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Meaning of the Decreased HPV Normalized Viral Load Marker in Clinical Evolution of Women with HPV Infection

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Abstract: (1) Background: HPV infection can progress over the years to become cervical cancer. In this study, genotype and a normalized viral load were evaluated as surrogate markers of progression to cancer. (2) Methods: A total of 558 endocervical swabs were collected from 120 women (mean, 40.1 ± 11.8 years old). Seventy-eight of the women underwent clinical intervention (CI) to clear the infection during the course of the study, while forty-two did not (NCI). Normalized viral load (NVL) was calculated using a COBAS 4800 system. The INNOLIPA genotyping system was used to classify HPV which was neither type 16 or 18. (3) Results: The mean age of CI women was 41.1 ± 11.4 (22–68) years old and that of the NCI group was 37.7 ± 12.13 (23–65) (p: 0.104). HPV16 was present in 11 (25%) NCI and 30 (35.2%) CI patients, HPVα9non16 in 20 (45%) NCI and 34 (40%) CI, and HPVnonα9 in 13 (29.5%) NCI and 21 (24.7%) CI (p = 0.48). In NCI women there was an average NVL decrease of 0.95 log after two years and a further decrease of 2.35 log at the end of the third year. At the end of the study, 34 (80%) of the NCI patients were clear of HPV. However, NVL of CI women remained at around 5 log until intervention (p < 0.001). (4) Conclusions: Viral load decreased in NCI women at follow-up in the second year. In contrast, in CI women, their viral load did not fall over the follow-up period. This work thus demonstrates that a reduction in normalized viral load was associated with good evolution.

Keywords: HPV; viral load; predictor; evolution; progression; cervical cancer

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1. Introduction

Numerous factors associated with the host, such as smoking, oral contraceptives and coinfection with other microorganisms, as well as alterations of the vaginal microbiota, among others, contribute to the development of cervical carcinoma [1–3]. However, in all circumstances HPV must be present [4,5]. HPV infection takes around 10 years to progress to cancer, passing through a series of lesions: LSIL (low-grade lesion, including CIN I) and HSIL (high-grade lesion, including CIN II and CIN III). Current WHO clinical guidelines recommend that women with LSIL should be monitored, while those with HSIL are usually referred for therapy. However, between 40 and 68% of HSIL patients may spontaneously regress, suggesting some women are over-treated [6,7]. To find a marker that evaluates the infection in each step would thus be very useful, especially when spontaneous regression is possible. It seems logical that certain viral factors are also involved in carcinoma development, such as that high-risk genotypes such as HPV16 or 18 have been shown more implicated than low-risk ones, due to variant or more active viral replication. The monitoring and evaluation of HPV replication has been highlighted as a way of helping to understand and predict the progression of the infection [8,9], as is also the case with other chronic viral infections, where change in viral load is a useful marker to evaluate the evolution of the infection (for instance, HIV). The aim of this study

was to establish the utility of normalized viral load as a viral marker which can be used throughout HPV infection in order to predict the evolution of infected women.

2. Materials and Methods

Between 2014 and 2018, 558 endocervical swabs from a total of 120 women were collected. The women were all seen annually for a cervical pathology consultation because of HPV infection, according to clinical protocols. The mean age of patients was 40.1 ± 11.8 (22–68) years old. At the beginning of the study, 63 women did not present intraepithelial lesion (they developed throughout the study) and 57 had a lesion suggestive of HPV infection.

The study was approved by the Principado de Asturias Ethics Committee, and all methods were carried out in accordance with relevant guidelines and regulations. Informed consent was obtained from all subjects included in the study.

Patients were grouped according to the evolution of their HPV infection: those whose infection resolved without the need for clinical intervention (no clinical intervention, NCI) but were followed up for at least 3 years; and those where viral lesions needed to be eliminated by different procedures (clinical intervention, CI). In the second case, only viral loads prior to surgery were considered in the analyses.

Samples were collected by endocervical brushing during the cervical pathology appointment, stored in 20 mL of STE buffer (10 mM Tris-HCl (pH: 8), 0.1 M NaCl, 1 mM EDTA) and sent to the Virology laboratory. Once in the lab, samples were stored at room temperature for no more than one week. An automatic COBAS 4800 system (ROCHE Diagnostics, Mannheim, Germany) was used to detect HPV according to the manufacturer's instructions. This system allows, in one step, the extraction of DNA from the sample and the amplification of a fragment of the HPV L1 gene, as well as the detection of the human Betaglobin gene in order to check the quality of the sample. In addition, it individually distinguishes HPV16 and HPV18, as well as a pool of 12 other high-risk HPV genotypes (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68).

This system, besides providing a report of the positive/negative result, allows the amplification cycle (Ct) of HPV-positive cases to be obtained, as well as those of the Betaglobin gene. The relationship between the two results can be used to estimate normalized viral load, since the betaglobin Ct indicates the number of cells present in the sample while the HPV Ct is an indication of the amount of virus present. Comparison of these data with their respective standard curves enabled the number of viral copies per cell to be calculated as it was described previously by Alvarez-Argüelles et al. [10]. Normalized viral load was thus expressed as the number of copies of HPV per 1000 cells.

To identify which high-risk (HR) genotypes were detected by the COBAS 4800 system, the INNOLIPA HPV genotyping extra II hybridization system (IINOGENETICS N.V., Ghent, Belgium) was performed according to the manufacturer's instructions. In addition, those HPV16 samples which were the T350G variant were identified using an in-house PCR previously described [11].

For analysis purposes, the genotypes found were then grouped as HPV16, HPVα9non16 (HPV31, 33, 35, 51, 52 and 58) and HPVnonα9.

The statistical analysis, consisting of the parametric Student's t-test and contingency tables, were carried out using the R Studio software [12]. In order to know whether lower viral load is a good marker to predict patient evolution, an ROC study was used. Results with a p value < 0.05 were considered to be statistically significant.

3. Results

Of the 57 patients who presented a lesion at the beginning of the study, 41 underwent surgery during the study, as did 36 of the negative for intraepithelial lesion or malignancy (NILM) patients. Table 1 shows the data for each patient when they entered the study and at each follow-up, which in the case of CI patients ceased post-surgery.

Table 1. Clinical and virological characteristics of patients studied.

Patient	Age	Lesion	Genotype(s)	Genotype Group	Variant T350G	VL0	VL1	VL2	VL3	VL4	GROUP
1	32	NILM	53	Nonα9		4.4	3.5				CI
2	65	NILM	18	Nonα9		3.7	4.3	6.4	2.7		NCI
3	45	HSIL	31/33	α9		4.8	4.9	3.9			CI
4	61	LSIL	44/66	Nonα9		5.1	4.9	4.2			CI
5	39	HSIL	16	HPV16	YES	5.1	2				CI
6	30	LSIL	16/51	HPV16/α9	NO	4.8	8	8.7	3.8	0	NCI
7	37	LSIL	52	α9		4	4.1	6.4			CI
8	30	HSIL	33/31	α9		5.3	6.1				CI
9	23	LSIL	53/66	Nonα9		2.4	3.1	0			NCI
10	63	NILM	16	HPV16	YES	4	3.8	5.9	0		NCI
11	30	HSIL	16	HPV16	YES	4.2	4				CI
12	57	HSIL	66	Nonα9		6	4.5	4.5			CI
13	44	LSIL	31	α9		5.4	4.9	0	0		NCI
14	49	HSIL	16	HPV16	NO	3.7	3.4	3.1	4.4	4.1	CI
15	38	HSIL	33	α9		5.3	5.3				CI
16	33	NILM	31	α9		3.1	4.3	0			NCI
17	44	LSIL	16/66	HPV16	YES	9	8.6	8.5	7.8		CI
18	29	NILM	31	α9		4.9	6.1	3.8	0		NCI
19	38	NILM	16/45	HPV16/nonα9	NO	5.2	7.3	5.9			CI
20	64	NILM	52	α9		3.4	3.5	4.2			CI
21	60	NILM	16	HPV16	NO	4.2	3.6	3.9	3.4		NCI
22	39	NILM	18	Nonα9		4.2	4	4.6			CI
23	68	HSIL	16/52	HPV16/α9	YES	6.5	10.8	6.4			CI
24	56	LSIL	53/56	Nonα9		5.4	5.8	5.6	4.8		CI
25	57	NILM	31	α9		3.8	2.4	3.3	0		NCI
26	30	NILM	52/56	α9		5.1	5.2	3.6	0	0	NCI
27	43	NILM	31	α9		5.6	5.7	5.4	0	0	NCI
28	26	NILM	52	α9		3	3.8	4.6	4.3		CI
29	35	NILM	52	α9		4.7	4.8	5.2	0	0	NCI
30	27	NILM	56	Nonα9		4.9	3.9	4.1	4.6		CI
31	35	HSIL	16	HPV16	NO	5	4				CI
32	24	LSIL	16	HPV16	NO	4.9	5.8	0	0		NCI
33	67	NILM	31	α9		3.6	3.2	3.4			CI
34	46	HSIL	16	HPV16	YES	3.8	3.9	4.3			CI
35	37	NILM	16	HPV16	YES	4	2.7				CI
36	52	NILM	16	HPV16	YES	3.9	2.5	2.5	2.7	0	NCI
37	34	LSIL	31	α9		4.2	2.9	4.7	4.1	0	NCI
38	32	NILM	16/59	HPV16/nonα9	YES	10.9	9.2	11.5	9.6		CI
39	27	LSIL	31	α9		6.7	3.4	3.2	0		NCI
40	58	HSIL	16	HPV16	YES	3.5	3.5				CI
41	43	LSIL	56	Nonα9		4.9	5.2				CI
42	62	NILM	52	α9		4.6	3.6				CI
43	37	NILM	33	α9		4.9	4.6	3.6			CI
44	39	NILM	31	α9		3.1	5.3	6	5.3	3.6	NCI
45	26	LSIL	16	HPV16	NO	4.4	4.5	0	0		NCI
46	36	NILM	58/66	α9		5.6	5.3	5.2			NCI
47	30	ASCUS	31/66	α9		6.9	6.1				CI
48	37	NILM	45/52	α9		3.2	3.7	5.5			CI
49	23	ASCUS	58	α9		5.5	6.3	3.9	0	0	NCI
50	31	HSIL	16	HPV16	NO	5	3.9	3.1	3.1		CI
51	28	NILM	35	α9		5.7	4.8				CI
52	30	HSIL	18	Nonα9		5.1	6.2	5.4	5.2		CI
53	49	LSIL	52	α9		5.4	3.6	5.1			CI
54	35	NILM	59	Nonα9		4.2	5.7	4.5	2.7		CI
55	56	LSIL	16	HPV16	NO	4.6	4.8				CI
56	53	NILM	51	Nonα9		5.3	2				CI
57	59	HSIL	31	α9		4.1	2.8				CI
58	49	HSIL	16/66	HPV16/nonα9	NO	8.4	10.4				CI
59	38	NILM	39	Nonα9		6.5	6	0			NCI
60	36	LSIL	16	HPV16	YES	2.9	5.4	5.8	3.3		CI
61	40	NILM	51	Nonα9		4.8	4.7	3.4	0		NCI
62	26	LSIL	16	HPV16	YES	5.4	5.4	5.3	0	0	NCI
63	28	ASCUS	56	Nonα9		4.2	5.4	0			NCI
64	26	NILM	31/33	α9		5.6	6	3.7	0	0	NCI
65	47	HSIL	16	HPV16	YES	2.8	4				CI
66	53	NILM	31	α9		7.1	5.8	5.7	3		CI
67	37	LSIL	35	α9		5.7	4.4				CI
68	22	ASCUS	16	HPV16	NO	4.9	4.7	4.4			CI
69	30	HSIL	33	α9		5	4.2				CI
70	39	ASCUS	18	Nonα9		3.3	5.9	0			NCI
71	30	LSIL	52	α9		3.8	4.5	4	3.2	0	NCI
72	47	NILM	39	Nonα9		6.1	6.1	3.8	0		NCI
73	40	NILM	16	HPV16	YES	2.1	3	2.1			CI
74	33	NILM	18	Nonα9		4.9	4.2	3.2	3.7		NCI
75	28	NILM	16	HPV16	NO	4.7	3.5	3.5	4.3		CI
76	37	HSIL	35	α9		4.2	3	3.3			CI
77	29	NILM	35	α9		5.6	5.7	5.8			CI
78	45	NILM	35	α9		3	4.6	4.6			CI
79	40	NILM	31	α9		6.3	3.1	2.7	0	0	NCI
80	48	NILM	16	HPV16	NO	4.6	5.2	4.1			CI

Table 1. *Cont.*

Patient	Age	Lesion	Genotype(s)	Genotype Group	Variant T350G	VL0	VL1	VL2	VL3	VL4	GROUP
81	42	NILM	31/70	α9		3.4	3.3				NCI
82	33	LSIL	51	Non α9		2.1	2.2	5	0		NCI
83	34	LSIL	66	Non α9		5.7	5.9	5.6			CI
84	51	NILM	16	HPV16	NO	4.2	4.2	4.5	6.7		CI
85	33	NILM	51	Non α9		2.8	6.8	5.2	3.2		CI
86	24	NILM	52	α9		2.5	2.7				CI
87	59	HSIL	58	α9		2.7	2.6				CI
88	39	NILM	16	HPV16	NO	3.7	4	3.5			CI
89	53	NILM	16	HPV16	NO	5.1	5.4	4.4	4.7	4.5	NCI
90	42	HSIL	39/58	α9		4.1	4.7	4.7	3.8	4.3	CI
91	27	LSIL	16/52	HPV16/α9	YES	11.2	10.5	8.6	8.1	4.6	NCI
92	50	NILM	31	α9		2.9	4.5	3			CI
93	42	LSIL	51	Non α9		5.6	5.4	5.6	6.6	4.6	CI
94	55	NILM	51	Non α9		4.6	7.1	7.6			CI
95	60	NILM	35	α9		4	2.9	3.3	3.6		NCI
96	34	NILM	31	α9		5.4	3	2.7			CI
97	54	NILM	16	HPV16	YES	4.4	4.5				CI
98	33	LSIL	18	Non α9		6.9	6	6	6.9	6.9	CI
99	31	NILM	16	HPV16	YES	2.9	3.3	3.8			CI
100	30	ASCUS	33/61	α9		3.9	4.3	6	0		NCI
101	27	HSIL	16	HPV16	NO	5.8	6.2	5.8	4		CI
102	33	NILM	16	HPV16	YES	6	3.3				CI
103	29	LSIL	56	Non α9		7	4.1	3.6			CI
104	43	NILM	52	α9		3.7	3.9	3.8	3.2		NCI
105	28	NILM	16	HPV16	YES	4.2	3.8	0			NCI
106	29	NILM	56	Non α9		5.9	5	0			NCI
107	58	NILM	31/53	α9		4.7	4.3	5.2			CI
108	31	NILM	39	Non α9		6.2	4.6	5	0		NCI
109	49	NILM	16	HPV16	NO	5.1	4.7				CI
110	33	ASCUS	56	Non α9		7.1	5.6	3.6	4.8		CI
111	46	LSIL	31/51	α9		3.6	6.7	6.1	6.1		CI
112	38	NILM	16	HPV16	NO	5.4	3.6	4.3			CI
113	28	HSIL	16/31	HPV16/α9	YES	7.9	7.8	3.4			CI
114	27	NILM	16	HPV16	NO	2.1	4.3	3.4	3.2	0	NCI
115	61	NILM	45	Non α9		3.2	4.4	4.5	0		NCI
116	53	NILM	66	Non α9		6	3.3	4.3			CI
117	48	NILM	16	HPV16	YES	4.9	3.2	5.3			CI
118	49	LSIL	16/52	HPV16/α9	NO	8.8	9.3	9.6			CI
119	30	LSIL	51	Non α9		5.8	7.1	4.3	5.4	5.2	CI
120	26	NILM	31	α9		5.6	5.2	5.6			CI

VL: Viral load; CI: Clinical intervention. Data in grey correspond to the last viral load measurement prior to clinical intervention; NCI: No clinical intervention; NILM: Negative for intraepithelial lesion or malignancy; ASCUS: Atypical squamous cells of undetermined significance; LSIL: Low grade intraepithelial lesion; HSIL: High grade intraepithelial lesion. Results of VL are expressed in copies of HPV/1000cells.

3.1. Genotype

The influence of age, genotype and the presence of single or mixed infections on the evolution of patients was studied.

There was no difference in age between those patients who received clinical intervention and those that did not when looking at the amalgamated data for single and mixed infections. The picture was, however, different when mixed and single infections, and the different genotype groups, were examined separately. In mixed infections and for the HPVα9non16 group, women were younger than in the respective CI group.

In terms of genotype group, HPVα9 genotypes (either HPV16 or HPVα9non16) were found in 95 cases (73.6% of total), of which 41 were HPV16 (43.1% of the subgroup). Consideration of genotype and disease evolution data showed that HPV16 was found in 30 (35.2%) CI patients compared to 11 (25%) NCI patients, while HPVα9non16 was present in 34 (40%) CI and 20 (45%) NCI cases, and HPVnonα9 was detected in 21 (24.7%) CI and 13 (29.5%) NCI patients. The HPV16 variant T350G was present in 16 (20.5%) CI patients and in 5 (11.9%) NCI ($p = 0.31$). Furthermore, all of the 24 patients with mixed infection (20% of the total) were positive for HPVα9 genotypes (either HPV16 or HPVα9non16) and of these, 16 received CI (20.5% of the CI subgroup) while 8 did not (19% of the NCI subgroup) (Table 2).

Table 2. Age distribution according to genotype and evolution.

	n	Age CI Patients x ± σ (Range)	CI95	*n*	Age NCI Patients x ± σ (Range)	CI95	*p*
Total	78	41.4 ± 11.49 (22–68)	38.8–43.9	42	37.7 ± 12.13 (23–65)	33.9–41.4	0.104
Single	62	40.66 ± 11.34(22–67)	37.9–43.7	34	39.41 ± 12.63 (23–65)	34.5–43.6	0.52
Mixed	16	44.56 ± 11.93 (28–68)	38.2–50.9	8	30.5 ± 6 (23–42)	25.4–35.5	0.005
HPV16	30	41.03 ± 10.72 (22–68)	37.02–45.03	11	37.82 ± 15.55 (24–63)	27.3–48.2	0.53
HPVα9non16	34	43.82 ± 13.32 (24–68)	39.1–48.4	20	34.50 ± 10.19 (23–60)	29.7–39.2	0.019
HPVnonα9	21	39.1 ± 9.42 (27–57)	34.8–43.3	13	38.77 ± 12.05 (28–65)	31.4–46.05	0.934

3.2. Viral Load

Of the 42 NCI patients, viral load became undetectable in 34 while, in contrast, all 78 of the patients who needed surgery to eliminate the infection had a detectable viral load throughout the study period ($p = 0.0003$). The average viral load at each follow-up according to treatment condition (CI/NCI) and the number of patients who cleared the infection spontaneously (NCI group) or through surgery (CI group) is shown in Table 3.

Table 3. Number of patients clear of HPV or that were intervened at each follow-up.

	Initial Test (0)		Follow-Up 1		Follow-Up 2		Follow-Up 3		Follow-Up 4		
	n	VL	*n*	VL	*n*	VL	*n*	VL	n	VL	*p*
NCI											
VL	42	4.66 ± 1.55 (2.1–11.2)	42	4.71 ± 1.53 (2.2–10.5)	41	3.45 ± 2.37 (0.0–8.7)	32	1.61 ± 2.17 (0.0–8.1)	8	1.58 ± 2.21 (0.0–4.6)	<0.0001
CI95%		4.17/5.14		4.23/5.18		2.7/4.19		0.82/2.39		−0.26/3.4	
Undetectable (number and %)			0		1	2.3%	10	23.8%	23	54.7%	
CI											
VL	78	4.96 ± 1.57 (2.1–10.9)	78	4.83 ± 1.79 (2.0–10.8)	54	4.9 ± 1.65 (2.1–11.5)	21	4.98 ± 1.76 (2.7–9.6)	5 **	5.02 ± 1.13 (4.1–6.9)	0.989
CI95%		4.6/5.31		4.42/5.23		4.44/5.35		4.15/5.8		3.61/6.42	
Surgery *			0		24	30.7%	33	42.3%	16	20.5%	
p		0.318		0.713		0.0007		<0.0001		0.0086	

* Number and % of women receiving surgery between previous and current follow-up. ** In these women clinical intervention took place after this control.

As shown in Table 3, viral load was maintained in both groups during the first year. In the NCI group, viral load decreased (1 log) throughout the second year of follow-up, while it remained constant in the CI group. This decrease was more pronounced along the follow-up.

Because the COBAS HPV (Roche) detects a pool of 12 HR genotypes in the same channel, viral load of mixed infections was treated globally for this analysis.

A further analysis of differences in viral load for the NCI and CI group in terms of the different genotype groups at each follow-up was carried out (Table 4). The amount of data for the fourth year of follow-up was not sufficient for any statistical analysis in terms of genotype.

Figure 1 shows in graphical form the data from Table 4. The difference in viral load (all genotypes) between the NCI and the CI group (A), and also by HPV genotype group (B, C, D), are shown for the annual follow-up tests (C1 to C4 in A, but C1 to C3 in the rest).

In order to establish whether reduction in viral load was a good patient outcome marker, the ROC curves were studied. Figure 2 shows these curves for all patients (A) and by genotype for the second and third year of follow-up. The 0.95 logarithm decrease in viral load at follow-up in the second year and the 2.35 logarithm drop in the third year of follow-up indicate that the test is reliable.

Table 4. Variation in viral load by genotype group over the course of patient follow-up tests.

	Follow-Up 1			Follow-Up 2			Follow-Up 3			Follow-Up 4		
	n	**x ± σ (Range)**	**CI95**	***n***	**x ± σ (Range)**	**CI95**	***n***	**x ± σ (Range)**	**CI95**	***n***	**x ± σ (Range)**	**CI95**
						Total						
CI	78	−0.13 ± 1.44 (−3.3/4.3)	−0.4/0.19	54	−0.11 ± 1.53 (−4.5/3)	−0.52/0.30	20	−0.43 ± 1.5 (−4.1/2.5)	−1.13/0.27	5	−0.3 ± 0.62 (−1/0.4)	−1.28/0.68
NCI	42	0.05 ± 1.29 (−3.3/3.2)	−0.35/0.62	41	−1.22 ± 2.55 (−6.5/3.9)	−2.02/0.41	32	−3.26 ± 2.42 (−6.7/2.2)	−4.07/−2.29	8	−3.35 ± 2.22 (−6.6/0.5)	−5.05/−1.64
p		0.46			0.017			0.0000016			0.02	
						HPV16						
CI	30	−0.17 ± 1.27 (−3.1/2.5)	−0.64/0.30	19	0 ± 1 (−1.9/2.9)	−0.48/0.48	8	−0.1 ± 1.42 (−1.9/2.5)	−1.28/1.08	1	0.4	
NCI	11	0.09 ± 0.91 (−1.4/2.2)	−0.52/0.70	11	−1.66 ± 2.44 (−4.9/1.9)	−3.29/−0.02	9	−2.37 ± 2.29 (−5.3/1.1)	−4.1/−0.60	2	−1.2	
p		0.469			0.052			0.026				
						HPVα9non16						
CI	34	−0.11 ± 1.05 (−2.4/3.1)	−0.47/0.25	24	0.24 ± 1.30 (−2.7/2.6)	−0.30/0.78	5	−0.24 ± 2.49 (−4.1/2.5)	−3.33/2.85	0		
NCI	20	−0.21 ± 1.35 (−3.3/2.2)	−0.84/0.42	19	−1.12 ± 2 (−5.4/2.9)	−2.09/−0.16	18	−3.71 ± 2.5 (−6.7/2.2)	−4.96/−2.47	3	−2.8 (−4.7–0.5)	
p		0.77			0.014			0.03				
						HPVnonα9						
CI	21	−0.20 ± 1.75 (−3.3/4)	−0.99/0.59	17	−0.31 ± 1.76 (−3.5/3)	−1.21/0.59	9	−0.34 ± 1.16 (−2.3/1.4)	−1.23/0.55	3	−0.53 (−1–0)	
NCI	13	0.47 ± 0.99 (−0.9/2.6)	−0.1/1.06	13	−1.42 ± 3.05 (−6.5/3.3)	−3.26/0.42	10	−3.52 ± 2.27 (−6.5–/0.6)	−5.14/−1.89	1	−3.8	
p		0.155			0.25			0.0017				

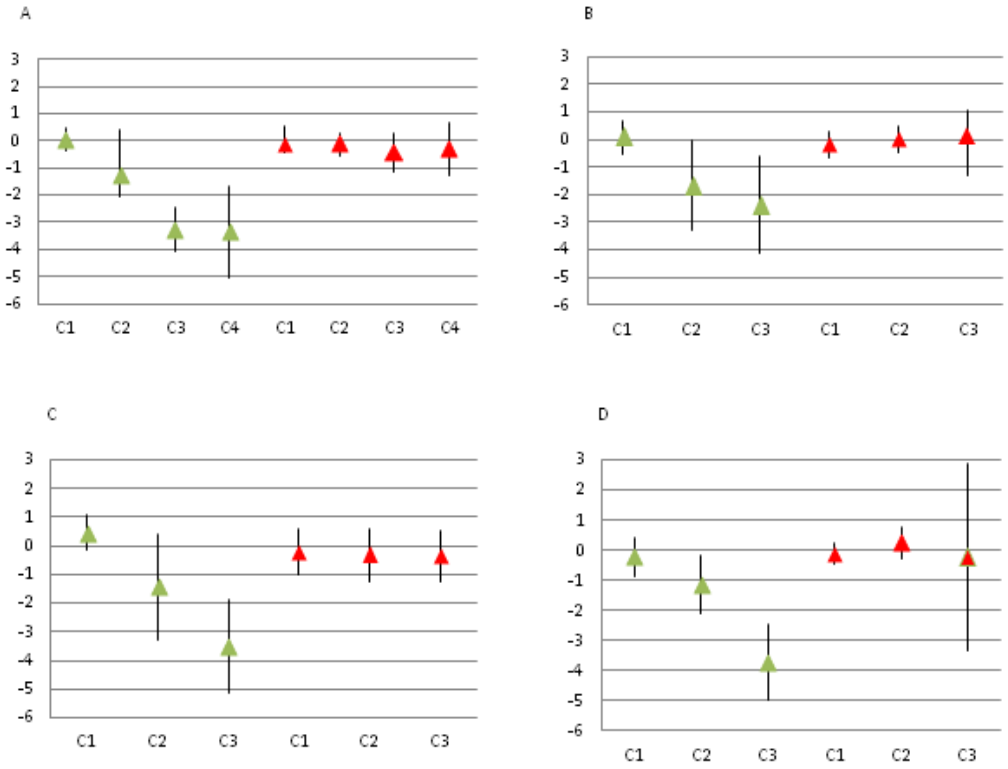

Figure 1. Evolution of viral load CI95 at each follow-up for NCI (green) and CI (red) patients. (**A**) All genotypes; (**B**) HPV16; (**C**) HPVα9non16; (**D**) HPVnonα9.

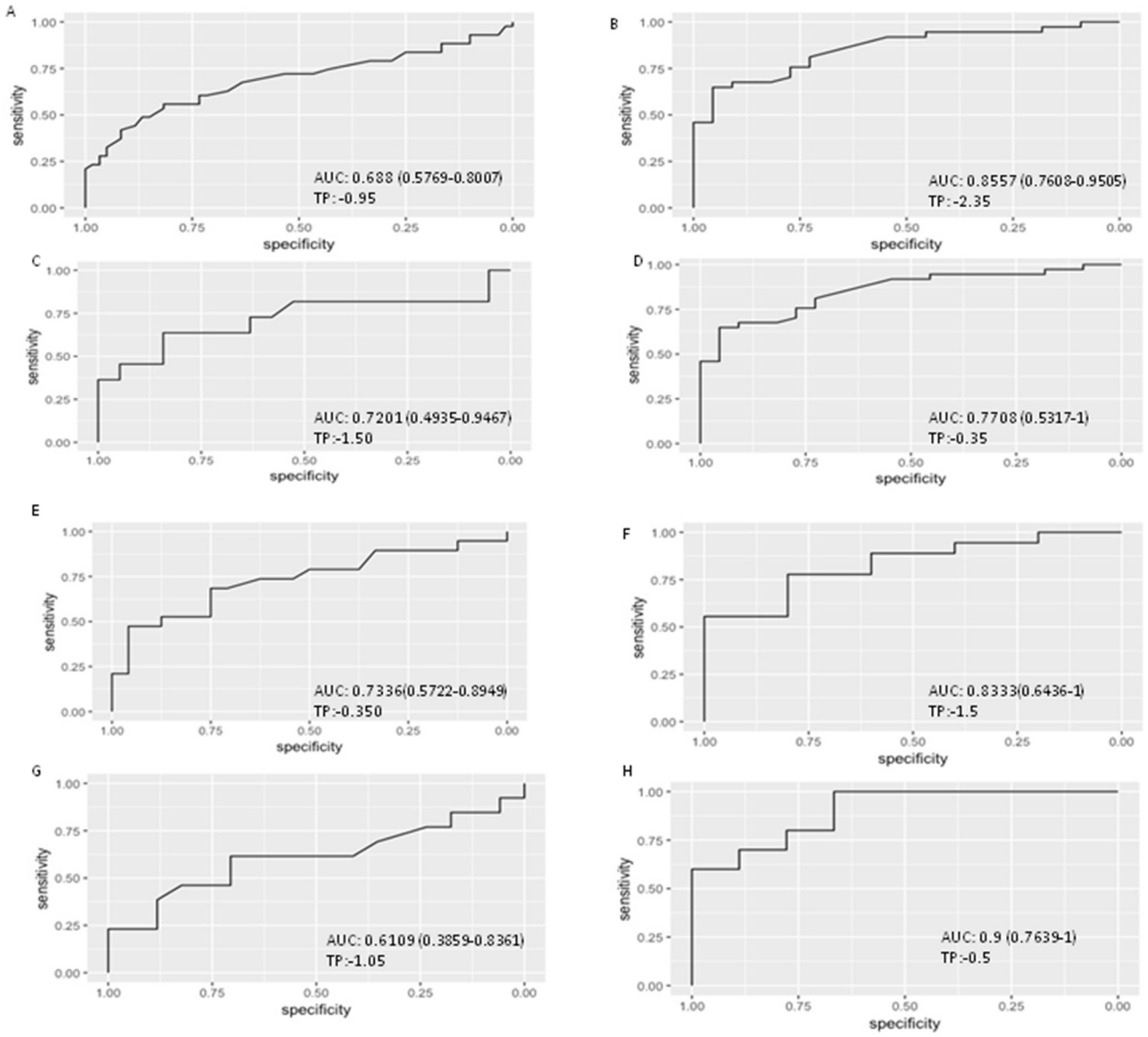

Figure 2. Variation in viral load ROC curves at 2 years (**A**,**C**,**E**,**G**) and 3 years (**B**,**D**,**F**,**H**). **A** and **B** show summed data for all patients; **C** and **D**, data for HPV16 infected patients; **E** and **F**, for those with HPVα9non16; and **G** and **H** relate to HPVnonα9 patients. TP: threshold point.

4. Discussion

HPV infection is a necessary condition for the development of cervical cancer, although other factors also influence this process. HPV features are, however, important in disease progression. HPV-infected women may develop a series of cervical cancer precursor lesions. Fortunately, a large number of women regress spontaneously, but others need to be treated to eliminate these lesions as well as the virus. Techniques that are able to clinically distinguish between these two types of infection are important in order to avoid unnecessary surgical interventions and to reassure women.

HPV infection is believed to clear spontaneously within 2 years in more than 90% of cases [13,14]. However, other authors have described a much lower rate, around 40% [15,16]. This regression is a slow process because HPV evades the immune system, and this delays adaptive immunity [17].

In terms of spontaneous regression, none of the patients became undetectable for the virus before the first year of follow-up, and clearly none of the CI group achieved spontaneous regression. However, by the end of the follow-up, 80% of NCI patients had a

viral load of zero, but only 26.1% of NCI patients had cleared the virus by the third year of follow-up, which indicates that virus removal is slow and controls should be performed for years. Despite this, in studies carried out in younger patients, it has been seen that most infections became undetectable within 1–2 years [18,19] and it occurs rapidly among infections destinated to clear [20].

Many studies have evidenced that virus replication control occurs more frequently in younger women [7,21], but this was not the case here. Furthermore, the CI group included women in their 30s, and even one 22-year-old woman. This highlights the fact that the initiation of HPV-based cervical cancer screening at 35 years old, as proposed by most guidelines, should perhaps be reconsidered, and that beginning when women are in their early 30s or before might be a better alternative.

Numerous authors have studied the influence of genotype on the severity of HPV infection and its influence on progression to cancer. The most frequent genotype found in this study was HPV16 (41), followed by HPV31 (15), HPV52 (9) and HPV56 (6), similar results to those found by Kjaer [22]. Other authors have, however, found HPV18 and HPV45 to be the most frequent after HPV16, although here, these genotypes were only occasionally detected. Finally, here, HPVnonα9 genotypes were found in the same proportion as in other studies [22–25]. In this study, no link was found between HPV16, HPVα9non16 or HPVnonα9 and surgical intervention.

Within HPV16, the T350G variant was present in some patients, but no relationship with CI was found.

The incidence rates of mixed infections described in the literature vary widely, ranging from 20–30% to 79.2% [26–30]. What is more, the implications of coinfection remain unknown. According to the one virus one lesion hypothesis, it seems that it is unlikely that several different HPV genotypes infect the same cell, but that each one is associated with a different lesion [31]. The rate of mixed infections in this study was 20%, and the same percentage of women infected with more than one type of HPV received surgery as those who did not (20.5%). However, a potentially important finding of this study was that in all cases of coinfection, one of the genotypes always belonged to the HPVα9 family. In addition, a trend was discerned that women with mixed infection in the CI group were older than those in the NCI group, although the low number of patients in these subgroups limits the interpretation of these results.

Some studies have attempted to establish a relationship between a single viral load and the severity of lesions [32–34]. While it might seem logical to think that a high viral load could be translated into a greater degree of injury [35] and, in consequence, poorer prognosis, it must also be remembered that at the beginning of any viral infection, replication rate is always high because no immune defense is yet present. In this study, we did not find a significant difference in viral load between CI and NCI women in the initial test, with average VL being around 5 log copies of HPV per 1000 cells across both groups.

Other authors have asserted that such decreases in VL for different HPV types during the follow-up period can be a good clinical biomarker [36–38]. In line with this, and in order to add to current knowledge on this aspect of the evolution of HPV, variation in viral load at a series of follow-up appointments was studied here. In this study, where women were followed and treated by expert gynecologists in cervical pathology, a significant decrease in the VL of NCI patients in the second year of follow-up was observed, specifically, an average reduction of 0.95 log copies/1000 cells compared to mean VL in the initial test. The trend continued, and was in fact more pronounced, in the third follow-up, where mean VL dropped by a further 2.35 log copies/1000 cells. In the fourth control, 80% of NCI patients had undetectable levels of HPV. Considering the genotype groups separately, the decrease was found to be slower in HPVnonα9 types, as indicated by the ROC curves, and faster for HPVα9 genotypes. Furthermore, our results show that the drop in VL for HPV16 patients was greatest between the first and second follow-up, while for the other genotype groups the reduction in VL was greater at each follow-up. Thus, it would seem that, in spite of

being aggressive, HPV16 seems to be cleared (when it happens) faster than other members of the HPVα9family.

The main limitation of this study was that it worked with patients in follow-up and a random design was not developed. A study with a greater number of patients in both groups and for a long time should be carried out to verify the results obtained.

Obtaining biopsies is undoubtedly necessary to see the degree of the lesion and for decision-making by the gynecologist. This study tried to find an easy and non-invasive marker that could help to determine the evolution of the HPV infection, avoiding biopsies as much as possible. In any case, the results obtained in this study indicate that monitoring the variation in normalized HPV viral load during the course of follow-up could help to understand the evolution of this disease. It would allow, in the case of a viral load decrease, surgical interventions to be postponed for up to two years (or as long as the severity of the lesion permits) as well as avoid the adverse effects of these interventions. Moreover, VL can be useful in screening programs for follow-up patients before they are referred for pathology consultation.

In summary, normalized viral load should be used as a determining marker in women with HPV infection. A decrease in normalized VL appears to be a better indicator to predict good prognosis than other markers such as genotype or lesion grade. Further studies, however, are needed to confirm our findings.

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