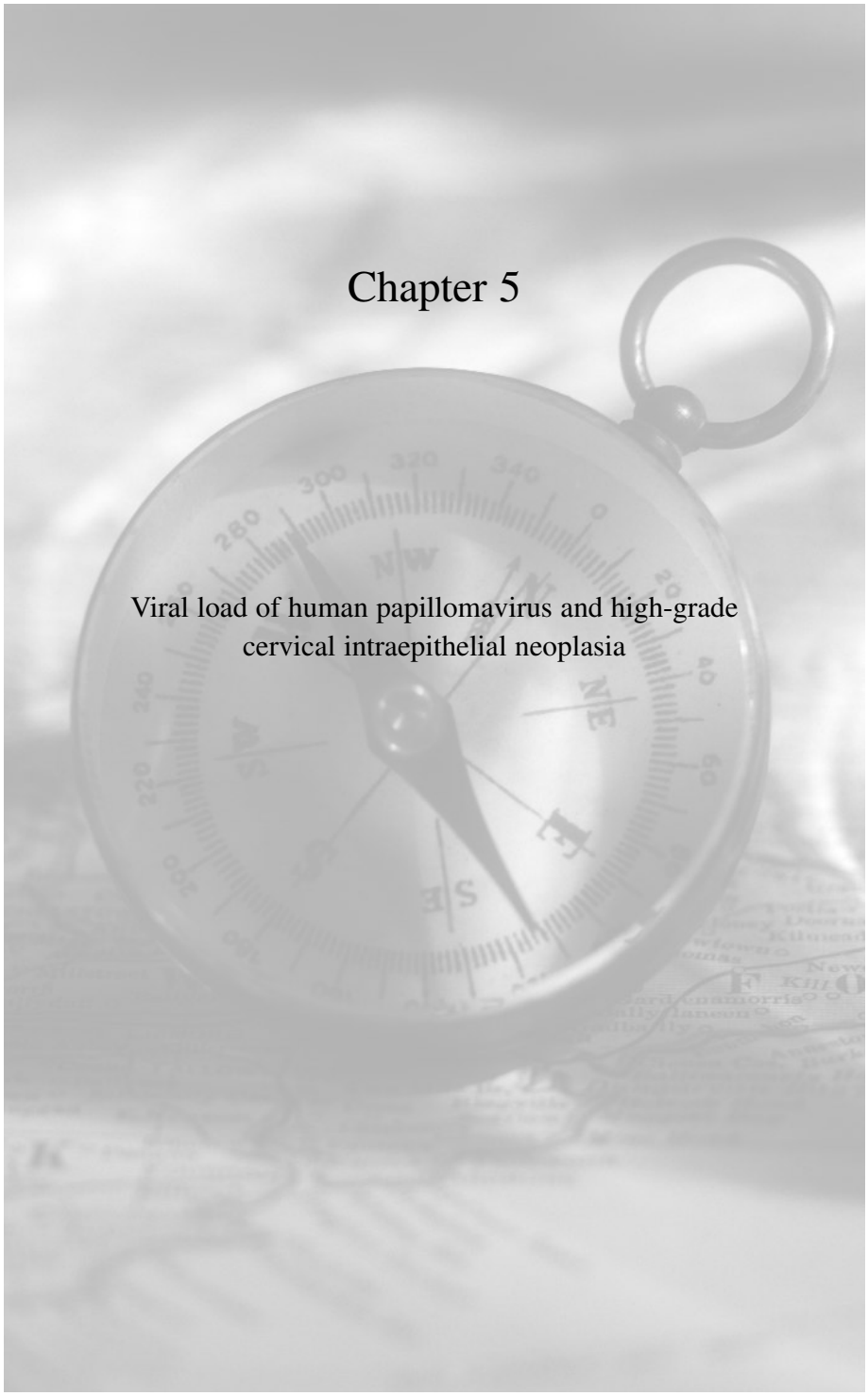
Triaging equivocal cytology of the cervix : identyfying women at risk for high-grade cervical lesions

Wensveen, C.W.M.



Chapter 5

Viral load of human papillomavirus and high-grade cervical intraepithelial neoplasia

Abstract

Objective: To assess the association between viral load and cervical intraepithelial neoplasia and to explore alternative human papillomavirus test cut-off points for detecting high-grade lesions.
Method: In this cohort study we evaluated the viral load of the human papillomavirus (HPV) semi-quantitatively by the relative light units (RLU) values provided by the Hybrid Capture II HPV assay in 473 women with abnormal cytology from a hospital based population, and followed them up for cervical intraepithelial neoplasia (CIN).
Result: There was no significant difference in the mean viral load among the grades of CIN and overlapping of viral loads was seen among the grades of CIN. The highest accuracy of the standard HPV test was found in women with borderline or mild dyskaryosis, older than 30 years of age, and a less than six months interval between HPV and histology. Cut-off point of 1.0 (standard) and 3.0 relative light units/ positive control (RLU/PC) of the HPV test for detecting high-grade CIN in these women showed a sensitivity of 95% and 93%, respectively. A cut-off point of 3.0 resulted in a more specific diagnostic test with a specificity of 37% compared to 20% for the standard.
Conclusion: Viral load is not a useful marker over HPV positivity alone to identify women at risk for high-grade lesions of the cervix. In this study the accuracy of the HPV test was better for women over 30 years of age and low-grade cytology. The possibility of a higher cut-off point of the HPV test can be considered.

Introduction

Epidemiological and molecular data suggest that persistent infections with high-risk (oncogenic) human papillomavirus (HPV) are obligatory for cervical intraepithelial neoplasia (CIN) and cervical cancer (odds ratio: 60, 95% CI 49-73)[1-3]. The association between HPV and CIN is very strong, independent of other risk factors, and is consistently found in many countries[4]. However, only a small fraction of women with persistent HPV infection will eventually develop cervical cancer. Most HPV infections in young women are transient (80%)[5] and clear within 8.2-13.5 months[6]. However, occasionally (for poorly understood reasons) infection progresses to viral integration into the chromosomes of host cells, which is necessary for cell transformation and the development of CIN[7, 8].
Oncogenic HPV is found almost universally in cervical cancer[9, 10]. The sensitivity of the standard (≥ 1.0 cut-off point) HPV Hybrid capture II test for detection of high-grade CIN varies from 55.7% to 93.3%. However, the studies reporting the highest sensitivity rates showed the lowest specificity rates of 24.2% to 66.8%[11].
It has also been reported that viral load as estimated by the intensity of the hybridization signal positively correlates with the risk of high-grade CIN[2, 12-14]. Nevertheless, the

clinical usefulness of measuring viral load is still moot, as earlier studies used a wide range of designs, laboratory assays, and analytical methods. One study showed that viral loads overlapped considerably among grades of CIN and did not increase with severity of disease, and no definition of an optimal cut-off value was given[15].

In this cohort study we used the relative light units of the Hybrid Capture II HPV test to assess the semi-quantitative viral load of HPV in women with abnormal cytology in relation to cytology at enrolment and histological outcome over time. To improve the HPV test for predicting high-grade CIN (CIN II/ III) different cut-off points were compared with the standard ($\geq$ 1.0 RLU/PC).

Materials and Methods

In this prospective study, between June 1998 and July 2002, a Hybrid Capture II (HC-II) HPV test was done in 1565 women with an abnormal liquid-based Pap test from a hospital based population in Rotterdam, the Netherlands. Abnormal Pap test was defined as borderline, mild, moderate, or severe dyskaryosis, carcinoma in situ or cervical cancer[16]. The Pap smear was diagnosed in 5.6% as borderline, 1.1% as mild dyskaryosis, 0.9% as moderate dyskaryosis, 0.4% as severe dyskaryosis, 0.1% as carcinoma in situ, and in 0.02% as cervical cancer. All liquid-based cytology was sent to the Cyto-diagnostic Research Laboratory Rotterdam. Two pathologists of this laboratory reviewed all abnormal Pap smears. The Hybrid Capture II HPV tests were done conforming to the standard protocol (see "detection of HPV" below). In 582 of the 1565 women with abnormal Pap smear histology was obtained during follow-up. Follow-up biopsies of treated CIN lesions (109) were excluded. All 473 histology samples were diagnosed by two experienced pathologists and were classified according to CIN classification (WHO). Biopsy specimens revealing koilocytotic atypia were included in the CIN I classification. Histological diagnoses of squamous metaplasia, immature squamous metaplasia, reactive changes, and inflammatory atypia were classified as no CIN. Of the 473 included abnormal cytology specimens 208 were diagnosed as borderline, 103 as mild dyskaryosis, 99 as moderate dyskaryosis, and 51 as severe dyskaryosis, eight as carcinoma in situ, and four as cervical cancer at enrolment.

Detection of HPV

Specimens for HPV were collected from the cervix. Thirteen high/ intermediate-risk types of HPV DNA (16/18/31/33/35/39/45/51/52/56/58/59/68) were detected using the Hybrid Capture II™ technology, a signal amplified hybridization antibody capture microplate assay using chemiluminescence for the quantitative detection of human papillomavirus DNA in cervical specimens (Digene Corporation, Gaithersburg, Maryland,

USA). Signal strengths in relative light units were compared to 1 pg/ml HPV positive controls (RLU/PC), and specimens with ratios ≥ 1.0 were deemed positive (as described in detail previously)[17]. The relative positive light units provided by the HC-II assay semi-quantitatively were used to measure viral load. Different cut off points of RLU/PC were compared to the standard of ≥ 1.0 RLU/PC cut-off point.

Statistics

The Mann Whitney test was used to compare viral loads among the classes of cytology (Bethesda classification) and also among the grades of CIN (WHO). The Spearman's correlation co-efficient (Rho) was computed to explore the association between the viral load and CIN. Two-sided p-values of ≤0.05 were considered as significant.

Sensitivity, specificity, and the area under the receiver operating characteristic curve (aROC) were calculated to express the accuracy of the HPV test[18].

The receiver operating characteristic curve was evaluated to estimate an optimal test-positive cut-off point for the hybrid capture test to predict high-grade CIN. Stratification for age and interval between HPV test and histology were done.

Results

The mean age and the mean viral load of the 473 Pap women at enrolment and of the histological outcome over time are shown in table 1. Mild dyskaryosis showed a significantly higher mean viral load than borderline cytology (457 and 342 RLU, respectively, Mann-Whitney test, p=0.002). There was a non-significant difference of mean viral load between the other cytological classes and the viral loads overlapped considerably among these classes (Table 1, Figure 1).

Table 1. Mean age and mean viral load of women with abnormal cytology at enrolment (N=473).

	Mean Age in years	Mean Viral Load RLU*
Cytology at enrolment		
Borderline (N=208)	36 (17-63)	342 (0.1-2793)
Mild dyskaryosis (N=103)	37 (17-63)	457 (0.2-2853)
Moderate dyskaryosis (N=99)	37 (22-60)	553 (0.3-3487)
Severe dyskaryosis (N=51)	38 (25-66)	494 (1.4-2267)
Carcinoma in situ (N=8)	43 (34-65)	349 (5.5-1109)
Carcinoma (N=4)	54 (39-60)	409 (0.5-1627)
Histological outcome		
No CIN† (N=114)	40 (19-66)	204 (0.1-2793)
CIN I (N=112)	35 (17-60)	441 (0.2-2493)
CIN II (N=139)	35 (17-60)	529 (0.2-3487)
CIN III (N=97)	37 (18-66)	573 (0.9-2526)
Cervical cancer (N=11)	45 (33-60)	67 (0.5-238)
Total (N=473)	37 (17-66)	428 (0.1-3487)

*RLU= relative light units. †CIN= cervical intraepithelial neoplasia

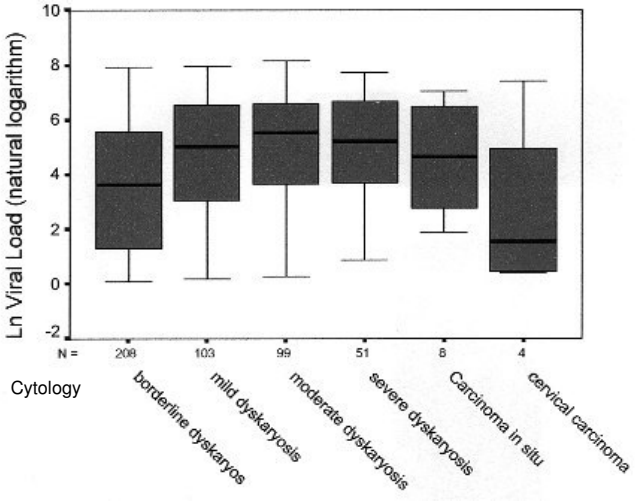


Figure 1. Boxplot of viral load and cytology

CIN showed a significantly higher mean viral load than normal histology (513 and 204 RLU, respectively, Mann-Whitney test, $p<0.0005$). Although the number of cervical cancers was small, we found a significantly lower mean viral load for cervical cancer than for CIN III (67 and 573 RLU, respectively, Mann-Whitney test, $p=0.001$). Unfortunately, there was no significant difference in the mean viral load among the grades of CIN and overlapping of viral loads was seen among the grades of CIN (Table1, Figure 2).

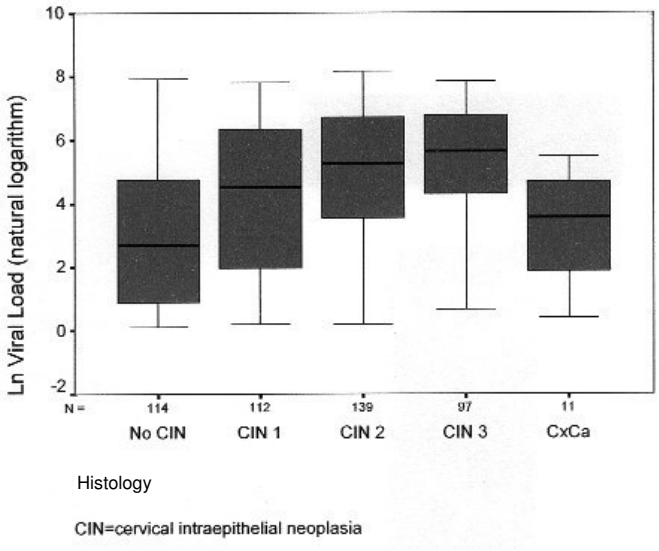


Figure 1. Boxplot of viral load and cytology

However, of the 234 women with a viral load of ≥ 100 RLU 63% showed histological CIN II or III compared to 32% of the 74 women with viral load between ≥1 and 10 RLU at enrolment.

Of the 311 women with borderline or mild dyskaryosis 37% showed CIN II/ III over time compared to 74.7% of the 162 women with moderate dyskaryosis or more at enrolment. In table 2 we show the risk of CIN II or III in women with borderline or mild dyskaryosis stratified into four groups of viral load (0-<1, ≥1-10, ≥10-100, ≥100 RLU/PC respectively). In women with borderline or mild dyskaryosis we found that the risk of CIN II/ III increased with viral load (Table 2).

Table 2. Histological outcome in women with borderline or mild dyskaryosis at enrolment for four categories of viral load (N=311).

Viral Load	Histology					
RLU*	No CIN†	CIN I	CIN II	CIN III	CxCa	Total
	N (%)	N(%)	N(%)	N(%)	N(%)	N(%)
0-1	25 (59.5)	12 (28.6)	4 (9.5)	1 (2.4)		42 (100)
≥1-10	23 (40.4)	21 (36.8)	11 (19.3)	2 (3.5)		57 (100)
≥10-100	25 (33.3)	21 (36.8)	22 (29.3)	7 (9.3)		75 (100)
≥100	22 (16.1)	46 (33.6)	50 (36.5)	18 (13.1)	1 (0.7)	137 (100)
Total	95 (30.5)	100 (32.2)	87 (28)	28 (9)	1 (0.3)	311 (100)

* RLU= relative light unit. †CIN= cervical intraepithelial neoplasia

We could not find such a trend for women with moderate dyskaryosis or more at enrolment (Table 3)

Table 3. Histological outcome in women with moderate dyskaryosis or more at enrolment for four categories of viral load (N=162).

Viral Load	Histology					
RLU*	No CIN†	CIN I	CIN II	CIN III	CxCa	Total
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
0-1	1 (20)		2 (40)		2 (40)	5 (100)
≥1-10	3 (17.6)	1 (5.9)	4 (23.5)	7 (41.2)	2 (11.8)	17 (100)
≥10-100	8 (18.6)	3 (7)	17 (39.5)	12 (27.9)	3 (7)	43 (100)
≥100	7 (7.2)	8 (8.2)	29 (29.9)	50 (51.5)	3 (3.1)	97 (100)
Total	19 (11.7)	12 (7.4)	52 (32.1)	69 (42.6)	10 (6.2)	162 (100)

* RLU= relative light unit. †CIN= cervical intraepithelial neoplasia

To explore the association between viral load and CIN we computed the Spearman's Correlation Coefficient (Rho). There was a marked association between viral load and CIN (Rho 0.37, p<0.0001). The highest strength of association between viral load and CIN was seen in women between 30 and 40 years of age (Rho 0.43, p<0.0001). The association was less strong for women younger than 30 years of age (Rho 0.28, p<0.005). Stratification by the interval between the HPV test and histological sample showed that there was no significant association between viral load and CIN, for intervals longer than six months (Rho 0.22, P=0.07).
The purpose of the HPV test is to accurately predict high-grade CIN (CIN II/ III). We used a ROC-curve to explore the sensitivity and specificity at different cut-off points of the HPV test for predicting high-grade CIN (Figure 3).

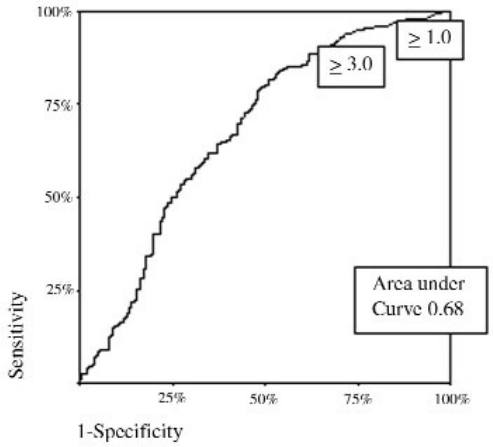


Figure 3. ROC-curve for Hybrid Capture II HPV test to predict high-grade CIN

For women with abnormal cytology the area under the ROC-curve for the Hybrid Capture II HPV test to predict ≥ CIN II was 0.68. To predict CIN III only, the area under the ROC-curve was 0.62. The HPV test for women younger than 30 years of age appeared to be less accurate for predicting ≥ CIN II (aROC 0.60). Stratification by the interval between HPV test and histology did not significantly change the accuracy of the HPV test to predict high-grade CIN, for intervals less than six months. For longer than six months intervals a decrease in the accuracy of the HPV test was seen (aROC 0.58). The sensitivity of the Hybrid Capture II HPV test at the standard cut-off point of 1.0 RLU/PC was 96% and the specificity was 17% for predicting high-grade CIN. Increasing the standard cut-off point to 3.0 tdecreased the sensitivity to 93% and increased the specificity to 29%. The sensitivity and specificity of the HPV test at the 1.0 and the 3.0 cut-off point for predicting CIN III was not significantly different. The sensitivity and specificity of the HPV test for the cytology subclasses to predict CIN II/ III are shown in table 4.

Table 4. Sensitivity and Specificity of the Hybrid Capture II HPV test at a cut-off point of ≥1.0 RLU/PC (standard) and ≥3.0 RLU/PC* to predict CIN# II/ III in women with borderline/ mild dyskaryosis and moderate/ severe dyskaryosis.

	Human papillomavirus test			
	≥1.0 cut-off point		≥3.0 cut-off point	
Diagnosis of cytology	Sensitivity	Specificity	Sensitivity	Specificity
Borderline/ mild dyskaryosis	95%	19%	94%	31%
Moderate/ severe dyskaryosis	98%	4%	94%	15%

*RLU=relative light units/PC=positive controls, # CIN=cervical intraepithelial neoplasia.

The highest accuracy of the HPV test to predict high-grade CIN was found in women with low-grade cytology (borderline or mild dyskaryosis), over 30, and a ≤ 6 months interval between the HPV test and histology. The area under this ROC-curve was 0.74. The HPV test at a ≥ 3.0 cut-off point showed a sensitivity of 93% and a specificity of 37% compared to 95% and 20%, respectively, for the standard HPV test (cut-off point at ≥ 1.0 RLU/PC).

Discussion

In this study we found significantly higher viral loads in CIN lesions than in normal cervical tissues, but cervical cancer showed significantly lower viral loads than CIN III. Like others[12;14;15], we also found a considerable overlap of viral loads among the grades of CIN. The life cycle of the papillomavirus within the infected host cells can be divided into early and late stages, which are linked to the differentiation state of the epithelial cells. In the early stage, a low level of viral replication is maintained in the nucleus of the basal cell. The late stage occurs in terminally differentiated epithelial cells and is associated with vegetative viral DNA replication[19-21]. Integration of viral DNA into the chromosomes of the host-cells occurs in the basal cells, which is necessary for cell transformation and the development of CIN and cervical cancer[22]. Although not all cervical carcinomas contain only integrated viral genomes[8], a HPV test that only measures the viral load of integrated viral DNA would probably improve detection of high-grade CIN. Unfortunately, the hybrid Capture II HPV test measures all replicated viral DNA, which may partly explain the wide overlap of viral load among CIN. Therefore viral load measured by HC-II seems not to be a useful marker to identify women at risk for high-grade CIN.

Studies have suggested that higher viral loads of HPV evaluated by sensitive quantitative PCR assay are associated with progression to CIN III over time[13, 14]. In this study we found no significant association between semi-quantitative viral load and CIN for intervals longer than 6 months. Apparently, viral loads cannot accurately predict the

long-term risk of CIN. This is consistent with findings by Lorincz et al[23], who found that the relative risk for CIN III in the first 9 months increased with semi-quantitative viral load, but not for longer intervals. The median retention time (8 months) of a high/intermediate risk HPV infection may explain the waning of association with time[24, 25].

The purpose of the HPV test is to predict high-grade CIN. To determine the accuracy of the HPV test for predicting high-grade CIN we computed the area under the ROC-curve (aROC)[18;19]. We found that the accuracy of the HPV test improved for women over 30 years of age. The accuracy of the Hybrid Capture II test for predicting high-grade CIN decreased with the duration between the HPV test and histology.

In this study the specificity at the standard cut-off point (≥ 1.0) was only 4% in women with moderate or severe dyskaryosis, and the false-positive rate was 17%. These findings indicate the futility of a HPV test in women with moderate or severe dyskaryosis. The highest accuracy of the standard HPV test was seen in women over 30 with low-grade cytology (borderline or mild dyskaryosis) and less than 6 months interval between HPV and histology. Increasing the cut-off point to 3.0 RLU/PC improved the specificity from 20% to 37%, with only two percent decrease of the sensitivity (95% to 93%). Thus, fewer women with low-grade cytology would have a false-positive HPV test at a cut-off point of ≥ 3.0, thereby reducing the number of colposcopies without substantially decreasing the risk of missing high-grade CIN lesions.

In conclusion, there is a considerable overlap of viral loads among the grades of CIN and viral loads cannot predict the long-term risk of CIN. Therefore, viral load is not a useful marker over HPV positivity alone. In this study the accuracy of the HPV test was better in women over 30 years of age and low-grade cytology. The possibility of a higher cut-off point of the HPV test can be considered.

References

(1) Nobbenhuis MA, Walboomers JM, Helmerhorst TJ, Rozendaal L, Remmink AJ, Risse EK, van der Linden HC, Voorhorst FJ, Kenemans P, Meijer CJ. Relation of human papillomavirus status to cervical lesions and consequences for cervical-cancer screening: a prospective study. Lancet 1999 Jul 3;354(9172):20-5.

(2) Ho GY, Palan PR, Basu J, Romney SL, Kadish AS, Mikhail M, Wassertheil-Smoller S, Runowicz C, Burk RD. Viral characteristics of human papillomavirus infection and antioxidant levels as risk factors for cervical dysplasia. Int J Cancer 1998 Nov 23;78(5):594-9.

(3) Bosch FX, Munoz N, de Sanjose S, Navarro C, Moreo P, Ascunce N, Gonzalez LC, Tafur L, Gili M, Larranaga I, . Human papillomavirus and cervical intraepithelial neoplasia grade III/carcinoma in situ: a case-control study in Spain and Colombia. Cancer Epidemiol Biomarkers Prev 1993 Sep;2(5):415-22.

(4) IARC Working Group. Human Papillomaviruses. IARC Monograph on the evaluation of carcinogenic risks to humans. 65 ed. Lyon, France: International Agency for Research on Cancer; 1995.

(5) Hildesheim A, Schiffman MH, Gravitt PE, Glass AG, Greer CE, Zhang T, Scott DR, Rush BB, Lawler P, Sherman ME, . Persistence of type-specific human papillomavirus infection among cytologically normal women. J Infect Dis 1994 Feb;169(2):235-40.

(6) Franco EL, Villa LL, Sobrinho JP, Prado JM, Rousseau MC, Desy M, Rohan TE. Epidemiology of acquisition and clearance of cervical human papillomavirus infection in women from a high-risk area for cervical cancer. J Infect Dis 1999 Nov;180(5):1415-23.

(7) Ferenczy A, Franco E. Persistent human papillomavirus infection and cervical neoplasia. Lancet Oncol 2002 Jan;3(1):11-6.

(8) Cullen AP, Reid R, Campion M, Lorincz AT. Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasm. J Virol 1991 Feb;65(2):606-12.

(9) Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Munoz N. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 1999 Sep;189(1):12-9.

(10) Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J, Schiffman MH, Moreno V, Kurman R, Shah KV. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. J Natl Cancer Inst 1995 Jun 7;87(11):796-802.

(11) Kaufman RH, Adam E. Is human papillomavirus testing of value in clinical practice? Am J Obstet Gynecol 1999 May;180(5):1049-53.

(12) van Duin M, Snijders PJ, Schrijnemakers HF, Voorhorst FJ, Rozendaal L, Nobbenhuis MA, van den Brule AJ, Verheijen RH, Helmerhorst TJ, Meijer CJ. Human papillomavirus 16 load in normal and abnormal cervical scrapes: an indicator of CIN II/III and viral clearance. Int J Cancer 2002 Apr 1;98(4):590-5.

(13) Josefsson AM, Magnusson PK, Ylitalo N, Sorensen P, Qwarforth-Tubbin P, Andersen PK, Melbye M, Adami HO, Gyllensten UB. Viral load of human papilloma virus 16 as a determinant for development of cervical carcinoma in situ: a nested case-control study. Lancet 2000 Jun 24;355(9222):2189-93.

(14) Ylitalo N, Sorensen P, Josefsson AM, Magnusson PK, Andersen PK, Ponten J, Adami HO, Gyllensten UB, Melbye M. Consistent high viral load of human papillomavirus 16 and risk of cervical carcinoma in situ: a nested case-control study. Lancet 2000 Jun 24;355(9222):2194-8.

(15) Sherman ME, Schiffman M, Cox JT. Effects of age and human papilloma viral load on colposcopy triage: data from the randomized Atypical Squamous Cells of Undetermined Significance/Low-Grade Squamous Intraepithelial Lesion Triage Study (ALTS). J Natl Cancer Inst 2002 Jan 16;94(2):102-7.

(16) Hanselaar AG. Criteria for organized cervical screening programs. Special emphasis on The Netherlands program. Acta Cytol 2002 Jul;46(4):619-29.

(17) Schiffman M, Herrero R, Hildesheim A, Sherman ME, Bratti M, Wacholder S, Alfaro M, Hutchinson M, Morales J, Greenberg MD, Lorincz AT. HPV DNA testing in cervical cancer screening: results from women in a high-risk province of Costa Rica. JAMA 2000 Jan 5;283(1):87-93.

(18) Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, Moher D, Rennie D, de Vet HC, Lijmer JG. The STARD statement for reporting studies of diagnostic accuracy: explanation and elaboration. Ann Intern Med 2003 Jan 7;138(1):W1-12.

(19) Howley PM. Papillomavirinae: The viruses and their replication. In Fields BN, et al (eds): Fields Virology.Philadelphia: Lippencott-Raven; 1996. p. 2045-76.
(20) Frattini MG, Lim HB, Laimins LA. In vitro synthesis of oncogenic human papillomaviruses requires episomal genomes for differentiation-dependent late expression. Proc Natl Acad Sci U S A 1996 Apr 2;93(7):3062-7.
(21) Syrjanen SM, Syrjanen KJ. New concepts on the role of human papillomavirus in cell cycle regulation. Ann Med 1999 Jun;31(3):175-87.
(22) Bosch FX, Munoz N. The viral etiology of cervical cancer. Virus Res 2002 Nov;89(2):183-90.
(23) Lorincz AT, Castle PE, Sherman ME, Scott DR, Glass AG, Wacholder S, Rush BB, Gravitt PE, Schussler JE, Schiffman M. Viral load of human papillomavirus and risk of CIN3 or cervical cancer. Lancet 2002 Jul 20;360(9328):228-9.
(24) Eduardo CE, Jose CM, Kasumi SN, Araujo MF. Papillomavirus in cervicovaginal smears of women infected with human immunodeficiency virus. Rev Paul Med 1995 Nov;113(6):1009-11.
(25) Bory JP, Cucherousset J, Lorenzato M, Gabriel R, Quereux C, Birembaut P, Clavel C. 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 Dec 10;102(5):519-25.