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Dynamics of human papillomavirus serology in women followed up for 36 months after pregnancy

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We determined L1 antibodies for human papillomavirus (HPV) types 6, 11, 16, 18 and 45 by multiplex serology in our prospective HPV family study. We report seroprevalence, seroconversion and antibody decay in 290 women (mean age, 25.5 years) sampled before delivery and at 12, 24 and 36 months of follow-up. Multiplex HPV genotyping of the baseline oral and genital scrapings was performed. At baseline, seroprevalence of HPV 6, 11, 16, 18 and 45 was 53.3, 21.5, 34.9, 21.5 and 9.0 %, respectively. Seropositivity for low-risk HPV (LR-HPV) was associated significantly with age at onset of sexual activity (P=0.001), number of sexual partners until age 20 (P=0.018), lifetime number of sexual partners (P=0.0001), history of genital warts (P=0.0001) and being seropositive for high-risk (HR) HPV (P=0.0001). The same covariates also predicted seropositivity for HR-HPV. During follow-up, 26.7, 13.9, 17.0, 16.8 and 6.6 % of the women seroconverted to L1 antigen of HPV 6, 11, 16, 18 and 45, respectively, between 18.2 and 23.8 months. Independent predictors of seroconversion to LR-HPV were unemployment (P=0.019) and absence of anal sex practice (P=0.031), and to HR-HPV, absence of smoking history and lifetime number of sexual partners. Decay of HPV 6, 11, 16, 18 and 45 antibodies was observed in 2.3, 4.0, 5.3, 4.5 and 1.5 % of the women, respectively, with decay time varying from 27.2 to 35.8 months. These data imply that (i) a substantial proportion of young women are seropositive for both LR- and HR-HPV types, (ii) they frequently undergo seroconversion within 18–24 months, predicted by common covariates, and (iii) antibody decay over 3 years is rare.

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INTRODUCTION

Acquisition of human papillomavirus (HPV) infection is common, particularly among young, sexually active women. It has been estimated that approximately 80 % of Finnish women will acquire clinically detectable genital HPV infection at least once during their lifetime (Syrjänen *et al.*, 1990). Natural history of genital HPV infections in women has been monitored widely by serial sampling for Pap smears and HPV DNA testing. Most genital HPV infections are transient. Of young women, <10 % are still infected after 2–5 years of follow-up (Ho *et al.*, 1998; Molano *et al.*, 2003). Median duration of genital HPV infections varies from 8 to 15 months (Ho *et al.*, 1998; Muñoz *et al.*, 2004), with an approximate monthly clearance rate of 1.6 % (Franco *et al.*, 1999; Syrjänen *et al.*, 2005a). In contrast to acquisition, HPV clearance seems to be age-independent (Franco *et al.*, 1999; Syrjänen *et al.*, 2005b). High-risk (HR) HPV infections are shown to last longer than infections with low-risk (LR) types (Ho *et al.*, 1998; Kjaer *et al.*, 2002; Richardson *et al.*, 2003; Muñoz *et al.*, 2004). The data on persistence of multiple-type infections are controversial (Ho *et al.*, 1998; Rousseau *et al.*, 2001; Woodman *et al.*, 2001; Molano *et al.*, 2003; Kulmala *et al.*, 2007).

Cervical HPV DNA testing has limitations in that it only detects HPV infection in a sampled area, and does not assay any past HPV exposure or infection at other anatomical sites. Since virus-like particles (VLPs) and their use in vaccination were introduced, interest in seroepidemiological studies has increased rapidly. Several studies suggest that HPV serology based on VLP ELISA can detect past and present exposures to HPV at different mucosal sites. However, there is some evidence that transient HPV infections do not always elicit seroconversion and, also, some women with persistent infection fail to seroconvert (Carter *et al.*, 2000). Only about half of all HPV 16

DNA-positive women tested seropositive for the corresponding HPV type using available assays (Kirnbauer *et al.*, 1994; Wideroff *et al.*, 1996; Carter *et al.*, 1996, 2000; Viscidi *et al.*, 1997; Ho *et al.*, 2004; Skjeldestad *et al.*, 2008). In addition, abnormal cytology could not be correlated with positive serology (Rama *et al.*, 2006). In the literature, HPV 16 seroprevalence in the healthy female population seems to vary from 3.4 to 44.0 % (af Geijersstam *et al.*, 1998; Stone *et al.*, 2002; Wang *et al.*, 2003; Lehtinen *et al.*, 2006; Villa *et al.*, 2006; Marais *et al.*, 2007; Skjeldestad *et al.*, 2008; Wang *et al.*, 2008). Seroprevalence seems to be more common among cervical intraepithelial neoplasia or sexually transmitted disease (STD) patients, as well as among women with cervical cancer or human immunodeficiency virus infection (Thompson *et al.*, 2004; Viscidi *et al.*, 2005). The seroprevalence data must be read with caution, because different studies use different assays with varying cut-off definitions.

Controversy exists on the stability of HPV antibody titres over time. IgG antibodies after natural HPV infection have been reported to be stable (af Geijersstam *et al.*, 1998; Carter *et al.*, 2000; Lehtinen *et al.*, 2006; Villa *et al.*, 2006), whilst Ho *et al.* (2004) reported that approximately 50 % of initially seropositive subjects had undetectable IgG antibody levels within 36 months. Antigenic re-exposure is likely to affect HPV seropersistence (Ho *et al.*, 2004). Not all studies have included HPV DNA data parallel with the serology (af Geijersstam *et al.*, 1998; Thompson *et al.*, 2004; Wang *et al.*, 2004). However, it must be noted that the presence of HPV DNA cannot be interpreted directly as a sign of antigenic re-exposure to L1, because it is not known whether the L1 protein is invariably expressed from the L1 gene.

In 1998, we designed a prospective cohort study, the Finnish Family HPV Study, to understand the HPV dynamics in parents and their newborn offspring. The present study focused on describing HPV seroprevalence, seroconversion and antibody decay for five mucosal HPV types (HPV 6, 11, 16, 18 and 45) among 290 women followed up for 3 years after delivery. A time-dependent generalized estimating equation (GEE) was used to explore predictors of seroconversion to either LR- or HR-HPV types in univariate and multivariate modes.

METHODS

Women of the study. The Finnish Family HPV Study is a cross-sectional and prospective cohort study to understand the dynamics of HPV infection in mothers, fathers and their newborn infants. Between 1998 and 2001, 329 pregnant women in their third trimester and their spouses were enrolled at the Maternity Unit of the Turku University Hospital, Turku, Finland, as described previously (Rintala *et al.*, 2005a, b, 2006). The study design was approved by the Research Ethics Committee of Turku University Hospital before initiation (#3/1998) and informed consent was obtained from all parents participating in the study. The HPV status of the woman at enrolment was not part of the inclusion criteria. The families have been subsequently followed up until the present; the current study reports the results until follow-up of 36 months. Of the 329 women enrolled, 290 women were eligible in the present study, all having at least two serum samples taken during the study period. Mean age of the mothers at study entry was 25.5 years (range, 18–38 years). Most deliveries (95 %) were at term (mean gestational age, 40.1 ± 1.4 weeks) and 77.9 % were by the vaginal route. The mean length of the follow-up period for the present analysis was 37.5 months (range, 32.7–49.3 months).

Questionnaire. All women filled in a standardized questionnaire during their first post-partum visit (mean, 3.6 months; range, 1.5–4.9 months after delivery), providing information on demographics, sexual behaviour, gynaecological and obstetric history and risk factors for HPV infections. No demographic data were recorded during the later follow-up visits, except for data on the presence of HPV-induced clinical lesions, such as oral papilloma and skin and genital warts at the 1 and 3 year control visits.

Pap smear. A routine Pap smear was taken from all women at baseline, 12, 24 and 36 months, using the conventional three-sample technique (vagina, exocervix, endocervix) with two wooden spatulas and a cytobrush (MedScand).

HPV testing. HPV DNA-testing procedures and results have been described previously (Rintala *et al.*, 2005b). Briefly, cervical and oral scrapings for HPV testing were taken from the women at baseline. DNA was extracted from scrapings by the high-salt method (Miller *et al.*, 1988). HPV testing was done with PCR using GP05+/GP06+ primers (Snijders *et al.*, 1990). For oral DNA, nested PCR was performed with MY09/MY11 (Manos *et al.*, 1989) and GP05+/GP06+ primers. The PCR products were hybridized with digoxigenin-labelled HR-HPV oligoprobe cocktail (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 54, 56 and 58) to determine whether the sample was HR-HPV+ or HR-HPV− (Anttila *et al.*, 1999).

HPV genotyping. The baseline samples were also HPV-genotyped with a fluorescent bead array on 24 HPV types in PCR-amplified samples according to Schmitt *et al.* (2006), using a kit from Multimetrix. This method combines PCR with hybridization to fluorescence-labelled polystyrene bead microarrays (Luminex suspension array technology). The assay can detect the following HPV types: (LR-HPV) 6, 11, 42, 43, 44, 70; (HR-HPV) 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82; and also probable HR-HPV types 26, 53, 66 and 70. The manufacturer's instructions were followed, but the reactions were done in 50 µl instead of 100 µl. At the final step before reading in a Luminex analyser, 100 µl blocking buffer was used. As target DNA, the PCR products from HPV testing, which were now biotinylated by reamplification with GP05+/biotin–GP06+ primers, were used. The median reporter fluorescence intensity (MFI) of at least 100 beads was computed for each bead set in the sample. The cut-off value was defined for each HPV probe individually as follows: $1.5 \times$ background MFI + 5 MFI. If the sample tested HPV 16+, the original sample DNA was retested with an in-house, bead-based HPV 16 genotyping assay. This retesting was done to identify samples that might have become contaminated with HPV 16 during the previous testing or reamplification.

Serology. Blood samples were taken at baseline and at 12, 24 and 36 months of follow-up. After collection, the blood samples were centrifuged at 2400 r.p.m. for 10 min (Sorvall GLC-2; DuPont Instrument); the serum was divided into three 1 ml aliquots and stored first at −20 °C for no longer than 1 week and then at −70 °C until sent for analysis at the DKFZ, Heidelberg, Germany.

Antibodies to the major capsid protein L1 of HPV types 6, 11, 16, 18 and 45 were analysed by multiplex HPV serology based on glutathione *S*-transferase fusion-protein capture on fluorescent beads, as described previously (Waterboer *et al.*, 2005, 2006).

Sera were scored as positive when the antigen-specific MFI values were greater than the cut-off level of 200 or 400 MFI (stringent) for L1 antigen of individual HPV types (Michael *et al.*, 2008). Seroconversion was defined by two conditions: at least a 2-fold increase of the previous serum value and an MFI value over the cut-off of 200 or 400 MFI (stringent). Similarly, antibody decay was defined by two conditions: at least a 2-fold decrease of the previous serum value and fall of the MFI value below the cut-off of 200 or 400 MFI (stringent).

Statistical analysis. All statistical analyses were run by using the SPSS for Windows (version 16.0.1; SPSS, Inc.) and STATA (STATA/SE 10.1; Stata Corporation) software packages. Frequency tables were analysed by using the χ^2 test, with the likelihood-ratio test or Fisher's exact test for categorical variables. Differences in the means of continuous variables were analysed by using a Mann–Whitney test or Kruskal–Wallis test for two and multiple independent samples, respectively. Univariate survival analysis for the outcome measures (seroconversion, antibody decay) was based on the Kaplan–Meier method, where stratum-specific outcomes were compared by using log-rank statistics.

To analyse the influence of covariates on seroprevalance and seroconversion, a GEE model approach was used, clustered by woman ID and stratified by the five HPV types (Silins *et al.*, 2000; Diggle *et al.*, 2002; Hardin & Hilbe, 2003). GEE adjusts for the serial correlation within subjects due to the longitudinal nature of the data by modelling the covariance structure within subjects. The dependent variable was binomial (seropositive, +/−; seroconversion, +/−), and hence the logit link function was used. The exchangeable working correlation structure with a robust variance estimator to account for within-subject correlation was selected as the best-fitting covariance pattern, using the quasi-likelihood information criterion (Silins *et al.*, 2000). In this analysis, we assumed that HPV seroconversion depends on time since the previous sample and, therefore, a time variable was included as a covariate in these GEE models. In multivariate GEE models, we entered several covariates shown or implicated previously as risk factors for HPV infections in our cohort (Rintala *et al.*, 2005a). All statistical tests performed were two-sided and declared to be significant when $P<0.05$.

RESULTS

The rationale of this study was to assess seroprevalence, seroconversion and antibody decay in women participating in the Finnish Family HPV Study. The study was planned to understand the dynamics of HPV in families followed for 3 years.

The key demographic characteristics of the 290 mothers included in the study are shown in Table 1. Of the 286 mothers who were HPV DNA-tested at baseline, 47 (16.4 %) were carriers of genital HR-HPV and 51 (17.8 %) had HR-HPV DNA in their oral samples. At the same time, 34 (11.8 %) had atypical cells of undetermined significance (ASCUS) or a higher level of abnormality in their Pap smear and, of those, 27 % (nine of 33) were genital HR-HPV+. Altogether, 135 (50.2 %) were current or past smokers. Only seven (2.6 %) had started their sexual activity by 13 years of age, whilst the majority (56.8 %) had their first sexual intercourse between 14 and 16 years of age. As to the number of lifetime sexual partners, 68 (25.2 %) women reported only one or two partners, and 56 (20.7 %) reported more than 10 partners. The corresponding figures for partners before age 20 were 43.2 and 6.6 %, respectively. Nearly half of the women (42.4 %) had initiated the use of oral contraception (OC) between 14 and 16 years of age, and 23 (8.5 %) had never used OC. Of the 290 women, 231 (79.7 %) reported no history of any STD (excluding genital warts), whilst 73 (27.3 %) reported a history of genital warts (exophytic condylomata). Oral warts were significantly rarer (eight cases only), whereas skin warts were reported by 160 women (60.6 % of those who answered this question), with 37.5 % of the warts appearing on hands, 38.8 % on feet and 23.8 % on multiple sites.

Seroprevalence

Fig. 1 illustrates the frequency of seropositivity at both cut-offs, 200 and 400 MFI, for L1 antigens of HPV 6, 11, 16, 18 and 45 at baseline and the three follow-up visits. The latter were originally scheduled at 12, 24 and 36 months, the actual mean intervals being 12.9 months (range, 10.2–16.3 months), 25.2 months (range, 21.7–32.9 months) and 37.6 months (range, 32.7–49.3 months), respectively. At baseline, 53.3, 21.5, 34.9, 21.5 and 9.0 % of the women were seropositive for HPV 6, 11, 16, 18 and 45, respectively, at 200 MFI cut-off ($P=0.0001$). As compared at each of the four visits (single time point), the difference in HPV seropositivity between HPV types remained practically identical ($P=0.995$). On the other hand, when compared between visits (longitudinal), the difference in seroreactivity of individual HPV types was statistically significant except for HPV 18 and 45 (HPV 6, $P=0.0001$; HPV 11, $P=0.004$; HPV 16, $P=0.027$; HPV 18, $P=0.542$; HPV 45, $P=0.557$). There was a slight increase in seropositivity to HPV 6, 11 and 16 at a cut-off of 200 MFI at the 12 and 24 months follow-up visits, reverting back to the level of the baseline status at the 36 months visit (53.3, 15.1, 28.6, 23.6 and 7.3 %, respectively) (trend; not significant) (Fig. 1).

When using the more stringent cut-off (400 MFI), the antibody profile remains similar, with no difference between the four visits ($P=0.942$), but the level of prevalence of each L1 antibody drops (except for HPV 18) on average by 15–20 %, compared with the prevalence at a cut-off of 200 MFI (Fig. 1). The profile of the seropositivity among individual types during the follow-up remained significant only for HPV types 11 ($P=0.022$) and 16 ($P=0.013$). Also, the same trend for increased seroprevalance at the 12 and 24 months visits persists with this stringent cut-off, except for HPV 18.

Predictors of seropositivity

Table 2 summarizes HPV serostatus as related to the baseline genital, oral or combined HPV DNA positivity (positive at any site, genital and/or oral) for the same HPV genotypes. Collectively, HPV 6, 11, 16, 18 or 45 were detected in 16.1 and 21.5 % of genital and oral baseline

Table 1. Key demographic characteristics of the women and their HPV DNA and Pap smear status at enrolment

Data were recorded at enrolment (Pap, HPV DNA) and 2 months after delivery (demographics).

Variable (no. of responders)	*n* (%)
Baseline cervical HR-HPV DNA (286)	
Positive	47 (16.4)
Negative	239 (83.6)
Baseline oral HR-HPV DNA (287)	
Positive	51 (17.8)
Negative	236 (82.2)
Baseline Pap smear cytology (288)	
ASCUS or higher	34 (11.8)
<ASCUS	254 (88.2)
Age:	
2 months after delivery (290)	25.6±3.1*
At menarche (262)	12.3±2.3*
When first pregnant (271)	22.9±3.4*
Marital status (271)	
Single	19 (7.0)
Living with partner	122 (45.0)
Married	128 (47.2)
Divorced	2 (0.7)
Education (271)	
Elementary school	23 (8.5)
Vocational training	72 (26.6)
Secondary school graduate	50 (18.5)
College graduate	86 (31.7)
Academic degree	40 (14.8)
Employment status (265)	
Employed	161 (60.8)
Student	42 (15.8)
Unemployed	62 (23.4)
Allergic symptoms (268)	
No	150 (56.0)
Yes	118 (44.0)
Recognized atopia (262)	
No	220 (84.0)
Yes	42 (16.0)
Smoking history (269)	
Current or past smoker	135 (50.2)
Never smoked	134 (49.8)
Age at first sexual intercourse (271)	
⩽13 years	7 (2.6)
14–16 years	154 (56.8)
17–19 years	97 (35.8)
⩾20 years	13 (4.8)
No. of lifetime sexual partners (270)	
1–2	68 (25.2)
3–5	87 (32.2)
6–10	59 (21.9)
>10	56 (20.7)
No. of sexual partners before 20 years of age (271)	
0–2	117 (43.2)
3–5	92 (33.9)
6–10	44 (16.2)
>10	18 (6.6)
Sexual intercourse month^{-1} (270)	
0–1	6 (2.2)
2–4	85 (31.5)
5–10	147 (54.4)
>10	32 (11.9)
Practice of oral sex (271)	
Regularly	35 (12.9)
Occasionally	181 (66.8)
Never	55 (20.3)
Practice of anal sex (271)	
Regularly	3 (1.1)
Occasionally	49 (18.1)
Never	219 (80.8)
Age starting oral contraception (271)	
Never used	23 (8.5)
⩽13 years	3 (1.1)
14–16 years	115 (42.4)
17–19 years	104 (38.4)
⩾20 years	26 (9.6)
History of STDs (genital warts excluded) (290)	
No	231 (79.7)
Yes	59 (20.3)
History of genital warts (267)	
No	194 (72.7)
Yes	73 (27.3)
Age at diagnosis of genital warts (71)	
<20 years	34 (48)
20–24 years	29 (41)
⩾25 years	8 (11)
Treatment of genital warts (99)	
No treatment	37 (37)
Topical treatment	27 (27)
Electrocautery	4 (4)
Cryotherapy	3 (3)
Laser therapy	12 (12)
Surgery	1 (1)
Several treatments	15 (15)
History of oral warts (264)	
Never	256 (97.0)
Yes, no treatment	7 (2.7)
Yes, surgical treatment	1 (0.4)
Location of skin warts (160)	
Hands	60 (37.5)
Feet	62 (38.8)
Multiple sites	38 (23.8)
Number of previous:	
Abortions (263)	0.19 (0–3)†
Miscarriages (262)	0.17 (0–3)†
Deliveries (271)	1.29 (0–4)†

*Mean±SD (years).
†Mean (range).

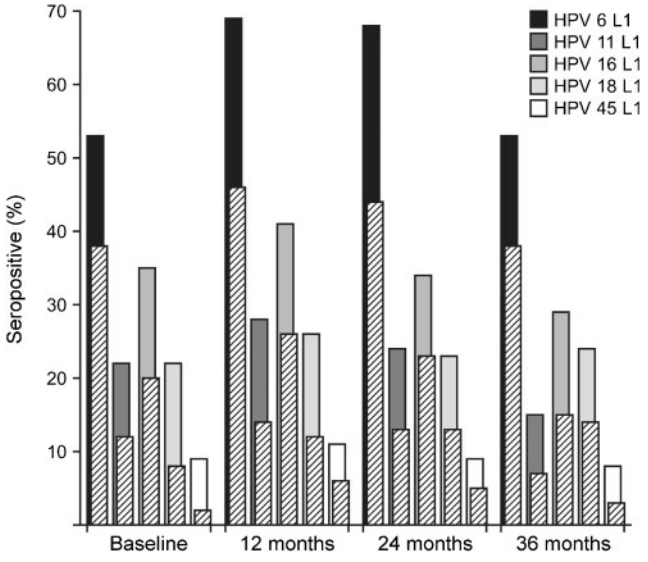

Fig. 1. Prevalence of antibodies to L1 of HPV types 6, 11, 16, 18 and 45 (cut-off, 200 MFI) at baseline and follow-up visits. The hatched columns at the front present the corresponding figures under stringent conditions (cut-off, 400 MFI).

samples, respectively. The type-specific concordance between HPV DNA detection and seropositivity was poor to modest. The highest concordance was detected for HPV 16, being 4.9 and 9.0 % for genital and oral samples, respectively. By combined DNA positivity (positive at any site, genital and/or oral), the agreement between seropositivity and DNA positivity for HPV 16 increased to 12.1 %. As HPV DNA status did not explain much of the detected seropositivity, the next logical step was to assess the other determinants of seropositivity and seroconversion.

Table 3 shows the determinants of seropositivity for LR-HPV and HR-HPV types at the baseline visit both in univariate analysis and in GEE adjusted for other covariates. In univariate GEE, five covariates were associated significantly with seropositivity to LR-HPV: age at onset of sexual activity (P=0.001), number of sexual partners until age of 20 years (P=0.018), lifetime number of sexual partners (P=0.0001), history of genital warts (P=0.0001) and being seropositive for HR-HPV (P=0.0001). For seropositivity for HR-HPV, these predictors were the same except for onset of sexual activity, which was not related significantly to HR-HPV seropositivity.

When adjusted for all other covariates, four independent predictors of seropositivity to LR-HPV remained in the GEE model: age at onset of sexual activity (P=0.002), lifetime number of sexual partners (P=0.047), history of genital warts (P=0.023) and being seropositive for HR-HPV (P=0.0001). In a similar model for HR-HPV seropositivity end point, only three independent predictors were disclosed: lifetime number of sexual partners (P=0.006), history of genital warts (P=0.021) and being seropositive for LR-HPV (P=0.0001).

Seroconversion

Fig. 2 shows Kaplan–Meier analysis of cumulative seroconversion to the five HPV types. During the median follow-up time of 37.2 months, 26.7 % of the women showed seroconversion to HPV 6, 13.9 % to HPV 11, 17.0 % to HPV 16, 16.8 % to HPV 18 and 6.6 % to HPV 45 (P=0.0001). Calculated from enrolment (baseline visit), the mean (95 % confidence interval) times to seroconversion were as follows: 20.3 months (18.2–22.3 months), 20.8 months (17.7–23.9 months), 18.2 months (15.3–

Table 2. HPV serostatus as related to genital and oral HPV DNA status at baseline

Data are given as *n* (%).

Status	HPV 6	HPV 11	HPV 16	HPV 18	HPV 45
Genital samples (*n*=322)					
DNA−/serology−	143 (44.4)	251 (77.9)	200 (62.2)	253 (78.6)	287 (89.1)
DNA−/serology+	172 (53.4)	67 (20.9)	92 (28.6)	63 (19.6)	30 (9.3)
DNA+/serology−	4 (1.2)	4 (1.2)	14 (4.3)	4 (1.2)	5 (1.6)
DNA+/serology+	3 (1.0)	0 (0.0)	16 (4.9)	2 (0.6)	0 (0.0)
Oral samples (*n*=297)					
DNA−/serology−	131 (44.1)	235 (79.1)	159 (53.5)	237 (79.8)	269 (90.5)
DNA−/serology+	159 (53.5)	61 (20.5)	74 (25.0)	58 (19.5)	28 (9.5)
DNA+/serology−	5 (1.7)	1 (0.4)	37 (12.5)	2 (0.7)	0 (0.0)
DNA+/serology+	2 (0.7)	0 (0.0)	27 (9.0)	0 (0.0)	0 (0.0)
Combined genital and oral samples (*n*=323)					
DNA−/serology−	139 (43.0)	250 (77.4)	166 (51.4)	252 (78.0)	288 (89.2)
DNA−/serology+	171 (52.9)	68 (21.1)	69 (21.4)	63 (19.5)	30 (9.3)
DNA+/serology−	8 (2.5)	5 (1.5)	49 (15.2)	6 (1.9)	5 (1.5)
DNA+/serology+	5 (1.5)	0 (0.0)	39 (12.1)	2 (0.6)	0 (0.0)

Table 3. Predictors of baseline seropositivity to LR-HPV 6 and/or 11 and to HR-HPV 16 and/or 18 and/or 45 in time-dependent GEE modelling run in univariate mode and adjusted for other covariates

Results were obtained from time-dependent GEE with logit link for binary outcomes clustered by woman ID number. Binary outcome (seropositive/seronegative) was detected at baseline visit (cut-off, 200 MFI). Statistically significant covariates ($P<0.05$) are indicated in bold.

Covariate	Seropositive for LR-HPV (HPV 6 and/or 11)						Seropositive for HR-HPV (HPV 16 and/or 18 and/or 45)					
	Crude OR	95 % CI	*P*	Adjusted OR*	95 % CI	*P*	Crude OR	95 % CI	*P*	Adjusted OR*	95 % CI	*P*
Age (categorical)	1.9	0.81–4.33	0.145	2.2	0.67–7.20	0.192	1.3	0.59–3.03	0.489	0.9	0.29–2.81	0.868
Baseline genital HR-HPV DNA status	1.0	0.54–1.81	0.979	1.6	0.73–3.41	0.248	0.7	0.38–1.27	0.233	0.8	0.37–1.69	0.540
Baseline oral HR-HPV DNA status	1.0	0.57–1.92	0.886	1.5	0.66–3.66	0.319	1.0	0.53–1.83	0.966	1.0	0.43–2.45	0.950
Baseline Pap smear (ASCUS cut-off)	0.5	0.24–1.10	0.086	0.4	0.14–1.09	0.073	0.9	0.42–1.76	0.673	1.3	0.60–2.87	0.492
Marital status	1.0	0.67–1.43	0.925	1.3	0.80–2.11	0.292	0.8	0.55–1.21	0.303	0.8	0.46–1.29	0.319
Age at menarche	0.9	0.84–1.07	0.398	0.9	0.77–1.11	0.416	1.0	0.90–1.12	0.949	1.0	0.89–1.17	0.783
Age at onset of sexual activity (inverse relationship)	**0.5**	**0.35–0.77**	**0.001**	**0.4**	**0.25–0.74**	**0.002**	1.0	0.65–1.42	0.846	1.6	0.93–2.76	0.090
Age at first pregnancy	1.0	0.91–1.05	0.525	1.0	0.87–1.09	0.648	1.0	0.95–1.10	0.601	1.0	0.88–1.09	0.718
Employment status (employed; ref†)	1.0	0.73–1.29	0.843	1.2	0.86–1.69	0.286	0.9	0.71–1.27	0.722	0.8	0.57–1.15	0.237
Smoking status (non-smoker; ref†)	1.1	0.79–1.41	0.698	0.7	0.50–1.11	0.147	1.1	0.79–1.41	0.730	0.8	0.54–1.14	0.209
Oral contraceptive use	0.9	0.70–1.15	0.405	0.8	0.59–1.15	0.261	1.1	0.89–1.44	0.313	1.0	0.71–1.45	0.918
No. of sexual partners until age 20 years (linear)	**1.4**	**1.06–1.83**	**0.018**	0.7	0.44–1.14	0.155	**1.4**	**1.04–1.77**	**0.025**	1.0	0.61–1.65	0.985
Lifetime no. of sexual partners (linear)	**1.6**	**1.30–2.09**	**0.0001**	**1.5**	**1.01–2.31**	**0.047**	**1.7**	**1.34–2.18**	**0.0001**	**1.8**	**1.17–2.62**	**0.006**
Practice of anal sex (never; ref†)	1.1	0.66–2.00	0.625	1.4	0.74–2.65	0.308	1.1	0.61–1.87	0.813	1.0	0.48–2.00	0.958
History of genital warts (no history; ref†)	**2.6**	**1.93–3.60**	**0.0001**	**2.4**	**1.13–4.94**	**0.023**	**3.1**	**1.76–5.39**	**0.0001**	**2.2**	**1.12–4.27**	**0.021**
Seropositive for HR- or LR-HPV types	**3.3**	**2.00–5.47**	**0.0001**	**3.2**	**1.72–5.95**	**0.0001**	**3.3**	**2.00–5.47**	**0.0001**	**3.1**	**1.66–5.75**	**0.0001**

*Adjusted for all other covariates in the model.
†ref, Used as reference.

21.1 months), 23.8 months (20.5–27.1 months) and 20.7 months (16.1–25.4 months) for HPV 6, 11, 16, 18 and 45, respectively [$P=0.107$ (ANOVA); $P=0.069$ (Kruskal–Wallis exact test)]. In total, 39.7 % of the women had seroconverted to HPV 6 and/or 11. Half of the women with HPV seroconversion (67 of 134) had seroconverted to only one HPV type, whilst the other half of the women showed seroconversion to multiple HPV types.

In post-hoc LSD (least significant difference) tests, the time to seroconversion to HPV 16 was significantly shorter than that for HPV 18 ($P=0.007$), the difference from seroconversion times of other HPV types being not statistically significant. In Kaplan–Meier analysis, the cumulative seroconversion to the five HPV types is highly significant (log-rank, $P=0.0001$). Most notably, the Kaplan–Meier curve of cumulative seroconversion to HPV 6 deviates markedly from all the others, reaching levels that are almost twice as high as for the L1 of any other HPV.

Predictors of seroconversion

GEE with time-dependent variables was used to analyse the predictors of seroconversion to HPV L1 antigens stratified by LR-HPV (HPV 6 and/or 11) and HR-HPV (HPV 16, 18 and/or 45), tested both in univariate mode and adjusted for other covariates (Table 4). Being unemployed versus a student or employed was the only significant predictor of seroconversion to LR-HPV. When adjusted for other

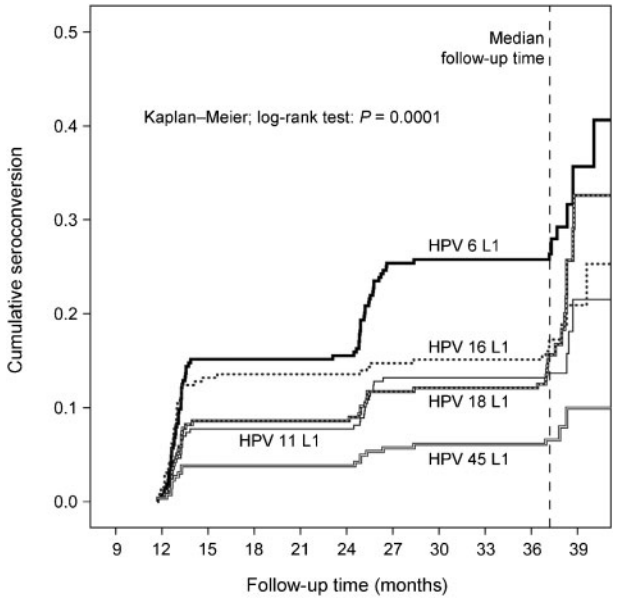

Fig. 2. Cumulative seroconversion for HPV 6, 11, 16, 18 and 45 during the 36 months of follow-up.

covariates in the model, the status of employment (P=0.019) and practice of anal sex (P=0.031) were the only independent protective predictors: those who were employed or practiced anal sex were less likely to convert than those who never practised anal sex. In addition to the variables in Table 2, we also tested all of the other variables listed in Table 1, but none of those were significant predictors of seroconversion to HPV 6 or 11.

For HR-HPV in univariate GEE, three variables were significant predictors: baseline genital HR-HPV status (HR-HPV− women were less likely to convert) (P=0.031), marital status (unmarried women were more likely to convert) (P=0.023) and lifetime number of sexual partners (linear relationship) (P=0.004). In GEE adjusted for other covariates, two independent predictors appeared: smoking history (smokers were less likely to convert) (P=0.021) and lifetime number of sexual partners (a linear increase in conversion frequency, from 16.2 % among women with between zero and two partners up to 39.3 % among those with more than 10 lifetime partners) (P=0.003).

Decay of HPV antibodies

Fig. 3 shows Kaplan–Meier analysis of cumulative decay of L1 antibodies to the five HPV types during the 36 months of follow-up. During the median follow-up time, decay of antibodies to HPV 6 was observed in 2.3 % of the initially HPV 6 antibody-positive women, to HPV 11 in 4.0 % of the initially HPV 11-antibody positive women, to HPV 16 in 5.3 %, to HPV 18 in 4.5 % and to HPV 45 in 1.5 % (P=0.091, Fisher's exact test). Calculated from enrolment, the mean (95 % confidence interval) times for antibody decay were 34.1 months (27.6–40.7 months), 28.9 months (21.8–36.1 months), 32.9 months (27.1–38.6 months), 27.2 months (19.3–35.1 months) and 35.8 months (32.4–39.2 months) for HPV 6, 11, 16, 18 and 45, respectively [P=0.393 (ANOVA); P=0.503 (Kruskal–Wallis exact test)]. In Kaplan–Meier analysis, the cumulative antibody decay between the five HPV types is not significantly different (log-rank, P=0.130). In fact, the Kaplan–Meier curves for individual HPV types run an almost-parallel course, with no such deviations as illustrated in Fig. 2, e.g. for HPV 6 L1.

DISCUSSION

Most serological studies of women with genital HPV infections have been cross-sectional in design, which precludes the analysis of seroconversion or antibody decay over time. One of the obstacles of HPV serology studies is that the presence and duration of HPV infection are difficult to assess, because HPV DNA testing cannot be used to estimate the first exposure to HPV. Fluctuation in HPV DNA positivity might represent a new infection, recurrence of an earlier HPV infection or just a sampling error. Thus, longitudinal studies on HPV serology are needed. So far, most of the seroepidemiological data on HPV infections have been gathered from the placebo groups of ongoing HPV vaccine trials, in addition to studies on HPV 16 serology among college girls, STD patients and pregnant women (Carter *et al.*, 1996; Hagensee *et al.*, 1999, 2000; Ho *et al.*, 2004; Thompson *et al.*, 2004; Lehtinen *et al.*, 2006; Villa *et al.*, 2006). We report here a longitudinal study on serology to L1 of HPV 6, 11, 16, 18 and 45 in a cohort of 290 young women (mean age, 25.5 years; range, 18–38 years), subjected to four serial testings during a mean follow-up period of 37.6 months.

First, we must stress that our study population showed markers of high sexual activity compared with populations analysed in other serological studies. This is even true when we compare our data with those published recently on sexual habits of Finnish women. Nikula *et al.* (2007) composed a module of questions on sexual behaviour integrated into a population-based general-health survey in Finland. A representative sample of 1894 individuals aged between 18 and 29 years drawn from the population registry in 2001 was studied. The mean number of sexual partners of the women was 3.4 (SD 2.1), whereas in our study, the women had about five lifetime sexual partners.

The oral HPV DNA-detection rate is higher in our study than reported previously. In addition to the probably increased sensitivity of the nested PCR, the sexual behaviour of our study population (in general, and especially the 80 % with at least occasional oral sex practice) could result in high oral HPV exposure and, thus, high HPV DNA prevalence in oral samples. The concordance between oral and genital HPV DNA status

Table 4. Predictors of seroconversion to LR-HPV 6 and/or 11 and to HR-HPV 16 and/or 18 and/or 45 during the follow-up in time-dependent GEE modelling run in univariate mode and adjusted for other covariates

Results were obtained from time-dependent GEE with logit link for binary outcomes clustered by woman ID number. Binary outcome (seroconversion/no seroconversion) was detected during the 36 months follow-up (cut-off, 200 MFI; >2× increase of MFI values in two subsequent samples). Statistically significant covariates ($P<0.05$) are indicated in bold.

Covariate	Seroconversion to LR-HPV (HPV 6 and/or 11)						Seroconversion to HR-HPV (HPV 16 and/or 18 and/or 45)					
	Crude OR	95 % CI	*P*	Adjusted OR*	95 % CI	*P*	Crude OR	95 % CI	*P*	Adjusted OR*	95 % CI	*P*
Age (categorical)	0.5	0.22–1.15	0.102	0.5	0.19–1.49	0.225	0.9	0.59–1.50	0.802	0.7	0.28–1.96	0.537
Baseline genital HR-HPV DNA status (HPV+; ref†)	0.9	0.54–1.46	0.647	1.1	0.61–1.91	0.794	0.6	**0.36–0.95**	**0.031**	0.7	0.41–1.25	0.241
Baseline oral HR-HPV DNA status	0.7	0.50–1.02	0.066	0.7	0.49–1.10	0.138	1.3	0.84–1.90	0.268	1.3	0.80–1.97	0.323
Baseline Pap smear (ASCUS cut-off)	0.8	0.51–1.33	0.415	0.6	0.37–1.06	0.079	0.6	0.39–1.04	0.071	0.7	0.39–1.26	0.236
Marital status (unmarried; ref†)	0.9	0.56–1.31	0.471	0.7	0.43–1.19	0.194	**0.6**	**0.40–0.94**	**0.023**	0.6	0.37–1.02	0.062
Age at menarche	1.0	0.91–1.19	0.596	1.0	0.89–1.19	0.696	1.0	0.90–1.16	0.719	1.0	0.89–1.23	0.605
Age at onset of sexual activity	1.3	0.87–1.96	0.193	1.1	0.60–1.96	0.787	1.2	0.77–1.78	0.450	1.8	0.95–3.34	0.072
Age at first pregnancy	1.0	0.90–1.04	0.309	0.9	0.86–1.05	0.311	1.0	0.89–1.04	0.326	0.9	0.85–1.05	0.273
Employment status (employed; ref†)	**1.5**	**1.08–1.97**	**0.014**	**1.5**	**1.07–2.09**	**0.019**	0.9	0.66–1.28	0.616	0.9	0.60–1.26	0.453
Smoking status (non-smoker; ref†)	1.2	0.74–2.07	0.412	0.9	0.61–1.29	0.530	0.8	0.61–1.17	0.308	**0.6**	**0.40–0.93**	**0.021**
Oral contraceptive use	1.1	0.84–1.48	0.439	1.2	0.85–1.78	0.277	0.9	0.70–1.22	0.592	0.9	0.61–1.21	0.388
No. of sexual partners until age 20 years	0.9	0.66–1.18	0.411	0.8	0.50–1.33	0.414	1.1	0.84–1.49	0.430	0.8	0.51–1.37	0.475
Lifetime no. of sexual partners (linear)	0.9	0.69–1.13	0.329	1.0	0.68–1.51	0.964	**1.4**	**1.13–1.85**	**0.004**	**2.0**	**1.26–3.03**	**0.003**
Practice of anal sex (never; ref†)	0.6	0.33–1.05	0.071	**0.5**	**0.24–0.94**	**0.031**	0.8	0.44–1.40	0.410	0.8	0.40–1.61	0.535
History of genital warts (no history; ref†)	1.4	0.77–2.53	0.269	1.5	0.80–2.99	0.194	0.6	0.32–1.02	0.060	0.7	0.35–1.33	0.259

*Adjusted for all other covariates in the model.
†ref, Used as reference.

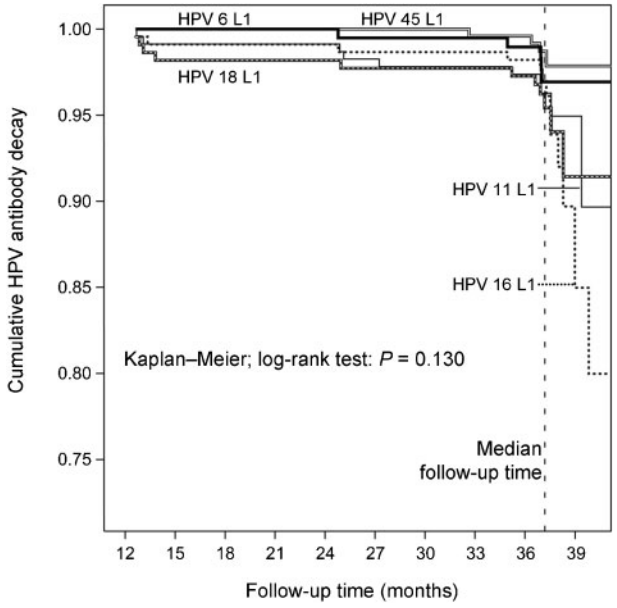

Fig. 3. Cumulative decay of antibodies against HPV 6, 11, 16, 18 and 45 during the 36 months of follow-up.

was modest or poor in the present study and lower than reported previously. By combined DNA positivity (positive at any site, genital and/or oral), we could obtain a better agreement between seropositivity and DNA positivity, especially for HPV 16, indicating that both sites could contribute to seropositivity and that seropositivity cannot distinguish at which site the immunogenic infection occurred.

The value of comparisons of absolute HPV type-specific seroprevalence figures published previously is very limited because of the use of different assays and, most importantly, different cut-off definitions. Only the use of a common standard would allow a comparison. In addition, comparative methodological studies, which would give a crude estimation of the specificity and sensitivity of different assays as related to each other, are lacking. There is recent evidence that ELISA is a less-sensitive method than the bead-based multiplex assay used in our study (Waterboer *et al.*, 2005). In general, we can conclude that the baseline seroprevalence figures for all HPV types in our study were higher than reported previously (Lehtinen *et al.*, 2006; Rama *et al.*, 2006; Jit *et al.*, 2007; Skjeldestad *et al.*, 2008; Wang *et al.*, 2008). When the more stringent (400 MFI) cut-off value for L1 antibodies was used, seroprevalence declined by 15–20 % and, in particular, seroprevalence of HPV 16 (20 %) and HPV 18 (8 %) was in line with earlier reports (Viscidi *et al.*, 1997; af Geijersstam *et al.*, 1998; Stone *et al.*, 2002). So far, the highest prevalence of HPV 16 seropositivity has been reported in South Africa, where 44 % of healthy 18–59-year-old women (median age, 44 years) were seropositive by using the VLP ELISA (Marais *et al.*, 2007).

In the present study, the seroprevalence of HPV 6 was significantly higher than those of the other four types tested and also higher than reported previously, except for the study of van Doornum *et al.* (1998), who reported seropositivity for HPV 6 and 11 in 58 and 48 %, respectively, of heterosexuals with multiple partners. The same group also reported that 4 and 17 % of teenagers without any evidence of sexual contact were seropositive for HPV 6 and 11, respectively. This might indicate that part of HPV 6 and 11 immunoreactivity is caused by HPV exposure on non-genital sites of the body and prior to the beginning of sexual activity. Thus, the wide variation in HPV seroprevalence can be explained not only by the different sensitivity and specificity of HPV serological assays, but also by the age of the subjects and their sexual behaviour, as well as the presence of active HPV lesions in the genital tract. The history of genital warts reported by women in our study cohort was also quite high, i.e. 27 %. This high prevalence may not be typical for the whole of Finland (Nikula *et al.*, 2007). However, women enrolled in our study were all from the Turku area and were enrolled during 1998–2001. For this particular area, Lehtinen *et al.* (2006) showed an increase in seroprevalence for HPV 6 and 11 among 23–32-year-old women from about 10 % in 1983–1988 to 16 % in 1989–1994, and about 30 % prevalence had already been reached in another region. It is conceivable that these seroprevalence increases, due to changes in sexual behaviour, continued further. Our study population showed markers of high sexual activity compared with populations analysed in other serological studies (mean age at first intercourse, $<$16 years; mean number of lifetime sexual partners, about five; history of genital warts, 27 %). The characteristics of our study population, together with sensitive serology and different cut-off definitions, might explain the high overall, and especially the very high HPV 6, seroprevalence.

Only two previous studies have used the same multiplex HPV serology method as the present study: one to assess serological response to HR-HPV types among university students in South Korea and the other in the German population (Clifford *et al.*, 2007; Michael *et al.*, 2008). In sexually active South Korean women, a higher prevalence for HPV 18 and 45 antibodies, but lower prevalence for HPV 16 antibodies, were found than reported in our cohort (HPV 18, 12 versus 8 %; HPV 45, 9 versus 2 %; HPV 16, 20 versus 10 %). In young adults (15–34 years) of the German population sampled in 1987 and 1988, the seroprevalence for HPV 16 and 18 was 4.1 and 7.8 %, respectively (Michael *et al.*, 2008). The method being the same, this difference probably reflects differences in the sexual habits and/or distinct distribution of different HPV types in these geographical regions, as well as different sampling times.

The predictors of HPV antibodies, i.e. covariates of HPV seropositivity, have recently attracted increasing attention. In the current study, the covariates were analysed by using both univariate and multivariate GEE approaches. We

clearly confirmed the results reported in several studies that the lifetime number of sexual partners is an independent predictor of seropositivity for HR-HPV (Carter *et al.*, 1996; Olsen *et al.*, 1997; Viscidi *et al.*, 1997; Castle *et al.*, 2002; Stone *et al.*, 2002; Clifford *et al.*, 2007; Skjeldestad *et al.*, 2008). In addition, we identified two additional independent predictors: being seropositive for LR-HPV and history of genital warts. These same covariates were also among the predictors of seropositivity for LR-HPV, in addition to age at onset of sexual activity. Although several studies have addressed the risk factors for being seropositive for HR-HPV (especially for HPV 16), there are very few previous data on predictors for serological response to LR-HPV. Interestingly, we found that serological response to LR-HPV types was also a risk for seropositivity to HR-HPV types, a finding also reported by Silins *et al.* (2000). They also found an association with condyloma history and seroconversion, which we could clearly confirm here for both LR- and HR-HPV. This might indicate that the same people are exposed to many different HPV types, and the LR-HPV types are probably the first. This would be in agreement with the detection of higher baseline seroprevalence of HPV 6 and 11 than HPV 16 and 18 in our cohort. Similarly, women who have seroconverted to one HPV type have a higher probability of also seroconverting to other HPV types and, in this series, 50 % of the seroconverted women showed seroconversion to two or more HPV types.

In multivariate GEE, the only independent predictors for HR-HPV seroconversion were smoking history (smokers were less likely to convert) and lifetime number of sexual partners. These factors were also reported by Wang *et al.* (2004) as the only predictors of HPV 16 seroconversion. In contrast to these, the only predictors of LR-HPV conversion were status of employment and anal sex. Those who practiced anal sex were less likely to convert than those who never practiced anal sex. Interestingly, we noticed that those who regularly practiced anal sex had no history of genital warts, whilst both the women who had never practiced anal sex and those who had occasional anal sex frequently reported a history of genital warts. One possible explanation could be that frequently practicing anal sex would expose the recipient continually to HPV, resulting steady levels of antibodies. Thus, using the settings of the current study, even repeated antibody measurements would not give significantly different HPV antibody titres, which, by definition, makes these individuals less likely to be seroconverters. However, additional studies on LR-HPV seroconversion are needed.

It is well established that HPV antibody levels are likely to wane over time, but detailed studies are lacking and some controversy exists (af Geijersstam *et al.*, 1998; Villa *et al.*, 2006). The ongoing vaccination trials have shown that HPV 6-, 11-, 16- and 18-seropositive subjects in the placebo group have had stable antibody levels during a follow-up time of up to 3 years (Villa *et al.*, 2006). Contradictory to that, several studies have reported that seropersistence seems to be related to the initial antibody levels and continuous antigen exposure (Carter *et al.*, 2000; Ho *et al.*, 2004; Wang *et al.*, 2004). In our study, decay of HPV antibodies was a rare event, ranging from 1.5 to 5.3 % of those initially seropositive for HPV 45 and 16, respectively, when the same criteria for HPV decay as for seroconversion were used. Also, the mean time for decay was nearly the same for all HPV types examined, which is contradictory to the report by Carter *et al.* (2000), who found differences between HPV 6 and HPV 16/18 antibody-decay times. Compared with the seroconversion times, however, decay was a much more delayed event.

In summary, we found that a substantial proportion of young Finnish women are seropositive for both LR- and HR-HPV types. Seroconversion to both groups is frequent, occurs within 18–24 months and is predicted by several covariates in common. In contrast, HPV antibody decay is a rare and more delayed event.

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