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HPV detection and genotyping of FFPE head and neck cancer biopsies by molecular testing to address new oropharyngeal squamous cell carcinoma classification based on HPV status.

Running title: HPV molecular tests comparison on FFPE HNSCC biopsies.

David Veyer[1], Maxime Wack[4,5], Ophélie Grard[1], Pierre Bonfils[3,5], Stéphane Hans[3a], Laurent Belec[1,5], Cécile Badoual[2,5], Hélène Péré[1,5,#]

[1]Laboratoire de virologie, Hôpital Européen Georges Pompidou, and Assistance Publique - Hôpitaux de Paris, Paris, France ;
[2]Laboratoire d'anatomo-cytopathologie, Hôpital Européen Georges Pompidou, and Assistance Publique - Hôpitaux de Paris;
[3]Service d'ORL et chirurgie cervico-faciale, Hôpital Européen Georges Pompidou, and Assistance Publique - Hôpitaux de Paris;
[4]Département d'Informatique Médicale, Biostatistiques et Santé Publique, Hôpital Européen Georges Pompidou, and Assistance Publique - Hôpitaux de Paris
[5]Faculté de Médecine Paris Descartes, Université Paris Descartes (Paris V), Sorbonne Paris Cité, Paris

Key words: Anyplex II HPV28 Seegene® HPV Genotyping assay; Inno-Lipa® HPV Genotyping Extra II ; HPV16 E6 viral load; HNSCC; FFPE biopsy

Corresponding author :

Dr Hélène Péré
helene.pere@aphp.fr

[a] Stéphane Hans is currently affiliated to the Service d'ORL et de chirurgie cervico-faciale at Hôpital FOCH, UFR Simone Veil, Université Versailles Saint Quentin-en-Yvelines.

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Abstract

Recently, both the WHO/IARC (World Health Organisation/International Agency for Research on Cancer) and the American Joint Committee on Cancer (AJCC) have classified the oropharyngeal squamous cell carcinoma (OPSCC) on the basis of HPV status. For this purpose, the WHO/IARC recommended direct molecular HPV testing. In practice, formalin-fixed, paraffin-embedded (FFPE) biopsy specimens are frequently the only available samples. We herein compared in parallel two commercially available molecular assays that were firstly designed for cervical HPV detection and genotyping: Inno-Lipa® HPV genotyping extra II assay and Anyplex™ II HPV 28.

A total of 55 samples were tested. By IL assay, chosen as reference assay, 27 (49.1%) biopsies were positive for HPV16, 10 (18.2%) were positive for HPV but negative for HPV16, and 18 (32.7%) were negative for HPV. A valid result with AP28 was obtained for 51 biopsy samples (92.7%). Among 37 HPV-positive samples by IL, 33 (89.2%) were positive by AP28. The agreement between both assays was good (Cohen's $\kappa = 0.78$). Among the six discrepancies between assays, always associated with low HPV16 viral load, four biopsies positive for HPV16 by IL could not be detected by AP28.

Taken together, these observations demonstrate that both assays could be used in routine for HPV detection and genotyping on FFPE-biopsy samples of head and neck tumour.

54 **Introduction**

55 With 600,000 cases per year, head and neck cancer was estimated to be the sixth most
56 common cancer worldwide [1]. Head and neck squamous cell carcinoma (HNSCC) represents
57 90% of them. Since 2007, human papillomavirus (HPV) was considered as an independent
58 risk factor for HNSCC by the World Health Organization's International Agency for Research
59 on Cancer (WHO/IARC) [2]. Broad genetic distribution of HPV has been reported in
60 oropharyngeal squamous cell carcinoma (OPSCC), including HPV16 in 85% of cases,
61 followed by HPV18, 31, 33, 35, 45, 51, 52, 56, 58, 59, 68 and 82 [3,4]. Finally, OPSCC is
62 currently considered as an epidemic viral-induced carcinoma, since the incidence of HPV-
63 positive carcinoma of the tonsil nearly doubled every 10 years [5–8].

64 Recently, the major modification in the eight edition of the American Joint Committee
65 on Cancer (AJCC) Staging Manuel, Head and Neck Section was the introduction of a specific
66 staging algorithm for high-risk (HR) HPV-associated OPSCC [9]. Furthermore, the 2017
67 edition of the WHO/IARC on Head and Neck tumor classified the OPSCC on the basis of
68 HPV status [10–12]. HPV-positive OPSCC constitutes a tumor entity with better prognosis, a
69 distinct epidemiological profile, with specific genetic features and clinical presentations and
70 outcomes. Immuno-histochemical detection of HPV using p16 staining as surrogate marker of
71 HPV has been until now widely carried out [13]. The 2017-revised WHO/IARC
72 recommendations introduced direct HPV testing based on *in-situ* hybridization and/or PCR in
73 order to classify the OPSCC according to HPV status [12]. Until now, several commercially
74 available assays have been clinically validated for the detection and genotyping of HPV-
75 associated cervical cancer [14,15], whereas to our knowledge none have been validated for
76 OPSCC. Furthermore, formalin-fixed, paraffin-embedded (FFPE) biopsy samples are
77 frequently the only available ones for molecular testing after pathological examination. Such

78 FFPE samples however necessitate specific processing before PCR analysis because formalin
79 fixation induces fragmentation of nucleic acids [16–18].

80 Taken together, the aim of the present study was to evaluate a new multiplex real-time
81 PCR-based assay (Anyplex™ II HPV 28, Seegene, Seoul, South Korea) (AP28), allowing to
82 detect and genotype a wide range of high-risk HPV genotypes and previously tested for
83 cervical samples [19,20], in a prospective series of OPSCC FFPE samples, by reference to Inno-
84 Lipa® HPV genotyping extra II assay (Fujirebio, Gent, Belgium) (IL) chosen as reference
85 assay [21–23].

86

87 **Materials and methods**

88 **Collection of biopsy samples and processing.** Head and neck biopsy samples received
89 before treatment at the European Georges Pompidou hospital were prospectively included
90 between 2014 and 2017 for routine pathologic examination. Patient had not received any
91 treatment for their cancer at the time of the biopsy. The biopsies were fixed in formalin 10%
92 overnight and included in paraffin. FFPE-biopsies diagnosed as HNSCC were selected by a
93 pathologist for further cutting in 5- to 20- µm sections. Five sections were sent to the ISO
94 15189-accredited virology laboratory of the hospital for DNA extraction prior to HPV
95 detection and genotyping by IL and, in parallel, by in-house quantitative real-time PCR
96 targeting E6 gene from HPV16 (HPV16 qPCR). Afterwards, same DNA extracts were
97 subjected to multiplex HPV PCR by AP28.

98 **DNA extraction procedures.** Sections of FFPE-biopsies were deparaffinised overnight at
99 +56°C with 40 µl of proteinase K (Qiagen, Hilden, Germany) and 360 µl of ATL buffer
100 (Qiagen). Afterwards, 200 µl of ATL buffer were added and incubated 10 min at +70°C.
101 DNA was further extracted using QiaAmp DNA Mini Kit (Qiagen), and eluted in 50 µl of
102 PCR-grade water. For discordant results between IL and AP28, 5 new sections of biopsy

103 samples were also subjected to DNA extraction procedure optimized for FFPE-biopsies, as
104 previously described by Steinau *et al* [17].

105 **HPV detection and genotyping in routine.** Two molecular HPV assays are used in parallel
106 for routine HPV detection and genotyping on FFPE-biopsies. The IL assay consists of PCR
107 amplification of a small 65 bp-fragment of the L1 gene using SPF10 primers sets and the
108 ubiquitary gene human leukocyte antigen-DPB1 as internal control, followed by hybridization
109 of specific HPV probes in a dedicated automat according to manufacturer's instructions. The
110 IL assay detects 13 HR HPV (HPV -16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -
111 68), 9 low-risk (LR) HPV (HPV -06, -11, -40, -42, -43, -44, -54, -61, -81), 7 genotypes
112 reported as possibly carcinogenic (HPV -26, -53, -66, -67, -70, -73, -82) and 3 genotypes not
113 described as carcinogenic (HPV -62, -83, -89) [24].

114 HPV16 qPCR was also systematically carried out, as previously described [25], in order
115 to double check every sample for HPV16 which constitutes the most prevalent HPV genotype
116 in HNSCC [26], as well as to assess lack of contamination by IL. For quantification, serial
117 dilutions of titrated Caski cells (Amplirun, Orgentec, France) were used to plot external
118 standard curve.

119 Positive controls for HPV16 and HPV18 consisted in DNA extracted from SiHa and
120 HeLa cell lines, respectively; water was used as negative control.

121 **HPV detection and genotyping by multiplex PCR.** The AP28 assay that distinguishes 28
122 HPV genotypes, by amplifying 100 to 200 bp-fragments of the L1 gene [including 13 HR
123 types (HPV -16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, and -68), 8 LR types (HPV
124 -6, -11, -40, -42, -43, -44, -54, -61) and 7 genotypes reported as possibly carcinogenic (HPV -
125 26, 53, -66, -69, 70 -73 and -82)], and human gene β-globin in two different reactions was
126 used for multiplex HPV molecular testing [27]. Melting curves were obtained at 30, 40, and 50
127 cycles. Results were first automatically analysed using the Seegene Viewer software, version

128 2.0 (Seegene) and raw data of results were checked by the virologist. The results were
129 considered as invalid when the negative controls were negative and no HPV was found. An
130 estimation of the viral load was approached by indicating the cycle number at which the
131 positivity was detected: + (50 cycles), ++ (40 cycles) and +++ (30 cycles).

132 **Ethical clearance.** All included patients belonged to a cohort declared and approved by the
133 Ethics Committee (Comité de Protection des Personnes Ile de France II, no. 2015-09-04)

134 **Statistical analysis.** IL assay was chosen in our laboratory as the reference technique for
135 HPV detection and genotyping, because the assay was reported to show high analytical
136 sensitivity and specificity on FFPE samples [28,29]. Results strictly similar by AP28 and IL
137 assays were defined as identical; results giving at least one identical HPV genotype by AP28
138 and IL assays were defined as compatible; other results were defined as discordant.
139 Agreement between IL and AP28 assays was assessed by the Cohen's κ test: 1 indicating
140 perfect agreement; 1 to 0.81, very good agreement; 0.80 to 0.61, good agreement; 0.60 to
141 0.21; moderate to poor agreement. The Mann-Whitney test was used to compare HPV16 viral
142 loads between samples showing concordant or discordant results. A linear regression model
143 was used to assess the relation between the viral load and the semi-quantitative result of
144 AP28.

145

146 **Results**

147 Fifty-five biopsy samples from patients followed for HNSCC were prospectively selected. By
148 the IL assay, 27 (49.1%) biopsies were HPV16-positive and 10 (18.2%) were HPV-positive
149 but not HPV16-positive, as depicted in the Table 1. Among the 37 positive samples, 3
150 (biopsies #8, #19 and #33) were positive for more than one HPV. Finally, 18 (32.7%) biopsy
151 samples were HPV-negative.

152

153 **Comparison of HPV detection and genotyping results between IL and AP28 assays.**
154 The same DNA extracts from the 55 selected biopsy samples tested by IL were further
155 subjected to AP28 assay. Among 7 discordant samples, one that was initially negative when
156 analyzed with the Seegene Viewer software was finally classified as positive after raw data
157 analysis; the raw data analysis of the remaining discordant samples (corresponding to 10.9%
158 of samples) gave similar results to the ones obtained by automatic analysis. Final results are
159 shown in the Tables 1 and 2. A valid result with AP28 was obtained for 51 biopsy samples
160 (92.7%) (Table 2). Among the 37 HPV-positive biopsy samples by IL, 33 (89.2%) were
161 found positive by AP28, including 90.9% of identical results and 9.1% of compatible results,
162 the remaining results being either invalid (n=2) or negative (n=2). Finally, the vast majority
163 (n=16; 88.9%) of 18 biopsy samples negative by IL were also negative by AP28; only 2 IL-
164 negative biopsies were found invalid by AP28. The overall agreement between both assays
165 was good (Cohen's κ coefficient = 0.78). Among the 27 samples that were HPV16-positive
166 with IL, AP28 detected HPV16 in 23 (85.2%), 2 (7.4%) were negative and 2 (7.4%) invalid
167 (Table 1). AP28 did not detect any HPV16 in samples that were negative for HPV16 with IL.
168 Regarding HPV16 detection, the agreement between both assays was good (Cohen's
169 κ coefficient=0.75).
170 **HPV16 viral load.** HPV16 qPCR was carried out on the 55 biopsy samples. All HPV16-
171 positive samples with IL were positive by HPV16 qPCR and all HPV16-negative samples
172 with IL were also negative with HPV16 qPCR (Table 1). The median of HPV16 viral loads
173 was higher in concordant than in discordant samples [20,800 copies/µl (4.32 log/µl), range,
174 734-595000 *versus* 503 copies/µl (2.70 log/µl), range, 133-512; P=0.00077]. Interestingly, the
175 HPV16 viral loads of IL-positive/AP28-negative results were low (Table 1). As expected,
176 using a linear regression model, a significant relation (P=0.000388) was observed between the
177 viral load (expressed in $\log_{10}$) and the AP28 semi-quantitative results with a linear coefficient

178 of 0.79 log between each level of AP28 (negative, +, ++ and +++). The Figure 1 depicts the
179 results of HPV16 viral loads and AP28 semi-quantitative results.
180

181 **Discussion**

182 We herein compared on a large series of FFPE-biopsy samples of HNSCC two
183 commercially available molecular assays targeting L1 gene for routine HPV detection and
184 genotyping. Nearly half of FFPE-biopsy samples from patients followed for HNSCC in our
185 hospital was found positive for HPV DNA using IL as reference assay, emphasizing the need
186 to strictly follow the recent 2017-revised WHO/IARC recommendation of direct HPV testing
187 in case of OPSCC. Both assays showed good agreement for HPV detection as well as for
188 genotyping. However, rare discrepancies between assays could be observed with positive
189 samples for HPV16 by IL which were not detected by AP28. Interestingly, these
190 discrepancies were always associated with low HPV16 viral load. These observations
191 demonstrate that both assays could be used in routine for HPV detection and genotyping on
192 FFPE-biopsy samples of HNSCC, keeping in mind that a few biopsy samples with low
193 HPV16 viral load could be missed. Taken together, our findings clearly emphasize the
194 necessity to validate commercially available molecular assays on patients' biopsy samples,
195 and to confront the results with other molecular technics in case of low HPV viral load.

196 In the present series, the calculated agreement between IL and AP28 results was good,
197 both for HPV16 and other genotypes. However, around ten percent of biopsy samples gave
198 discrepancies between both assays, including biopsies positive for HPV16 by IL which were
199 not detected by AP28. Using HPV16 qPCR, low HPV16 viral load was clearly associated
200 with the misdetection of HPV16 by AP28 assay. Although HPV16 viral load was not
201 normalized on extracted DNA quantity, the experimentations were carried out on the same
202 tissue extract allowing accurate comparison of the results obtained by AP28 and HPV16

203 qPCR. The capability of IL assay to detect HPV16 in samples harboring low HPV16 viral
204 load is likely due to sufficient amplification efficiency in samples containing fragmented
205 DNA. Indeed, it is well reported that DNA recovering in FFPE specimens may be influenced
206 by several factors, such as formalin quality and concentration, length of fixation, paraffin
207 quality and temperature [30]. As a consequence, DNA in FFPE biopsy is either completely or
208 partially degraded into DNA fragments of 200 bp or less [16]. The IL assay relies on the
209 amplification of shorter fragments than AP28 assay, a feature which could partly explain the
210 discrepancies observed between both assays in our series. Furthermore, discordant results
211 could also be associated with extract quantity used for experiment. Thus, the IL assay requires
212 more quantity of tissue extract (10 µl) than AP28 (5 µl), according to manufacturers'
213 instructions. The possibility may be also envisioned that mutations affecting the priming sites
214 of AP28 primers could have led to misdetection. This hypothesis should be however ruled out
215 because the correlation between the efficiency of detection by AP28 and HPV16 viral load
216 was marked. Finally, we checked that observed discrepancies were not due to extraction
217 procedures.

218 In our series, one negative sample by AP28 which was positive by IL was finally
219 diagnosed as positive for HPV16 after analysis of the raw data. This finding indicates that raw
220 data of every AP28 negative samples need to be analyzed in order to detect some of the false-
221 negative results. Indeed, the automatic analysis and cut-off used by AP28 test may miss out
222 some HPV-positive samples since the assay was primarily designed to detect HPV on either
223 cervical swab or liquid based cytology specimen and not on FFPE samples from
224 oropharyngeal origin. In practice, the automatic Seegene software interpretation should be
225 used with caution for negative or invalid FFPE biopsies.

226 Our observations indicate that IL as well as AP28 assays could be used in clinical
227 laboratory to detect and genotype HPV in FFPE OPSCC biopsy samples. To our knowledge,

228 only one study compared the performance of the AP28 assay and the CLART system from
229 Genomica (Madrid, Spain) on only 3 FFPE HNSCC samples [27]. In our series, the IL assay
230 appeared as sensitive as in-house HPV16 qPCR, in keeping with previous reports showing
231 that IL is highly reliable on FFPE archival samples. Nevertheless, the AP28 technic could be
232 preferred depending on the recruitment of the laboratory, particularly if the number of
233 samples is important. Indeed, AP28 relies on real-time PCR, which allows easier and faster
234 procedure as compared with IL technic which appears more cumbersome and requires
235 specific material for hybridization on membrane following PCR amplification. In addition, the
236 risk of cross contamination between samples is less important with AP28 than with IL.
237 However, it should be kept in mind that misdetection of HPV16-positive biopsies could occur
238 with AP28 assay. Since HPV16 is known to be the most prevalent HPV genotype in OPSCC,
239 diagnosed in 85% of cases [26], one could strongly recommend confirming the negative results
240 by AP28 with HPV16 genotype-specific molecular assay validated on FFPE samples. In our
241 laboratory, we have chosen to screen first for HPV16 by in-house HPV16 qPCR, and then to
242 test further the negative samples with IL or AP28 to potentially detect less common genotypes
243 in OPSCC, or confirm the negativity for HPV.

244 In conclusion, our observations demonstrate that both commercially available IL and
245 AP28 assays could be used in clinical laboratories with similar performances in routine on
246 OPSCC FFPE samples, in order to address the recent recommendations for direct HPV testing
247 in OPSCC [12]. Nevertheless, low HPV viral load in FFPE biopsy samples could be a limiting
248 factor, rendering in medical practice the diagnosis of HPV in OPSCC sometimes difficult.
249 Finally, HPV diagnosis and genotyping in OPSCC may necessitate several complementary
250 technics, to overcome the limitation of some commercially available assays.

251

252

Authors' contributions. DV, OG, CB and HP have conceived and designed the research; OG, has performed the experiments; MW performed statistical analyses; DV, PB, SH, CB and HP analyzed the results ; DV, LB and HP drafted the manuscript.

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389

390 **Table 1.** HPV detection and genotyping by IL, AP28 and E6 HPV-16 detection and quantification by in-
391 house PCR of 37 IL-positive FFPE-biopsies diagnosed as HNSCC. HPV 16-positive samples are presented
392 by increasing viral load.

ID	HPV genotyping results		In-house E6 HPV16 PCR	
	IL	AP28 (semi-quantitative results[a])	Qualitative results	Viral load [copies/µl (log)]
#3	HPV16	Invalid[b]	Positive	ND
#5	HPV16	Invalid[b]	Positive	133 (2.12)
#7	HPV16	Negative[b]	Positive	503 (2.70)
#4	HPV16	Negative[b]	Positive	512 (2.72)
#30	HPV16	HPV16 (+)	Positive	734 (2.87)
#50	HPV16	HPV16 (++)	Positive	3,910 (3.59)
#33	HPV16 ; HPV82	HPV16 (+)	Positive	4,000 (3.60)
#9	HPV16	HPV16 (++)	Positive	4,860 (3.69)
#14	HPV16	HPV16 (++)	Positive	5,010 (3.70)
#46	HPV16	HPV16 (++)	Positive	5,070 (3.70)
#10	HPV16	HPV16 (++)	Positive	5,360 (3.73)
#45	HPV16	HPV16 (++)	Positive	5,650 (3.75)
#35	HPV16	HPV16 (++)	Positive	7,730 (3.89)
#13	HPV16	HPV16 (+)	Positive	9,230 (3.97)
#49	HPV16	HPV16 (++)	Positive	20,200 (4.31)
#44	HPV16	HPV16 (++)	Positive	20,800 (4.32)
#12	HPV16	HPV16 (+)	Positive	35,200 (4.55)
#2	HPV16	HPV16 (+)	Positive	40,200 (4.60)
#48	HPV16	HPV16 (++)	Positive	78,300 (4.89)
#11	HPV16	HPV16 (++)	Positive	78,400 (4.89)
#32	HPV16	HPV16 (++)	Positive	80,200 (4.90)
#16	HPV16	HPV16 (++)	Positive	82,400 (4.92)
#6	HPV16	HPV16 (++)	Positive	112,000 (5.05)
#27	HPV16	HPV16 (++)	Positive	128,000 (5.11)
#39	HPV16	HPV16 (++)	Positive	183,000 (5.26)
#1	HPV16	HPV16 (++)	Positive	323,000 (5.51)
#42	HPV16	HPV16 (+++)	Positive	595,000 (5.77)
#34	HPV6	HPV6 (++)	Negative	NA
#43	HPV6	HPV6 (++)	Negative	NA
#18	HPV11	HPV11 (++)	Negative	NA
#31	HPV11	HPV11 (++)	Negative	NA
#40	HPV11	HPV11 (++)	Negative	NA
#19	HPV18 ; HPV39p	HPV18 (++)	Negative	NA
#47	HPV18	HPV18 (+++)	Negative	NA
#8	HPV33 ; HPV52p	HPV33 (+++)	Negative	NA
#38	HPV59	HPV59 (++)	Negative	NA
#22	HPV82	HPV82 (+++)	Negative	NA

393 [a] Semi-quantitative results are given in brackets (detection of signal at 30 cycles: +++ ; 40 cycles: ++ ; 50 cycles: +);

394 [b] Results checked manually without the automated analysis software (Seegene Viewer version 2.0, Seegene).

395 IL: Inno-Lipa® HPV genotyping extra II assay (Fujirebio, Gent, Belgium); AP28: Anyplex™ II HPV 28, Seegene (Seoul, South
396 Korea); NA: Not attributable; ND: Not done; p: probable
397
398

399

400 **Table 2.** Qualitative detection of HPV by IL and AP28

401 asays in 55 FFPE-biopsies diagnosed as HNSCC.

402

IL/AP28 results	N (%)
Concordant results	
. Positive/Positive	33 (60.0)
o **Identical**	30 (90.9)[a,b]
o **Compatible**	3 (9.1)[c]
. Negative/Negative	16 (29.1)
Discordant results	
. Positive/Negative	2 (3.6)
. Positive/Invalid	2 (3.6)
. Negative/Invalid	2 (3.6)
Total	55 (100.0)

[a] In brackets: percentage of IL/AP28 concordant out of total Positive/Positive;

[b] Among discordant samples, only one initially negative by Seegene Viewer software (Seegene) showed raw data compatible with positivity, and was classified as positive sample;

[c] In brackets: percentage of IL/AP28 compatible out of total Positive/Positive.

IL: Inno-Lipa® HPV genotyping extra II assay (Fujirebio, Gent, Belgium);

AP28: Anyplex™ II HPV 28, Seegene (Seoul, South Korea)

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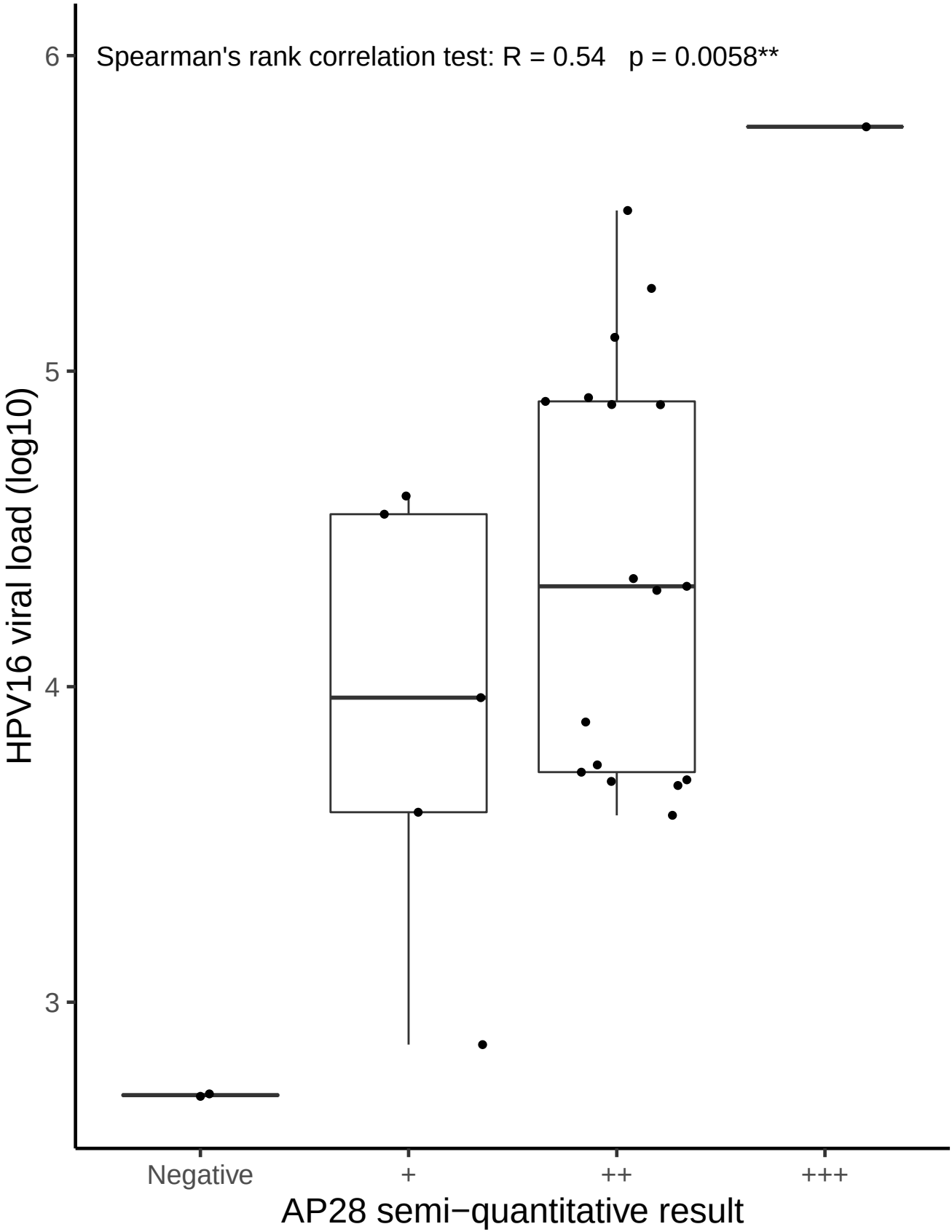
427

428 **Legend for the figure**

429 **Figure 1.** Comparison of HPV16 viral loads (in-house qPCR) and HPV16 AP28 semi-
430 quantitative results. HPV16 semi-quantitative results are presented as negative, +, ++ and +++
431 according to the cycle at which the signal is detected (no detection, detection at 50, 40 and 30
432 cycles respectively). The Spearman's rank correlation test is shown on the figure (R=0.54,
433 p=0.0058).

434


Spearman's rank correlation test: R = 0.54 p = 0.0058**
HPV16 viral load (log10)
6
5
4
3
Negative
+
++
+++
AP28 semi−quantitative result