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Original Research

Large contribution of human papillomavirus in vaginal neoplastic lesions: A worldwide study in 597 samples

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Abstract ***Aim:*** This work describes the human papillomavirus (HPV) prevalence and the HPV type distribution in a large series of vaginal intraepithelial neoplasia (VAIN) grades 2/3 and vaginal cancer worldwide.
Methods: We analysed 189 VAIN 2/3 and 408 invasive vaginal cancer cases collected from 31 countries from 1986 to 2011. After histopathological evaluation of sectioned formalin-fixed paraffin-embedded samples, HPV DNA detection and typing was performed using the SPF-10/DNA enzyme immunoassay (DEIA)/LiPA$_{25}$ system (version 1). A subset of 146 vaginal cancers was tested for p16^{INK4a} expression, a cellular surrogate marker for HPV transformation. Prevalence ratios were estimated using multivariate Poisson regression with robust variance.
Results: HPV DNA was detected in 74% (95% confidence interval (CI): 70–78%) of invasive cancers and in 96% (95% CI: 92–98%) of VAIN 2/3. Among cancers, the highest detection rates were observed in warty-basaloid subtype of squamous cell carcinomas, and in younger ages. Concerning the type-specific distribution, HPV16 was the most frequently type detected in both precancerous and cancerous lesions (59%). p16^{INK4a} overexpression was found in 87% of HPV DNA positive vaginal cancer cases.
Conclusions: HPV was identified in a large proportion of invasive vaginal cancers and in almost all VAIN 2/3. HPV16 was the most common type detected. A large impact in the reduction of the burden of vaginal neoplastic lesions is expected among vaccinated cohorts.

1. Introduction

Vaginal cancer is a rare malignancy, with an estimation of 13,000 new cases diagnosed worldwide in 2008 and accounting for about 2% of all gynaecologic cancers [1,2]. Most vaginal invasive cancer cases occur in patients older than 60 years, except for adenocarcinomas which occur in younger ages [2,3].The squamous cell carcinoma (SCC) is the most frequently diagnosed histological type (80–90%), followed by adenocarcinomas [2]. As for cervical cancer, squamous cell vaginal cancer is preceded by premalignant lesions. They are referred as vaginal intraepithelial neoplasia (VAIN) of grades 1, 2 or 3 on the basis of features similar to the cervical intraepithelial neoplasia.

Several risk factors have been described for vaginal cancer and in particular for the SCC type which resemble those of cervical cancer like smoking, immunosuppression, high number of sexual partners, and also history of cervical precancerous and cancerous lesions [4–6]. In contrast, the vaginal adenocarcinomas, particularly clear cell adenocarcinomas, have been largely related in the past to *in utero* exposure to diethylstilbestrol (DES), which was prescribed as an anti-abortive until the early 1970's [7–9].

Human papillomaviruses (HPVs) have been causally linked to vaginal cancers in few case–control studies [6,10,11]. HPV DNA has been detected in a large proportion of vaginal SCC and, as in other anogenital cancers, HPV16 has been shown to be the predominant HPV type identified [12,13]. However, data remain scarce due to the rarity of this cancer and little is known about the contribution of other HPV types and their geographical variability. A meta-analysis on HPV prevalence and type distribution in different anogenital cancer sites included a small number of cases from vaginal lesions (191 VAIN 2/3 and 136 invasive vaginal cancer cases). Furthermore, due to the diversity of the study protocols and of the HPV DNA detection techniques used in the studies, HPV prevalence varied from 50% to 100% in VAIN 2/3 and from 25% to 89% in invasive cancers of the vagina [12].

In the present study, a standard protocol for collection and histological evaluation of specimens and a highly sensitive SPF-10 polymerase chain reaction (PCR), DNA enzyme immunoassay (DEIA) HPV detection

combined with the $LiPA_{25}$ genotyping technique was used to analyse the HPV DNA prevalence and type-specific distribution in 597 vaginal specimens (189 VAIN 2/3 and 408 invasive vaginal cancer cases) from 31 countries. This systematic approach will give a wider representation of the HPV type specific burden in vaginal lesions in the world and to better assess the potential impact of HPV vaccination on these lesions.

2. Materials and methods

2.1. Study design

A retrospective cross-sectional study was designed and coordinated by the Catalan Institute of Oncology (ICO), Barcelona, Spain, and DDL Diagnostic Laboratory, Rijswijk, Netherlands, to estimate the HPV DNA prevalence and type distribution in women with VAIN 2/3 and invasive vaginal cancers diagnosed from 1986 to 2011. Formalin-fixed paraffin-embedded (FFPE) specimens from cases were obtained from hospital pathology archives in 31 countries: Europe (Austria, Belarus, Czech Republic, France, Germany, Greece, Poland, Spain and United Kingdom); North America (United States of America); Latin America (Argentina, Brazil, Chile, Colombia, Ecuador, Guatemala, Mexico, Paraguay, Uruguay and Venezuela); Africa (Mozambique, Nigeria); Asia (Bangladesh, India, Israel, South Korea, Kuwait, Philippines, Taiwan and Turkey); and Oceania (Australia). Centres were requested to provide non-selected series of primary cancer and pre-neoplastic cases from their archives preferably consecutive in time. Information about age at diagnosis, year of diagnosis and original histological diagnosis was also obtained from the participating centres.

2.2. Histopathological evaluation

FFPE blocks were processed under strict conditions to avoid potential contamination as previously described [14]. At least four FFPE sections were obtained from each block. Briefly, first and last sections were used for histopathological evaluation after haematoxylin and eosin (HE) staining. The intermediate sections were used for HPV DNA testing. Processing of FFPE and pathology reassessment of the initial histopathological diagnosis was done by the reference pathology laboratory for the study at ICO, and was performed following the consensus criteria established by an expert panel of pathologists based on the WHO classification on female genital organs [2]. A block was determined to be adequate for further HPV DNA testing if invasive cancer or VAIN 2/3 lesion was observed in the two HE stained sections of the specimen. In case of discrepancies between the local and the reference pathology laboratories, the results obtained at the reference lab prevailed. To control for possible sources of contamination during tissue preservation, blocks containing non-HPV related tissue processed at the same time as the included specimens in the local pathology lab were blindly included and processed (5% of the total vaginal lesions).

2.3. HPV DNA detection and typing

For each specimen, a paraffin tissue section was digested with 250 μL of Proteinase K solution (10 mg/mL proteinase K in 50 mM Tris–HCI, pH 8.0) to release DNA. SPF-10 PCR was performed using 10 μL of the extracted DNA that was diluted ten times in a final reaction volume of 50 μL. The PCR products were tested for the presence of HPV DNA trough DEIA as previously described [15,16]. Amplimers testing positive for viral DNA by DEIA were used to perform reverse hybridization line probe assay ($LiPA_{25}$) (version 1: produced at Laboratory Biomedical Products, Rijswijk, The Netherlands) [16]. The $LiPA_{25}$ detection system allows for genotyping of 25 HPVs categorised by the International Agency for Research on Cancer (IARC) within the Group 1 (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59), Group 2A (HPV68), Group 2B (HPV34, 53, 66, 70 and 73), Group 3 (HPV6 and 11), as well as other HPVs (HPV40, 42, 43, 44, 54 and 74) [17]. All these 25 types belong to nine species within *Alphapapillomaviruses*. The sequence variation within the SPF-10 inter-primer region allows for the recognition of these different HPV genotypes, except for types 68 and 73, as their inter-primer regions are identical and cannot be distinguished by $LiPA_{25}$. Specimens that tested positive for HPV DNA by DEIA but that could not be genotyped by $LiPA_{25}$ were further analysed by direct Sanger sequencing of PCR products [18]. HPV DNA positive cases that could not be sequenced were labelled as '*HPV undetermined*'. In addition, specimens with an inconclusive probe line pattern by $LiPA_{25}$ (i.e. HPV68/73 or HPV39/68/73) were also sequenced to distinguish the specific HPV types. Cases that could not be distinguished were labelled as HPV68/73 or HPV39/68/73. In order to evaluate DNA quality, all HPV DNA negative samples were subjected to a PCR targeting the human tubulin gene (forward primer: TCCTCCACTGGTACACAGGC; reverse primer: CATGTTGCTCTCAGCCTCGG), which generates a 65 bp amplicon, the same size as the SPF-10 amplicon used for assessing presence of HPV DNA. Samples that were both negative for HPV and tubulin DNA were considered to be of inadequate quality and were therefore excluded from the final analyses (Supplemental Fig. 1).

2.4. $p16^{INK4a}$ expression

Immunohistochemical cellular $p16^{INK4a}$ expression evaluation was performed on a random selection of HPV negative and positive cases (total $n = 146$).

$p16^{INK4a}$ was detected using the CINtec histology kit (clone E6H4, Roche mtm laboratories AG, Germany), following the manufacturer's protocol. A pattern of diffuse staining of more than 25% stained tumoral cells (nuclear and cytoplasmic staining) was considered positive [19,20].

2.5. Statistical analysis

Variables included in the analysis were country, age at and year of diagnosis, histopathological diagnosis, HPV DNA presence, HPV genotype, and $p16^{INK4a}$ expression. Histological subtypes in vaginal cancers were grouped into four categories: (1) SCC 100% warty-basaloid (i.e. exclusively or combinations of warty, basaloid or papillary basaloid histologies), (2) SCC 100% non-warty-basaloid (i.e. SCC without any warty-basaloid morphological feature), (3) SCC mixed histologies (i.e. mix of previous histological subtypes); or (4) other (i.e. undifferentiated carcinomas, neuroendocrine, adenocarcinomas, and adenosquamous carcinomas).

HPV DNA prevalence and HPV type-specific detection percentages were determined for the different geographical regions, histopathological categories, year of and age at diagnosis. Prevalence ratios (PRs) were estimated using bivariate and multivariate Poisson regression models with robust variance [21]. In the adjusted model we included region, year of and age at diagnosis. Histological diagnosis was not included in the regression analysis since it was considered as an intermediate variable in the carcinogenic process and not a potential confounding factor. The best fitting model was selected based on the log-likelihood ratio test.

HPV DNA prevalences were estimated among included cases and HPV type-specific relative contributions were calculated among HPV DNA positive cases. Multiple infections were added to single types under a weighting attribution proportional to the detection found in cases with single types as previously described [13]. In order to evaluate the increase or decrease of the HPV type-specific relative contributions between type of lesions, relative contribution ratios and their 95% confidence intervals (CI) were estimated (ratio of type-specific relative contribution: percentage of a specific type in vaginal cancer/percentage of the same type in high-grade pre-neoplastic lesions).

Agreement between HPV DNA and $p16^{INK4a}$ results within the samples was assessed by Kappa score. The McNemar chi-squared test for matched pair data was used for assessing unequal distribution of discordant results.

Statistical significance for all analyses was set at the two-sided 0.05 level. Data analyses were performed with STATA version 10.0 (Stata Corporation, Computing Resource Center, College Station, Texas).

2.6. Ethical consideration

Specimens were received anonymously and allocated a unique identification number upon reception. All protocols were approved by international and ICO ethics committees and all the study progress was overseen by an international steering committee specifically formed for the supervision and advising in critical issues of the project.

3. Results

Initially, 830 FFPE tissue samples were collected. From these, 84 were classified as controls and used for contamination control, and 149 cases were excluded from the analysis. Reasons for exclusion were: 126 cases were not suitable for HPV DNA testing based on the centralised histopathological evaluation and 23 were finally excluded for being both HPV and tubulin DNA negative. Therefore, 189 VAIN 2/3 and 408 vaginal invasive cancers were included in the final analysis (Supplemental Fig. 1). Mean age at diagnosis was 50 years (standard deviation-SD: 14) for VAIN 2/3 cases and 61 years (SD: 15) for invasive cancer cases (p-value < 0.001).

HPV DNA prevalence for VAIN 2/3 was 96% (95% CI: 92–98%) and 74% (95% CI: 70–78%) for invasive cancer cases (Tables 1 and 2, respectively). In invasive cancer cases, the highest HPV DNA prevalence was observed in the American cases (78%) and the lowest in the African specimens (68%) (Table 2). However, no statistically significant associations between HPV prevalence and either the geographical region or the period of diagnosis could be established. Younger cancer patients showed the highest HPV DNA detection rates. These findings remain valid even after excluding the adenocarcinomas from the analysis (data not shown). Regarding histology, SCC with 100% warty-basaloid features showed a higher HPV DNA prevalence (86%) than the SCC 100% non-warty-basaloid (77%) (Table 3). 'Other' histological diagnosis had a much lower HPV DNA positivity (30%) than SCCs combined altogether (81%) (p-value < 0.001). The 'other' category included: 35 adenocarcinomas, twelve undifferentiated carcinomas, four adenosquamous cell carcinomas, and three neuroendocrine tumours. HPV positivity among adenocarcinomas was 26%, and the most frequent histological subtype was the clear cell, thirteen cases were identified and two were HPV DNA positive. In none of the two positive cases $p16^{INK4a}$ was overexpressed. Other adenocarcinoma subtypes and their HPV DNA positivity were: mucinous 2/12 (17%), not otherwise specified 3/6 (50%), and endometrioid 2/4 (50%). Regarding the other histological types, HPV positivity was found in 4/12 (33%) of the undifferentiated cases, 1/4 (25%) of adenosquamous cell carcinomas, and 2/3 (67%) of neuroendocrine tumours.

Table 1
Sample description and HPV DNA prevalence in VAIN 2/3 cases.

			HPV prevalence			Prevalence ratios (PR)		
	n	%	*n*	%	95% CI	PR	95% CI	*p*-Value
Region								
Europe[a]	96	51%	94	98%	[93–100%]	1		
Latin America	80	42%	74	93%	[84–97%]	**0.91**	**[0.83–0.99]**	**0.038**
Asia and Oceania	13	7%	13	100%	[75–100%*]	1.00	[0.97–1.03]	0.975
Period of diagnosis								
1986–1999	22	12%	22	100%	[85–100%*]	**1.10**	**[1.02–1.19]**	**0.019**
2000–2011[a]	167	88%	159	95%	[91–98%]	1		
Age at diagnosis								
<55 yo[a]	117	62%	113	97%	[92–99%]	1[b]		
55–75 yo	63	33%	61	97%	[89–100%]	1.00	[0.94–1.05]	0.862
>75 yo	8	4%	6	75%	[35–97%]	0.77	[0.52–1.14]	0.196
Missing information	1	<1%	1	100%	[3–100%*]	–	–	–
Total	**189**	**100%**	**181**	**96%**	**[92–98%]**			

'VAIN 2/3': vaginal intraepithelial neoplasia 2/3; 'HPV prevalence': HPV DNA positivity; 'yo': years old; '95% CI': 95% confidence interval.
[a] Reference category for the multivariate analysis. Model adjusted for region, period of diagnosis and age at diagnosis.
[b] *p*-trend test, 0.204 (excluding missing category). In bold numbers are highlighted PRs with a *p*-value < 0.05.
* One-sided, 97.5% confidence interval.

Table 2
Sample description and HPV DNA prevalence in invasive vaginal cancer cases.

			HPV prevalence			Prevalence ratios (PR)		
	n	%	*n*	%	95% CI	PR	95% CI	*p*-Value
Region								
Europe	152	37%	108	71%	[63–78%]	0.95	[0.84–1.08]	0.466
America[a,*]	191	47%	149	78%	[72–84%]	1		
Africa	19	5%	13	68%	[44–87%]	0.88	[0.64–1.20]	0.411
Asia and Oceania	46	11%	33	72%	[57–84%]	0.95	[0.78–1.16]	0.593
*Period of diagnosis***								
1986–1999	91	22%	66	73%	[62–81%]	0.99	[0.86–1.14]	0.915
2000–2011[a]	316	78%	236	75%	[70–79%]	1		
Age at diagnosis								
<55 yo[a]	137	34%	107	78%	[70–85%]	1[b]		
55–75 yo	181	44%	137	76%	[69–82%]	0.97	[0.86–1.09]	0.597
>75 yo	75	18%	47	63%	[51–74%]	**0.81**	**[0.66–0.99]**	**0.043**
Missing information	15	4%	12	80%	[52–96%]	1.01	[0.76–1.33]	0.951
Total	**408**	**100%**	**303**	**74%**	**[70–78%]**			

'HPV prevalence': HPV DNA positivity; 'yo': years old; '95% CI': 95% confidence interval.
[a] Reference category for the multivariate analysis. Model adjusted for region, period of diagnosis and age at diagnosis.
[b] *p*-trend test, 0.052 (excluding missing category). In bold numbers are highlighted PRs with a *p*-value < 0.05.
* All cases are from Latin American countries, except from three cases from United States of America.
** One case with missing information regarding year of diagnosis.

The most common HPV type was HPV16, which was detected in 59% of either VAIN 2/3 lesions and invasive vaginal cancer, among the HPV DNA positive cases (Table 4). In VAIN 2/3, HPV16 was followed by HPV18 (6%), HPV52 (6%); and HPV73 (5%). Other HPV types accounted for less than 5% each. The proportion of multiple infections was 11%. In invasive cancer cases, HPV16 was followed by HPV18, 31 and 33 (5% each); and other HPV types accounted for less than 5% each. Undetermined HPV types were found in <1% and multiple infections in 4%.

When analysing the relative contribution of HPV16 or HPV18 in VAIN 2/3 and in invasive cancer samples, we did not find statistically significant differences according to the different available information from cases: age groups, time at diagnosis, the histology or the region (data not shown. For type specific data by variables please see Supplemental Tables from 1 to 7).

A high proportion of HPV DNA positive cases showed a $p16^{INK4a}$ overexpression pattern (96/110 = 87%). Concordance between $p16^{INK4a}$ and HPV DNA detection was observed in 87% of the cases (95% CI: 80–92%; Kappa score = 0.677; *p*-value < 0.001) (Table 5). The McNemar test was not statistically significant ($p = 0.064$); indicating that the discordant results were equally distributed.

Table 3
Histological diagnosis in invasive vaginal cancer cases.

			HPV prevalence and prevalence ratios (PR)					
	n	%	*n*	%	95% CI	PR	95% CI	*p*-Value
Histological diagnosis								
SCC 100% warty-basaloid[a]	128	31%	110	86%	[79–92%]	1		
SCC 100% non-warty-basaloid	209	51%	161	77%	[71–83%]	**0.90**	**[0.81–0.99]**	**0.035**
SCC mixed histologies	17	4%	16	94%	[71–100%]	1.10	[0.95–1.26]	0.196
Other[b]	54	13%	16	30%	[18–44%]	**0.34**	**[0.22–0.51]**	**<0.001**
Total	**408**	**100%**	**303**	**74%**	**[70–78%]**			

'HPV prevalence': HPV DNA positivity; 'SCC': squamous cell carcinoma; '95% CI': 95% confidence interval.
[a] Reference category for univariate analysis.
[b] Other histological diagnosis includes: 35 adenocarcinoma, 12 undifferentiated carcinomas, four adenosquamous cell carcinomas; and three neuroendocrine tumours. In bold numbers are highlighted PRs with a *p*-value <0.05.

Table 4
HPV type-specific relative contribution among HPV DNA positive VAIN 2/3 and invasive vaginal cancer cases.

HPV Type	VAIN 2/3 (HPV+, $n = 181$)				Invasive vaginal cancer (HPV+, $n = 303$)				Relative contribution ratio (cancer:VAIN)*	
	Single		Single + Multiple		Single		Single + Multiple			
	n	%	*n*	%	*n*	%	*n*	%	Ratio	(95% CI)
HPV6	2	(1%)	3	(1%)	2	(<1%)	3	(1%)	0.60	(0.12–2.93)
HPV11	–	–	–	–	1	(<1%)	1	(<1%)	–	–
HPV16	95	(53%)	106	(59%)	174	(57%)	178	(59%)	1.00	(0.86–1.17)
HPV18	10	(6%)	10	(6%)	15	(5%)	15	(5%)	0.90	(0.41–1.95)
HPV26	1	(<1%)	1	(<1%)	1	(<1%)	1	(<1%)	0.60	(0.04–9.49)
HPV30	2	(1%)	2	(1%)	–	–	–	–	–	–
HPV31	1	(<1%)	1	(<1%)	16	(5%)	16	(5%)	**9.56**	**(1.28–71.47)**
HPV33	7	(4%)	8	(4%)	14	(5%)	15	(5%)	1.12	(0.49–2.59)
HPV35	3	(2%)	3	(2%)	3	(1%)	3	(1%)	0.60	(0.12–2.93)
HPV39	–	–	–	–	6	(2%)	6	(2%)	–	–
HPV39/68/73	–	–	–	–	1	(<1%)	1	(<1%)	–	–
HPV42	–	–	–	–	1	(<1%)	1	(<1%)	–	–
HPV45	3	(2%)	3	(2%)	10	(3%)	11	(4%)	2.19	(0.62– 7.75)
HPV51	3	(2%)	4	(2%)	7	(2%)	7	(2%)	1.05	(0.31– 3.52)
HPV52	9	(5%)	10	(6%)	8	(3%)	9	(3%)	0.54	(0.22– 1.30)
HPV53	3	(2%)	3	(2%)	–	–	–	–	–	–
HPV56	5	(3%)	5	(3%)	4	(1%)	5	(2%)	0.60	(0.18–2.04)
HPV58	2	(1%)	2	(1%)	11	(4%)	11	(4%)	3.29	(0.74–14.66)
HPV59	4	(2%)	7	(4%)	2	(<1%)	5	(2%)	0.43	(0.14–1.32)
HPV66	2	(1%)	2	(1%)	1	(<1%)	1	(<1%)	0.30	(0.03–3.27)
HPV67	1	(<1%)	1	(<1%)	–	–	–	–	–	–
HPV68	–	–	–	–	2	(<1%)	2	(<1%)	–	–
HPV68/73	–	–	–	–	1	(<1%)	1	(<1%)	–	–
HPV69	–	–	–	–	3	(1%)	3	(1%)	–	–
HPV73	7	(4%)	9	(5%)	6	(2%)	6	(2%)	0.40	(0.14–1.10)
HPV82	–	–	–	–	1	(<1%)	1	(<1%)	–	–
HPV89	1	(<1%)	1	(<1%)	–	–	–	–	–	–
HPV Undetermined	–	–	–	–	1	(<1%)	1	(<1%)	–	–
Multiple	20	(11%)	–	–	12	(4%)	–	–	**0.36**	**(0.18–0.72)**

'VAIN 2/3': vaginal intraepithelial neoplasia; 'HPV + ': HPV DNA positive; 'Single': single infections; 'Single + Multiple': multiple infections were added to single types under a weighting attribution proportional to the detection found in cases with single types as described in the methodology; '95% CI': 95% confidence interval.
* Considering single + multiple columns estimation. 95% CI relative contribution ratio that does not contain 1 is highlighted in bold numbers.

4. Discussion

To our knowledge, the present study is the largest dataset of VAIN 2/3 and invasive vaginal cancer cases published so far. We described here the HPV DNA prevalence and type distribution in a large series of 189 VAIN 2/3 and 408 vaginal invasive cancer cases from 31 countries.

HPV prevalence in VAIN 2/3 lesions and invasive vaginal cancer was of 96% and 74%, respectively; similar to that found in a previous meta-analysis, 90% and 70%, and in a recently published report of vaginal cancers from US, 75% [12,22]. The lower positivity among invasive vaginal cancers compared to precursor lesions is consistent with previous reports [6,12]; and has been also described in a higher magnitude in HPV related

Table 5
Concordance of HPV and $p16^{INK4a}$ results in invasive vaginal cancer cases.

	$p16^{INK4a}$		Total
HPV DNA	Negative	Positive	
Negative	31 (86%)	5 (14%)	36
Positive	14* (13 %)	96 (87%)	110
Total	45	101	146

Overall concordance: 87% (95% confidence interval (CI): 80–92%); Kappa score = 0.677, $p < 0.001$ (95% CI: 0.541–0.812); Concordant cells are highlighted in grey.%: Row% (p16 results among each HPV results category).
* HPV types with a p16 negative result-cases with single HPV infection: HPV6 (1), HPV16 (5), HPV31 (2), HPV33 (2), HPV39 (1), HPV56 (1), HPV69 (1), HPV Undetermined (1)

precancerous and cancerous lesions from other anatomical sites like in the vulva [12,23]. The explanation of these findings is still unclear and could be related to the existence of two different aetiopathogenic pathways one HPV related and the other independent of HPV, and that the HPV related cancers have more clear preneoplastic stages and/or easier to be diagnosed than non-HPV related lesions. Moreover, HPV positivity was associated with age at diagnosis such as for other HPV related cancers, the youngest the case the highest HPV detection was observed. Although we cannot discard a cohort effect, this repeated finding through different anogenital sites could suggest that HPV related cancers evolve faster; and that cancers with a late onset may be related to other risk factors that may need more years of carcinogenic exposition [12,23]. This age association with HPV detection is stronger in vulvar cancers than in anal, vaginal or cervical cancers [12,23]. Interestingly, in a subset of invasive cases, $p16^{INK4a}$ was performed showing overexpression in 87% of HPV DNA positive invasive cases, confirming the concordance of this molecular surrogate marker with HPV diagnosis in HPV related tumours and suggesting an E7 effect on the pRb pathway [19,20].

In most of VAIN 2/3 and invasive cancer cases, HPVs were found as a single type, and multiple types were detected in a higher proportion in VAIN 2/3 than in invasive cases. As for other anogenital cancers, the decrease in the detection of multiple infections from precancerous to cancerous lesions and the differences in HPV type distributions might be explained by the selection of the most carcinogenic HPV types and the clearance of those that are less carcinogenic during the tumorigenic process [12,23,24]. For both VAIN 2/3 and invasive cancer cases, the most common HPV type was HPV16, which was detected in 59% of the viral DNA positive cases, similar to that found in the cervix (Table 6) [14], and lower to that observed in other HPV related cancers like vulva, anal or oropharyngeal cancers [12,23,25]. In Table 6 a comparison of HPV type-specific relative contributions between cervical, vulvar and vaginal cancers is shown. The most frequent HPV types detected in cervical and vaginal cancers are the same except for HPV35 in the cervix and 51 in vagina, and the contributions are similar except for HPV18, which is more frequent in cervical cancer than in vaginal. The difference in HPV18 was lower when selecting only cervical squamous cell carcinomas, due to the higher detection of this type in glandular lesions. Conversely, in vulvar cancer there is a higher detection of alpha-9 types, particularly HPV16 compared to cervical and vaginal cancers [23]. These differences may be due to a higher tropism of these types for vulvar epithelial or immunological differences between the different anatomical tissues. There were not statistical differences in the HPV16/18 relative contribution counted together by the available information (i.e. age at and year of diagnosis, geographical region and histological diagnosis), neither for VAIN 2/3 nor for invasive vaginal lesions. The global HPV16 and HPV18 relative contribution in both VAIN 2/3 and invasive vaginal cancer was 64%.

HPV prevalence in the 'other' histological category was much lower than the one observed for SCC lesions. This low prevalence has been already reported in other HPV related cancer sites like in the vulva [23], and also but with a lower magnitude in the cervix [14]. This could suggest that HPV is more prone to develop neoplastic lesions in the squamous epithelial cells, particularly in HPV related cancers other than cervix. Interestingly, the most frequent 'other' diagnosis was the adenocarcinoma and within this type, the clear cell was the most frequent histological subtype. Only two of the clear cell adenocarcinomas were positive and $p16^{INK4a}$ was not overexpressed in none. Clear cell adenocarcinomas have been strongly linked with *in utero* exposure to the antimiscarriage pill DES [7–9]. It was recently shown that DES, which was prescribed from the forties until the early seventies, is still increasing the risk of clear cell adenocarcinoma of the vagina in women that were exposed *in utero* [26,27]. Although women exposed to DES were at an increased risk, only one in one thousand women exposed would develop such cancers [23]. Furthermore, DES is not the only cause since women 'not exposed' to DES can also develop clear cell adenocarcinomas, like in the cervix [28].

The strengths of the study include the high number of samples, even though the rarity of the disease, and participating centres, the centralised and standardised histological assessment and classification of lesions; and the use of a uniform protocol to process and analyse the specimens under strict contamination control, involving a highly sensitive and well-characterised assay suitable for FFPE materials. One of the possible limitations of the study is the potential misclassification of the

Table 6
Comparison of HPV relative contribution of most frequent types in cervical, vulvar and vaginal cancers.

	Cervical cancer series* (*n* HPV+ = 8977)		Vulvar cancer series** (*n* HPV+ = 429)		Vaginal cancer series (*n* HPV+ = 303)	
	n HPV+	%	*n* HPV+	%	*n* HPV+	%
The most frequent HPV types[a]						
HPV16	5439	61	311	73	178	59
HPV18	918	10	20	5	15	5
HPV45	528	6	14	3	11	4
HPV33	345	4	28	7	15	5
HPV31	335	4	4	1	16	5
HPV52	253	3	8	2	9	3
HPV58	203	2	4	1	11	4
HPV35	175	2	0	0	3	1
HPV types by species[b]						
Alpha-9	6776	75	356	83	232	77
Alpha-7	1786	20	41	10	41	14

* Data extracted from Ref. [14] (HPV+: HPV DNA+).
** Data extracted from Ref. [23] (HPV+: HPV DNA+ and p16+).
[a] Selection of the eight most frequent types based on findings in cervical cancer.
[b] Including single and multiple infections, Alpha-9: 16, 31, 33, 35, 52, 58, 67, Alpha-7: 18, 39, 45, 59, 68, 70, 85.

anatomical site since it has been described that vaginal cancers are associated to previous history of anogenital cancers and that up to 30% cancer patients are reported to have had previous precancerous and/or cancerous lesions of the cervix. We have tried to minimise this potential bias by selecting only primary tumours, thus as stated by the International Federation of Gynaecology and Obstetrics (FIGO), a tumour of the vagina involving the uterine cervix or the vulva should be classified as a primary cervical or vulvar cancer, respectively. Regarding geographical region interpretation of the results, we must be cautious since the samples recruited do not cover the entire countries within a given region, and the inclusion of institutions follows a convenient selection.

The majority of the cancers of the vagina and almost all VAIN 2/3 analysed in this large study were HPV DNA positive. Furthermore, the HPV cell transforming activity was confirmed in most of the HPV positive cancer cases by $p16^{INK4a}$ overexpression. With HPV16, and HPV18 to a lesser extent, being the most common type in VAIN 2/3 and in invasive vaginal cancers, HPV vaccination could prevent around 64% of both HPV related lesions.

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Conflict of interest statement

The corresponding author has received occasional travel fund to attend scientific meetings from Merck and Sanofi Pasteur MSD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejca.2014.07.018.

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