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Changes in type-specific human papillomavirus load predict progression to cervical cancer

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Abstract

Persistent high-risk human papillomavirus (HPV) infection is strongly associated with the development of high-grade cervical intraepithelial neoplasia or cancer (CIN3+). However, HPV infection is common and usually transient. Viral load measured at a single time-point is a poor predictor of the natural history of HPV infection. The profile of viral load evolution over time could distinguish HPV infections with carcinogenic potential from infections that regress. A case-cohort natural history study was set-up using a Belgian laboratory database processing more than 100,000 liquid cytology specimens annually. All cytology leftovers were submitted to real-time PCR testing identifying E6/E7 genes of 17 HPV types, with viral load expressed as HPV copies/cell. Samples from untreated women who developed CIN3+ ($n = 138$) and women with transient HPV infection ($n = 601$) who contributed at least three viral load measurements were studied. Only single-type HPV infections were selected. The changes in viral load over time were assessed by the linear regression slope for the productive and/or clearing phase of infection in women developing CIN3+ and women with transient infection respectively. Transient HPV infections generated similar increasing (0.21 copies/cell/day) and decreasing (−0.28 copies/cell/day) viral load slopes. In HPV infections leading to CIN3+, the viral load increased almost linearly with a slope of 0.0028 copies/cell/day. Difference in slopes between transient infections and infections leading to CIN3+ was highly significant ($P < .0001$). Serial type-specific viral load measurements predict the natural history of HPV infections and could be used to triage women in HPV-based cervical cancer screening.

Keywords: Viral doubling time • virologic model • cervical intraepithelial neoplasia • liquid-based cytology leftover • real-time quantitative PCR

Introduction

Momentum builds towards the understanding and awareness that persistent infection with high-risk human papillomavirus (HPV) is the primary risk factor for the development of high-grade cervical intraepithelial neoplasia (CIN) and cancer (CIN3+) [1–3]. Although many questions remain unanswered regarding the natural history of HPV infection and CIN3+ [4], it is now generally accepted that loss of the episomal form of the viral DNA and its integration with the host cell genome is probably a key event in carcinogenesis [5].

Cervical cancer screening programmes have been based on cytologic Papanicolaou (Pap) tests, which show moderate cross-sectional sensitivity for detection of CIN3+. High-risk HPV-based screening considerably increases sensitivity [ratio: 1.28 (95% CI 1.12–1.47)], but its specificity is significantly lower compared with cytology-based screening [0.93 (95% CI 0.91–0.95)] [6]. The lower specificity is due to the high number of transient HPV infections and associated low-grade cytological abnormalities. At the current turning point in screening procedure, cytology is making way for primary HPV screening [7, 8]. In this setting, adequate triage of HPV-positive women remains a key problem.

Until now, most viral load studies focused on HPV 16 or HPV 18 [9–18], and few on additional high-risk HPV types [9, 10]. Also, single-point studies on HPV viral load measurement added little or no

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information about the probability of developing cervical cancer [11, 16, 19] and some studies suggested that serial viral load measurements are indicated [11, 16].

Repeated non-quantitative HPV testing was shown to be associated with a significantly higher probability of developing CIN3+, particularly with high-risk HPV types 16, 18, 31 and 33 [20, 21]. In the majority of cases, studies using sequential quantitative viral load measurements showed a significant decrease in HPV or clearance; viral load increase occurred only in a minority of individuals and was associated with a modest increased risk of acquiring a cytologic abnormality [11, 16, 19, 22, 23].

This large case-cohort natural history study analyses the variations in type-specific HPV loads over time for single HPV types in a control group with transient infections (no CIN3+; HPV clearance) and a patient group developing CIN3+ lesions. Considering the change in viral load over time, expressed as HPV copies per cell in single-type infections, a model is proposed distinguishing transient from persistent progressive infections leading to CIN3+.

Materials and methods

Samples for HPV analyses

This laboratory-based, case-cohort natural history study was set-up using the RIATOL cervical cancer screening and diagnostic follow-up database. The laboratory for clinical pathology (RIATOL), Antwerp, Belgium, is processing more than 100,000 liquid-based cytology samples annually, representing approximately one quarter of women participating in cervical cancer screening in Belgium. Since June 2006, all liquid-based cytology leftovers (n = 582,781 samples from 291,883 women) were subjected to HPV measurements to enable HPV-guided cytology [24]. Therefore, cytology is not considered an independent parameter and was not used in the building of the virologic model. Cervical samples were collected in the framework of cervical cancer screening. In Belgium, costs for collection and interpretation of Pap smears are partially reimbursed. This was done without interval constraint until mid-2009 and, since then, on biennial basis for screening unless previous samples were abnormal. Local follow-up guidelines for management of

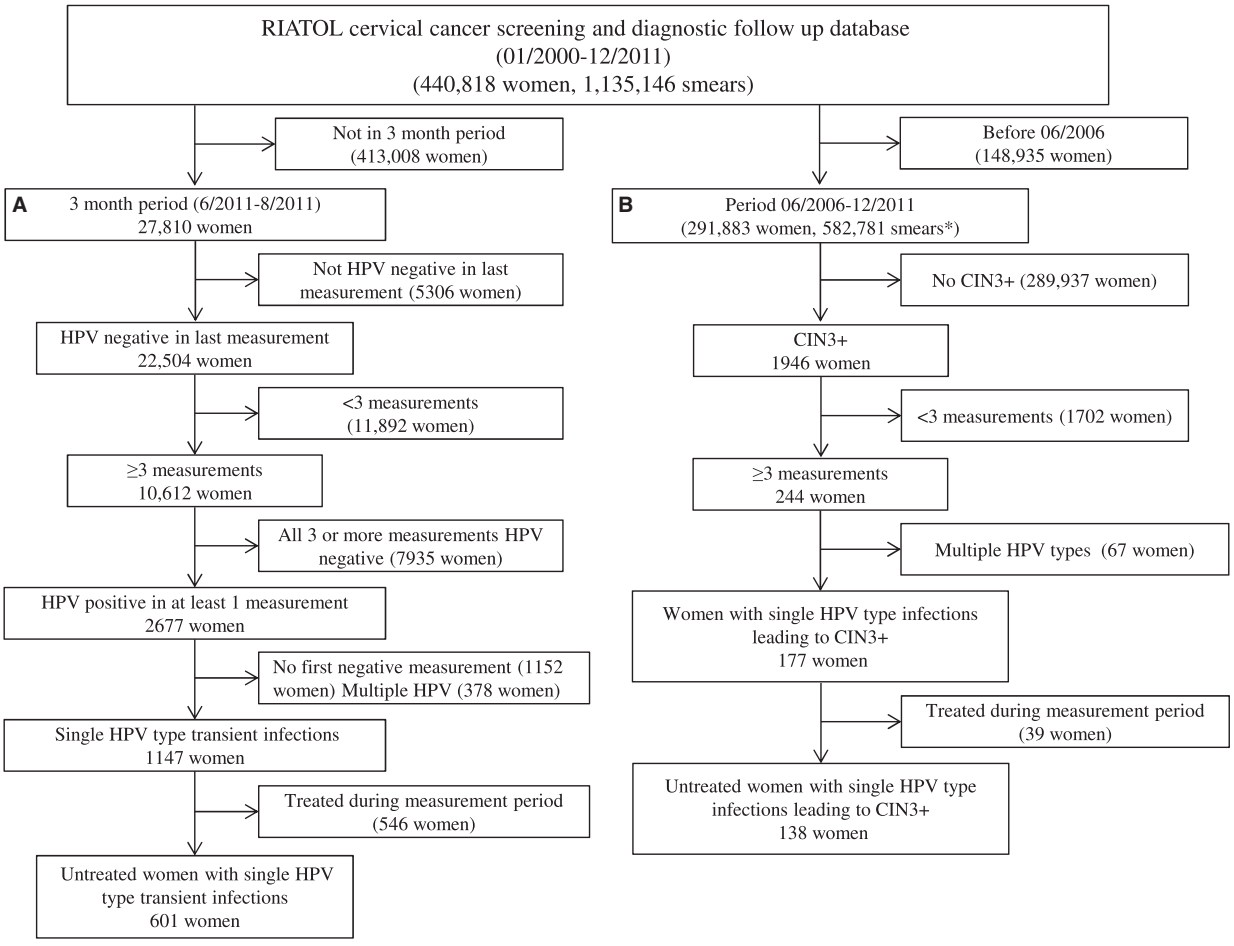

Fig. 1 Study Profile. (**A**) Selection of women with transient single-type HPV infections. (**B**) Selection of women with single-type HPV infections leading to CIN3+. * = Since June 2006 HPV genotyping was always performed before cytology reading.

cervical abnormalities are in agreement with EU guidelines [25, 26]. The RIATOL cervical cancer screening and diagnostic follow-up database contains all the cytologic, histologic, treatment and virologic data, which are linked using a unique case identification (ID) number. Patients were selected by the following inclusion criteria: women with a single-type HPV infection and who have not received any treatment of the cervix during the measurement period. For transient infections all consecutive patients presenting in a 3-month period (June to August 2011) with at least three consecutive measurements that start with a negative HPV result on time t_{0-1}, followed by one or more positive results, with t_{max} being the time with the highest viral load, and ending with a negative result on time t_{0+1} were included (Figs 1and 2).

For persistent infections leading to CIN3+ the whole database was searched. Only patients with at least three consecutive viral load measurements followed by histologically proven CIN3+ were included (Fig. 1). This study was approved by the local ethical committee (University Hospital Antwerp, Antwerp, Belgium).

Isolation of DNA from cervical cells for HPV DNA testing

Cervical cells were collected using the Cervex-Brush® Combi (Rovers, Oss, The Netherlands) as recommended in EU guidelines [27]. After collection, the head of the brush was left in the vial containing the ethanol-based BD SurePath™ Preservative Fluid (BD SurePath™; BD Diagnostics – TriPath, Burlington, NC, USA). The vials were then transported to RIATOL, Department of Molecular Diagnostics, Sonic Healthcare Benelux, Antwerp, Belgium, where all samples were prepared. A density sedimentation method (BD PrepMate™; BD Diagnostics – Tripath) was used to enrich the cell samples by removing obscuring elements such as blood, inflammatory cells, necrotic debris and mucus.

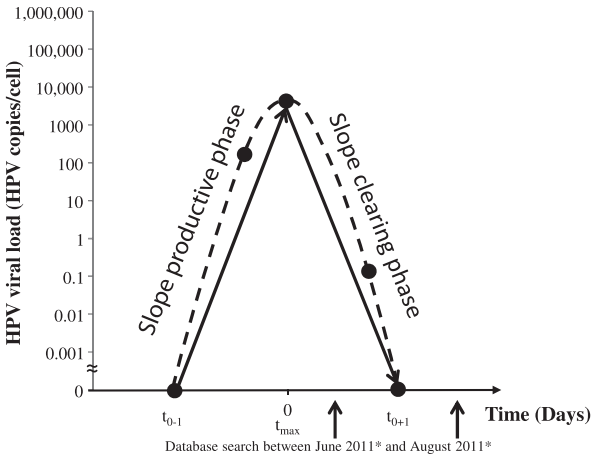

Fig. 2 Transient Infections. t_{0-1} = time of the last negative measurement before the transient infection. t_{0+1} = time of the first negative measurement after the transient infection. t_{max} = time of highest measured viral load. Circles represent viral load measurement on liquid-based cytology leftover. The slopes were calculated between the lowest and highest viral load measurement on t_{max}. To calculate logarithms at t_{0-1} and t_{0+1}, a value of 1E-99 was used as viral load. * = database search identified consecutive transient infections between June and August 2011.

DNA was isolated from the cellular pellet remaining after cytologic processing as previously described in earlier work [28].

Real-time type-specific PCR analysis of HPV DNA

Each DNA extract was subjected to real-time quantitative PCR for the detection of 17 different HPV types: HPV6 E6, HPV11 E6, HPV16 E7, HPV18 E7, HPV31 E6, HPV33 E6, HPV35 E6, HPV39 E7, HPV45 E7, HPV51 E7, HPV52 E7, HPV53 E6, HPV56 E7, HPV58 E7, HPV59 E7, HPV66 E6 and HPV68 E7, as previously described by Micalessi *et al.* [29]. A β-globin real-time quantitative PCR was used to assess the DNA quality and to estimate the number of cells [29]. The number of HPV copies was divided by the number of cells to calculate the viral load (HPV copies/cell).

Statistical analysis

For each case, the successive viral loads (HPV copies/cell) were plotted on a logarithmic scale against time (expressed in days). For transient infections, the change in type-specific viral load per unit of time was projected by estimating two slopes, considering (i) the calendar time (t_{0-1}) corresponding with the negative HPV result preceding a transient infection, and the highest subsequent observed load measurement (t_{max}) for the productive phase and (ii) the load at t_{max} and the time (t_{0+1}) at the first subsequent time-point with a negative HPV result for the clearing phase. The slopes were defined as $\log_{10}$ (type-specific HPV load on date 1) – $\log_{10}$ (type-specific HPV load on date 2) divided by the number of days occurring between date 1 and date 2. To calculate logarithms at t_{0-1} and t_{0+1}, a value of 1E-99 was used as viral load.

For infections leading to CIN3+, the slope was calculated between the consecutive viral load measurements using a simple linear regression model [$y = a + bx$, where y is the predicted $\log_{10}$ (viral load), a is the intercept, x is the time interval, b is the slope (change in $\log_{10}$ viral load per unit of time)]. For each regression the coefficient of determination R^2 was calculated, which is a measure of deviation between the regression line and the observed points. For the productive phase of transient infections and persistent infections leading to CIN3+, the viral doubling time (V_{DT}) in days was calculated for each HPV type by (ln 2)/slope. To calculate the starting point of the linear increase leading to CIN3+ a viral load of 0.00001 HPV copies/cell was used.

The slopes and viral doubling times were compared between the transient cases and infections leading to CIN3+ using the MedCalc® program (MedCalc Software, Mariakerke, Belgium) [30]. For abnormally distributed variables, median values and interquartile range (IQR) are given. To compare differences in slopes between transient and progressive infections a two-sided Mann–Whitney *U* test was used.

Results

Transient HPV infections

The database search identified 601 monotypic transient infections in a 3-month period during 2011 (Fig. 1A). The median age of

Table 1 Type-specific median HPV slope and viral doubling times in transient infections

HPV Type	*n*	Productive Phase				Clearing Phase	
		Slope	IQR	V_{DT} (days)	IQR	Slope end	IQR
6	15	0.24	0.14–0.32	2.9	2.2–5.0	−0.43	−0.53 to −0.28
11	7	0.28	0.26–0.36	2.5	2.0–2.7	−0.39	−0.50 to −0.30
16	73	0.19	0.14–0.28	3.6	2.5–5.0	−0.28	−0.49 to −0.17
18	21	0.22	0.16–0.27	3.2	2.5–4.3	−0.25	−0.35 to −0.18
31	44	0.21	0.14–0.27	3.4	2.6–4.9	−0.26	−0.34 to −0.19
33	19	0.17	0.14–0.34	4.1	2.0–5.0	−0.25	−0.40 to −0.20
35	21	0.16	0.13–0.51	4.3	1.4–5.5	−0.32	−0.51 to −0.27
39	56	0.26	0.16–0.32	2.7	2.2–4.4	−0.25	−0.44 to −0.14
45	23	0.25	0.17–0.38	2.8	1.8–4.1	−0.32	−0.45 to −0.27
51	67	0.19	0.13–0.27	3.7	2.6–5.2	−0.26	−0.46 to −0.18
52	46	0.21	0.14–0.29	3.3	2.4–5.1	−0.25	−0.34 to −0.18
53	41	0.24	0.15–0.27	2.9	2.6–4.6	−0.24	−0.37 to −0.16
56	46	0.20	0.14–0.28	3.4	2.5–5.0	−0.28	−0.33 to −0.21
58	39	0.23	0.13–0.32	3.0	2.2–5.2	−0.30	−0.46 to −0.22
59	32	0.16	0.12–0.24	4.4	2.9–5.8	−0.27	−0.35 to −0.20
66	40	0.21	0.14–0.26	3.3	2.7–4.8	−0.29	−0.47 to −0.20
68	11	0.27	0.17–0.36	2.5	2.0–4.1	−0.37	−0.56 to −0.31
All	601	0.21	0.14–0.28	3.3	2.4–5.0	−0.28	−0.45 to −0.20

Slope: ΔHPV copies/cell/day; V_{DT}: viral doubling time; IQR: interquartile range.

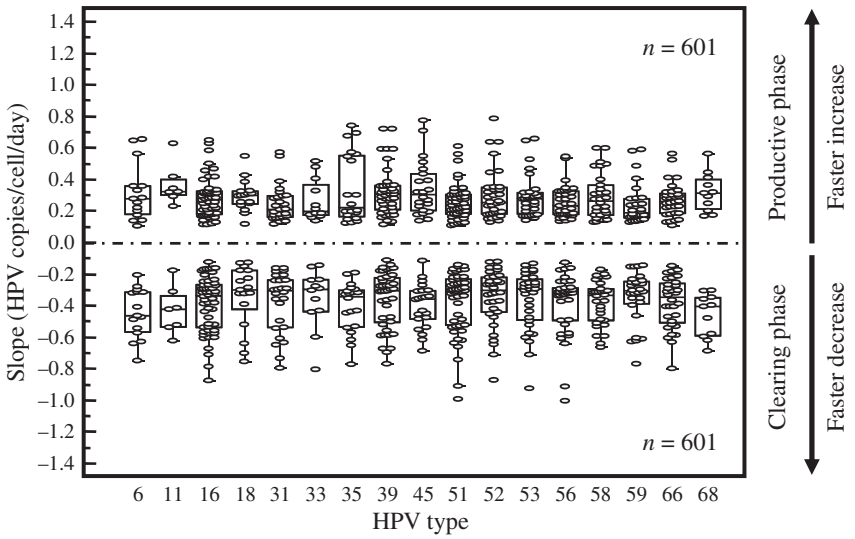

Fig. 3 Slope in Productive and Clearing Phase of Transient Infections by HPV type.

Table 2 Type-specific Median HPV Slope and V_{DT} in HPV Infections Leading to CIN3+

HPV type	*n*	Slope ($\geq$3 points)	R^2	V_{DT} CIN3+ (days)	Constant Rate of Cell Division *P*	*n*	Slope (2 points)	*n*	Slope (2 points and $\geq$3 points)
6	1	0.0026	0.999	263.5	0.0061			1	0.0026
11	ND								
16	48	0.0029	0.966	289.0	0.0069	13	0.0034	61	0.0030
18	4	0.0019	0.938	408.5	0.0046			4	0.0019
31	14	0.0025	0.977	336.3	0.0061	5	0.0029	19	0.0026
33	14	0.0032	0.962	276.8	0.0069	1	0.0013	15	0.0031
35	3	0.0025	0.938	304.6	0.0062			3	0.0025
39	4	0.0028	0.979	291.7	0.0063			4	0.0027
45	1	0.0018	1.000	397.1	0.0040			1	0.0018
51	7	0.0032	0·983	249.4	0.0074	1	0.0037	8	0.0032
52	9	0.0033	0.982	244.0	0.0081	1	0.0053	10	0.0035
53	ND								
56	2	0.0038	0.953	187.7	0.0087			2	0.0038
58	8	0.0034	0.948	246.0	0.0078			8	0.0034
59	1	0.0029	0.961	242.3	0.0066			1	0.0029
66	1	0.0032	0.993	213.7	0.0075			1	0.0032
68	ND								
All	117	0.0029	0.984	286.3	0.0069	21	0.0035	138	0.0028

Slope: ΔHPV copies/cell/day; V_{DT}: viral doubling time; ND: not detected.

women sampled was 36 years (IQR 27–45 years) with no significant age difference between the groups infected with different HPV types.

The median number of days between the first negative measurement (t_{0-1}), the viral load maximum (t_{max}) and the last negative measurement (t_{0+1}), irrespective of the HPV type, was 477 days (IQR 353–725 days) and 363 days (IQR 223–511 days) respectively. The median viral load at t_{max} was 62 HPV copies/cell (IQR 1–1765 HPV copies/cell).

The slopes for the productive and clearing phases were also calculated for each HPV type separately. Table 1 records the slopes for the individual and all HPV types. There was no significant heterogeneity in the slopes for the transient infection between different HPV types ($P = .434$; Fig. 3). For all HPV types the median slope for the productive phase of the transient infection was 0.21 HPV copies/cell/day (IQR 0.14–0.28), and −0.28 HPV copies/cell/day (IQR −0.45 to −0.20) for the clearing phase.

For all HPV types, the median viral doubling time was 3.3 days (IQR 2.4–5.0). There was no difference in median viral doubling time for the different HPV types (Table 1).

HPV Infections progressing to CIN3+

The database search revealed 1946 cases of CIN3+. Only 244 cases showed three or more virologic measurements before the histologic diagnosis, whereas only 138 (seven with cervical cancer and 131 with CIN3) were infected by a single HPV type and untreated for cervical disease during these measurements (Fig. 1B). The median age of women sampled was 33 years (IQR 28–43 years) with no significant age difference between the groups infected with different HPV types.

The median viral load just before detection of CIN3+ was 2094 HPV copies/cell (IQR 548–11 642 HPV copies/cell). The viral load was not significantly higher in cancer cases (71191 HPV copies/cell) compared with CIN3 cases (36913 HPV copies/cell). In 117 of the 138 cases (84.8%), before CIN3+ detection, at least three viral load measurements followed a rising straight line with a median R^2 of 0.984 (IQR 0.951–0.996) and a median linear slope of 0.0028 HPV copies/cell/day (IQR 0.0020–0.0036) (Table 2). The evolution of the viral loads over time for the 24 progressive cases with at least four viral load measurements on a rising straight line is shown in Figure 4.

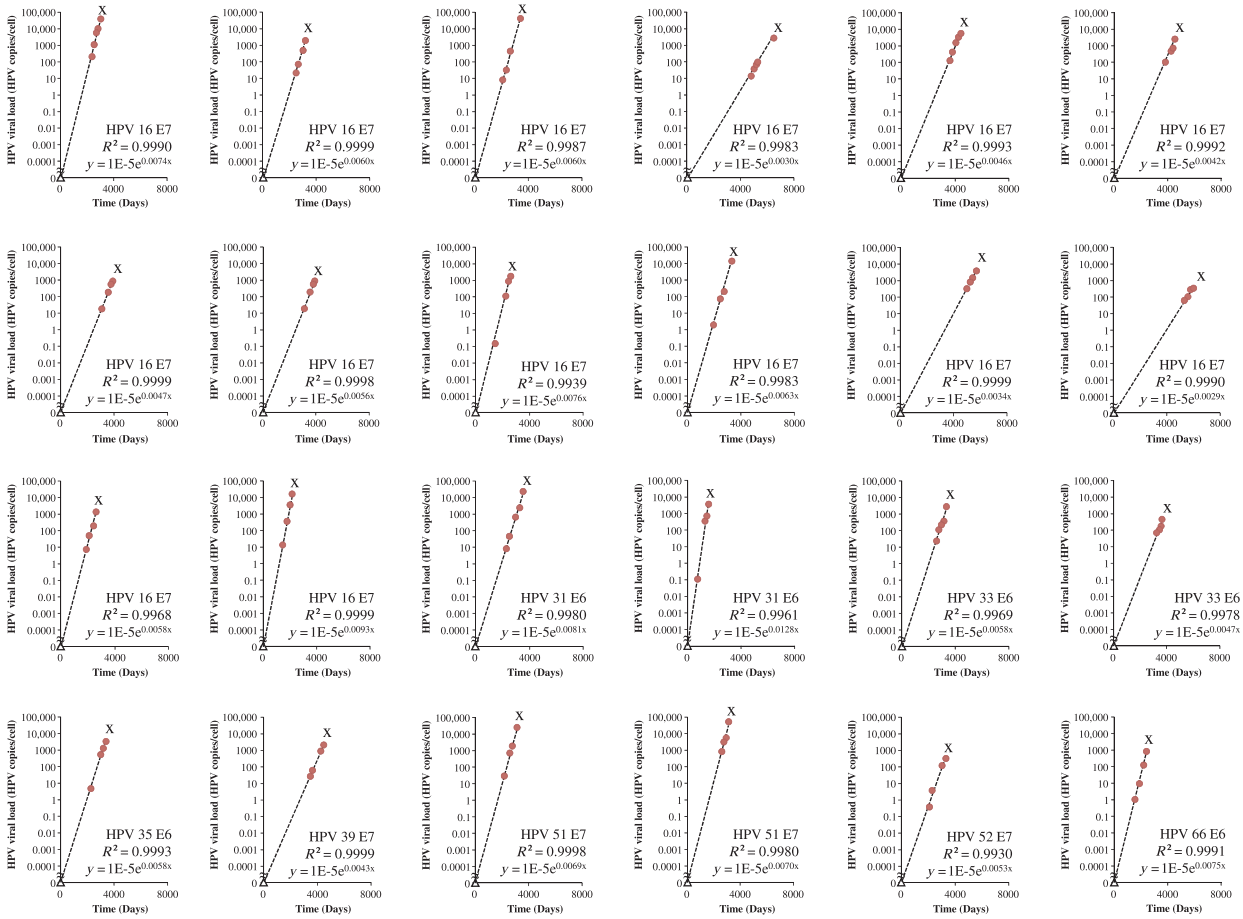

Fig. 4 Evolution of the Viral Load (HPV Copies/Cell) Over Time (in Days) in Cases Developing CIN3+. Circles = viral load measurements. Triangle represents the calculated starting point of the linear increase leading to CIN3+ with viral load of 0.00001 HPV copies/cell. The dotted line represents the least-squares line. X = detection of CIN3+. Only cases with more than three viral load measurements are shown.

The individual viral doubling time for CIN3+ was calculated from the individual mean slopes ($n = 117$). The mean viral doubling time was 286.3 days, and not significantly different for the different HPV types. In the remaining 21 CIN3+ cases (15.2%), R^2 was <0.9. In 14 cases, the third from last measured viral load was above the regression line, which could be caused by a coinciding transient infection (Fig. 5A). The slope of the straight line between the last two measurements was 0.0037 HPV copies/cell/day (IQR 0.0027–0.0047 HPV copies/cell/day). In seven cases, the second last measured point was above the regression line. The slope of the straight line between the third from last point and the last point was 0.0030 HPV copies/cell/day. There was no difference between the slopes of the two groups. The median slope for these 21 cases was 0.0035 HPV copies/cell/day (IQR 0.0019–0.0051 HPV copies/cell/day), which was not statistically different from the other 117 cases reported above. The median slope for linear (≥ 3 points) and transient-linear (2 points) infections leading to CIN3+ was 0.0028 HPV copies/cell/day (IQR 0.0020–0.0037 HPV copies/cell/day). The equation for predicting exponential growth in CIN3+ was y (type-specific viral load) = $1E\text{-}5\ e^{0.0069x}$ (number of days). Using this equation, the mean calculated time to a diagnosis of the CIN3+ lesion was 9.4 years (SD 4.1 years).

The hinge visualization of a transient prophase during linear persistent infection

In 24 cases from the total group of 138, more than one viral load measurement was available before the linear course preceding CIN3+, showing a transient infection preceding the hinge. The median slope of the clearing phase of transient prophase was −0.0025 HPV copies/cell/day (IQR −0.0040 to −0.0020 HPV copies/cell/day). The differences in slope of the clearing phase between transient infections and infections in a transient prophase but leading to CIN3+ was highly sig-

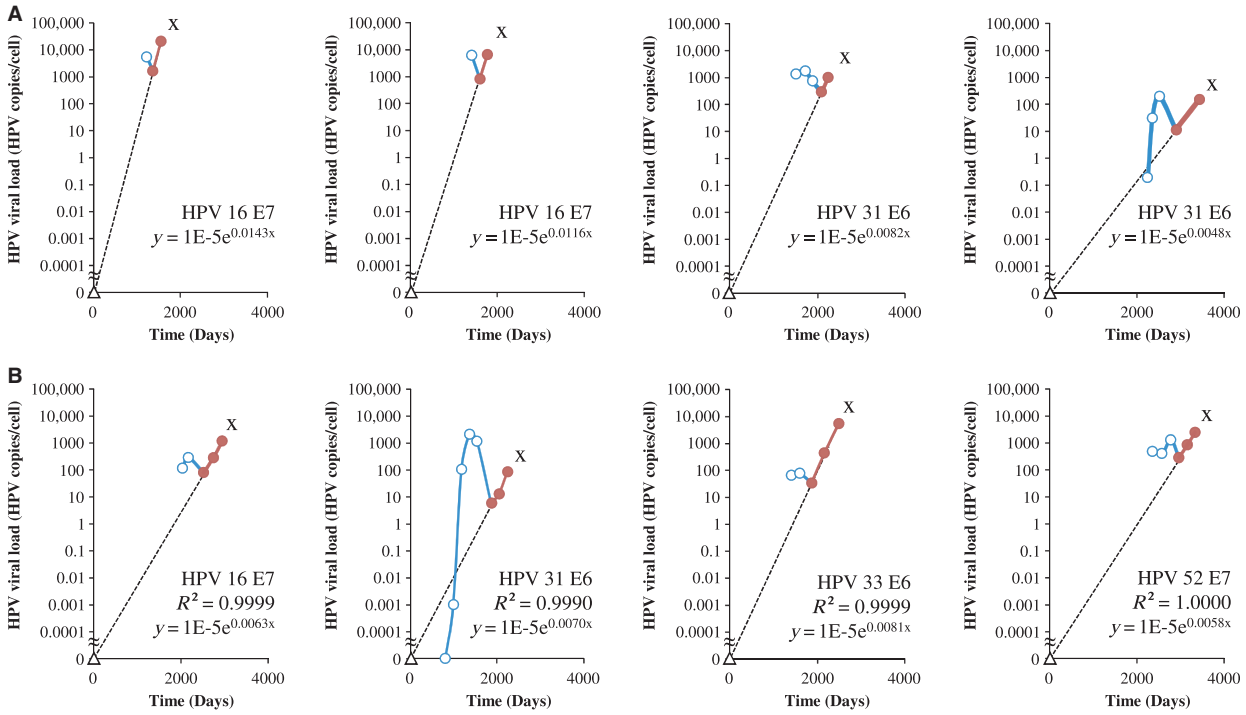

Fig. 5 The Hinge Virological Turning Point: Transient Prophase Infections Occurring During a Progressive Linear Phase Increase in Infections Leading to CIN3+. (**A**) Slope calculated with last two measurements. (**B**) Slope calculated with last three measurements. Circles represent viral load measurements prior to detection of CIN3+. X = detection of CIN3+. Open circles = measurements during transient phase. Closed circles = measurement in the linear phase with $R^2 > 0.9$. Triangle represents the calculated starting point of the linear increase leading to CIN3+ with viral load of 0.00001 HPV copies/cell. Solid line represents the transient course (blue) and the linear course (red) of the infection between the viral load measurements. The dotted line represents the calculated slope before reaching the hinge.

nificant ($P < .0001$). Representative cases that had one or more measurements before the linear phase are shown in Figure 5. The differences in slope kinetics between the productive phase of transient infections and infections leading to CIN3+ were highly significant ($P < .0001$).

Discussion

In this large population-based observational study (291,883 women) on HPV as the primary risk factor for the development of cervical cancer, all cytology leftovers were analysed for the HPV type-specific viral load in an attempt to predict whether an HPV infection is transient or will lead to CIN3+. The number of HPV type-specific E6 or E7 copies adjusted for number of cells was measured. The use of a PCR assay targeting E6 and E7 viral oncogenes avoids the risk of missing a number of L1-negative cases [31]. To avoid a possible cytology reading bias, HPV viral load testing was always performed initially [24].

In this study, 601 transient infections and 138 progressing infections with a single HPV type leading to histologically confirmed CIN3+ in untreated women were analysed. For the analysis of the increase and decrease in the viral load, the slope of a straight line between two or more measurement points and the viral doubling time were calculated for each HPV type.

In transient infections, a typical time-dependent course with a steep rise (0.21 HPV copies/cell/day) and decline (−0.28 HPV copies/cell/day), and a very short viral doubling time (3.3 days) was found. This is in accordance with previous studies showing that HPV infection is common in screening but that a large majority of the infections clear rapidly [11, 13, 16, 17, 19, 23]. This quick viral replication is probably the reflection of HPV infecting the basal cell(s) followed by the production of a large number of virions during differentiation and desquamation.

This rapid viral replication in transient infections contrasts with the 100 times slower increase (0.0028 HPV copies/cell/day) found in progressing infections during the linear phase preceding CIN3+ and the much longer viral doubling time (286.3 days). This is consistent with recent studies measuring HPV 16 viral load [13, 15, 16], and studies showing that high loads may be predictive of the transition of CIN 2-3 to cervical cancer [10, 15], or future risk for CIN3+ [9, 15]. However, others did not find a high viral load to be a clinically useful biomarker [4, 11, 14]. We hypothesize that, during progressing infections leading to CIN3+, the infected basal cell divides with a number of HPV copies inside. This would correspond to an exponential linear growth over time.

The blatant difference in the kinetics of viral replication between transient and persistent infections leading to CIN3+ enables prediction of the clinical outcome of an HPV infection. A steady linear increase with a small slope and long viral doubling time is found in progressive infections and is distinct from the rapid increase followed by a decline in viral load and short viral doubling time in transient infections. In addition, our study showed that this can be applied to the individual slopes of all high-risk HPV types and even low-risk HPV-type HPV 6.

On the basis of the 24 cases with measurements before the linear phase of persistent infections, we hypothesize that these are probably preceded by a transient prophase. The examples in Figure 5 illustrate the different phases during HPV infection, *i.e.* a steeply increasing slope in the productive phase in the case of transient infection(s) and a shallow increasing slope in the case of progressive infection with the hinge moment, at the end of the transient infection. This could, however, be as a result of the relatively short study period, only fully documented in a few cases. This study clearly shows the limitations of a single-point viral load measurement because similar viral loads can be found in the rising and declining parts of transient infections as well as in the progressive infections. A transient prophase might explain why, in 21 cases leading to CIN3+, R^2 was smaller than 0.9. It seems reasonable to assume that in 14 cases the third from last measurement and in seven cases the second from last measurement were all positively influenced by an underlying transient infection.

In conclusion, the measurement of viral load at different timepoints enables assessment of the kinetics of HPV infections over time. In transient infections, slopes (increase/decrease) are 100 times steeper compared with the steadily increasing slopes found in infections leading to CIN3+. This difference might enable the prediction of progression towards CIN3+ and could be applied as a triage tool for HPV-positive women in primary HPV screening. Further studies investigating the dynamic of viral load in patients with regression of CIN could contribute to the understanding of HPV infection.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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