Original Articles

Epidemiological, clinical and viral determinants of the increased prevalence of high-risk human papillomavirus (HPV) infections in elderly women

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Summary

Background: Population-based studies have reported a second peak of human papillomavirus (HPV) prevalence among women > 55 years, but reasons for this U-shaped HPV prevalence curve are poorly understood. *Objectives:* To analyse determinants of high-risk HPV (HR-HPV) infections among postmenopausal women. *Study design and Methods:* A cohort of 3,187 women was stratified into three age categories: i) youngest age group < 25 years (n = 1.103); ii) women between 26-55 years (n = 2.004), and iii) women > 55 years (n = 80), analysed for epidemiological, clinical and virological determinants of their HR-HPV infections. Real-time PCR was used for HPV genotyping, analysis of viral loads for HPV16, 18/45, 31, 33/52/58, 35 and 39, and load of integrated HPV16. *Results:* Age-standardised prevalence of HR-HPV infections showed a second peak among women > 55 years, with a perfect U-shaped curve ($R^2 = 0.966$). The factors explaining this increased HR-HPV prevalence among older women include: i) cohort effect, ii) higher viral loads for HR-HPV types with cubic model curve ($R^2 = 0.714$) for HPV16, iii) distinct shift ($p = 0.0001$) from multiple-type infections to single HR-HPV types, iv) transition from episomal to integrated HPV16 ($p = 0.009$), v) higher load of integrated HPV16 ($p = 0.009$), and, vi) higher proportion of incident infections, higher rate of viral persistence, and lower rate of HR-HPV clearance. *Conclusions:* These data suggest that in women who fail to eradicate their HR-HPV infection until menopause, selection of integrated viral clone has taken place, driving the process towards progressing disease. Consequent to this, most of the HR-HPV infections in women > 55 years were associated with high-grade CIN or invasive carcinoma.

Key words: High-risk HPV; Postmenopause; Prevalence; Second peak; Predictors; Sexual behavior; Viral load; Integration; CIN, cervical cancer; Follow-up.

Introduction

Since the recognition of human papillomavirus (HPV) as the causal agent of cervical cancer (CC) and its precursor (CIN) lesions, epidemiological data from different countries confirmed that the peak prevalence of cervical HPV infections (detected by Pap smear or DNA hybridisation techniques) occurs between 22-24 years of age, with a constant decline with progressing age [1-6]. This was neatly explained by the early studies (based on Pap smear screening data) implicating a particularly high (8%) annual incidence of cervical HPV infections among 22-year-old women [7, 8].

More recent studies on the natural history of HPV infections [9] have further refined the dynamics of these viral events in different populations. Accordingly, incident HR-HPV infections are clearly age-dependent, the 3-year cumulative incidence exceeding 50% among women under 20 years of age, following the onset of their sexual activity [10-12]. On the other hand, clearance of the virus did not show such strict age-dependence [13], but continued at a constant rate among women over 30 years of age [4, 14]. Using these age-specific incidence and clearance rates to estimate the age-specific preva-

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lence of HR-HPV infections models the true figures quite closely except for a small gap in each of the 5-year age groups (15-75 years) [15]. This gap between the true- and estimated age-specific prevalence rates is due to the fact that instead of clearance, some of the acquired infections remain persistent. These persistent HR-HPV infections are considered as a prerequisite for developing a progressive cervical disease and are currently the subject of intense study for their predisposing co-factors [16-18].

During the past few years, this dynamic model of HPV acquisition, clearance and persistence, explaining the linearly declining age-specific prevalence curve [1-6] has been challenged by the data from several population-based studies, reporting a second peak in HPV prevalence among women > 55 years of age [19-21]. In some studies a similar peak among older women has been reported for HPV incidence as well [22, 23]. Indeed, the recently published population-based studies report highly contradictory results from different geographic areas. There are populations, where the age-specific prevalence curve is clearly U-shaped, with a second peak among post-menopausal women [19-22, 24-28]. In other studies, no such U-shaped prevalence curve was established, but the shape was that of a declining linear curve [29-33]. The IARC HPV Prevalence Survey data failed to give one single explanation for these differences, and several key questions still remain unanswered [34, 35].

The present study sheds new light on most of these open issues related to the shape of the age-specific HPV prevalence curves, and in particular to the determinants of the second peak observed among women over 55 years of age.

Material and Methods

Patients and study design

The subjects and the study design of this European Commission (EC)-funded cross-sectional and cohort study have been published earlier [36, 37]. The study cohort comprises 3,187 consecutive women attending six different outpatient clinics in three New Independent States (NIS) of the former Soviet Union between 1998-2002. These women derive from three different groups: i) cervical cancer screening (= SCR patients); ii) attendants of gynaecology outpatient clinics (= GYN patients), and iii) patients examined at STD clinics (= STD patients). The mean age of the women was 32.6 (± 10.7 SD) years (median 30.6, range 15-85 years).

The study design has been detailed in a series of previous reports [12, 13, 15, 18, 36, 37]. All eligible women had Pap smears taken and were tested for HR-HPV using Hybrid Capture II (HCII) and the first 1,500 also with PCR and confirmative hybridisation [38]. Patients with ASC-US or higher Pap had biopsy confirmation [36, 37].

Follow-up

All women who presented with biopsy-confirmed low-grade lesions were assigned for prospective follow-up, while women with high-grade lesions were treated [36, 37]. Altogether, follow-up (FU) data are available on 887 women (median FU 16.7 months), divided into four sub-cohorts according to their baseline HPV/PAP smear status [12, 13, 15, 18]. Four possible outcomes were recorded: a) always Pap (or HPV) negative, b) incident Pap abnormality (or new HPV), c) persistent Pap abnormality (or HPV), and d) cleared disease (or HPV infection). The criteria for defining these four outcomes have been described in detail elsewhere [12, 13, 15, 18].

Age-group analysis

The present analysis was focused on assessing the epidemiological, clinical and viral predictors for HR-HPV infections in different age categories. In simple terms, we wanted to clarify the reasons for the U-shaped HPV prevalence curve, previously observed in this cohort [36, 37]. The whole cohort of 3,187 women was stratified into three age groups according to their different HR-HPV prevalence profiles established by HCII assay and PCR [36, 38]. These three age categories are: i) two youngest age groups (women > 20 years and those between 21-25 years; n = 1,103) with the peak HPV prevalence; ii) women between 26-55 years (n = 2,004) with linearly declining HPV prevalence; and iii) women > 55 years (n = 80) with a sharply increasing HR-HPV prevalence [36, 37]. In all analyses of this study, these three age categories were compared to each other.

Methods

Epidemiological questionnaire

At the first visit, all women who gave their consent to participate filled in a detailed inquiry concerning the risk factors of HPV, CIN and CC. This structured questionnaire contained questions exploring reproductive history, sexual history, current sexual practices, sexual hygiene, medical history, smoking habits and contraception [37, 39].

Papanicolaou (Pap) smears

Altogether, 3,097 women were subjected to conventional Pap smear, interpreted using the jointly agreed terminology [36].

Directed punch biopsy

On histological grading of the lesions, CIN nomenclature was used. The presence of HPV infection was recorded using the accepted morphological criteria [36].

Detection of HPV DNA by Hybrid Capture II assay

From 3,087 women, the sample for the Hybrid Capture II test was taken from the cervix using the HCII sampling kit (Digene, Silver Springs, MD, USA). The test was performed according to the provider's instructions using the probe Panel B which detects 13 high-risk HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). The RLU/CO value of 1 pg/ml was used as the cut-off for a positive test [36, 38].

Detection and quantification of HR-HPV by real-time PCR

The same samples were then processed for DNA extraction using the high salt method of Miller *et al.* [40]. HR-HPV detection, genotype analysis, and viral load quantification was performed with a real-time PCR-based assay described recently [41]. With this method, HR-HPV types 16, 31, 39 and members of the group 18/45 and group 33/52/58 were detected in two different reactions. The amplification conditions used were described recently [41]. A total of six non-template controls, where DNA was substituted by water were included in each run. The dynamic range of the assay is 102-107 copies of HR-HPV per assay [41]. HPV35 detection was performed only from the baseline samples.

Integration assay

All HPV16 positive samples at the baseline and all follow-up visits were further analysed for their physical status, using a real-time PCR method, recently developed in our laboratory [42]. The amplification conditions, primers and probes were as described earlier. Two standard curves were obtained by amplification of a dilution series of five million to 500 copies of a clone of HPV 16 in pBR322. There was a linear relationship between the threshold cycle values plotted against the log-copy number over the entire range of dilutions [42]. When no E2 PCR signal was detected, the HPV16 genome was interpreted as integrated. When the ratio of copies of E2:E6 was above 0.5, the physical status was interpreted as episomal. Otherwise the sample was interpreted to contain both episomal and integrated forms of HPV16 (mixed form) [42, 43].

Statistical analyses

Statistical analyses were performed using the SPSS® and STATA software packages (SPSS for Windows, Version 14.0.1., SPSS Inc., Chicago, IL, USA and STATA/SE 9.2., Stata Corp.,

Table 1. — *The key clinical and epidemiological variables in the three groups*.

Characteristic	Women under 25 years[1] (n = 1.103)	Women between 26 and 55 years[2] (n = 2.004)	Women over 55 years[2] (n = 80)	p
Mean Age (95%CI)#	21.7 (21.6-21.8)	37.3 (37.0-37.7)	61.6 (60.4-62.8)	0.0001
Patient category:				
STD	34.7% (383/1103)	16.9% (338/2004)	2.5% (2/80)	
GYN27.7% (305/1103)	27.7% (305/1103)	22.5% (451/2004)	15.0% (12/80)	0.0001
SCR	37.6% (415/1103)	60.6% (1215/2004)	82.5%(66/80)	
HPV positive (HCII test)	48.8% (522/1069)	24.5% (474/1938)	21.3% (17/80)	0.0001
HPV positive (TaqMan assay)	40.3% (420/1042)	26.6% (493/1856)	20.3% (16/79)	0.0001
Pap Smear:				
ASCUS or worse	18.5% (200/1079)	15.3% (297/1938)	22.5% (18/80)	0.030
LSIL or worse	8.0% (86/1079)	7.9% (154/1938)	17.5% (14/80)	0.025
HSIL or worse	0.2% (2/1079)	2.1% (40/1938)	13.8% (11/80)	0.0001
Cervical Biopsy:				
CIN1 or worse	38.8% (88/227)	49.2% (125/254)	81.3% (13/16)	0.0001
CIN2 or worse	13.2% (30/227)	34.3% (87/254)	68.8% (11/16)	0.0001
CIN3 or cancer	3.1% (7/227)4	21.3% (54/254)5	68.8% (11/16)6	0.0001
Ever been pregnant	67.3% (664/986)	81.2% (1485/1828)	88.8% (71/80)	0.0001
Number of deliveries (M±SD)#	0.65 (±0.80)	0.97 (±0.87)	1.14 (±0.79)	0.0001
Ever had miscarriages	12.3%(117/948)	17.3%(311/1798)	21.8%(17/78)	0.001
Ever had abortions	47.2% (448/950)	58.8%(1053/1792)	77.6% (59/76)	0.0001
Number of abortions (M±SD)#	1.88 (±1.35)	2.16 (±1.62)	2.58 (±1.88)	0.0001
Age at first sexual intercourse#	18.47 (±2.76)	19.52 (±2.92)	20.99 (±4.00)	0.0001
Sexual habits regular ever since	46.0% (439/954)	53.6% (945/1762)	67.5% (52/77)	0.0001
Currently, one sexual partner	84.6% (823/973)	83.8% (1500/1791)	62.0% (49/79)7	0.0001
No. of partners during past 2 yrs	2.12 (±3.42)	1.53 (±1.51)	1.33 (±1.62)	0.0001
Ever had venereal disease	20.0% (194/970)	12.7% (226/1780)	15.2% (12/79)	0.0001
Sexual practices: oral sex	57.2% (518/905)	52.2% (838/1605)	32.4% (22/68)	0.0001
Sexual practices: anal sex	14.7% (120/815)	11.5% (163/1421)	9.4% (6/64)	0.063
Casual sexual partners	19.0% (182/959)	13.2% (231/1745)	12.7% (10/79)	0.0001
Casual contacts domestic	51.9% (122/235)	45.1% (176/390)	28.6% (6/21)	0.055
Casual contacts abroad	5.9% (34/576)	5.8% (62/1065)	5.8% (3/52)	0.997
Mode of contraception:				
No contraception	46.4% (442/952)	49.9% (875/1755)	75.0% (57/76)	
No oral contraception	38.1% (363/952)	36.1% (634/1755)	19.7% (15/76)	0.0001
Oral contraception	15.4% (147/952)	14.0% (246/1755)	5.3% (4/76)	
Bide/douche at intercourse	94.3% (909/964)	96.3% (1704/1769)	86.8% (66/76)	0.001
Douche requested from the partner	86.0% (832/968)	89.6% (1585/1768)	78.9% (60/76)	0.001
History of skin or genital warts	28.1% (269/956)	24.6% (430/1748)	23.1% (18/78)	0.116
History of previous CIN	5.8% (50/863)	8.7% (127/1464)	12.1% (7/58)	0.018
Ever had Pap smear	35.5% (302/851)	43.4% (678/1563)	34.7% (25/72)	0.0001
Time since the last Pap test (months)#	11.35 (±11.69)	12.21 (±12.92)	12.44 (±7.61)	0.594
Previous Pap test normal	72.3% (219/302)	71.2% (501/704)	73.1% (19/26)	0.923
Current smoker	30.9% (301/974)	23.9% (430/1796)	21.8% (17/78)	0.0001
If yes, for how long (no. of yrs)	6.63 (±4.92)	9.58 (±7.04)	9.54 (±5.07)	0.0001
If not current, ever been smoker	22.4% (147/655)	19.2% (250/1300)	11.7% (7/60)	0.055
How long did you smoke (yrs)#	4.57 (±3.66)	5.43 (±4.41)	10.8 (±8.61)	0.003
Time since stopped smoking (months)#	31.49 (±37.73)	54.59 (±68.53)	81.60 (±93.10)	0.004
Sexual partner regular smoker	61.5% (575/935)	55.9% (951/1701)	43.5% (30/69)	0.001
Ever had cervical erosion	60.7% (589/970)	62.0% (1108/1787)	55.1% (43/78)	0.420
If yes, was erosion treated	42.6% (339/796)	53.5% (775/1449)	64.2% (34/53)	0.0001

#Kruskal-Wallis test; [1]Age group with peak HR-HPV prevalence; [2]Age groups with progressively declining HR-HPV prevalence; [3]Age groups with sharply increasing HR-HPV prevalence; [4]0/7 were SCC; [5]16/54 were SCC; [6]10/11 were SCC; [7]Negative response includes women with no current partner.

Table 2. — *Significant determinants of HR-HPV infection in the three groups.*

Covariate	Women under 25 years[1] (n = 1.103)		Women between 26 and 55 years[2] (n = 2.004)		Women over 55 years[3] (n = 80)	
	OR (95% CI)	p	(95% CI)	p	OR (95% CI)	p
Patient category:						
SCR	Reference		Reference		Reference	
STD	1.53 (1.15-2.03)	0.008	2.39 (1.82-3.12)	0.0001	NC	0.054
GYN	1.41 (1.04-1.91)		1.90 (1.48-2.44)		5.00 (1.35-18.41)	
HSIL PAP	NC	0.238	39.33 (12.0-128.5)	0.0001	34.3 (6.26-187.86)	0.0001
CIN3 or above	0.75 (0.08-6.74)	0.579	7.99 (1.87-34.16)	0.001	1.12 (0.08-16.30)	0.931
Ever been pregnant	0.76 (0.57-0.98)	0.041	0.77 (0.58-0.99)	0.048	0.93 (0.18-4.98)	0.940
No. of deliveries	0.86 (0.73-1.01)	0.068	0.84 (0.73-0.95)	0.007	1.95 (0.97-3.94)	0.060
Sexual habits regular since onset	0.94 (0.73-1.22)	0.689	0.78 (0.62-0.97)	0.029	8.84 (1.09-71.64)	0.028
Partner's good sexual hygiene (bide)	0.81 (0.56-1.17)	0.270	0.67 (0.48-0.94)	0.024	1.08 (0.26-4.41)	0.911
Ever had genital warts	1.08 (0.90-1.20)	0.588	0.93 (0.76-1.14)	0.502	2.33 (1.03-5.24)	0.045*
Previous CIN	1.08 (0.60-1.95)	0.789	1.44 (0.96-2.15)	0.081	5.62 (1.01-31.48)	0.033*
Previous Pap normal	1.01 (0.60-1.67)	0.985	0.56 (0.38-0.81)	0.002	1.45 (0.22-9.61)	1.000
Current smoker	1.14 (0.86-1.50)	0.349	1.39 (1.09-1.78)	0.009	2.78 (0.83-9.27)	0.103
Cervical erosion treated	0.80 (0.60-1.06)	0.148	0.75 (0.59-0.96)	0.022	0.65 (0.15-2.77)	0.706

[1]Age group with peak HR-HPV prevalence; [2]Age groups with progressively declining HR-HPV prevalence; [3]Age groups with sharply increasing HR-HPV prevalence; NC, non computable; *Pearson Chi-square.

Table 3. — *Viral loads, individual HR-HPV* types, and physical state of HPV16 in the three groups.*

Characteristics	Women under 25 years[1] (n = 1.103)			Women between 26 and 55 years[2] (n = 2.004)			Women over 55 years[3] (n = 80)			p
HPV positive (HCII test)	48.8% (522/1069)			24.5% (474/1938)			21.3% (17/80)			0.0001
Viral load HCII test***	207.4 (95% CI 177.0-237.7)			84.9 (95% CI 69.4-100.5)			120.8 (95% CI 31.3-210.2)			0.0001[2]
HPV positive (TaqMan assay)	40.3% (420/1042)			26.6% (493/1856)			20.3% (16/79)			0.0001
Viral load[3] (TaqMan assay):										
HPV16	-0.90 (-1.49- -0.32)			-1.16 (-1.65- -0.68)			2.32 (-0.96-5.62)			0.070[2]
HPV18/45	-0.63 (-1.32-0.06)			-1.85 (-2.67- -1.03)			0.88 (-53.4-55.22)			0.065[2]
HPV31	-1.21 (-1.97- -0.45)			-2.39 (-3.00- -1.77)			0.06 (-1.76-1.77)			0.010[2]
HPV33	0.98 (0.25-1.70)			0.51 (-0.25-1.27)			4.72 (-0.02-9.47)			0.204[2]
HPV35	1.12 (-1.09-3.34)			2.35 (-0.09-4.79)			NC			0.549[2]
HPV39	2.16 (1.04-3.28)			1.61 (0.37-2.86)			NC			0.464[2]
HR-HPV Types:	No.	Per Cent	HPV+	No.	Per Cent	HPV+	No.	Per Cent	HPV+	
HPV-negative	622	59.7	@	1363	73.4	@	63	79.7	@	0.0001#
HPV16	117	11.2	27.9	196	10.6	39.8	6	7.6	37.5	
HPV18/45	61	5.9	14.5	41	2.2	8.3	2	2.5	12.5	
HPV31	45	4.3	10.7	83	4.5	16.8	5	6.3	31.3	
HPV33	51	4.9	12.1	59	3.2	12.0	2	2.5	12.5	0.0001@
HPV35**	2	0.2	0.5	1	0.1	0.2	0	0.0	0.0	
HPV39	15	1.4	3.6	12	0.6	2.4	0	0.0	0.0	
Multiple	129	12.4	30.7	101	5.4	20.5	1	1.3	6.3	
HPV16 Integration Status:										
Episomal	112		50.3%	145		44.1%	0		0.0%	
Mixed	100		44.8%	151		45.9%	5		71.4%	0.005
Integrated	11		4.9%	33		10.0%	2		28.6%	
Integration:										
Yes	111	49.8%	184		55.9%	7		100.0%		0.009
No	112	50.2%	145		44.1%	0		0.0%		
[1]Integration load:	10.5 (95% CI 9.3-11.7)			10.0 (95% CI 9.2-10.9)			17.5 (95% CI 11.3-23.7)			0.019[2]

*HR-HPV types determined by real-time PCR (TaqMan) analysis; **HPV35 analysed in 1.500 samples only; ***HCII index values; #HPV-negative cases included; @HPV-negative cases excluded; [1]Integration load in logarithmic scale; [2]Kruskal-Wallis test; [3]Log-transformed copy/cell values; NC, no cases.

College Station, TX, USA). To adjust for the differences in age distribution in the three NIS countries, we calculated age-standardised HPV prevalence for 14 five-year age groups (15–84 years) of the European standard population [44]. Logistic regression modelling with a curve estimation procedure was used to assess the age profile in each three countries, by fitting a logistic regression model with either i) linear, ii) quadratic or iii) cubic terms for 14 five-year age groups. Curves with a significant ($p < 0.05$) quadratic term were classified as non-linear (U-shaped), those with significant cubic term as non-linear (bi-phasic or S-shaped), to distinguish from those with only a linear age term. All curve fit procedures were controlled by scatter plots, where the fit parameters (= predicted parameters) were plotted against the residuals.

Frequency tables for categorical variables were analysed using the chi-square test, with likelihood ratio (LR) or Fisher's

Table 4. — *Clinical outcome of cervical lesions and HR-HPV infections in the three groups.*

Characteristic	Women under 25 years[1] (n = 402)		Women between 26 and 55 years[2] (n = 439)		Women over 55 years[3] (n = 13)		p
Baseline Disease Status:							
HPV-/PAP-	10.0%	(40/400)	16.9%	(74/437)	38.5%	(5/13)	
HPV-/PAP+	10.3%	(41/400)	19.5%	(85/437)	7.7%	(1/13)	0.0001
HPV+/PAP-	24.0%	(96/400)	20.8%	(91/437)	7.7%	(1/13)	
HPV+/PAP+	55.8%	(223/400)	42.8%	(187/437)	46.2%	(6/13)	
[1]Clinical Outcome of Lesions:							
Always Pap-negative	17.8%	(68/383)	18.9%	(79/419)	33.3%	(4/12)	
Incident abnormal Pap	15.9%	(61/383)	18.6%	(78/419)	8.3%	(1/12)	
Persisting abnormality	36.3%	(139/383)	34.6%	(145/419)	33.3%	(4/12)	0.893
Cleared abnormal Pap	29.0%	(111/383)	27.0%	(113/419)	25.0%	(3/12)	
Fluctuating course	1.0%	(4/383)	1.0%	(4/419)	0.0%	(0/12)	
[1]Outcome of HR-HPV infections:							
Always HPV-negative	8.4%	(30/358)	26.8%	(99/369)	33.3%	(4/12)	
Incident HR-HPV	7.8%	(28/358)	3.0%	(11/369)	8.3%	(1/12)	
Persisting HR-HPV	43.9%	(157/358)	27.4%	(101/369)	33.3%	(4/12)	0.0001
Cleared HR-HPV	33.0%	(118/358)	37.7%	(139/369)	16.7%	(2/12)	
Fluctuating course	7.0%	(25/358)	5.1%	(19/369)	8.3%	(1/12)	

[1]Cases with only one test done were excluded.

exact test for significance. Differences in the means of continuous variables were analysed using non-parametric tests (Mann-Whitney, Kruskal-Wallis) or ANOVA, after careful control of the normal distribution. Logistic regression was used to analyse the power of different covariates as predictors of the outcome variables (CIN2/3, HR-HPV), calculating crude odds ratios (OR) and 95% confidence interval (CI). Significant variables in univariate analysis were entered into the multivariate regression models to calculate adjusted ORs (95% CI). Confounding was also controlled by calculating the weighted-average of the stratum-specific estimates using the Mantel-Haenszel test for common OR (95% CI). In all tests, the values $p < 0.05$ were regarded as statistically significant.

Results

The age-standardised prevalence rate (ASPR) of HR-HPV infections was very similar in Russia (18.3/100 women; 95% CI, 16.6-19.9), and Belarus (17.2/100 women; 95% CI, 14.1-20.3), but in Latvia as high as 24.6/100 women (95% CI, 20.60-28.65).

In the whole cohort, the HPV prevalence curve was clearly U-shaped, steadily declined from 55.6% among women < 20 years of age, down to 10.1% among those aged 51-55 years, followed by a deep increase among women > 55 years (Figure 1). In the whole cohort, the F statistic for model fit was significant both in the linear and quadratic equation ($p = 0.0001$), but substantially higher ($R^2 = 0.966$) for the quadratic model (U-shape curve) than ($R^2 = 0.809$) for the linear model. In the curve of Russia, the results mimic those for the whole cohort; $R^2 = 0.806$ for the linear model and $R^2 = 0.968$ for the quadratic model ($p = 0.0001$ for both). In the curve of Belarus, there was not much difference between the linear and quadratic models; $R^2 = 0.952$ and $R^2 = 0.995$, respectively. The age-specific HPV curve of Latvia showed the least obvious linearity and the most accentuated second peak; $R^2 = 0.647$ for linear and $R^2 = 0.915$) for the quadratic model.

These three groups differed at the $p = 0.0001$ level with regard to the majority of the recorded epidemiological variables (Table 1). Many of these variables can be directly explained by the age difference between the three age categories. On the other hand, however, there are some interesting variables that do not show any difference between the three groups; e.g., history of skin and genital warts, time since the last Pap smear, previous Pap normal, and ever had cervical erosion.

Of the determinants of HR-HPV infection in the three groups, patient category was significant only in the two groups of younger women, but not among the older ones (Table 2). HSIL Pap predicted HR-HPV only in the two older groups, whereas the CIN3 cut-off was a significant predictor only in women between 25-55 years of age. The same holds true with the number of deliveries, which had a protective effect among this age group (a surrogate of regular family life?). A history of previous CIN was significant only among the older women (OR = 5.62; 95% CI, 1.01-31.48).

HPV prevalence was highest among the youngest age groups, but not significantly different between the two older ones, either in HCII or TaqMan assay (Table 3). The quantitative viral loads for HPV16, 18/45, 31 and 33 were markedly higher among the older women. The most interesting is the curve of HPV16 loads, as shown in Figure 2. It shows the best fit with the cubic model ($R^2 = 0.714$), resulting in a distinct biphasic S-shaped curve (Figure 3), with a sharp second rise among women > 50 years.

The distribution of individual HPV types was significantly different among the three age categories (Table 3). As compared with the youngest age groups, there was a marked shift from multiple-type infections (from 30.7% to 6.3%) to the accumulation of HPV16 (37.5% of HPV+ cases) and HPV31 (31.3%) among the older women. There was a transition from episomal to mixed and integrated state from the youngest age groups to the women over 55 years, in whom, all HPV16 positive lesions

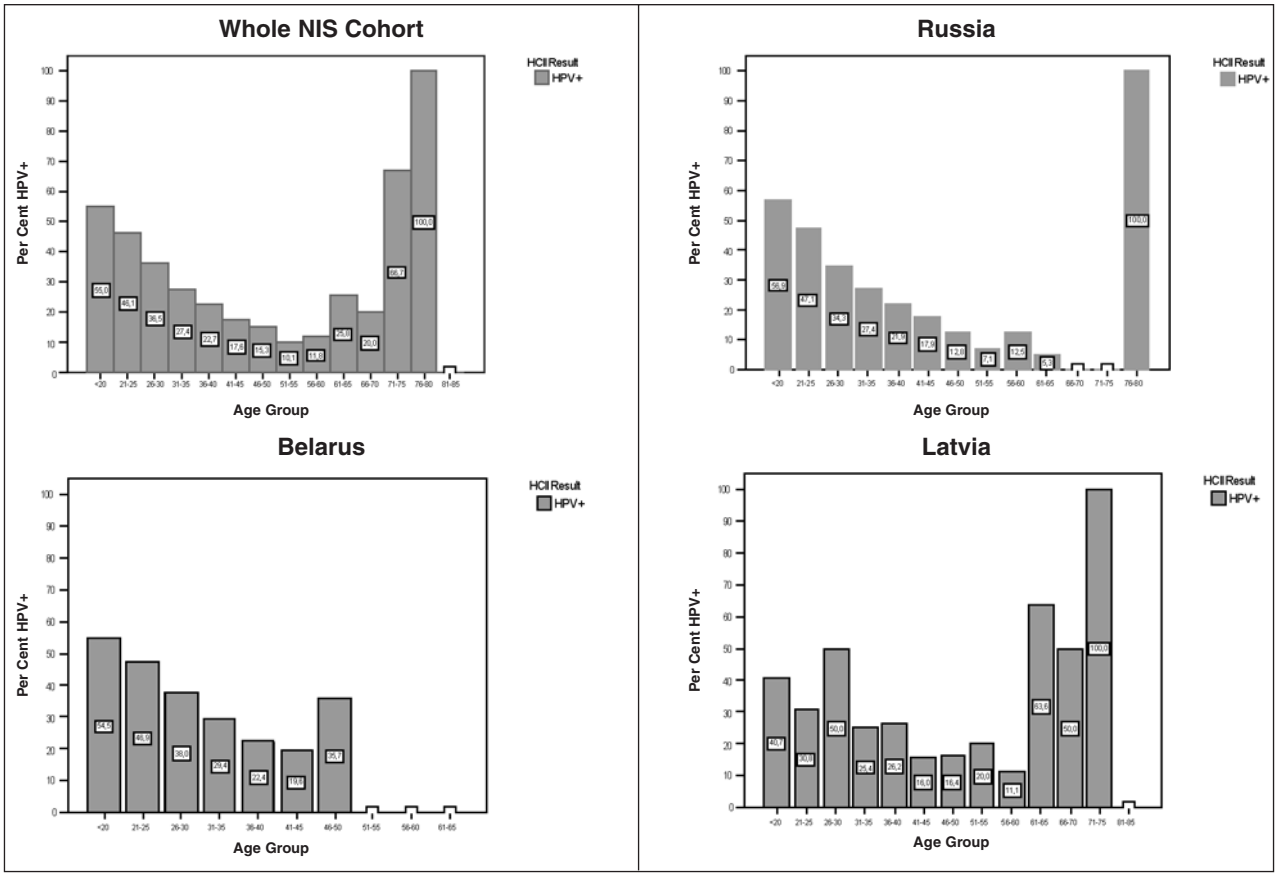

Figure 1. — Age-specific prevalence of HR-HPV infections in the three NIS countries.

showed viral integration (p = 0.009). Similarly, the viral load of integrated HPV16 was significantly higher (17.5) in the older women, as compared with the two other age categories, with practically identical loads of integrated HPV16.

There was no difference in the clinical course of the cervical disease as determined by the repeated Pap tests (Table 4). In contrast, the outcome of HR-HPV infections was significantly different between the three groups. As compared with the age group 26-55, in which a sharp decline of HR-HPV prevalence was characteristic (Figure 1), women over 55 years of age show i) higher proportion of incident infections, ii) higher rate of viral persistence, and particularly iii) lower rate of HR-HPV clearance (p = 0.0001).

Discussion

As emphasised in a recent editorial on this subject (35), several key questions await clarification to explain the observed increase in HPV prevalence among older women [19-22, 24-28]. These unanswered questions are: 1) Is the increased prevalence among elderly women due to i) viral persistence, or ii) acquisition of new infections? 2) How much of this increase is attributable to the cohort effect? 3) What is the role of changing sexual habits and other risk factors by age as determinants of acquisition or persistence of HPV infections? 4) Are the age-related differences between oncogenic- and non-oncogenic HPV types a potential cause of these differences? 5) Is there an age-dependence of other viral factors, particularly i) viral load, and ii) viral integration, and what is their contribution to increased prevalence among older women? 6) What is the influence of early (i.e., intrauterine, perinatal or early childhood) HPV exposure on the subsequent risk of HPV persistence in adult age? 7) Are there any age-specific differences in the outcome (persistence, progression, clearance) of HPV infections? In the present study, we provide answers to most of these questions, except for no. 6, which is being explored in our ongoing study on HPV transmission within families [45].

The age-specific prevalence curve for the entire cohort from the three different NIS countries was shown to be clearly U-shaped. This U-shaped curve fits almost perfectly (96.6%) with the quadratic model in logistic regression. This observation is consonant with the data reported in several other populations [19-22, 24-28]. However, the shape of these age-specific prevalence curves differed substantially among the three neighbouring countries. While the linear model fits best (95.2%) with the age-specific curve of Belarus, the quadratic model (U-curve) shows by far the closest fit in the two

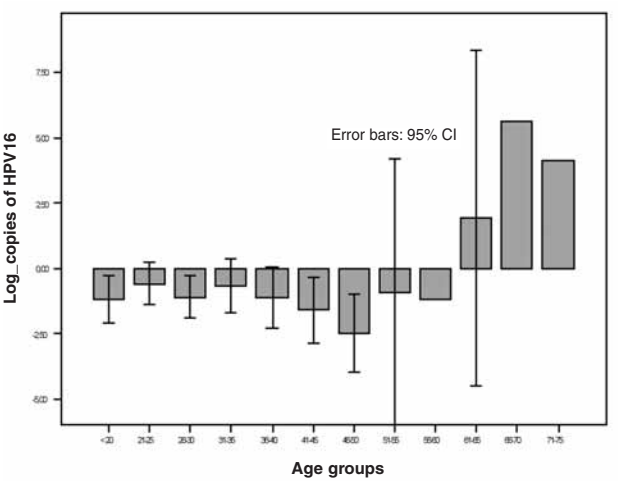

Figure 2. — Age-specific viral loads of HPV16.

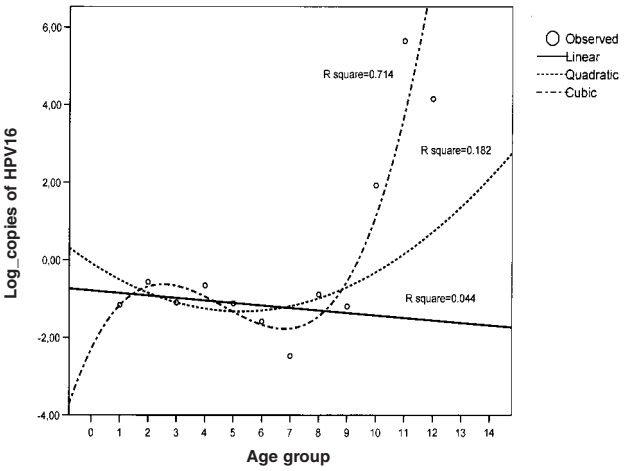

Figure 3. — Curve estimation for age-specific viral loads of HPV16.

others. Such a declining linear curve has been reported for a variety of populations in other geographic regions [29-34].

As to the implicated cohort effects [22, 35], our three birth cohorts (women born after 1974; those between 1973-1945; and those between 1914-1944) differed at the p = 0.0001 level with regard to the majority of the recorded epidemiological variables, including the patient category, Pap smear abnormalities and CIN lesions in the biopsy. These data clearly confirm that women born before 1945 did demonstrate a sexual behaviour and other risk factors markedly different from women born after 1974. Women between 26 and 55 years of age fall between these two extremes in many of the recorded variables, and it is frequently difficult to visualise whether they are closer to their younger or older counterparts. Yet, the age-specific HPV profile in these three groups was completely different. Our results lend support to the concepts discussed recently by Winer *et al*. [35], as to i) the different sexual habits in different birth cohorts (Table 1), and ii) the role of these divergent risk factors as determinants of HR-HPV among the different age groups. There was not a single predictive variable common to all three age categories, and few such predictors that were significant even in two of these categories. However, we could not provide direct evidence to support the notion that changing a sexual partner later in life might be the reason for increased HPV prevalence [25, 35]. In fact, the number of recent (past 24 months) sexual partners was almost identical (1.53 vs 1.33) among women between 26 and 55 years and those > 55 years, respectively. Similarly, although different in the three age categories, the use of oral contraception [11, 20, 25] could not be confirmed as a risk factor of HR-HPV in this cohort, as recently reported [39]. As to another implicated risk factor, i.e., current smoking [21, 25], it was significant only in the 26-55-year cohort, but not in the other age categories. This leaves little doubt that different factors are significant predictors in different birth cohorts.

This study provides essentially new data on the age-dependence of the key viral factors (type prevalence, viral load and viral integration status), which are on the short-list of the most pressing open issues [34, 35]. First of all, the prevalence of individual HPV types was significantly different among the three age categories. There was a marked shift from multiple-type infections (from 30.7% to 6.3%) among the younger women to accumulation of HPV16 (37.5% of HPV + cases) and HPV31 (31.3%) in the older women (p = 0.0001). A similar transition from multiple- to single-type infections has been reported in some previous studies [20, 21, 28], whereas in another one, multiple-type infections were shown to increase along with age [25].

We observed that the quantitative viral loads of HPV16, 18/45, 31 and 33 were markedly higher among the women > 55 years. The most interesting is the shape of the age-specific curve of HPV16 loads, showing the best fit (R^2 = 0.714) with the cubic model. This S-shaped curve of HPV16 viral load closely paralleled the age-specific HPV prevalence curve of the whole cohort. These data indicate that the second rise of HPV prevalence among the older women clearly coincides with the increased viral loads of all HR-HPV types analysed. In fact, all viral loads were highest among the older women.

Similarly, no previous data are available on the physical state and integration load of HR-HPV types in different age groups [35]. Such age-dependence of HPV integration was first suggested by us, while detecting that women with purely integrated HPV16 were almost ten years older than those with episomal HPV16 [44]. This was fully confirmed in the present analysis, where the physical state of HPV16 was significantly different among the three age categories (p = 0.005). There seems to be a distinct transition from episomal to integrated state with progressing age, and in women > 55 years, all HPV16 positive lesions showed viral integration. Importantly, also the quantitative load of integrated HPV16 seems to be significantly higher in these older women as

compared with the two other age categories (p = 0.009). This implicates that not only is viral integration increased, but also the load of the integrated virus is of different order of magnitude among the women > 55 years.

Finally, we demonstrated that the outcome of HR-HPV infections is significantly different between the three age categories. As compared with the age group 26-55 years, characterised by a sharp decline in HR-HPV prevalence, women over 55 years of age showed: i) higher proportion of incident infections, ii) higher rate of viral persistence, and particularly, iii) significantly lower rate of HR-HPV clearance. All this contributes to the fact that the prevalence of HR-HPV (21.3%) among women above 55 years is almost similar to that (24.5%) of the 26-55-year age group.

The present study casts more light on most of the unanswered questions to explain the differences in the age-specific prevalence of HR-HPV infections [35]. Accordingly, 1) the second peak in prevalence among women over 55 years seems to be equally contributed to by i) an increased viral persistence, ii) acquisition of new infections, and iii) decreased clearance of these infections. As to 2) the possible cohort effect, our data implicate that women born before 1945 did demonstrate a sexual behaviour and other risk factors markedly different from the women born after 1974. There is little doubt that 3) these changing sexual habits and other risk factors by age contribute to the different age-specific prevalence of HR-HPV infections. The present study fully confirmed 4) the age-dependence of the viral factors, i.e., i) type prevalence (shift from multiple- to single-type), ii) viral load, and iii) viral integration as explanatory factors of increased prevalence among the older women.

Taken together, the present results feasibly explain what was suggested by our in vitro studies some years back [42, 43, 46]. The rapid acquisition of HR-HPV infections after onset of sexual activity [12, 15] leads to an early peak of both HR-HPV prevalence and viral loads between 20 and 25 years of age. This is followed by a constant clearance (reduced viral loads) [13, 15] of the infections between 25 and 55 years of age. In women > 55, a sharp increase in both HPV prevalence and viral loads follows, shown by the U-shaped and S-shaped age-specific curves, respectively. These data implicate that in women who fail to eradicate their HR-HPV infection by menopause, selection of an integrated viral clone has likely taken place, driving the process towards an aggressively progressing disease. Consequent to this, most of the HR-HPV infections in women older than 55 years were associated with high-grade CIN or invasive carcinoma in the present cohort.

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