

1 **Droplet digital PCR quantification suggests that higher viral load correlates with improved survival**  
2 **in HPV-positive oropharyngeal tumours.**

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19 Short Title: HPV viral load risk stratifies Oropharyngeal cancer

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21 Key words: Oropharyngeal cancer, HPV, viral load, digital PCR

22 **Word count: 2927 (permission for this has been requested)**

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26 **ABSTRACT**

27

28 **BACKGROUND**

29 Although HPV-positive oropharyngeal cancer (OPC) patients have improved prognosis compared to  
30 HPV negative patients; there remains an HPV-positive group who have poor outcomes. Biomarkers  
31 to stratify discrete patient outcomes are thus desirable. Our objective was to analyse viral load (VL)  
32 by droplet digital PCR (ddPCR), in HPV-positive patients with OPC on whom clinical outcome data  
33 were available.

34 **METHODS**

35 In a cohort of patients that had previously tested HPV positive via conventional PCR, VL was  
36 determined using ddPCR assays for HPV16 L1 and E6 genes. VL was classed as “medium/high” if  
37 more than 5.57 copies or 8.68 copies of the HPV 16 L1 or E6 gene were detected respectively. Effect  
38 of VL on overall survival and hazard of death & disease progression was performed with adjustments  
39 made for sex, age, deprivation, smoking, alcohol consumption and stage.

40 **RESULTS**

41 L1 VL ranged from 0.0014 to 304 gene copies per cell with a mean of 30.9; comparatively E6 VL  
42 ranged from 0.0012 to 356 copies per cell with a mean of 37.9. Univariate analysis showed those  
43 with a medium/high VL had a lower hazard of death; this was significant for L1 ( $p=0.02$ ) but not for  
44 E6 ( $p=0.67$ ). The ratio of E6 to L1 deviated from  $n=1$  in most samples but had no influence on clinical  
45 outcomes.

46 **CONCLUSIONS** HPV viral load may be informative for the further stratification of clinical outcomes in  
47 HPV positive OPC patients

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52 **INTRODUCTION**

53 The incidence of oropharyngeal cancer (OPC) has increased dramatically over the last two decades  
54 including in the United Kingdom (1,2). A component of OPC is associated with HPV although the  
55 amount varies. For example, a recent global analysis indicated <10% of OPC cases in Brazil were  
56 positive for HPV compared to ~50% in the UK (3). While the extent of HPV driven OPC varies, data  
57 converge on the fact that HPV positive (versus negative) status is independently associated with  
58 better clinical outcomes (4,5). This has led to a recent change in tumour classification which  
59 incorporates HPV status and also trials to determine the efficacy of de-escalated therapy in HPV  
60 positive OPC patients (6,7).

61

62 Current approaches for determining HPV status of OPC are largely based on qualitative tests and  
63 include immunohistochemistry for p16INK4a or HPV PCR for DNA or mRNA (8). Unfortunately, some  
64 HPV-positive patients, so determined by these methods, still have very poor outcomes (9). Further,  
65 these approaches do not quantify levels of infection i.e. viral load (VL). Given the increased incidence  
66 of OPC, it is important to refine tools for improved risk stratification; one such candidate is the  
67 measurement of viral load.

68

69 Current evidence indicates that VL in HPV-positive head and neck cancers varies widely within and  
70 between anatomical sites (10-14). In addition, investigations into the physical status of HPV in OPC  
71 indicate a landscape of integrated and episomal forms within a single lesion (15,16). The implications  
72 of physical status on clinical outcome are not understood but given the fundamental influence of  
73 integration on viral gene expression, a simple measure of virus activity (such as VL in the lesion) may  
74 yield insight into clinical manifestations.

75

76

77 To date, small cohort studies have indicated VL measurement can delineate patient outcomes within  
78 HPV positive OPC (17). This reconciles with data that indicate VL may be prognostic for other HPV  
79 driven cancers, including cervix (18,19). Previous studies on VL have generally focussed on the  
80 amplification of one target and have employed real-time PCR. ddPCR allows absolute quantification  
81 of DNA targets and has been used for applied virology where knowledge of load can influence  
82 clinical management (20,21). Around 20,000 data points are generated per sample making it highly  
83 accurate and reproducible (20,21) and proof principle of this approach when applied to OPC for  
84 single HPV target-detection was reported recently (22). The present study builds on this work  
85 through the evaluation of a ddPCR approach to measure VL of two HPV gene targets: E6 and L1,  
86 within a well characterised cohort of OPC cases diagnosed in Scotland on which clinical and survival  
87 outcomes are known. Our overarching aim is to determine the utility of HPV viral load measurement  
88 for the risk-stratification of OPC

89

## 90 **METHODS**

### 91 **Dimensions of original, parent OPC cohort**

92 We focussed on a subsample of an OPC cohort from Scotland (n=235). Full details of the original  
93 cohort are available in Wakeham et al 2019 (23). In brief, patients with OPC diagnosed in the West of  
94 Scotland between April 2013 and December 2015 had PCR-based HPV typing, performed in a central  
95 reference laboratory in Scotland. The original PCR-based assay targeted L1 DNA and incorporated  
96 genotyping using luminex technology for 24 HPV types including all established high-risk HPV types.  
97 HPV positivity using this technology is associated with improved clinical outcomes; as described in  
98 Wakeham et al (2019) and concordance of the assay with p16INK4a staining is between 80-90% (2).  
99 Of the technically valid cases (n=229), 136 were HPV positive and 130/136 were positive for HPV 16.

100

### 101 **Sample(s) used for VL analysis**

102 An aliquot of the original nucleic acid extraction stored at -80°C, was obtained. Given the  
103 dominance of HPV 16 in OPC generally, including the present cohort, HPV 16-specific ddPCR  
104 protocols were generated. 113 HPV 16 positive samples were available for the ddPCR after  
105 exclusion for limiting volume or incomplete clinical data. Of these, 93 and 82 tested positive for L1  
106 and E6 respectively and were the focus of subsequent analysis. Sex, age, deprivation (represented as  
107 quintiles; 1=most deprived, 5 least deprived), smoking (ever/never), alcohol consumption (heavy vs  
108 not), TMN stage, ICON-S stage and treatment-type were recorded as per Wakeham et al 2019 (23).  
109 Study permissions were through NHS Greater Glasgow and Clyde research office, the clinical  
110 effectiveness team and a data sharing agreement with the West of Scotland Cancer network.

111

#### 112 **ddPCR methodology and optimisation**

113 ddPCR was carried out to conform to the MIQE guidelines for ddPCR (24,25). Copy number variant  
114 (CNV) analysis was used to determine average HPV16 VL/cell. ddPCR assays were set up as duplexes  
115 with the cellular RPP30 control probe primer set, supplied as standard for ddPCR (BioRad, UK), and  
116 either custom-designed HPV16 L1-specific primers and probe sets (HPV16 L1 Forward Primer 5'-  
117 GCCTCCTGTCCCAGTATCTAA-3', HPV16L1 Reverse Primer 5'-GGATGTCCAAGTAGTC-3',  
118 HPV16L1 Probe 5'-TGCCTGCAACATATTCATCCGTGC-3') (26) or HPV16 E6-specific primers and probe  
119 sets (E6 Forward Primer 5'-CAATGTTTCAGGACCCACAG-3', E6 Reverse Primer 5'-  
120 CTGTTGCTTGAGTACACACATTC-3', E6 Probe 5'-CCACAGTTATGCACAGAGCTGC-3') (27). For L1 and E6  
121 probes the reporter dye was FAM and the dark quencher was BHQ1 (IDT, Belgium). Primers and  
122 probes were designed using IDT's Primer Quest Tool  
123 (<https://eu.idtdna.com/PrimerQuest/Home/Index>). The probe/primer sets were optimised using a  
124 temperature gradient exactly as recommended in the Bio-Rad ddPCR manual. The endogenous  
125 control assay kit; Human RPP30 (reporter dye HEX) (Bio-Rad, UK) was included in every ddPCR  
126 reaction as a copy number reference, i.e. each ddPCR was a duplex reaction. All reaction runs  
127 contained negative control wells in triplicate. In-reaction digestion of the DNA with restriction

128 enzymes was performed to enhance the partitioning of DNA into droplets. We confirmed that the  
129 restriction enzymes selected for this (*EcoRI* and *HindIII*), would not cut within any of the viral or  
130 control target sequences. Primer and probe concentrations were optimised by titration. Reaction  
131 mixes were set up using ddPCR Supermix for Probes without dUTP (Bio-Rad), 0.7 µl of the RPP30  
132 endogenous control assay, HPV16 L1 or E6 specific primers and probes at 300 nM and 200 nM (final  
133 concentration) respectively, 10–100 ng of template DNA and 1 µl of restriction digest mix (consisting  
134 of 4 U of both *EcoRI* and *HindIII* in 1x NEB Cutsmart buffer (NEB, UK)). Reactions were mixed with  
135 Droplet Generation Oil on DG8 cartridges in the QX200 droplet generator (Bio-Rad) to generate  
136 droplets. Thermal cycling conditions were: 95°C for 10 minutes followed by 40 x 94°C for 30 s and  
137 60°C for 1 minute prior to final extension at 98°C for 10 minutes. Post amplification, droplets were  
138 analysed on a QX200 Droplet Reader (Bio-Rad) and output data files were analysed using QuantaSoft  
139 analysis software v1.7.4 (Bio-Rad).

140

#### 141 **Definition of low and high VL**

142 The individual viral loads were ranked from smallest to largest and separated using tertiles. A priori,  
143 the analysis planned to compare viral load in tertiles (low, medium and high) but low numbers of  
144 deaths in the medium and high viral load groups meant that analysis was performed for low VL(viral  
145 load in the lowest third) vs a combined medium/high VL category (viral load in the upper two-thirds);  
146 this was performed for both L1 and E6. The VL threshold(s) for “medium/high” E6 VL and L1 VL were  
147 >8.68 and >5.57 viral gene copies per cell respectively. Samples with VL lower than this were classed  
148 as having a low VL.

149

#### 150 **E6 and L1 VL and clinical outcome**

151 Analysis was performed on the cases which had ddPCR results (n=93 for L1 and n = 82 for E6).  
152 Kaplan Meier plots were constructed for overall and progression free survival, stratified according to  
153 the OPC cases having a low or medium/high VL. In addition, hazard to death and hazard to death or

154 recurrence were assessed and related to low or medium/high VL status using Cox's regression with  
155 Firth's penalised likelihood given the small denominators. Follow up data were censored as of  
156 November 2016. Univariate and adjusted results are presented with adjustments made for sex, age,  
157 deprivation, smoking, alcohol intake, TMN stage, ICON stage. Type of treatment was not included in  
158 the model given its high association with stage.

159

#### 160 **Ratios of E6 and L1 VL and clinical outcome**

161 The association(s) between E6/L1 ratio and the demographic and clinical variables were assessed  
162 with significance determined using the Fishers exact test. Further, we modelled the association  
163 between the distance of the E6/L1 ratio to 1 and the variables using a regression model (to avoid  
164 imposing an "arbitrary" cut off). As the effect may have been different for E6/L1 >1 vs <1, tests for  
165 interaction were performed to determine whether they could be included with the same model. All  
166 statistical analyses were performed in R version 3.6.1.

167

168

### 169 **Results**

#### 170 **Characteristics of cohort assessed for VL**

171 Demographic and clinical variables of the cohort are presented in Table 1 in addition to VL status  
172 separated as "low", "medium" or "high". The cohort contained 75 males and 18 females with an  
173 average age of 57 (interquartile range of 52-66). All were squamous cell carcinomas and most cases  
174 were from more deprived areas; 50/93 patients were SIMD 1 and 2. In relation to smoking and  
175 alcohol, 53 had "ever" smoked and 19 were heavy drinkers. A total of 84 cases were TMN 3 or above  
176 and most, (56/93), received chemo-radiotherapy as treatment.

177

#### 178 **VL and impact on clinical outcomes (L1)**

179 All original data files for this study have been uploaded to the University of Glasgow's open  
180 access data sharing platform "Enlighten" (<http://researchdata.gla.ac.uk/>) The DOI for accession  
181 to this data is <http://dx.doi.org/10.5525/gla.researchdata.1023>

182 Samples were analysed singly and any assay generating less than 10,000 droplets was  
183 discounted from analysis. Raw data for the average HPV16 VL per cell as deduced from ddPCR  
184 experiments are presented in Supplementary Table 3. This shows the VL (based on either the L1 or  
185 E6 DNA targets) calculated relative to the endogenous RRP30 cellular gene internal control, which is  
186 known to have two copies per cell. A threshold is set on the QuantaSoft analysis software (either  
187 automatically or manually as required [http://www.bio-](http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf)  
188 [rad.com/webroot/web/pdf/lsr/literature/Bulletin\\_6407.pdf](http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf)) and from this, the number of positive  
189 and negative droplets for both gene targets in each patient sample is measured. The software then  
190 fits the positive droplets to a Poisson algorithm to determine the target DNA concentrations. L1 VL  
191 ranged from 0.0011 to 304 copies of HPV L1 gene per cell with a mean of 30.9 L1 copies. Those who  
192 died from OPC had a median L1 VL of 5.21 (IQR 0.23-18.15); while those still alive had a median L1  
193 VL of 16.3 (IQR 4.13-33.65). Table 2 shows overall survival stratified by the clinical and demographic  
194 variables described in Table 1 in addition to viral load defined as medium/high (treated as a  
195 composite) or low. Medium/high viral load was associated with improved overall survival in the  
196 univariate analysis with a hazard ratio of 0.3 (95% CI 0.11-0.84),  $p=0.02$ . Variables associated with a  
197 worse overall survival were high alcohol consumption; HR 4.94 (95% CI 1.7-14.39)  $p<0.01$ , more  
198 advanced stage and treatment modality. Similar observations were noted when progression free  
199 survival was used as an outcome (Supplementary data 1). Figure 1 shows overall survival and  
200 progression free survival according to the binary VL categories.

201 In the adjusted analysis no individual variable, including viral load, influenced overall survival other  
202 than high alcohol consumption; HR 3.6 (95% CI 0.98-15.22) – although the confidence interval



203 spanned 1. Alcohol consumption was also associated with a significantly worse progression free  
204 survival; HR 5.48 (95% CI 1.56-19.22) in the adjusted analysis (supplementary Table 1).

205

206

#### 207 **VL and impact on clinical outcomes (E6)**

208 E6 VL ranged from 0.0012 to 356 copies of HPV E6 gene per cell. Mean viral load was 37.9 E6 gene  
209 copies per cell and those who died from OPC had a E6 VL of 12.19 (IQR 6.65-39.25), while those still  
210 alive had a median E6 VL of 15.69 (IQR 6.04-41.81).

211 Medium/high viral load was weakly associated with longer overall survival and progression free  
212 survival although the relationship was not as strong as that observed for L1 (Figure 2). In the  
213 univariate analyses, medium/high E6 VL was associated with a slightly higher overall survival  
214 although this was not significant; HR 0.76 (95%CI 0.21-2.68) p=0.67 (Table 3, Figure 2). High alcohol  
215 intake was associated with worse overall survival; HR 4.31 (95%CI 1.18-15.67) p=0.03, as was  
216 treatment type. These observations were unchanged when progression free survival was assessed  
217 (Supplementary table 2).

218

#### 219 **L1 v s E6 ratio**

220 All viral genes in an HPV genome have an expected copy number of one. Comparison of VL  
221 measured by L1 versus E6 gene copy number revealed a small deviance from “copy=one” in almost  
222 all cases. However, in 19 cases out of 82 positive for E6, there was almost a one log range of values  
223 (0.262 to 2.27 copies of E6/L1) (Figure 3). We modelled the distance of E6/L1 to 1 against  
224 demographic and clinical variables; none of the demographic or clinical variables were significantly  
225 associated with the distance of E6/L1 to 1.

226

227 **Discussion**

228 We have demonstrated that ddPCR is an accurate and rapid method for determining HPV VL in OPC  
229 patients, consistent with previous studies in smaller cohorts (28,29). The VL detected with both E6  
230 and L1 genes displayed a wide range but was much more restricted than the  $10^3$  to  $10^7$  range  
231 reported for 48 OPC patients (12) or  $1- >900$  copies in 45 clinical samples, (29), which both detected  
232 L1 gene copy numbers.

233

234 The ddPCR analysis indicated that a higher L1 VL was associated with better clinical outcomes. One  
235 explanation is that cancers with a higher VL are virus “driven” - whereas those with lower VLs may  
236 represent cancers where other drivers are responsible for the cancer due to impairment of viral  
237 function through integration and/or epigenetic mechanisms. Notably, we detected very low levels  
238 indeed of L1 with some as low as 0.001 copies per cell. Although such samples tested positive for  
239 HPV16 in the original HPV PCR/genotyping test, we accept that the presence of the virus might not  
240 drive tumorigenesis in these cases and that some tumours in the “low VL” group could be treated as  
241 functionally HPV-negative. We did not test cancers for p16INK4a or E6 RT-PCR or perform in-situ  
242 hybridisation for E6/E7 sequences, which can indicate transcriptional activity of virus. This would be  
243 of interest for future work and could better clarify the relevance and activity of the virus of the “low  
244 VL” group (30).

245

246 It is known that the mutational burden of HPV-positive OPC is lower than HPV negative cancer;  
247 making it amenable to non-surgical treatment options (7). This may be because the majority of OPCs  
248 retain viral episomes and there is a lack of insertional mutagenesis of cellular genes due to  
249 integration (31). Therefore, the association of a higher VL with better outcome could be that  
250 multiple viral episomes might allow full virus gene expression, particularly of the highly  
251 immunogenic L1 protein. Theoretically, this would allow for greater antigen presentation and  
252 immune checks, particularly in the tonsils, which are lymphoid tissue.

253

254 HPV genome status in OPC tumours can be episomal or integrated or exist as virus-human episomes  
255 or integrants (32). While the relationship between integration status and clinical outcome is not fully  
256 understood, only low copy numbers have been detected in cases with integrated HPV genomes (32).  
257 Our samples had a mean viral load of 30.9 (L1) or 37.9 (E6), and VL with L1 detection was similar to  
258 VL with E6 detection, suggesting the majority of samples had mostly episomal genomes, but this  
259 requires confirmation. A surprising finding was that in the majority of samples, the ratio of E6 to L1  
260 was not equal to one and some had greater numbers of E6 vs L1 copies; this may be explained by  
261 amplification of E6/E7 genes either in episomal or integrated viral genomes due to recombination  
262 events. Conversely, the samples which had more L1 than E6 copies may reflect the presence of full-  
263 length genomes alongside partially deleted viral genomes missing the E6 gene. We could not  
264 demonstrate a relationship between L1:E6 ratios and clinical/demographic variables or outcomes,  
265 but this may be an artefact of the small number of cases. Viral activity in OPCs, including level of HPV  
266 gene expression is likely to provide further insight on clinical outcomes. While sequencing is  
267 required to address this question, our data provide further proof of the instability of the HPV  
268 genome in OPC.

269

270 In the adjusted analysis, VL was not independently associated with improved outcomes. This is  
271 consistent with the fact that OPC is influenced by various behavioural, demographic and clinical  
272 factors and their complex interplay. Consequently, L1 VL may be a proxy of one or a combination of  
273 these but nevertheless represents a tool which can be applied objectively to ascertain risk-groups  
274 within the HPV positive category. It is also feasible that VL may be used to indicate/inform treatment  
275 options. Trials of therapy in OPC based on HPV status have generally relied on qualitative tests; it  
276 would be of value to include VL in such studies to determine if it serves as a complimentary  
277 biomarker of disease outcomes (33).

278

279 There are limitations to the study – although the sample set was well annotated it was still relatively  
280 small. In addition, we did not impose additional tools to determine viral activity & physical state  
281 which may have helped explain the findings, including the relationship between L1 and E6 ratios.  
282 Nevertheless, given the increasing burden of OPC globally and the desire for optimal management  
283 strategies - we would argue that assessment of viral load is worthy of further investigation. To this  
284 end we aim to confirm our findings in a larger patient cohort where complimentary biomarkers of  
285 viral activity including p16INK4a and E6/E7 mRNA detection (34) are imposed and where increased  
286 power would allow further categorisation of load beyond binary groups.

287

## 288 **ACKNOWLEDGMENTS**

289 We are grateful to staff at the Scottish HPV Reference laboratory for support with sample collation.

290 **Conflicts:** K Cuschieri (non-personal) K Cuschieri's institution has received research funding or gratis  
291 consumables to support research from the following commercial entities in the last 3 years:

292 Cepheid, Genomica, LifeRiver, Euroimmun, GeneFirst, SelfScreen, Qiagen, Abbott, Hiantis and  
293 Hologic. No other authors report a conflict of interest in relation to this work.

294

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