 (0.55-35.57) | 0.160 | 0.47 (0.10-2.08) | 0.320 |
| Between 6 and 12 months | 0.81 (0.31-1.83) | 0.614 | 0.40 (0.02-6.44) | 0.523 | 0.20 (0.04-1.09) | 0.063 |
| Less than 6 months | 0.19 (0.05-0.71) | 0.014 | 2.59 (0.30-21.92) | 0.381 | 0.26 (0.05-1.39) | 0.115 |
| History of previous CIN | | | | | | |
| No | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| Yes | 0.79 (0.19-3.22) | 0.746 | 1.88 (0.44-8.04) | 0.391 | 1.50 (0.19-11.62) | 0.693 |
| Ever been a smoker | | | | | | |
| Never | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| Ever (current and past) | 1.11 (0.60-2.06) | 0.719 | 5.86 (2.12-16.15) | 0.001 | 1.49 (0.48-4.67) | 0.492 |
| Duration of smoking | | | | | | |
| Less than 5 years | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| Between 5 and 10 years | 0.55 (0.15-1.99) | 0.362 | 1.64 (0.48-5.59) | 0.422 | 3.29 (0.30-36.06) | 0.329 |
| Longer than 10 years | 0.88 (0.29-2.57) | 0.811 | 1.58 (0.46-5.37) | 0.463 | 3.16 (0.29-34.62) | 0.346 |

@only HR-HPV positive women included; n=1,105; SHR, subhazard ratio; NC, not computable.

CI), adjusted for age and all significant univariates. These analyses were repeated for CIN1, CIN2 and CIN3 incident endpoints (1, 2, or 3; recorded at each FU-visit), using the other CIN endpoints and non-progression (endpoint = 0) as competing-risks events [32, 33]. To enable pair-wise comparisons between the CIN grades, the competing-risks event in the model was changed appropriately, i.e., CIN2 vs CIN1, CIN3 vs CIN1, and CIN3 vs CIN2. In all calculations, robust variance estimator (vce) was used, clustered by woman-ID, to account for the repeated sampling of each woman. All tests were 2-sided, and values $p < 0.05$ were regarded as statistically significant.

## Results

Table 1 summarizes the key characteristics of progression to CIN1, CIN2 and CIN3 histological outcomes during the follow-up of the NIS (n = 854) and LAMS (n = 1,011) cohorts. These two cohorts are markedly different as to the proportion of the progression events and the rates of incident CIN1, CIN2, and CIN3, reflecting the different baseline status of these women. However, all progression times to the three endpoints are very similar in both cohorts.

Table 2 lists the results of univariate competing-risks regression analysis of all HPV covariates associated with the CIN1, CIN2 and CIN3 incident endpoints among baseline HR-HPV+ women (to control for confounding by HR-HPV, i.e., the common risk factor of all CIN outcomes) [30]. While keeping the other CIN grades and non-progression as competing-risk events in the model, the covariates associated with incident CIN1, CIN2 and CIN3 were quite different. The single most powerful cofactor of incident CIN1 was ever having had a Pap smear (SHR = 5.65, 95% CI 2.03-15.73) (p = 0.001), followed by baseline LSIL+ smear (SHR = 3.59), baseline ASCUS+ smear (SHR = 3.0). Other significant covariates were current use of OC (SHR = 2.38, 95%CI 1.0-5.69) (p = 0.050) and time since last Pap test < 6 months (protective) (SHR = 0.19, 95% CI 0.05-0.71) (p = 0.014). Ever having been a smoker was the most powerful predictor of CIN2 outcome (SHR = 5.86, 95% CI 2.12-16.15) (p = 0.001), ever having used OC was another significant cofactor (p = 0.044). Also baseline ASCUS+ smear (but not LSIL+) was significantly associated with incident CIN2 (SHR = 3.93, 95% CI 1.48-10.45) (p = 0.006). Baseline ASCUS+, LSIL+ and HSIL Pap were all significantly (p = 0.001) associated with incident CIN3. Other significant (or borderline) covariates include number of live births, ever having used OC, and number of recent sexual partners (ambiguous).

All these significant univariate predictors were entered in multivariate competing-risk regression models, and only a few remained significant independent predictors of each CIN endpoint (Table 3). Again, these significant HPV covariates were different for the three CIN outcomes. For incident CIN1: previous Pap history; for CIN2: ever having been a smoker, baseline ASCUS+ Pap test; for CIN3: baseline HSIL Pap test, recent sexual partners.

Table 3. — *Covariates of HR-HPV associated with progression to CIN1, CIN2 and CIN3 in multivariate competing-risks regression model (with non-progression and other CIN grades as competing events)@.*

| Progressed to | *Adjusted SHR | 95% CI | | Significance |
|---|---|---|---|---|
| | | Lower Bound | Upper Bound | |
| CIN 1 | | | | |
| Age (35 yrs cut-off): | 1.91 | 0.91 | 4.04 | 0.087 |
| Baseline Pap test ASCUS+ | 1.99 | 0.73 | 5.35 | 0.173 |
| Baseline Pap test LSIL+ | 1.95 | 0.67 | 5.63 | 0.217 |
| Mode of contraception | 1.20 | 0.80 | 1.80 | 0.374 |
| Previous Pap smear taken[2] | **5.95** | **1.77** | **20.06** | **0.004** |
| CIN2 | | | | |
| Age (35 yrs cut-off): | 4.62 | 0.95 | 22.68 | 0.059 |
| Baseline Pap test ASCUS+ | **4.93** | **1.71** | **14.26** | **0.003** |
| Oral contraception ever[1] | 1.34 | 0.48 | 3.77 | 0.573 |
| Ever been smoker | **5.79** | **1.75** | **19.20** | **0.004** |
| CIN3 | | | | |
| Age (35 yrs cut-off): | 0.36 | 0.10 | 1.24 | 0.106 |
| Baseline Pap test HSIL[3] | **175.74** | **41.56** | **743.13** | **0.0001** |
| Number of live births | 0.99 | 0.62 | 1.56 | 0.971 |
| Number of recent (< 12-mo) partners | **0.22** | **0.10** | **0.45** | **0.0001** |
| Oral contraception ever[1] | 2.37 | 0.75 | 7.46 | 0.138 |

@only HR-HPV positive women included; n = 1,105; *Adjusted for all covariates that were significant in univariate model; [1]Mode of contraception dropped from the model because of collinearity; [2]Time since last Pap smear dropped from the model because of collinearity; [3]LSIL+ and ASCUS+ dropped from the model because of collinearity, SHR, subhazard ratio; significant covariates are in bold.

The three incident CIN endpoints were compared pairwise for their HPV covariates by changing the competing-risks events in the models. Only significant covariates are listed in Table 4. Three covariates made a significant distinction between incident CIN2 and CIN1; baseline ASCUS+ Pap test (SHR = 4.61), ever having used OC (SHR = 2.25) and ever having been a smoker (SHR = 5.99). Not unexpected, there were five HPV covariates that were significantly associated with incident CIN3 (with CIN1 as a competing-risk event): baseline Pap ASCUS+, LSIL+, HSIL, number of live births, and recent partners. The first four also distinguished between incident CIN3 and CIN2 (as competing events).

In the final multivariate model with all significant univariates entered (Table 5), ever having been a smoker and baseline ASCUS+ Pap were significant covariates of the CIN2 endpoint as compared with CIN1 as a competing event. HSIL baseline Pap was the single most powerful predictor of incident CIN3, when CIN1 was the competing event. The same was true (with a slightly lower SHR) also when CIN2 was used as the competing event for CIN3.

## Discussion

Data on HR-HPV persistence are of little help while assessing the differences between CIN1, CIN2 and CIN3, because HR-HPV seems to be involved in the development of practically all these endpoints [34, 41-44]. A powerful novel approach to gain further insights in the true biological differences between CIN1, CIN2 and CIN3 is to assess, whether the covariates of progression from normal epithelium to CIN1 are different from those required for progression to CIN2 and CIN3. To adequately control for residual confounding by HR-HPV, this assessment needs to be done on baseline HR-HPV positive women [44]. Until now, three such studies have been published [7, 28, 29], all using multinomial regression models to assess the risk profiles for prevalent CIN1, CIN2 and CIN3 endpoints. We recently provided such data in a prospective setting by analysing the HR-HPV covariates for incident CIN1, CIN2 and CIN3 [30].

To make our results [30] comparable with the published three studies [7, 28, 29], we used a similar statistical approach (multinomial logistic regression models) to analyse our longitudinal data. This technique has an advantage to the standard logistic regression or Cox models in that it enables alternating the dependent variable instead of the binomial (0/1) endpoint, which makes possible also the pair-wise comparisons between the three CIN grades [30]. Using this approach in the sub-cohort of 1,105 baseline HR-HPV-positive women, we demonstrated that different HPV covariates are associated with incident CIN1, CIN2 and CIN3, irrespective whether the comparisons are made to cases with no progression or to lower grades of CIN [30]. This suggests that each CIN grade represents a distinct biological entity, with distinct natural history and covariates associated with HPV in disease progression, persistence or regression [42-46].

In a recent review, the appropriate statistical techniques to be applied in the natural history studies on HPV were extensively discussed [31]. It seems obvious that the natural history of HPV has several characteristics that, at least from a statistical perspective, are infrequently encountered in other fields of infectious disease or cancer research [31, 42-44]. The same applies to the natural history of CIN, which also runs a complex natural history, with persistence, progression or spontaneous regression as potential outcomes [45, 46]. Despite the fact that multiple-type infections are common, prevalence, incidence, persistence and clearance of HPV can be measured at genotype level in longitudinal settings with repeated sampling [47-49]. In all settings where repeated measures involve the same subject, the results tend to be correlated [31]. In other words, the probability of detecting any given HPV genotype is greater among women who test positive for another genotype, and similarly, women with biopsy-confirmed CIN at a given visit are more likely to have the disease in the subsequent visit as well, if repeated within a reasonable time frame, e.g., at 6-month intervals. Statistical techniques that fail to take these correlations into account would be invalid, and methods that do not exploit all the collected data (in a repeated measures setting) would be inefficient [31]. Marginal (e.g., GEE, generalized estimating equation) and mixed-effects models are both capable of handling these issues, showing a greater efficiency as compared with standard logistic regression and Cox models for studying the natural history of HPV infections, which is fully confirmed by our recent experience as well [47-49].

Table 4. — *Significant covariates of HR-HPV associated with progression to CIN2 and CIN3 in univariate competing-risks regression model (with CIN1 and CIN2, respectively, as competing events)@.*

| HPV Covariate | Progressed to CIN2 (CIN1 as competing event) | | Progressed to CIN3 (CIN1 as competing event) | | Progressed to CIN3 (CIN2 as competing event) | |
|---|---|---|---|---|---|---|
| | SHR (95% CI) | *p* | SHR (95% CI) | *p* | SHR (95% CI) | *p* |
| Baseline Pap test ASCUS+ | | | | | | |
| PAP negative | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| PAP ASCUS+ | 4.61 (1.78-11.92) | 0.002 | 40.86 (5.22-319.42) | 0.0001 | 40.83 (5.22-319.30) | 0.0001 |
| Baseline Pap test LSIL+ | | | | | | |
| PAP < LSIL | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| PAP LSIL+ | 2.20 (0.62-7.73) | 0.217 | 22.24 (5.72-86.50) | 0.0001 | 22.28 (5.72-86.78) | 0.0001 |
| Baseline Pap test HSIL | | | | | | |
| PAP < HSIL | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| PAP HSIL+ | 5.07 (0.66-38.91) | 0.118 | 166.62 (43.68-635.50) | 0.0001 | 167.93 (43.79-644.00) | 0.0001 |
| Number of live births | | | | | | |
| 0 | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| 1 | 0.70 (0.27-1.81) | 0.470 | 0.40 (0.04-3.74) | 0.426 | 0.40 (0.04-3.74) | 0.426 |
| 2 | 0.26 (0.03-2.03) | 0.202 | 3.90 (1.04-14.54) | 0.043 | 3.90 (1.05-14.58) | 0.042 |
| 3 or more | 0.56 (0.14-2.28) | 0.426 | 1.86 (0.35-9.88) | 0.465 | 1.86 (0.35-9.90) | 0.464 |
| Number of partners during past 12 months | | | | | | |
| 0 | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| 1 | 0.88 (0.09-7.93) | 0.911 | NC | 0.946 | NC | NC |
| 2 or more | 0.88 (0.11-6.56) | 0.903 | 0.37 (0.20-0.66) | 0.001 | NC | NC |
| Oral contraception | | | | | | |
| Never | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| Ever (current and past) | 2.25 (0.99-5.11) | 0.052 | 2.97 (0.92-9.58) | 0.068 | 2.97 (0.92-9.59) | 0.068 |
| Ever been a smoker | | | | | | |
| Never | 1.00 (Reference) | | 1.00 (Reference) | | 1.00 (Reference) | |
| Ever (current and past) | 5.99 (2.16-16.65) | 0.001 | 1.46 (0.46-4.64) | 0.513 | 1.46 (0.46-4.62) | 0.517 |

@only HR-HPV positive women included; n = 1,105; SHR, subhazard ratio; NC, not computable.

Table 5. — *Covariates of HR-HPV associated with progression to CIN2 and CIN3 in multivariate competing-risks regression model (with CIN1 and CIN2, respectively, as competing events)@.*

| Progressed to | *Adjusted SHR | 95% CI | | Significance |
|---|---|---|---|---|
| | | Lower Bound | Upper Bound | |
| CIN2 (CIN1 competing event) | | | | |
| Age (35 yrs cut-off) | 4.59 | 0.97 | 21.74 | 0.054 |
| Baseline Pap test ASCUS+ | **5.54** | **1.96** | **15.67** | **0.001** |
| Oral contraception ever | 1.37 | 0.48 | 3.83 | 0.553 |
| Ever been smoker | **5.61** | **1.70** | **18.44** | **0.004** |
| CIN3 (CIN1 competing event) | | | | |
| Age (35 yrs cut-off) | 0.30 | 0.08 | 1.06 | 0.064 |
| Baseline Pap test HSIL[1] | **173.92** | **46.54** | **649.92** | **0.0001** |
| Number of live births | 1.13 | 0.76 | 1.67 | 0.541 |
| Number of recent partners | 2.03 | 0.98 | 4.22 | 0.056 |
| CIN3 (CIN2 competing event) | | | | |
| Age (35 yrs cut-off) | 0.33 | 0.10 | 1.09 | 0.069 |
| Baseline Pap test HSIL[1] | **146.16** | **40.24** | **530.82** | **0.0001** |
| Number of live births | 0.99 | 0.64 | 1.52 | 0.986 |

@only HR-HPV positive women included; n = 1,105; *Adjusted for all covariates that were significant or borderline significant in the univariate model; [1]LSIL+ and ASCUS+ dropped from the model because of collinearity; SHR, subhazard ratio.

As pointed out in our original report [30], the potential weaknesses of the multinomial regression model used in analysis of HPV covariates in incident CIN include the fact that this method i) fails to fully exploit these longitudinal data based on repeated testing of individual women, and ii) more importantly, fails to control for the dependence of these repeated measurements [30]. Incident CIN cases represent count variables (events per person time at risk), and as such would be perfectly suitable for analysis by Poisson regression models. However, useful as Poisson models are in analysing the incident endpoints in panel data [48, 49], this technique only accepts a binomial (0/1) dependent variable and thus would necessitate a separate analysis for each of the multiple comparisons between the three CIN grades, which is not feasible. To overcome the potential caveats of the multinomial regression, we ended up in selecting another method for analysing our data, by taking into account the fact that i) the longitudinal data be utilised in full, ii) dependence of the repeated measurements be taken into account, and iii) the multiple-endpoint (CIN1, CIN2, CIN3, no progression) variable be appropriately treated in a single model. There prerequisites are met by the competing-risks regression, here used to validate the previous results on HPV covariates in incident CIN1, CIN2 and CIN3, obtained by multinomial regression [30].

Based on the method of Fine and Gray (1999), competing-risks regression provides a useful alternative to standard Cox regression for survival data in the presence of competing risks [32]. In contrast to the usual survival analysis measuring time-to-failure as a function of observed cofactors, e.g. development of CIN3 in relation to HPV and other covariates, the term competing risk refers to the chance that instead of incident CIN3, one will observe a competing event, i.e., incident CIN1, CIN2 or no progression at all [32, 33]. During the observation period, detection of any of these com-

peting events impedes the occurrence of the event of interest (CIN3). This is basically different from the usual censoring that occurs in conventional survival analysis, i.e., loss to follow-up; while censoring obstructs you from observing the event of interest, a competing event prevents the occurrence of the event of interest. In simple terms, competing-risks regression generates hazard for (failure) events of interest, while simultaneously keeping the subjects who experience competing events still "at risk" so that they can be adequately counted as not a chance of failing. Different from the usual Cox regression models producing HR (hazard ratio), this technique reports exponentiated coefficients known as subhazard ratios (SHR) [32, 33]. Technically, the correlation within multiple records on the same subject is accounted for by using a robust variance estimator, clustered by patient-ID, so as to treat each observation within a patient as an own predictor and not as a set of overlapping predictors [32, 33].

In the present study, all previous calculations based on multinomial regression in the original report [30] were repeated using the competing-risks regression models (Table 2-5). Importantly, as compared with the original data, the key results did not substantially change with this new technique of analysing the data. A detailed discussion of the results concerning the different HPV covariates in CIN1, CIN2 and CIN3 was provided in the original report, and is not repeated here. In the present analysis, the HPV covariates associated with incident CIN1, CIN2 and CIN3 are clearly different. In general, the number of significant covariates declined from CIN1 towards CIN3, as was also the case with the original analysis [30]. Interestingly, practically the same covariates remained significant, with only slight changes in their relative risk (SHR), and 95% CIs (Table 2). However, the most dramatic difference to the original analysis appears among the HPV covariates of incident CIN3, where the role of baseline Pap smear becomes accentuated. Instead of only ASCUS+ in the original data [30], also LSIL+ and HSIL are significant HPV covariates, the latter having the single most powerful predictive value of incident CIN3 (SHR = 146.2). This emphasises the difference between the two techniques of data analysis; while multinomial regression fails to use all recorded data (resulting in several non-computable estimates for CIN3 covariates) [30], competing-risk regression is capable of utilising all the data collected by repeated sampling at all FU visits. This results in more efficient estimates, with only one (a sub-category) of all analysed covariates remaining non-computable (Table 2). Because of a larger number of incident CIN2 and CIN1 cases, the estimates in these two categories are not that dramatically affected by the different techniques of data analysis. Even here, however, the baseline Pap smear will appear among the significant covariates of both CIN1 (ASCUS+, LSIL) and CIN2 (ASCUS+), in contrast to the original analysis, where no significance was established for these covariates.

Like before [30], all significant univariate predictors were entered in multivariate models separately for CIN1, CIN2 and CIN3 (keeping all others as competing-risks events). Again, the significant independent HPV covariates were different for the three CIN outcomes (Table 3). Similar analysis was also completed for each CIN grade, using its immediate precursor as the only competing event (i.e., CIN1 vs non-regression, CIN2 vs CIN1, etc.) (Table 5). Ever having been a smoker and baseline ASCUS+ Pap were significant covariates of CIN2 endpoint (CIN1 as a competing event). HSIL baseline Pap was the single most powerful predictor of incident CIN3, when CIN1 was the competing event, and the same was true also when CIN2 was the competing event. None of these independent significant HPV covariates are exactly identical to those reported in the previous studies on stage-specific risk profiles of prevalent CIN [7, 28, 29]. Direct comparison of these studies is not straightforward, however, the present data is the only information based on a prospective setting (with incident CIN events), while the others used prevalent (baseline) CIN outcomes. However, more important than the individual covariates is the demonstration of the concept that the significant HPV covariates associated with progression from normal epithelium to CIN1 are different from those associated with progression to CIN2 and further to CIN3. All previously published studies are unanimous in demonstrating this [7, 28-30].

Taken together, the present analysis based on competing-risk regression models gives no evidence justifying us to change the original conclusions reached by multinomial logistic regression models, unequivocally demonstrating that different HPV covariates are associated with incident CIN1, CIN2 and CIN3 [30]. These conclusions remain the same, irrespective which competing-risk events are used for the failure event of interest, i.e., all other categories or just the immediate precursor category. Having been confirmed by two fundamentally different techniques of data analysis, these results substantiate the concept that each CIN grade represents a distinct biological entity, as also suggested by the extensive natural history data available for CIN [42, 43, 45, 46]. This should have important implications in at least two fields: 1) lumping together CIN2 and CIN3 in the histological classification of cervical cancer precursors should be revisited, and 2) using the combined CIN2/CIN3 endpoint in any studies assessing the risk factors of cervical cancer should be reconsidered. Of great interest will be to assess whether these different HPV covariate profiles are linked with individual HR-HPV genotypes.

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