Title: Association of human papillomavirus genotype 16 viral variant and viral load with cervical high-grade intraepithelial lesions

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Abstract

Human papillomavirus genotype 16 (HPV16) is by far the genotype most strongly associated with cervical cancer; viral variant and/or viral load of HPV16 could modulate this association. Objective: to determine the association between the viral variant and viral load of HPV16 and the presence of cervical high-grade lesions.

This cross-sectional study included all women in whom HPV infection was found by cervical smear during routine gynecological health checks. Women with single or multiple HPV16 infections (n=176) were selected for viral variant and viral load analysis. Smear results were classified using the Bethesda system. HPV types were classified according to the International Agency for Research on Cancer. Odds ratios (ORs) with their 95% confidence intervals (CI) were estimated by logistic regression, adjusted for age, immigrant status, and coinfection with other high-risk genotypes.

No statistically significant associations were found regarding the detected viral variants. A viral load above the median (>1367.79 copies/cell) was associated with a significant risk of high-grade epithelial lesion or carcinoma, after adjusting for age, immigrant status, coinfections, and viral variant: (adjusted OR 7.89; 95% CI: 2.75–22.68). This relationship showed a statistically significant dose–response pattern after categorizing by viral load tertiles: adjusted OR for a viral load greater than the third tertile was 17.23 (95% CI: 4.20–70.65), with adjusted linear p trend=0.001.

In patients infected with HPV16, viral load is associated with high-grade intraepithelial lesions or cervical carcinoma. This could be useful as prognostic biomarker of neoplastic progression and as screening for cervical cancer.

Introduction

Cervical cancer is the second most common cancer in women aged 15 to 44 years in the United States [1,2] and Europe [3,4]. The association between human papillomavirus (HPV) and cervical cancer has been clearly established [5-7], with persistent infection by viral genotypes of high oncogenic risk having been identified as the most important risk factor [8, 9].

HPV genotypes 16 and 18 are high-risk genotypes that are associated with 70% of cervical cancer cases and an even higher proportion of HPV-associated cancers such as of the vulva, vagina, penis, anus, and oropharynx [10, 11] HPV 16 is by far the genotype most frequently associated with cases of cervical cancer (between 50–70%) [12]. Thus, most knowledge about the relationship between HPV viral variant, viral load, and cervix cancer has been based on this viral type.

There are molecular factors related to HPV 16 that could specifically modulate this association. Among them, genetic variability within the same viral genotype [13-16] and HPV viral load stand out [17, 18].

The determination of the viral load became a methodological challenge since it has been suggested that the high number of copies correlates with an increased risk of developing cervical lesions associated with HPV. The quantification of HPV DNA in the biological sample can be achieved by PCR-based methods (Polymerase chain reaction) or by the HC2 test (capture and hybridization) in a semiquantitative manner Estimates of the number of viral copies depend directly on the total number of cells and, ultimately, on the amount of viral DNA. Therefore, the adjustment for cell loading is an absolute requirement that is frequently not met, as is the case of HC2 and some protocols based on PCR [19-24].

Previous studies have been conducted to determine the association between viral load and the persistence of infection [25, 26]; as well as the relationship between the viral load and the severity, progression and development of cervical lesions [17, 27]. The results of these publications show that the amount of viral DNA increases proportionally to the severity of the lesions and is detectable even before the development of cervical lesions [26, 28-30]; however, other studies did not show such association [31, 18].

With regard to the determination of papillomavirus viral subtypes, HPVs are known to mutate very slowly because they are double-stranded DNA viruses that utilize the excellent correction ability of their host DNA polymerase. However, nucleotide polymorphisms can occur through a random mutation and can be established in a population. This genetic drift has been observed among the variants of HPV 16, suggesting its coevolution with humanity [32].

The HPV subtypes have been identified by comparing the sequences of the E6 and L1 genes and the long control region (LCR) [14, 33-34] with consensus sequences described [35] Most of the articles described refer to the amplification of the sequence of interest, either conventional PCR or nested PCR followed by sequencing for the determination of the viral variant [14]. Some studies have performed hybridization with specific lineage probes against the L1, E6 regions [36]. In the case of HPV 16, the E6 region is a short and conserved gene, frequently used because it contains enough information to identify all the subtypes and variants that have been described so far.

HPV16 variants have been classified into four major lineages based upon common phylogenetic patterns of single-nucleotide polymorphisms: European Asian, including

the sublineages European (EUR), and Asian (As), African 1 (AFR1), African 2 (AFR2) and Asian American/North American (AA/NA), including the sublineages Asian American 1, Asian American 2 and North American [37, 38].

Non-European variants of HPV 16, particularly of the Asian-American (AA) lineage, have been shown to have a greater propensity for persistence [39];-perhaps for this reason, they have a stronger association with high-grade squamous intraepithelial lesions (HSIL) [40, 41]. A recent meta-analysis of worldwide HPV 16 lineage distribution data confirmed the association of certain lineages with increased risk of cervical disease; however, some geographical dependence of these associations was also noted [15]. Within the European variant lineage, a T350G substitution in the E6 gene leads to an altered amino acid residue (L83V); this has been associated with persistence of HPV 16 [42] and cervical disease [43], although this association has not been found in all cases [44-46]. Two meta-analyses demonstrated that the E350 codon is associated with cervical disease, and it is likely that this association is geographically dependent [15, 47].

The effect of HPV coinfections on the risk of developing cervical intraepithelial lesions and cervical cancer remains unclear [48-50]. Some authors have showed strong associations of some high-risk HPV genotypes with coinfection or multiple infections [49-51]. In contrast, other authors have found no association between coinfection and increased risk of intraepithelial lesions and cervical cancer [52, 53].

The aim of our study was to determine the association between viral variant, viral load, and the risk of high-grade lesions in women infected with HPV genotype 16 in Spain.

Materials and Methods

Study Design

Cross-sectional study.

Patients

We included women who were attended for the first time at the Gynecology Department of the Hospital General Universitario de Elche in Spain for a routine gynecological health check (in which an opportunistic screening of cervical cancer was carried out) and who tested positive for any HPV genotype by cervical smear between January 4, 2010 and December 30, 2011. The study population was 180 women with a single or multiple infection determined by genotype 16 of HPV. In 176 of these 180 women it was possible to study the variant or the viral load of the HPV genotype 16 infection in depth. Data was available on HPV variant 16 in 135 women and on viral load in 144 women (all of them had available histopathology data).
S1 Fig shows a flow diagram of the population and samples to be studied.

Data source

The HPV molecular study was initiated in August 2016, and the data of each patient were obtained from computerized hospital records of the gynecology and microbiology departments. The data were extracted from paper medical records and histopathology departments using CliniViewer when necessary.

Samples

All samples were tested for the presence of HPV DNA using the LINEAR ARRAY HPV genotyping test® (Roche Molecular Diagnostics, Pleasanton, CA, USA) protocol, which is capable of genotyping HPV 6, 11, 16, 18, 26, 31,33, 35, 39, 40, 42,

45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39, and CP6108. HPV types are classified according to the World Health Organization International Agency for Research on Cancer (IARC) Monographs Working Group assessment of the carcinogenicity of different HPV types [8, 54-55]: 13 genotypes are classified as high risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and 5 as probable high risk (53, 66, 70, 73 MM9 and 82 MM4). The presence of two or more genotypes (high-risk, likely high-risk HPV or low-risk genotypes) in the same woman was defined as multiple infection. [50].The presence of two or more high-risk genotypes in the same patient was defined as coinfection [56].

Cells taken from the transformation zone were fixed on a slide for the cytology study. The Papanicolaou staining technique was used to check for the presence of cytological abnormalities, and the results were classified according to the Bethesda 2014 system [57].

Molecular methods

(i) Extraction of DNA from cervical specimens

DNA extraction was performed after incubation overnight at 56°C with 50 µL proteinase K and 25 µL 10% sodium dodecyl sulfate (SDS), using the NucliSENS® easyMAG® (Biomerieux, Marcy l'Etoile, France) platform, following the manufacturer's instructions. The extracted DNA was quantified, and DNA purity was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

(ii) Quantification of HPV16

A system was designed in our laboratory using the Primer Express 3.0 software package to amplify a fragment of the E6 gene of HPV16 (GenBank K02718.1) with the following primers: forward, 5′- CACAGGAGCGACCCAGAAA -3′; reverse, 5′- CACGTCGCAGTAACTGTTGCTT-3′; FAM-labeled probe, 5′- ACCACAGTTATGCACAGAGCTGCAAACAA -3′. The reaction was performed using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). This was determined using a standard curve made with six points ten-fold serial dilutions of a commercial papillomavirus DNA standard (AMPLIRUN® PAPILLOMAVIRUS TYPE 16 DNA; Vircell, Granada, Spain). Each reaction was performed in a final volume of 20 µl, containing 10 µl of Taqman Universal Master Mix (Thermo Fisher Scientific, Massachusetts, USA) 7.6 µl sterile nuclease-free water (Thermo Fisher Scientific, Massachusetts, USA), 0.6 µl of each primer (12.5 pmol) (Isogen Life Science, The Netherlands), 0, 2 µl of probe (4 pmol) Taqman and 1 µl of total DNA from each sample. The PCR program consisted of three steps: 1st of 50ºC for 2 minutes, 2nd of 95ºC for 10 minutes and followed by 40 cycles of a denaturing temperature of 95ºC for 15 seconds and annealing and extension step at 60ºC for 1 minute.

(iii) Adjustment of results according to the number of human cells in each cervical specimen

The human albumin gene (GenBank AY728024.1) was amplified in all samples to verify DNA integrity and determine viral copy number per cell, using primers 5′- CTGCATTGCCGAAGTGGAA-3′ and 5′- CAAACATCCTTACTTTCAACAAAATCA-3′ plus the FAM-labeled probe 5′- TGCCTGCTGACTTGCCTTCATTAGCTG -3′. A standard curve for human cells was made from leukocytes extracted from peripheral

blood using Histopaque (Sigma-Aldrich, St. Louis, MO, USA) and quantified using a CEL-DYN 3600 (Abbott Laboratories, Chicago, IL, USA). A ten fold serial diluted standard curve was performed ranging from 1 million of cells to 10 cells per microliter and albumin PCR were run by triplicate for each standard curve point. A linear regression was performed with the data obtained from the albumin amplification representing for the Ct values versus the logarithm of the number of targets present at each point of the standard line. The results are expressed as the ratio between the number of HPV 16 and number of human cells in each cervical sample.

(iv) Viral variant

The viral variant was detected by nested PCR. Primers were designed to target specific HPV16 genome E6 regions (S1 Table). All PCR reaction mixtures contained: 5 µL 10x PCR buffer, 0.1 µL Taq Bioline® DNA Polymerase, 1.5 µL MgCl2 (50 Mm), 0.1 µmol/L deoxynucleotide triphosphates 50 mM (Thermo Fisher Scientific, Massachusetts, USA), 1 µL forward and reverse primer, 1 µL DNA solution for the first amplification and 5 µL for the second amplification, and nuclease-free water up to 50 µL final volume. PCR conditions were 94°C for 7 min; 40 cycles of 30 sec at 94°C, 30 sec at 54°C, 40 sec at 72°C; plus a 7-min final extension at 72°C. PCR products were Sanger-sequenced in an external company (Macrogen Inc. Seul, Rep. of Korea, www.dna.macrogen.com). The obtained sequences were aligned with the reference sequence NC_001526.2 (corresponding to an E6 350T sequence without mutations) using the CLC Sequence Viewer 6 program (QIAGEN, Redwood City, CA, USA) and thus look for the key positions described for the genetic classification of the viral variant [35].

Statistical analysis

For the categorical and discrete variables, proportions were estimated using the Pearson’s chi-squared test for comparisons and Fisher's exact test when appropriate. Quantitative variables were expressed as mean and standard deviation (SD), using the Student *t*-test for comparisons, after testing normality using the Shapiro–Wilk test.

Histological lesions were classified as "no high-grade lesion" (smear negative for intraepithelial lesion, atypical squamous cells of undetermined significance/atypical glandular cells (ASC-US/AGC), atypical squamous cells–cannot exclude high-grade squamous intraepithelial lesion (HSIL) (ASC-H), or low-grade squamous intraepithelial lesion (LSIL) versus "high-grade lesion" (HSIL, squamous cell carcinoma, adenocarcinoma in situ, or adenocarcinoma), and treated as a dependent variable in the logistic regression models.

Variant and viral load were treated as independent variables. Viral load was categorized using tertiles as ‘”cut-off” points into an ordinal variable (low, moderate, high viral load). To estimate the strength of association between variant and viral load and the risk of high-grade lesion, crude and adjusted odds ratios (ORs) with their 95% confidence intervals (CI) were estimated by unconditional logistic regression. The following potential confounders were pre-established for inclusion in the models: age (continuous variable), immigrant status, and coinfection. We calculated tests for OR trends of viral load using logistic models that included categorical terms as a continuous variable.

The alpha error was set at 0.05 and all *p*-values were two-sided. All statistical analyses were performed using IBM SPSS Statistics V22.0 (IBM Corp., Armonk, NY, USA).

The research protocol was approved by the Elche Clinical Research Ethics Committee and the data were anonymized prior to statistical analysis.

Results

One hundred and eighty women were included in the study, with mean age 34.34 years (SD=11.35), and 88.51% were Spanish. Coinfection with HPV 16 and another high-risk genotype was present in 30.68% (n=54) of the women. Coinfection with HPV 16 and two high oncological-risk genotypes was found in 10.23% of women.

More than half (51.14%) of women had at least one cytology smear showing a morphological anomaly (ASC-US/ASC-H, LSIL, HSIL, or carcinoma). The prevalence of ASC-US/ASC-H was 7.39% (95% CI: 3.24–11.54), that of LSIL was 17.61% (95% CI: 11.70–23.53), and that of HSIL was 21.59% (95% CI: 15.23–27.95). The prevalence of cancerous lesions was 4.55% (95% CI: 1.18–7.91); see Table 1.

Age and immigrant status were both independently associated with greater risk of a high-grade intraepithelial lesion or carcinoma. For each 10-year increase in age, the risk of a lesion was 2.35 times greater (95% CI: 1.40–3.94), regardless of the viral load or HPV 16 variant. Furthermore, being an immigrant was independently associated with a 4.31 times greater (95% CI: 1.06–17.51) risk of a lesion (S2 Table). Multiple infection or coinfection were not significantly associated with HSIL or carcinoma (Table 2).

More than one-third (34.7%) of women infected with HPV 16 had a single infection with this genotype. The most prevalent viral variants in single infections, as well as in

multiple ones, were European E350G and European E350T, present in 42.1% and 33.5% of women, respectively. Regarding HPV viral load, the median was 1367.78 copies/cell, with an interquartile range of 127.41–35645.39 (Table 3).

None of the analyzed HPV 16 variants was specifically associated with a statistically significant increase in the risk of high-grade intraepithelial lesion or carcinoma. See S3 Table.

The box plot in Fig 1 shows the viral load of HPV 16 on a logarithmic scale, according to whether there is high-grade intraepithelial lesion or carcinoma. The median, as well as the first and last tertiles, were higher in patients with a high-grade intraepithelial lesion or carcinoma than patients without high-grade intraepithelial lesion or carcinoma.

Having a viral load above the median (>1367.79 copies/cell) was associated with an unadjusted statistically significant increased risk of high-grade intraepithelial lesion or carcinoma: crude OR 8.17 (95% CI: 3.13–21.31). The viral load also showed a statistically significant dose–response pattern on categorizing the viral load ordinally based on tertiles, linear p trend <0.001, last tertile crude OR 16.67 (95% CI: 4.55–61.03).

These associations were maintained after adjusting for age and immigrant status (adjusted ORs 7.35 and 16.34, respectively).

The association between viral load and risk of intraepithelial lesion was also independent of the HPV 16 variant (variant-adjusted ORs 8.43 and 17.25, respectively).

Lastly, the associations of viral load were maintained after including all the covariates in the multivariate regression models, with an OR adjusted for age, immigrant status, HPV 16 variant, and coinfection for viral load greater than the median of 7.89 (95% CI: 2.75–22.68) and an adjusted OR for viral load greater than the third tertile (P75) of 17.23 (95% CI: 4.20–70.65); adjusted linear p trend <0.001 (Table 4).

Fig 1. Box plot of HPV 16 viral load (logarithmic scale), according to histopathology lesion.

Discussion

Regarding analysis of the European variants, some studies have shown an association between the E350G variant (which has an altered amino acid residue in gene E6) and cervical disease [43] Others found a higher association with persistence for the E350T variant [46]. In our study, we did not find any association between cervical disease and the European variants E350T or E350G, which coincides with previously reported results [45-47].

Regarding an association between the AA variant of HPV genotype 16 and the presence of HSIL or cervical carcinoma, Xi et al. observed that the risk of developing HSIL in women infected with the HPV 16 AA variant was 3.1 times greater than women infected with European variants [40]. The association reported by Xi et al. is similar to our crude OR of 2.93. However, our crude association was not statistically significant because it was based in very few cases of AA variants: 2 cases in the "No HSIL/Carcinoma" group and 2 cases in the "HSIL/Carcinoma" group. Moreover, our

association did not maintain when adjusting for age, immigrant status, coinfection or age, so we cannot claim an association. Some authors have pointed out that the differences observed in these associations might be geographically dependent [15]. A metaanalysis on this topic could reach a conclusion with more precision.

The viral load of HPV genotype 16 was the most important independent predictor of a high-grade epithelial lesion or carcinoma in our study. We found that viral loads above 1,367 copies/cell (median) were associated with an 8 times higher risk of having a high-grade histopathological lesion. In a cohort with 1,728 women followed-up for 9 years, Muñoz et al. [9] found that viral load was the main determining factor for persistence of HPV 16. High viral loads are associated with a greater risk of developing high-grade lesions, thus establishing that viral load is a determining factor for the persistence of infection.

Wu et al. [58] found a strong association between viral load and women with cancer of the cervix (OR 68; 95% CI: 17.8–259.7). They showed that a high viral load could predict future development of cervical cancer, and raised the possibility of using additional markers for the early identification of women at risk.

Our results show relatively lower values than the studies mentioned [9, 58], but we should point out that we focused on HPV genotype 16 and did not take into account the weight of other detected high oncological-risk genotypes.

Among studies that have used the real-time PCR technique, Ylitalo et al. [29] used a case-control design and found that cases had consistently higher viral loads for HPV 16 than controls; in addition, higher viral loads could be detected up to 13 years before the diagnosis of cervical cancer. In that study, women with high viral loads of HPV 16 had a 30 times greater risk of cervical cancer compared with women who

were HPV negative, and this increased risk was consistent over time. A second study conducted in the same population showed that 20% of women with the highest HPV 16 viral loads had a 60 times higher risk of developing carcinoma in situ than those who were negative for HPV [30].

The relationship between HPV 16 viral load and risk of a cervical intraepithelial lesion showed a clear dose–response pattern in our study. After adjusting for age, immigrant status, coinfection, and detected viral variant, we found an OR of 17.23 for the last tertile (viral count greater than 11,792 copies/cell). Moberg et al. [59] also found a dose–response pattern, observing maximum ORs (OR=51) in the higher viral load percentile.

In the literature, different cut-off points have been adopted for categorizing viral load. However, the different methods used (real-time PCR, Hybrid Capture II) for quantifying viral load precludes comparisons between studies. Marks et al. [60] categorized the cut-off point at 2000 copies/10^4 cells, although they concluded that individual measurements of viral load were not useful. Saunier et al. [17] quantified viral load using the same method as in our study and proposed that a viral load of greater than 22,000 copies /10^3 cells could be used to identify women at greater risk of high-grade lesions. In one study evaluating the clinical correlation of HPV 16 and HPV 18, it was found that the highest predictive value for a grade 2 cervical epithelial lesion or higher was observed with a HPV 16 viral load cut-off of 3.0×10^6 copies per million cells [61]. Taking the aforementioned information into account, with the aim of ensuring the quality of the analysis and a higher statistical power, the viral load in our study was categorized according to the median and the distribution in tertiles.

The results obtained corresponded to a single evaluation of HPV viral load. As our design is cross-sectional and there is no follow-up, our study cannot analyze the association between a higher viral load and an increased viral persistence supported by longitudinal published studies [9, 27-30, 62].

On the other hand, another caveat is that cervical lesion was performed on exfoliated cervical cells instead of biopsy samples with laser dissection, so we did not have confirmation of high-grade lesions by histological study (biopsy). However, studies evaluating the category HSIL compared to biopsy as gold standard support a very high probability of an accurate diagnosis [63, 64].Therefore, several authors have used a methodology similar to ours, assessing the cervical lesion with cervical cytology [63].

In conclusion, in patients infected with HPV genotype 16, the viral load of this genotype was the most important independent predictor of high-grade intraepithelial lesion or cervical carcinoma. In addition, a strong dose–response pattern was observed. The viral load of genotype 16 was associated with higher grades of cervical intraepithelial lesion or carcinoma, especially when it was above 1,367 copies/cell. Higher HPV-16 viral loads may indicate viral persistance, progression to cervical dysplasia, and may even serve as a prognostic biomarker for screening tests of cervical cancer; however, longitudinal studies are needed to confirm these hypotheses.

List of abbreviations

AOR: odds ratios adjusted

ASC-US: atypical squamous cells of undetermined significance

CI: confidence intervals

COR: crude odds ratios

HPV: human papillomavirus

HSIL: high grade squamous intraepithelial lesion

IARC : World Health Organization International Agency for Research on Cancer

LSIL: low grade squamous intraepithelial lesion

OR: odds ratios

PCR: polymerase chain reaction

SD: standard deviation

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Table 1. Sociodemographic variables, HPV coinfections and histopathologic findings in women infected with HPV genotype 16.

	HPV 16 (n=176)		
	n	**%***	**95% CI**
Age, years; mean (SD)	34.34 (11.35) Range: 17-76		
Nationality			
Spanish	154	88.51	83.48–93.53
Immigrant	20	11.49	6.47–16.52
Missing	2		
No. of high-risk genotypes detected**			
Only 1 genotype (HPV 16)	92	52.27	44.61–59.94
2 genotypes	54	30.68	23.58–37.78
3 genotypes	18	10.23	5.47–14.99
4 genotypes	12	6.82	2.81–10.83
No. of probable high-risk genotypes detected besides HPV 16**			
No genotypes	140	79.55	73.30–85.79
1 genotype	30	17.05	11.21–22.89
2 genotypes	6	3.41	0.44–6.37
No. of low-risk genotypes besides HPV 16**			
No genotypes	119	67.61	60.42–74.81
1 genotype	37	21.02	14.72–27.33
2 genotypes	15	8.52	4.11–12.93
3 genotypes	4	2.27	0.62–5.72
4 genotypes	1	0.57	0.01–3.13
Cytology results***			
Negative****	49	27.84	20.94–34.75
Inflammation	28	15.91	10.22–21.60
ASC-US	13	7.39	3.24–11.54
LISL	31	17.61	11.70–23.53
HISL	38	21.59	15.23–27.95
Carcinoma in situ	8	4.55	1.18–7.91
Specimen not evaluable	9	5.11	1.58–8.65

HPV, human papillomavirus; CI, confidence interval; SD, standard deviation; ASC-US, atypical squamous cells of undetermined significance; LISL, low-grade squamous intraepithelial lesion; HISL, high-grade squamous intraepithelial lesion.

*Percentage valid with no unknown values.
** Classification of papillomavirus genotypes of high oncological risk according to the latest international guidelines (Muñoz N et al., 2003; IARC Group, 2007; Schiffman M et al., 2009).
***Cytology results classified according to the Bethesda 2014 system (Nayar R et al., 2015).
****Negative for intraepithelial lesion or malignancy.

Table 2. Characteristics of HPV 16 infection and risk of high-grade intraepithelial lesion or carcinoma.

	No HSIL or carcinoma* (n=121)	**HSIL or carcinoma* (n=46)**				
HPV 16 infection	n	n	COR	95% CI	AOR	95% CI
Only HPV 16	43	15	1		1	
Multiple infection **	78	31	1.14	0.55–2.34	1.42	0.64–3.13
p-value			0.723		0.384	
No coinfection	63	25	1		1	
Coinfection***	58	21	0.91	0.46–1.80	1.11	0.53–2.32
p-value			0.792		0.775	

HPV, human papillomavirus; CI, confidence interval; HSIL, high-grade squamous intraepithelial lesion; COR, crude odds ratio; AOR, odds ratio adjusted for age (continuous) and immigrant status.
*Cytology results classified according to the Bethesda 2014 system (Nayar R et al., 2015).
**Two or more genotypes in the same woman was defined as multiple infection (Goldman et al., 2013).
***Two or more high-risk genotypes in the same woman was defined as coinfection (Trigo-Daporta et al., 2014).

Table 3. Characteristics of HPV16 infection: single or multiple infection, HPV 16 variant, and HPV 16 viral load.

HPV 16 (n=176)	n	%	95% CI
Infection			
Only HPV 16	61	34.66	27.34–41.97
HPV 16 + other genotypes*	115	65.34	58.03–72.66
HPV 16 + other high-risk genotypes**	84	47.73	40.06–55.39
HPV 16 variant			
European (E350T)	59	33.52	26.26–40.78
European (E350G)	74	42.05	34.47–49.62
Asian-American (AA)	4	2.27	0.62–5.72
African lineage (Af1)	1	0.57	0.01–3.13
African lineage (Af2)	3	1.71	0.35–4.9
Missing	35	19.89	13.71–26.07
HPV 16 viral load			
First quartile (P25)	127.41		
Second quartile (median)	1367.78		
Third quartile (P75)	35645.39		

HPV, human papillomavirus; CI, confidence interval.
*Two or more genotypes in the same woman was defined as multiple infection (Goldman et al., 2013).
**Two or more high-risk genotypes in the same woman was defined as coinfection (Trigo-Daporta et al., 2014).

Table 4. Associations between viral load in infection with HPV 16 and risk of high-grade intraepithelial lesion or carcinoma.

	No HSIL or carcinoma	HSIL or carcinoma*								
HPV 16	**(n=108)**	**(n=36)**	**COR**	**95% CI**	**AOR1**	**95% CI**	**AOR2**	**95% CI**	**AOR3**	**95% CI**
Viral load										
Median										
≤1367.78	67	6	1		1		1		1	
1367.79+	41	30	8.17	3.13–21.31	7.35	2.69–20.07	8.43	3.16–22.45	7.89	2.75–22.68
p-value			<0.001		<0.001		<0.001		<0.001	
Tertiles										
≤305.30	46	3	1		1		1		1	
305.31–11792.11	39	8	3.15	0.78–2.68	2.74	0.65–11.60	3.22	0.79–13.20	2.54	0.58–11.11
11792.12+	23	25	16.67	4.55–61.03	16.34	4.21–63.49	17.25	4.60–64.74	17.23	4.20–70.65
p-value			<0.001		<0.001		<0.001		<0.001	

HPV, human papillomavirus; CI, confidence interval; HSIL: high-grade squamous intraepithelial lesion; COR, crude odds ratio; AOR1, odds ratio adjusted for age (continuous) and immigrant status; AOR2, odds ratio adjusted for viral variant; AOR3, odds ratio adjusted for age (continuous), immigrant status, and coinfection.

* Cytology results classified according to the Bethesda 2014 system (Nayar R et al., 2015)

Fig 1. Box plot of HPV 16 viral load (logarithmic scale), according to histopathology lesion.