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

Authors: Sadie EF. Jones (1), Samantha Hibbitts (1), Christopher N. Hurt (2), Dean Bryant (3), Alison N. Fiander (1), Ned Powell[*](1), Amanda J. Tristram[*](1)

Corresponding author: Dr Sadie EF. Jones, School of Medicine, Cardiff University, Cardiff, CF14 4XW, UK jonessef@cardiff.ac.uk

[*]Joint senior authorship

Affiliations:

1 - School of Medicine, Cardiff University, Cardiff, CF14 4QZ, UK

2 - Wales Cancer Trials Unit (WCTU), School of Medicine, Cardiff University, Cardiff, CF14 4XW, UK

3 - University of Southampton, SO17 1BJ, UK

Running title:

HPV methylation in VIN3; response to cidofovir and imiquimod

Keywords:

Vulval intraepithelial neoplasia, cidofovir, imiquimod, DNA methylation, HPV

Funding

The RT3 VIN trial was funded by Cancer Research UK (CRUK/06/024) and CRUK core funding to the Wales Clinical Trial Unit (WCTU) at Cardiff University. The Tom Owen Memorial Fund (Cardiff University) also contributed toward the cost of consumables for the viral methylation analyses.

Current affiliation for A.N. Fiander: Royal College of Obstetricians and Gynecologists, London, UK.

The authors declare no conflict of interest

Statement of translational relevance (120-150 words)

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

Abstract

Purpose

Response rates to treatment of vulval intraepithelial neoplasia (VIN) with imiquimod and cidofovir are approximately 57% and 61% respectively. Treatment is associated with significant side effects and, if ineffective, risk of malignant progression. Treatment response is not predicted by clinical factors. Identification of a biomarker that could predict response is an attractive prospect. This work investigated HPV DNA methylation as a potential predictive biomarker in this setting.

Experimental design

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

Results

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

Conclusions

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

Introduction

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

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

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

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

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

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

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

Methods

Patients and samples

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

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

HPV detection

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

HPV DNA methylation

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

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

Biomarker development and statistics

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

Results

Variability in HPV DNA methylation

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

HPV DNA methylation and response to treatment

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

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

E2 Methylation

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

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

L1/L2 Methylation

For cidofovir treated patients with clinical outcome data (n=54), the L1L2 methylation assay generated a result in 39/54 (72.2%) of cases; 17/39 (43.6%) responded to treatment and 22/39 (56.4%) did not. Median *L1/L2* methylation was found to be non-significantly higher (59.03%, IQR = 11.17% - 86.15%) in patients who responded to cidofovir than patients who did not respond (9.62%, IQR = 5.25% - 28.41%), (U = 113.00, p = 0.04).

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

Promoter Methylation

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

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

Sensitivity and specificity of E2 methylation

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

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

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

Cases without E2 methylation data

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Acknowledgements

We would like to acknowledge all the members of the HPV research group in Cardiff University for their invaluable support and contributions to this work. The trial was run independently at WCTU. Gilead Sciences supported the study by provision of cidofovir at a discounted price, which was funded by a central subvention from the Department of Health (England) and the National Institute for Social Care and Health Research (Wales). Finally, we thank all patients who participated in the trial and the principal investigators and their colleagues for recruitment and treatment of patients.

Funding

The RT3 VIN trial was funded by Cancer Research UK (CRUK/06/024) and CRUK core funding to the Wales Clinical Trial Unit (WCTU) at Cardiff University. The Tom Owen Memorial Fund (Cardiff University) also contributed toward the cost of consumables for the viral methylation analyses.

457

References

1. De Vuyst H, Clifford GM, Nascimento MC, Madeleine MM, Franceschi S. Prevalence and type distribution of human papillomavirus in carcinoma and intraepithelial neoplasia of the vulva, vagina and anus: a meta-analysis. Int J Cancer **2009**;124(7):1626-36.
2. Sykes P, Smith N, McCormick P, Frizelle FA. High-grade vulval intraepithelial neoplasia (VIN 3): a retrospective analysis of patient characteristics, management, outcome and relationship to squamous cell carcinoma of the vulva 1989-1999. Aust N Z J Obstet Gynaecol **2002**;42(1):69-74.
3. Husseinzadeh N, Recinto C. Frequency of invasive cancer in surgically excised vulvar lesions with intraepithelial neoplasia (VIN 3). Gynecol Oncol **1999**;73(1):119-20.
4. Shylasree TS, Karanjgaokar V, Tristram A, Wilkes AR, MacLean AB, Fiander AN. Contribution of demographic, psychological and disease-related factors to quality of life in women with high-grade vulval intraepithelial neoplasia. Gynecol Oncol **2008**;110(2):185-9 doi 10.1016/j.ygyno.2008.04.023S0090-8258(08)00327-2 [pii].
5. van Seters M, van Beurden M, de Craen AJ. Is the assumed natural history of vulvar intraepithelial neoplasia III based on enough evidence? A systematic review of 3322 published patients. Gynecol Oncol **2005**;97(2):645-51.
6. Kaushik S, Pepas L, Nordin A, Bryant A, Dickinson HO. Surgical interventions for high-grade vulval intraepithelial neoplasia. The Cochrane database of systematic reviews **2014**(3):CD007928 doi 10.1002/14651858.CD007928.pub3.
7. Kaushik S, Pepas L, Nordin A, Bryant A, Dickinson HO. Surgical interventions for high grade vulval intraepithelial neoplasia. The Cochrane database of systematic reviews **2011**(1):CD007928 doi 10.1002/14651858.CD007928.pub2.
8. Stier EA, Goldstone SE, Einstein MH, Jay N, Berry JM, Wilkin T, *et al.* Safety and efficacy of topical cidofovir to treat high-grade perianal and vulvar intraepithelial neoplasia in HIV-positive men and women. Aids **2013**;27(4):545-51 doi 10.1097/QAD.0b013e32835a9b16.
9. Tristram A, Fiander A. Clinical responses to Cidofovir applied topically to women with high grade vulval intraepithelial neoplasia. Gynecol Oncol **2005**;99(3):652-5.
10. Iavazzo C, Pitsouni E, Athanasiou S, Falagas ME. Imiquimod for treatment of vulvar and vaginal intraepithelial neoplasia. International journal of gynaecology

and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics **2008**;101(1):3-10 doi 10.1016/j.ijgo.2007.10.023.

11. Tristram A, Hurt CN, Madden T, Powell N, Man S, Hibbitts S*, et al.* Activity, safety, and feasibility of cidofovir and imiquimod for treatment of vulval intraepithelial neoplasia (RT(3)VIN): a multicentre, open-label, randomised, phase 2 trial. Lancet Oncol **2014**;15(12):1361-8 doi 10.1016/S1470-2045(14)70456-5.
12. Andrei G, Snoeck R, Piette J, Delvenne P, De Clercq E. Inhibiting effects of cidofovir (HPMPC) on the growth of the human cervical carcinoma (SiHa) xenografts in athymic nude mice. Oncol Res **1998**;10(10):533-9.
13. Johnson JA, Gangemi JD. Selective inhibition of human papillomavirus-induced cell proliferation by (S)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl]cytosine. Antimicrob Agents Chemother **1999**;43(5):1198-205.
14. Donne AJ, Hampson L, He XT, Rothera MP, Homer JJ, Hampson IN. Cidofovir induces an increase in levels of low-risk and high-risk HPV E6. Head Neck **2009**;31(7):893-901 doi 10.1002/hed.21043.
15. Stanley MA. Imiquimod and the imidazoquinolones: mechanism of action and therapeutic potential. Clinical and experimental dermatology **2002**;27(7):571-7.
16. Doorbar J. Molecular biology of human papillomavirus infection and cervical cancer. Clin Sci **2006**;110(5):525-41.
17. Lorincz AT, Brentnall AR, Vasiljevic N, Scibior-Bentkowska D, Castanon A, Fiander A*, et al.* HPV16 L1 and L2 DNA methylation predicts high-grade cervical intraepithelial neoplasia in women with mildly abnormal cervical cytology. Int J Cancer **2013**;133(3):637-44 doi 10.1002/ijc.28050.
18. Mirabello L, Schiffman M, Ghosh A, Rodriguez AC, Vasiljevic N, Wentzensen N*, et al.* Elevated methylation of HPV16 DNA is associated with the development of high grade cervical intraepithelial neoplasia. Int J Cancer **2013**;132(6):1412-22 doi 10.1002/ijc.27750.
19. Collins SI, Constandinou-Williams C, Wen K, Young LS, Roberts S, Murray PG*, et al.* Disruption of the E2 gene is a common and early event in the natural history of cervical human papillomavirus infection: a longitudinal cohort study. Cancer Res **2009**;69(9):3828-32 doi 10.1158/0008-5472.CAN-08-30990008-5472.CAN-08-3099 [pii].
20. Bryant D, Tristram A, Liloglou T, Hibbitts S, Fiander A, Powell N. Quantitative measurement of Human Papillomavirus type 16 L1/L2 DNA methylation correlates with cervical disease grade. J Clin Virol **2014**;59(1):24-9 doi 10.1016/j.jcv.2013.10.029.

21. Lioumi M, Newall D. CR-UK biomarker roadmaps. Clinical Cancer Research **2010**;16(19):B33 doi 10.1158/DIAG-10-B33.
22. Bryant D, Onions T, Raybould R, Jones S, Tristram A, Hibbitts S*, et al.* Increased methylation of Human Papillomavirus type 16 DNA correlates with viral integration in Vulval Intraepithelial Neoplasia. J Clin Virol **2014**;61(3):393-9 doi 10.1016/j.jcv.2014.08.006.
23. Gros C, Fahy J, Halby L, Dufau I, Erdmann A, Gregoire JM*, et al.* DNA methylation inhibitors in cancer: recent and future approaches. Biochimie **2012**;94(11):2280-96 doi 10.1016/j.biochi.2012.07.025.
24. Gall T, Kis A, Feher E, Gergely L, Szarka K. Virological failure of intralesional cidofovir therapy in recurrent respiratory papillomatosis is not associated with genetic or epigenetic changes of HPV11: complete genome comparison of sequential isolates. Antiviral Res **2011**;92(2):356-8 doi 10.1016/j.antiviral.2011.09.007.
25. Andrei G, Topalis D, De Schutter T, Snoeck R. Insights into the mechanism of action of cidofovir and other acyclic nucleoside phosphonates against polyoma- and papillomaviruses and non-viral induced neoplasia. Antiviral Res **2015**;114:21-46 doi 10.1016/j.antiviral.2014.10.012.
26. De Schutter T, Andrei G, Topalis D, Duraffour S, Mitera T, Naesens L*, et al.* Cidofovir treatment improves the pathology caused by the growth of human papillomavirus-positive cervical carcinoma xenografts in athymic nude mice. Cancer letters **2013**;329(2):137-45 doi 10.1016/j.canlet.2012.10.036.
27. Diaz-Arrastia C, Arany I, Robazetti SC, Dinh TV, Gatalica Z, Tyring SK*, et al.* Clinical and molecular responses in high-grade intraepithelial neoplasia treated with topical imiquimod 5%. Clin Cancer Res **2001**;7(10):3031-3.
28. Edwards L. Imiquimod in clinical practice. The Australasian journal of dermatology **1998**;39 Suppl 1:S14-6.
29. Bryant D, Onions T, Raybould R, Flynn A, Tristram A, Meyrick S*, et al.* mRNA sequencing of novel cell lines from human papillomavirus type-16 related vulval intraepithelial neoplasia: consequences of expression of HPV16 E4 and E5. J Med Virol **2014**;86(9):1534-41 doi 10.1002/jmv.23994.
30. Oka N, Kajita M, Nishimura R, Ohbayashi C, Sudo T. L1 gene methylation in high-risk human papillomaviruses for the prognosis of cervical intraepithelial neoplasia. Int J Gynecol Cancer **2013**;23(2):235-43 doi 10.1097/IGC.0b013e31827da1f6.
31. Brandsma JL, Sun Y, Lizardi PM, Tuck DP, Zelterman D, Haines GK, 3rd*, et al.* Distinct human papillomavirus type 16 methylomes in cervical cells at different

stages of premalignancy. Virology **2009**;389(1-2):100-7 doi S0042-6822(09)00231-1 [pii]10.1016/j.virol.2009.03.029.

32. Kalantari M, Chase DM, Tewari KS, Bernard HU. Recombination of human papillomavirus-16 and host DNA in exfoliated cervical cells: a pilot study of L1 gene methylation and chromosomal integration as biomarkers of carcinogenic progression. J Med Virol **2010**;82(2):311-20 doi 10.1002/jmv.21676.
33. Mirabello L, Sun C, Ghosh A, Rodriguez AC, Schiffman M, Wentzensen N, *et al.* Methylation of Human Papillomavirus Type 16 Genome and Risk of Cervical Precancer in a Costa Rican Population. J Nat Cancer Inst **2012**;104(7):556-65 doi 10.1093/jnci/djs135.
34. Turan T, Kalantari M, Cuschieri K, Cubie HA, Skomedal H, Bernard HU. High-throughput detection of human papillomavirus-18 L1 gene methylation, a candidate biomarker for the progression of cervical neoplasia. Virology **2007**;361(1):185-93 doi S0042-6822(06)00839-7 [pii]10.1016/j.virol.2006.11.010.
35. Wiley DJ, Huh J, Rao JY, Chang C, Goetz M, Poulter M, *et al.* Methylation of human papillomavirus genomes in cells of anal epithelia of HIV-infected men. Journal of acquired immune deficiency syndromes **2005**;39(2):143-51.
36. Lorincz AT, Brentnall AR, Scibior-Bentkowska D, Reuter C, Banwait R, Cadman L, *et al.* Validation of a DNA methylation HPV triage classifier in a screening sample. Int J Cancer **2016**;138(11):2745-51 doi 10.1002/ijc.30008.

Figure Legends:

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

Figure 2.Variation of regional HPV methylation in the RT3 VIN cohort. The median value was calculated for each region from all CpG sites tested and is represented by a horizontal bar. Six CpG sites were tested for the *E2* region (nt 3411, nt 3414, nt 3416, nt 3432, nt 3435, nt 3447), four CpG sites were tested for the *L1/L2* region (nt 5615, nt 5606, nt5609, nt 5600) and five CpG sites were tested for the 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

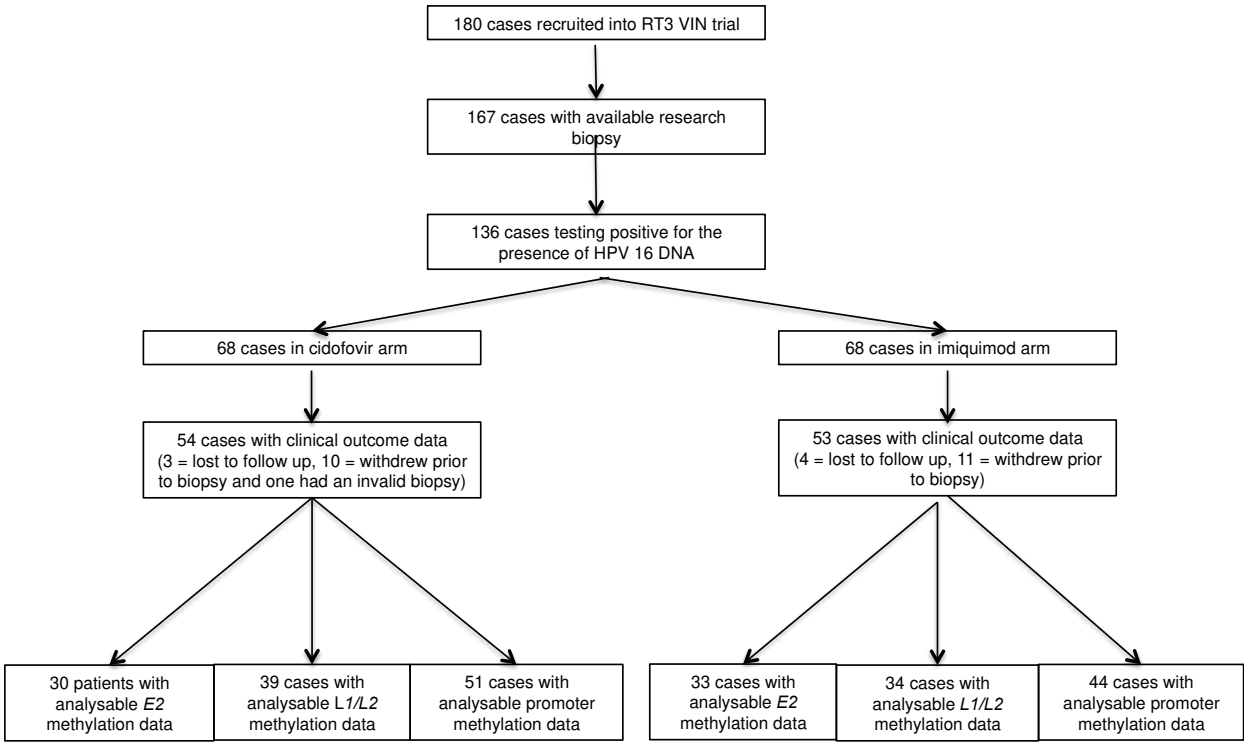
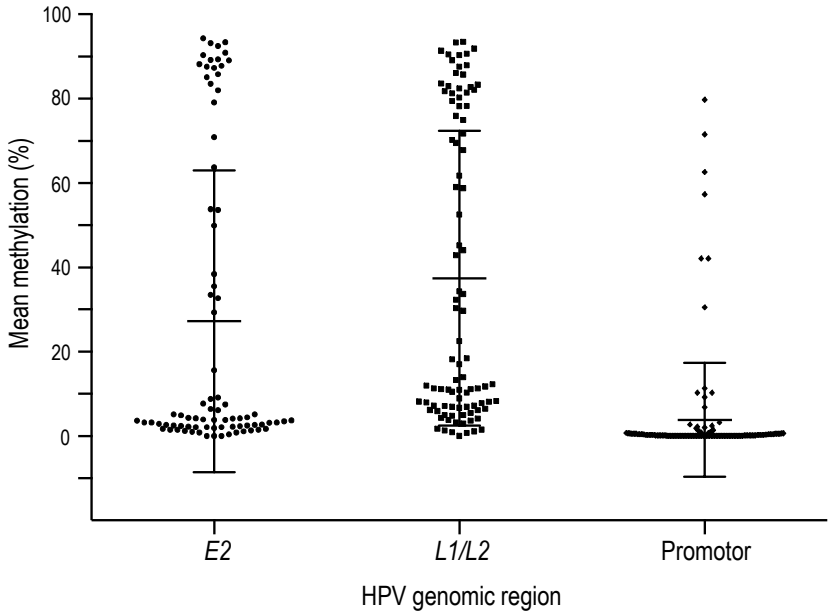
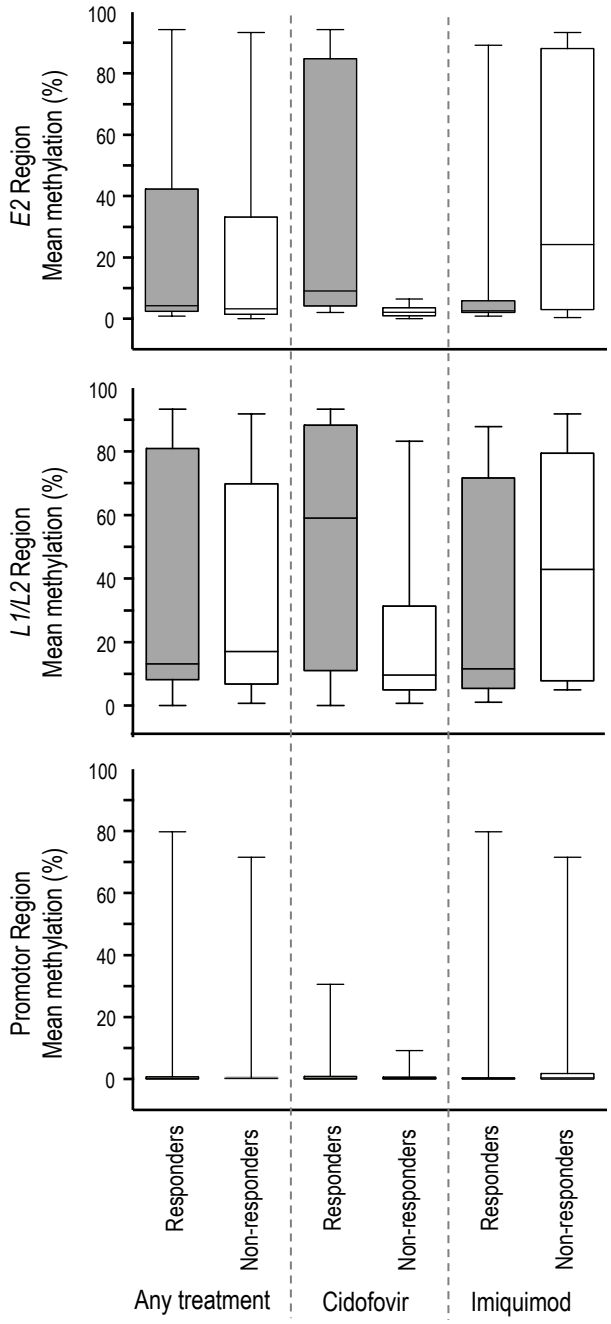
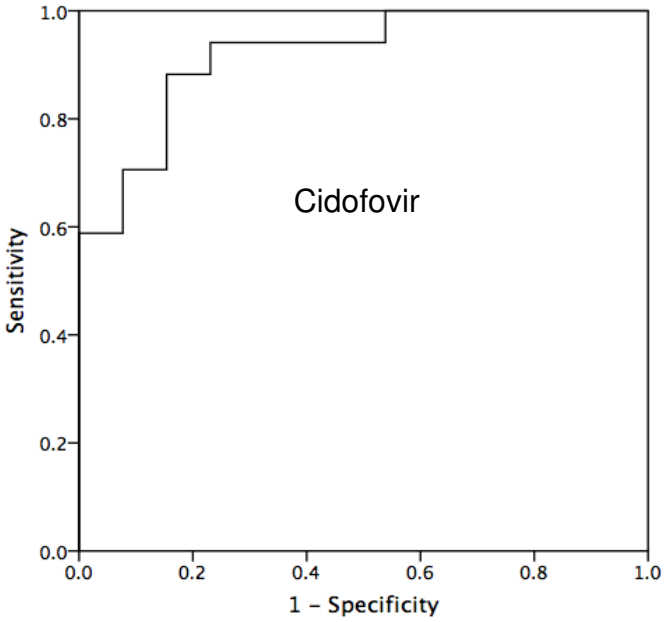


Figure 2







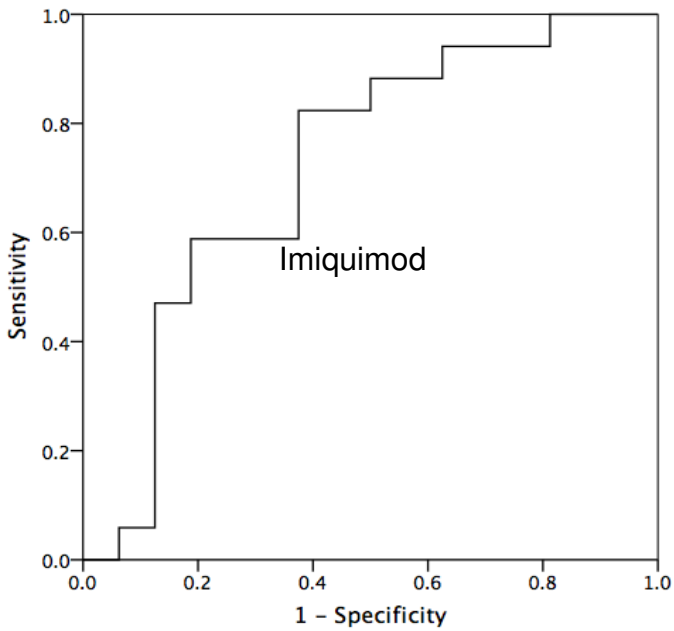


Table 1. Sensitivity and specificity of different *E2* methylation cut-off levels to distinguish responders and non-responders.

Response to cidofovir and E2 methylation		
Responds to treatment if E2 methylation greater than or equal to	sensitivity (%)	specificity (%)
-1.00	100	0
0.43	100	15.4
0.94	100	23.1
1.07	100	30.8
1.32	100	38.5
1.81	100	46.2
2.15	94.1	46.2
2.41	94.1	53.8
2.89	94.1	61.5
3.21	94.1	69.2
3.56	94.1	76.9
3.87	88.2	76.9
3.94	88.2	84.6
4.12	82.4	84.6
4.26	76.5	84.6
4.62	70.6	84.6
5.04	70.6	92.3
5.13	64.7	92.3
5.81	58.8	92.3
7.68	58.8	100
9.01	52.9	100
22.36	47.1	100
44.73	41.2	100
62.44	35.3	100
76.51	29.4	100
84.84	23.5	100
88.48	17.6	100
90.92	11.8	100
93.44	5.9	100
95.36	0	100

Response to imiquimod and E2 methylation		
Responds to treatment if E2 methylation less than or equal to	Sensitivity (%)	Specificity (%)
-0.59	0	100
0.65	0	93.7
1.06	5.9	93.7
1.41	5.9	87.5
1.67	11.8	87.5
1.97	17.6	87.5
2.19	23.5	87.5
2.25	29.4	87.5
2.34	35.3	87.5
2.41	41.2	87.5
2.47	47.1	87.5
2.53	47.1	81.2
2.61	52.9	81.2
2.80	58.8	81.2
3.09	58.8	75
3.23	58.8	68.7
3.40	58.8	62.5
3.64	64.7	62.5
3.96	70.6	62.5
5.88	76.5	62.5
7.64	82.4	62.5
11.69	82.4	56.2
22.49	82.4	50
31.05	88.2	50
33.17	88.2	43.7
35.99	88.2	37.5
60.97	94.1	37.5
85.68	94.1	31.2
88.05	94.1	25
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

AACR American Association for Cancer Research

Clinical Cancer Research

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

Sadie Esme Fleur Jones, Samantha Hibbitts, Christopher N Hurt, et al.

Clin Cancer Res Published OnlineFirst June 9, 2017.