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1 **Title: Human Papillomavirus DNA methylation predicts response to treatment**
2 **using cidofovir and imiquimod in Vulval Intraepithelial Neoplasia 3**

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13 **Running title:**

14 HPV methylation in VIN3; response to cidofovir and imiquimod

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23 Gynecologists, London, UK.

24 **The authors declare no conflict of interest**

25 **Statement of translational relevance (120-150 words)**

26 Treatment for the premalignant condition Vulval Intraepithelial Neoplasia (VIN) is
27 primarily surgical, however topical therapy offers many advantages. In a recent
28 clinical trial, we evaluated treatment of VIN using the antiviral nucleoside analogue
29 cidofovir, and TLR- agonist imiquimod. Both agents were effective in approximately
30 half the patients treated. We now report a strong association between methylation of
31 HPV DNA in pre-treatment biopsies and response to treatment. High levels of
32 methylation were associated with response to cidofovir and low levels with response
33 to imiquimod. This suggests that the two treatments may be effective in two
34 biologically distinct patient groups. These findings have two major implications.
35 Firstly, that a high proportion of patients could be successfully treated using a non-
36 surgical approach if, after further prospective validation, HPV DNA methylation was
37 used as a predictive biomarker. Secondly, that similar success rates might be
38 achievable using cidofovir and imiquimod in combination.

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42

43 **Abstract**

44 **Purpose**

45 Response rates to treatment of vulval intraepithelial neoplasia (VIN) with imiquimod
46 and cidofovir are approximately 57% and 61% respectively. Treatment is associated
47 with significant side effects and, if ineffective, risk of malignant progression.

48 Treatment response is not predicted by clinical factors. Identification of a biomarker
49 that could predict response is an attractive prospect. This work investigated HPV
50 DNA methylation as a potential predictive biomarker in this setting.

51 **Experimental design**

52 DNA from 167 cases of VIN 3 from the RT3 VIN clinical trial was assessed. HPV
53 positive cases were identified using: Greiner PapilloCheck and HPV 16 type-specific
54 PCR. HPV DNA methylation status was assessed in three viral regions: *E2*, *L1/L2*,
55 and the promoter, using pyrosequencing.

56 **Results**

57 Methylation of the HPV *E2* region was associated with response to treatment. For
58 cidofovir (n=30), median *E2* methylation was significantly higher in patients who
59 responded ($p = <0.0001$); *E2* methylation $>4\%$ predicted response with 88.2%
60 sensitivity and 84.6% specificity. For imiquimod (n=33), median *E2* methylation was
61 lower in patients who responded to treatment ($p = 0.03$ (not significant after
62 Bonferroni correction)); *E2* methylation $<4\%$ predicted response with 70.6%
63 sensitivity and 62.5% specificity.

64 **Conclusions**

65 These data indicate that cidofovir and imiquimod may be effective in two biologically
66 defined groups. HPV *E2* DNA methylation demonstrated potential as a predictive
67 biomarker for the treatment of VIN with cidofovir and may warrant investigation in a
68 biomarker-guided clinical trial.

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71

72 **Introduction**

73 Vulval intraepithelial neoplasia (VIN) is a chronic condition of vulval skin that is
74 diagnosed histologically by the identification of cellular changes associated with a
75 pre-malignant state. VIN is commonly caused by Human Papillomavirus (HPV),
76 which is present in around 85% of cases (1). VIN can be very distressing for patients
77 and often takes a long time to diagnose. If untreated, VIN may progress to vulval
78 cancer.

79 Currently, most cases of VIN are managed surgically. The aims of management are
80 reduction in risk of malignant progression, symptom alleviation (2) and confirmation
81 of the absence of stromal invasion (as occult malignancies are reported in up to 20.5%
82 of cases (3)). The extent of surgery required depends on the extent of disease and can
83 therefore range from local excision, to partial or complete vulvectomy with
84 reconstructive surgery. Due to the location of disease, rates of wound infection and
85 breakdown are high. These procedures affect both the anatomy and function of the
86 vulva and may be associated with significant psychosocial distress (4). Despite the
87 excision of disease, recurrence rates are unacceptably high. A systematic review
88 performed in 2005 revealed recurrence rates of 19% following complete vulvectomy,
89 18% following partial vulvectomy and 22% following local excision (5). This results
90 in repeated surgical procedures, and causes significant distress to patients (6). A
91 growing number of younger women are presenting with VIN, and surgical excision is
92 an increasingly unattractive option for both patients and clinicians (7).

93 Management options that preserve vulval tissue are urgently needed. Two compounds
94 with antiviral activity: the nucleoside analogue cidofovir, and the TLR7 agonist
95 imiquimod, are topical therapies that have been investigated with this aim. In small
96 studies, cidofovir demonstrated response rates ranging from 40%-79% (8,9) and
97 imiquimod from 26%-100% (10). Recently, the CRUK-funded RT3 VIN clinical trial
98 randomised patients with VIN 3 to treatment with either cidofovir or imiquimod (11).
99 Histologically confirmed, complete response rates were seen in 41/72 (57%) cidofovir
100 patients and 42/69 (61%) imiquimod patients. A predictive biomarker that could
101 identify patients likely to respond to specific treatments would facilitate optimal
102 management of these patients. The RT3 VIN study provided valuable bio-resources to
103 investigate potential biomarkers for response to topical therapy.

104 The limited research available indicates that not all patients with VIN respond to
105 treatment with cidofovir (9,11). In vitro studies have demonstrated that cidofovir
106 causes selective inhibition of proliferation in HPV infected cells compared with HPV
107 negative cell lines (12,13), and also that cidofovir is more effective in cells containing
108 specifically a high-risk HPV infection (14). However, consideration of the data on
109 HPV prevalence in VIN and response to cidofovir suggest that only a subset of HPV-
110 positive VIN responds to cidofovir.

111 It is plausible therefore that a more refined knowledge of HPV status and biology,
112 prior to treatment with cidofovir, is required to identify the patients most likely to
113 respond.

114 Imiquimod is a non-nucleoside heterocyclic amine, which acts as an immune-
115 response modifier. It induces activity of interferon α (IFN α), tumour necrosis factor α
116 (TNF α) and interleukin-6 via stimulation of TLR7 (15). The mechanism of action of
117 imiquimod is hence linked to the direct stimulation of the innate immune system and
118 requires a host response to HPV infection in the first instance. HPV infection is likely
119 to be most immunogenic in the context of a productive infection, when new viral
120 particles are produced. Previous literature suggests that productive infections may be
121 associated with low levels of methylation of viral DNA (16). This is consistent with
122 high levels of HPV DNA methylation being associated with more advanced disease
123 (17,18). It was therefore hypothesised that levels of HPV DNA methylation in VIN
124 might correlate with response to topical therapy with imiquimod.

125 The primary objective of this study was to quantify HPV DNA methylation in VIN,
126 and assess the association with response to topical treatment in the RT3 VIN clinical
127 trial cohort. The ultimate aim was to determine whether quantification of viral DNA
128 methylation had potential as a predictive biomarker to identify patients likely to
129 benefit from topical therapy for VIN.

130

131 **Methods**

132 **Patients and samples**

133 The study utilised bio-resources and clinical data from the RT3VIN clinical trial, the
134 design and eligibility criteria of this trial have been reported previously (11). Briefly,
135 180 women with histologically confirmed VIN 3 were randomised to receive topically
136 administered cidofovir or imiquimod for 24 weeks. The primary endpoint was
137 histologically confirmed complete response in baseline lesions 6 weeks after
138 completion of treatment. Response to treatment with either cidofovir or imiquimod
139 was determined by the absence of VIN in a tissue biopsy taken from the previously
140 affected area 6 weeks following the completion of treatment. The presence of VIN 1
141 or greater was considered persistent disease indicating failure to respond.

142 HPV testing was carried out on punch biopsies (4mm) available at baseline from the
143 site of disease in 167 patients (93%). Biopsies were stored in ThinPrep media
144 (Hologic, Marlborough, MA, USA) prior to processing. DNA was extracted using the
145 Qiagen DNA mini kit (Qiagen, Hilden, Germany).

146 **HPV detection**

147 A type-specific PCR targeting the HPV 16 *E6* region (19) was used to detect cases of
148 HPV 16. The Greiner PapilloCheck HPV genotyping assay (Greiner Bio-One,
149 Frickenhausen, Germany), which tests for 24 HPV genotypes (HPV 6, 11, 40, 42, 43,
150 44, 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73 and 82), was used
151 as per manufacturers instructions to test for the presence of non-HPV 16 genotypes.
152 HPV DNA methylation was only investigated in cases that tested positive for HPV 16
153 (defined as testing HPV 16 positive using HPV 16 *E6* PCR and/or PapilloCheck).

154 **HPV DNA methylation**

155 DNA methylation was quantified in the HPV promotor, *E2* and *L1/L2* regions. These
156 regions were assessed due to the possible functional significance of methylation in
157 regulating *E6* and *E7* oncogene expression (promotor and *E2* region) and their
158 established association with cervical neoplasia (*L1/L2*) (17,18). Positioning of primer
159 sequences reflected sequence constraints and the desire to amplify the maximum
160 number of CpG sites within a single reaction. Viral targets were assessed rather than
161 cellular ones, because the putative mechanisms of action of cidofovir and imiquimod

162 imply specificity to virus-infected cells. DNA (500 ng) was sodium bisulfite treated
163 using the EZ-DNA methylation kit (Zymo Research Corp, CA, USA). DNA
164 methylation was assessed by pyrosequencing of the *E2* ORF, *L1/L2* overlap, and
165 promoter regions using a Qiagen PyroMark Q96 ID system as previously described
166 (20). Each assay targeted multiple CpGs and all assays were performed in duplicate.
167 Methylation levels are reported as means for each region. These assays were specific
168 for HPV 16 only. Stringent quality assurance checks were applied to the methylation
169 data, including assessment of bisulphite conversion and primer extension; additional
170 quality control assessments were performed by the pyrosequencing software, and any
171 sample classed a ‘fail’ was excluded from the analysis. All samples were run in
172 duplicate and the standard deviation was calculated for each CpG site analysed. This
173 data was used to demonstrate the intra-run reproducibility of the assay and provided
174 an additional quality control step; samples were excluded from further analysis if a
175 value was beyond 3 standard deviations of the mean standard deviation calculated for
176 all CpG sites for each region. This final step was performed to enhance the quality of
177 the data set by excluding any samples generating dissimilar duplicate readings.

178

179 **Biomarker development and statistics**

180 Guidelines for predictive biomarker development were adhered to
181 (http://www.cancerresearchuk.org/sites/default/files/prognostic_and_predictivepdf),
182 (21). A statistical analysis plan was developed a priori and the laboratory team were
183 blinded to clinical outcomes. The distribution of HPV DNA methylation level in the
184 RT3 VIN baseline cohort was first established (biomarker discovery –stage 1).
185 Retrospective correlation with response to treatment of patients in the RT3 VIN
186 clinical trial was then assessed (biomarker discovery – stage 2). Mann-Whitney U
187 tests were used to identify statistically significant differences between methylation
188 levels in responders and non-responders. A Bonferroni correction to account for
189 multiple comparisons was incorporated making a p value of $p = 0.016$ significant.
190 Significant findings for any biomarker in either treatment cohort were further
191 investigated in both cohorts using ROC curve analysis to find optimum cut offs for
192 sensitivity and specificity.

193 **Results**

194 *Variability in HPV DNA methylation*

195 One-hundred-and-thirty-six cases (136/167) tested positive for HPV 16 DNA (Figure
196 1). The proportion of cases yielding analysable data in HPV DNA methylation assays
197 varied depending on the region examined ($E2 = 82$, $L1/L2 = 93$ and promoter = 122).
198 The higher rates of inadequate data in the $E2$ and $L1/L2$ regions most likely reflect
199 disruption of these regions associated with viral integration. The degree of
200 methylation of HPV DNA varied between the regions (Figure 2). A bimodal
201 distribution of values was observed for the $E2$ and $L1/L2$ regions, contrasting with
202 more uniformly low levels of methylation in the promoter region.

203 *HPV DNA methylation and response to treatment*

204 Correlation between methylation levels and response to treatment was retrospectively
205 assessed (Figure 3). Of the 136 cases that tested positive for HPV 16, twenty-nine
206 cases did not have post-treatment clinical outcome data; therefore 107 cases were
207 available for analysis.

208 For the $E2$ region 63/107 cases gave analysable data; for the $L1/L2$ region 73/107
209 cases; and for the promoter region 95/107 cases. A flow chart depicting how the final
210 numbers of patients suitable for analysis were derived is shown in Figure 1. Levels of
211 $E2$, $L1/L2$ and promoter region methylation were then compared between patients
212 who responded to treatment, and those who did not.

213 *E2 Methylation*

214 For patients treated with cidofovir with clinical outcome data ($n=54$), the $E2$
215 methylation assay generated a result in 30/54 (55.6%) of cases; 17/30 (56.7%)
216 responded to treatment and 13/30 (43.3%) did not. Median $E2$ methylation was
217 significantly higher in patients who responded (9.14%, inter-quartile range (IQR) =
218 4.28% - 82.03%) to cidofovir than in patients who did not (1.85%, IQR = 1.01% -
219 3.26%), ($U = 18.00$, $p = <0.0001$) (Figure 1.3).

220 For patients treated with imiquimod with clinical outcome data ($n=53$), the $E2$
221 methylation assay generated a result in 33/53 (62.3%) of cases; 17/33 (51.5%)
222 responded and 16/33 (48.5%) did not. Median $E2$ methylation was lower (2.57%, IQR
223 = 2.21% - 4.20%) in patients who responded to treatment than in patients who did not
224 (24.22%, IQR 3.15% - 87.94%), although this finding did not reach the required
225 statistical significance ($U = 196.00$, $p = 0.03$).

226 *L1/L2 Methylation*

227 For cidofovir treated patients with clinical outcome data (n=54), the L1/L2
228 methylation assay generated a result in 39/54 (72.2%) of cases; 17/39 (43.6%)
229 responded to treatment and 22/39 (56.4%) did not. Median *L1/L2* methylation was
230 found to be non-significantly higher (59.03%, IQR = 11.17% - 86.15%) in patients
231 who responded to cidofovir than patients who did not respond (9.62%, IQR = 5.25% -
232 28.41%), ($U = 113.00$, $p = 0.04$).

233 For patients treated with imiquimod with clinical outcome data (n=53), the L1/L2
234 assay generated a result in 34/53 (64.2%) of cases; 19/34 (55.9%) responded to
235 treatment and 15/34 (44.1%) did not. Median *L1/L2* methylation was non-
236 significantly lower in patients who responded to imiquimod (11.72% IQR = 6.81% -
237 62.13%) than in those patients who did not (37.60%, IQR = 12.49% - 77.69%), ($U =$
238 181.00 , $p = 0.34$).

239 *Promoter Methylation*

240 For cidofovir treated patients with clinical outcome data (n=54), the promoter
241 methylation assay generated a result in 51/54 (94.4%) cases; 26/51 (51.0%) responded
242 to treatment and 25/51 (49.0%) did not. Median promoter methylation was similar
243 between patients who responded to cidofovir (0.20%, IQR = 0.04% - 0.73%) and
244 patients who did not (0.24%, IQR = 0.00% - 0.55%), ($U = 295.5$, $p = 0.57$).

245 For patients treated with imiquimod with clinical outcome data (n=53), the promoter
246 methylation assay generated a result in 44/53 (83.0%). A complete response to
247 treatment was seen in 24/44 (54.5%) and 20/44 (45.5%) did not respond completely.
248 Median promoter methylation was non-significantly lower (0.16%, IQR = 0.00% -
249 0.44%) in patients who responded to imiquimod than in those patients who did not
250 (0.26%, IQR = 0.10% - 1.07%) ($U = 292.5$, $p = 0.21$).

251 *Sensitivity and specificity of E2 methylation*

252 ROC curve analysis was performed to investigate the ability of methylation of the *E2*
253 region to discriminate between patients who responded to treatment and those who
254 did not (figure 4). Quantification of *E2* methylation was able to discriminate between
255 responders and non-responders, with an AUC of 0.919 (95% CI 0.822-1.000).
256 Quantification of *E2* methylation also demonstrated the ability to distinguish
257 imiquimod responders from non-responders, with an AUC of 0.721 (95% CI = 0.538-
258 0.903).

259 Table 1 shows the sensitivity and specificity achievable at various cut-off levels of
260 methylation. This demonstrated that high sensitivity and specificity (88.2 and 84.6%)
261 to identify potential responders to treatment with cidofovir, could be achieved using a
262 cut-off value of 4% methylation. For imiquimod, a cut-off of 4% *E2* methylation
263 showed sensitivity and specificity of 70.6 and 62.5%. Use of a higher cut-off of 10%
264 would make the assay more sensitive but substantially less specific.

265 In the population treated with cidofovir, in both univariable and multivariable
266 (including the randomisation stratification factors of unifocal or multifocal disease,
267 and first presentation or recurrent disease) logistic regression models there was strong
268 evidence that the odds of response were significantly higher in patients with $\geq 4\%$ *E2*
269 methylation compared to those with $< 4\%$ *E2* methylation (n=30; univariable odds
270 ratio: 25.67, 95% CI: 3.63-181.44, p=0.001; multivariable odds ratio: 52.51, 95% CI:
271 3.88-709.90, p=0.003). In the population treated with imiquimod, there was weaker
272 evidence that the odds of response were lower in patients with $\geq 4\%$ *E2* methylation
273 compared to those with $< 4\%$ *E2* methylation (n=33; univariable odds ratio: 0.25,
274 95% CI: 0.06-1.07, p=0.062; multivariable odds ratio: 0.27, 95% CI: 0.06-1.19,
275 p=0.083).

276 *Cases without E2 methylation data*

277 Further analysis was undertaken of those cases for which *E2* methylation data was not
278 obtained. *E2* methylation data was not obtained for 85/167 (50.9%) of the research
279 samples from the RT3 VIN trial, of which seventy-two had clinical outcome data.
280 Thirty-eight cases were treated with cidofovir and 34 cases were treated with
281 imiquimod. Of the 38 cases treated with cidofovir, 19/38 (50.0%) responded to
282 treatment and 19/38 (50.0%) failed to respond to treatment. Of the 34 cases treated
283 with imiquimod, 21/34 (61.8%) and 13/34 (38.2%) failed to respond.

284 The cases without *E2* methylation were separated into cases in which there was no
285 detectable HPV 16 DNA and cases that failed the HPV 16 assay quality controls. Of
286 the HPV 16 negative cases (n=31), 28 had clinical outcome data and two approaches
287 were taken in their analysis. Firstly, there were 14 patients treated with cidofovir of
288 which, more patients responded 9/14 (64.3%) to treatment than did not 5/14 (35.7%).
289 Similarly, there were 14 patients treated with imiquimod and again, these patients
290 were more likely to respond to treatment than not (10/14 (71.4%) vs. 4/14 (28.6%)).
291 The second approach was to consider cases displaying complete absence of HPV

292 DNA, in comparison with those in which an HPV type other than HPV 16 was
293 detected. Of the 28 cases, 14 had no HPV DNA detected and 14 had a non-HPV 16
294 genotype detected. In cases with no detectable HPV DNA, 12/14 (85.7%) responded
295 to treatment (six in the cidofovir arm and six in the imiquimod arm) and 2/14 (14.3%)
296 failed to respond (one in each treatment arm). In cases where an HPV type other than
297 HPV 16 was detected, 7/14 (50.0%) responded to treatment (three in the cidofovir arm
298 and four in the imiquimod arm) and 7/14 (50.0%) failed to respond to treatment (four
299 in the cidofovir arm and three in the imiquimod arm).

300 HPV 33 was the second most common genotype, detected in 8 (non-HPV 16)
301 samples. Of these cases, 7/8 had clinical data (five cases were treated with cidofovir
302 and two cases with imiquimod). For the cidofovir cases, 3/5 failed to respond to
303 treatment and 2/5 responded. For the imiquimod case, one case responded and one
304 case failed to respond.

305 The remaining 54/85 (63.5%) cases without *E2* DNA methylation data were excluded
306 as they did not meet assay quality controls standards; 44 of these cases had clinical
307 outcome data. Twenty-four cases were treated with cidofovir, and 10/24 (41.7%)
308 responded to treatment while 14/24 (58.3%) failed to respond. Twenty cases were
309 treated with imiquimod, of which 11/20 (55.0%) responded to treatment and 9/20
310 (45.0%) failed to respond.

311 **Discussion**

312 The principle finding of this work was that DNA methylation of the HPV *E2* gene,
313 assessed in pre-treatment biopsies from patients with VIN 3, significantly correlated
314 with response to treatment with cidofovir. There was weaker evidence (not significant
315 after Bonferroni correction) of an association between *E2* DNA methylation and
316 response to treatment with imiquimod. High levels of methylation were highly
317 predictive of a clinical response to cidofovir, and conversely, low levels of
318 methylation were associated with a clinical response to treatment with imiquimod.

319 Several previous studies have demonstrated a strong association between *L1/L2*
320 methylation and cervical neoplasia (17,18). Increased methylation of the *E2* and
321 *L1/L2* regions is also observed in cervical cancers (20). It was notable that in the
322 current study, while *L1/L2* methylation showed some correlation with treatment
323 response, a stronger correlation was observed between response and methylation of

324 the *E2* region (median *E2* methylation was 9.14% in patients who responded to
325 treatment with cidofovir and 2.85% in patients who did not respond).

326 It has been proposed that in cervical HPV infections, increased methylation of the
327 *L1/L2* region may indicate the duration of an infection. It has also been shown that
328 increased methylation correlates with integration of the virus into the host genome
329 (22). It is not clear why *E2* methylation should correlate with response to treatment
330 with cidofovir. It is unclear if it is the level of methylation per se that is important or
331 if methylation is a surrogate marker of another relevant process. This is partly due to
332 the exact mechanism of action of cidofovir in HPV infected cells being poorly
333 defined. It is possible that the action of cidofovir in this context is as a de-methylating
334 agent. This is a somewhat speculative suggestion but is consistent with cidofovir
335 being a nucleoside analogue with similar structure to the established demethylating
336 agent decitabine (used in treatment of myelodysplastic blood conditions (23)). This
337 possibility is further supported by a study of cases of failed cidofovir treatment in
338 recurrent respiratory papillomatosis (caused by HPV 11), which correlated treatment
339 failure with uniformly low levels of methylation (24). Alternatively *E2* methylation
340 maybe a surrogate marker of another relevant process, e.g. it may be associated with
341 more advanced infections with lower levels of p53 protein. This would be consistent
342 with the suggestion that that the selectivity of cidofovir for transformed cells is due to
343 the absence, or perturbation, of normal DNA repair pathways associated with
344 dysfunctional p53 mediated signalling (25). Cidofovir has been shown to generate
345 double-stranded breaks in cellular DNA, which can be repaired in normal cells, but
346 not in tumour cells (26). In HPV infected cells the level of p53 is reduced through
347 ubiquitination and proteosomal degradation mediated by the HPV E6 oncoprotein,
348 expression of which can become deregulated as a result of HPV integration and/or
349 HPV DNA methylation (16). HPV integration and increased methylation could
350 therefore be more common in cells that have lower levels of p53/pRb, and may be
351 more likely to respond to cidofovir. The strong correlation between increased *E2*
352 methylation and response to treatment could therefore be because *E2* methylation is a
353 surrogate marker of absent/low level p53/pRb.

354 Contrary to the case with cidofovir, mean *E2* methylation was lower in patients who
355 responded to imiquimod (11.6% vs. 40.0%), although this finding was not statistically
356 significant. Imiquimod acts as an immunomodulator by activating TLR7, which in
357 turn, enhances the innate immune system by stimulating the synthesis of pro-

358 inflammatory cytokines, especially IFN α , which enhance cell-mediated cytolytic
359 activity against viral targets (15,27,28). However, the enhanced host immune
360 response needs direction in order to be effective and it is plausible that a proliferative
361 HPV infection provides this direction.

362 The success of HPV is often attributed to its ability to hide from normal host defence
363 mechanisms permitting persistent infection (16). Persistent infection can be associated
364 with development of high-grade intraepithelial neoplasia, in which HPV integration
365 and increased HPV DNA methylation are common (17,18,29). Similarly, low levels
366 of HPV DNA methylation strongly correlate with the presence of episomal HPV (30).
367 Hence HPV DNA methylation may be higher in infections that successfully evade
368 host immunity. Conversely, cases of early, episomal HPV infections with lower levels
369 of HPV DNA methylation, are more likely to stimulate an immune response that can
370 then be enhanced by the action of imiquimod. The values obtained for HPV DNA
371 methylation of the *E2* and *L1/L2* regions showed a bimodal distribution. Reports in
372 the literature exist correlating higher levels of *E2* and *L1/L2* methylation with high-
373 grade cervical and vulval disease (20,31-35). Based on this, it is perhaps surprising that
374 we observed consistently high levels of methylation in HPV 16 positive cases in this
375 cohort of VIN 3. It is possible that these higher levels of methylation reflect the
376 influences of a small number of other influences such as viral integration.

377 This is the first study investigating the potential role of viral methylation as a
378 predictive biomarker in the treatment of VIN. HPV *E2* DNA methylation meets the
379 criteria required for early predictive biomarker assay discovery and development. *E2*
380 methylation varied in the RT3 VIN cohort, which is highly representative of the
381 cohort to which the biomarker would apply. Strong correlations between high *E2*
382 methylation and response to treatment with cidofovir and low *E2* methylation and
383 response to treatment with imiquimod were identified retrospectively. However, prior
384 to further qualification in the context of a clinical trial utilising *E2* methylation as a
385 biomarker in the randomisation process, its 'fitness for purpose' needs to be
386 addressed. These criteria may include cost efficiency, ease of incorporation into the
387 clinical setting, efficiency of the assay testing the biomarker and patient coverage.
388 Incorporating the assay into the clinical setting is feasible. Testing could be carried
389 out on remaining biopsy material following histological assessment (the assay has
390 previously been successfully applied to DNA from fixed pathology blocks (22)).
391 Assessment of methylation state using bisulphite conversion and pyrosequencing is a

392 relatively standard assay and this equipment is likely to be widely available if HPV
393 DNA methylation is adopted as a triage assay in a cervical screening workflow (36).

394 The bio-resources used were obtained within a randomised clinical trial and were
395 associated with robust clinical endpoints (11). The material was rigorously quality
396 assured and controlled. Viral characteristics were assessed using well-validated
397 assays, with stringent quality assurance and control.

398 A significant concern was the 85/167 (50.9%) patients for whom an *E2* methylation
399 result was not available, potentially hindering the clinical application of the test. It
400 was also a concern that these cases could potentially represent a specific subset of
401 patients, and their exclusion might introduce bias into the findings. However, the
402 overall response rates for patients with no *E2* data were similar to the response rates
403 seen in the main clinical trial, which suggests that the risk of bias appears minimal.
404 The majority of excluded cases, were associated with failure to meet stringent assay
405 quality controls (n=54); this was most likely attributable to insufficient DNA in the
406 sample used for bisulphite conversion, or poor DNA quality. The methylation assay
407 requires a specific DNA concentration in the input sample, but the relative
408 concentration of human vs. viral DNA was not determined. The assay failures
409 associated with insufficient DNA appear likely to be attributable to relatively low
410 concentrations of viral DNA. In order to improve coverage in future studies, efforts
411 would need to be made to improve the quality and quantity of DNA through
412 optimisation of sample collection, processing and storage.

413 The remaining (n=31) for which *E2* data was unavailable did not have detectable
414 HPV 16 DNA in the sample. In the presence of HPV DNA of another genotype,
415 response rates were 50.0% in each treatment arm, however a clinical response was
416 seen more frequently in cases with no detectable HPV DNA (85.7%). The number of
417 cases is too small to draw any significant conclusions from this, however it raises the
418 possibility that management of HPV negative patients should be perhaps considered
419 separately. The data suggest that topical therapy may still be highly effective in this
420 group of patients. HPV 33 was the second most common detected HPV genotype. In
421 order to improve coverage of the assay, it may be of benefit to develop the *E2*
422 methylation assay for this genotype. Although it is important to note that an HPV 33
423 assay might not confer the same predictive value as observed with HPV 16.

424 Potential biomarkers were investigated in all patients enrolled in the trial for whom
425 pre and post treatment biopsies were available, even if they did not adhere to the
426 treatment regime. In the cidofovir arm, 78/89 patients adhered to the treatment
427 regime; in the imiquimod arm, 78/91 patients adhered. Patients who did not adhere to
428 the treatment regime, typically reduced dosing due to side-effects. Inclusion of all
429 patients allowed more accurate estimation of real-world clinical utility but may mean
430 that the performance of the biomarkers in the optimum setting may have been
431 underestimated.

432 The findings of this research indicate that imiquimod and cidofovir may be effective
433 in two biologically distinct groups. This observation invites a re-evaluation of how
434 topical treatment for VIN is conceived and delivered. To ensure that individual
435 patients receive an effective therapy, treatment could be personalised through use of a
436 biomarker. Further development of *E2* methylation as a predictive biomarker in the
437 treatment of VIN with cidofovir and imiquimod should be considered. This would
438 require validation in an independent cohort, and efforts would need to be made to
439 further optimise the *E2* methylation assay to reduce the number of failed results;
440 additionally, a pragmatic approach would be required to manage women with invalid
441 data.

442

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457

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587

588

589 **Figure Legends:**

590 **Figure 1. Flow chart indicating how final numbers of patients suitable for**
591 **analysis were derived.**

592 **Figure 2. Variation of regional HPV methylation in the RT3 VIN cohort.** The
593 median value was calculated for each region from all CpG sites tested and is
594 represented by a horizontal bar. Six CpG sites were tested for the *E2* region (nt 3411,
595 nt 3414, nt 3416, nt 3432, nt 3435, nt 3447), four CpG sites were tested for the *L1/L2*
596 region (nt 5615, nt 5606, nt5609, nt 5600) and five CpG sites were tested for the
597 promoter region (nt 31, nt 37, nt 43, nt 52, nt 58). Bars represent inter-quartile range.

598 **Figure 3. HPV DNA methylation in treatment responders and non-responders.**
599 Upper panel shows *E2* region methylation, middle panel *L1/L2* region, and lower
600 panel promotor region. Any treatment represents combined data from both cidofovir
601 and imiquimod treatment arms. Boxes represent the interquartile range, the central bar
602 represents the median value, and whiskers represent minimum and maximum values.

603 **Figure 4. ROC curve analysis.** The upper panel demonstrates the ability of *E2*
604 methylation levels to distinguish cidofovir responders from non-responders. N = 30.
605 Increasing level of *E2* methylation demonstrates ‘excellent’ ability to distinguish
606 cidofovir responders from non-responders with AUC 0.919 (95%CI 0.882–1.00). The
607 lower panel demonstrates the ability of *E2* methylation to distinguish imiquimod
608 responders from non-responders. N = 33. Decreasing *E2* methylation demonstrated
609 ‘fair to good’ ability to distinguish imiquimod responders from non-responders with
610 an AUC of 0.721 (95%CI 0.538–0.903).

611 **Table 1.** The level of methylation in the first column is based on the average
612 methylation found from the multiple CpG’s tested in the *E2* region. The smallest cut-
613 off value represents the minimum *E2* methylation value obtained -1 and the largest
614 cut off point represents the maximum value +1. Cut-off values between these are the
615 average of two consecutive ordered observed test values, generated by SPSS ROC
616 analysis.

617

618

619

Figure 1

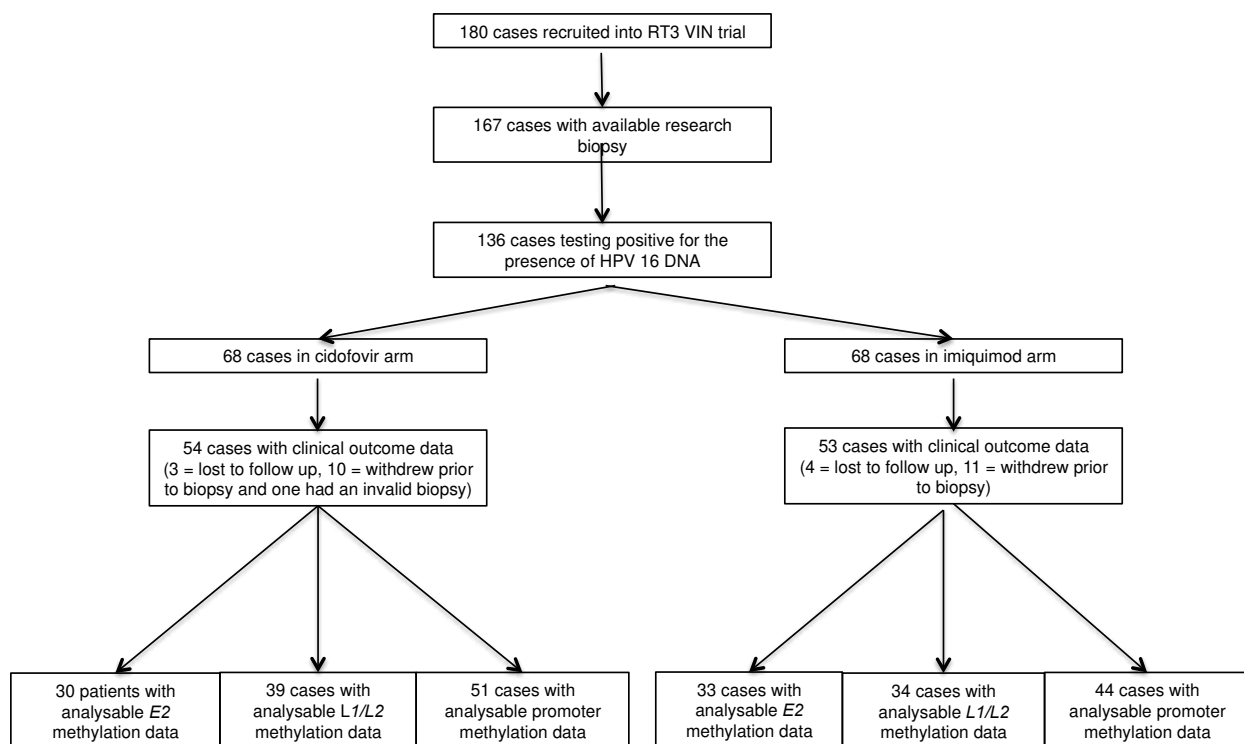
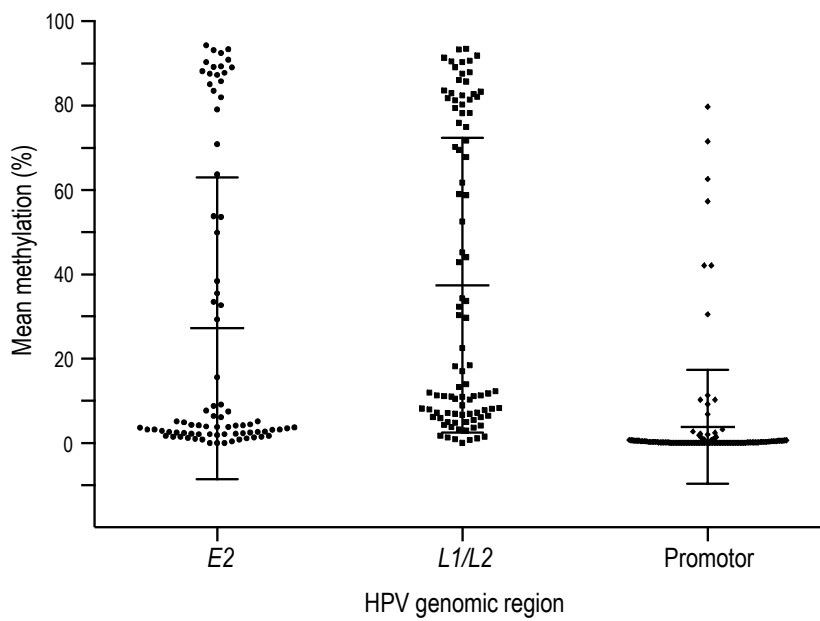
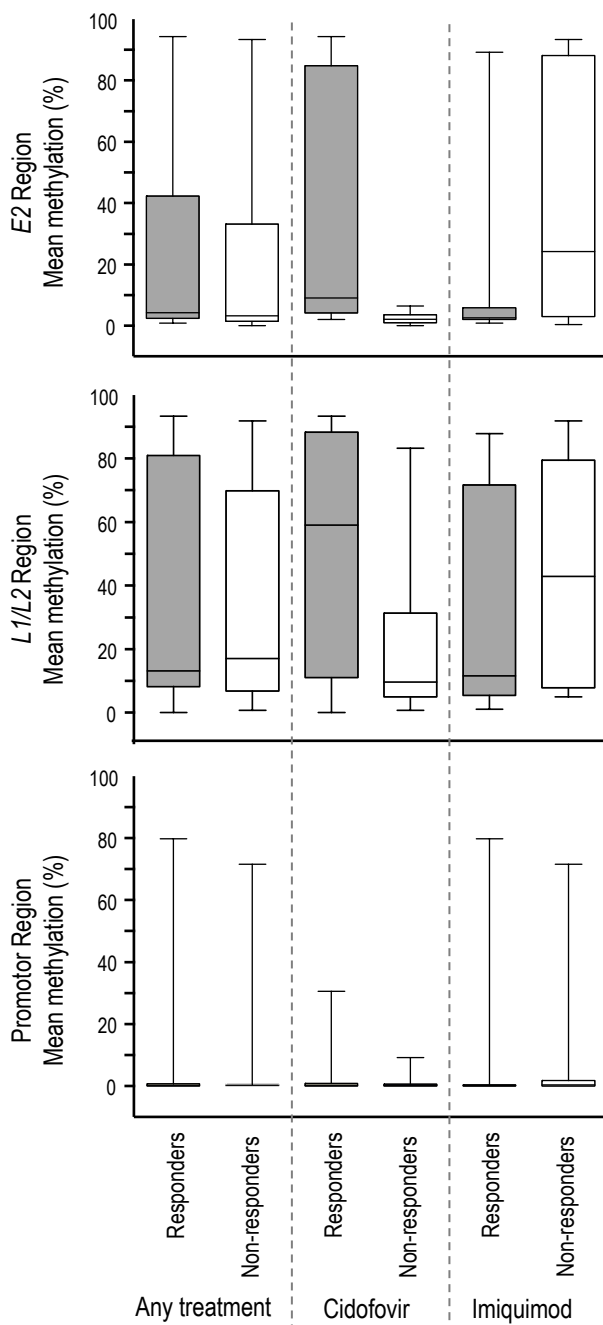


Figure 2





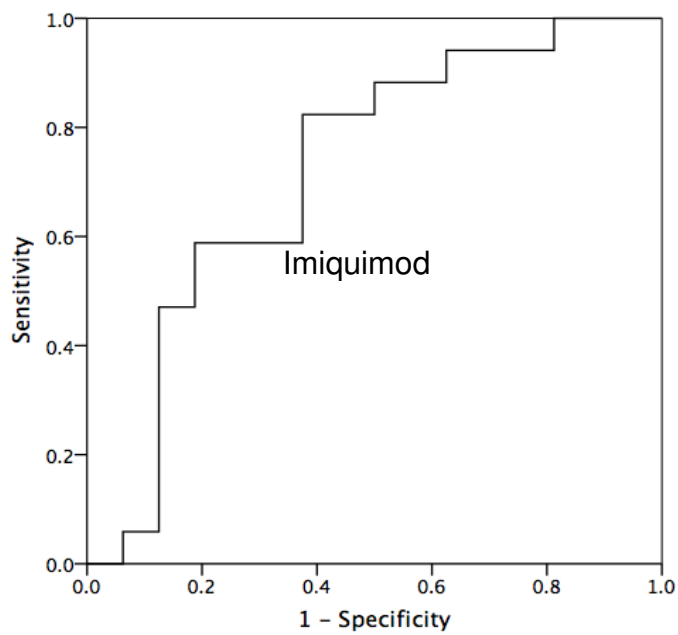
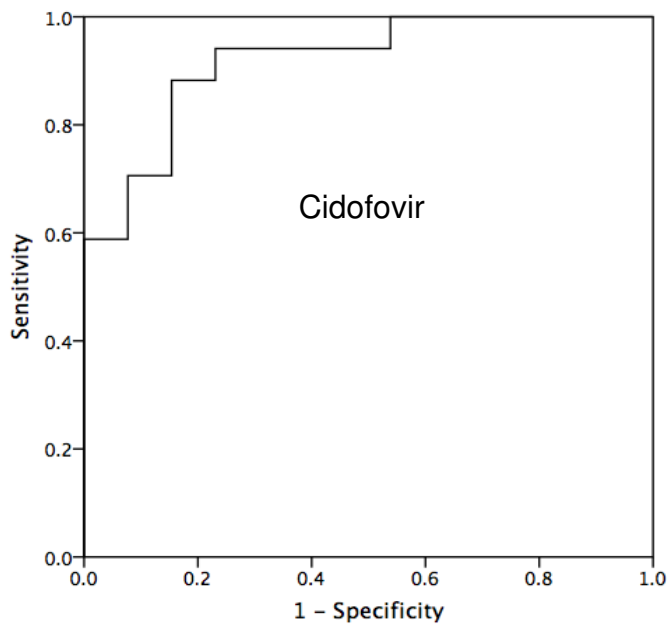


Table 1. Sensitivity and specificity of different *E2* methylation cut-off levels to distinguish responders and non-responders.

Response to treatment with <i>E2</i> methylation greater than or equal to			Response to treatment with <i>E2</i> methylation less than or equal to		
	sensitivity (%)	specificity (%)		Sensitivity (%)	Specificity (%)
-1.00	100	0	-0.59	0	100
0.43	100	15.4	0.65	0	93.7
0.94	100	23.1	1.06	5.9	93.7
1.07	100	30.8	1.41	5.9	87.5
1.32	100	38.5	1.67	11.8	87.5
1.81	100	46.2	1.97	17.6	87.5
2.15	94.1	46.2	2.19	23.5	87.5
2.41	94.1	53.8	2.25	29.4	87.5
2.89	94.1	61.5	2.34	35.3	87.5
3.21	94.1	69.2	2.41	41.2	87.5
3.56	94.1	76.9	2.47	47.1	87.5
3.87	88.2	76.9	2.53	47.1	81.2
3.94	88.2	84.6	2.61	52.9	81.2
4.12	82.4	84.6	2.80	58.8	81.2
4.26	76.5	84.6	3.09	58.8	75
4.62	70.6	84.6	3.23	58.8	68.7
5.04	70.6	92.3	3.40	58.8	62.5
5.13	64.7	92.3	3.64	64.7	62.5
5.81	58.8	92.3	3.96	70.6	62.5
7.68	58.8	100	5.88	76.5	62.5
9.01	52.9	100	7.64	82.4	62.5
22.36	47.1	100	11.69	82.4	56.2
44.73	41.2	100	22.49	82.4	50
62.44	35.3	100	31.05	88.2	50
76.51	29.4	100	33.17	88.2	43.7
84.84	23.5	100	35.99	88.2	37.5
88.48	17.6	100	60.97	94.1	37.5
90.92	11.8	100	85.68	94.1	31.2
93.44	5.9	100	88.05	94.1	25
95.36	0	100	88.78	94.1	18.7
			89.83	100	18.7
			91.82	100	12.5
			93.36	100	6.2
			94.45	100	0

Clinical Cancer Research

Human Papillomavirus DNA methylation predicts response to treatment using cidofovir and imiquimod in Vulval Intraepithelial Neoplasia 3

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