HPV Research Group

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HPV Biology in VIN: Viral Biomarkers to Predict Response to Treatment

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Summary

Vulval intraepithelial neoplasia (VIN) is a condition of vulval skin that is often chronic in nature. The diagnosis is made histologically and can be delayed due to VINs variable clinical presentation. Frequently reported symptoms of pain and itching can be very distressing for patients. If left untreated, VIN has the potential to become malignant. The current standard treatment for VIN is surgical excision. However this approach is not ideal; it can be disfiguring and lead to significant psychosexual morbidity. There are also potentially, significant post-operative complications (e.g. infection) and a high rate of recurrence. Alternative treatments are needed. The efficacy of cidofovir and imiquimod treatment was investigated in the RT3 VIN clinical trial, and complete responses were observed in 57% and 61% of patients respectively.

The Human Papillomavirus (HPV) plays a major aetiological role in the development of VIN. To further understanding of HPV pathogenesis in VIN, HPV prevalence, HPV integration, HPV methylation and HPV gene expression were investigated in 167 tissue biopsies from patients participating in the RT3 VIN trial. High HPV prevalence of 98.2% was detected. HPV biology was found to be heterogeneous: HPV integration was detected in 71/136 (52.5%) cases; HPV DNA methylation ranged from 0.0% - 94.4%, and there was variable expression of HPV genes. Strong correlations were found between HPV integration, high levels of methylation, low levels of E2 gene expression and deregulated oncogene expression.

Variable HPV biology in VIN 3 meant that HPV characteristics had a potential role as predictive biomarkers. HPV *E2* region methylation demonstrated greatest potential. *E2* methylation >4% predicted response to cidofovir with 88.2% sensitivity and 84.6% specificity; while *E2* methylation <4% predicted response to imiquimod with 70.6% sensitivity and 62.5% specificity. Further investigation of this biomarker in a large prospective study is justified.

Declaration

This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidature for any degree or other award.

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I have had additional support from all members of the Cardiff University HPV research group, but particularly Dr Dean Bryant who took time to teach me laboratory techniques. Finally, I would like to thank Dr Kate Cushieri in Edinburgh who kindly ran the DiaMex assay on a small subset of my samples for part of this work.

Personally, I would like to thank my husband, Rhidian Jones and my children Molly and Harri. This would not have been possible without their support and understanding over the last few years.

Contributions

For the purpose of clarification, my specific involvement in the initial RT3 VIN trial included the recruitment of a number of patients at the Cardiff centre; weekly attendance in the VIN specialist clinic for patient follow up including biopsy collection and completion of all trial documentation; transportation of the majority of the Cardiff research biopsies (and bloods) from the clinic to the laboratory (between two hospitals in Cardiff); completion of adverse reaction paperwork in the absence of the chief investigator; regular attendance at the trial management meeting and recognition of the need to change the trial protocol regarding collection of research samples and devising the appropriate minor amendment. I personally carried out all the HPV testing for the clinical trial, and was responsible

its data processing and writing a small section of the final RT3 VIN paper and its proof reading.

Regarding the additional laboratory work that was undertaken for this thesis, aside from the DiaMex HPV testing that was undertaken on 24 samples by Dr Kate Cushieri's laboratory in Edinburgh, I carried out all the laboratory work, all the data processing, analysis and statistics and of course wrote the final thesis.

Abbreviations

αIFN	Alpha interferon
bp	Base pair
APOT	Amplification of papillomavirus oncogene transcripts
APS	Adenosine 5'phosphosulphate
ATM-ATR	Ataxia telangiectasia mutated – RAD3 associated protein
ATP	Adenosine triphosphate
CCD	Charged couples device
CCE	Cornified cell envelope
CIN	Cervical intraepithelial neoplasia
CpG	Cytosine-phosphate-guanine
CT	Threshold cycle
cDNA	Copy deoxyribse nucleic acid
DNA	Deoxyribose nucleic acid
DNMT	DNA methyltransferases
dNTP	Dinucleotide triphosphate
DTT	Dithiothreitol
dVIN	Differentiated vulval intraepithelial neoplasia
E2BS	E2 binding site
E6AP	E6 associated protein
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMA	European medicines agency
FAK	Focal adhesion kinase
FS	Fast start
GCP	Good clinical practice
GTP	Guanosine triphosphate
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HPV	Human papillomavirus
I3C	Indol-3-carbinol
IARC	International agency for research on cancer
ISSVD	International society for the study of vulvovaginal disease

Kbp	Kilobase pair
LBC	Liquid based cytology
LCR	Long control region
MHC	Major histocompatibility complex
ND	Nuclear domain
ORF	Open reading frame
PAMPs	Pathogen associated molecular patterns
PCR	Polymerase chain reaction
PDT	Photodynamic therapy
PPi	Inorganic phosphate
PV	Papillomavirus
qRT-PCR	Quantitative reverse transcription - polymerase chain reaction
RNA	Ribose nucleic acid
RRP	Recurrent respiratory papillomatosis
RT	Reverse transcriptase
RT3 VIN	Randomised trial of topical treatment of vulval intraepithelial neoplasia
TBP	TATA-binding protein
TERT	Telomerase reverse transcriptase
TLR	Toll-like receptor
TNFR1	Tumour necrosis factor receptor 1
TNFα	Tumour necrosis factor alpha
uVIN	Usual vulval intraepithelial neoplasia
VIN	Vulval intraepithelial neoplasia
WCTU	Wales cancer trials unit

Table of Contents

Chapter 1

Background

1 Background

1.1 Vulval Intraepithelial Neoplasia (VIN)

VIN is a chronic condition of vulval skin diagnosed histologically by the identification of cellular changes associated with a pre-malignant state. It has a variable clinical presentation, can be very distressing for patients and can often take a long time to diagnose. If left untreated, VIN has the potential to become malignant.

1.1.1 Classification

Evidence of intraepithelial neoplasia was probably first reported by Williams *et al*. 1886 who observed that the epithelium adjacent to cervical carcinomas was frequently abnormal. Early methods of classification of these abnormalities in the cervix developed in 1955 when Raegans *et al*. suggested the terminology atypical hyperplasia of slight, moderate and marked degree. The current classification system used for cervical intraepithelial neoplasia was first established in 1967 by Richart *et al*. who defined cervical intraepithelial neoplasia grade I, II and III. The classification was extended to vulval disease much later and presently, two methods of classification of VIN exist; both based on histologically identifiable characteristics in a tissue biopsy. The first method was established in 1986 when the International Society for the Study of Vulvovaginal Disease (ISSVD) developed the term VIN to describe the precursor lesions of vulval squamous cell carcinoma on the basis of the terminology used for cervical disease (CIN). Histological features, comparable to those seen in CIN, including epidermal thickening with hyperkeratosis/parakeratosis, loss of cell maturation, increased nuclear to cytoplasmic ratio with nuclear hyperchromasia, pleomorphism and numerous mitotic cell are required to make the diagnosis. Lesions are further classified based on the degree of epithelial involvement as, VIN 1, 2 or 3 (Ridley *et al*. 1989), (Figure 1.1).

It was thought that the natural history of disease was progressive from VIN 1 to VIN 3 and in some cases invasive cancer. Recently, use of the term VIN 1 has been discouraged based on the lack of evidence supporting the morphologic continuum of VIN 1-3 synonymous with CIN (van Seters *et al*. 2005). The histological changes previously identified as VIN 1 are now thought to represent the early reactive atypia associated with new HPV infection and are, more often than not, totally reversible making labelling as a pre-malignant state misleading (Preti *et al*. 2015; Del Pino *et al*. 2013; Reyes & Cooper 2014; McCluggage 2009).

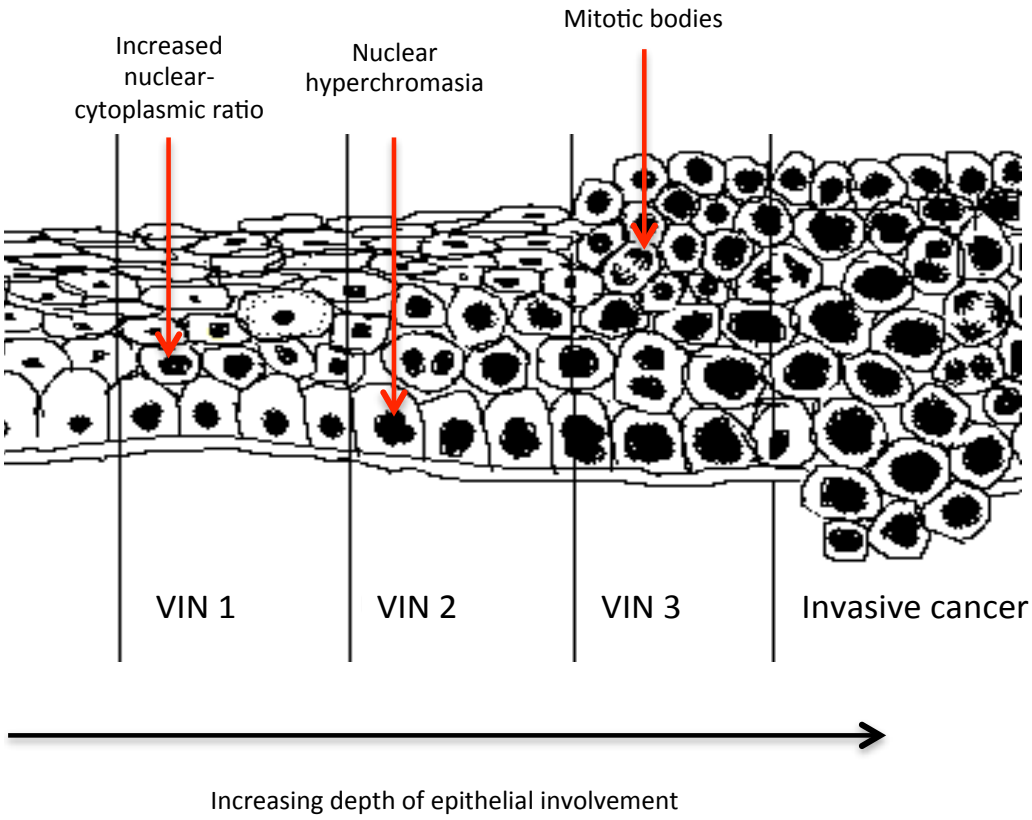

Figure 1.1 Traditional VIN classification. Figure represents the vulval epithelium with increasing grade of VIN moving from left to right. VIN 1 illustrated involving <1/3 of epithelium, VIN 2 involving up to 2/3 of epithelium and VIN 3 involving>2/3 of epithelium. Histological features labelled. Invasive cancer displays invasion through the basement membrane. Adapted from figure drawn by Dr Amanda Tristram.

The classification was modified in 2004 by the ISSVD upon the recognition of two different modes of pathogenesis leading to disease; the more common usual VIN (uVIN) being HPV dependent and the less common differentiated VIN (dVIN), which is HPV independent (Sideri et al. 2005). The subtypes are differentiated histologically. Histological features of uVIN remain the same as those used for CIN. The histological features for dVIN are subtle, and include:

- Atypical keratinocytes in the context of a fully differentiated vulval epithelium
- Thickened epidermis with parakeratotic surface reaction
- Elongated rete ridges, frequently branched
- A significant number of abnormal enlarged squamous cells with large vesicular nuclei and macronuclei.

- Abundant and brightly eosinophilic cytoplasm (Figure 1.2).

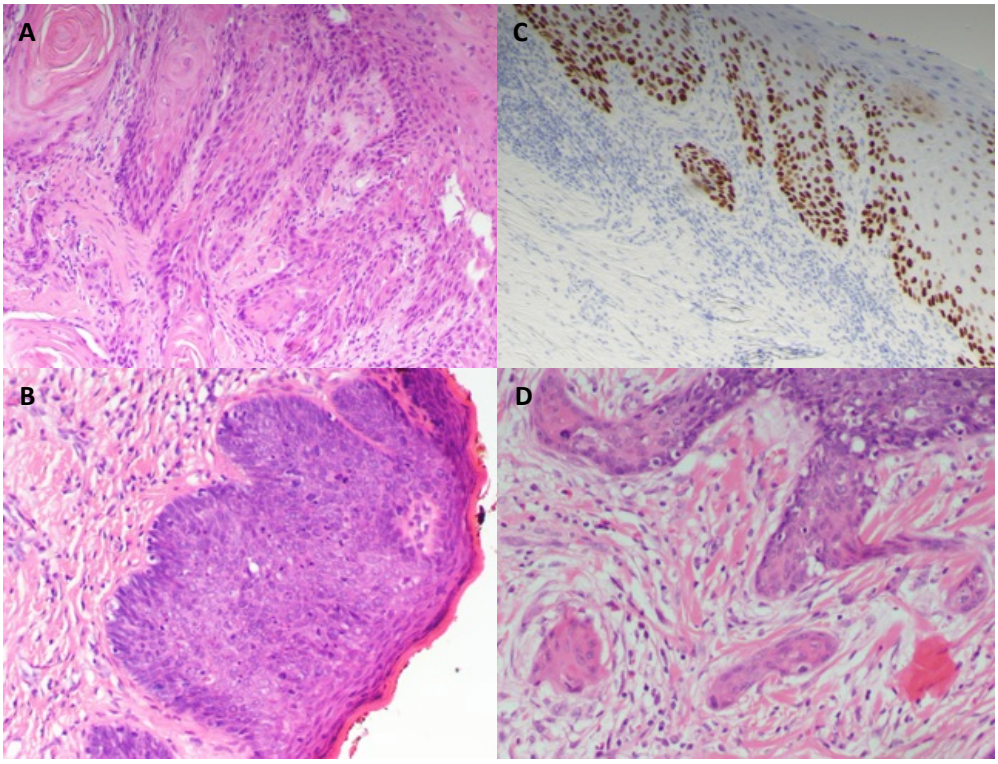

Figure 1.2 VIN histological subtypes. Images A and C are of dVIN demonstrating histological features including abnormally, enlarged squamous cell nuclei, elongated rete ridges and thickened epidermis. Image C shows diffuse p53 staining. Images B and D are of uVIN demonstrating histological features such as loss of cell maturation, increased nuclear-cytoplasmic ratio and numerous mitotic bodies. Photographs kindly provided by Dr Gareth Rowlands, Consultant Histopathologist, University Hospital of Wales, Cardiff.

The new terminology (uVIN, dVIN) has not been broadly adopted in the UK yet, with many departments preferring the traditional method analogous to CIN (VIN 1, 2 and 3). This may be a result of the histological characteristics of dVIN being subtle and less well defined than its uVIN counterpart leading to increased likelihood of intra-observer variation (Preti *et al*. 2000). It may also be a result of several studies reporting HPV positivity in cases of VIN histologically defined as dVIN (see section 1.2.4). Based on both these factors, it seems unlikely that the disease can be easily classified into these subtypes and further; the clinical utility of such a classification system is questionable.

The biopsies used for this research came from a multi-centre UK-based clinical trial (RT3 VIN), which adopted the traditional classification method in order to ensure consistency and familiarity by all participating centres. For this reason, the traditional terminology is used in this thesis. However, HPV DNA testing was performed on all biopsies as a proxy for uVIN (HPV positive) and dVIN (HPV negative).

1.1.2 Epidemiology

Vulval intraepithelial neoplasia (of either subtype) is an uncommon condition with incidence rates of approximately 2 per 100,000 with a peak at 30-49 years of age (De Vuyst *et al*. 2009; van Seters *et al*. 2005; R. W. Jones *et al*. 2005). Incidence has been rising over recent decades particularly in younger women. Usual VIN is the most common VIN type. In some countries the incidence of uVIN in particular has almost doubled over the last 10 years (Preti *et al*. 2015). Interestingly, despite this, the incidence of vulval cancer has remained relatively stable. Possible explanations for this disparity include: an increased awareness of the disease by healthcare professionals increasing the number of diagnoses; treatment of lesions prior to the development of malignancy; and, increased prevalence of HPV infection leading to more cases of uVIN which has a lower risk of malignant progression (Preti *et al*. 2015).

1.1.2.1 Usual VIN

Usual VIN is strongly associated with HPV infection and, in particular, HPV 16 infection. The largest single study of HPV prevalence in VIN to date examined 587 paraffin embedded vulval specimens histologically diagnosed with VIN and found the overall HPV prevalence to be 88.7%, of which 77.3% were HPV 16 positive. They further differentiated cases as either uVIN or dVIN and reported HPV prevalence as 90.3% in uVIN (de Sanjosé *et al*. 2013). A meta-analysis performed in 2009 reported overall HPV prevalence in VIN 2/3 as 87.7% (De Vuyst *et al*. 2009). Usual VIN presents in younger women than dVIN and this reflects the differing aetiologies (Trimble *et al*. 1996; Del Pino *et al*. 2013; McCluggage 2009). Human papillomavirus is a sexually transmitted infection and is therefore more common in younger women. Factors that increase the likelihood of contracting and maintaining HPV infection, including increased number of sexual partners, smoking and impaired immunological status, are all associated with the development of uVIN (De Vuyst *et al*. 2009; Sykes*et al*. 2002; Preti *et* al. 2005; van der Avoort *et al*. 2006; Fox &Wells 2003).

1.1.2.2 Differentiated VIN

Differentiated VIN is less common than uVIN. Differentiated VIN occurs more frequently in older women with a peak incidence seen in the 5-6thth decades of life, and often in association with chronic skin disease such as lichen sclerosus and lichen planus (Sideri *et al*. 2005; Jones 2001; van de Nieuwenhof *et al*. 2009; Jones *et al*. 2005). There are reports of HPV being found in specimens from patients diagnosed with dVIN; the significance of the HPV in these cases is uncertain and could be incidental.

1.1.3 Clinical Presentation

Vulval intraepithelial neoplasia may be symptomless and diagnosed as an incidental finding during examination of the female genital tract, but in 60% of cases, symptoms exist. Clinical symptoms are variable and include: pain, itching, irritation, dyspareunia, discharge and bleeding. The lesions can be variable in their colour, number, size, shape, and location on the vulva (Figure 1.3). Lesions can be subtle and, in combination with the symptom profile, disease can be confused with vulval candidiasis, which may result in delayed diagnosis and treatment (Rodolakis *et al*. 2003; Reyes & Cooper 2014).

Usual VIN is often multifocal and multi-centric in its presentation and the likelihood of concurrent disease at other regions of the genital tract such as the anus, vagina and cervix is high (approximately 40% of cases) (Preti *et al*. 2015). For this reason, upon diagnosis, examination of the complete lower genital tract should take place. Differentiated VIN is often discovered as a solitary lesion within a field of lichen sclerosus or adjacent to invasive disease (van de Nieuwenhof *et al*. 2011).

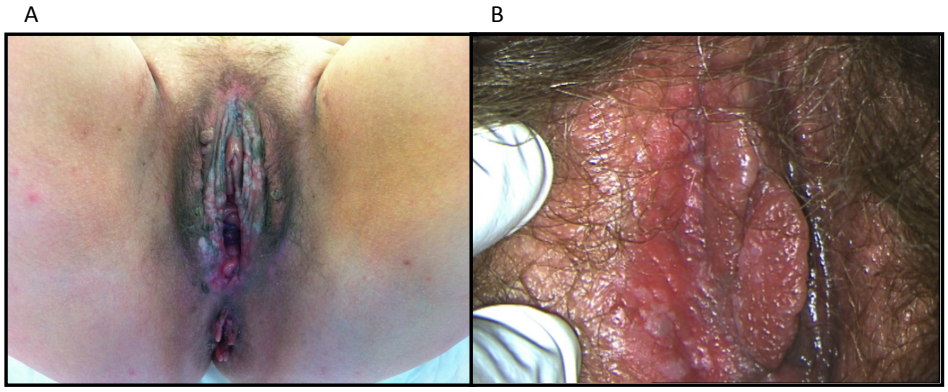

Figure 1.3 Clinical appearance of VIN. Image A - extensive VIN 3 covering all aspects of the vulval epithelium. Lesions are raised, warty, white, brown and red. Image B - Less extensive VIN 3 affecting the skin fold between the right labia. Lesions are flat and white with occasional brown patches

1.1.4 Malignant Potential

Establishing the malignant potential of VIN is challenging. Studies published vary in duration of follow up, treatment modes, treatment regimes and inclusion and exclusion criteria making comparisons difficult. Overall, the malignant potential of VIN is relatively low. Not all cases of VIN progress to cancer; spontaneous regression occurs in 1%-2% of women (Jones *et al*. 2005). Without treatment, rates of malignant progression range from 9% to 18.5% (Jones & Rowan 2000; Jones & Rowan 1995; Jones *et al*. 2005). An additional

study reported malignant progression rates of 87.5% in untreated women, however, this study cohort consisted of patients with unusually high levels of co-morbidities (Jones & Rowan 1995). This range may be explained by variation in the duration of follow up of patients between studies as well as differing proportions of dVIN and uVIN in the cohorts; dVIN has a higher risk of progression to malignancy than usual VIN (33% versus 5.7% respectively). Even with treatment, the rates of recurrence are relatively high and progression to malignancy varies from 1% to 6.5% (van Seters *et al*. 2005; Hillemanns *et al*. 2006; Jayne & Kaufman 2002).

1.1.5 Management

The aims of clinical management of VIN include: reduction in risk of malignant progression; symptom alleviation (Sykes *et al* 2002); and, confirmation of the absence of stromal invasion as occult malignancies are reported in 3.2% of cases (van Seters *et al*. 2005). Decisions regarding the management of VIN should also consider patient desires, co-morbidities, and extent, location and duration of disease. Low-grade disease, termed VIN 1 using the traditional classification method, is generally managed conservatively with regular vulvoscopy and biopsy of any suspicious area. For high-grade disease (VIN 2/3, or uVIN and dVIN in new classification) treatment is usually recommended due to the higher chance of malignant progression.

1.1.5.1 Surgery

The overall aim of surgery is to relieve symptoms and exclude any underlying malignancy, whilst at the same time, preserving vulval anatomy and function as much as possible. Surgical options are either excisional or ablative techniques. Most cases of VIN are managed with excisional surgery in order to remove the diseased tissue and allow histological evaluation to ensure there is no underlying malignancy. The degree of surgical excision required is dependent on the extent of disease and can range from local excision to partial or complete vulvectomy with reconstructive surgery. Figure1.4 shows a case of extensive recurrent VIN 3 requiring complete vulvectomy with reconstructive surgery before, during and after surgery.

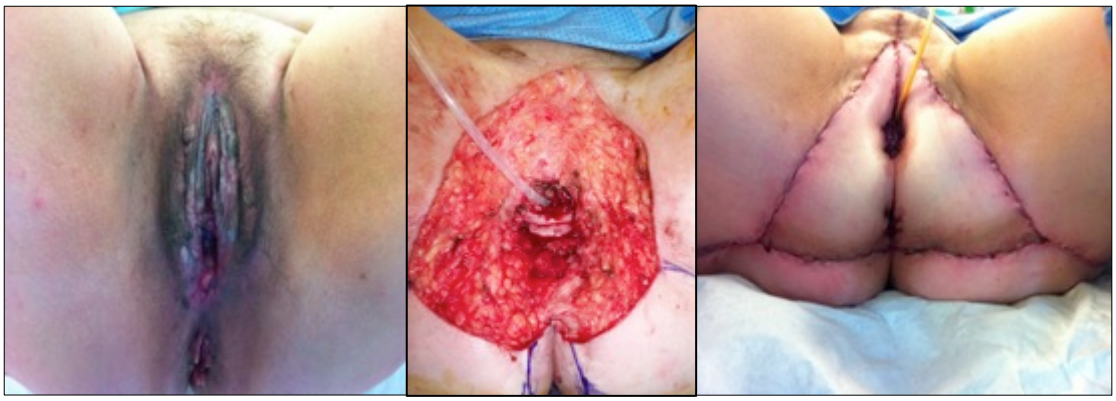

Figure 1.4 Surgical vulvectomy images. The image on the left is of a patient with extensive, recurrent VIN 3 having had multiple excisional procedures previously. The central image was taken following the vulvectomy, demonstrating the extent of surgery required. The image on the right was taken following the reconstructive surgery performed by the plastic surgeon (both aspects of procedure take place on the same day). Skin flaps were taken from the patient's buttocks. A urinary catheter is in-situ to aid functional healing of the urethra.

Due to the location of disease, even after small excisions, rates of wound infection and wound breakdown are high. Wound breakdown can be very problematic requiring further surgery, prolonged hospital stays and poorer cosmetic outcomes. These procedures can be disfiguring affecting the anatomy and occasionally the function of the vulva. With an increasingly younger population presenting with the disease, surgical excision is an increasingly unattractive option for both patients and clinicians due to the significant psychosexual and surgical morbidity that potentially follows. Studies have shown that following vulval surgery, women report a reduction in sexual function and quality of life (Corney *et al*. 1993; Andersen & Hacker 1983; Aerts *et al*. 2012; Likes *et al*. 2006; Likes *et al*. 2007). Despite the excision of disease, recurrence rates are unacceptably high, meaning repeated procedures are often required (Kaushik *et al*. 2014). For example, a systematic review performed in 2005 revealed recurrence rates of 19% following complete vulvectomy, 18% following partial vulvectomy and 22% following local excision (van Seters *et al*. 2005).

Ablative surgery such as carbon dioxide (CO2) laser ablation, cryocoagulation and loop electrosurgical excision procedure (LEEP) are alternative techniques involving destruction of diseased tissue. These techniques have become less favoured over recent years in the UK due to the lack of an adequate tissue sample to send for histological evaluation, meaning that underlying malignancy can never be excluded with certainty. Furthermore disease recurrence rates following laser vaporization have been reported as 23% and following cryocoagulation up to 56% (van Seters *et al*. 2005) conferring no therapeutic advantage in terms of reduced surgical episodes.

1.1.5.2 Medical

Efforts are being made to develop alternative, less invasive medical treatment options for VIN to reduce the impact the treatment has on the patient. There are however, currently no medical treatments approved by the European Medicines Agency (EMA) for treatment of VIN. In the 1980s chemotherapeutic interventions such as 5-Flourouracil (Sillman *et al*. 1985;), Bleomycin (Roberts *et al*. 1980) and trinitrochlorobenzene (Foster & Woodruff 1981) were investigated; these medications were associated with intolerable side effects and are no longer used (Pepas *et al*. 2014). More recently, imiquimod, cidofovir, photodynamic therapy, interferon alpha (α-IFN), phytochemical indole-3-carbinol (I3C) and therapeutic vaccines have been investigated with varying degrees of success and are discussed in more detail below.

1.1.5.2.1 Imiquimod

Imiquimod, 1-(2-methylpropyl)-1H-imidazo[4, 5-c]quinolin-4-amine, 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, and has demonstrated both anti-tumour and anti-viral activity in animal studies (Schön & Schön 2007; Stanley 2002). Imiquimod 5% is a topical treatment licensed for use in the treatment of anogenital warts and basal cell carcinoma. There are several studies into its efficacy in the treatment of VIN demonstrating success rates ranging from 20.0%-80.9% (see Table 1 4). Imiquimod is one of the two treatments that this thesis focuses on and is discussed in depth in section 1.4.

1.1.5.2.2 Cidofovir

Cidofovir is an acyclic nucleoside analogue with broad-spectrum antiviral activity. Its licenced use is in the treatment of cytomegalovirus retinitis in HIV patients but has demonstrated efficacy in the treatment of VIN 3 in pilot work. In a pilot study of cidofovir treatment in 12 patients with VIN, 10/12 women completed follow up, four of whom demonstrated complete response, three patients had a reduction in lesion size >50% and three failed to respond (Tristram & Fiander 2005). Cidofovir treatment is a focus of this thesis and is discussed in depth in section 1.3.

1.1.5.2.3 Photodynamic Therapy

Photodynamic therapy (PDT) is a two-step process. Firstly, a non-toxic, photosensitiser (e.g. 5-aminolaevulinic acid) is applied to the visibly affected tissue. Secondly, a visible light of appropriate wavelength is applied to activate the photosensitizer that leads to the production of reactive oxygen species that cause cell death. The benefit of PDT lies in its ability to treat multi-focal disease without tissue loss; there is minimal tissue destruction

and excellent cosmesis. An additional advantage of this procedure is its suitability for administration in the outpatient setting. Challenges associated with this treatment option include pain (some patients are unable to tolerate it); the absence of histology to confirm diagnosis and risk of damage to the surrounding tissue should the photosensitiser leak. Several studies have been performed to investigate the efficacy of treating VIN with PDT, and reported histological response rates varying from 0%-67% (Hillemanns *et al*. 2000; Martin-Hirsch *et al*. 1998; Zawislak *et al*. 2009; Daayana *et al*. 2011; Fehr *et al*. 2001).

1.1.5.2.4 Interferon Alpha (α-IFN)

The immunotherapy α-IFN, was investigated as a potential treatment modality for VIN in the 1980s and 1990s with no work having been published more recently. Interferons have broad antiviral activity and also possess immunoregulatory and antiproliferative properties (Cirelli & Tyring 1994). Interferon has been shown to directly inhibit viral replication and proliferation in infected cells. It has also been demonstrated to stimulate the expression of HPV associated cell surface antigens making HPV infected cells more recognizable by the immune system (Beglin *et al*. 2009). A study of 21 patients investigating the use of α-IFN, within a randomized trial setting, assigned patients to receive α-IFN with or without the addition of 1% nonoxynol-9 (thought to enhance the effect of α-IFN). No benefit of the addition of 1% nonoxynol-9 was found, a 37.5% complete response rate was reported with a further 29.5% of patients experiencing a partial response (Spirtos *et al*. 1990).

1.1.5.3 Phytochemical indole-3-carbinol (I3C)

Indole-3-carbinol is a substance found in cruciferous vegetables such as broccoli, sprouts, cabbage and cauliflower. Animal models have demonstrated I3C to have a role in the prevention of oestrogen dependent cancers (Jin *et al*. 1999). Although one small, randomised placebo-controlled trial showed an effect in the treatment of premalignant lesions of the cervix (CIN), this effect was not demonstrated in a larger randomised trial of its dimer, diindolylmethane (DIM), (Bell *et al*. 2000; Castanon et al 2012). A single study investigating the efficacy of I3C in the treatment of VIN found that despite improvement in clinical appearance and symptomatology of VIN, there was no histological evidence of response to treatment (Naik *et al*. 2006).

1.1.5.4 Prophylactic vaccines

A program of prophylactic HPV vaccination has been implemented in the UK. As this prevents infection with HPV types 16 and 18, it is projected that with good uptake, it could potentially prevent 75% of VIN that is associated with these HPV types (De Vuyst *et al*. 2009). Benefits from the vaccine are likely to start being seen approximately 20 years

from implementation. However, the longer the vaccine program is in place, the more benefit that that will be seen (including that obtained from the 'herd effect'). By 50 years post implementation, it is likely that significant benefits will be seen with present uptake rates.

1.1.6 Conservative

Conservative management involves regular outpatient follow-up and biopsy of any lesions suspicious of invasion. Patient counselling regarding the risks of invasion and signs to look out for is paramount as well as an assurance that the patient will attend follow up appointments. This approach is generally reserved for women with VIN 1and women with asymptomatic high-grade disease who wish to avoid the morbidity of treatment. VIN 1 is more likely to regress without medical intervention, however, even high-grade VIN will spontaneously regress in a minority of women (Sideri *et al*. 2005; Jones & Rowan 2000; Stephenson & Denehy 2012).

1.1.7 Recurrent VIN

One of the difficulties in managing VIN is its recurring nature. Rates of recurrence are reported up to 79% (Küppers *et al*. 1997; Herod *et al*. 1996; Rodolakis *et al*. 2003; van Seters et al. 2005) and are strongly influenced by treatment modality. The recurring nature of the disease poses problems both in terms of optimum follow up and management. Kuppers *et al*. 1997 followed 102 patients with VIN and discovered that disease grade and disease focality were risk factors for recurrent disease (higher disease grade and multifocal disease being more likely to recur). To support this, Rodalakis *et al*. 2003, found a strong correlation between multi-focality and recurrence but did not find a relationship between grade of disease and recurrence.

Regarding mode of treatment, in terms of surgical approaches a systematic review of 68 studies revealed recurrence rates of 19% following vulvectomy, 18% following partial vulvectomy, 22% following local excision, 23% following laser vaporisation and 56% following cryocoagulation (van Seters *et al*. 2005). Data on recurrence rates following medical management of the condition are limited.

In summary, current management of VIN is associated with high rates of recurrence. Alternative treatment options offering more definitive clearance of disease are urgently required.

1.2 Human Papillomavirus

The papillomaviruses (PV) are a diverse group of viruses that infect many mammals and birds. Human papillomaviruses (HPV) have circular, double-stranded DNA genomes of approximately 8 kb. Their genomes consist of six early (E) open reading frames (ORFs) (E1-E2 and E4-E7), two late (L) ORFs (L1 and L2) and a long non-coding region known as the long control region (LCR), (Figure 1 8). Functions of individual proteins are discussed in more detail below. The L1 ORF is the most conserved region within the genome and is therefore used for classification of PV. The PV family consists of 18 genera (different genera share less than 60% nucleotide sequence identity in the L1 ORF) and more than 210 different genotypes (based on >10% sequence variation in the L1 ORF) (Doorbar*et al*.2012). There are more than 150 human papillomavirus genotypes that belong to five of the 18 different PV genera: Alpha, Beta, Gamma, Nu and Mu; the majority of HPV's belong to the Alpha and Beta genera. Alpha viruses are the most researched genera as these viruses are those associated with the development of anogenital and oropharyngeal malignancies (Doorbar *et al*. 2012). Beta papillomaviruses are commonly associated with unapparent, cutaneous infections but, can be associated with skin cancer development in immunocompromised patients (Doorbar 2006). Gamma, Mu and Nu viruses are more typically associated with more benign clinical conditions such as warts and verrucae (Doorbar 2006). HPVs are further categorized as high and low risk genotypes depending on the strength of their association with cancers. The International Agency for Research on Cancer (IARC) has identified 12 high-risk HPV 'cancer-causing' genotypes: HPV 16, 18, 31, 33, 35, 39, 45,51, 52, 56, 58 and 59. HPV 68 and 73 have been identified as possible cancer-causing types. The remaining HPV types are categorised as low risk (World Health Organisation International Agency for Research on Cancer 2005).

1.2.1 HPV Infection and Life Cycle

The HPV lifecycle and the functions of the majority of the HPV gene products are discussed in this section.

1.2.1.1 The Target Tissue – The Squamous Epithelium:

The natural host tissue for the complete infection cycle of all HPVs is the squamous epithelium. The squamous epithelium is a multi-layered structure, each layer possessing a particular profile of gene expression, protein composition and keratinocyte cellular architecture that changes as the keratinocytes progress through differentiation. Most body openings are lined with stratified squamous epithelium but more internally the epithelium

becomes columnar. The junction between the two epithelia is referred to as the squamo-columnar junction.

1.2.1.2 HPV Transmission and Primary Infection

Transmission from person to person requires direct mucosal/dermal contact e.g. sexual intercourse. The risk of contracting HPV infection is increased with:

- Early-age onset of sexual activity
- Increased number of sexual partners
- Lack of use of barrier contraception
- Smoking
- Immunodeficiency
- Young age
- Use of oral contraception (Braaten & Laufer 2008; Frazer 2009)

Upon transmission, HPV needs access to immature dividing basal cells in the basal epithelial layer. This access is typically achieved through small deficiencies in the integrity of the superficial layers of the epithelium (micro-wounds) present as a result of minor trauma through sexual intercourse etc. It is hypothesized that the increased susceptibility of cells within the transformation zone to infection and subsequently, malignant transformation, is linked to the increased accessibility and proliferation of the basal cell layers at this metaplastic epithelial site (Doorbar *et al*. 2012). Interaction with heparin sulphate proteoglycans (Johnson *et al*. 2009) results in conformational changes in the viral capsid leading to internalization and subsequent transfer to the nucleus (Doorbar *et al*. 2012). The HPV life cycle is closely linked to cellular differentiation, and is discussed below.

1.2.1.3 HPV Genome Maintenance and Cell Proliferation

The HPV early promoter (P97), located at the 3' end of the long control region (LCR) regulates the transcription of the early genes. Various cellular transcription factors are involved in its regulation as well as the viral E2 gene products (Thain *et al*. 1996; Kämmer & Warthorst 2000). In the early stages of infection, P97 is activated by cellular transcription factors that lead to the expression of low levels of E1 and E2 gene products (Kadaja 2009; Steger & Corbach 1997). The E1 protein works in conjunction with E2 and binds to the viral origin of replication where it forms an active hexamer complex (Sanders & Stenlund 1998). This complex exhibits ATPase activity and helicase activity causing the unwinding and separation of the DNA double helix with exposure of base sequences necessary for DNA replication (Longworth & Laimins 2004; Lin *et al*. 2002; Conger *et al*.

1999). The E2 protein plays an important role in establishing productive infection through the segregation of episomal genomes into daughter cells during mitosis (You et al. 2004; Van Tine et al. 2004). The E2 protein is also involved in the regulation of P97; at low levels E2 positively regulates P97 whereas at high levels, E2 negatively regulates P97. Stable levels of E2 control P97 activity such that a stable environment of balanced *E6* and *E7* expression is created, promoting relatively stable genome replication in undifferentiated cells.

HPV does not encode its own DNA polymerases and therefore requires cellular polymerases, produced in mitotically active cells, for its own replication. As keratinocytes begin to differentiate, the *E6* and *E7* genes become expressed at low levels. E7 binds the pRb (retinoblastoma protein) family of proteins targeting them for degradation, this disrupts associations between pRb and E2F transcription factors which in turn, leads to the transactivation of certain cellular factors required for viral DNA replication (Dyson *et al*. 1989). E6 targets p53 for degradation, which results in the inhibition of apoptosis what would have resulted from E7 mediated cell cycle entry in differentiated epithelial layers (Doorbar 2006). In summary, low levels of E6 and E7 enable on-going cell cycling in differentiated keratinocytes thereby maintaining an environment conducive to on-going viral genome replication and production of viral progeny. In these early stages of the lifecycle, viral copy number is maintained at approximately 50 copies per cell (Middleton *et al*. 2003; Stanley *et al*. 2007)

1.2.1.4 Genome Amplification

The basal cells form the reservoir of infection, and in these cells, the viral genome is maintained in episomal form at a low copy number expressing the early genes at low levels (Stoler & Broker 1986; Frattini *et al*. 1996). As the basal cells divide and differentiate, they produce daughter cells that are detached from the basement membrane and pushed outwards towards the epithelial surface (Stanley *et al*. 2007). Upon detachment differentiation begins and in more terminally differentiated cells, activation of the HPV late promoter (E2 independent) P670 takes place. This results in increased levels of the proteins involved in viral DNA replication (E1, E2, E4 and E5) increasing the rate of viral DNA replication resulting in increased viral copy number to approximately 1000 copies per cell (Flores & Lambert 1997).

1.2.1.5 Viral Assembly and Distribution

The L1 and L2 proteins begin to accumulate following genome amplification and viral assembly is the result of a series of coordinated interactions between L1, L2 and E2

proteins (Doorbar 2006). HPV virions are then distributed and spread via epithelial desquamation (Woodman *et al*. 2007).

1.2.1.6 E4 and E5

1.2.1.6.1 E4

This is the most divergent protein in sequence and length among the different types of HPV. The exact functions of this highly abundant protein remain to be elucidated. Most research to date indicates that it is most active during the vegetative viral DNA replication phase. The functions appear to be regulated by post-transcriptional modification – oligomerisation, phosphorylation and proteolytic cleavage. The majority of evidence indicates that E4's role is primarily to facilitate viral shedding (Doorbar *et al*. 1991).

E4 has been shown to interact with and disrupt the keratin cytoskeleton as well as the cornified cell envelope (CCE), both of which compromise the structural integrity of the cell. This reduced structural integrity facilitates easy cell rupture during desquamation, leading to effective viral shedding at the end of cellular differentiation (Roberts *et al*. 1994; Roberts *et al*. 1997; Brown & Bryan 2000; Doorbar *et al*. 1991). E4 has also been shown to interact with cellular mitochondria causing a reduction in their membrane potential that leads to apoptosis (Raj *et al*. 2004). This supports the hypotheses that E4 facilitates viral shedding not only through disruption to cell structural integrity but also through the induction of apoptosis. Additional functions of E4 include its involvement with the dispersal of nuclear domain (ND) 10s. Dispersal of ND10s may be relevant to virion assembly, as they have been shown to recruit the structural proteins L1 and L2. Finally, the expression of E4 has been shown to induce G2 cell cycle arrest. This may have the advantage of keeping the cell in a metabolically active state without competing with host DNA synthesis and may therefore boost viral genome replication (Davy *et al*. 2002; Nakahara *et al*. 2002)

1.2.1.6.2 E5

The precise role of E5 in the viral life cycle is poorly understood. E5 is now considered, along with E6 and E7, to be a transforming protein. It is a small multifunctional membrane protein, predominantly localized to the endoplasmic reticulum. It interacts with vacuolar ATPase and prevents acidification of early endosomes, thereby altering the trafficking, turnover, and signal transduction or epidermal growth factor receptor (EGFR) and related receptor tyrosine kinases, hence modulating cell growth. It is therefore thought that E5 may have an important role in establishing and expanding the infected basal/parabasal cell population during the tissue repair phase after the episode during which the virus first

gained entry (Doorbar*et al*.2012). Other functions of E5 indicating its role as a transforming protein have been demonstrated in mouse work and include:

- The transformation of cultured murine fibroblasts and keratinocytes.
- The enhanced immortalization potential of E6 and E7 proteins.
- Stimulated proliferation of mouse primary cells in conjunction with E7.
- Enhanced activity of the epidermal growth factor receptor (EGFR) interfering with several signalling pathways.
- Inhibition of gap-junction intercellular communication isolating cells from homeostatic control of neighbouring cells.
- Inhibition of apoptosis (World Health Organisation International Agency for Research on Cancer 2005).

The HPV genome is illustrated in **Figure 1.5** along with a summary of gene functions.

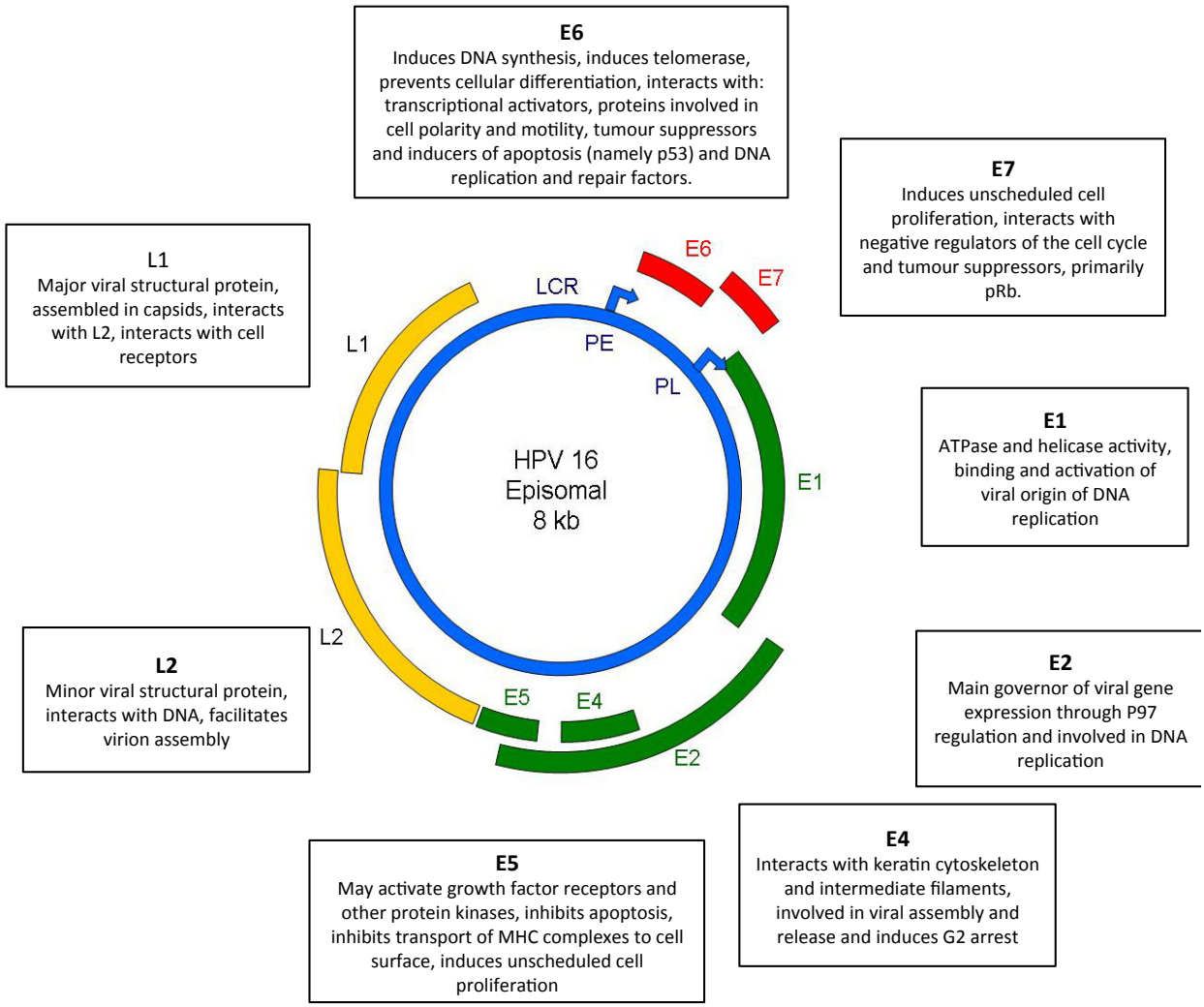

Figure 1.5 The HPV 16 genome. HPV 16 is broadly representative of all HPV genomes and is composed of circular, double-stranded DNA with six early genes (E1, E2, E4, E5, E6 and E7) and two late genes (L1 and L2). The grey box represents the long control region. Green lines represent polyadenylation sites. The main, known functions of each protein are described in the boxes. Central image of genome from Dr Ned Powell

1.2.2 Transforming HPV Infection

The malignant transformation of a host cell by a persistent HPV infection is an accidental event (Ho et al. 1995). No evolutionary benefit is gained by HPV as a result of malignant transformation, as the cell is rendered incapable of viral amplification and the late gene expression required for the production of virions (Münger & Howley 2002). Deregulated expression of the viral oncogenes, *E6* and *E7* plays a central role in HPV carcinogenesis (Stanley 2002; Kessis *et al*. 1993; Duensing & Münger 2004; Pierry *et al*. 2012; Doorbar 2006; Tsimplaki *et al*. 2012). Deregulated expression of these viral oncogenes alters the typical cell biology in several ways leading to neoplastic transformation.

1.2.2.1 Induction of Proliferation

The E6 and E7 oncoproteins induce inappropriate cellular proliferation in four main ways. These are described below and summarised in Figure 1.6.

1.2.2.1.1 Activation of E2F

The E2F family of transcription factors regulates the expression of genes involved in G1/S phase cell cycle progression. Additional roles include regulation of cellular differentiation and regulation of apoptosis (Johnson & DeGregori 2006). In a normal cell, the retinoblastoma protein (pRb) binds to E2F preventing it from binding to the E2F dependent promoters of many genes involved in cell cycle progression. Specifically, for normal cell cycle progression, at the end of G1, pRb becomes phosphorylated by cyclin-dependent kinases, freeing the E2F, enabling binding to E2F dependent promoters (Classon & Harlow 2002). In transforming HPV infections, the E7 protein binds to and disrupts the pRb-E2F complex prematurely, causing release of E2F, constitutive expression of E2F dependent genes, and inappropriate cell cycle progression.

1.2.2.1.2 Increased Cyclin-Dependent Kinase Activity

Appropriate cell cycle progression is also reliant on the interaction between cyclin-dependent kinases (CDK) and their inhibitors, particularly p21 and p27. In the presence of p21 and p27 (triggered by noxious stimuli), G1/S cell cycle progression is prevented through inhibition of CDK. In HPV infection the E7 protein is able to bind p21 and p27, neutralising their inhibitory effect leading to persistently high CDK activity and inappropriate cellular proliferation (Jones, Alani *et al*. 1997; Jones, Thompson *et al*. 1997; Funk *et al*. 1997).

1.2.2.1.3 Ubiquitination of p53

Ubiquitination of the tumour suppressor protein, p53, by the E6 oncoprotein represents the third mechanism by which HPV infection leads to inappropriate cellular proliferation. In the absence of E6, the cellular effects of E7 would lead to increased levels of p53 leading to growth inhibition and apoptosis (Demers *et al*. 1994). However, E6 is able to recruit cellular E3 ubiquitin ligase E6-associated protein (E6AP) and cause ubiquitylation and degradation of p53 excluding it from the cells normal surveillance machinery (Scheffner *et al*. 1990; Scheffner *et al*. 1993).

1.2.2.1.4 Increased Telomerase/TERT Activity

The E6 protein has been shown to activate telomerase and telomerase reverse transcriptase (TERT) through its interaction with E6AP enabling the replication of telomeres; another key step in immortalization (Howie *et al*. 2009; Wise-Draper & Wells 2008).

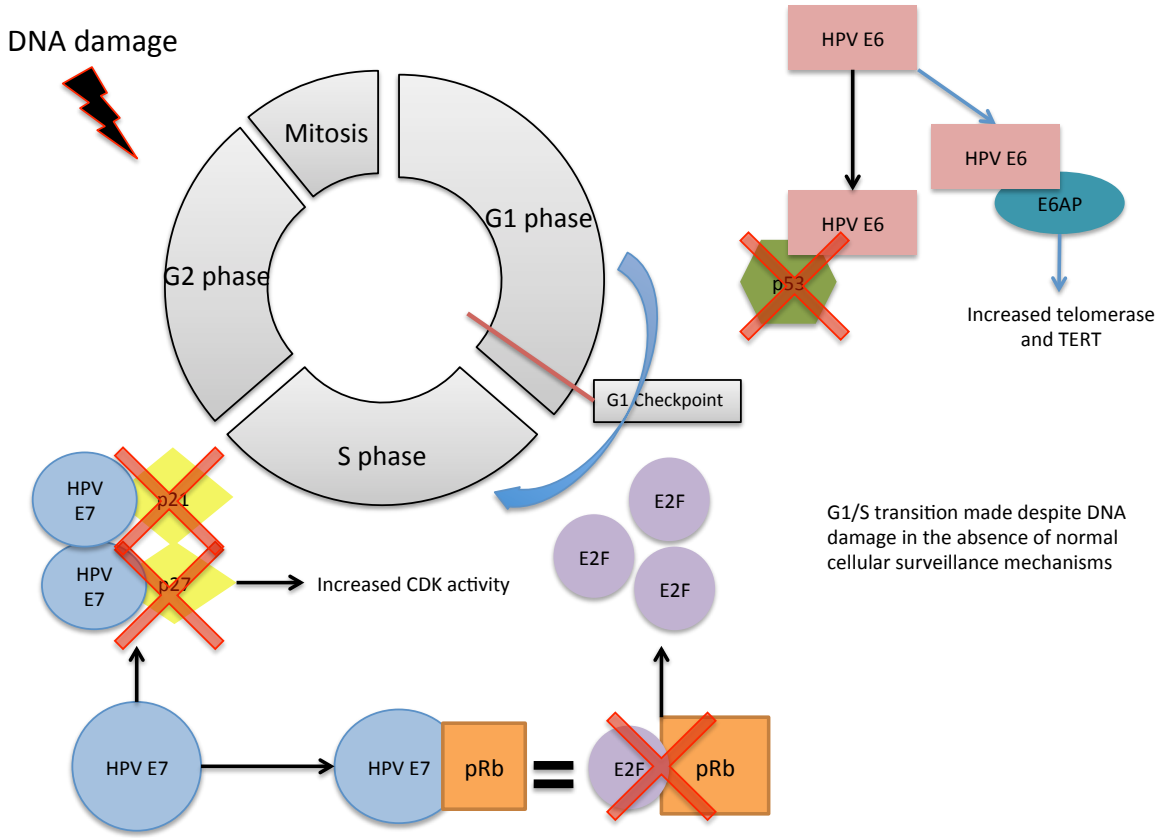

Figure 1.6 HPV induced cellular proliferation during transforming infection. The tumour suppressor protein p53 plays a critical role at the G1/S checkpoint, at which time it is activated following stress exposures, such as DNA damage, in the normal cell. P53 determines whether a cell is fit for transition from G1 to S phase. The retinoblastoma family of proteins (pRb) are also involved in G1/S phase transition through regulation of the E2F transcription factors. Binding of E2F by Rb prevents progression from G1 to S phase. In HPV infected cells, the E6 oncoprotein causes the ubiquitination of p53 rendering it inactive and unable to halt cell cycle progression in the event of cellular stress. E6 also acts to increase the expression of telomerase and telomerase reverse transcriptase (TERT) to enable cellular proliferation. The E7 oncoprotein binds to pRb and disrupts the Rb/E2F complex resulting in constitutive expression of E2F responsive genes also leading to on-going cell cycle progression despite cellular stress. It also binds the CDK inhibitors p21 and p27 increasing CDK activity thereby driving cell cycle progression.

1.2.2.2 Genomic Instability

Although E6 and E7 proteins are necessary for the development of a transforming infection, they are not sufficient alone. The E6 and E7 proteins contribute to the development of genomic instability (discussed below) however, genomic instability is also detected in HPV associated lesions prior to HPV integration, indicating that alternative causes of genomic instability also play a role (White *et al.* 1994). The concept of the presence of alternative causes of genomic instability is supported firstly by the fact that significant clinical disease (pre-cancer and cancer) only develops in approximately 10% of

newly acquired HPV infection (Schiffman *et al*. 2007). Secondly, it is supported by the >5 year lag time between initial HPV infection and appearance of first precancerous lesions (Woodman *et al*. 2001) and crude estimates from large studies of pre-cancers indicating a 20-30% risk of invasion over a 5-10 year period (Schiffman *et al*. 2007; Chang *et al*. 1990). Additional causes of genomic instability include exposure to exogenous genotoxic agents such as tobacco smoke; smoking is a well-established risk factor for the development of persistent HPV infection and cervical cancer (Collins *et al*. 2010).

HPV infection contributes to genomic instability through the effects of the E6 and E7 proteins, which work together to induce centrosome abnormalities that have the potential to lead to chromosomal missagregation and aneuploidy. The E6 and E7 proteins have been shown to cause bypass of mitotic checkpoints; this is likely to be important in viral replication but could also lead to genomic instability in infected cells. Accumulation of genomic alterations over years of infection may eventually lead to a growth advantage and ultimately malignant progression (Duensing & Münger 2002a; Duensing & Münger 2002b; Moody & Laimins 2010). Activation of certain DNA damage repair pathways such as the ATM-ATR pathways (ataxia telangiectasia-mutated-ATM and RAD3-related DNA damage repair pathway) have also been implicated in HPV transformation promoting differentiation-dependent genome amplification. Degradation of claspin, a key regulator of the ATM-CHK1 DNA damage pathway by the E7 protein, alters G2/M checkpoint recovery (Spardy *et al*. 2009) permitting cell cycle progression in the presence of damaged DNA (Moody & Laimins 2010).

1.2.2.3 Apoptosis

HPV has been shown (as detailed above) to block apoptosis through the E6 mediated degradation of p53. Other mechanisms by which E6 and E7 proteins lead to the avoidance of apoptosis have also been reported. One such mechanism is the inhibition of anoikis (Chiarugi & Giannoni 2008). Anoikis is an apoptotic pathway induced by the detachment of anchorage-dependent cells from the surrounding extracellular matrix (ECM) leading to loss of essential growth signal stimulation required for cell survival. In normal cells integrins on the cell membrane interact with the ECM and regulate growth signal transduction through focal adhesion kinase (FAK). Anoikis occurs when this communication is lost. HPV positive cells express high levels of FAK, which enable cell proliferation despite the loss of adherence to the ECM (McCormack *et al*. 1997). Another action of the E6 protein is its ability to bind tumour necrosis factor receptor-1 (TNFR1). TNFR1 is a cell membrane 'death' receptor capable of activating extrinsic apoptotic pathways when stimulated by the inflammatory cytokines tumour necrosis factor (TNFα). Upon binding, E6 blocks the transduction of apoptotic signals promoting cell survival.

Finally, the E6 and E7 interact with the normal cellular response to interferon (IFN). Interferon is expressed as part of the innate immune response to viral infection. The E6 and E7 proteins are able to repress the transcription of signal transducer and activator of transcription 1 (STAT1), which is a key transcription factor regulating cellular response to interferon (Beglin *et al*. 2009). A summary of mechanisms of HPV induced cellular transformation is show in Figure 1.7.

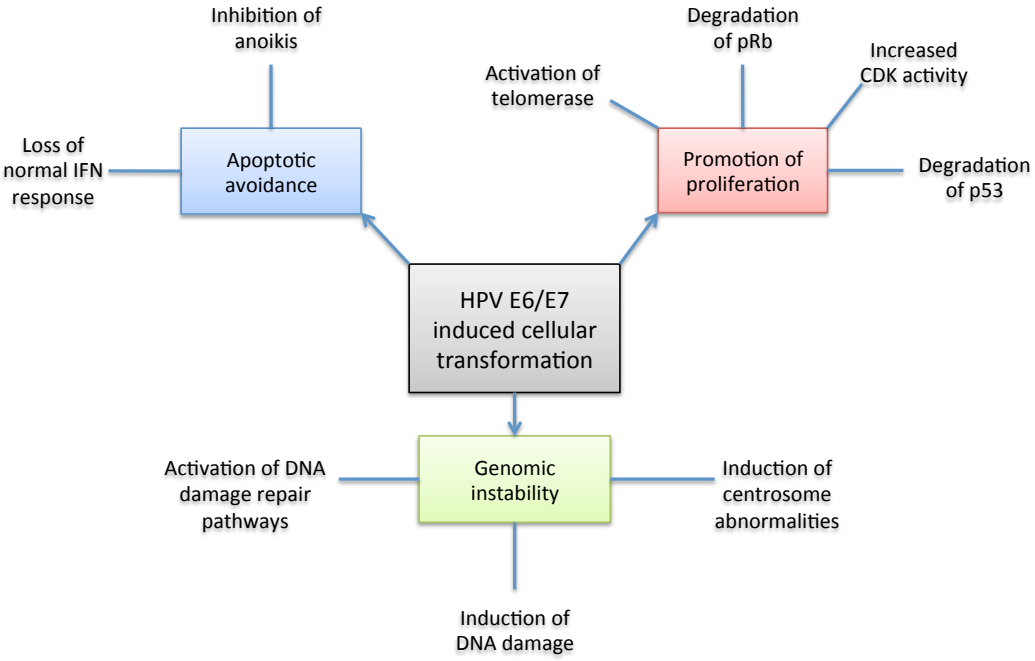

Figure 1.7 Summary of mechanisms of HPV induced cellular transformation.

1.2.2.4 Mechanisms of Deregulated *E6* and *E7* Expression

Integration of HPV DNA into the host genome leading to disruption of the E2 ORF is broadly acknowledged to lead to deregulated expression of the E6 and E7 ORF's (Stanley 2002; Doorbar 2006; Pett & Coleman 2007;). However, there are a proportion of cases of advanced CIN (CIN II/III) and cervical carcinoma in which HPV does not exist in an integrated form, suggesting that other factors controlling E6/E7 expression must exist (Doorbar 2006; Deng *et al*. 2012; Häfner *et al*. 2008; Gray *et al*. 2010). HPV DNA methylation is one such epigenetic mechanism that has been shown to correlate with a high risk of cervical cancer development (Mirabello, Sun*et al*. 2012; Ding *et al*. 2009; Badal *et al*. 2003; Ghosh *et al*. 2012). The biology of HPV integration and methylation specifically in VIN is discussed in more detail below.

1.2.2.4.1 HPV Integration

Following infection, HPV viral genomes are typically maintained as intact episomes independent of the human genome. In a small proportion of cases, the HPV genome integrates into the host cell genome and this is thought to represent a key event in HPV associated carcinogenesis (Pett & Coleman 2007; Hillemanns & X. Wang 2006; Ho et al. 2011). Cells containing integrated HPV have a selective growth advantage over cells containing episomal HPV in vivo (Jeon & Lambert 1995). Integration of HPV has been found to frequently disrupt the *E1*/*E2* region of the virus, altering expression of these genes, leading to deregulated expression of the *E6* and *E7* oncogenes (Romanczuk & Howley 1992). No specific 'hot-spots' for HPV integration have been identified in human DNA however; regions of known host genomic instability known as common fragile site (CFS) have been shown to be commonly involved (Ziegert *et al*. 2003; Smith *et al*. 1992). HPV 16 integration has been found to be present in >50% of cervical cancer cases and in nearly all HPV 18 associated cases (Choo *et al*. 1987). The presence of HPV integration has also been correlated with the risk of CIN progression (Arias-Pulido *et al*. 2006). Incidence of HPV integration in vulval intraepithelial neoplasia and vulval cancer is less well characterized.

1.2.2.4.2 HPV Integration in VIN

HPV integration was studied in 30 cases of VIN using a PCR based protocol for the amplification of papillomavirus oncogene transcripts (APOT). Viral integration was detected in 8/21 (38.1%) ofHPV 16/18 positive cases (Hillemanns & Wang 2006). Van de Nieuwenhof *et al*. investigated the aetiological role of HPV in 130 cases of vulval cancer. Having defined cases histologically as uVIN ordVIN in advance, in situ hybridization was carried out to determine the physical state of any HPV infection present. 25/130 (19.2%) cases were found to be associated with uVIN, of which all were high-risk HPV positive and 24/25 (96%) contained HPV in an integrated from. 105/130 (80.8%) were found to be dVIN associated, of which 11 (10.5%) were found to be high-risk HPV positive, none of which were found to contain HPV in an integrated form. They concluded that these findings indicated that although high-risk HPV may be present in cases of squamous cell vulval cancer, its role is unlikely to be aetiological (van de Nieuwenhof, van Kempen*et al*. 2009). Another study conducted by Bryant *et al*. found HPV integration events present in 8/25 cases of HPV 16 positive intraepithelial neoplasia (22 cases of VIN 3, one case of VaIN 3, one case of AIN 2 and one normal vulval biopsy). The *E1* ORF was most frequently disrupted (3/8 (37.5%) cases) followed by the *E2* and *L2* ORF's (each in 2/8 (25.0% cases) and *E4* involvement in 1/8 (12.5%) cases (Bryant, Onions, *et al*. 2014).

1.2.2.4.3 HPV DNA Methylation

DNA methylation is a common form of epigenetic modification (Feinberg 2007; Yamada & Watanabe 2010; Gonzalo *et al*. 2010; Ikegami *et al*. 2009). DNA methylation frequently occurs at CpG dinucleotides where a methyl group is covalently bound to the C-5 position of cytosine (Figure 1.8). Methylation represents an important mechanism regulating gene expression. Methylation regulates gene expression by preventing transcription factors from recognising their binding sites or by attracting proteins that bind methylated DNA and recruit chromatin modifying enzymes to condense adjacent chromatin (Bird & Wolffe 1999; Thain *et al*. 1996; Jones & Takai 2001; Kuroda *et al*. 2009).

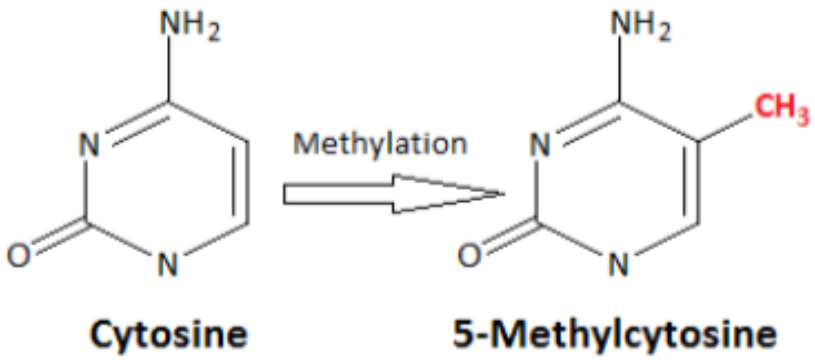

Figure 1-8 Schematic drawing of cytosine methylation. Figure courtesy of Dr Dean Bryant.

Both viral and host genes can be targeted by the cellular methylation machinery. In vitro studies have shown that methylation of HPV genomes leads to silencing of transcription. This de novo methylation of HPV DNA could be a host defence mechanism to suppress transcription of foreign DNA, or a viral strategy to maintain a long-term infection (Doorbar*et al*. 2012; Badal *et al*. 2003). Evidence supporting the latter comes from the ability of the HPV E7 protein to directly activate DNA methyltransferase (DNMT) a crucial enzyme involved in methylation (Doorbar*et al*.2012; Burgers *et al*. 2007).

A series of publications investigating the role of HPV DNA methylation in HPV associated disease have discovered that methylation of HPV genomes is consistently higher in carcinomas compared to pre-cancerous lesions (Badal *et al*. 2003; Ding *et al*. 2009; Kalantari *et al*. 2004). There is also a growing body of evidence showing that increased HPV genome methylation is found in association with viral integration (Fernandez *et al*. 2008; Kalantari *et al*. 2008; Kalantari *et al*. 2010; Bryant, Onions, *et al*. 2014). This could

indicate the susceptibility of HPV integrants to methylation induced silencing upon recognition by the host.

A small number of studies have specifically investigated methylation of the E2 binding site (E2BS), located within the HPV upstream regulatory region. Methylation of the E2BS has been shown to inhibit E2 protein binding (Thain *et al*. 1996). This could inhibit the transcriptional control E2 has on *E6* and *E7* gene expression (Kim *et al*. 2003) however, no studies have yet demonstrated a direct correlation between methylation state and *E6/7* transcription. A study conducted by Bryant *et al*. aimed to determine if E2BS HPV DNA methylation could act as an alternative transforming event in the absence of HPV integration (Bryant, Onions *et al*. 2014). Methylation levels of the E2BS were compared between integrated infections and episomal infection in same disease grade; no difference was found.

In summary HPV DNA methylation is correlated with viral integration and increasing disease grade but has not yet been shown to correlate with increased expression of *E6* or *E7* genes. This thesis describes investigation of methylation levels of the E2BS, the *E2* ORF and the *L1* and *L2* ORF in the largest studied cohort of VIN 3 to date. Relationships with viral integration and viral gene expression were explored to investigate the hypothesis that methylation provides an alternate means of deregulating *E6/E7* expression in the absence of HPV integration. Finally, the suitability of HPV DNA methylation as a potential biomarker predictive of response to treatment of VIN 3 with cidofovir and imiquimod therapy was assessed.

In this thesis, the term 'transforming HPV infection' is used to denote an infection where the HPV infection displays one of the characteristics described above (integrated DNA, highly methylated DNA or deregulated oncogene expression).

1.2.3 HPV and Disease Burden

HPV is the most common sexually transmitted infection worldwide (Centers for Disease Control 2013). The majority of sexually active individuals will acquire an HPV infection at some point; however, only in the minority of individuals will significant disease ensue (Forman *et a*l. 2012). Benign diseases resulting from HPV infection are genital warts (the commonest sexually transmitted disease in the UK), verrucae and a rare condition known as recurrent respiratory papillomatosis (RRP) where benign growths develop in the respiratory tract often requiring repeated surgery. Genital warts (predominantly caused by HPV types 6 and 11) pose a significant disease burden worldwide. In the UK in 2012 there were 220, 875 cases of genital warts, which cost £58.4 million to treat (Coles *et al*.

2015). HPV also causes pre-malignant change of the cervix, vulva, vagina and penis known as intraepithelial neoplasia, as well as malignancies of the cervix, vulva, vagina, anus, penis and oropharynx. HPV infection accounts for approximately 2% of the cancer burden in developed countries and for approximately 7% in less developed countries. In Europe in 2008, there were estimated 3.2million new cases of cancer in total, of which, 110,000 cases occurred in HPV affected sites, of which, 80,000 were estimated to be directly attributable to HPV infection. This equates to a 2.5% contribution by HPV to the total cancer burden in Europe. Breakdown per affected site is illustrated in Figure 1.9 (Forman *et al*. 2012).

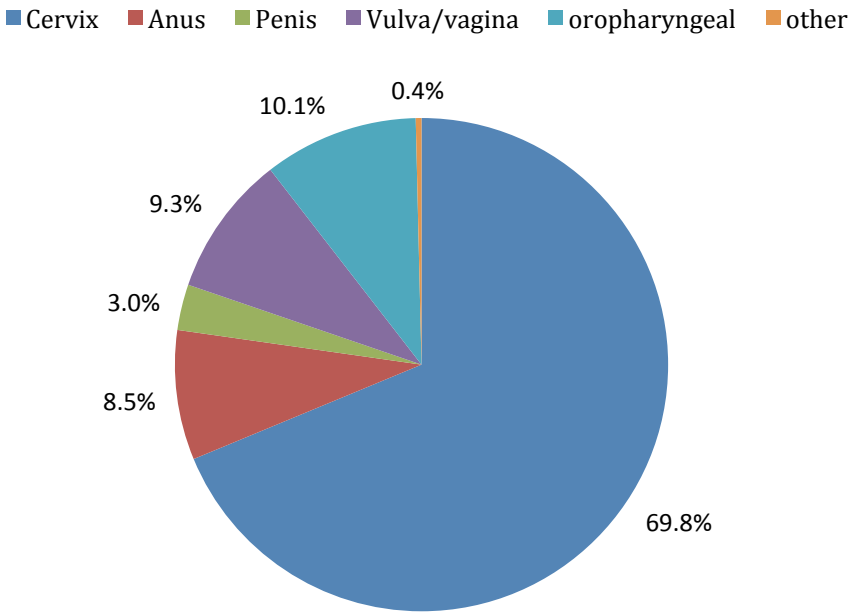

Figure 1-9 HPV associate cancer burden Europe 2008. Of all cancer cases directly attributed to HPV infection in Europe in 2008 (80,000), 55.000 cases were cervical cancer, 8,100 were oropharyngeal cancer, 7,400 were vulva/vagina cancer, 6,800 cases were anal cancer and 2,400 were penile cancer (Foreman *et al*. 2012).

1.2.4 HPV Prevalence in VIN

Establishing accurate HPV prevalence rates in VIN is challenging but important. Knowledge of HPV prevalence in VIN contributes to vaccination program planning on a global scale, but is likely to become increasingly important at the level of the individual. Two aetiologies for VIN are now established, and are defined as two different histological subtypes: uVIN being directly attributed to HPV infection and dVIN being HPV independent (section 1.1). HPV prevalence rates will be directly related to the nature of the VIN being studied. The importance of defining study cohorts based on the new

histological classification is arguable and likely guided by the nature of the study and its specific aims. However, the two disease aetiologies follow different courses in terms of malignant progression, and will be affected differently by HPV vaccination. They are also likely to respond differently to therapies. It can be presumed therefore that in most contexts, knowledge of the histological subtype being studied will be informative.

A review of the literature discovered 32 studies reporting HPV prevalence in high-grade VIN (Table 1.1). The mean HPV prevalence across all studies was 85.1% with a range of 30.8% - 100%. A meta-analysis conducted by De Vuyst *et al*. reported a similar overall HPV prevalence of 85.3% in 1061 cases of high-grade VIN (De Vuyst *et al*. 2009). Although the overall HPV prevalence reported by studies is generally high, the range is broad (30.8% - 100%). A study conducted by Van Seters *et al*. reported a particularly low HPV prevalence (30.8%); however, this may be explained by selection of cases with diagnoses of both VIN and lichen sclerosus, which conferred a selection bias towards HPV negative cases. It is interesting that all the VIN in the van Seters study was found to be undifferentiated subtype (n=26). Lichen sclerosus is traditionally thought to be associated with dVIN but van Seters *et al*. discovered that this relationship is supported, in the majority, by research involving cases of coexistent vulval SCC (van Seters *et al*. 2007). Reports of lichen sclerosus and dVIN in the absence of SCC at the time of the Van Seters study were limited to five cases. Van Seters et al. hypothesised that their study indicated the possibility of a different aetiology or classification for VIN with perhaps a different prognosis. This highlights the first challenge that arises when establishing HPV prevalence in VIN, which comes from the two aetiologies. Very few studies reporting HPV prevalence rates define the VIN sub-type. Many studies adhere to the traditional VIN classification, reporting VIN as high-grade or VIN 2/3, which amalgamates the uVIN and dVIN subtypes. HPV prevalence rates reported by studies including cases of VIN of the dVIN sub-type will likely be lower than studies reporting on uVIN alone. It is important to account for this when considering HPV prevalence rates in VIN.

It is of worthy note that although it is not thought that HPV directly contributes to dVIN pathology, several studies do report presence of HPV in dVIN. For example, the de Sanjose study (de Sanjosé *et al*. 2013) reported HPV prevalence rates of 48.9% in 48 cases of dVIN, and reported HPV prevalence rates of 90.3% in uVIN. It is thought that in these cases, HPV is an incidental finding. This is based on the lack of the viral physical features associated with disease e.g. viral integration, methylation and altered gene expression profiles. More research investigating the exact role of HPV in dVIN would be worthwhile.

Samples used in this study were all classified as VIN 3 based on the traditional histological classification. This decision was made to ensure that all centres participating in the study were familiar with the classification system and also to reduce the effect of inter-observer variability (Preti *et al*. 2000).

Table 1-1 HPV prevalence in VIN

Ref	Year	n=	Assay	uVIN or dVIN	HPV Prev. (%)	HPV 16 prev. (%)**
Hording *et al*. 1991	1991	19	HPV 16 type-specific PCR	N/S	78.9	78.9
Nuovo *et al*. 1991	1991	22	MY09/11	N/S	59.1	40.9
Park *et al*. 1991	1991	30	9/11 and type-specific HPV 6/11/16/18	N/S	53.3	53.3
Torre *et al*. 1992	1992	7	HPV 16 type-specific PCR	N/S	100	100
Pilotti *et al*. 1995	1995	5	HPV 16 type-specific PCR	N/S	100	100
Junge *et al*. 1995	1995	58	HPV 6/11/16/18/31/33 type-specific PCR	N/S	88	77.6
Van Beurden *et al*. 1995	1995	46	CpI/IIG	N/S	95.7	89.1
Trimble *et al*. 1996	1996	54	Digene	N/S	89	N/S
Nagano *et al*. 1996	1996	6	L1 PCR	N/S	100	66.7
Madeleine *et al*. 1997	1997	253	MY09/11 and TS HPV 6/11/16/18/45/31	N/S	71.5	61.7
Pinto *et al*. 1999	1999	16	PCR L1 Consensus primers	uVIN	67	N/S
Carter *et al*. 2001	2001	18	PGMY9/11	N/S	91	74.6
Abdel-Hady *et al*. 2001	2001	19	GP5+/6+ and TS HPV 6/11/16/18/31/33	N/S	73.7	64.3
Logani *et al*. 2003	2003	6	SPF10	uVIN	100	66.6
Riethdorf *et al*. 2004	2004	39	GP5+/6+ and HPV 16 E6/E7 ISH	N/S	100	100
Bryndorf *et al*. 2004	2004	11	SPF10	N/S	81.8	63.6
Todd *et al*. 2004	2004	10	GP5+/6+	N/S	90.0	50.0
Bonvicini *et al*. 2005	2005	25	MY09/11	uVIN (n=18)dVIN (n=7)	61.1 0	50 0
Rufforny *et al*. 2005	2005	21	HPV 16 type-specific	N/S	100	100
Van der Avoort *et al*. 2006	2006	37	SPF10	uVIN	66	65

Srodon *et al*. 2006	2006	34	PGMY09/11 and SPF10	N/S	100	91
Hample *et al*. 2007	2007	49	GP5+/6+ and MY09/11	N/S	91.8	67.3
Skapa *et al*. 2007	2007	33	GP5+/6+	uVIN	100	76.1
Van Seters *et al*. 2007	2007	26	GP5+/6+	uVIN	30.8	26.9
(van Seters, Beckman*et al*. 2008)	2008	52	GP5+/6+	N/S	96.2	78.8
Van de Nieuwenhof, van Kempen *et al*. 2009	2009	13	PCR L1	uVIN	100	44
Garland *et al*. 2009	2009	62	PCR	uVIN	84	42
Smith *et al*. 2009	2009	65	PGMY9/11	N/S	98	50
Gargano *et al*. 2012	2011	66	PGMY9/11	N/S	94	48
Tachezy *et al*. 2011	2011	46	GP5+/6+	uVIN	100	71.7
Tsimplaki *et al*. 2012	2012	28	PapilloCheck	uVIN	71	65
De Sanjose *et al*. 2013	2013	583	SPF10	uVIN n=535dVIN n=48	90.3 48.9	Overall 77.3
Bryant, Onions *et al*. 2014	2014	10	GP5+/6+	N/S	80.0	N/S

ers et al

2007 only studied cases of VIN associated with lichen sclerosus. **HPV 16 prevalence is reported as percentage prevalence of all cases, not just HPV positive. N/S = Not specified.

It is somewhat surprising that even in studies that do define the cohort based on the new histological subtype, HPV prevalence in uVIN is not always 100%. Studies reporting HPV prevalence in uVIN specifically, quote rates varying from 67% - 100%. This may reflect the difficulties of histological diagnosis of VIN. Distinguishing VIN from atypical inflammatory changes in normal skin can lead to over-diagnosis and the diagnosis of dVIN can be easily missed because of its highly differentiated features and absence of widespread architectural disarray (Reyes & Cooper 2014). These challenges inevitably play some role in the lower than expected HPV prevalence figures. However, limitations of the biological methods used to detect HPV are likely to be more important, and are discussed in the following section.

1.2.4.1 HPV Detection Methods

Because of the link between the viral lifecycle and epithelial differentiation, HPV is very difficult to propagate in culture so traditional microbiological techniques are of limited use (Doorbar 2006). Current methods of HPV detection predominantly use molecular biology techniques that rely upon the detection of nucleic acids through nucleic acid hybridization followed by signal amplification, or nucleic acid amplification (Abreu *et al*. 2012). The majority of assays target the well-conserved L1 ORF of the virus, some target the E1 and others target E6/E7 mRNA (Table 1.2).

Nucleic acids are prone to degradation due to exposure to DNases/RNAses in the environment and freeze thawing. The quality of material being investigated in terms of DNA/RNA integrity, concentration and purity will vary from study to study and inevitably affects the ability of any molecular biology technique to detect the presence/absence of HPV accurately. It is rarely possible to obtain optimum fresh tissue samples from the clinical setting for laboratory analysis but efforts should be made to ensure that the best quality DNA/RNA is obtained from what is available. When assessing reports of HPV prevalence rates from the literature, consideration should be given to the quality of tissue/sample.

An additional challenge posed by detecting HPV nucleic acids is the reliance on the targeted DNA or RNA not being subject to recombination. Integration of HPV DNA into the host genome is a significant step in HPV pathogenesis. Studies have demonstrated the incidence of HPV integration is 11% - 60% in cases of VIN/CIN (van de Nieuwenhof, van Kempen *et al*. 2009, Hillemanns, Wang 2006; Häfner *et al*. 2008; Bryant, Onions *et al*. 2014). Although these integration events can involve any part of the HPV DNA, the *E1*, *E2*, *L1* and *L2* ORF's are most frequently involved (Hillemanns, Wang 2006; Häfner *et al*. 2008; Choo *et al*. 1987; Wang *et al*. 2013; Corden *et al*. 1999). The *L1* ORF is the most frequently used target region (Table 1.3), probably because sequence variation in this region is used to define HPV genotypes. Further to this, HPV integration is more common in higher-grade disease (e.g. CIN 3 and VIN 3) making disruption to the DNA more likely in more advanced disease. One of the hypotheses investigated in this thesis is that HPV assays may falsely identify a sample as HPV negative if integration has disrupted the targeted viral region, and that this is more likely to happen in cases of more advanced disease.

Table 1-2 HPV detection methods.

Assay	Manufacturer	HPV target region	Target size	Target type (DNA/RNA)
GP5+ GP6+ PCR EIA	N/A	L1	150 bp	DNA
MY09/11 and PGMYop/11	N/A	L1	450 bp	DNA
PapilloCheck®	Greiner Bio One	E1	350 bp	DNA
ProDect® HPV Chip	Bcs Biotech	L1 and E6/E7	N/S	DNA
CLART® HPV 2	Genomica	L1	450 bp	DNA
Cobas® 4800 system	Roche	L1	200 bp	DNA
Digene HPV genotyping PS test	Qiagen	L1 (GP5+ GP6+ above)	150 bp	DNA
Cobas® Amplicor	Roche	L1	165 bp	DNA
Aptima®	Gen-Probe	E6/E7	N/S	mRNA
AID HPV Screening kit	GenID GMBH	E1	N/S	DNA
Pretect HPV-Proofer	Norchip	E6/E7	N/S	mRNA
NucliSENS EasyQ® HPV	BioMerieux	E6/E7	N/S	mRNA
HPV OncoTect™	IncellDx	E6/E7		mRNA
INNO Lipa	Innogenetics	L1	65 bp	DNA
SPF10-LiPA25	Labo Biomedical Products	L1	65 bp	DNA
HPV DNA Chip	BioMedLab	L1 (GP5+ GP6+ above)	150bp	DNA
Linear Array® HPV genotyping kit	Roche	L1	450 bp	DNA
Full Spectrum HPV Assay	GenoID	L1	N/S	DNA
Digene HC2 HPV DNA test	Qiagen	N/S	N/S	DNA
RealTi*m*e High Risk HPV	Abbott Molecular	L1	N/S	DNA
f-HPV typing™	GENOMED	E6/E7	N?S	mRNA
Cervista® HPV	Hologic	N/S	N/S	DNA

N/A = not applicable, N/S = not specified. Target size describes the length of region of DNA or RNA targeted by the primers in the assay.

1.3 Cidofovir:

(S)-1-[3-hydroxy-2-(phosphonomethoxy)-propyl]cytosine dihydrate, HPMPC or cidofovir (CDV) (Figure 1.10) is a cytosine analogue that inhibits viral replication in a broad spectrum of DNA viruses including herpesviruses, poxviruses and papillomaviruses (Tempesta *et al*. 2008; Andrei, Snoeck 2010; Snoeck *et al*. 2001; Tristram, Fiander 2005). Its only licensed use however, is for treating cytomegalovirus (CMV) retinitis in human immunodeficiency virus (HIV) positive patients (Plosker, Noble 1999). At the time of writing, cidofovir was no longer available in the UK, having been withdrawn from the UK in October 2014 due to poor cost efficacy. It was however, previously supplied by Gilead Sciences Ltd, Cambridge at a price of £653.22 for a vial of 75mg/ml.

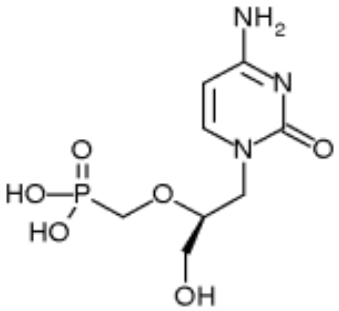

Figure 1.10 Chemical structure of cidofovir

1.3.1 Metabolism and Mechanism of Action

Cidofovir enters cells through fluid-phase endocytosis. Once inside the cell, cidofovir undergoes two phosphorylation steps, mediated by host enzymes, to reach its active diphosphate derivative, CDVpp. Firstly, cidofovir is phosphorylated to CDVp by nucleoside monophosphate kinase (NMK). A second phosphorylation then occurs, primarily by pyruvate kinase (PK) and to a lesser degree, nucleoside diphosphate kinase (NDK) (Johnson & Gangemi 1999) resulting in CDVpp. In disease associated with viruses that encode their own DNA polymerase, during viral DNA replication, CDVpp acts as a competitive/alternative substrate to the normal substrate (dCTP) leading to its incorporation into the growing DNA strand and ultimately to chain termination (De Clercq 1996), (Figure 1.11). The selective antiviral activity of cidofovir is thought to result from its higher affinity for viral DNA polymerase than cellular DNA polymerase (De Clercq

2007). HPV does not encode its own DNA polymerase therefore the mechanism of action in this context must differ; this is discussed in the following sections.

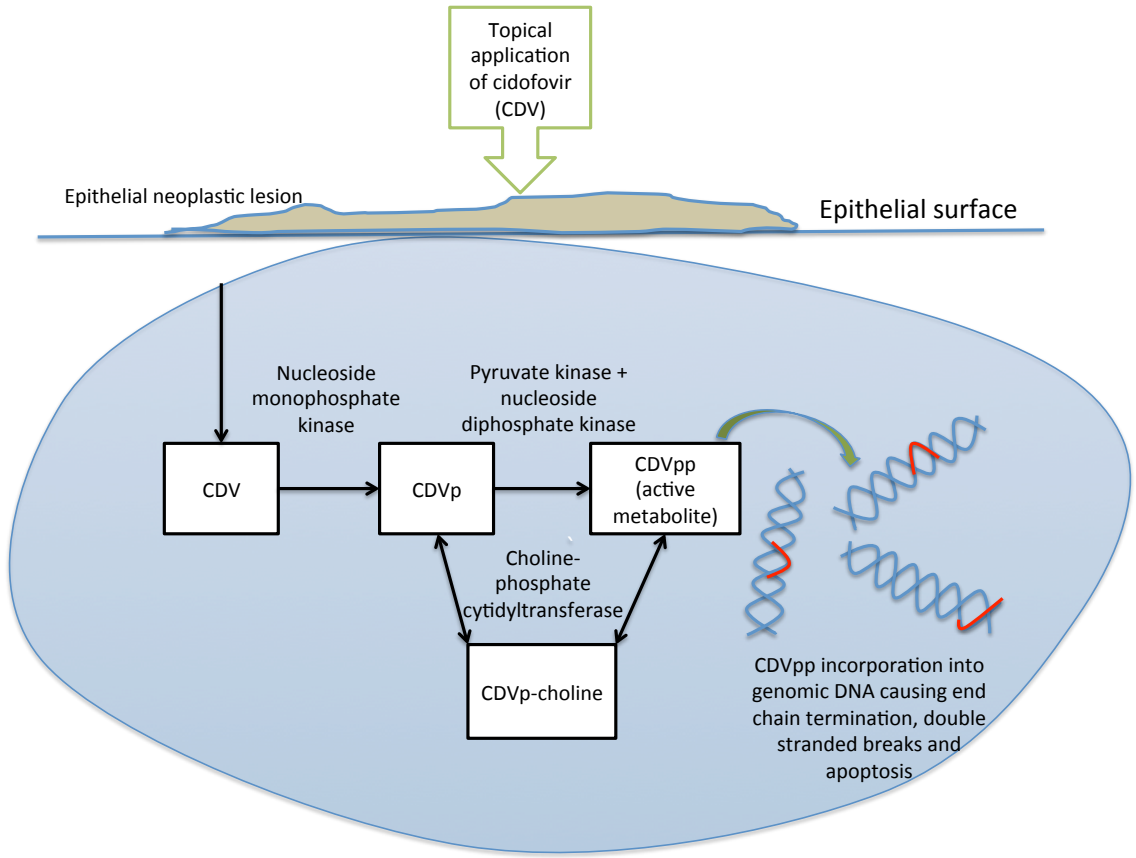

Figure 1-11 Cellular metabolism of cidofovir. CDV=cidofovir for the purposes of this figure. Upon cell entry, the host enzyme nucleoside monophosphate kinase phosphorylates CDV to its monophosphate derivative CDVp. CDVp is then further phosphorylated to its biologically active form CDVpp by pyruvate kinase and to a lesser degree, nucleoside diphosphate kinase. Choline phosphate cytidyltransferase is an enzyme responsible for regulating phosphatidylcholine content in cell membranes but is also responsible for the conversion of CDVp and CDVpp to CDVp-choline, which can act as a reversible reservoir of the drug or be excreted.

1.3.2 Cidofovir and VIN

1.3.2.1 Efficacy

There are few clinical trials investigating the role of cidofovir in the treatment of VIN. A prospective study of 12 women with histologically confirmed VIN 3 conducted by Tristram, Fiander *et al*. assessed use of cidofovir 1% topical treatment, applied by the patient on alternate days for 16 weeks. 10 women completed follow up, of whom, four

(40.0%) patients had complete resolution of all symptoms and of visible lesion(s), confirmed by histological examination and viral clearance. Three (30.0%) women had a partial response to treatment (reduction in lesions size by at least 50%). Of the three remaining women, one showed a complete symptomatic response but the biopsy of the remaining lesion demonstrated invasive disease; she underwent radical excision of the remaining disease. The final two patients did not respond. Potential reasons for non-response were: involvement of hair follicles, non-compliance due to pain, and previously undiagnosed invasive disease. Side effects of treatment were limited to pain, which usually lasted for a few days after application and was occasionally associated with ulceration of the diseased area but not the surrounding tissue (Tristram, Fiander 2005). A phase IIa prospective study (Stier *et al*. 2013) investigated the efficacy of treatment of high-grade VIN and AIN in HIV positive patients. Of the 33 patients enrolled in the trial, 26 (79%) completed the treatment protocol. Of these, five (15.0%) had a complete response, 12 (36.0%) had a partial response, seven (26%) had stable disease and two patients (6%) had progressive disease. It is possible that the deficient host immunity caused by HIV in these patients, impacted on the efficacy of cidofovir treatment.

In a study of 15 women with CIN treated with 3 g of 1% cidofovir gel (applied by a gynaecologist) every other day for six days, 7/15 (46.7%) patients completely responded to treatment and a further 5/15 (33.3%) patients had a partial response to treatment (Snoeck *et al*. 2000).

A randomised, controlled study (Snoeck *et al*. 2001) evaluated cidofovir (1% gel applied 5 days a week, every other week for a maximum of 6 cycles) as a topical treatment for genital warts. Of 30 patients, 19 received cidofovir and 11 received placebo. The most frequently reported adverse events included pain, pruritus and a rash at the site of application. A complete response was seen in 9/19 (47%) patients treated with cidofovir; no patients had a complete response in the placebo group. There were 7/19 (36.8%) patients in the cidofovir group who had a partial response compared to 2/11 (18%) of the placebo group. Of all the patients who had a complete response 1/9 (11.1%) had a recurrence of disease during the follow up period (168 days).

1.3.2.2 Treatment Regime

Cidofovir is licensed for intravenous use in the treatment of CMV retinitis in HIV positive patients. No license exists for its use in topical form. Due to the limited research, the treatment regime is not yet optimized. Cidofovir has been formulated in a variety of ointments and gels and applied using regimes varying in both frequency and duration

(Table 1 3). Should cidofovir show utility in the management of VIN, the optimum-dosing regime will need to be determined.

Table 1-3 Summary of treatment regimes reported in studies using topical cidofovir for HPV related anogenital disease.

Study	Formulation	Strength	Dosing
Snoeck et al. 2001	Gel (20% propylene glycol, 2% hydroxyethylcellulose, 0.18% methylparaben, 0.02% propylparaben, 0.02% edetate disodium and 1% cidofovir	1%	Daily for 5 consecutive days every other week for 12 weeks.
Koonsaeng et al. 2001	Beeler base	1%	5 months of intermittent cycles of 5 days of treatment and 5 days of rest
Tristram, Fiander 2005	Unguentum Merck	1%	Alternate days for 16 weeks
Stier **et al**. 2013	Cream base – an emulsion of mineral oil, deionized water, acetyle alcohol, ceresin wax, beeswax and sodium borate	1%	6 cycle of 5 daily applications followed by 9 rest days

1.3.2.3 Recurrence Rates

The limited research available makes it impossible to determine recurrence rates of VIN following treatment with cidofovir.

1.3.2.4 Side Effects of Cidofovir

The European Medicines Agency (EMA) reports neutropenia, headache, nausea and vomiting, alopecia, rash, proteinuria and fever to be very common (<1:10) side effects and iritis, dyspnoea, diarrhoea and renal failure to be common (>1:10 but <1:100) side effects (EMA, Vistide 2015). It is important to remember that these reports are based on the intravenous systemic administration of the drug, and it is likely that the side effect profile associated with topical, localized treatment will differ. Side effects commonly reported in the literature following topical treatment include pain, pruritus, itching, irritation and ulceration at the site of treatment.

1.3.3 Cidofovir and HPV

The anti-papillomavirus activity of cidofovir was first demonstrated in the cottontail rabbit model and has since been confirmed in patients with severe recurrent laryngeal papillomatosis, anogenital disease and cervical intraepithelial neoplasia (CIN) (Andrei *et al*. 1998; Abdulkarim *et al*. 2002; Johnson & Gangemi 1999; Snoeck *et al*. 2001; Grasso *et al*. 2014; Stragier *et al*. 2001; Stier *et al*. 2010). The mechanism of action of cidofovir in HPV associated disease is not defined. Cidofovir has been shown to have higher affinity for viral DNA polymerase compared to human DNA polymerase; this results in its preferential incorporation into viral DNA in viruses, such as HSV, which encode their own DNA thereby explaining its mechanism of action. However, HPV does not encode its own DNA polymerase and therefore, this mechanism of action cannot be attributed. A small number of studies exist investigating the mechanism of action of cidofovir in HPV associated disease in vitro. Analysis of these studies is discussed below and has resulted in two proposed mechanisms of action being hypothesised.

1.3.3.1 Altered Drug Metabolism in HPV Infected Cells

High concentrations of cidofovir (>1 uM) inhibit proliferation of normal human keratinocytes, albeit to a lesser degree than in HPV-transformed keratinocytes. However, below 1 uM, cidofovir had no effect on normal cells whilst inhibition of proliferation still occurred in the HPV-transformed cells (Johnson, Gangemi 1999). These findings indicate increased sensitivity to cidofovir in HPV transformed cells. The differential response was not related to increased uptake of the drug by HPV-transformed cells, which was similar in all cell types studied. Instead, the results indicate that HPV transformed cells metabolized CDV differently. There was an increased accumulation of the biologically active metabolite, HPMPCpp, in HPV-transformed cells. In contrast, in normal cells the end product of cidofovir metabolism, HPMPCp-choline, was present at higher levels. This suggests that the presence of HPV may influence metabolism of CDV.

In-vitro, anti-proliferative effects were seen in HPV transformed cell lines (HeLa, SiHa) and immortalised cell lines from human malignancies that were HPV negative (breast, colon, lung, skin) but not in human primary keratinocytes (Andrei *et al*. 1998). The cellular environment that leads to uncontrolled proliferation could therefore be an important common characteristic shared by cells sensitive to cidofovir. In order to proliferate, cellular machinery involved in energy metabolism and cell cycle progression needs to be activated. Key enzymes, such as pyruvate kinase, are crucial in cell proliferation, and are abundant in rapidly proliferating cells. In the case of HPV infection, the E7 oncoprotein is responsible for driving cellular proliferation and therefore is

associated with the presence of high levels of pyruvate kinase. As illustrated in Figure 1.14, pyruvate kinase is also involved in the metabolism of cidofovir into its active metabolite – CDVpp. It is therefore possible that the increased sensitivity of HPV transformed cells to cidofovir is not directly related to the virus itself but a result of the altered cellular environment created by the proliferating cell.

Another study investigated the effects of cidofovir on the HPV negative cervical cancer cell line C33A and two derivative lines (Donne *et al*. 2009): one transfected with high-risk HPV 16 E6 and the other transfected with low-risk HPV 6 E6. A marked growth inhibitory effect was seen in the high-risk E6 expressing cell line but not in the low-risk E6 expressing cells line. The findings of this study indicate that despite cidofovir's increased activity in rapidly dividing cells from non-HPV immortalized cell lines, the presence of high-risk HPV further enhances this effect. It is possible that the presence of high-risk HPV enhances the effects of cidofovir by facilitating its ability to cause DNA damage (see below).

1.3.3.2 Transforming HPV Infection Permits Genomic DNA Damage by Cidofovir

A study suggesting that the action of cidofovir in HPV transformed cells is mediated through apoptosis found that treatment of HPV transformed cells with cidofovir resulted in:

- Induction of CPP32 (caspase-3) protease activity
- Translocation of phosphatidylserine (PS) from the inner part of the plasma membrane to the outer layer.
- Disintegration of the nuclear matrix protein
- DNA fragmentation
- Increased number of cells in apoptotic phase following cell cycle analysis.

Further to this, treatment of HPV positive cervical carcinoma cell lines with cidofovir has been shown to:

- Arrest cells in the S-phase of the cell cycle
- Increase levels of p53 and pRb
- Increase levels of the cyclin-dependent kinase inhibitor p21/WAF-1
- Induce apoptosis (Andrei *et al*. 2000; Abdulkarim *et al*. 2002).

It would therefore appear that treatment with cidofovir is able to overcome some of the cellular sequelae that result from a transforming HPV infection (i.e. reduced p53)

The viral characteristics of a transforming HPV infection and the altered cellular environment created discussed in section 1.2.2 and summarised in Figure 1.6 are re-

considered at this stage. In normal cells, the tumour suppressor protein p53 responds to a variety of stresses, including DNA damage, and in response to stress, may prevent progression through the cell cycle. P53 activity regulates multiple genes, including genes involved in the induction of cell-cycle arrest, apoptosis, senescence, DNA repair and altered metabolism. Specifically p53 controls the G1/S transition at the G1 checkpoint by inducing expression of cyclin inhibitors p16, p21 and p27, which block the activities of cyclin dependent kinase complexes, thus promoting cell cycle arrest. In HPV transformed cells, these cell surveillance mechanisms are diminished as a result of the de-regulated expression of the E6 and E7 oncogenes. HPV E6 oncoprotein results in the ubiquitination of p53 through its association with the ubiquitin protein ligase E6AP (E6 associated protein) disrupting its control of cell cycle progression. HPV E7 protein binds to several cellular factors including the retinoblastoma (Rb) family of proteins. The Rb family control the G1/S phase transition by regulating the activity of the E2F family of transcription factors, which are critical regulators of G1/S phase transition. The Rb/E2F complex is a transcriptional repressor specific to G1. The binding of the HPV E7 protein to Rb disrupts the Rb-E2F complexes and leads to the constitutive expression of E2F-responsive genes resulting in cell cycle progression (Kessis *et al*. 1993; Duensing, Münger 2004; Andrei *et al*. 2015).

Reduced functional p53 is a characteristic shared by many non-HPV related tumour cells and HPV transformed cells; more than 50% of human tumours contain mutations in the TP53 gene, rendering p53 inactive. Aberrant P53 specifically could therefore be linked to the mechanism of action of cidofovir.

Gene expression changes have been evaluated following cidofovir treatment in different cell types in order to reveal molecular mechanisms underlying cidofovir's selectivity for tumour cells and HPV transformed cells (De Schutter *et al*. 2013). Response to cidofovir in HPV transformed cells was linked to expression profiles indicative of altered response to DNA damage, DNA replication rate as well as incorporation of cidofovir into the genome. They also revealed the presence of micro-array gene expression signatures indicating the activation of DNA double-stranded break repair mechanism in normal primary human keratinocytes in response to treatment with cidofovir. It is therefore possible that the absence of functional p53 in HPV transformed cells facilitates the incorporation of cidofovir into genomic DNA and the resultant DNA damage, which then activates alternative apoptotic pathways leading to cell death (Figure 1.12).

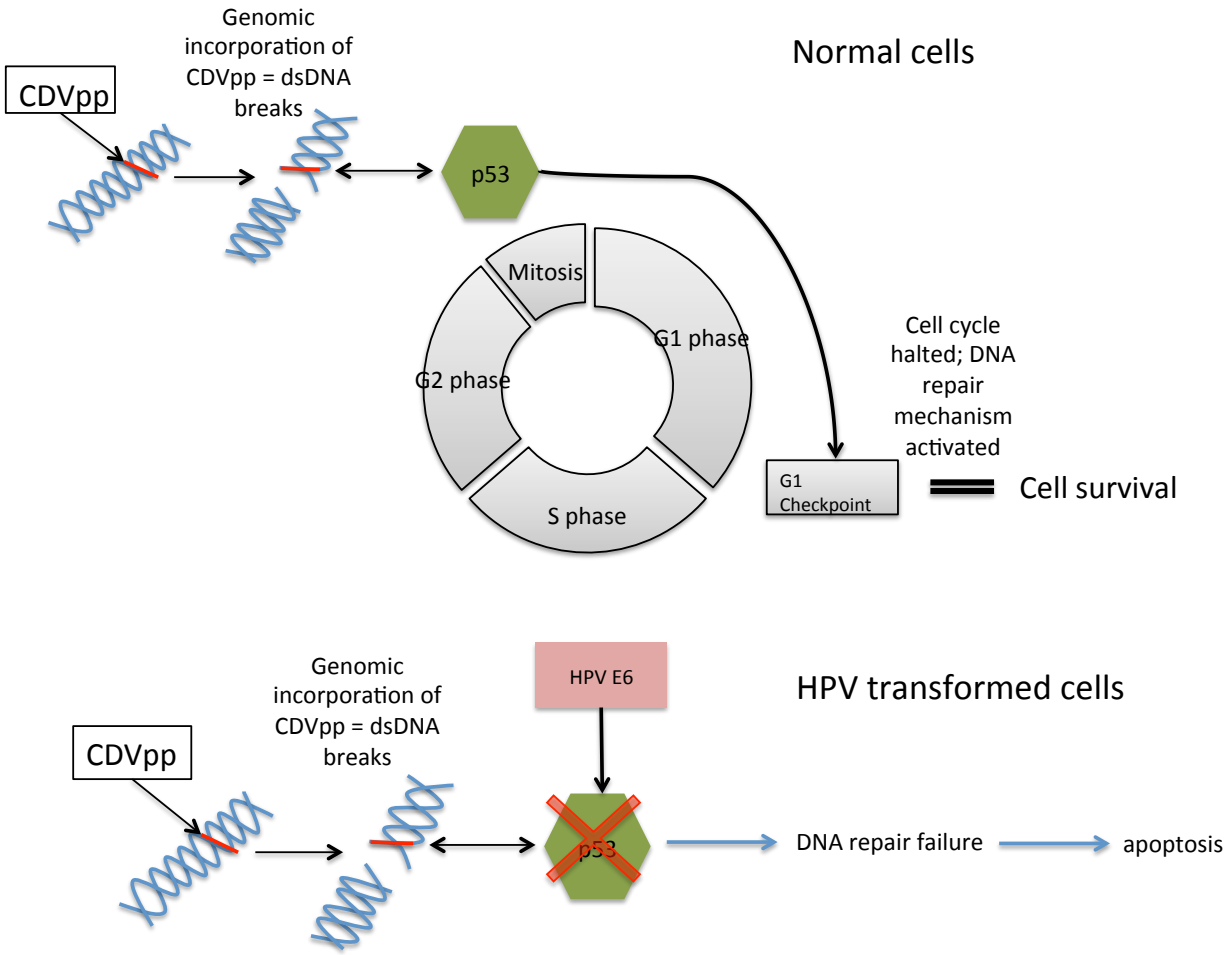

Figure 1.12 Proposed cellular responses to cidofovir in normal and HPV transformed cells. Top: In response to the DNA damage caused by genomic incorporation of cidofovir, normal cells activate DNA damage response pathways via p53 leading to G1/S cell cycle arrest. DNA repair mechanisms are then employed by the cell resulting in genomic stabilization and cell survival. Bottom: in HPV transformed cells, cell surveillance machinery, including p53 are rendered inactive through viral oncoprotein expression. DNA damage caused by cidofovir goes undetected, DNA repair mechanisms are not activated and consequently cell death ensues via apoptosis. Need to include explanation of how CDV causes double stranded breaks

1.3.4 Targeting Cidofovir Therapy in VIN

The limited research available indicates that not all patients with VIN respond to treatment with cidofovir (Tristram, Fiander 2005). In vitro studies have demonstrated that cidofovir causes selective inhibition of proliferation in HPV infected cells compared with HPV negative cell lines (Johnson, Gangemi 1999; Andrei *et al*. 1998). Reports have also shown that topical treatment causes visible reaction in the skin affected by VIN whilst conserving the normal, surrounding skin and that those patients with visible reaction are those women more likely to respond (Tristram, Fiander 2005). It is plausible therefore that knowledge of HPV status prior to treatment with cidofovir could be used to identify patients more likely to respond.

In *vitro* studies have also demonstrated increased efficacy of cidofovir in cells containing a high risk HPV infection (Donne *et al*. 2009). As described in section 1.4.2, HPV integration, DNA methylation and deregulated oncogene expression are all viral characteristics associated with high-risk, transforming HPV infection. It is hypothesized that patients in whom these viral characteristics are identified will be more likely to respond to treatment with cidofovir.

1.4 Imiquimod:

Imiquimod (Aldara, 3M Pharmaceuticals) is an imidazoquinoline amine (Figure 1.13) licensed for the topical treatment of external genital and perianal warts. It exhibits antiviral and anti-tumour effects in vivo by stimulation of the innate immune response through induction of cytokines and the cellular arm of acquired immunity (Schön, Schön 2007). It is used broadly across the UK for the treatment of genital wart and is priced at £48.60 for a 12-sachet pack.

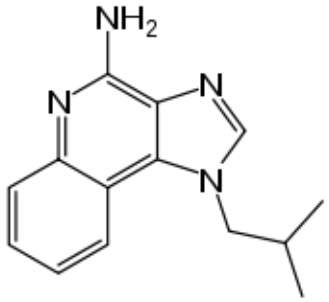

Figure 1.13 Molecular structure of imiquimod. 1-(2-methylpropyl)-1 H –imidazo [4,5-c]quinolin-4-amine. Molecular formula: $C_{14}H_{16}N_4$. Molecular weight: 240.3

1.4.1 Innate Immune System:

The innate immune system is composed of mononuclear phagocytes, dendritic cells, the complement system and epithelial barriers (Alberts *et al.* 2002). It is the pre-programmed immune system of all multicellular organisms that quickly detects harmful pathogens the body may encounter. It does this through the recognition of pathogen associated molecular patterns (PAMPs). PAMPs are essential microbial products and include molecules such as lipopolysaccharides and peptidoglyans. The PAMPs do not tend to be specific to a pathogen but are generally shared by groups of pathogens. Single, membrane spanning, toll-like receptors (TLRs) on the cells of the innate immune system recognise PAMPs. Activation of TLRs stimulates the innate immune system. Activation of TLRs in monocytes such as dendritic cells, results in phagocytosis of the stimulant of its receptor. The cell then combines peptides of the microbe with major histocompatibility complexes forming peptide/MHC that it then presents on its surface. Antigen presentation in turn leads to stimulation of T-lymphocytes. Dendritic cells (and macrophages) are also involved in cytokine production. Cytokines are the communication proteins involved in the inflammatory response and include immunomodulating agents such as interleukins,

tumour necrosis factor and interferon. The production of these cytokines stimulates the differentiation of naïve T cells and promote an amplified positive

autocrine feedback process enhancing immunity to the point that microbial invasion is resolved (Alberts *et al*. 2014; Stanley 2002).

1.4.2 Mechanism of Action of Imiquimod:

Imiquimod is an immunomodulator through its binding and activation of toll-like receptor 7 (TLR7). TLR7 is found on plasmacytoid dendritic cells and macrophages when activated, enhances the innate immune system by stimulating the synthesis of pro-inflammatory Th1 cytokines (IL-12) and interferon α (IFNα). This in turn enhances a CD4+T cell-mediated response and leads to cytolytic activity against viral targets (Stanley 2002; Edwards 1998) through the activation of CD8+ T cells natural killer cells, macrophages and B-lymphocytes (Miller 1999; Stanley 2002; Edwards 1998).

1.4.3 Imiquimod and VIN:

1.4.3.1 Efficacy

The role of imiquimod in the treatment of VIN has been researched more extensively than Cidofovir. Imiquimod is used across the UK as an off-license treatment for VIN, with varying success. A review of 17 studies investigating the efficacy of imiquimod in the treatment of VIN reported complete response rates ranging from 26% to 100%, partial response rates ranging from 0%-60% and non-response rates from 0% -69% (Iavazzo *et al*. 2008). A review of the literature to date reveals complete response rates ranging from 20.0%-100.0% (Table 1.4).

Analysing the literature is challenging due to: variation in treatment regimes and follow-up periods between studies; small patient numbers; variable inclusion/exclusion criteria and varied study design. The two studies reporting 100.0% complete response rates are from case reports on only a few patients (Davis *et al*. 2000; McQuillan, Morgan 2007). One randomized controlled, double blind, clinical trial of 52 patients using 5% Imiquimod versus placebo has been performed (van Seters *et al*. 2008). It reported complete histological response as significantly higher in the imiquimod group compared to the placebo group ($P<0.001$), with complete histological response in 9/26 (34.6%) patients in the treatment arm. A further five (19.2%) patients experienced a significant reduction in lesion size (>75%). No patients in the placebo arm had a complete histological response.

One study compared the efficacy of imiquimod 5% with surgical excision in the treatment of VIN (Frega *et al*. 2013). This study had the added advantage of 5-year follow-up data;

one of the longest follow-up periods reported in this area. Thirty-two patients were treated with imiquimod and 38 patients were treated with surgical excision. In the imiquimod arm, 13/32 (40.6%) patients had a complete response to the first cycle of treatment and a further two patients had complete response following a second course of treatment. Eight patients had a partial response followed by surgical excision and nine patients failed to respond. Of the complete responders, 5/15 (33.3%) developed recurrence; mean time to recurrence was 25.6±19 months. In the surgical arm, 21/38 (55.3%) of patients had no recurrence in the 5-year follow up period and 17/38 (44.7%) did. Mean time to recurrence was 29±26 months. At the end of the 5-year follow up period, 10/32 (31.3%), imiquimod patients remained disease free and so did 21/38 (55.3%) surgically managed patients. This study found the complete response rate to surgical excision to be significantly higher than the response rate following treatment with imiquimod ($p=0.04$), but observed that recurrence rates following treatment were significantly lower following treatment with imiquimod ($p=0.009$).

A study reported by Gentile et al. (Gentile *et al*. 2014) compared efficacy and recurrence rates between patients treated with either excisional surgery alone or excisional surgery followed by a 16-week course of topical imiquimod. Patients were followed up for 5 years. In patients treated with surgery alone, 21/38 (55.2%) had a complete response, and 17/38 (44.8%) had a recurrence (mean time to recurrence 29±26 months). In patients treated with combined surgery and imiquimod, 17/33 (51.6%) had a complete response with 16/33 (48.4%) patients experiencing a recurrence (mean time to recurrence 31±30months). They concluded that there was no added benefit in combining imiquimod with surgical excision in the management of VIN. However, it could also be hypothesised, based on the response/recurrence rates reported by other studies of imiquimod therapy alone, that current optimal management of VIN may be achieved using surgery as an adjunct to initial imiquimod therapy in patients with a partial response or repeated recurrent episodes.

Table 1-4 Summary of studies investigating use of imiquimod in treatment of VIN

Study	n=	Complete response n=(%)	Partial response (any) n=(%)	Recurrence in complete responders n=(%)	Follow-up	Notes
Diaz-Arrastia *et al*. 2001	8	4/8 (50.0)	2/8 (20.0)	2/4(50%)	Mean 33 months	
Jayne *et al*. 2002	13	8/13 (61.5)	4/13 (30.8)	Not reported	5.5 months	
Todd *et al*. 2002	15	3/15 (20.0)	1/15 (6.7)	Not reported	5 months	Local side effects limited treatment application so that 7 patients only applied the cream once a week, 6 patients applied in twice a week and only 2 patients adhered to the treatment protocol of three times a week.
Davis *et al*. 2000	4	4/4 (100.0)	0/4 (0.0)	2/4 (50.0%)	12 months	In the 2 cases of recurrence, one cases has a recurrence in the perianal region and another had a recurrence in a region outside of the previously treated field.
Van Seters *et al*. 2002	15	4/15 (26.6)	9/15 (60.0)	0	Not reported	
Marchitelli *et al*. 2004	8	7/8 (87.5)	1/8 (12.5)	0	Range 10-30 months	
Wendling *et al.* 2004	12	3/12 (25.0)	4/12 (33.3)	0	Mean 9.7 months	
Mathiesen *et al.* 2004	32	17/21 (81.0%)	2/21(10.0 %)	Not reported	Not reported	14/21 patients had to reduce the frequency of cream applications due to side

						effects.
McQuillan, Morgan 2007	1	1/1 (100.0%)	N/A	Not reported	Not reported	
Le *et al*. 2007	39	21/33 (63.6%)	9/33 (27.3%)	8/21(38.1%)	Mean 16 months	
van Seters *et al*. 2008; Terlou *et al*. 2011	26	9/26 (34.6%)	12/26 (46.2%)	0	12 months	7 year follow up data presented by linked study Terlou et al of 24/26 patients. 1/9 (11.1%) complete responders developed recurrence.
Frega *et al.* 2013	40	10/32 (31.0%)	10/32 (31.3%)	5/15 (33.3%)	5 years	
Westermann *et al*. 2013	62	47/62 (75.8%)	14/62	17/47 (36.2%)	Median 21 months	

1.4.3.2 Treatment Regime

Imiquimod is supplied in the form of 5% cream. The European Medicines Agency's (EMA) licenced treatment regime for genital warts is three applications per week until resolution of the lesions for a maximum of 16 weeks per episode (European Medicines Agency, Aldara 2008). The optimal treatment regime for VIN specifically, is yet to be determined. There has been significant variability in methods of administration reported in studies to date. Additional clinical trials will be needed if this treatment option proves useful in the treatment of VIN.

1.4.3.3 Recurrence Rates

Recurrence rates of VIN following treatment with imiquimod are difficult to determine accurately due to the varied nature of the studies reported in the literature. A review of the literature is illustrated in Table 1.4, and shows recurrence rates range from 0% - 50% with a range of follow-up period of 9 months – 7 years.

The longest follow-up period (7 years) following treatment of VIN with imiquimod is reported by Terlou *et al*. 2011 on the initial RCT performed by Van Seters *et al*. 2008 discussed above. In this study, patients were randomized to treatment with imiquimod or placebo. Nine of twenty-six patients (34.6%) had a complete response to treatment of whom, 1/9 (11.1%) experienced a recurrence in the 7-year follow-up period.

A study published by Wallbillich *et al*. 2012 compared disease recurrence rates between different treatment modalities including surgical excision and laser ablation and found imiquimod to have the lowest recurrence rates at 13.6% (laser ablation 41.9% and excision 26.4%).

1.4.3.4 Side Effects

The imiquimod (Aldara) product information approved by the European Medicines Agency (European Medicines Agency, Aldara 2008) regarding side effects states that local side effects in response to treatment with imiquimod are very common (>1 in 10) and include: erythema, ulceration, swelling, burning, pain and itching. These local effects are usually mild and tolerable but in some instances require a reduction in treatment intensity. Headaches are commonly (>1 in 10) reported and flu-like symptoms as well as joint and muscle pains are uncommonly (<1 in 100) reported. Very rarely (<1 in 10,000) women have reported severe and painful skin reactions making passing urine very difficult. The largest single study of imiquimod treatment in VIN to date, a retrospective study of 62 women conducted by Westermann *et al*. 2013, found that 30/62 (48.4%)

reported local adverse effects (mainly burning and itching), 22/62 (35.5%) reported local and systemic (flu-like symptoms) and 2/62 (3.2%) reported exclusive systemic symptoms. Interestingly, complete response to treatment was significantly higher among women who reported adverse events than among those who did not (p=0.049).

1.4.4 Imiquimod and HPV:

HPV related disease is more common among patients with immunodeficiency (e.g. organ transplant, HIV and diabetes) (Doorbar 2006; Palefsky 2006). The high prevalence of HPV infection can be partly attributed to its ability to cause minimal inflammation in the host. HPV has been shown to alter transcriptional activity of IFNβ and NFkβ pathways which results in the decreased ability of keratinocytes to produce the necessary cytokines and chemokines to activate adaptive immunity (Muñoz, Jacquard 2008; Huang, McCance 2002; Ronco *et al*. 1998). It has been found that in comparison with other opportunistic infections, HPV stimulates less local inflammation with lower antigen levels detectable by the innate immune system (Doorbar 2006; Huang, McCance 2002; van der Burg, Palefsky 2009). This evasion of host immunity is essential for the survival of the virus, which requires full keratinocyte differentiation in order to produce new viral particles. Treatment of HPV related disease with immune modifying agents designed to enhance innate immunity such as imiquimod is therefore an obvious course to follow.

Imiquimod has demonstrated efficacy in various HPV associated diseases including cutaneous warts, genital warts and intraepithelial conditions of the anogenital tracts. Imiquimod has been shown to significantly reduce viral load as well as HPV *E7* and *L1* mRNA expression (Tyring *et al*. 1998; Arany *et al*. 1999; Stanley 2002). It also has the ability to generate and maintain effective HPV-specific immunity as suggested by low wart recurrence rates.

Evidence that HPV clearance is driven by imiquimod and that this is important in VIN disease resolution comes from a trial by van Seters *et al*. 2008 (van Seters, van Beurden *et al*. 2008). There were 25 HPV positive patients in each treatment arm (imiquimod and placebo) of this study. After 20 weeks of treatment, 15/25 (60%) patients and 2/25 (8%) patients were HPV negative in the imiquimod arm and placebo arm respectively (p=<0.001). There was a strong association between HPV clearance and disease clearance (p=<0.001); of the 14 lesions in the study that regressed, 13/14 were cleared of an original HPV infection and the other was HPV negative at baseline. This data, along with knowledge of the mechanism of action of imiquimod, gives rise to the hypothesis that HPV could act as a predictive biomarker to response to treatment with imiquimod in patients with VIN.

In-vitro investigations have demonstrated that when integrated and episomal forms of HPV coexist, expression of viral *E2* from the episomes results in repression of transcription of *E6* and *E7* from the integrated HPV and it is only when there is complete loss of episomal HPV that *E6* and *E7* expression becomes fully deregulated. Episome loss is associated with expression of antiviral response genes induced by the type I interferon (IFN) pathway. This has been confirmed by studies using exogenous IFN, which resulted in clearance of episomes. Interestingly, the consequence of this was up-regulation of oncogene expression from previously latent HPV infection (due to the loss of the episomal E2) (Herdman *et al*. 2006; Pett *et al*. 2006). Clinical studies have shown that treatment of HPV tissue with imiquimod causes an increase in the production mRNA for IFN-α at the application site. IFN-α and IFN-β are both type I IFN's signalling through the IFN-α receptor, it is likely that treatment with Imiquimod may cause episome clearance in the same manner as exogenous IFN-β (Stanley 2002). If is the case, then apparent clinical response could well be followed by emergence of a more aggressive lesion with enhanced expression of viral oncogenes. This important parameter will be assessed within the clinical trial (RT3 VIN) that is linked to this study.

1.4.5 Targeting Imiquimod Therapy in VIN

Not all patients with VIN respond to treatment with imiquimod; non-response rates are reported between 0% - 69% (Iavazzo *et al*. 2008). Imiquimod also carries a significant side effect profile and can be difficult to tolerate. A means of targeting therapy to those patients in whom a response is more likely is an attractive prospect, and could avoid unnecessary side effects in patients in whom an alternative approach would be better. Cases that are driven by HPV have been shown to respond better to treatment with imiquimod than cases that are HPV negative (van Seters, van Beurden*et al*. 2008). It is therefore possible that identification of patients with HPV driven disease could also identify patients more likely to respond to therapy. It is also the case that not all cases of HPV positive VIN respond to treatment with imiquimod (10/25 (40%) HPV positive cases failed to respond to treatment in the van Seters study mentioned above). Three explanations have been proposed as to why some patients fail to respond to treatment with imiquimod (Stanley 2002):

1. Persistent disease may result in tolerance to viral infection.
2. Presence of signalling defects in the T cells.
3. Antigen loads being too low for effective priming.

Points 1 and 3 above are of particular interest to this thesis. The mechanism of action of imiquimod is linked to the direct stimulation of the innate immune system and specifically

to the target infection. Therefore, a requirement of successful imiquimod treatment would be a degree of host response to the HPV infection in the first instance. The likelihood of treatment failure is potentially higher in patients in whom the HPV infection is more successfully evading host immunity and who are therefore lacking the directed immune response (van Seters, Beckmann *et al*. 2008). Successful evasion of host immunity increases the likelihood of persistent HPV infection, which in turn increases the likelihood of developing a transforming infection. Therefore viral markers of persistent/transforming HPV infection could potentially identify those patients in whom treatment with imiquimod is destined to fail. As described in section 1.2.2, the presence of viral integration, increased viral DNA methylation and de-regulated E6/E7 gene expression are all associated with persistent transforming infections. By creating a physical 'profile' of the HPV infection upon diagnosis, it might be possible to predict patients in whom treatment is more likely to be successful.

1.5 Biomarkers

A biological marker (biomarker) is a characteristic than can be objectively measured such as DNA, RNA, protein, protein modification or metabolite that reflects the biological state of interest. Biomarkers can be used to indicate normal biological processes, pathogenic processes or pharmacological responses to therapeutic interventions and are being increasingly used in day-to-day medical practice.

Biomarkers may be used in a variety of settings, including:

- **Diagnostic**– indicating the probability that a disease is present
- **Prognostic** – indicating how disease may develop in individual cases, regardless of treatment
- **Predictive** – indicating the probable effect of an intervention on the disease

1.5.1 Predictive Biomarker Development

The aim of the biomarker investigation in this thesis was to determine whether HPV characteristics have potential to identify patients whose VIN is more likely to respond to topical treatment of VIN 3 with cidofovir or imiquimod. Cancer Research UK has published guidelines in the form of a 'roadmap' to aid in development of predictive biomarkers. This roadmap breaks down biomarker development into three broad sections starting with establishing the rationale for developing the biomarker. Questions to be asked at this stage include:

- Is there an unmet clinical need for the biomarker?
- Does the work primarily focus on the discovery/development of a biomarker for application to clinical material?
- Is there a sample collection available for retrospective biomarker-outcome correlation?

If the answer to the above questions is 'yes' then progress can be made through several stages of 'biomarker discovery' and 'assay development' before the final stages of 'biomarker qualification' are addressed. Figure 1.14 illustrates the CRUK prognostic/predictive biomarker roadmap.

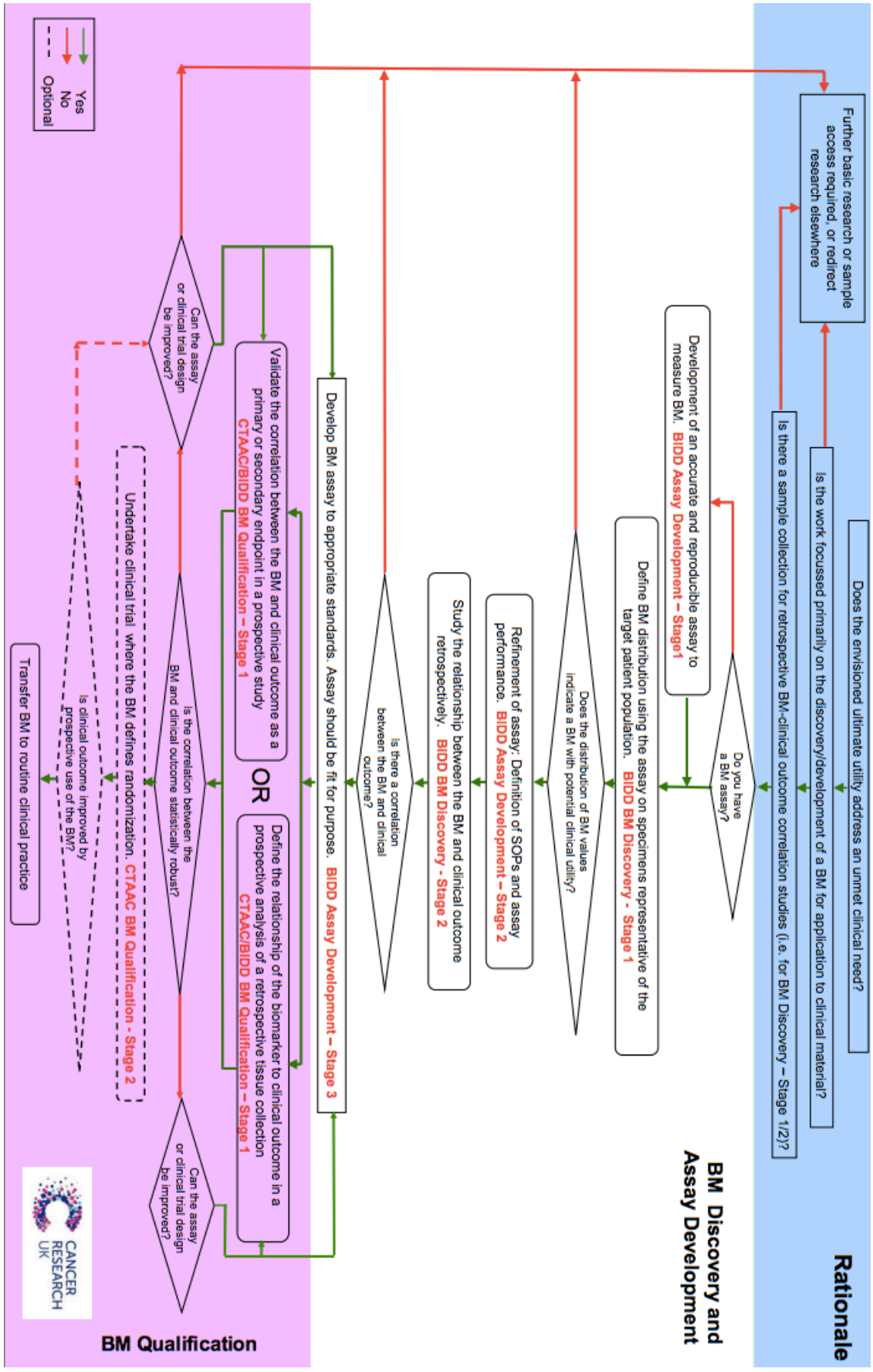

Figure 1.14 CRUK Prognostic/Predictive Biomarker Roadmap

1.6 Study aims and Hypotheses

This study had two broad aims: firstly, to investigate HPV biology in VIN and, secondly, to assess HPV characteristics as potential predictive biomarkers to direct treatment of VIN with cidofovir and imiquimod. HPV pathogenesis in VIN is in largely presumed to be similar to HPV pathogenesis in CIN. Investigation of HPV biology in VIN is important in view of the lack of evidence to support this assumption. This study is the largest investigation of multiple aspects of HPV biology in VIN to date. Based on the literature described, several hypotheses were generated (Figure 1.15). The results and discussion of this part of the investigation are detailed in chapter 5.

The second aim of this study was to investigate HPV characteristics as potential predictive biomarkers in the treatment of VIN with cidofovir and imiquimod. This thesis describes translational work associated with the RT3 VIN clinical trial (chapter 3). This phase II trial confirmed early reports in the literature that approximately 50% of patients treated with either cidofovir or imiquimod respond and also found no correlation between clinical markers (smoking status, disease episode and disease focality) and response to treatment. These findings satisfied the 'unmet clinical need' requirement in the rationale for developing a biomarker laid out in the CRUK biomarker development roadmap (Figure 1.14). Regarding the two remaining questions asked in the rationale for biomarker development in the roadmap, this work was focused primarily on the discovery and development of a biomarker for application to clinical material. The RT3 VIN cohort provided a sample collection for retrospective biomarker-clinical outcome correlation studies. Based on what was known about the pharmacology of the two drugs being studied, several hypotheses were generated (Figure 1.16). The results of this part of the investigation are detailed in chapter 6.

The following chapter describes the methods used to carry out this work. Chapter 3 provides a summary of the RT3 VIN clinical trial (the trial with which, this translational work was associated), which is followed by two results chapters with their relevant discussions: 'HPV biology in VIN' (Chapter 4) and 'viral characteristics as predictive biomarkers in response to treatment' (chapter 5). A short conclusion of the work overall can be found at the end.

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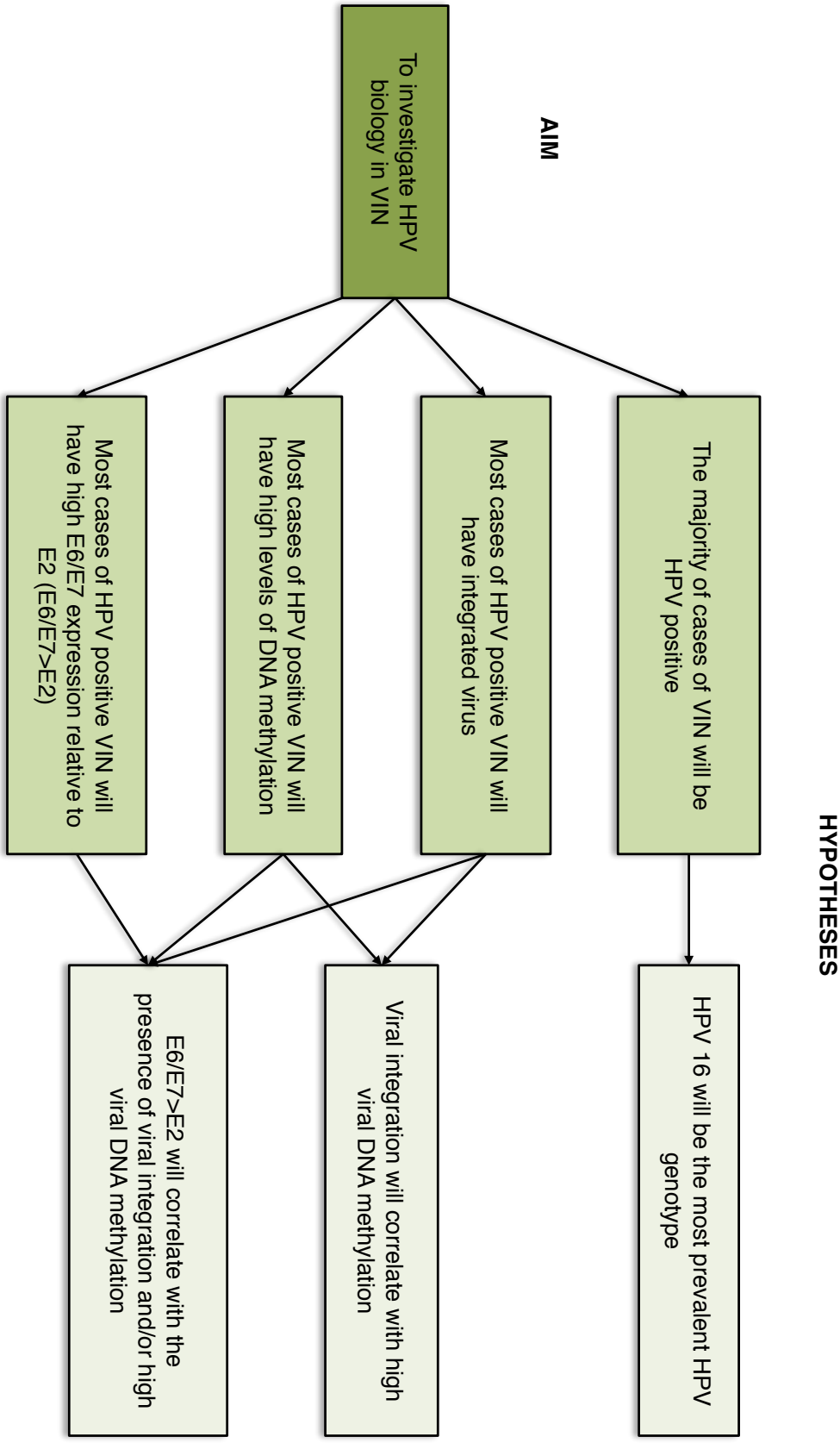


Figure 1.15 Study Aim 1 and Associated Hypotheses

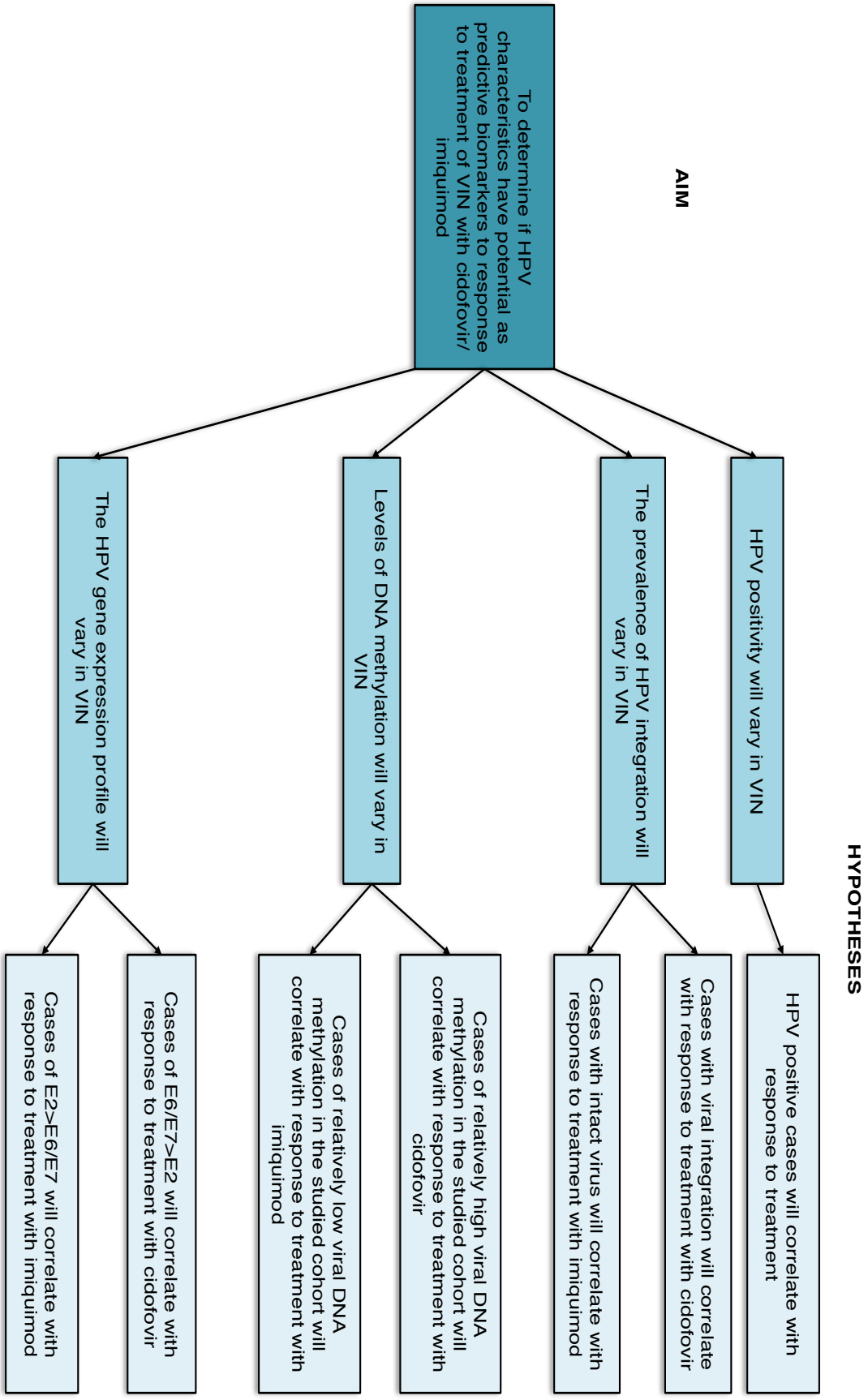


Figure 1.16 Study Aim 2 and Associated Hypotheses

Chapter 2

Materials and Methods

2 Methods

2.1 Study Population

The biopsies used for this study were collected from patients in the RT3 VIN clinical trial; for detailed trial information see chapter 3. In summary, patients with histologically confirmed VIN 3 were recruited for treatment with one of two novel topical treatments (imiquimod and cidofovir) in a phase II setting with curative aims over a maximum course of 24 weeks. HPV biopsies were taken prior to treatment ('baseline') and 6 weeks following the completion of treatment ('post-treatment'). In some cases, patients consented to have an additional biopsy taken at 6 weeks into treatment ('6 week').

The trial was run across 32 centres in the UK. HPV biopsies were initially collected in ThinPrep Liquid Based Cytology (LBC) media (Hologic®, Bedford, MA, USA) for transportation and then stored at -20°C prior to processing. Later, in order to improve RNA quality, all samples were stored at -80°C prior to extraction and samples from the main recruiting centre were collected and stored in RNAlater® storage media (Life Technologies Ltd, Paisley, UK). Laboratory researchers were blinded to clinical outcomes until all scientific assays were complete to prevent biased data interpretation.

2.2 DNA and RNA Extraction

A modified version of the Qiagen RNeasy® Fibrous Tissue Mini Kit and Qiagen® DNA Mini kit (Qiagen, Hilden, Germany) was used. The RNeasy Fibrous Tissue Mini Kit was chosen because the tissue of the vulva, particularly when VIN 3 disease is present, can be quite fibrous. Additionally, fixation of the tissue in LBC media resulted in more physically robust samples. Tissue samples were first lysed and homogenised (TissueRuptor®, Qiagen, Hilden, Germany) for 20secs in 360 µl Buffer RLT solution before being treated with 728 µl of proteinase K solution (708 µl of RNAse free water and 20 µl of proteinase K). The mixture was centrifuged at 13,000rpm for 3 minutes to create a pellet of debris; 650 µl of the supernatant was removed for RNA extraction. The remaining 350 µl and the pellet were re-suspended and incubated at 56 °C for 16 hours in preparation for DNA extraction the following day. The 650 µl supernatant was mixed by repeat pipetting with 325 µl of ethanol and then centrifuged at 13,000 rpm for 15 secs through the provided RNeasy spin column, where the RNA binds to a silica membrane. Potential contaminants were washed away with one 350 µl buffer RW1 wash followed by 15 secs centrifuge at 13,000 rpm, and two 500 µl buffer RPE washes; the first followed by a 15 sec centrifuge at 13,000 rpm and the second followed by a 2 min centrifuge at 13,000 rpm. The remaining RNA was eluted using 100 µl RNase-free water.

The suspension previously left to incubate at 56 °C was used for DNA extraction. 350 µl of Buffer AL was added to the suspension and mixed by pulse vortex for 15 secs then left to incubate at 70 °C for 10 mins and centrifuged at 13,000 rpm for 15 secs. This was followed by the addition of 350 µl of 100% ethanol, a mix by pulse vortex for 15 secs and 15 secs in the centrifuge at 13,000 rpm to precipitate the DNA. The mixture was then added to the spin column and residual contaminants were washed away using 2 wash buffers, AW1 and AW2. Initially 500 µl of AW1 was added to the column and centrifuged at 13,000 rpm for 1 minute, then, 500 µl of AW2 was added and centrifuged at 13,000 rpm for 3 minutes. Purified DNA was eluted from the QIAmp Mini spin column in a concentrated form in 200 µl of AE buffer.

2.3 Nucleic Acid Quantification Analysis

DNA and RNA elutions were quantified using a NanoDrop-1000 spectrophotometer (FisherScientific UK Ltd, Loughborough, UK) 2 µl of DNA or RNA was tested against a 'blank' of the appropriate elution buffer (AE buffer in the case of DNA and RNase free water in the case of RNA). Sample purity was also assessed as the presence of impurities negatively affects the reliability of the quantification.

The ratio of absorbance at 260 nm and 280 nm assesses purity and a ratio of ~1.8 was accepted as 'pure' for the DNA and a ratio of ~2.0 was regarded as 'pure' for the RNA sample. These figures are taken from guidelines provided by the NanoDrop manufacturers.

2.4 HPV Testing

Four different HPV detection/genotyping assays targeting different regions of the virus were used during this study. **Figure 2.1** is a representation of the HPV genome with the regions specific to each assay highlighted. The concentration of sample DNA was standardised in all HPV detection/genotyping assays to 10 ng/µl, except for the PapilloCheck® assay which requires 2 µl of sample DNA.

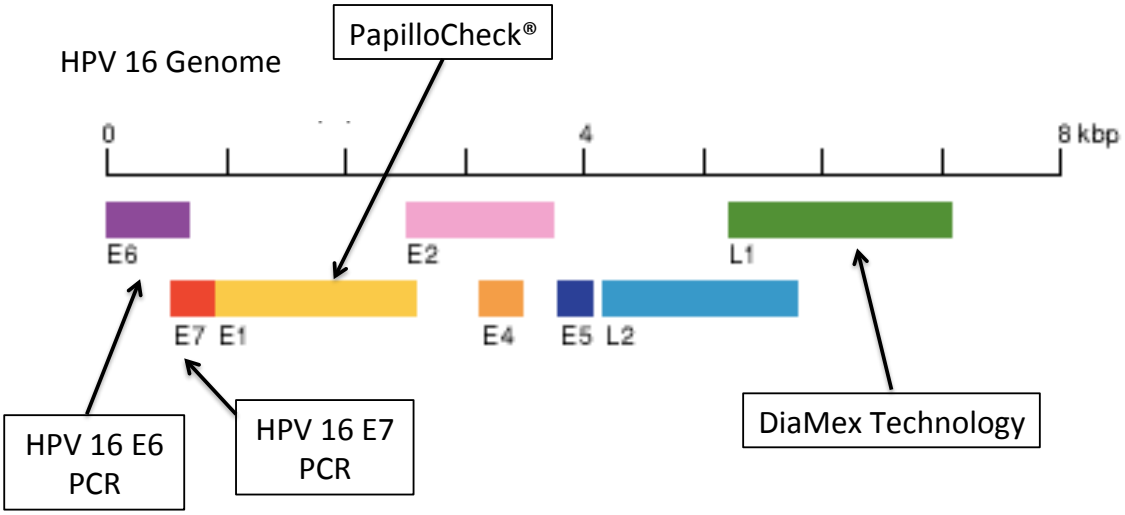


Figure 2.1HPV detection assay target regions in the HPV genome. Demonstrating that all four HPV detection methods target different regions of the HPV genome.

2.4.1 Greiner PapilloCheck® HPV Genotyping

All HPV samples received from the RT3 VIN trial underwent the Greiner PapilloCheck®(Greiner Bio-one GmbH, Frickenhausen, Germany) assay for HPV genotyping as per manufacturer's instructions. It simultaneously tests for 24 different 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) through the detection of a 350bp fragment of the *E1* gene.

PCR is used to amplify the target sequence of the *E1* gene using a subset of fluorescently labelled HPV specific primers. The assay incorporates several control steps including the amplification of the human gene, *ADAT1* (Adenosine deaminase 1) to avoid false negatives due to sample inadequacy. Amplification products hybridise to HPV-specific probes attached to a DNA chip as well as built in controls to check for adequate amplification and hybridisation. Un-hybridised DNA is removed in later wash steps. The DNA chip is then scanned and analysed using CheckScanner and CheckReport software generating a data output indicating samples that failed or passed internal controls and any identified genotypes in the samples that passed internal controls. Positive (DNA from HPV16 positive CaSki cells) and negative (H_2O) controls were included on each chip.

2.4.1.1 Quality Assurance

For the purpose of quality assurance, 25% of samples were repeated in duplicate from the point of PCR set-up to test for inter-run variation. 98% of results were concordant. In cases of discrepant results, the first result was used due to the potential for DNA degradation to be caused by any subsequent freeze-thaw cycles.

2.4.2 HPV 16 E6 PCR

The HPV 16 E6 PCR assay amplified a 161 bp region of the E6 gene of HPV 16 using the primers described by Collins et al. (Collins *et al*. 2009). All PCR primers used for detection of different fragments of HPV are listed in Table 2.1. This assay was run on all samples to identify those that were HPV 16 positive. This method of HPV 16 detection was chosen due to its benefits in terms of cost, ease of technique, quick generation of results and most importantly because the E6 gene is highly conserved in HPV infection independent of the physical state of the virus and therefore reduces the likelihood of false negatives. Detection of HPV 16 was the primary focus due to data from existing literature indicating it to be the most prevalent genotype in VIN (de Sanjose *et al*. 2013). The PCR mastermix was setup in a final volume of 20 µl including template DNA at 10 ng/µl, 10x PCR buffer, forward and reverse primers at 10 µM, MgCl2 at 15 mM, dNTP at 2 mM, Taq polymerase at 1 U and 4.8 µl of H2O. The Thermocycling (Applied Biosystems® GeneAmp® PCR system 9700) conditions used were: initial denaturation of 94 °C for 3 minutes; a touchdown primer annealing stage of 10 cycles of 94 °C for 30 secs, 65 °C (decreased by 1 °C increments every cycle to 55 °C) and 72 °C for 1 minute; an amplification stage of 30 cycles of 94 °C for 30 secs, 55 °C for 30 secs and 72 °C for 1 minute followed by a final extension of 72 °C for 10 minutes.

2.4.2.1 Quality Assurance

The assay was repeated on 20% of samples from the point of PCR set up for the purpose of inter-run quality control. 100% of samples generated the same result on both occasions. In addition each run included a positive control (CaSki) and a negative control (H_2O).

2.4.3 HPV Genotyping using the Optiplex HPV Testing Kit

All RT3 VIN baseline samples that tested negative for any HPV genotype using either the HPV 16 *E6* PCR assay or the PapilloCheck® assay were sent to the Scottish Human Papillomavirus Reference Laboratory in Edinburgh for further testing using the Optiplex HPV testing kit (DiaMex^{GmbH}, Heidelberg, Germany). This assay was chosen because it amplifies a region of the *L1* gene of HPV, generating amplimers ranging from 145 bp to 148 bp. The kit is a multiplex assay using polymerase chain reaction to test for the presence of 24 different genotypes (HPV 6, 11, 42, 43, 44, 70, 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68, 73 and 82). The test utilises Luminex technology, which is based on a suspension of polystyrene beads that are labelled with various ratios of two spectrally distinct red fluorophores. Each of the beads in the suspension is coupled to a specific oligonucleotide probe. The target sequences are indirectly labelled with a third fluorophore. The bead sets can be differentiated and the bound fluorescence can be quantified using the Luminex analyser (Luminex®, Austin, USA).

2.4.3.1 Optiplex Method in Summary

The assay was performed according to the manufacturer's instructions. Sample DNA at 10 ng/µl was subjected to PCR amplification using biotinylated primers provided in the Optiplex kit. Quantifast Multiplex Mastermix (Qiagen, Hilden, Germany) was used for the reaction to reduce the need for optimisation of reaction cycling conditions. At the PCR stage, the DiaMex kit incorporates an internal control for sample adequacy in the form of primers for the amplification of a fragment of the human*β-Globin* gene. The PCR product is then added to the Luminex bead mix containing the 24 different HPV genotype bead populations and the *β-Globin* bead population. An additional control bead population is present to ensure that correct hybridization conditions were applied to the reaction.

Following thermal denaturation and the hybridization of the target sequences to the bead-bound probes, labelling of the hybridised biotinylated PCR products is achieved by addition of R-Phycoerythin labelled Streptavadin. Finally, the Luminex instrument analyses the reported fluorescence signal of at least 70 beads of each individual bead set per well. The fluorescence intensity is used to evaluate the output data using pre-defined 'cut-off' values for the median fluorescence intensity (MFI).

2.4.3.2 Quality Assurance

Positive (CaSki) and negative (H_2O) controls were used with each run. The assay incorporates internal controls including the amplification of the human gene *β-Globin* and hybridization controls.

2.4.4 HPV Type-Specific E7 PCR

In order to further investigate the baseline RT3 VIN cohort for HPV prevalence, all samples that generated a negative result from PapilloCheck®, HPV 16 E6 PCR and Luminex, were put through a HPV type-specific PCR targeting the E7 region of the virus for genotypes HPV 16, 18, 31, 33, 35 and 51. This was done in attempt to reduce the risk of false negative results as a result of the previous assay target regions being potentially disrupted in non-HPV 16 genotypes. These genotypes were selected based on their relative high prevalence in HPV related disease (de Sanjose *et al*. 2013). Primers were taken from Walboomers *et al*. 1999 and are specified in Table 2 1. Primer-specific 20 µl mastermixes were made up using 5 µl of template DNA at 10 ng/µl, 2 µl of 10x concentration PCR buffer (Invitrogen), 2 µl of forward and reverse primers at 10 µM, 2 µl of MgCl2 at 15 mM, 2 µl of dNTP's at 2 mM, 0.2 µl of Taq polymerase at 5 U/µl and water to make up the final volume (4.8 µl). The thermocycling conditions (Applied Biosystems®GeneAmp® PCR system 9700) were initial denaturation of 5 min at 94 °C, followed by 40 cycles of 94 °C for 1 min, 55 °C for 2 min and 72 °C for 1.5 min, with a final extension of 72 °C for 10min.

2.5 HPV Disruption Assay

An assay was developed that combined the *E2* tiling PCR assay originally described by Collins *et al*. 2009 with a novel two-fragment *E1* tiling PCR. The two tiling PCRs were run separately and the results combined for final interpretation. The assay aimed to detect the presence of small, breakdown fragments of two of the larger HPV genes: E1 and E2 (1954 and 1097 bp respectively). Figure 2.2 illustrates the combined HPV disruption assay.

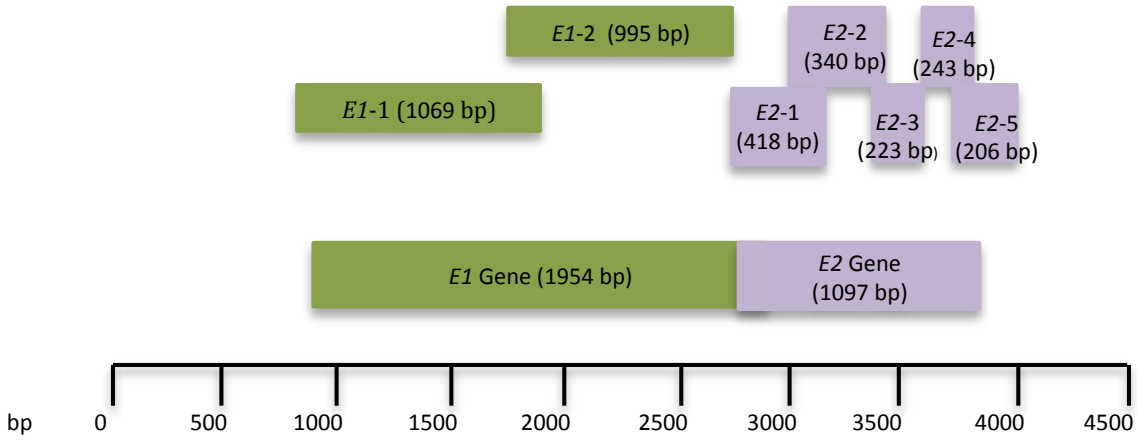


Figure 2.2 Combined *E1/E2* gene tiling PCR. Assay used to detect the presence of viral disruption as a surrogate marker for the presence of viral integration. Dark grey boxes are *E1* gene fragments; light grey boxes are *E2* gene fragments. bp = base pair. Bottom line represents base pair number.

This method was chosen because it is highly reproducible, easy to run (reducing the impact of human error) and relatively inexpensive.

2.5.1 The *E1* Tiling PCR

A primer was designed for two fragments of the *E1* gene to determine the presence of intact HPV 16 *E1* using primer blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). PCRs were performed comprising 5 μl of DNA at 10 ng/ μl, 2.5 μl of PCR buffer with 15 mM MgCl2 (10x) (Invitrogen™), 1 μl of primers at 10 μM, 2.5 μl of 2 mM dNTP, 0.125 μl of 1 U Taq Polymerase and 12.875 μl of distilled water to a final volume of 25 μl. Details of primers can be found in Table 2.1.

The initial denaturation step was 95 °C for 15 minutes followed by an amplification step of 30 cycles consisting of 30 seconds at 94 °C, 30 seconds at 59 °C and 180 seconds at 72 °C. This was followed with a final extension of 1 min at 72 °C for 7 minutes.

2.5.2 The *E2* Tiling PCR

This assay consisted of five tiling PCRs using primers previously described by Collins *et al*. 2009. The reaction used 5 μl of DNA at 10 ng/μl, 2 μl of 10x concentration PCR buffer (Invitrogen), 2 μl of primers at 10 μM (Table 2.1), 2 μl of dNTP at 2 mM, 2 μl of MgCl2 at 15 mM, 0.2 μl of Taq Polymerase at 5 U/μl and filtered water to a final volume of 20 μl (4.8 μl). Cycling conditions were initial denaturation of 94°C for 3 minutes followed by a touchdown annealing stage (10 cycles of 94 °C for 30 seconds, 65 °C decreasing by 1 °C every cycle for 30 seconds, and 72 °C for 1 minute), followed by 30 cycles of 94 °C 30 seconds, 55 °C for 30 seconds and 72 °C for 1 minute. The last cycle was followed by a 10 minute extension at 72 °C.

2.5.2.1 Data Interpretation

A sample was considered to have intact HPV when all fragments of the *E1* and *E2* gene were present. If one or more fragments were absent, the sample was considered disrupted (and therefore likely to have HPV integration). A significant limitation of this assay is its inability to identify those samples with 'mixed' HPV infection i.e. those cases whereby HPV is present in both episomal and integrated form in which case the more abundant HPV form would be represented by the assay. An additional limitation of this assay is its inability to detect the presence of viral disruption in regions of the virus other than *E1* and *E2.*

2.5.2.2 Quality Assurance

For the *E1* tiling PCR 61% of baseline RT3 VIN samples were run in duplicate at separate points in time with 93.8% concordance. 30% of all E2 fragments were repeated with the following concordance: *E2*-1 fragment 95.1%, *E2*-2 fragment 95.1%, *E2*-3 fragment 92.6%, *E2*-4 fragment 95.1% and *E2*-5 fragment 94.8%. Where there was a discrepancy between repeats, the result from the first run was used (due to the potential impact of time and multiple freeze-thaw cycles on the DNA). Positive (CaSki) and negative (H_2O) controls were included in each run. Runs were only analysed if the controls gave appropriate results.

Table 2-1 PCR primers used for HPV detection assays

Primer Type	**HPV 16 *E6* Primer sequence**	**Product size**
HPV 16 *E6* Forward primer HPV 16 *E6* Reverse primer	GAACAGCAATACAACAAACC GATCTGCAACAAGACATACA	161 bp
Primer Type	**HPV 16 *E7* Primers sequence**	**Product size**
HPV 16 *E7* forward primer HPV 16 *E7* reverse primer	GATGAAATAGATGGTCCAGC GCTTTGTACGCACAACCGAAGC	100bp
HPV 18 *E7* forward primer HPV 18 *E7* reverse primer	AAGAAAACGATGAAATAGATGGA GGCTTCACACTTACAACACA	100 bp
HPV 31 *E7* forward primer HPV 31 *E7* reverse primer	GGGCTCATTTGGAATCGTGTG AACCATTGCATCCCGTCCCC	100 bp
HPV 33 *E7* forward primer HPV 33 *E7* reverse primer	TGAGGATGAAGGCTTGGACC TGACACATAAACGAACTGTG	100 bp
HPV 35 *E7* forward primer HPV 35 *E7* reverse primer	CTATTGACGGTCCAGCT TACACACAGACGTAGTGTCG	100 bp
HPV 51 *E7* forward primer HPV 51 *E7* reverse primer	TACGTGTTACAGAATTGAAG AACCAGGCTTAGTTCGCCCATT	100 bp
Primer Type	**HPV 16 *E2* Primers sequence**	**Product size**
HPV 16 *E2*-1 forward HPV 16 *E2*-1 reverse	AGGACGTGGTCCAGATTAAG TCAAACTGCACTTCCACTGT	418 bp
HPV 16 *E2*-2 forward HPV 16 *E2*-2 reverse	TAACTGCACCAACAGGATGT GCCAAGTGCTGCCTAATAAT	340 bp
HPV 16 *E2*-3 forward HPV 16 *E2*-3 reverse	ATCTGTGTTTAGCAGCAACG TAAATGCAGTGAGGATTGGA	223 bp
HPV 16 *E2*-4 forward HPV 16 *E2*-4 reverse	ACAGTGCTCCAATCCTCACT TCACGTTGCCATTCACTATC	243 bp
HPV 16 *E2*-5 forward HPV 16 *E2*-5 reverse	GGCATTGGACAGGACATAAT CAAAAGCACACAAAGCAAAG	206 bp
Primer Type	**HPV 16 *E1* Primers sequence**	**Product size**
HPV 16 *E1*-1 forward HPV 16 *E1*-1 reverse	CTAGGAATTGTGTGCCCATCTG CTTTCTATCCATTCTGGCGTGTCT	1069 bp
HPV 16 *E1*-2 forward HPV 16 *E1*-2 reverse	GATAGAGCCTCCAAAATTGCGT ACGTTGGCAAAGAGTCTCCATC	995 bp

Note: all primer sequences are given in 5'-3' orientation.

2.6 HPV DNA Methylation Analysis

Bisulfite conversion of HPV DNA followed by PCR and pyrosequencing was used for the analysis of HPV DNA methylation. Bisulfite conversion is widely used for methylation

analysis and relies on the ability of sodium bisulfite to deaminate unmethylated cytosines to produce uracil whilst leaving methylated cytosines unchanged (Figure 2.3). In subsequent PCR amplification, ATP is incorporated opposite the uracil base. Sequencing of the PCR product then allows differentiation between products derived from templates containing the original methylated and unmethylated cytosines.

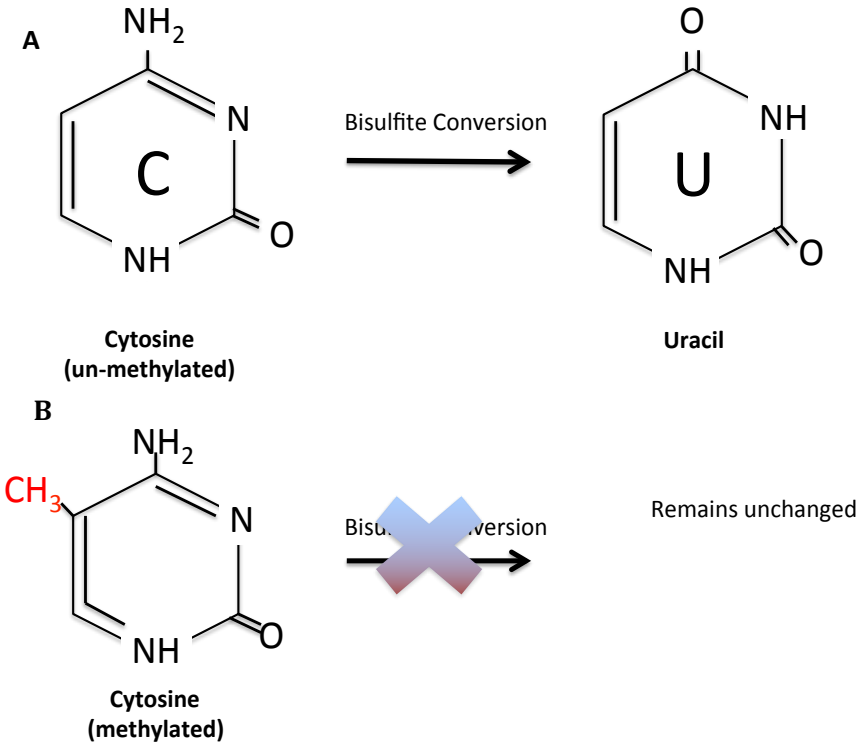


Figure 2.3 Bisulfite conversion. 'A' shows the result of sodium bisulfite conversion of unmethylated cytosine to uracil through deamination and 'B' illustrates maintenance of cytosine due to methylation

Bisulfite conversion and PCR were followed by pyrosequencing (Figure 2.4). Pyrosequencing involves the photon-based detection of released inorganic phosphate (PPi) during nucleotide incorporation. This method was chosen because it allows for quantitative analysis of the methylation status of multiple CpG sites within relatively short reads. Initially, the DNA segment is amplified by PCR using a biotinylated primer. The biotinylated strand is then isolated and hybridized with the sequencing primer. The hybridized primer is then incubated with DNA polymerase, ATP sulfurylase, luciferase and apyrase and the substrates adenosine 5'phosphosulphate(APS) and luciferin. As the dNTPs are added to the reaction, incorporation is catalysed by DNA polymerase (if complimentary to the base template), which, is accompanied by the release of pyrophosphate (PPi) in a quantity that is equimolar to the amount of incorporated

nucleotide. The ATP sulfurylase then converts the PPi to ATP in the presence of APS, which then drives the luciferase-mediated conversion of luciferin to oxyluciferin, which generates visible light proportional to the amount of ATP.

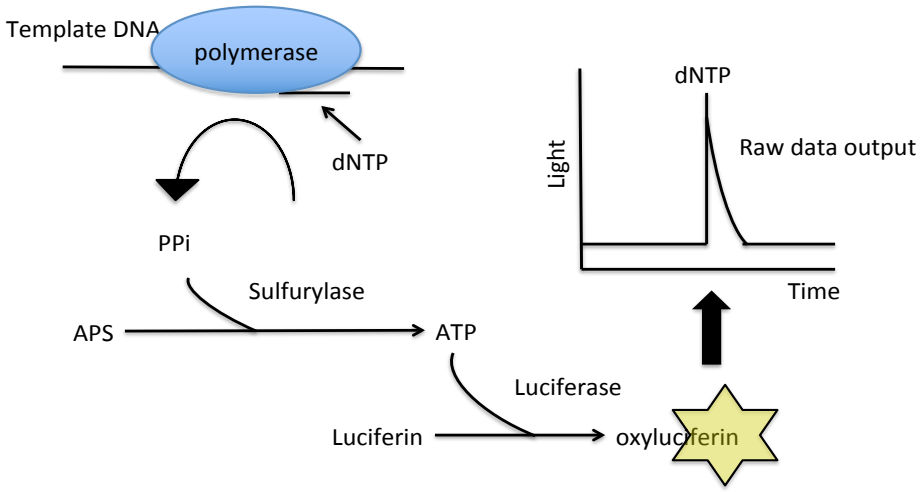


Figure 2-4 Schematic representation of pyrosequencing reaction.
APS=5'phosphosulphate, ATP=Adenosine triphosphate, PPi=inorganic pyrophosphate, dNTP=Deoxynucleotide triphosphate.

The light produced is detected by a charged couples device (CCD) camera and is seen as a peak in the raw data output, the height of which is proportional to the number of nucleotides incorporated. The purpose of the apyrase in the reaction is to degrade unincorporated nucleotides and ATP prior to the addition of another nucleotide.

2.6.1 Bisulfite Conversion

The EZ DNA Methylation Kit (Zymo Research Corporation, CA, USA) was used for sodium bisulfite conversion. Kit reagents were prepared according to manufacturer's instructions. Briefly, 500 ng of DNA was made up to a final volume of 32.5 µl with distilled H_2O. M-dilution Buffer (7.5 µl) was added to each reaction and then incubated at 42 °C for 30 minutes. 97.5 µl of pre-prepared CT Conversion Reagent was added to each reaction and incubated overnight at 50 °C (12-16 hours). Negative controls were included in each bisulfite treatment stage whereby the CT conversion reagent was replaced with sterile H_2O.

Samples were then cooled on ice for 10 minutes and loaded into Zymo-Spin columns containing 400 µl of M-Binding Buffer, mixed by inversion and centrifuged at 13,000 rpm for 30 seconds. Two consecutive wash steps were undertaken where 100 µl of M-Wash

Buffer was added to each column and centrifuged in the same manner described previously. DNA was eluted into 10 µl of M-Elution Buffer.

2.6.2 PCR

The bisulfite converted sample DNA was diluted 1:10; CaSki HPV positive cell line DNA was used as a positive control for the PCR and was diluted 1:100; molecular grade water (Sigma) was used as a negative. ZymoTaq hot start DNA polymerase premix was used for the PCR reaction (Zymo Research Corporation, CA, USA).

Three PCR primers were used; two (E2 and L1/L2) targeting viral genes and one targeting the promoter region of the virus. Mastermixes and PCR conditions are shown in Table 2.2 and Table 2.3).

Table 2-2 Mastermix setup for Pyrosequencing PC

		Primer		
		E2	**L1/L2**	**Promoter**
Reagent	**Concentration**	**Volume required for 1 x 50 µl reaction (µl)**		
Zymotaq premix	-	25	25	25
Forward primer	5 µM	2	2	2
Reverse primer	5 µM	2	2	2
H_2O	-	17	16	16
$MgCl_2$	25 mM	2	3	3
Template DNA	-	2	2	2

Table 2-3 PCR Thermocycling conditions for pyrosequencing PCR

	Temp (˚C)	**Time (secs)**	**Cycles**
Initial Denaturation	95	600	1
Denaturation	95	30	40
Primer Annealing	48 (E2)	45	
	54 (L1/L2)		
	51 (Promoter)		
Extension	72	30	
Final Extension	72	600	1

2.6.3 Pyrosequencing Reaction

The majority of equipment and reagents required for pyrosequencing were supplied by Qiagen (Qiagen, Hilden, Germany) including the:

- PyroMark Q96 ID instrument
- PyroMark CpG Software v1.0.11
- PSQ96 Reagent cartridges
- PSQ Reaction plates
- PyroMark Vacuum Prep Workstation
- PyroMark Gold Q96 Reagents
- PyroMark Buffer solutions.

The streptavidin Sepharose beads were supplied by GE Healthcare Life Sciences (Buckinghamshire, UK).

PCR products were diluted by adding 13 µl of water to 27 µl of PCR product. The PyroMark Vacuum Prep Workstation was prepared by filling the 4 trays with the appropriate solution (tray 1 = 70% ethanol, tray 2 = denaturation buffer, tray 3 = 1:10 diluted wash buffer and tray 4 = de-ionised water).

Pyrosequencing was performed using a biotin labelled primer. PCR products were initially immobilised by adding 1.75 µl of streptavidin sepharose bead suspension and 38.25 µl of PyroMark Binding buffer to each reaction. The samples were then placed on a heated shaking plate for 5 minutes at 22 °C (1,400 rpm). Sequencing primers were made up by diluting 1.5 µl of 10 µM sequencing primer with 43.5 µl of PyroMark Annealing Buffer per reaction. This was then dispensed into each well of a PSQ reaction plate, which was in turn placed on the 'PSQ plate' compartment on the workstation. The PCR product was removed from the shaking plate and placed on the 'PCR plate' compartment of the workstation.

The vacuum tool was activated within 30 seconds of the PCR product being removed from the shaking plate and used to capture the PCR products (immobilised with sepharose beads) by lowering it into the PCR plate. The vacuum tool was then placed into each of the trays in the correct order for 5 seconds (tray 1-4). Finally the vacuum switch was closed and the tool lowered into the PSQ reaction plate (containing the sequencing primer) and agitated in order to release the captured PCR products. The result of these steps was denatured, single stranded PCR products. The PSQ plate, containing the beads and sequencing primers, was heated at 80 °C for 2 minutes and then cooled to room temperature.

Enzyme and substrate from the PyroMark Gold Q96 Reagent kit were reconstituted with the manufacturer's specified volume of water 10 minutes prior to use. The PSQ96 Reagent

Cartridge was loaded using the volumes specified by the pyrosequencing software taking care to ensure the appropriate solution went into the appropriate well. The cartridge was then loaded into the PyroMark Q96 ID instrument and the run was initiated. The software analysed the data on run completion, automatically generating a pyrogram for each pyrosequencing reaction. Following run completion, the workstation, vacuum tool and cartridge were cleaned as per the manufacturer's guidelines.

2.6.4 Primer Sequences

For each pyrosequencing assay, the primer sequences, regions amplified and dispensation order for sequencing are listed in Table 2.4.

Table 2-4 **Primer sequences, regions amplified and CpGs sequenced for the pyrosequencing assays.**

E2	
Forward primer	GTGAAATTATTAGGTAGTATTTGG
Reverse primer	*BTN-CAACAACTTAATAATATAACAAAAA
Sequencing primer	GTGAAATTATTAGGTAGTA
Dispensation order	ATTGCTATGATTCTGTCAGTCGATTATATAATGTCAGTCGTTTGGTGATCGAGAA TATAGTATCGATATTATGTCGA
Amplified DNA	148 bp (nt 3378-3525 of **NC001526.1)
Sequenced CpGs	***nt 3411, 3414, 3416, 3432, 3435, 3447 (targeting E2 binding sites 3 and 4)
Reference	Dr T Liloglou, University of Liverpool, personal communication (Dec 7th 2010)
Promoter	
Forward primer	GTAAAATTGTATATGGGTGT
Reverse primer	BTN-TAAAATATCTACTTTTATACTAACC
Sequencing primer	TAATTTATGTATAAAATTAAGG
Dispensation order	AGTCGTCGATCGTAATCGTGTATCGTAATCGTA
Amplified DNA	156 bp (nt 7832-83 of NC001526.1)
Sequenced CpGs	nt 31, 37, 43, 52, 58
Reference	Dr T Liloglou, University of Liverpool, personal communication (Dec 7th 2010)
L1/L2	
Forward primer	BTN-TTATTGTTGATGTAGGTGATTT
Reverse primer	CCCAATAACCTCACTAAACAACC
Sequencing primer	TAACCTCACTAAACAACCAA
Dispensation order	GCAAAAACAATCTAAAAAAATGATAATAAATCGATTATCGATACGATTTACGATAC
Amplified DNA	118 bp (nt 5551-5668 of NC001526.1)
Sequenced CpGs	nt 5615, 5609, 5606, 5600
Reference	(Bryant, Tristram *et al*. 2014)

*BTN refers to the position of a biotin label. **NC001526.1 is the HPV 16 reference sequence. The dispensation order is the order in which, nucleotides were dispensed. ***nt = nucleotide. Primer sequences are listed in 5'-3' orientation. All primers used were HPLC purified.

2.6.5 Data Analysis and Quality Assurance

Each sample was repeated in duplicate within a run in order to improve data reliability and to achieve a 'pass' result for as many samples as possible. The pyrosequencing software runs internal control checks and the output report defines each sample run as either a 'pass' (blue), a 'check' (yellow) requiring human analysis in order to determine acceptability of data, or 'fail' (red). All failed runs were excluded from analyses. 'Check' runs were included following manual analysis if the generated histogram matched the expected pre-defined histogram.

A standard deviation was calculated for each sample with duplicate runs for each CpG site analysed. This data was used to demonstrate the intra-run reproducibility of the assay and also to further improve the quality of the data; samples were excluded from further analysis if this value was beyond 3 standard deviations of the mean standard deviation calculated for all CpG sites for each region (Table 2.5).

Table 2-5 Quality control analysis for methylation data.

	Mean SD for each CpG site	SD of the mean
E2 CpG1	1.56	1.71
E2 CpG2	0.84	1.15
E2 CpG3	0.92	1.03
E2 CpG4	1.06	1.17
E2 CpG5	1.09	1.39
E2 CpG6	1.37	1.9
L1L2 CpG1	1.39	1.98
L1L2 CpG2	2.49	7.44
L1L2 CpG3	2.04	3.15
L1L2 CpG4	1.56	2.28
Promoter CpG1	0.32	0.72
Promoter CpG2	0.37	0.79
Promoter CpG3	0.51	1.03
Promoter CpG4	0.64	0.87
Promoter CpG5	0.43	1.27

2.7 Quantitative Real-Time Reverse Transcriptase PCR (qRT-PCR)

Quantitative real-time, reverse transcriptase PCR was used for gene expression analysis. Two human genes, *HPRT* and *TBP* and five HPV genes, *E2*, *E4*, *E5E6* and *E7*, were assessed

in all RT3 VIN baseline samples. The HPV genes were selected based on their role in the pathogenesis of HPV associated disease and their role as potential biomarkers in treatment with the trial medications (cidofovir and imiquimod).

2.7.1 Reverse Transcription (cDNA Synthesis)

Reverse transcription was performed using random primers, and the SuperScript® III Reverse Transcriptase (RT) kit (Invitrogen, Paisley, UK). Reagents for the first stage of the RT reaction included 1 µl of random primers at 200 ng/µl, 1 µl of dNTP's at 10 mM, 0.5 µg of RNA (DNase treated) and RNase free water to make up the final volume of 13 µl. The mix was incubated at 65 °C for 5 minutes and then kept on ice for at least 1 minute. This was followed by the addition of 1 µl of Dithiothreitol (DTT) at 0.1M, 4 µl of FS buffer at 5 x concentration, 1 µl of RNase OUT and 1 µl SuperScript® III RT (1 µl) to the mixture and mixed by repeat pipetting to complete the RT reaction. The mixture was incubated at 25 °C for 5 minutes, 50 °C for 60 minutes and then inactivated at 70 °C for 15 minutes. In order to assess amplification due to undigested genomic DNA, which could affect the accuracy of results later in the experiment, all reaction were performed in triplicate, 2 reactions with complete RT reaction and 1 reaction in which the SuperScript® III RT was replaced by H_2O.

2.7.2 Quantitative Real Time Reverse Transcriptase (qRT-PCR) Reaction Protocol

qRT-PCR was performed using the LightCycler carousel-based qPCR system, LightCycler® DNA Master SYBR Green I reagent kits and LightCycler® reaction capillary tubes (Roche Applied Science, Mannheim, Germany). Initially the FS mix was made by adding 10 µl of reagent 1a to a full vial of defrosted reagent 1b (kept at 4 °C and never re-frozen). Primer sequences are described in Table 2.6. The qRT-PCR mastermixes (Table 2.7) were prepared in a cooling block. 18 µl of the mastermix was then dispensed into the appropriate number of glass capillary tubes and 2 µl of the appropriate cDNA added. The capillaries were briefly centrifuged (2,000 rpm for 5 seconds) to ensure the sample was collected in the stem of the tube. Each run included a water negative control and a triplicate repeat of 1:100 diluted CaSki cDNA. RT negative controls were included for each sample.

Capillaries were loaded into the LightCycler® and subjected to the appropriate reaction conditions, the conditions differed in terms of the primer annealing temperature for each primer set but otherwise were constant (Table 2.8).

Table 2-6 Description of primers used for RT-qPCR

HPRT Primer		E6 Primer		E4 Primer	
Name	Hypoxanthine guanine phosphoribosyl transferase	**Sense Primer**	CTGCAATGTTTCAGGACCCA	**Sense Primer**	AACGAAGTATCCTCTCCTGAAATTATTAG
Sense Primer	TGACACTGGCAAAACAATGCA	**Antisense Primer**	TCATGTATAGTTGTTTGCAGCTCTGT	**Antisense Primer**	CCAAGGCGACGGCTTTG
Antisense Primer	GGTCCTTTTCACCAGCAAGCT	**Amplified DNA/RNA**	80 bp(NT 99-178 of NC001526.1***)	**Amplified DNA/ RNA**	82 bp (NT 3362-3426 of NC001526.1)
Amplified DNA	4982 bp* (NT 133627546-133632438 of chr 6)	**Reference**	Wang-Johanning, L *et al* 2002(6)	**Reference**	Roberts, Ng *et al* 2008(7)
Amplified RNA	94 bp (NT** 496-589 of M31642.1)	**E7 Primer**		**E2 Primer**	
Reference	Allen *et al*, 2008(4)	**Sense Primer**	AAGTGTGACTCTACGCTTCGGTT	**Sense Primer**	AACGAAGTATCCTCTCCTGAAATTATTAG
TBP Primer		**Antisense Primer**	GCCCATTAACAGGTCTTCCAAA	**Antisense**	CCAAGGCGACGGCTTTG
Name	TATA binding protein, 2nd pair of primers (TBP2)	**Amplified DNA/RNA**	78 bp (NT 739-816 of NC001526.1)	**Amplified**	82 bp (nt 3362-3426 of NC001526.1)
Sense Primer	TCAAACCCAGAATTGTTCTCCTTAT	**Reference**	Wang-Johanning, Lu *et al* 2002(6)	**Reference**	(Roberts N *et a*l. 2008)
Antisense Primer	CCTGAATCCCTTTAGAATAGGGTAGA	**E5 Primer**			
Amplified DNA	803 bp (NT 170880539-170881341 of chr 6)	**Sense Primer**	CCGCTGCTTTTGTCTGTGTC		
Amplified RNA	122 bp (NT 1128-1224 of M55654.1)	**Antisense**	GCAGAGGCTGCTGTTATCCA		
Reference	Minner and Poumay 2008(5)	**Amplified**	76 bp (nt 3939-4015)		

bp = base pair, nt = nucleotide, NC001526.1 – HPV 16 genbank reference sequence.

Table 2-7 qRT-PCR mastermixes

		Volume per Primer Pair (µl)			
Reagent	**Concentration**	**HPV *E2/E6/E4* HPRT**	**HPV *E5***	**HPV *E7***	***TBP***
Forward Primer	5 µM	2	2	2	2
Reverse Primer	5 µM	2	2	2	2
FS Mix	-	2	2	2	2
$MgCl_2$	25mM	1.6	1.2	2	2.4
Water	-	10.4	10.8	10	9.6
Template cDNA	(1:10 dilution cDNA, 1:100 dilution for Caski positive control cDNA)	2	2	2	2

Table 2-8 PCR Thermocycling conditions used for qRTPCR

	Temp (°C)	**Time (secs)**	**Cycles**
Initial Denaturation	95	600	1
Denaturation	95	10	60
Primer Annealing	58 (*E2,E4*) 60 (*E5, E6, HPRT, TBP*) 62 (E7)	5	
Extension	72	5	
	Temp (°C)	**Rate of Change (°C/sec)**	**Cycles**
Melting Curve	65-95	0.1	1

2.7.3 Data Analysis and Quality Assurance

CT (threshold cycle) values were exported from the LightCycler software. All data analysis was done using qBase+ software (Biogazelle NV, Zwijnaarde, Belgium) and expression levels were calculated relative to the average for the specific gene in question across the entire cohort. Samples were excluded from analysis if they generated a non-specific PCR product or if the crossing point threshold of the RT negative control was within 5 points of the RT positive reaction. Relative quantification was performed using the two human reference genes (HPRT and TBP) using the PCR efficiency corrected equation described by Vandesompele *et al*. 2002.

Crossing point values for *E2* and *E6* expression for each sample were used to calculate the *E2:E6* expression ratio. This ratio was then utilised in an exploratory capacity to represent cases with regulated (*E2>E6*) expression and deregulated (*E6>E2*) expression. This was done on the basis that expression of *E6* in the cohort studied was relatively constant but the expression of *E2* varied significantly. With the understanding that *E2* regulates the expression of *E6*, relatively low or absent *E2* expression could represent deregulated oncogene expression and relatively high *E2* expression could represent regulated oncogene expression.

The qBase+ software incorporates internal quality controls, which were strictly adhered to. To further improve data reliability all RT positive reactions were repeated in duplicate within a run. All samples that generated data indicative of inadequate RT reaction were excluded from analysis.

Chapter 3

Results

RT3 VIN

(A Randomised Trial into Topical Treatment of Vulval Intraepithelial Neoplasia)

3 RT3 VIN– A randomised Trial of Topical Treatment in Vulval Intraepithelial Neoplasia

3.1 Introduction

This chapter details the methods and clinical results from the phase II clinical trial that the work described in this thesis contributed to. The RT3 VIN trial (Tristram *et al*, 2014), aimed to investigate the safety and efficacy of topical treatment of VIN with cidofovir and imiquimod (Appendix2).

This trial was funded by Cancer Research UK (CRUK/06/024). All patients provided written informed consent prior to randomization. Appropriate regulatory approvals were obtained from the UK Medicines and Healthcare products Regulatory Agency (21323/0020/001-0001), the Office for Research Ethics Committees Northern Ireland (08/NIR03/82) and NHS Research and Development departments at participating sites. The RT3VIN trial was sponsored by Cardiff University and coordinated by the Wales Cancer Trials Unit (WCTU) at Cardiff University. This trial is registered with International Standard Randomized Controlled Trial Number 34420460.

I was a member of the trial management group, and contributed to patient recruitment and follow-up .The work described in this thesis comprised the majority of the HPV testing and analysis conducted for the trial. The clinical trial design and methods are first outlined followed by the results. Specifically, this chapter describes response rates to treatment with both cidofovir and imiquimod and the relevant safety data. It also details correlations between clinical outcome and certain characteristics of the patient and disease, performed to determine whether they demonstrate potential as clinical markers to predict response to treatment. Results of the HPV testing in relation to clinical outcome are discussed in later chapters.

3.2 Methods

3.2.1 Trial Design

To assess the activity, safety and feasibility of treatment of VIN 3 with topical cidofovir and 5% concentration imiquimod, a phase II randomised controlled trial was designed. UK-wide, multi-centre recruitment was established and in total, 32 centres were involved. Ethical approval was obtained from the Research Ethics Committees Northern Ireland (08/NIR03/82). Regulatory approval was also obtained from the Medicines and Healthcare Products Regulatory Agency (MHRA) under the Medicines for Human Use (Clinical Trials) Regulations 2004 S.I 2004/1031 (reference number 21323/0020/001-

0001). Approval was also obtained from Research and Development at each of the 32 participating hospitals. The RT3VIN trial was sponsored by Cardiff University and co-ordinated by the Wales Cancer Trials Unit (WCTU) at Cardiff University. This trial was registered with International Standard Randomised Controlled Trial Number 34420460.

The primary endpoint for the trial was histologically confirmed complete response by 6 weeks from termination of treatment. Secondary endpoints included treatment toxicity as well as treatment compliance.

3.2.2 Patient Recruitment and Randomisation

All patients were required to provide informed consent in order to participate in the trial. **Table 3-1** describes the criteria used for patient inclusion and exclusion. The WCTU carried out randomisation. Patients were allocated to a treatment arm at a 1:1 ratio and stratified by hospital, unifocal or multi-focal disease and first-episode or recurrent disease.

3.2.3 Trial Schema

Once written consent was obtained and eligibility criteria checked, a series of baseline assessments were conducted prior to the commencement of treatment including a medical history, assessment of toxicities, clinical assessment of lesions and urinanalysis to check kidney function. Urinanalysis was undertaken due to the potential nephrotoxicity of cidofovir. A 4mm punch biopsy stored in ThinPrep media for HPV testing was also taken. Patients then commenced a course of treatment with the assigned trial medication for a maximum of 24 weeks. Patients were seen every 6 weeks during treatment at which time treatment concordance was checked, clinical assessment of lesions was performed, toxicities were assessed, and urinanalysis was undertaken. Six weeks after completion of treatment, another two 4mm punch biopsies were taken, one for histology and one for HPV testing, clinical assessment of the lesions, kidney function tests, haemoglobin and white blood cell count tests were also performed. Patients who showed a complete response to their originally allocated treatment were then assessed at 6 monthly intervals for a further 2 years, at which time assessment of toxicities, clinical examination +/- biopsy took place (**Figure 3.1**). All histology samples were examined and reported on by the histopathology department at the relevant participating site for each patient.

Table 3-1 RT3 VIN inclusion/exclusion criteria

Inclusion Criteria	Exclusion Criteria
• Female >16 years • Biopsy proven VIN 3 (including visible peri-anal disease not extending into the anal canal), biopsy to have been taken within the previous three months • At least one lesion that could be accurately measured (using RECIST criteria) in at least one dimension with longest diameter ≥ 20mm or two perpendicular dimension that when multiplied together came to greater than 120mm^2 • Using a reliable method of contraception if of childbearing age (excluding condoms) • Written informed consent to participate in trial (Participant Information Sheet and Consent Form 2)	• Any patient with impaired renal function (defined as serum creatinine> 133 µmol/l or > 1.5 mg/dl) • Any patient with current anogenital carcinoma or any patient who, in the PIs opinion, is at a high risk of developing invasive disease (patients in whom invasive disease or micro-invasive disease is suspected should have adequate biopsies to exclude this prior to entry) • Pregnant, breast feeding or trying to conceive • Active treatment for VIN within the previous four weeks • Known allergy to either of the topical treatments or any components in either the imiquimod or cidofovir gel • Unable to comply with protocol treatment • Previous failure of imiquimod or cidofovir following treatment three times a week for a minimum of 12 weeks.

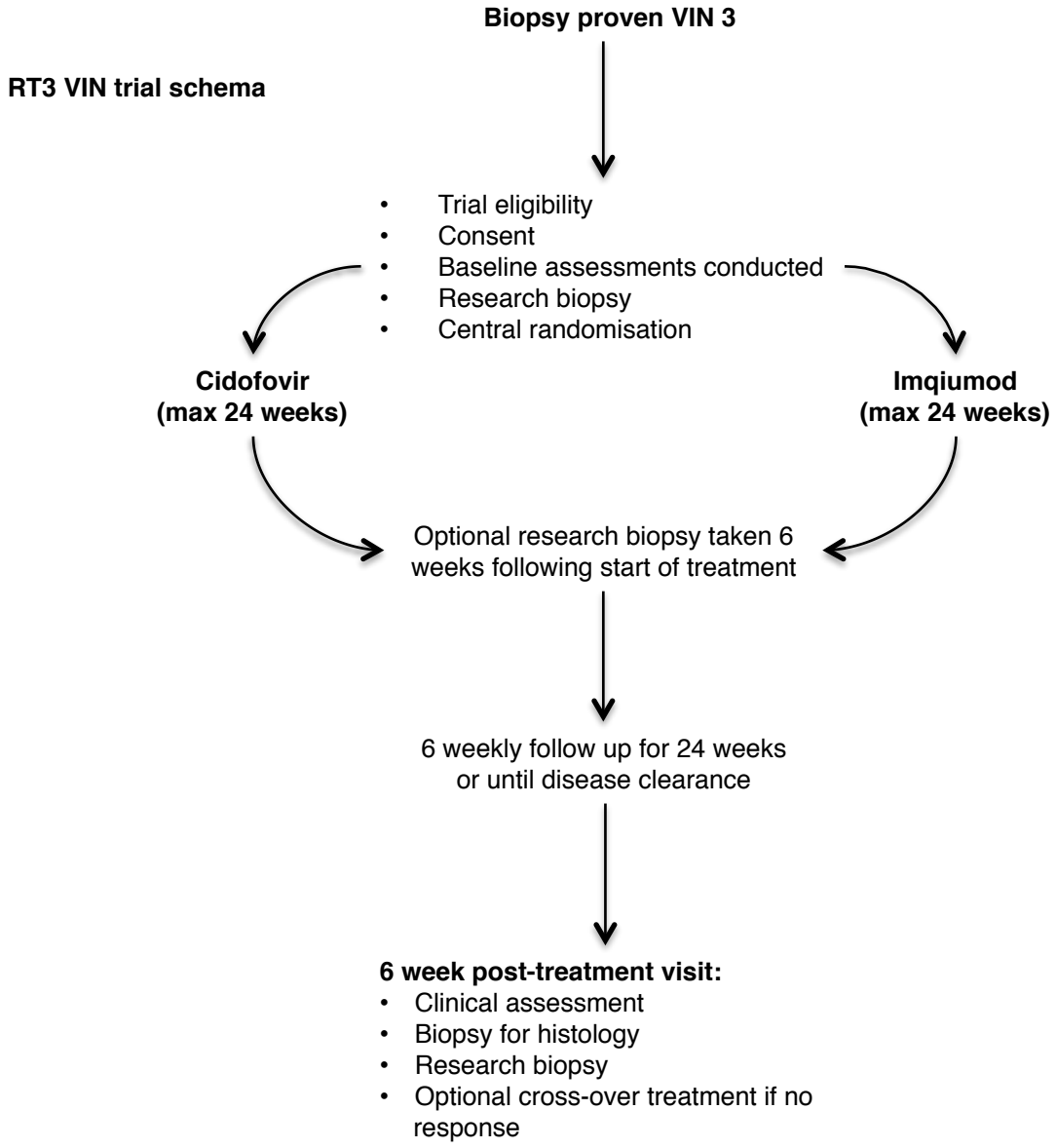


Figure 3.1 RT3 VIN trial schema

3.2.4 HPV Biopsies

Biopsies for HPV testing were taken before the start of treatment and 6 weeks following cessation of treatment. In some cases, patients consented to have an additional biopsy taken at 6 weeks into the course of treatment for translational research.

The 4mm punch biopsies were taken from the site of disease adjacent to the biopsy taken for histology and, in the majority of cases (n=330), were placed into a transportation pot containing ThinPrep media. Upon arrival in the laboratory sample were stored at -20 °C prior to DNA and RNA extraction. Extracted DNA and RNA was then stored at -20 ready for further testing. Towards the end of the trial the sample collection protocol was amended and collection media was changed to RNAlater with pre and post extraction storage at -80 °C, four samples included in this work were subject to this protocol.

3.3 Results

180 patients were recruited and randomised from a total of 32 UK based centres.

3.3.1 Patient Demographics

Data was collected from all 180 cases for demographic analysis including age, smoking status and immunocompromise. The mean age of patients in this cohort was 47.2 years (range 20-81 years) (Figure 3.2). Current smoking was reported by 106/180 (58.8%) patients, 42/180 (23.3%) patients were previous smokers and 32/180 (17.7%) had never smoked (Figure 3.3). A history of immunocompromise was recorded in 9/180, four of these patients were HIV positive and the five remaining patients had another form of immunocompromise.

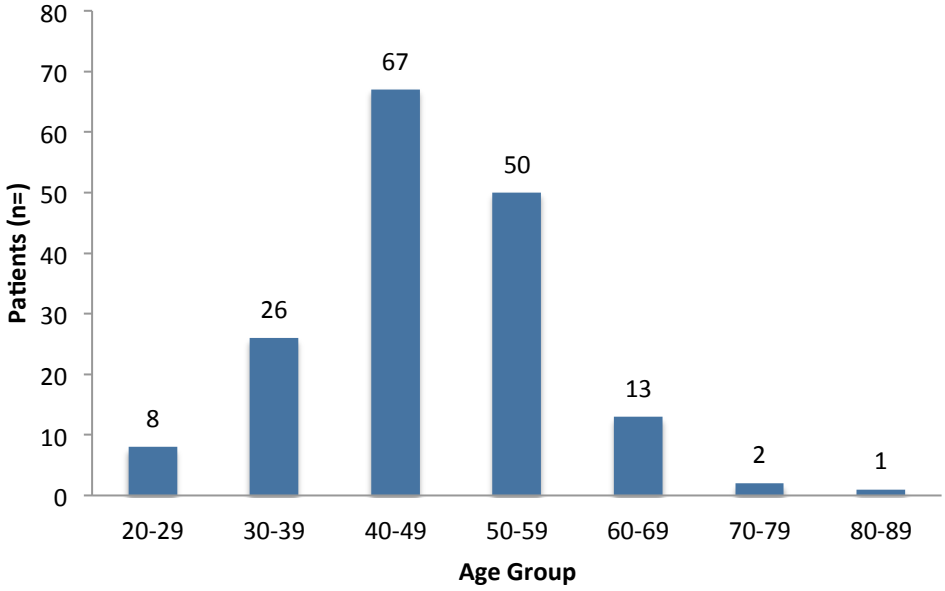


Figure 3.2 RT3 VIN study cohort age distribution (n=180)

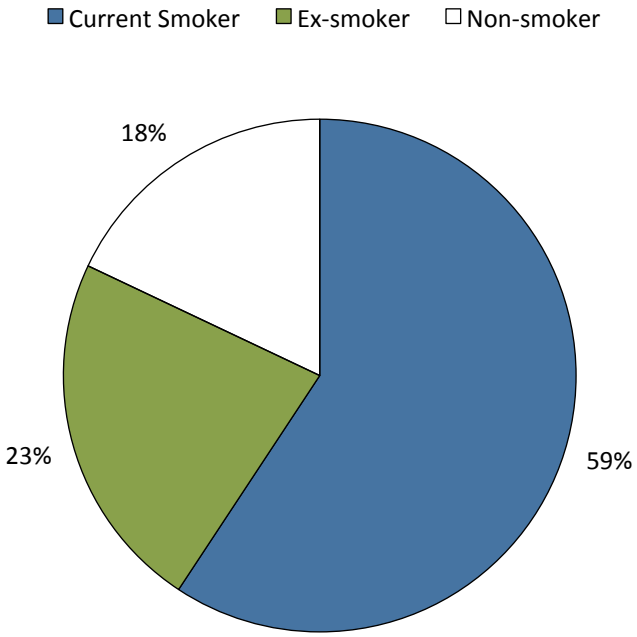


Figure 3.3 RT3 VIN study cohort smoking status (n=180)

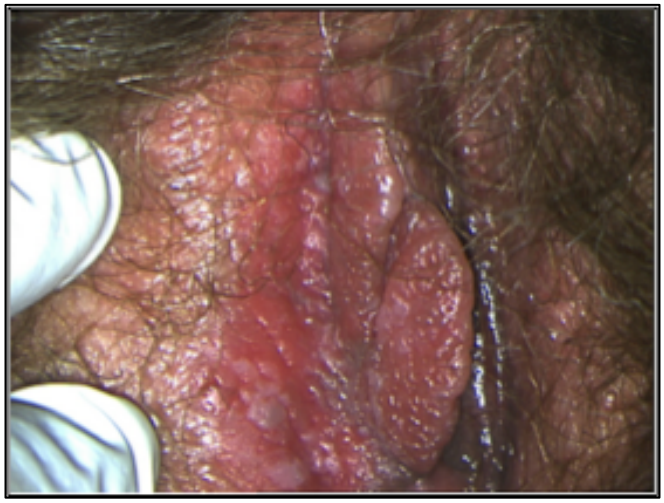
3.3.2 Clinical Characteristics of VIN 3

Data was collected on clinical characteristics of the disease for all 180 patients recruited and comprised unifocal or multi-focal lesions, first episode or recurrent episode and additional anogenital site (single site/multiple site). Unifocal disease was present in 89/180 (49.4%) of patients; 91/180 (50.6%) patients had multi-focal disease. First presentation episodes were seen in 98/180 (54.4%) and recurrent presentations were seen in 82/180 (45.6%).

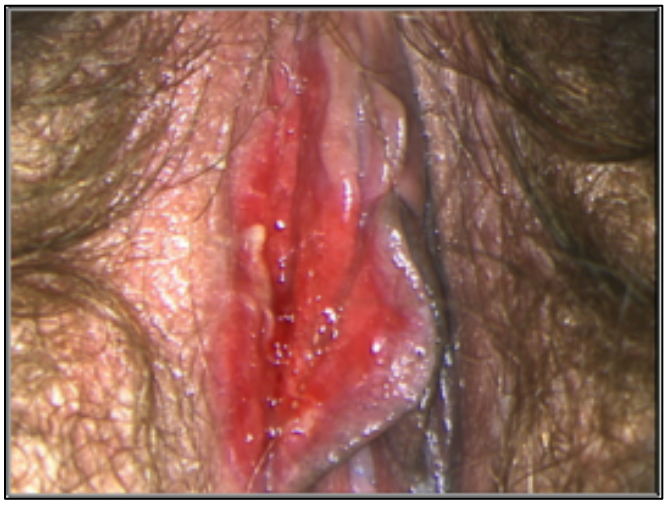
3.3.3 Response to treatment

Of the 180 patients, 89 were randomised to treatment with cidofovir and 91 patients were randomised to treatment with imiquimod. Patient demographic factors and clinical disease characteristics were equally distributed between the two treatment arms as demonstrated in Table 3-2. The flow chart in **Figure 3.5** demonstrates the clinical outcome of the two arms of the trial. Clinical outcome data was available for 72 patients treated with cidofovir (three patients were lost to follow up, 12 patients withdrew from the study and no post-treatment biopsy was available for two patients) and 69 patients treated with imiquimod (five patients were lost to follow up, 15 patients withdrew from the study and no post treatment biopsy was available for two patients). In the per protocol analysis, a complete response was reported in 41 of 72 (56.9%, 90% CIs: 46.6-66.9) women treated with cidofovir and 42 out of 69 (60.9%, 90% CIs: 50.3-70.7) women treated with imiquimod. Figure 3.4 shows photographs of disease prior to treatment with cidofovir (a), during treatment (B) and having healed following treatment (c).

It is of note that a history of smoking (present or previous) was reported by the majority of patients participating in the trial (148/180 82.2%). This is significantly higher than the 17% average smoking rates for the UK, female population reported for 2014 (www.ash.org.uk). It is likely that smoking plays a role in the aetiology of VIN. Data was not collected on change in smoking status over the course of the trial to correlate with response/resolution of VIN. This would be a worthy line of investigation in the future.



A



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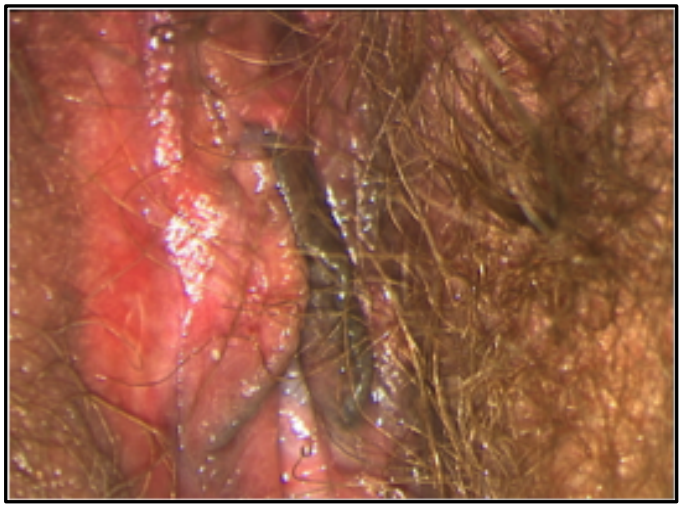


C

Figure 3.4 Photographic images of treatment with cidofovir. A = VIN prior to treatment, B = disease during treatment, C = healed tissue (disease-free) following treatment.

Table 3-2 Baseline characteristics by treatment arm in RT3 VIN

Baseline characteristic		Cidofovir Arm		Imiquimod Arm	
		n=	%	n=	%
No. Patients		89	49.4	91	50.6
Median age (range) in years		48 (26-74)		46 (20-81)	
Focality	Unifocal	44	49.4	45	49.5
	Multifocal	45	50.6	46	50.5
Recurrent disease	Yes	42	47.2	40	44
	No	47	52.8	51	56
Immunocompromise	Yes	3	3.4	6	6.6
	No	86	96.6	85	93.4
Smoking status	Current	50	56.2	56	61.5
	Previous	24	27	18	19.8
	Never	15	16.9	17	18.7

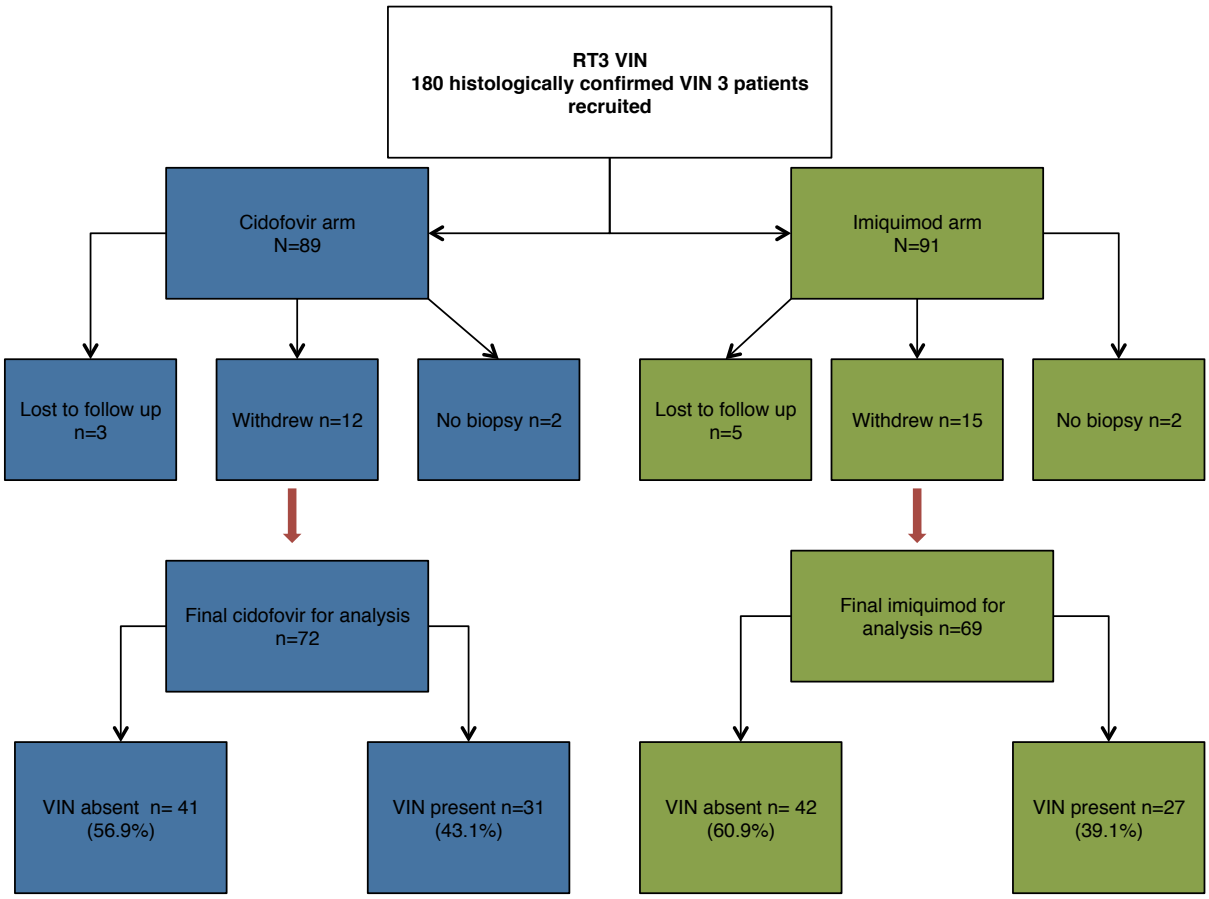


Figure 3.5 Flow chart depicting the outcome of 180 patients recruited into the RT3 VIN trial as per protocol analysis. Final analysis possible for 72 patients treated with cidofovir and for 69 patients treated with imiquimod. Histological presence of VIN was determined by a vulval punch biopsy of the previously affected area, 6 weeks following the termination of treatment.

3.3.4 Clinical markers predictive of response

Relationships were investigated between clinical characteristics and response to treatment to determine if a correlation was present indicating the potential of a clinical measure as a predictive marker of response. No correlation was found between focality of disease, episode type or smoking status and response to treatment in either treatment arm (Table 3.3).

3.3.5 Adverse events

Adverse events were recorded as per the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 (NCI CTCAE v.3.0). Overall, adverse events were commonly reported in both groups. Adverse events of grade 2 (moderate) or above were less common in the cidofovir arm than the imiquimod arm (72.6% (61/84) vs. 86.9% (73/84) respectively) p=0.021. Within the adverse event reports, most events were grade 2. Grade 3 (severe) toxicities were reported in 31/84 (36.9%) of patients in the cidofovir arm and 39.84 (46.4%) in the imiquimod arm. There were no deaths during the course of the trial.

Table 3-3 Clinical characteristics and smoking status correlation with treatment outcome

	Cidofovir arm			Imiquimod arm		
	n (%)			n (%)		
Patients randomised	89			91		
Per protocol population	72 (80.9)			69 (75.8)		
Unifocal/multifocal	**VIN absent (n)**	**VIN present (n)**	**p-value**	**VIN absent (n)**	**VIN present (n)**	**p-value**
Unifocal	24	13	0.163	20	13	0.966
Multifocal	17	18	X^2=1.95	22	14	X^2=0.00
First/recurrent disease	**VIN absent (n)**	**VIN present (n)**	**p-value**	**VIN absent (n)**	**VIN present (n)**	**p-value**
First	22	17	0.921	24	13	0.465
Recurrent	19	14	X^2=0.01	18	14	X^2=0.53
Smoking status	**VIN absent (n)**	**VIN present (n)**	**p-value**	**VIN absent (n)**	**VIN present (n)**	**p-value**
Current smoker	24	15	0.392	23	16	0.713
Previous or non-smoker	17	16	X^2=0.73	19	11	X^2=0.14

3.4 Conclusion

The clinical response data in the RT3 VIN trial has demonstrated adequate efficacy and safety of both cidofovir and imiquimod as potential alternatives to surgery in women with VIN. This data warrants further clinical research in the context of a phase III trial.

No patient demographic variable or disease characteristics demonstrated roles as potential biomarkers. The clinical response rates of approximately 50% in both of the treatment arms justifies further investigation of predictive biomarkers in order to better target therapy in the future. Viral factors would be an obvious parameter for investigation.

Chapter 4

Results

HPV Biology in VIN 3

4 HPV biology in VIN 3

4.1 Introduction

The aim of this work was to contribute to worldwide research improving the understanding of HPV driven disease, aiding in the development of health policies and novel therapies in the future. A growing body of research, investigating HPV in VIN exists but this study represents the largest, high-grade disease, single-study cohort to date investigating multiple aspects of HPV biology.

The specific aims of this chapter were to determine HPV genotype specific prevalence in VIN 3; to investigate viral characteristics (gene expression, HPV disruption and HPV methylation) in VIN 3; to establish relationships between viral characteristics and to investigate HPV methylation and integration as mechanisms leading to deregulated oncogene expression. Investigation into the biomarker potential of these viral characteristics in the treatment of VIN is presented in the chapter 5.

4.2 HPV Prevalence in VIN 3

Two methodologies were chosen to investigate HPV prevalence in VIN 3. Firstly, an HPV 16 type-specific *E6* PCR (HPV 16 *E6* PCR) was used to identify cases of HPV 16, as this is well documented as the most prevalent genotype in VIN. In addition, the Greiner PapilloCheck® assay was used to determine non-HPV 16 genotype prevalence. Biopsies for analysis were from 167 patients with histologically confirmed VIN 3 participating in the RT3 VIN clinical trial, as described in chapter 3. HPV 16 was detected in 135/167 (80.8%) cases using HPV 16 *E6* PCR, (Figure 4.1 is an example gel image). The PapilloCheck® assay identified an additional 18 HR HPV positive cases. HPV 33 was the second most prevalent genotype, detected in 10/167 cases; followed by HPV genotypes 18, 44/55 (low risk) and 70, each detected in 3/167 cases. The majority of HPV positive cases contained a single HPV genotype 124/136 (91.2%) whereas multiple genotypes were only detected in 12/136 (8.8%) cases (11 of which included HPV 16). The non-HPV 16-genotype prevalence and distribution amongst cases of single and multiple genotype infections is shown in (Figure 4.2). There were three cases (1.8%) in the cohort that only tested positive for a low-risk HPV genotype. Importantly, it was also noted that HPV 16 was only detected in 110/167 (65.9%) cases by the PapilloCheck® assay (25 cases less than the HPV 16 *E6* PCR).

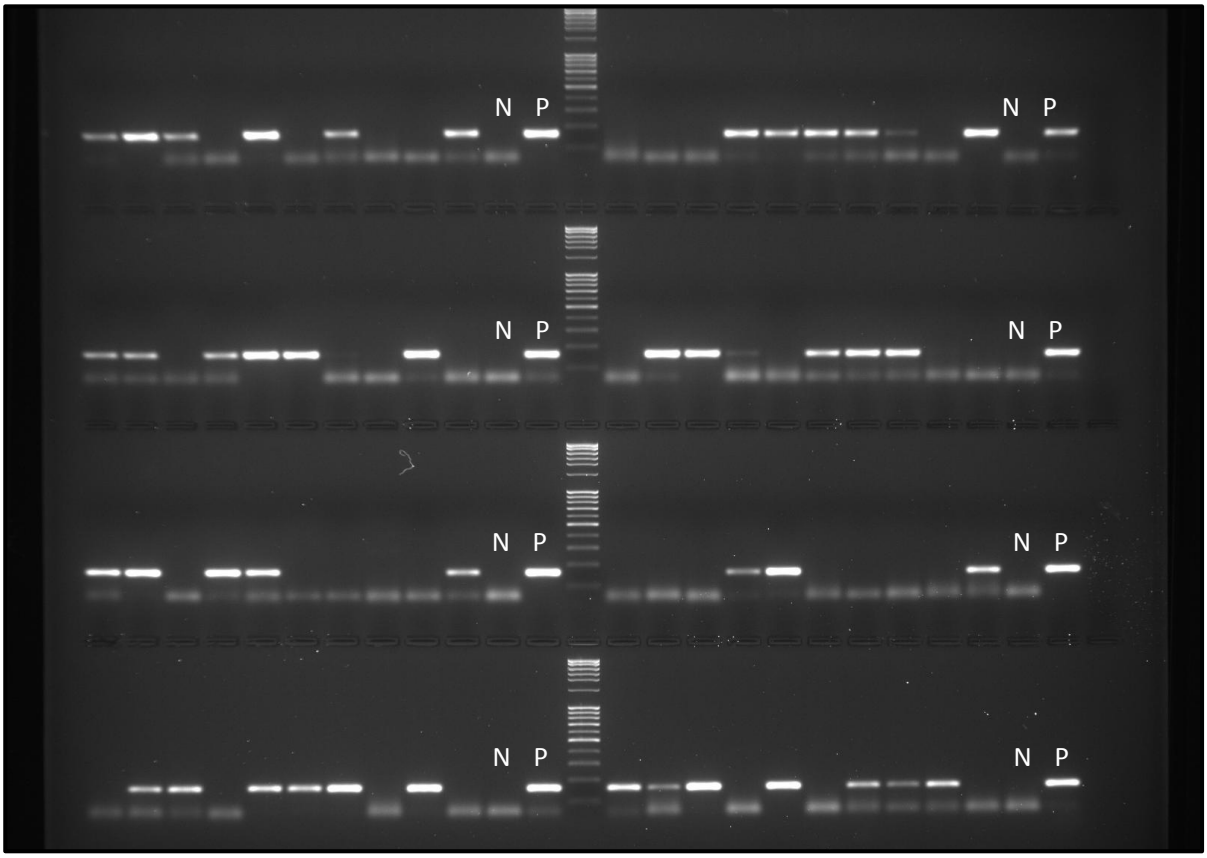


Figure 4.1 HPV 16 E6 PCR electrophoresis gel image. A 100 bp ladder was used (centre of the gel). The HPV 16 E6 fragment is 161 bp N=negative control (H_2O) and P=positive control (CaSki).

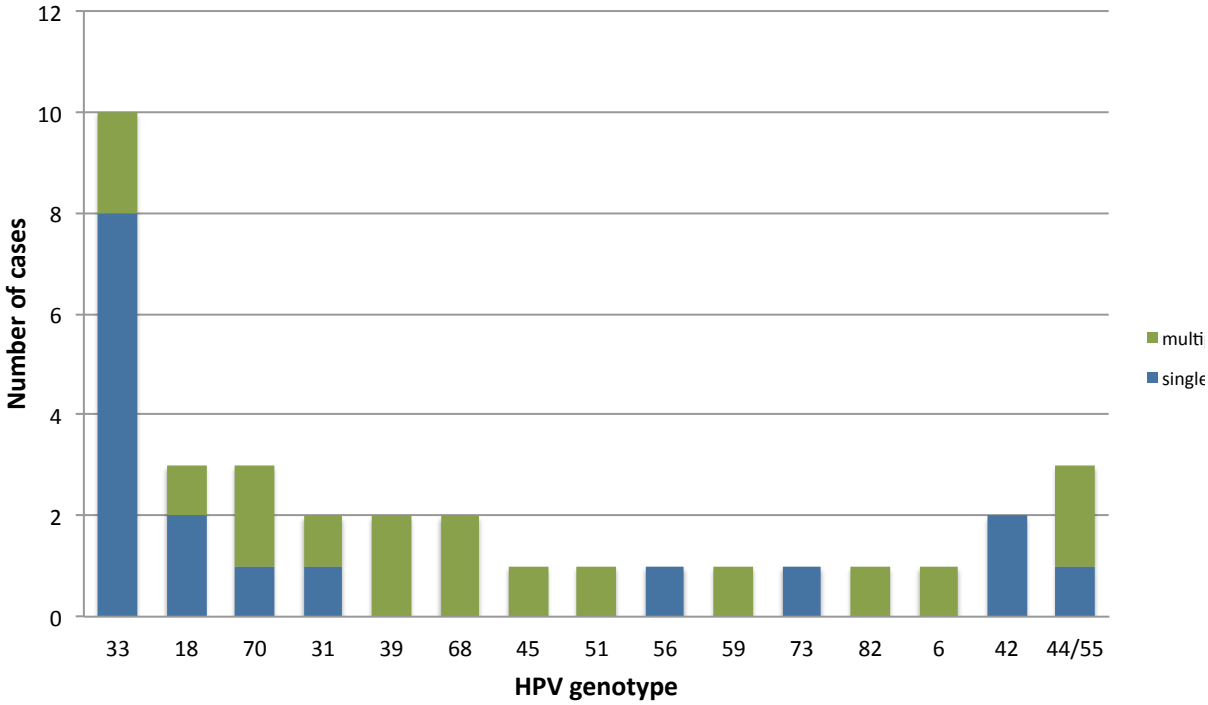


Figure 4.2 Non HPV 16 genotype prevalence in VIN 3 - PapilloCheck® data. Showing the number of cases of each genotype detected in a cohort of 167 cases of histologically confirmed VIN 3. HPV 44/55, 42, and 6 are low-risk genotypes, all remaining genotypes are high-risk. Green areas of columns represent genotype prevalence in cases testing positive for multiple HPV genotypes and blue areas represent genotype prevalence in cases testing positive for a single genotype. 12 cases testing positive for multiple genotypes were identified in the cohort, 11 of which contained HPV 16. Nb for HPV 44/55, the PapilloCheck® assay is unable to differentiate between HPV 44 and HPV 55 therefore this result indicates the presence of either genotype.

4.2.1 PCR Target Region Disruption Leads to False Negative HPV Results

Comparison of the HPV 16 prevalence data generated by the PapilloCheck® assay and the HPV 16 *E6* PCR highlighted a disparity. The HPV 16 *E6* PCR detected 25 more cases of HPV 16 than the PapilloCheck® assay. Quality assurance analysis of each assay revealed good reproducibility. Twenty-five per cent of cases were randomly selected for duplicate testing using the PapilloCheck® assay and generated data that was 98% concordant; and 20% of cases were randomly selected for the HPV 16 *E6* PCR generating 100% concordant results (see methods, chapter 2). Additionally, the human *ADAT-1* gene was successfully amplified in all cases (this provides a positive control for DNA integrity within the PapilloCheck® assay). It was hypothesised that disruption in the HPV *E1* open reading frame (the PCR target region of the PapilloCheck® assay) would be present in the 25 discordant cases, confirming them to be false negative results. A two-fragment HPV 16 specific *E1* tiling PCR was designed to identify disruption in the PCR target region and test this hypothesis (chapter 2). The *E1* region was deemed intact if both fragments were amplified, and disrupted if one or no fragments were amplified.

4.2.1.1 HPV 16 *E1* Tiling PCR Data

E1 disruption was detected in 77/136 (56.6%) HPV 16 positive cases identified by PapilloCheck® and HPV 16 *E6* PCR combined. In all 25 cases where HPV 16 was detected by the type-specific *E6* PCR but not by the PapilloCheck® assay, disruption of the *E1* region was present. This finding confirmed that disruption in the *E1* region of the virus in VIN 3 is a common event and an important cause of false negative results from the PapilloCheck® assay. It is notable that *E1* disruption did not always prevent the PapilloCheck assay from detecting the virus. In 21 cases, PapilloCheck® detected the presence of HPV 16 despite *E1* disruption; in 20 of these cases, the HPV 16 *E6* PCR assay also generated a positive result and in one it generated a negative result (thought to be a false positive HPV 16 result from PapilloCheck®, discussed later). In all cases where both PapilloCheck® and HPV 16 *E6* PCR were HPV 16 negative (n=31), neither *E1* fragment was amplified (Table 4.1).

Table 4–1 *E1* disruption data in RT3 VIN baseline cohort

HPV 16 test results	n=	*E1* intact (%)**	*E1* disrupted (%)*
PapilloCheck® +ve and HPV 16 *E6* PCR +ve	110	90 (81.8)	20 (18.2)
PapilloCheck® +ve and HPV 16 *E6* PCR -ve	1	0	1 (100)
PapilloCheck® -ve and HPV 16 *E6* PCR +ve	25	0	25 (100)
PapilloCheck® -ve and HPV 16 *E6* PCR -ve	31	0	31 (100)
Total	167	90	77

**E1* disruption was defined as absence of one or more fragments of the E1 tiling PCR; ***E1* intact defined as the presence of both tiling fragments of *E1* tiling PCR.

4.2.2 Further analysis of HPV negative results

Combined data generated from the PapilloCheck® assay and the HPV 16 *E6* PCR gave an overall (all genotypes) HPV prevalence rate of 153/167 (91.6%). Results of the *E1* analysis demonstrated a false negative rate of 14.9% for the detection of HPV 16 specifically using PapilloCheck®. It was hypothesised that non-HPV 16 genotypes could also go undetected by the PapilloCheck® assay as a result of viral disruption. Two additional HPV detection assays, targeting different regions of the virus were sequentially run on the HPV negative samples to try to minimise the number of false negative results. The DiaMex HPV typing kit utilising Luminex technology targeting the *L1* region was run on the initial 14 HPV negative samples followed by an HPV *E7* PCR for genotypes 16, 18, 31, 33, 35 and 51 on any negative samples thereafter. Figure 4.3 summarises the method used to minimise false negative results. Results are detailed below.

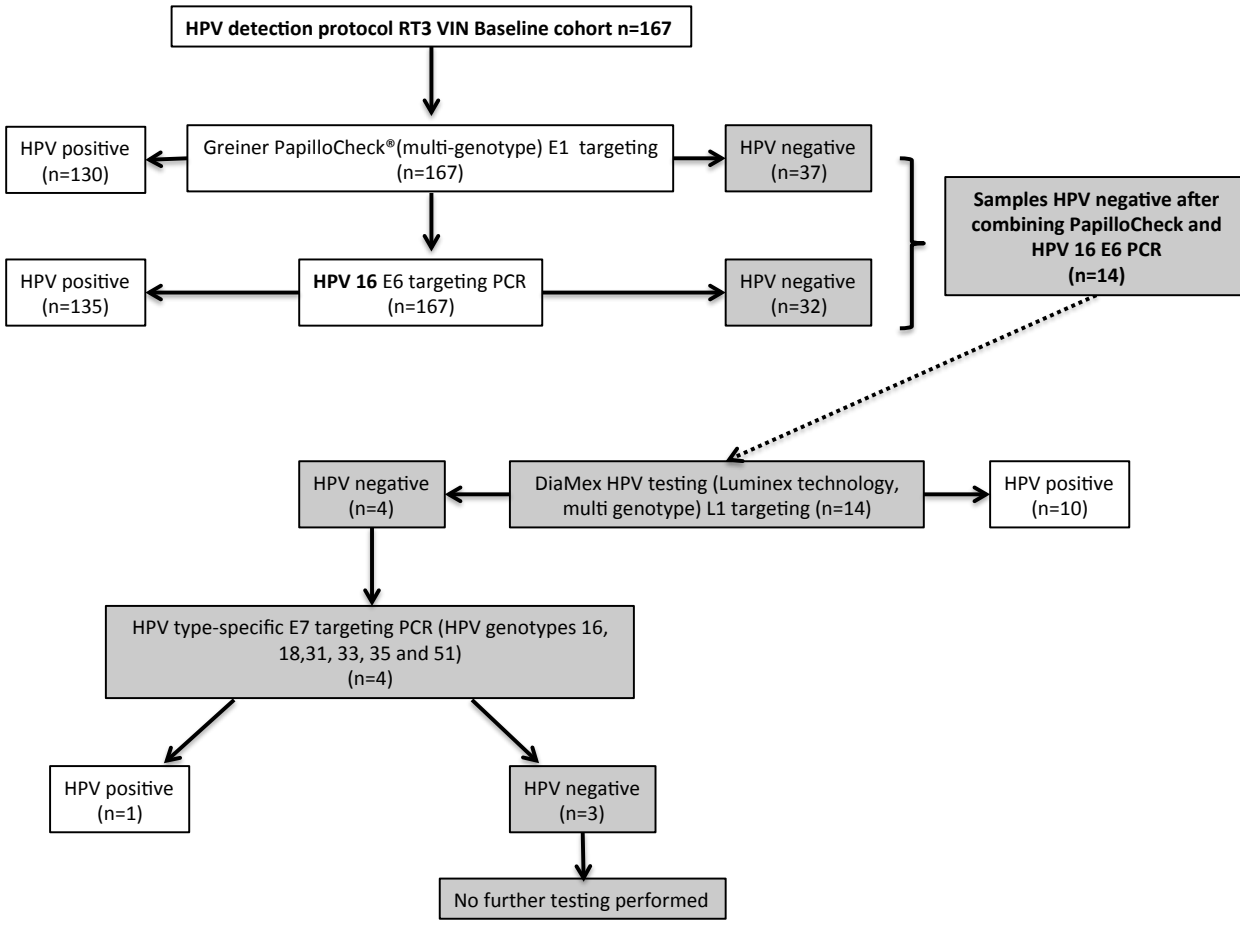


Figure 4-3Summary of results and protocols used for HPV testing of the RT3 VIN baseline cohort (n=167). Results include high risk and low risk genotypes when tested for. Final HPV prevalence = 164/167 (98.2%)

4.2.2.1 DiaMex HPV genotyping data

Samples testing HPV negative following PapilloCheck® and HPV 16 *E6* PCR (n=14) were sent to the Scottish Human Papillomavirus Virus Reference laboratory in Edinburgh for further testing using the DiaMex HPV typing kit. This assay targets the *L1* region of the virus and can identify multiple genotypes (HPV 6, 11, 42, 43, 44, 70, 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82) (see methods chapter 2). It was chosen at this stage to ensure that remaining DNA was utilised efficiently, conserving as much as was possible for downstream analyses. Ten additional samples were sent for analysis that had already generated a positive HPV result with either the PapilloCheck® and/or the HPV 16 *E6* PCR assay.

The DiaMex assay detected HPV in 10/14 (71.4%) of the previously HPV negative samples. This included two cases of HPV 16, three cases of HPV 33, one case of HPV 51, one case of HPV 73 and three cases of mixed infection (two of which contained HPV 16 and 33 and the other contained HPV 42 and 51), (Table 4.2). Of the ten control samples that were sent, eight generated results that were concordant with previous genotyping. Two cases (samples 23 and 24 in Table 4.2) generated disparate results. Both these samples tested HPV 16 positive with PapilloCheck® and HPV 16 *E6* PCR. However, DiaMex identified Sample 23 as HPV 42 positive. This could be explained by the presence of HPV 16 integration disrupting the L1 region with conserved *E1* and HPV 42 integration disrupting *E1* with conserved *L1*. Sample 24 generated a positive HPV 16 result with all three assays but only the DiaMex assay detected the presence of HPV 42, similarly this could be explained by HPV 42 integration disrupting the *E1* region with conserved *L1*.

Table 4–2 HPV genotyping data using the DiaMex assay

RT3 VIN Sample*	PapilloCheck®	HPV 16 *E6* PCR	DiaMex HPV result
Sample 1	Negative	Negative	16
Sample 2	Negative	Negative	16
Sample 3	Negative	Negative	33
Sample 4	Negative	Negative	33
Sample 5	Negative	Negative	33
Sample 6	Negative	Negative	51
Sample 7	Negative	Negative	73
Sample 8	Negative	Negative	16,33
Sample 9	Negative	Negative	16,33
Sample 10	Negative	Negative	42,51
Sample 11	Negative	Negative	Negative
Sample 12	Negative	Negative	Negative
Sample 13	Negative	Negative	Negative
Sample 14	Negative	Negative	Negative
Sample 15	42	Negative	42
Sample 16	42	Negative	42
Sample 17	16	16	16
Sample 18	16	16	16
Sample 19	16	16	16
Sample 20	16	16	16
Sample 21	16	16	16
Sample 22	16	16	16
Sample 23**	16	**16**	42
Sample 24***	16	**16**	16,42

*Results obtained from a total of 24 samples sent for DiaMex HPV testing from the RT3 VIN cohort. Samples 1-14 tested HPV negative by PapilloCheck® and HPV 16 E6 PCR; samples 15-24 tested HPV positive and were sent as positive controls. **, *** Samples 23 and 24 generated disparate results likely due to disruption in assay target regions.

4.2.2.2 HPV E7 type specific PCR for genotypes 16, 18, 31, 33, 35 and 51

To this point, 4/167 (2.4%) cases consistently tested HPV negative. It was possible that viral disruption affecting multiple viral regions was still preventing HPV being detected. Type-specific PCRs targeting the well-conserved *E7* region of the virus, using primers described by Walboomers *et al*, 1999 for HPV genotypes 16, 18, 31, 33, 35 were applied to these samples. HPV 33 was detected in one of the four samples, the remaining 3 samples (3/167, 1.8%) continued to test HPV negative.

4.2.3 Overall HPV Genotype specific prevalence

Combined data from the four assays applied to the cohort indicated an overall HPV prevalence of 164/167 (98.2%). HPV 16 was the most common genotype, present in 140/164 (85.4%) cases. A single genotype was detected in 150/164 (91.5%) cases and multiple genotypes were detected in 14/164 (8.54%) cases. Of the cases with single genotypes, 128/150 (85.3%) were HPV 16, HPV 33 was the second most prevalent genotype with 12/150 (8%) infections (Figure 4.4). Of the 14 cases of multiple genotypes, all but two cases included HPV 16; HPV 33 was the next most prevalent, present in 4/14 in conjunction with HPV 16. There were three cases of HPV positivity in the cohort that could only be attributed to a low-risk genotype: two cases of HPV 42 and one cases of HPV 44/55 (PapilloCheck®).

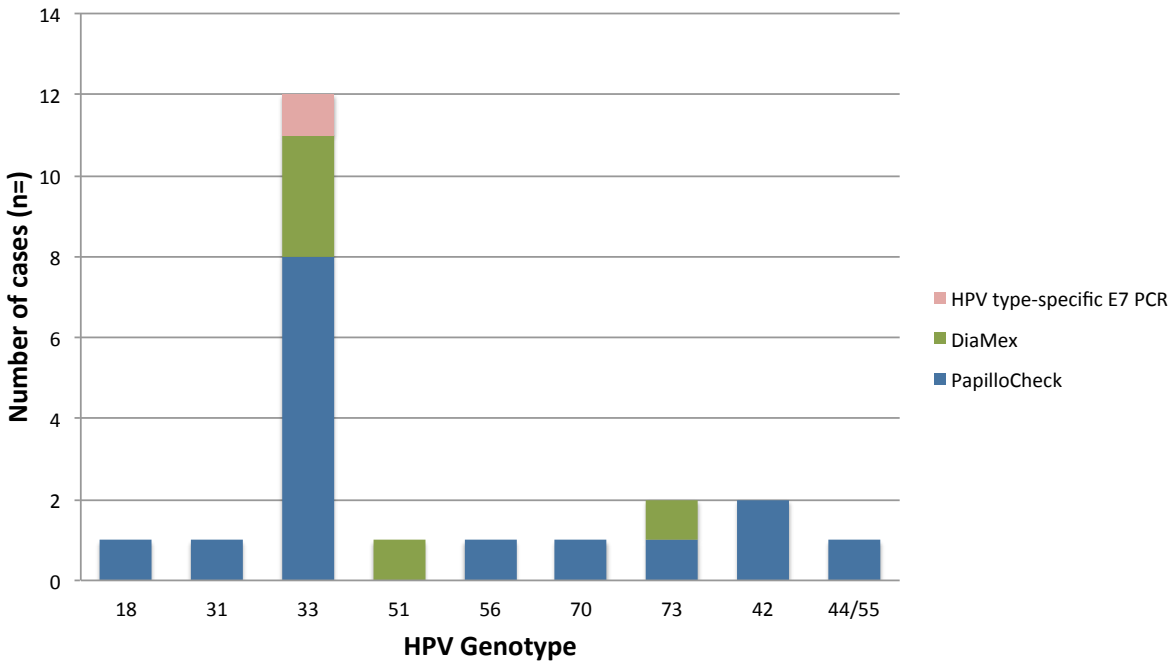


Figure 4.4 Non-HPV 16 genotype prevalence in single infections in RT3 VIN baseline cohort. Segments on bars represent the number of cases detected by each assay. HPV 44/55 are grouped as the PapilloCheck® assay is unable to distinguish these genotypes and therefore a positive result could indicate the presence of either genotype. HPV genotypes 42 and 44/55 represent low-risk HPV genotypes, the remaining genotypes are high-risk.

4.2.4 Summary of Findings

- The majority of cases of VIN 3 in the RT3 VIN cohort tested positive for the presence of HPV (164/167, 98.2%)
- HPV 16 was the most prevalent genotype, detected in 140/164 (85.4%) cases
- HPV 33 was the second most prevalent detected in 16/164 (9.8%) cases
- A single genotype was detected in the majority of cases 150/164 (91.5%)
- In 3/164 (1.8%) cases, only a low-risk genotype was identified

4.3 HPV Integration in VIN 3

HPV 16 positive cases detected by either PapilloCheck® or HPV 16 *E6* PCR were investigated for the presence of HPV integration (n=136). HPV 16 positive cases identified by these assays were selected, as these assays were those applied to all cases in the cohort. A novel test combining a two-fragment, tiling *E1* PCR and a commonly used five-fragment *E2* tiling PCR (Collins *et al*. 2009) was used to detect the presence of viral disruption. Addition of the *E1* tiling PCR was made on the premise that integration does not always affect the *E2* region of the virus (Bryant, Onions *et al*. 2014). A sample was considered to contain intact HPV if all seven PCR fragments were amplified; if ≥ 1 fragment failed to amplify, the sample was deemed to contain disrupted HPV. Cases of viral disruption were judged to represent cases of viral integration.

4.3.1 Prevalence of HPV Integration in VIN 3

Of the 136 cases tested, 71 (52.2%) failed to amplify all seven *E1/E2* fragments indicating the presence of viral integration. Intact virus was present in 65/136 (47.8%) cases (Table 4.3). Figure 4.5 and Figure 4.6 show example gel images.

E6 PCR was performed on all samples to confirm HPV DNA adequacy and was present in all cases except case number 1 in Table 4.3. Case number 1 was identified as HPV 16 positive using PapilloCheck® genotyping (*E1* targeting) but did not amplify *E6*, *E1* or *E2* and is therefore likely to represent a false positive PapilloCheck® result.

In cases with viral disruption present, 12/71 had disruption in the *E1* region and intact *E2*. Hence assessment of *E1* disruption, increased the number of cases of presumed viral integration detected from 59/136 (43.4%) to 71/136 (52.5%), an almost 10% increase in rate of detection (Table 4.4). No discernible pattern of HPV integration was apparent in terms of the location of HPV disruption within the *E1/E2* regions.

Table 4–3 HPV disruption in the RT3 VIN baseline cohort.

	HPV DNA control	Fragment								HPV DNA control	Fragment						
Case number	E6 result	E1-1	E1-2	E2-1	E2-2	E2-3	E2-4	E2-5	Case number	E6 result	E1-1	E1-2	E2-1	E2-2	E2-3	E2-4	E2-5
case 1*	0	0	0	0	0	0	0	0	case 69	1	1	0	1	1	1	1	1
case 2	1	0	0	0	0	0	0	0	case 70	1	0	1	1	1	1	1	1
case 3	1	0	0	0	0	0	0	0	case 71	1	0	1	1	1	1	1	1
case 4	1	0	0	0	0	0	0	0	case 72	1	1	1	1	1	1	1	1
case 5	1	0	0	0	0	0	0	0	case 73	1	1	1	1	1	1	1	1
case 6	1	0	0	0	0	0	0	0	case 74	1	1	1	1	1	1	1	1
case 7	1	0	0	0	0	0	0	0	case 75	1	1	1	1	1	1	1	1
case 8	1	0	0	0	0	0	0	0	case 76	1	1	1	1	1	1	1	1
case 9	1	1	0	0	0	0	0	0	case 77	1	1	1	1	1	1	1	1
case 10	1	1	0	0	0	0	0	0	case 78	1	1	1	1	1	1	1	1
case 11	1	1	0	0	0	0	0	0	case 79	1	1	1	1	1	1	1	1
case 12	1	1	0	0	0	0	0	0	case 80	1	1	1	1	1	1	1	1
case 13	1	1	0	0	0	0	0	0	case 81	1	1	1	1	1	1	1	1
case 14	1	1	0	0	0	0	0	0	case 82	1	1	1	1	1	1	1	1
case 15	1	1	0	0	0	0	0	0	case 83	1	1	1	1	1	1	1	1
case 16	1	1	0	0	0	0	0	0	case 84	1	1	1	1	1	1	1	1
case 17	1	1	0	0	0	0	0	0	case 85	1	1	1	1	1	1	1	1
case 18	1	1	0	0	0	0	0	0	case 86	1	1	1	1	1	1	1	1
case 19	1	1	0	0	0	0	0	0	case 87	1	1	1	1	1	1	1	1
case 20	1	1	0	0	0	0	0	0	case 88	1	1	1	1	1	1	1	1
case 21	1	1	0	0	0	0	0	0	case 89	1	1	1	1	1	1	1	1
case 22	1	1	1	0	0	0	0	0	case 90	1	1	1	1	1	1	1	1
case 23	1	1	1	0	0	0	0	0	case 91	1	1	1	1	1	1	1	1
case 24	1	1	1	0	0	0	0	0	case 92	1	1	1	1	1	1	1	1
case 25	1	1	1	0	0	0	0	0	case 93	1	1	1	1	1	1	1	1
case 26	1	1	1	1	0	0	0	0	case 94	1	1	1	1	1	1	1	1
case 27	1	1	1	1	0	0	0	0	case 95	1	1	1	1	1	1	1	1
case 28	1	1	1	1	0	0	0	0	case 96	1	1	1	1	1	1	1	1
case 29	1	1	0	0	1	0	0	0	case 97	1	1	1	1	1	1	1	1
case 30	1	1	1	1	1	0	0	0	case 98	1	1	1	1	1	1	1	1
case 31	1	1	1	1	1	0	0	0	case 99	1	1	1	1	1	1	1	1
case 32	1	1	1	0	0	0	1	0	case 100	1	1	1	1	1	1	1	1
case 33	1	0	0	1	1	1	1	0	case 101	1	1	1	1	1	1	1	1
case 34	1	0	0	1	1	1	1	0	case 102	1	1	1	1	1	1	1	1
case 35	1	1	0	1	1	1	1	0	case 103	1	1	1	1	1	1	1	1
case 36	1	1	1	1	1	1	1	0	case 104	1	1	1	1	1	1	1	1
case 37	1	1	1	1	1	1	1	0	case 105	1	1	1	1	1	1	1	1
case 38	1	1	1	1	1	1	1	0	case 106	1	1	1	1	1	1	1	1
case 39	1	1	1	1	1	1	1	0	case 107	1	1	1	1	1	1	1	1
case 40	1	1	1	1	1	1	1	0	case 108	1	1	1	1	1	1	1	1
case 41	1	1	1	1	1	1	1	0	case 109	1	1	1	1	1	1	1	1
case 42	1	1	1	1	1	1	1	0	case 110	1	1	1	1	1	1	1	1
case 43	1	1	1	1	1	1	1	0	case 111	1	1	1	1	1	1	1	1
case 44	1	1	1	1	1	1	1	0	case 112	1	1	1	1	1	1	1	1
case 45	1	1	1	1	1	1	1	0	case 113	1	1	1	1	1	1	1	1
case 46	1	1	1	1	1	1	1	0	case 114	1	1	1	1	1	1	1	1
case 47	1	0	0	0	0	0	0	1	case 115	1	1	1	1	1	1	1	1
case 48	1	0	0	0	0	0	0	1	case 116	1	1	1	1	1	1	1	1
case 49	1	0	0	0	0	1	0	1	case 117	1	1	1	1	1	1	1	1
case 50	1	1	0	0	0	1	0	1	case 118	1	1	1	1	1	1	1	1
case 51	1	1	0	1	1	1	0	1	case 119	1	1	1	1	1	1	1	1
case 52	1	1	0	0	0	0	1	1	case 120	1	1	1	1	1	1	1	1
case 53	1	1	1	1	0	0	1	1	case 121	1	1	1	1	1	1	1	1
case 54	1	0	0	0	0	1	1	1	case 122	1	1	1	1	1	1	1	1
case 55	1	1	1	0	0	1	1	1	case 123	1	1	1	1	1	1	1	1
case 56	1	1	1	0	0	1	1	1	case 124	1	1	1	1	1	1	1	1
case 57	1	0	0	0	1	1	1	1	case 125	1	1	1	1	1	1	1	1
case 58	1	1	0	0	1	1	1	1	case 126	1	1	1	1	1	1	1	1
case 59	1	1	1	0	1	1	1	1	case 127	1	1	1	1	1	1	1	1
case 60	1	0	0	1	1	1	1	1	case 128	1	1	1	1	1	1	1	1
case 61	1	0	0	1	1	1	1	1	case 129	1	1	1	1	1	1	1	1
case 62	1	0	0	1	1	1	1	1	case 130	1	1	1	1	1	1	1	1
case 63	1	0	0	1	1	1	1	1	case 131	1	1	1	1	1	1	1	1
case 64	1	0	0	1	1	1	1	1	case 132	1	1	1	1	1	1	1	1
case 65	1	0	0	1	1	1	1	1	case 133	1	1	1	1	1	1	1	1
case 66	1	1	0	1	1	1	1	1	case 134	1	1	1	1	1	1	1	1
case 67	1	1	0	1	1	1	1	1	case 135	1	1	1	1	1	1	1	1
case 68	1	1	0	1	1	1	1	1	case 136	1	1	1	1	1	1	1	1

E1-1 and *E1*-2 refer to the two tiling fragments of the *E1* gene PCR and *E2*-1 to *E2*-5 refers to the five tiling fragments of the E2 gene PCR. Any case with at least one fragment absent ('0' in the table, highlighted red) was regarded as containing integrated HPV. A number '1' (highlighted green) indicates successful amplification of the fragment in question. Case number 1* represents a case that repeatedly tested positive for HPV 16 using the PapilloCheck® but failed to amplify or any of the *E1* or fragments. It is likely that this is a false positive PapilloCheck result.

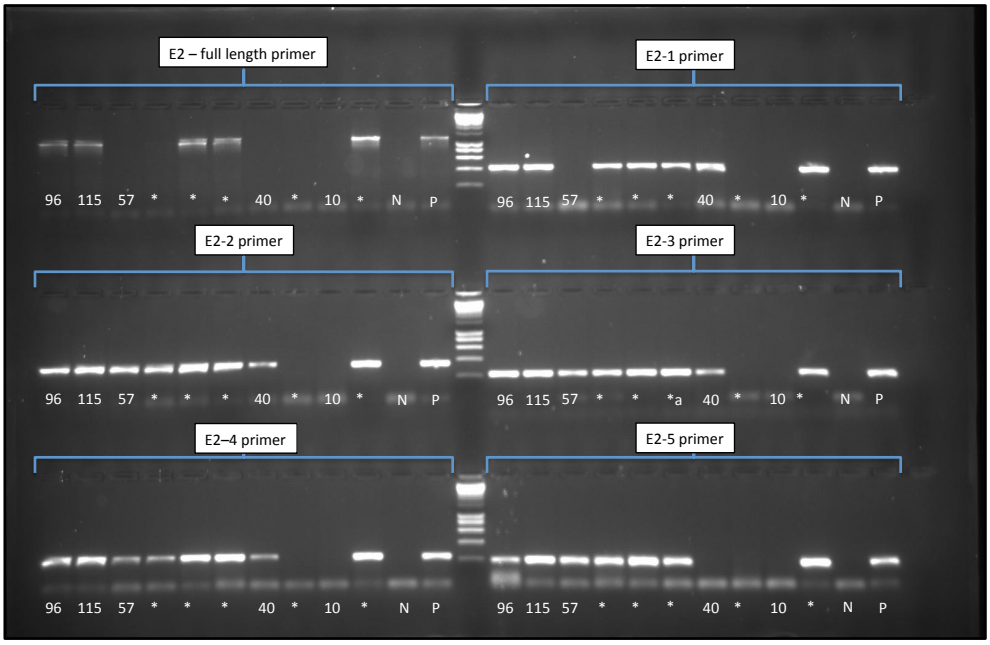


Figure 4.5 Example E2 tiling PCR gel image. Results for cases 96, 115, 57, 40, and 10 are shown. Cases marked with * indicate samples not part of the study. N=negative control (H_20), P=positive control (CaSki)

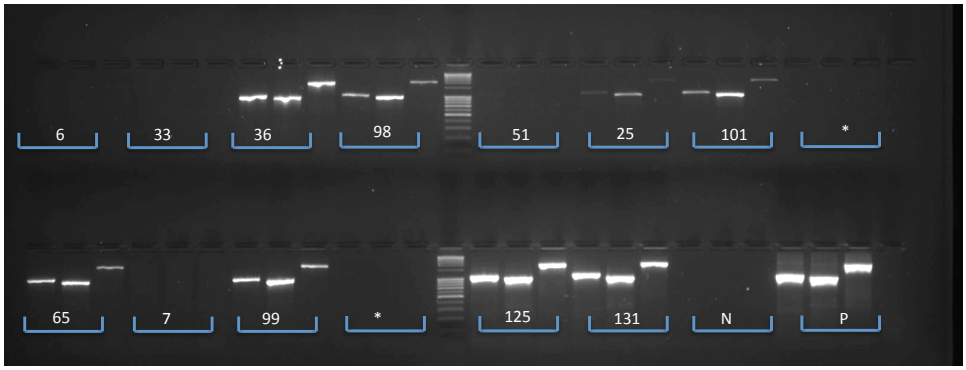


Figure 4.6 Example E1 tiling PCR gel image. The first fragment band generated for each sample represents the E1-1 fragment, the second represents the E1-2 fragment and the third represents the full-length fragment that was not included in this analysis. Results for cases 6, 33,36,51,25,101, 65,7,99,125 and 131 are shown. Cases marked with an * represent samples that were part of this study.

Table 4–4 Comparison of E1 and E2 tiling result

		E2 result		
		E2 intact	**E2 disrupted**	**Total**
E1 result	**E1 intact**	65	25	90
	E1 disrupted	12	34	46
	Total	77	59	136

4.3.2 Viral Integration, Patient Age, Smoking Status and Disease Episode

It was hypothesised that viral integration would be more common with increased age, positive smoking history and recurrent disease. A Bonferroni correction was applied to account for multiple comparisons; a p-value of <0.01 was considered significant. The median patient age for cases identified as containing integrated HPV (absence ≥ one *E1/E2* tiling fragments) was 47 years and with intact HPV was 45 years; this difference was not significant (U = 1,823, z = -2.11 and p = 0.05). A positive smoking history was found in 57/71 (80.3%) and 55/65 (84.6%) of women with integrated virus and intact virus respectively. This finding was not statistically significant (χ^2 = 0.439, p=0.508). Finally, no statistically significant relationship was identified between viral integration and disease episode with viral integration being present in 32/63 cases of recurrent disease and 39/73 cases of first episode disease, (χ^2 = 0.094, p = 0.759).

4.3.3 Summary of Findings

- HPV integration defined by the absence of ≥1 *E1/E2* PCR tiling fragments was present in 71/136 (52.2%) HPV 16 positive cases of VIN 3
- Adding an *E1* tiling PCR to the more commonly used *E2* assay increased detection of integrated HPV by 9.1%
- No statistically significant relationships were identified between smoking status, age or recurrent disease and HPV integration

4.4 HPV DNA Methylation in VIN 3

HPV DNA methylation levels were investigated using pyrosequencing in all HPV 16 positive cases identified by PapilloCheck® and/or HPV 16 *E6* PCR (n=136). Three regions of the virus were of interest: the *E2* region, the *L1/L2* overlapping region, and the promoter region. The *E2* and *L1/L2* regions were chosen due to reports in the literature of their methylation being potential biomarkers of high-grade disease (Mirabello, Schiffman *et al*. 2012). The promoter region was investigated due to promoter methylation being a potential alternative mechanism leading to deregulated oncogene expression. Six CpG sites were analysed in the *E2* region, four in the *L1/L2* region and five sites in the promoter region. Analysable data was available for a different number of cases for each region (*E2* = 82, *L1/L2* = 93 and promoter = 122). This was a result of quality control checks applied to the data and impacted more on the *E2* and *L1/L2* regions due to the increased likelihood of disruption in these PCR target regions; this is discussed further in the discussion of this chapter.

4.4.1 Regional HPV DNA Methylation

For each case, the methylation level at each region (*E2*, *L1/L2* and promoter) was calculated by taking the mean value generated from the CpG sites within the region in question. A minimum of two valid CpG readings for each region was required following the application of quality assurance checks (see methods chapter 2). To determine if methylation at each region varied significantly between cases within the cohort, a one-sample T-test was used. Kolmogorov-Smirnov (KS) and Shapiro-Wilk (SW) statistics were used to see if the data was normally distributed. Despite not following normal distribution for any of the analysed regions (*E2* methylation KS=0.35, p=<0.000 and SW=0.68, p=<0.000; L1L2 methylation KS=0.33, p=<0.000 and SW=0.72, p=<0.01; promoter methylation KS=0.42, p=<0.002 and SW=0.29, p=<0.02), the one-sample t-test was still used due to its robustness at coping with breaches in required assumptions (Markowski, Markowski 2012). A Bonferroni correction to account for multiple comparisons was applied and a p-value of < 0.01 was considered significant.

Methylation at each region was found to be variable in the cohort with the *E2* and *L1/L2* exhibiting the most variation and the promoter region, the least. For the *E2* region (n = 82) mean methylation was 27.2% ± 35.8% (p = <0.000), for the *L1/L2* region (n = 93), mean methylation was 37.5% ± 34.9% (p = <0.000) and for the promoter region (n = 122), mean methylation was 3.9% ± 13.44% (p = <0.002), (Figure 4.7).

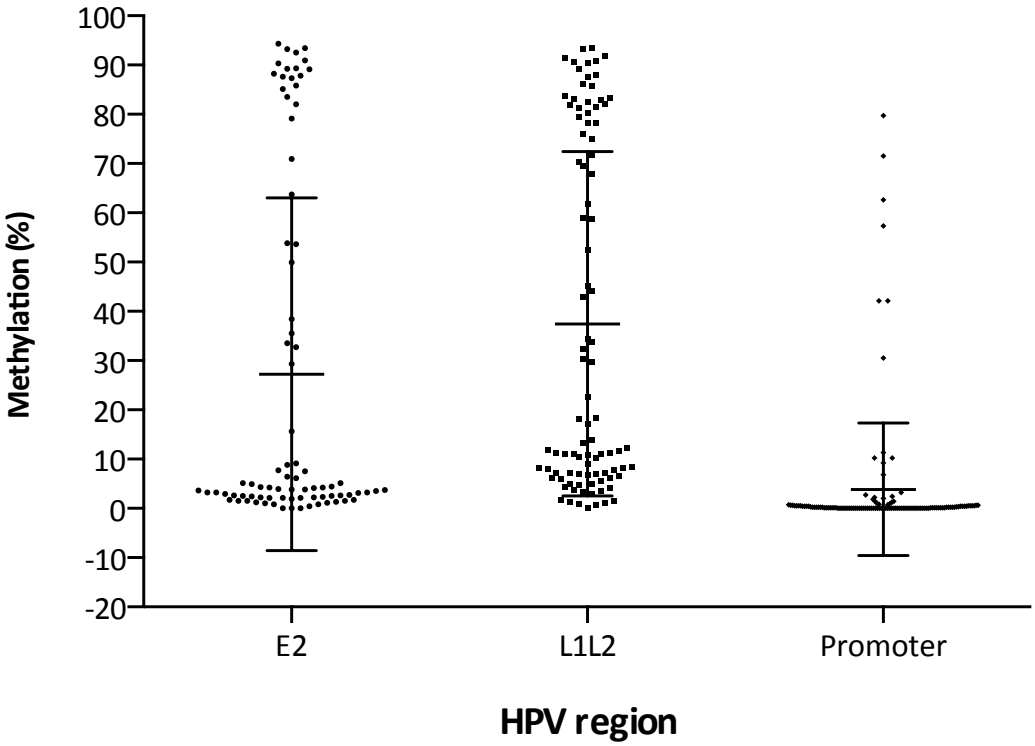


Figure 4.7.Variation of regional HPV methylation in the RT3 VIN cohort. The mean value was calculated for each region from all CpG sites tested. Error bars represent the mean and standard deviation. *E2* mean = 26.3%±35.6, L1L2mean = 28.0%±32.1, promoter mean = 3.1±11.6.

4.4.2 Inter-regional Methylation

Sixty-one cases generated data for all three tested HPV regions and were therefore suitable for investigating differences in inter-regional HPV DNA methylation. The median level of methylation was 4.2% for the *E2* region, 6.2% for the *L1/L2* region and 0.16% for the promoter region. In order to determine if the methylation level varied significantly between regions, a Friedman's two-way analysis of variance was used to compare the mean rank at each region. There was a statistically significant difference in methylation between the three regions $\chi^2(2)=92.96$, p=<0.001. Post hoc analysis with Wilcoxon signed-rank tests were conducted with a Bonferroni correction applied, resulting in a significant level set at $p < 0.016$. Statistically significant differences in methylation existed between all three regions: between the *E2* and *L1/L2* region Z = -3.519, p = <0.001, between the *E2* and promoter region Z= -7.404, p = <0.001 and between the *L1/L2* and promoter regions Z = -8.101, p = <0.001. Methylation was typically higher in the *E2* and *L1/L2* region and lower at the promoter region (Figure 4.8).

It was also hypothesised that trends would exist in the methylation levels of the different regions i.e. as methylation of the *E2* region increased so would methylation of the *L1/L2* region. Pearson's correlation coefficient was used to investigate this. A strong positive correlation was shown between methylation of the *L1/L2* region and the *E2* region as illustrated in Figure 4.9 (Pearson Correlation = 0.97, p=<0.000).A weakly positive correlation was found between *E2* methylation and promoter region methylation (Pearson's correlation = 0.42, p=<0.000) and *L1L2* methylation and promoter region methylation respectively (Pearson's Correlation = 0.32, p=<0.002).

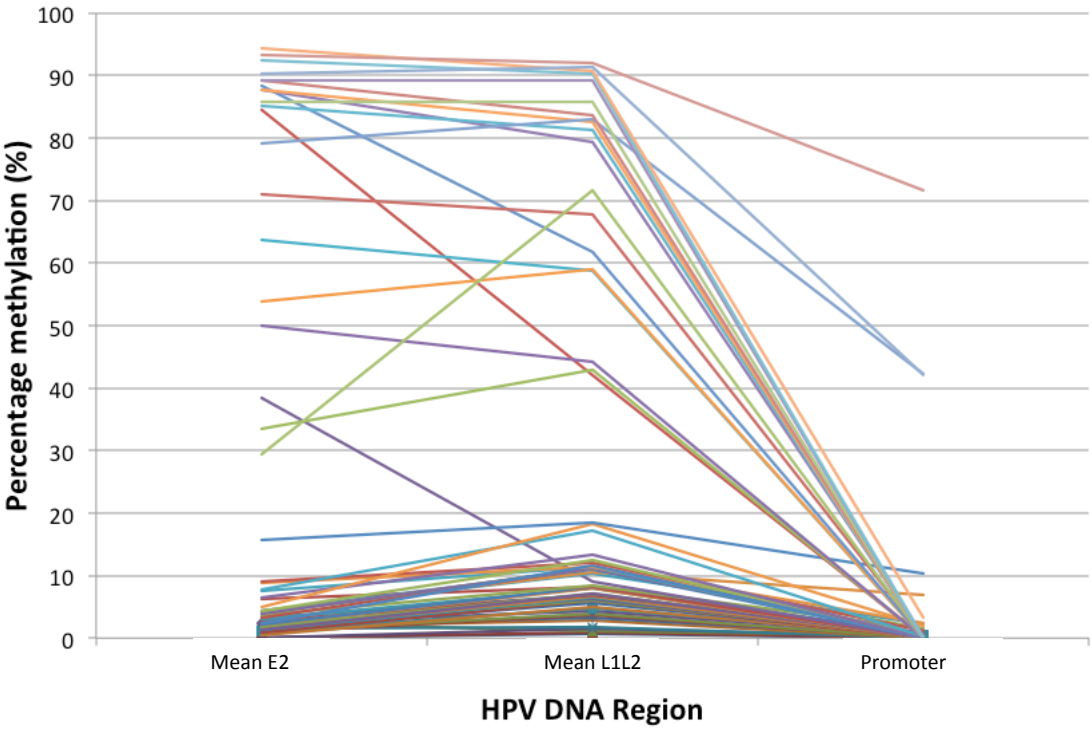


Figure 4.8 Case-by-case analysis of inter-regional variation of HPV DNA methylation. Mean methylation for each case was calculated from all CpG sites within the region in question. Points are linked to aid visualisation of trends within samples. Methylation of the *E2* and *L1/L2* regions was generally similar in samples whereas promoter region methylation was lower. Friedman's two way analysis of variation found the differences in mean rank of methylation between the three regions to be statistically significant p=<0.000.

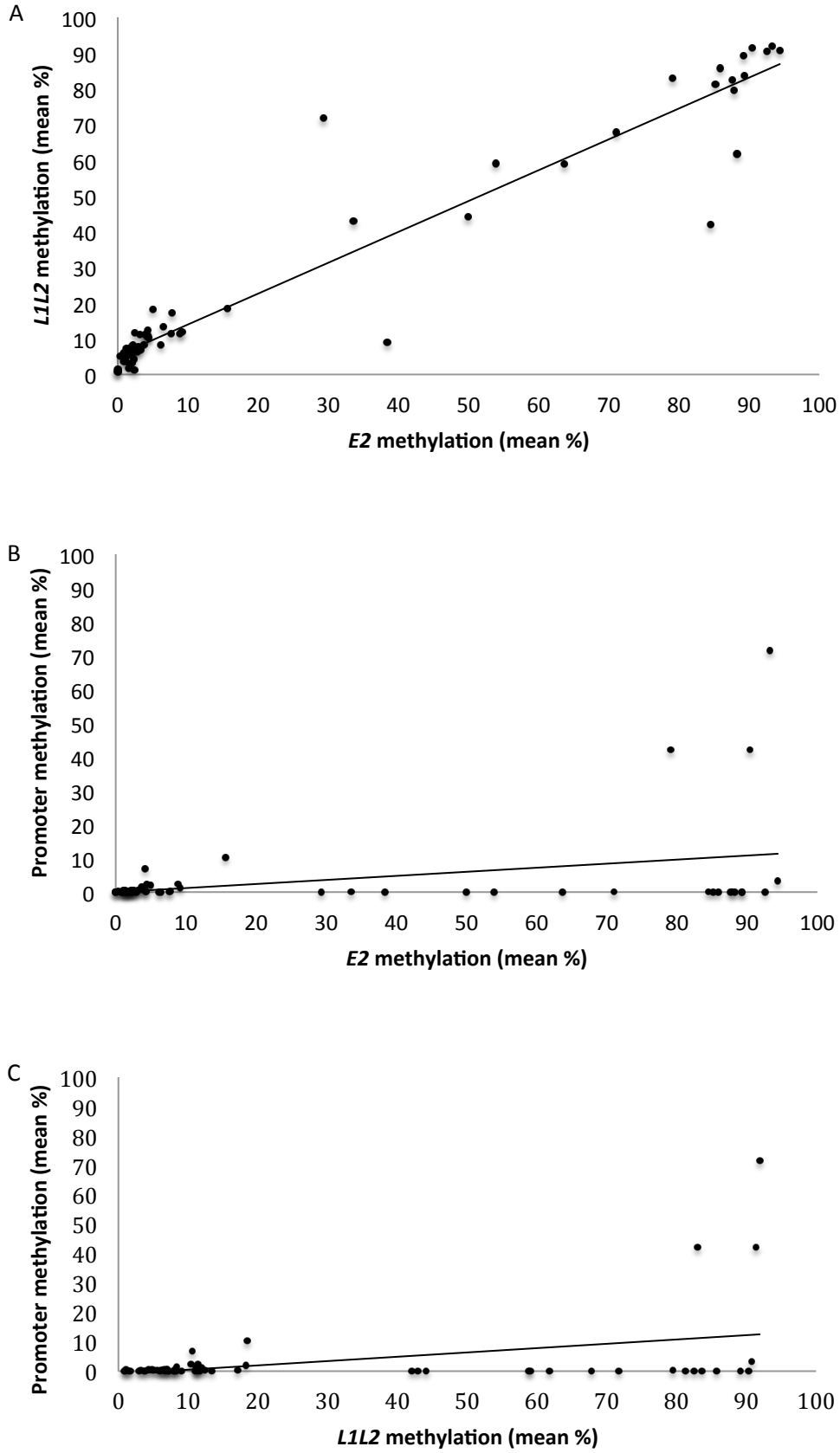


Figure 4.9 Relationships between HPV DNA methylation at different regions. Image A = *E2* and *L1L2* methylation, image B = *E2* and promoter methylation and image C = *L1L2* and promoter methylation. Methylation measured using pyrosequencing and recorded as mean percentage. N=61.

4.4.3 Intra-Regional HPV DNA Methylation

It was hypothesised that variation would exist between the methylation levels at each CpG site within a region. The graphs in Figure 4.10, Figure 4.11 and Figure 4.12 show conserved patterns of methylation at each region. Mean methylation levels with corresponding range and standard deviations, at each CpG site, within each region are shown in Table 4.5. To determine if this intra-regional variation was significant, a Friedman's two-way analysis of variance was used to compare the mean ranks for each CpG site for each region. Statistically significant differences in methylation levels existed between the CpG sites at the *E2* region ($\chi 2$ (2) = 177.65, p=<0.001), the *L1/L2* region ($\chi 2$ (2) = 148.48, p=<0.001) and the promoter region ($\chi 2$ (2)= 49.6, p=<0.001). Post hoc analysis with Wilcoxon signed-rank tests were conducted with a Bonferroni correction applied, resulting in a significant level set at $p < 0.003$. Results described in Table 4.6 show that the differences in methylation levels between adjacent CpG sites in the *E2* and *L2/L2* region were significant. For the promoter region, the differences in methylation between CpG3 and CpG4 and also between CpG4 and CpG5 were significant.

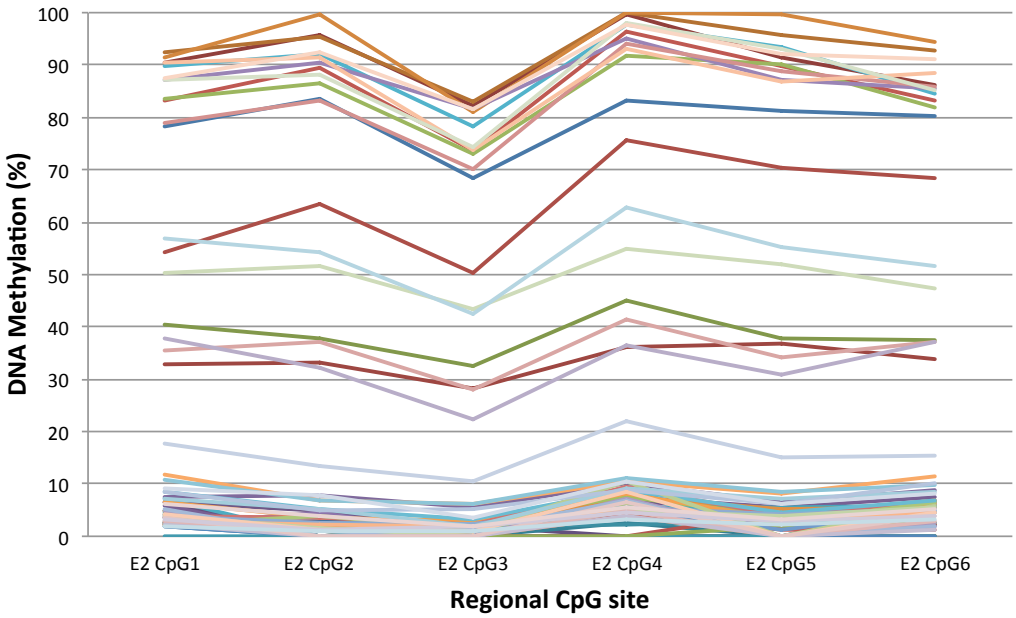


Figure 4.10 Pattern of E2 intra-regional methylation n=68. Nucleotides sequenced as follows: E2 CpG1 = NT 3411, CpG2 = NT 3414, CpG3 = 3416, CpG4 = 3432, CpG5 = 3447, CpG6 = 3447. Points are linked to aid visualisation of trends within samples.

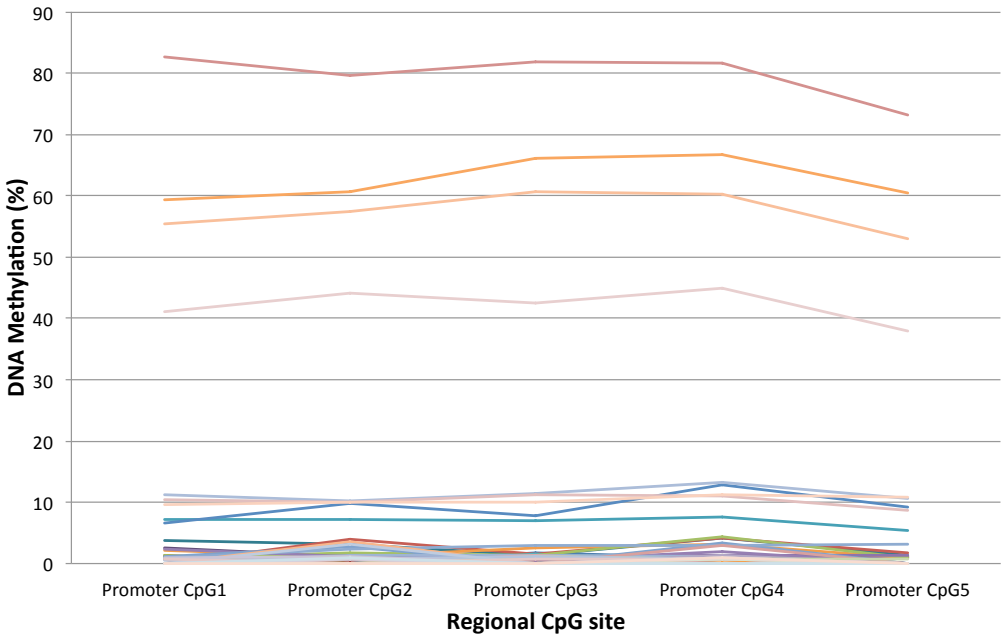


Figure 4.11 Pattern on promoter intra-regional methylation n=114. Sequenced nucleotides as follows: CpG1 = 31, CpG2 = 37, CpG3 = 43, CpG4 = 52, CpG5 = 58. Points are linked to aid visualisation of trends within samples.

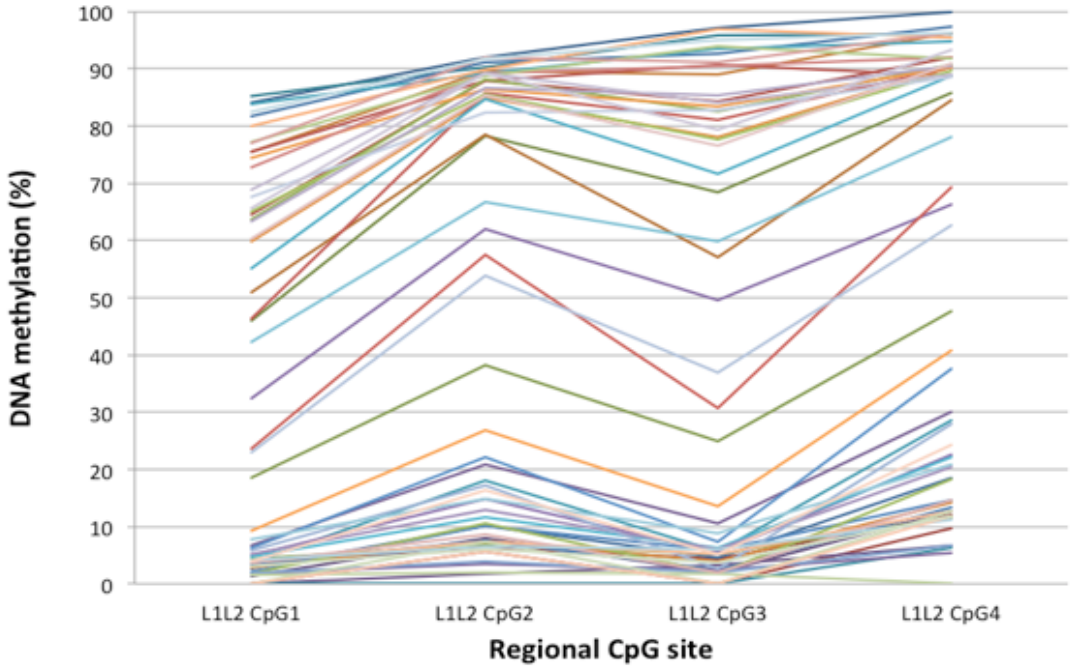


Figure 4.12Patterns of L1/L2 intra-regional methylation n=60. Sequenced nucleotides as follows: CpG1 = 5615, CpG2 = 5606, CpG3 = 5609, CpG4 = 5600. Points are linked to aid visualisation of trends within samples.

Methylation at CpG sites within each tested viral region: *E2*, *promoter* and *L1/L2*

***E2* region methylation(n=68)**				
CpG site	**Mean (%)**	**Min (%)**	**Max (%)**	**Std. Dev**
***E2* CpG1** (nt* 3411)	23.25	0.00	92.37	32.16
***E2* CpG2** (nt 3414)	22.03	0.00	99.81	34.80
***E2* CpG3** (nt 3416)	18.31	0.00	83.00	29.37
***E2* CpG4** (nt 3432)	26.46	0.00	100.00	35.36
***E2* CpG5** (nt 3435)	22.70	0.00	99.57	34.68
***E2* CpG6** (nt 3447)	23.31	0.00	94.24	32.28
***Promoter* region methylation(n=114)**				
CpG site	**Mean (%)**	**Min (%)**	**Max (%)**	**Std. Dev**
Promoter CpG1 (nt 31)	2.72	0.00	82.60	11.41
Promoter CpG2 (nt 37)	2.86	0.00	79.68	11.47
Promoter CpG3 (nt 43)	2.87	0.00	81.83	11.94
Promoter CpG4 (nt 52)	3.29	0.00	81.58	12.01
Promoter CpG5 (nt 58)	2.51	0.00	73.17	10.71
***L1/L2* region methylation(n=60)**				
CpG site	**Mean (%)**	**Min (%)**	**Max (%)**	**Std. Dev**
L1/L2 CpG1 (nt 5615)	32.77	0.00	85.25	32.65
L1/L2 CpG2 (nt 5606)	46.08	0.00	92.05	37.77
L1/L2 CpG3 (nt 5609)	40.82	0.00	97.19	39.46
L1/L2 CpG4 (nt 5600)	52.06	0.00	100.00	37.23

*nt = nucleotide position in sequence NC 001526.1 (HPV genbank accession number).

Table 4–5 Statistical significance of difference in methylation levels between intra-regional CpG sites

***E2* Region**					
	CpG1–CpG2	**CpG2–CpG3**	**CpG3–CpG4**	**CpG4–CpG5**	**CpG5–CpG6**
Z statistic	-3.070	-4.677	-6.942	-6.610	-2.094
P value	0.002	<0.001	<0.001	<0.001	0.036
***L1/L2* region**					
	CpG1-CpG2	**CpG2-CpG3**	**CpG3-CpG4**		
Z statistic	-6.680	-5.110	-6.552		
P value	<0.001	<0.001	<0.001		
Promoter region					
	CpG1-CpG2	**CpG2-CpG3**	**CpG3-CpG4**	**CpG4-CpG5**	
Z statistic	-1.292	-0.158	-4.359	-5.857	
P value	0.196	0.875	<0.001	<0.001	

4.4.4 HPV DNA Methylation, Patient Age, Smoking and Disease Episode

Increased levels of methylation were anticipated in older patients, those with a smoking history and those with recurrent disease. Bonferroni correction was applied to account for multiple comparisons and a p-value of <0.01 was considered significant. Pearson's rank found no significant correlation between increasing age and increased HPV DNA methylation at any of the three tested regions (*E2* r=0.206 p=0.063, *L1/L2* r=0.236 p=0.022 and *promoter* r=0.022 p=0.81). Median methylation level in smokers versus non-smokers for the *E2* region were 4.33% and 3.71%, for the *L1/L2* region were 15.22% and 29.76% and for the promoter region were 0.19% and 0.19%. Results of a Mann-Whitney U test did not find differences between groups at any region to be significant. Finally, median methylation levels in patients with first episode versus recurrent disease for the *E2* region were 27.48% and 26.95%, for the *L1/L2* region were 38.42% and 36.46% and for the promoter were 3.58% and 4.20%. A Mann-Whitney U test did not find significant differences between the groups at any region.

4.4.5 Summary of Findings

- HPV DNA methylation in VIN 3 varied significantly:
    - Between cases for each tested region
    - Between the three tested regions
    - Methylation levels were generally higher at the *E2* and *L1/L2* regions than the promoter region
    - Between CpG sites within regions (with patterns of methylation found at each region)
- A strong positive correlation existed between *E2* and *L1/L2* region methylation
- No relationships were identified between HPV DNA methylation and patient age, smoking status and disease episode

4.5 HPV Gene Expression Data

HPV gene expression levels were estimated in the 136 HPV 16 positive cases detected by PapilloCheck® and/or HPV 16 *E6* PCR (n=136) using RT-qPCR. Three HPV genes were analysed: *E2, E6* and *E7* as well as two human reference genes: *HPRT* and *TBP*. Of the 136 HPV 16 positive cases, two cases had insufficient RNA to perform any expression analyses, nine had insufficient to perform the *E2* analysis and three cases for the *E6* analysis. The data was analysed in two ways. In the first instance, crossing point data was analysed using Biogazelle qBase+ software, which generates gene expression levels for each gene relative to the cohort being tested. This method enabled comparisons in gene expression to be made between samples. Secondly, direct comparison was made between the crossing point values generated by the LightCycler software for the *E2* and *E6* gene for each case. This method meant that comparisons could be made between the expression levels of each gene within a sample and permitted exploratory work to be conducted using E2:E6 expression ratio to identify those cases with and without deregulated oncogene expression. Cases with *E2*>*E6*expression were judged to represent cases with regulated oncogene expression, and cases with *E6*>*E2* were judged to represent cases with deregulated oncogene expression.

4.5.1 Variation of HPV Gene Expression in VIN 3

Calibrated, normalised, relative quantities (CNRQ) of expression of each gene were generated by qBase+ software. The expression value was normalised to the two stably expressed reference genes (*TBP* and *HPRT*) and then made relative, to the minimum value generated in the cohort, for the particular gene in question. Samples that generated a non-specific PCR product (NSP) (determined by visual inspection of the melt curve generated by the LightCycler) were not put through analysis using the qBase+ software. These samples were cases in which, no gene expression could be detected. These samples were included in downstream statistical analysis using SPSS with a value of '0' (equal to the minimum value generated for the gene in the cohort). This was done to prevent inaccurate analysis of gene expression by qBase+ software whilst including them in overall analysis to ensure that data was not biased towards those samples with intact HPV. Following quality control measures, 115 samples were available for *E2* gene expression analysis, 120 cases for *E6* and 119 cases for *E7*.

The range of *E2* gene expression was CNRQ 0.00 – 3.68, with a mean CNRQ of 1.88 ± 1.30. The range of *E6* expression was 0.00 - 2.19 with a mean CNRQ of 1.01 ± 0.48 and the range of *E7* expression was 0.00 – 2.32 with a mean CNRQ of 1.2 ± 0.49 Table 4.7. Figure 4.13

shows the variation in expression of each gene in the cohort and enables visual comparisons to be made between expression levels of each gene. The range of both *HPRT* and *TBP* expression was CNRQ 0.00 – 0.83, the mean *HPRT* and *TBP* CNRQ were 0.43 ± 0.23 and 0.40 ± 0.23 respectively. Expression of each HPV gene was more variable than the expression of the human reference genes, with *E2* expression demonstrating the most variation.

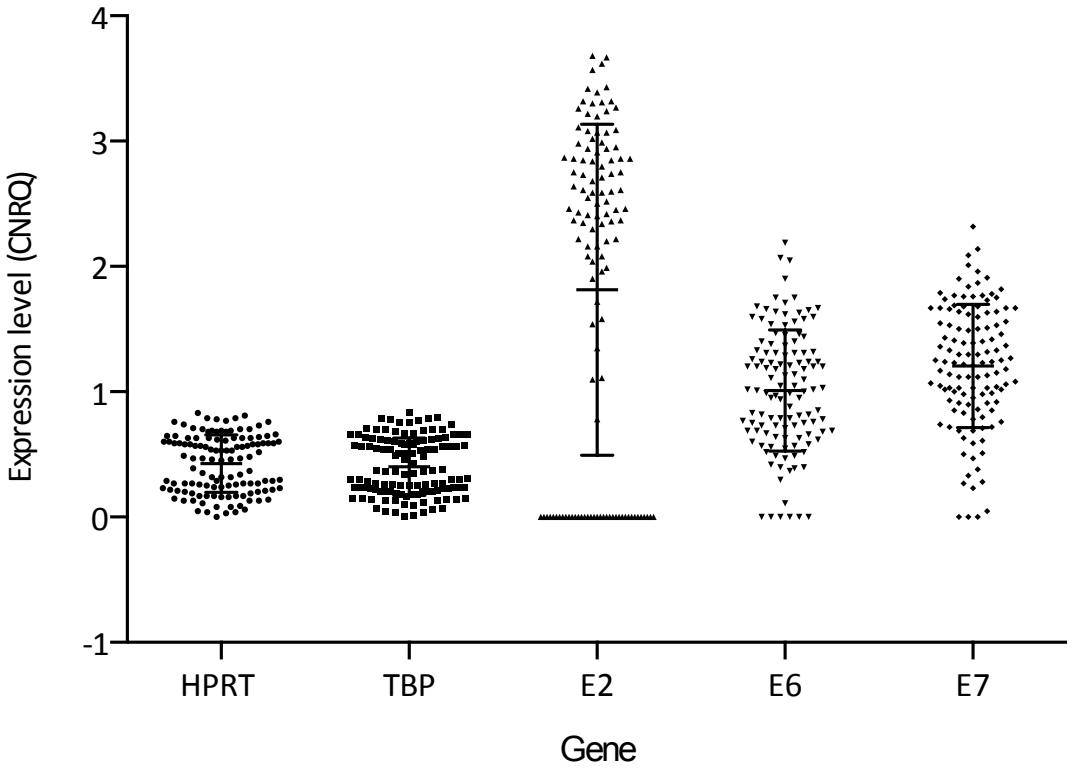


Figure 4-13 Variation in CNRQ values for HPV genes and two human reference genes. CNRQ = corrected, normalised, relative quantity, data generated using qBase+ software analysing the relative quantity of each gene to the minimum value generated in the cohort. Mean and standard deviation for each gene shown Presence of variation determined by visual comparison with the *HPRT* and *TBP* human reference genes.

Table 4–6 CNRQ analysis for each gene

	E2	*E6*	*E7*	*HPRT*	*TBP*
Valid results n=	115	120	119	122	122
Missing*	21	16	17	14	14
Mean	1.88	1.01	1.20	0.43	0.40
Median	2.40	1.02	1.23	0.47	0.36
Std. Deviation	1.30	0.48	0.49	0.23	0.23
Range	3.68	2.19	2.32	0.83	0.83
Minimum	0.00	0.00	0.00	0.00	0.00
Maximum	3.68	2.19	2.32	0.83	0.83

*Missing values include those samples for which there was insufficient RNA to run the assay and those samples that failed quality assurance parameters.

4.5.2 HPV E2:E6 Gene Expression in VIN 3

Direct comparisons of the crossing point values generated by the LightCycler software were made and a ratio of *E2:E6* expression generated. Full data was available for 112 cases in total; for 9 cases, there was insufficient DNA to run the assay, 2 cases were not included because both the *E2* and the *E6* assay generated a non-specific product and 13 samples failed quality control. This exploratory analysis found that in the majority of cases (83/112, 74.1%) *E6* expression was greater than *E2*indicating the presence of deregulated gene expression and in 29/112 (25.9%) *E2*expression was greater than *E6*indicating those cases with regulated oncogene expression.

4.5.3 HPV Gene Expression and Patient Age, Smoking Status and Disease Episode

It was hypothesised that age and recurrent disease would correlate with decreasing *E2* expression. For this section of analyses, a Bonferroni correction was applied to account for multiple comparisons, and a p-value of < 0.008 was considered significant. *E2* expression was found to decrease with increasing age as demonstrated by Pearson's correlation coefficient (x=-0.228, p=0.014), (Figure 4.14). Similarly, patients with *E2*>*E6* (regulated oncogene expression) were on average younger than women with *E6*>*E2* (deregulated oncogene expression) with a mean age of 43 years vs. 48 years. However, neither of these findings reached statistical significance (p=0.014 and 0.057 respectively).

Regarding disease episode, *E2* expression was found to be higher in patients with first episode disease (CNRQ 2.65) than in patients with recurrent disease (CNRQ 2.22); however, a Mann-Whitney U test did not find this difference to be statistically significant (U = 1332, Z = -1.8, p = 0.072). The majority of cases of both recurrent and first episode disease had greater *E6* expression than *E2* expression however; it was statistically significantly more likely to be the pattern of expression in recurrent disease. In recurrent disease 46/53 (86.8%) cases had *E6*>*E2* expression and 7/53 (13.2%) had *E2*>*E6*; in first episode disease 37/59 (62.7%) had *E6*>*E2* and 22/59 (37.3%) had *E2*>*E6* (p=0.004) See Figure 4.15.

No statistically significant difference in *E2* expression and was found between patients with and without a history of smoking (*E2* CNRQ 2.39 and *E2* CNRQ 2.41 respectively, p = 0.681). E6 expression was more likely to be greater than E2 expression in patients with and without a history of smoking (74.4% and 72.2% respectively), (Figure 4.16).

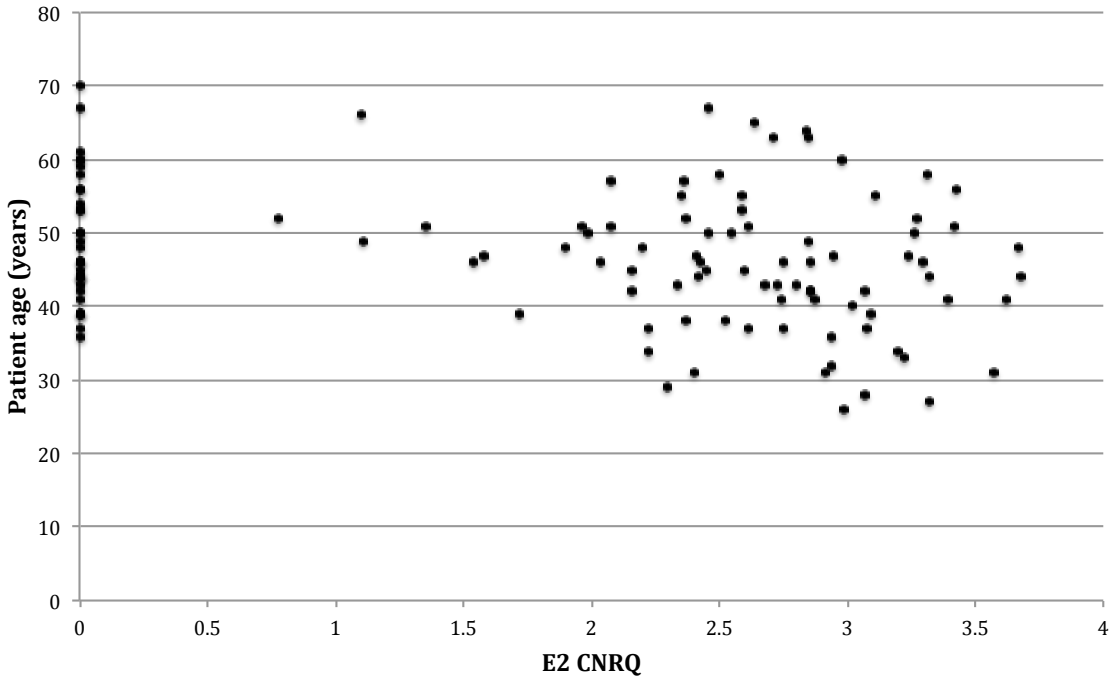


Figure 4.14 Relationship between *E2* expression (CNRQ) and patient age (years) in the RT3 VIN baseline cohort. Multiple values at CNRQ 0 represent those samples generating a non-specific PCR product and therefore exhibiting no gene expression. A negative correlation was identified but did not reach statistical significance (p=0.014) following the application of a Bonferroni correction to allow for multiple comparisons making a p-value of <0.008 significant.

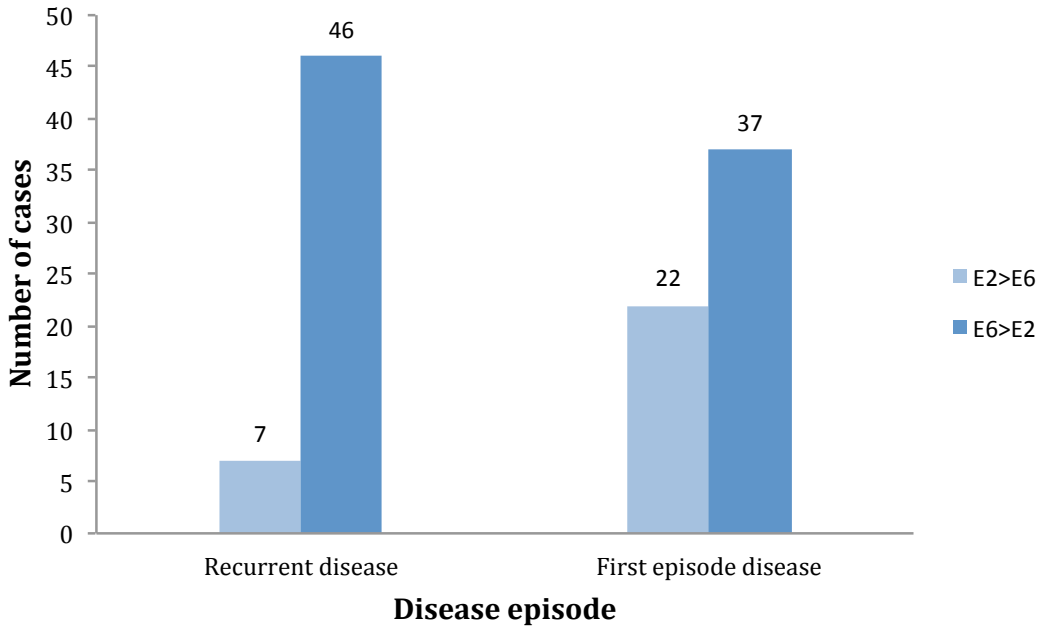


Figure 4.15 Relationship between disease episode and viral gene expression pattern. *E6* expression was greater than *E2* expression in 86.8% of recurrent disease cases and 62.7% of first episode disease cases (p=0.004).

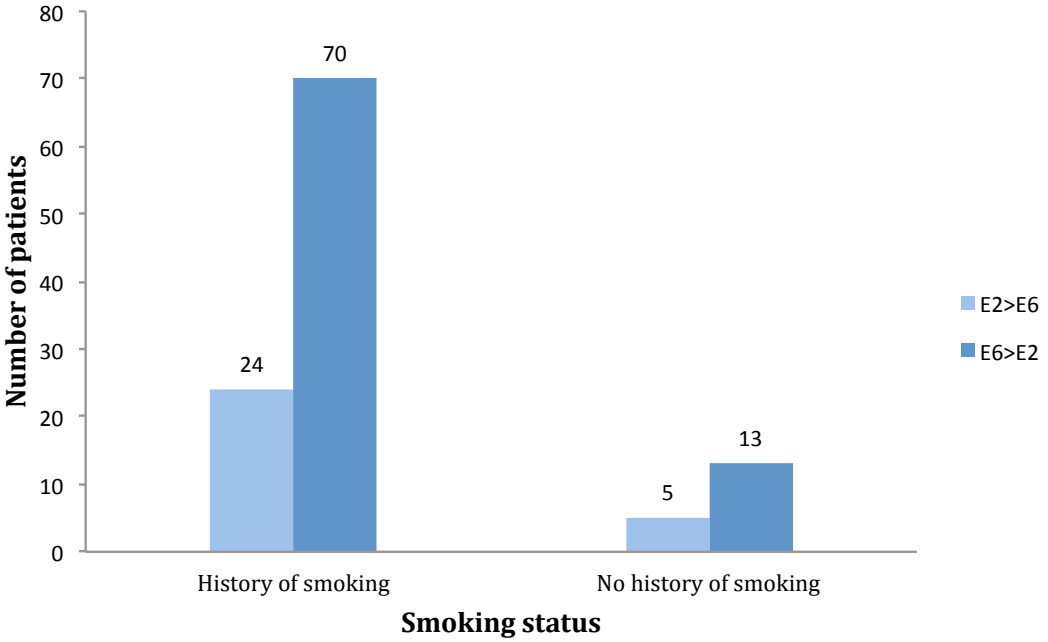


Figure 4.16 Relationship between smoking status and viral gene expression pattern. ***E6***expression was higher than *E2* expression in 74.4% of patients with a smoking history and in 72.2% of patients with no history of smoking.

4.5.4 Summary of Findings

- Expression of each HPV gene (*E2*, *E6* and *E7*) was more variable in VIN 3 than the human reference genes *HPRT* and *TBP* (broadly accepted to be stably expressed)
- The majority of VIN 3 expressed more *E6* than *E2*
- No statistically significant relationship between patient age or smoking status and gene expression was identified
- *E6*>*E2* expression was statistically more likely to be the pattern of gene expression in cases of recurrent disease than first-episode disease

4.6 Relationship Between Viral Characteristics

HPV integration (represented by absence of ≥ 1 *E1/E2* tiling PCR fragments), high HPV DNA methylation (determined by mean of *E2* and *L1/L2* CpG sites >50%) and deregulated oncogene expression (part of exploratory analysis represented by *E6>E2* expression), have all been associated with transforming HPV infection. Mean methylation with a cut-off, of >50% for the *E2* and *L1/L2* CpG sites was used to represent 'high' methylation for three reasons. Firstly, VIN 3 cases appeared to either be highly methylated or lowly methylated and rarely in between; secondly *E2* and *L1/L2* methylation levels were strongly correlated with one another and varied widely whereas promoter methylation was almost invariably low and finally, a similar cut-off has been used by other studies (Bryant, Onions *et al*. 2014). The first aim of this section was to explore relationships between these characteristics. The second aim of this section was to investigate viral characteristics leading to deregulated oncogene expression (*E6>E2*).

4.6.1 Viral Integration and HPV Gene Expression

HPV was more likely to be integrated in cases of *E6>E2* (deregulated oncogene expression) (49/83, 59%) and more likely to be intact in patients with *E2>E6* (regulated oncogene expression) (20/29, 69%) (p = 0.009), (**Figure 4.17**). Similarly, the median expression of *E2* was lower (CNRQ 0.0) when the virus was integrated and higher (CNRQ 2.8) when intact. This difference reached statistical significance (p = <0.0001) with a Mann-Whitney U test incorporating a Bonferroni correction (p=<0.01) to allow for multiple comparisons. No statistical significance was identified between the expression levels of *E6* and *E7* in cases of integrated and intact virus (CNRQ 1.02 vs. 0.96 and CNRQ 1.12 vs. 1.3 respectively).

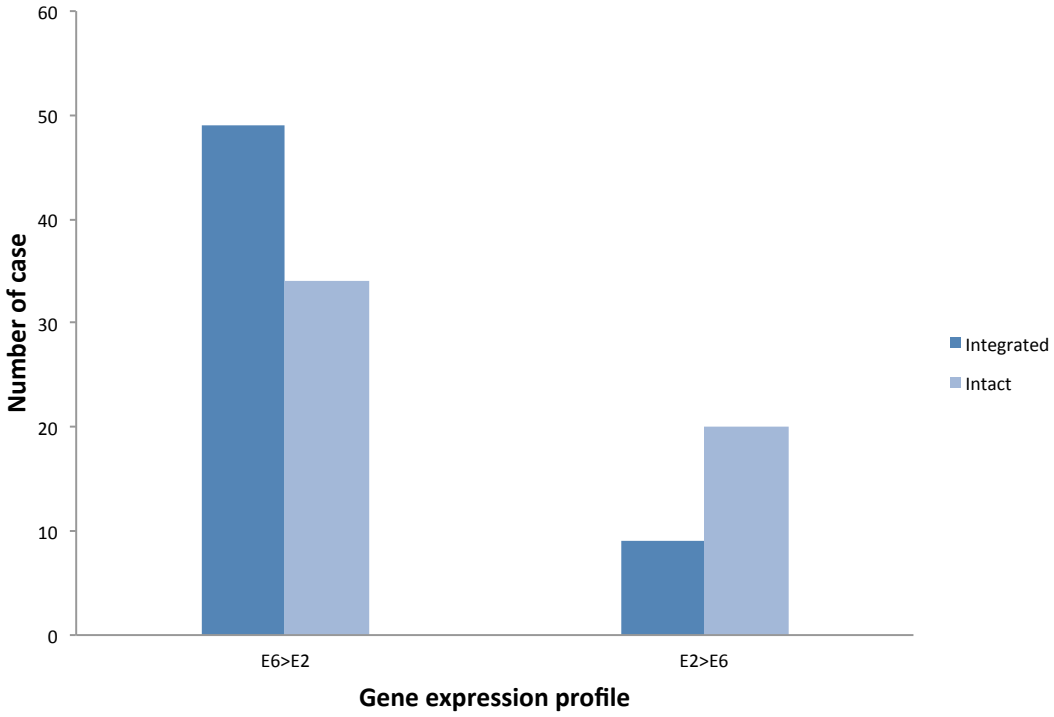


Figure 4.17 Relationship between HPV gene expression profile and viral integration. Full data was available for both assays for 112 cases. Integration status was determined by an *E1/E2* tiling PCR. HPV gene expression profile determine by ratio of expression of *E2:E6*. *E2>E6* used to represent regulated oncogene expression and if *E6>E2* used to represent deregulated oncogene expression.

4.6.2 Viral Gene Expression and Viral DNA Methylation

High HPV DNA methylation of the *E2* and *L1/L2* regions was strongly correlated with decreased *E2* gene expression using Pearson's rank ($r = -0.679$, $p = <0.0001$ and $r = -0.511$, $p = <0.0001$ respectively), (Figure 4.18 and Figure 4.19). No correlation was found between promoter region methylation and *E2* gene expression. Similarly, mean *E2* methylation was higher (36.4%) in cases of deregulated oncogene expression compared to regulated (12.04%) cases ($p = 0.008$). Likewise, mean *L1/L2* methylation was higher (46.3%) in cases with deregulated oncogene expression compared to 21.0% in cases with regulated oncogene expression ($p = 0.004$). No correlation was found between promoter region methylation and *E2:E6* expression (Table 4.8).

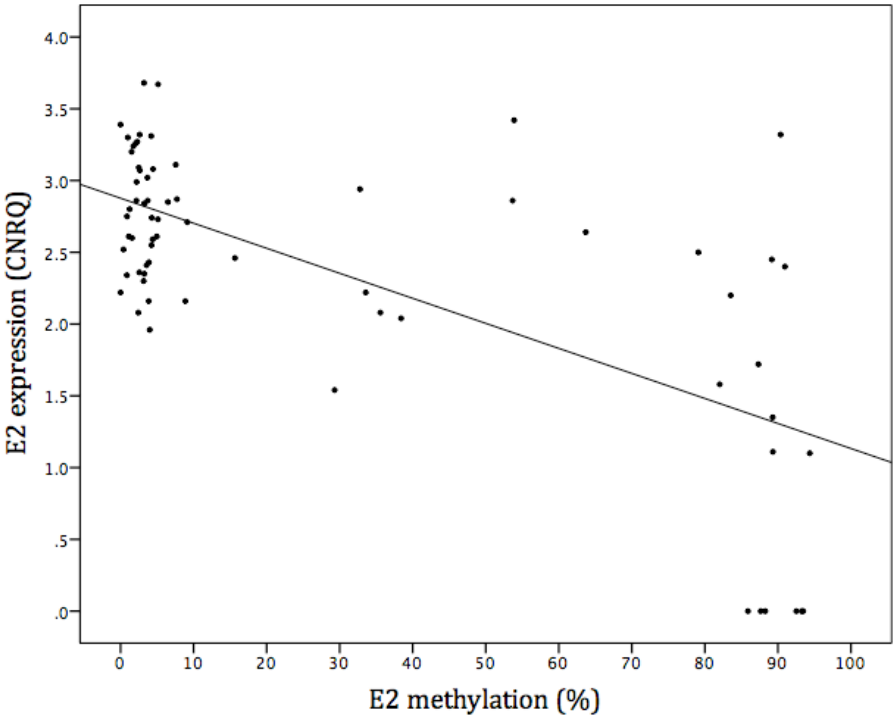


Figure 4.18 *E2* methylation and *E2* expression. Increased *E2* DNA methylation strongly correlated with reduced *E2* gene expression (p=<0.000). *E2* methylation represents the mean methylation of all tested CpG sites. *E2* expression is generated as the corrected normalised relative quantity within the study cohort. Line of best fit applied.

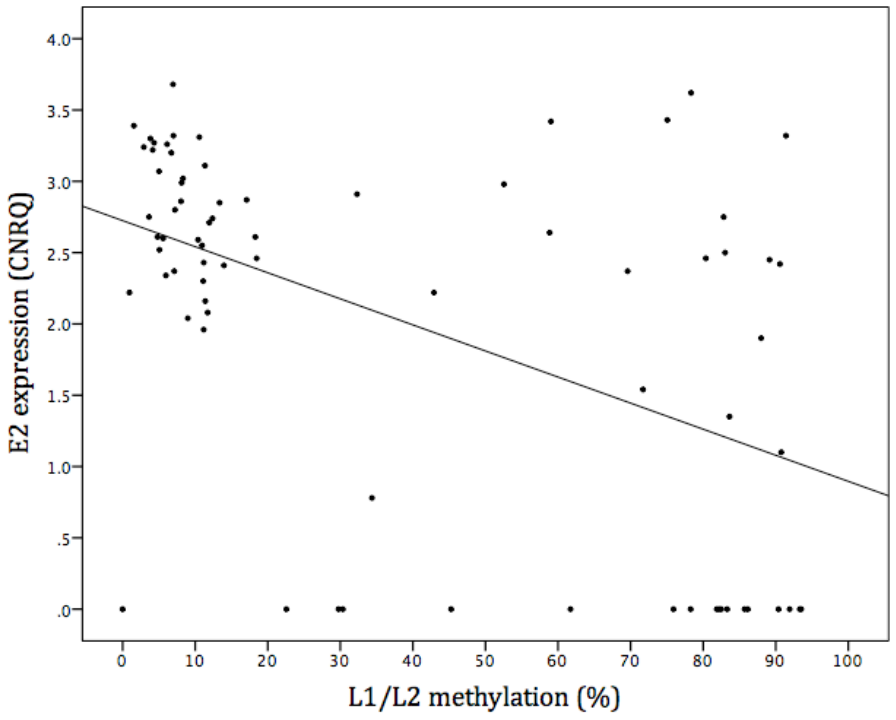


Figure 4.19 *L1/L2* methylation and *E2* gene expression. Increased *L1/L2* DNA methylation strongly correlated with reduced *E2* gene expression (p=<0.000). *L1/L2* methylation represents the mean methylation of all tested CpG sites. *E2* expression is generated as the corrected normalised relative quantity within the study cohort. Line of best fit applied.

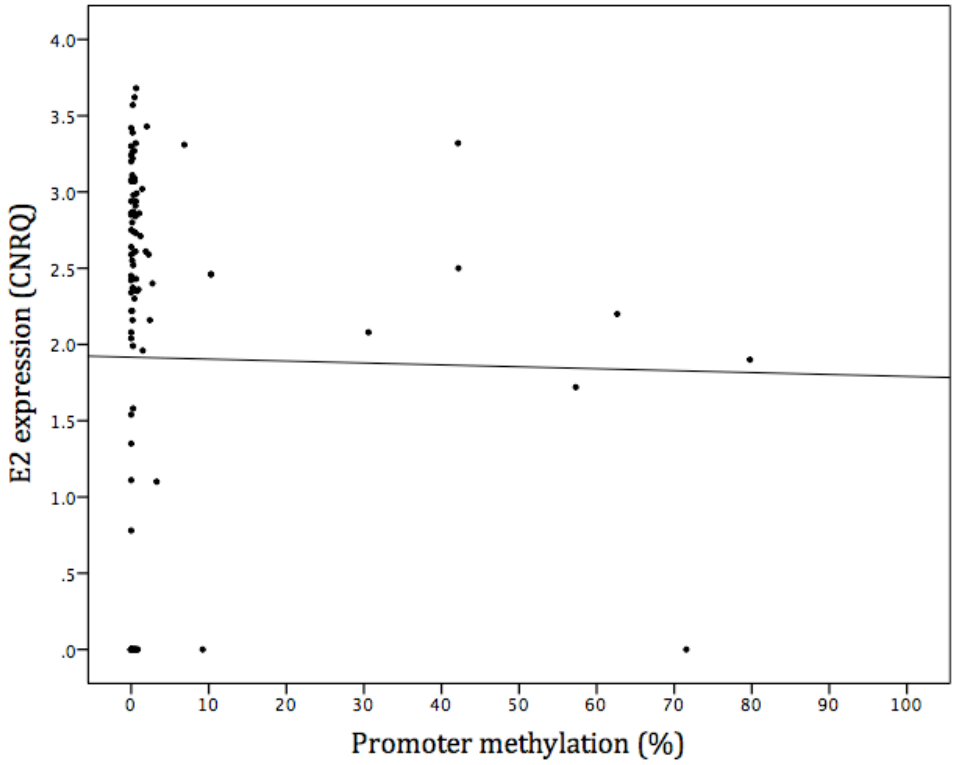


Figure 4.20 Promoter methylation and *E2* gene expression. No correlation identified. Promoter methylation represents the mean methylation of all tested CpG sites. *E2* expression is generated as the corrected normalised relative quantity within the study cohort. Line of best fit applied.

Table 4–7**HPV gene expression and DNA methylation**

***E2* methylation**			
	Mean	**Median**	**Significance**
E2>E6	12.04	3.2	P=0.008
E6>E2	36.4	7.7	
***L1/L2* methylation**			
	Mean	**Median**	**Significance**
E2>E6	21.0	8.05	P=0.004
E6>E2	46.3	42.9	
Promoter methylation			
	Mean	**Median**	**Significance**
E2>E6	3.7	0.4	P=0.232
E6>E2	4.7	0.18	

E2>E6 represents cases of regulated oncogene expression and *E6>E2* represents cases of deregulated oncogene expression. Significance calculated using Mann - Whitney U test incorporating a Bonferroni correction for multiple comparisons making a p value of < 0.03 significant.

4.6.3 Viral Integration and HPV DNA Methylation

Methylation of the *E2* and *L1/L2* regions was higher in patients with integrated virus. For the *E2* region, median methylation was 80.6% in disrupted cases and 3.19% in intact cases (p = <0.0001). For the *L1/L2* region median methylation was 75.9% in disrupted cases and 7.89% in intact cases (p=<0.0001). Levels of methylation at the viral promoter were found to be similarly low at 0.17% and 0.28% in cases of integrated and intact virus respectively. This difference was not statistically significant (p = 0.25), (Figure 4.21).

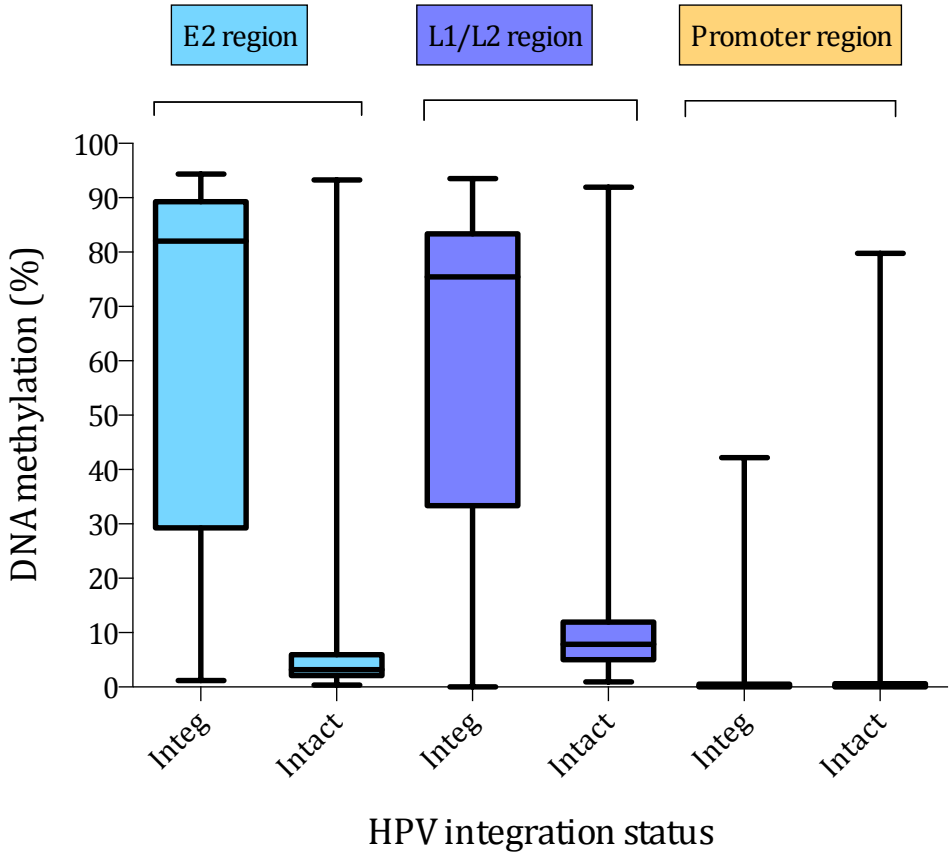


Figure 4.21 **Regional HPV DNA methylation in cases of disrupted and intact virus**. Whiskers of the box plot represent the minimum and maximum value in the range. Based on the results of a Mann Whitney U test, for the *E2* region and the *L1/L2* region, HPV DNA methylation was significantly higher in cases with integrated virus than in cases with intact virus (p=<0.0005). Methylation of the promoter region was found to be similar in disrupted and intact cases.

4.6.4 Relationships Between Viral Disruption, Methylation and Oncogene Expression

Ninety-two cases had data available for all three viral characteristics. Correlations between all three characteristics are illustrated in Figure 4.22. In 19/92 cases (20.7%), no transforming HPV characteristics were detected. Conversely, in 73/92 (79.3%) cases, at least one transforming characteristic was present. Deregulated oncogene expression (*E6>E2*) was the most common transforming characteristic present in the cohort, detected in 66/92 (71.7%) cases. High HPV DNA methylation was found in 35/92 (38.0%) and viral disruption in 43/92 (46.7%) cases. All three transforming characteristics were present in 25/92 (27.2%) cases. In all 35 cases of high HPV DNA methylation and 40/43 (93.2%) cases of HPV disruption, at least one (normally both) of the other transforming characteristics was also detected. Regarding deregulated oncogene expression (*E6>E2*), the majority (42/66, 63.3%) of cases showed the presence of ≥1 other transforming characteristic, however, in 24/66 (36.7%) cases, no other transforming characteristic was detected.

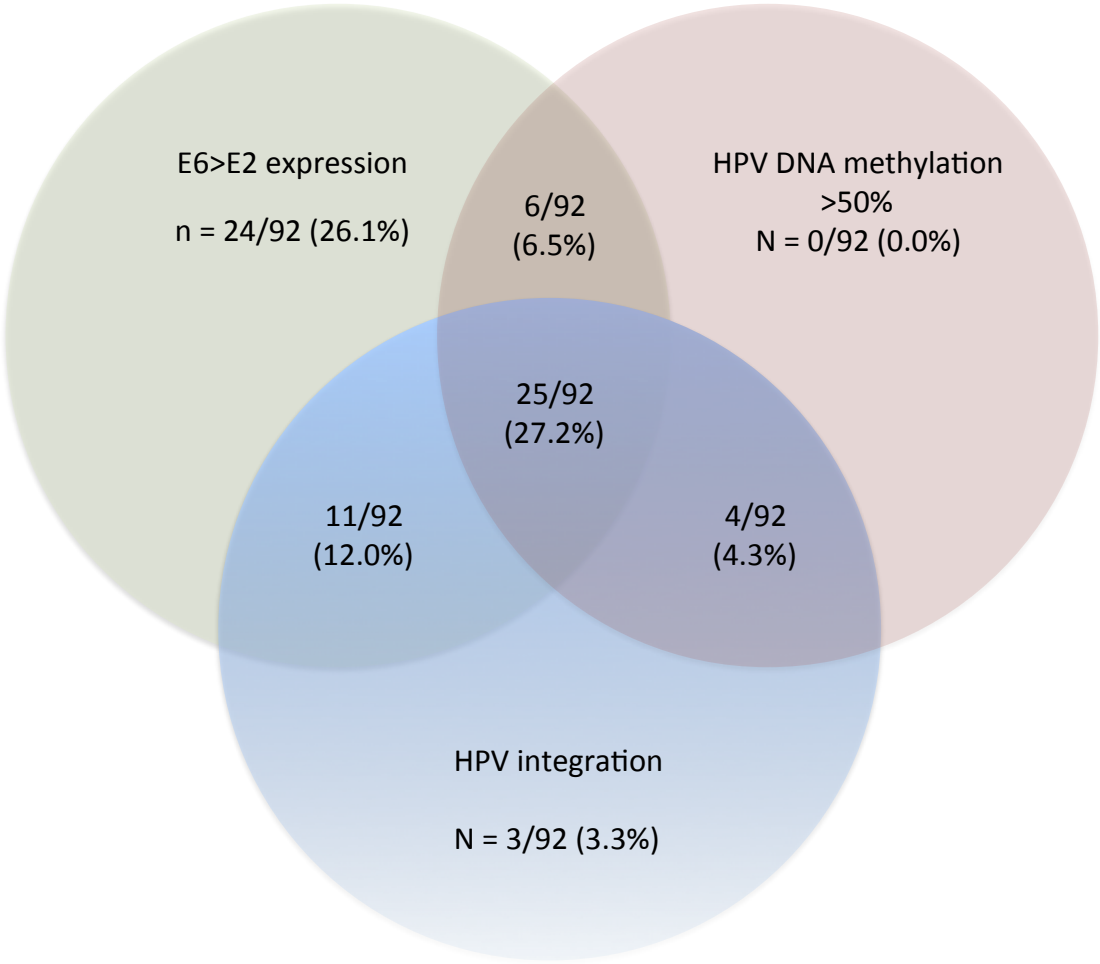


Figure 4.22 Correlations between HPV gene expression, high methylation and HPV disruption in 92 cases of HPV 16 positive VIN 3. Among the 92 cases, there were 66 cases of *E6>E2* expression, 43 cases of viral integration and 35 cases of high HPV DNA methylation in total. The sum of all proportions in each circle represents these values. Sample methylation was calculated by taking the mean value from the CpG sites in the *E2* and *L1/L2* regions

4.6.5 Mechanisms of Deregulated Oncogene Expression

Figure 4.23 demonstrates the presence of heterogeneous HPV biology in the 92 HPV 16 positive cases that had data available for all three tested viral characteristics. The left-hand-side of the figure (highlighted blue) describes the physical status of the HPV infection in the 66/92 cases of deregulated oncogene expression. In the majority of cases (42/66, 63.6%) HPV integration, high methylation (>50%) or both are present. In 25/66 (37.9%) there is both viral integration and high methylation. In 11/66 (16.7%) cases there is viral integration without increased methylation and in 6/66 (9.1%) cases; there is increased HPV DNA methylation without viral integration. This finding indicates the possibility that both HPV integration and HPV DNA methylation lead to deregulated oncogene expression independently but that deregulated oncogene expression is increasingly likely when these characteristics are present together. In 24/66 (36.4%) there is neither high HPV DNA methylation nor viral integration. This finding indicates the possibility that alternative mechanisms to those investigated in this study may play an important role in HPV pathogenesis in VIN (see discussion). The right side of the figure (highlighted green) represents the 26/92 (28.3%) cases in which *E2* expression was higher than *E6* expression (regulated oncogene expression). In the majority of these cases (19/26, 73.1%) HPV was intact and lowly methylated, in 7/26 (26.9%) cases the virus was integrated, three of which were also highly methylated. It is of note that the location of viral disruption in these seven cases was variable: four cases failed to amplify the *E2*-5 fragment; one case failed to amplify fragments *E2*-2 to *E2*-5; one cases failed to amplify fragments *E2*-3 to *E2*-5 and the last cases failed to amplify fragment *E1*-2.

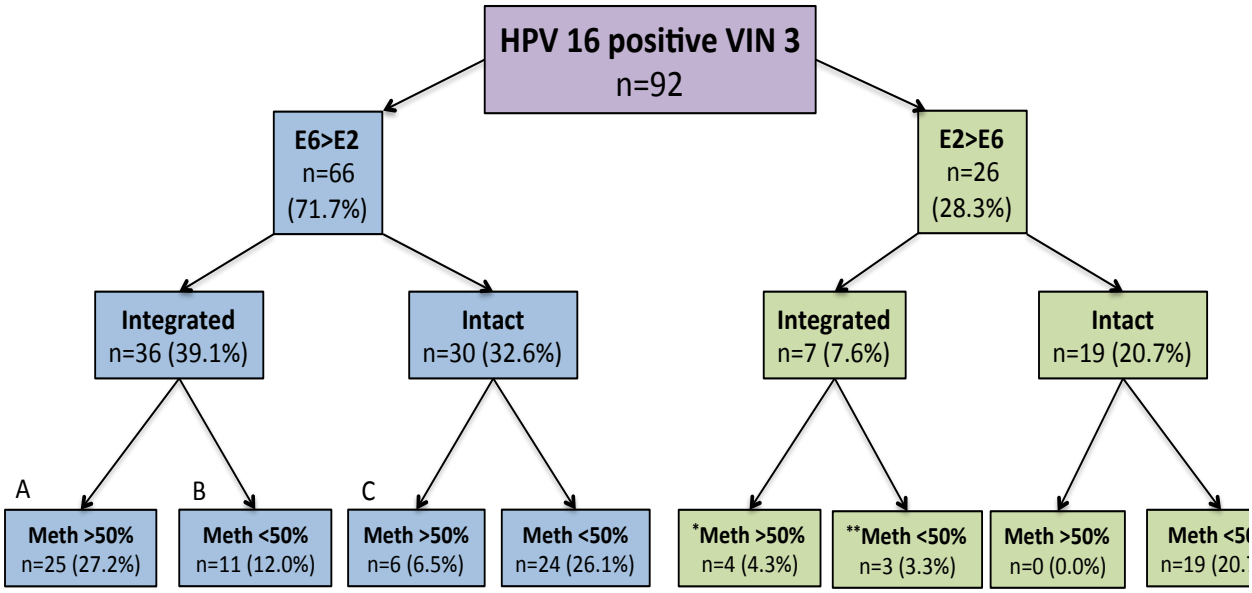


Figure 4.23 HPV characteristics in 92 cases of HPV 16 positive VIN 3. Exploratory work used E expression ratio to represent cases of deregulated oncogene expression and E2>E6 ratio to repres regulated oncogene expression. Viral integration was determined by the failure of amplification of fragments of an E1/E2 tiling PCR. High HPV DNA methylation was determined by a mean methylat E2 and L1/L2 CpG sites of >50%. Heterogeneous HPV biology in VIN 3 was present. Figure shows c sectional evidence of multiple mechanisms potentially causing oncogene expression; viral integrat methylation together as well as each independently (A, B and C). *, ** Evidence also indicating in a m of cases, that HPV pathogenesis is occurring in the absence of deregulated oncogene expression

4.6.6 Summary of Findings

- HPV disruption correlated with deregulated oncogene expression and relatively low *E2* gene expression in the cohort overall
- No relationships were identified between HPV disruption and *E6* or *E7* gene expression
- High *E2* and *L1/L2* methylation (>50%) strongly correlated with decreased *E2* gene expression, and mean methylation was higher in cases of deregulated oncogene expression than in cases of regulated oncogene expression
- Methylation of the promoter region did not correlate with *E2* gene expression or deregulated oncogene expression
- High *E2* and *L1/L2* DNA methylation strongly correlated with viral disruption
- At least one of three transforming viral characteristics was present in the majority (73/92, 79.3%) of cases of VIN 3, and 27.2% of VIN tested positive for all three.
- Deregulated oncogene expression was present independently of other transforming HPV characteristics in 24/66 (36.4%), whereas viral disruption and high HPV DNA methylation almost exclusively occurred in combination with at least one other transforming characteristic.
- Evidence exists for the potential presence of multiple mechanisms that could lead to deregulated oncogene expression.

4.7 Discussion

This discussion firstly gives an overview of the principal findings of this chapter and its overall strengths and limitations. The principal findings are then discussed individually in more detail.

4.7.1 Principal Findings

- High HPV prevalence in VIN 3, with HPV 16 being the most common genotype followed by HPV 33
- Strong correlations among viral characteristics associated with transforming HPV infection (deregulated oncogene expression, viral integration and high HPV DNA methylation)

4.7.2 Overall Study Strengths

The large cohort size investigated in this study is a major strength; this is the largest cohort of VIN 3, with investigation of multiple HPV characteristics to date. The quality of the tissue samples obtained from the patients is another strength. The tissue samples were collected as 4 mm punch biopsies from the disease affected area and immediately transferred into ThinPrep storage media for transportation and storage at -20 °C prior to DNA extraction. The DNA obtained from the samples was of good quality, the mean concentration was 89 ng/µl (range: 3.8-337.9 ng/µl) and the mean purity based on the A_{260}/A_{280} ratio was 2.0 (range: 1.5-2.2) (ThermoScientific, NanoDrop 2008). The quality of the DNA investigated has contributed to the reliability of the data.

4.7.3 Overall Study Limitations

It is possible that the RT3 VIN trial recruited a biased study cohort. This trial offered women access to novel topical therapies that could obviate the need for excisional surgery. It is possible therefore that recruitment could be biased towards women particularly keen to avoid surgical excision and its potential risks e.g. scarring, altered anatomy and potential psychosexual morbidity. It could be argued that these women are more likely to be younger women of child-breading age and therefore women in whom HPV driven disease is more likely (de Sanjose *et al*. 2013).

Presently, two histological classifications of VIN exist: usual VIN (uVIN), which is associated with HPV infection, and differentiated VIN (dVIN), which is independent of HPV and driven by chronic skin conditions such as lichen sclerosus. Even when disease is driven by lichen sclerosus, HPV may still be present as a bystander (de Sanjose *et al*. 2013). It is not possible to definitively distinguish cases of HPV-driven disease and non-

HPV driven disease. This must be considered when conclusions are drawn; the physical state of the virus is likely to be different between cases driven by HPV and those that are not. Grouping the two aetiologies together could complicate analyses aiming to investigate HPV biology in **HPV-driven** disease. Incorporating histological classification into dVIN and uVIN as well as incorporating use of P16 expression (Riethdorf *et al*. 2004) as a marker of HPV driven disease could be advantageous in future work developing understanding of HPV biology in VIN further.

Investigation of HPV biology in this study was limited to HPV 16 positive cases. HPV 16 was the most prevalent HPV genotype however; the HPV biology in the 28 non-16 HPV genotypes remains unknown. It is possible that biological variation exists between genotypes that could explain why certain genotypes are more prevalent than others.

Finally, conclusions drawn from analyses of relationships between viral characteristics are limited by the cross sectional nature of this data. Correlations can be made and biological explanations can be suggested but ultimately, no firm conclusions can be made regarding causal relationships.

4.7.4 HPV Prevalence in VIN

A high HPV prevalence was found in this cohort of histologically confirmed VIN 3(164/167, 98.2%). In agreement with the published literature, HPV 16 was the most common genotype detected (140/164, 85.4% cases), the second most common genotype was HPV 33, detected in 16/164 (9.8%) cases, followed by HPV 42 and 18, which were each detected in 4/164 cases (2.4%). In most HPV positive cases (150/164, 91.5%), a single genotype was detected and in 14/164 (8.5%) cases, multiple genotypes were present. High-risk genotypes were identified in 161/164 (96.9%) HPV positive cases and low-risk only genotypes were detected in 3/164 (3.1%) (two cases of HPV 42 and one case of HPV 44/55).

This study found that disruption in PCR target sequence was an important source of false negative HPV results obtained using the *E1* targeting, PapilloCheck® assay. HPV 16 was detected in 110/167 (65.9%) cases using PapilloCheck® and in 135/167 (80.8%) cases using an HPV 16 type-specific PCR that targets the *E6* viral region. Poor sample integrity was unlikely to be the cause of this disparity, as all 167 cases successfully amplified the *ADAT-1* human gene during PapilloCheck® assay quality control checks. Additionally, a standardised concentration of DNA (5 ng/µl) was used for all assays to ensure that variation in DNA concentration was not a source of disparate results. Similarly, the risk of false positive results was minimised by using positive and negative controls in every PCR

run and scrupulous technique. The *E1* ORF was disrupted in all 25 discordant cases and loss or disruption of the PCR target region for the PapilloCheck® assay was therefore the most likely explanation. Following this, the remaining HPV negative cases (n=14) were sequentially tested with two additional assays targeting different viral regions (*L1* and *E7*) and different genotypes in order to minimise the number of false negatives. This methodology resulted in overall HPV prevalence for all genotypes increasing from 77.8% with PapilloCheck® data alone to 98.2%. This approach was by no means infallible; non-HPV 16 genotypes with viral disruption were particularly vulnerable to being missed and HPV prevalence of 100% cannot be excluded. These findings are similar to those in the seminal study of HPV prevalence in cervical cancer published by Walboomers *et al*. in 1999. HPV was detected in nearly all cases of cervical cancer (99.7%, n=921) following sequential testing of HPV negative cases with assays targeting different regions; from this study, the principal that HPV infection is necessary for the development cervical cancer was established.

Walboomers *et al*. also found that in cases initially identified as HPV negative (using MY09/11, *L1* targeting primers) which were then re-tested (=55), an *E7* type-specific PCR detected HPV in 38/55 (69.0%), L1 consensus primers in 6/55 (10.9%) and *E1* consensus primers in 14/55 (25.5%). HPV that was not detected using *E1/L1* but was detected by *E7*, were more likely to be non-HPV 16 genotypes, particularly HPV 18 and 31. Additional reports in the literature indicate that in cases of high-grade cervical intraepithelial neoplasia, 8.3% of HPV 16 and 27.9% of HPV 18 infections are missed by an *L1* targeting assay compared to an *E6/E7* targeting assay; a finding attributed to the fact that viral integration is reported more commonly in non-HPV 16 genotypes (Pett & Coleman 2007; Roberts *et al*. 2006; Vinokurova *et al*. 2008). Studies have also demonstrated that the cases undetected by an *L1* targeting assay are more likely to be cases of higher-grade disease, in which viral integration is more likely. The present study has similarly found that the proportion of non-HPV 16 genotypes was higher in cases with viral disruption, but contrary to cervical disease, HPV 33 was the second most prevalent non-16 genotype. Proportionally, more non-HPV 16 genotypes were identified in cases initially identified as HPV negative by PapilloCheck® (6/31, 19.4%) than in the initial HPV positive cases identified (16/119, 13.4%).

This study has found that, similar to CIN and cervical cancer, disruption in PCR target region is an important cause of false negative HPV results in high grade VIN. This finding, at least in part, provides explanation for the broad range of HPV prevalence in VIN reported in the literature to date (30.8% - 100%). Aside from the fact that the histological composition of the cohorts varied amongst these studies, the majority of these studies

used only an *L1* targeting PCR to identify cases of HPV (see table 1.2 in background). In addition, nearly all studies in a meta-analysis conducted by de Vuyst *et al*. 2009, which reported HPV prevalence of 85.3% in 1061 cases of VIN, used single region *L1* targeting assays.

4.7.4.1 HPV Prevalence: Strengths

4.7.4.1.1 Quality Assurance (QA) and Reproducibility

All HPV detection/genotyping assays used in this work included a positive (CaSki cell line DNA) and a negative (H_20) control for which, all runs had to generate the correct result. Additional quality assurance checks were applied by the Greiner PapilloCheck® assay which tested DNA integrity in all samples via the amplification of the *ADAT-1* gene; all samples used in this work passed this QA step.

The inter-run reproducibility of the PapilloCheck® and HPV 16 *E6* PCR assays was tested and found to be high. The PapilloCheck® assay was repeated in 25% of the baseline cohort with 98% concordance. For the HPV 16 *E6* PCR assay, 20% of the baseline cohort was retested with 100% concordance and for the HPV 16 *E1* PCR, testing was repeated in 61% of the baseline cohort with 93.8% concordance. The number of samples repeated was higher for the HPV 16 *E1* PCR as this was a new assay developed for this study. The stringent quality control measures were a significant strength of this study.

4.7.4.2 HPV Prevalence: Limitations

4.7.4.2.1 Is a Negative Result Truly a Negative Result in This Cohort?

The multiple assays used by this study could still have failed to detect certain cases of disrupted HPV. HPV 16 was the genotype most exhaustively tested for, and would have been identified by all 4 assays used should their specific target regions be intact (*E1*, *L1*, *E7* and *E6*). Non-HPV 16 genotypes were tested for by three of the assays: PapilloCheck®, DiaMex and HPV *E7* type specific PCR. PapilloCheck® tested for multiple, commonly reported genotypes but targeted the *E1* region, well documented to be commonly affected by viral integration (Cricca, Venturoli *et al*. 2009b; Wang *et al*. 2013; Romanczuk & Howley 1992; Bryant *et al*. 2014). The DiaMex assay also tests for multiple genotypes but specifically targets the *L1* region of the virus; high levels of disruption in this region in cervical disease have been reported. For example, Wang et al. in 2013 found integration events in 64/113 (56.64%) cases of CIN, of which, 73.52% involved the *L1* region of the virus (Wang *et al*. 2013). HPV *E7* type-specific PCR was only able to detect HPV 18, 31, 33, 35 and 51. Retrospectively, the *E6* and *E7* type specific PCRs were best suited to accurately determine the presence of HPV being almost invariably retained viral regions due to their

expression being necessary for conversion to, and maintenance of, the transformed state. Limited DNA availability prohibited extensive use of these assays and HPV 16, 18, 31, 33, 35 and 51 were the genotypes tested for. These limitations mean that the potential for the three HPV negative samples in this cohort being HPV positive should be considered. It cannot be ruled out that the true HPV prevalence in this cohort is 100%.

4.7.4.2.2 Does HPV Prevalence in this Cohort Equate to HPV Pathogenesis?

It is widely accepted that there are four stages in the progression of HPV related disease: HPV acquisition, HPV persistence, progression of persisting infection to transforming infection, and invasion (World Health Organisation International Agency for Research on Cancer 2005). The data presented in this study (and in most studies) assessed HPV status at a single point in time and although a histological diagnosis of VIN has been made, it cannot be said with certainty that the two are not independent of one another. Some studies have tried to tackle this by using additional tests to try to identify those cases where HPV is likely to be the driving force for the disease. For example de Sanjose *et al*. 2013 incorporated a $p16^{INK4a}$ immunohistochemistry-staining test; based on evidence that $p16^{INK4a}$ is commonly overexpressed in VIN caused by HPV infection. Application of p16 immunohistochemistry to the RT3 VIN cohort was considered, but was not performed, because of conflicting evidence in the literature regarding the specificity of $p16^{INK4a}$ over-expression in HPV associated disease; several studies have found high expression in non-HPV associated VIN (Rufforny *et al*. 2005; Hoevenaars *et al*. 2008; O'Neil, McCluggage 2006; Ordi *et a*l. 2009; Riethdorf *et al*. 2004). In the absence of a definitive method available to determine HPV driven disease and HPV independent disease (despite HPV positivity), this study investigated three physical characteristics of the virus associated with cellular transformation: viral integration, viral DNA methylation and viral gene expression. The presence of one or more of these characteristics in a sample can help identify HPV driven cases and HPV independent cases and is discussed further in section 4.7.5.4.

4.7.4.3 Broader Implications

This work emphasises the importance of using HPV detection/genotyping assays that target well-conserved regions of the virus (*E6* and *E7*) to accurately determine HPV presence. HPV presence is more likely to be missed in disease that is high-grade and/or caused by non-HPV 16 genotypes due to the increased likelihood of HPV integration in both scenarios. This is of particular importance if HPV is to be utilised as a biomarker in disease management. For example at the time of this study, the cervical screening programme in England has incorporated the addition of HPV testing to triage management

of patients with low-grade abnormal cytology found in cervical smear tests. In this setting, selection of the HPV test to be used is of paramount importance, in order to minimise the risk of missing high-grade disease, with its associated integrated HPV infection and disrupted DNA. Precision in estimating HPV genotype prevalence in all HPV related disease is also important to aid HPV vaccine design and development, in addition to ensuring accurate assessment of clinical benefit and cost effectiveness.

It is also possible that this work indicates that HPV prevalence in VIN (and perhaps vulval cancer where viral integration is even more likely) is under-represented worldwide due to extensive use of assays reliant on the presence of intact virus. The cohort used in this study was potentially biased towards HPV positive cases through its likely appeal to younger women, particularly keen to avoid the traditional surgical approaches to management. However, other studies reporting 100% prevalence do exist (Torre *et al*. 1992; Nagano *et al*. 1996; Logani *et al*. 2003; Riethdorf *et al*. 2004; Rufforny *et al*. 2005; Srodon *et al*. 2006; Skapa *et al*. 2007; van de Nieuwenhof, van Kempen, *et al*. 2009; Tachezy *et al*. 2011) and it is possible that, like CIN and cervical cancer, HPV may be necessary in the causation of VIN and vulval cancer but not in isolation. It is possible that the presence of lichen sclerosus (thought to drive disease in a proportion of cases of VIN) is one reason why some women with HPV infection develop VIN and others do not. This is potentially supported by findings of high HPV prevalence (48.9%) in 48 cases of histologically defined dVIN (de Sanjose *et al*. 2013). This concept is developed and discussed in more detail in the sections that follow. Finally, the cohort could have been biased as women with invasive disease were excluded and lichen sclerosus dVIN has been reported more commonly in associated with invasive disease (McCluggage 2009).

4.7.4.4 Future Work

It would be of interest to conduct a large study of HPV prevalence VIN. It would be useful to carry out histological assessment to define samples as usual (thought to be HPV dependent) or differentiated (thought to be HPV independent); undertake HPV testing using an assay, such as Hybrid Capture 2 that targets the full HPV genome (that would limit false negative results obtained through PCR target disruption); and apply P16 analysis to determine those cases in which HPV driven disease is present. Such a study could greatly improve understanding of HPV biology in VIN. Finally, the limitation of HPV detection/genotyping tests targeting non-conserved viral regions should be acknowledged and their appropriate use in HPV research carefully considered. It is important to note that the PapilloCheck® assay has clinical validation for use within the cervical screening program (Hesselink et al. 2014). From a clinical perspective, accurate detection of every

HPV positive cases is not always what is important, but recognition of those cases causing disease, is. The validity of the PapilloCheck® assay in this context may reflect this; nonetheless, its 95.8% (Hesselink et al. 2014) sensitivity could be a result of failure to detect cases with disruption in the *E1* region.

4.7.5 HPV Characteristics in VIN

4.7.5.1 HPV Integration

This study constitutes the largest study of HPV integration in VIN to date, and detected HPV integration in 71/136 cases (52.5% CI 45.4%-59.0%) of the HPV 16 positive cases. Lower rates of integration are reported by the two published studies specifically looking at HPV integration in VIN. One study investigated HPV integration in 21 cases of HPV 16/18 positive VIN 3 using the APOT technique (amplification of papillomavirus oncogene transcripts, (Klaes *et al*. 1999). Integrated transcripts were detected in 8/21 (38.1%) (90% CI 21.0%-55.0%) cases (Hillemanns, Wang 2006). The other study investigated a cohort comprising 22 cases of HPV 16 positive VIN 3, 1 case of vaginal intraepithelial neoplasia 3 (VaIN 3), one case of anal intraepithelial neoplasia 2 (AIN 2) and 1 case of a biopsy that contained no discernible vulval pathology. Two techniques were adopted: APOT and DIPS (detection of integrated papillomavirus sequences (Luft *et al*. 2001). Viral integration was detected in 8/25 cases (32%, 90% CI 16.6%-47.4%), (Bryant, Onions *et al*. 2014). Research investigating HPV integration in CIN is more abundant and reported rates range from 11% to 60% (Doorbar *et al*. 2012b). One possible explanation for this variation is the disease grade in the studied cohort. Particularly with regard to the CIN studies, disease grade is often not distinguished making direct comparison with this work difficult. Another possible explanation is the choice of assay used. Both the APOT and DIPS assays specifically target integrated sequences, which can improve the specificity of the assay by reducing the risk of false positive results. However, these assays may have reduced sensitivity by increasing false negative results should assay design not permit the detection of **all** possible integration events. Furthermore, these are technically demanding assays and the integration events should ideally be validated by cross-junction PCR, but this is not always performed.

The presence of viral integration within a cohort could also be affected by the choice of HPV detection assay used. As this study found, successful detection of HPV depends on genomic integrity at the PCR target region. Many HPV detection assays target the *L1* region of the virus and some the *E1* region; both of which are frequently involved in viral integration (Bryant, Onions *et al*. 2014; Cricca *et al*. 2009b; Kalantari *et al*. 1998;

Romanczuk & Howley 1992; Wang *et al*. 1992). Therefore, HPV positive cases detected by these assays are more likely to contain intact, rather than integrated virus, biasing a study towards selection of intact cases and under-representing cases of viral integration. This could be of particular importance when studying cohorts of known high-grade disease when viral integration is more likely to be present (Hudelist *et al*. 2004).

Viral integration is associated with HPV pathogenesis and cellular transformation (Hillemanns &Wang 2006; Jeon *et al*. 1995; Cricca *et al*. 2009b; Woodman *et al*. 2007; van de Nieuwenhof, van Kempen *et a*l. 2009); it is important therefore to consider why not all samples testing positive for HPV 16 contained integrated virus. It is important to understand that HPV integration is not essential for disease to progress and even when in episomal form, disease can exist. For example, Arias-Pulido *et al*. found episomal HPV to be present in 61.9% of 126 cases of cervical carcinoma in situ (Arias-Pulido *et al*. 2006). However, other factors should also be considered, firstly, it is possible that despite the high prevalence of HPV 16 in this study cohort (81.5%), HPV was only responsible for driving disease in the 52% containing integrated virus. The remaining cases could represent those in which viral integration is yet to occur; viral integration may be a late event in HPV transformation and not required development of high-grade disease (Schiffman & Wentzensen 2013; Cullen *et al*. 1991). The disease category VIN 3 could represent a spectrum of disease grade itself; those cases of viral integration in this cohort representing those cases closer to malignancy. It is also possible that in a significant number of cases, alternative means of viral transformation are taking place e.g. DNA methylation (Fernandez *et al*. 2008; Burgers *et al*. 2007; Bryant, Onions *et al*. 2014). Finally, another possibility is that HPV was present but not causative of disease in the presence of alternative disease aetiology e.g. lichen sclerosus; perhaps some cases would have been classified as dVIN if further histological analysis have been available. In reality, the likelihood is that a combination of these three explanations apply i.e. in some cases, the HPV infection is only present as a bystander to alternative disease aetiology; in some cases, integration has not yet occurred; and finally, in other cases, alternative means of viral transformation such as DNA methylation are taking place.

No statistically significant relationship was identified between viral integration and patient age, smoking status or disease episode in this study. Studies do exist reporting associations between increasing patient age and viral integration (Klaes *et al*. 1999; Bryant, Onions *et al*. 2014; Luft *et al*. 2001; Häfner *et al*. 2008; Hopman *et al*. 2004), which is thought to reflect longer duration of HPV infection. In the current study the median age of patients was 45 years in cases of intact HPV and 47 years in integrated (range 20-81), but this difference was not significant. Smoking is well established as a co-factor for the

development of CIN and cervical cancer (Mzarico *et al*. 2015). The high prevalence of smoking (58.8% current smokers and 23.8% previous smokers) reported by women in this study, and others (De Vuyst *et al*. 2009; Jones *et al*. 2005) would suggest that smoking plays a similar aetiological role in VIN. It was hypothesised that smoking could increase the likelihood of viral integration due to diminished local host immunity but such an association was not identified.

4.7.5.1.1 Strengths

The *E1/E2* tiling PCR methodology used in this study is a relatively simple and robust assay. The quantity of template DNA used was standardised; *E6* PCR was used to ensure the presence of amplifiable HPV DNA; and positive and negative controls were used in every run. The data was also subject to reproducibility quality control checks. For the *E1* tiling PCR 61% of baseline RT3 VIN samples were run in duplicate at separate points in time with 93.8% concordance. 30% of all *E2* fragments were performed in duplicate repeat with the following concordance: *E2*-1 fragment 95.1%, *E2*-2 fragment 95.1%, *E2*-3 fragment 92.6%, *E2*-4 fragment 95.1% and *E2*-5 fragment 94.8% (see methods chapter, section 2.5.2.2).It is important to note that, one case included in this study did not amplify HPV 16 *E6* but repeatedly tested positive using the PapilloCheck® assay. This case also failed to amplify any of the *E1/E2* fragments and was likely a false positive HPV 16 positive result. Finally, another strength of this work was the addition of a dual fragment *E1* tiling PCR to the more conventional *E2* tiling PCR assay (Collins *et al.* 2009). This method detected an additional 12 cases of HPV integration (an increase of 9% in the cohort overall) that would not have been detected if the tiling *E2* PCR had been used alone.

4.7.5.1.2 Limitations

An *E1/E2* tiling PCR can only specifically ascertain whether or not the *E1* and *E2* regions of the virus are intact or disrupted. Although it is very likely that, when disrupted, it is a consequence of viral integration, it is not the only possibility. The integrity of the DNA in the sample may be poor as a result of sample processing leading to an over-estimation of the presence of viral integration. The validity of the data was supported by the strong correlation found between viral integration and HPV DNA methylation (section 4.6.3) (p=<0.000). Alternatively, it is also possible that the *E1*/*E2*tiling PCR may under-represent viral integration as these are not the only regions of the virus that are vulnerable to disruption as a result of viral integration. Viral integration has been shown in numerous studies to involve non-*E1/E2* regions of the virus, particularly the *L1/L2* (Bryant, Onions *et al*. 2014; Corden 1999; Schiffman & Wentzensen 2013) region.

4.7.5.2 HPV DNA Methylation

With regard to HPV DNA methylation, this work generated three main findings. Firstly, there was statistically significant variation between samples in the level of HPV DNA methylation at each viral region within the cohort. Secondly, conserved patterns of intra-regional methylation were apparent, consistent with existing reports in other HPV associated disease. Finally, there was no correlation between increased methylation and increased age, recurrent disease or smoking.

4.7.5.2.1 Variable Levels of HPV DNA Methylation at Genomic Regions in VIN 3

The overall level of methylation at each region, for each sample was calculated by taking the mean methylation of all measured regional CpG sites. The level of methylation at each tested region was found to vary significantly amongst samples *(E2* mean = 26.25%±35.65, *L1L2* mean = 28.03%±32.13, promoter mean = 3.143±11.629). Interestingly, for the *E2* and *L1/L2* regions, levels of methylation identified two groups (Figure 4.7). Samples appear to be broadly split into those with low levels of methylation (approximately <10%) and those with high levels of methylation (approximately >50%) with relatively few samples falling between. The same pattern was not apparent in the promoter region.

This is the first study investigating regional HPV DNA methylation within a single disease grade. Reports in the literature exist correlating higher levels of *E2* and *L1/L2* methylation with high-grade cervical and vulval disease (Mirabello, Schiffman *et al*. 2012; Bryant, Tristram *et al*. 2014; Turan *et al*. 2007; Kalantari *et al*. 2010; Wiley *et al*. 2005; Brandsma *et al*. 2009). Based on this, it was not unreasonable to expect relatively consistently high levels of methylation in HPV 16 positive cases in this cohort of VIN 3. The possible explanations of variable regional methylation and, perhaps even two groups of methylation, are the same as those describing why all cases were not found to contain HPV integration above. Those cases highly methylated may represent:

a. Cases closer to malignant transformation within a spectrum of disease grade that may exist within the VIN 3 classification
b. Cases in which HPV DNA methylation is driving cellular transformation as opposed to those cases in which alternative viral characteristics are e.g. viral integration
c. Cases with HPV driven disease as opposed to those cases in which HPV is an incidental finding to alternative vulval disease aetiology e.g. lichen sclerosus

Again, it is most likely that it is a combination of these three reasons that explains the variation in reality. Correlations between viral characteristics exploring this further are discussed in section 4.8.

4.7.5.2.2 HPV DNA methylation of the promoter region is consistently low in VIN 3

It was hypothesised that HPV DNA methylation of the promoter region (containing the $E2BS_3$ and the $E2BS_4$) would be high in VIN, representing a mechanism leading to deregulated oncogene expression and viral transformation. This hypothesis was based on evidence that methylation of the E2BS inhibits binding of the E2 protein (Thain *et al*. 1996; Kim *et al*. 2003). However, methylation of this region was found to be consistently low (mean = 3.143±11.629). This finding suggests that low/no methylation of the promoter may confer a selective growth advantage. Research reporting HPV promoter region methylation in vulval intraepithelial neoplasia is contradictory, some studies report increased methylation with increased disease grade (Brandsma *et al*. 2009; Kalantari *et al*. 2004; Bhattacharjee & Sengupta 2006; Ding *et al*. 2009), and others report the opposite (Piyathilake *et al*. 2011; Badal *et al*. 2003; Hublarova *et al*. 2009; Patel *et al*. 2012). This current study found almost universally low levels of promoter region methylation in a large cohort of high-grade disease and therefore, supports the latter.

The higher levels of methylation seen in the *E2* and *L1/L2* regions could suggest that methylation of these regions confers a selective advantage to the cell. It is possible that intragenic methylation of *E2* directly represses transcription of the gene. *E2* expression is known to repress the expression of the *E6* and *E7* oncogenes and without it, *E6* and *E7* expression would be unopposed and disease more likely to ensue. The *L1* and *L2* genes are responsible for capsid formation enduring the later stages of the viral life cycle and are not known to play a significant role in transforming infection (Ozbun & Meyers 1997).

4.7.5.2.3 Conserved intra-regional patterns of methylation exist in the RT3 VIN cohort

The mean level of methylation at each CpG site within each region was found to be different and conserved patterns of methylation at each region were apparent, comparable to patterns reported by other studies (Bryant, Tristram *et al*. 2014; Kalantari *et al*. 2004). Methylation patterns at each genomic region were of interest in this work as this observation contributes to a small, but growing, body of evidence, regarding the mechanisms of HPV DNA methylation. It is proposed that this conserved pattern of methylation is a result of specific mechanisms of DNA methylation targeting each CpG differently. It is possible that this results from enzymatic or structural differences within each region that might affect the ability of DNMT's and meCpG binding proteins to bind the DNA. Relevant structural differences may include nucleosome position, nuclear matrix

attachment regions and DNA secondary structure. Methylation of genomic DNA is directly related to the nucleosomal structure of DNA, which has a helical pitch of 10-15 bp (Chodavarapu *et al*. 2010). DNMTs access the major groove of nucleosomal DNA which would allow better access to DNA on the outside of nucleosomes; consistent with differing methylation levels seen every 10 bp. Different methylation levels every 10 bp due to nucleosomal structure would account for the "N" shaped pattern for the *L1/L2* region seen in this work as well as the conserved pattern seen in the *E2* region (Figure 4.10, Figure 4.12).

4.7.5.2.4 Variation of Inter-Regional HPV DNA Methylation

The median rank methylation of each test region was found to be statistically different (*E2*, *L1/L2* and the promoter region). Mean methylation at *E2* and *L1/L2* was generally higher and more variable (26.25% ± 35.82 and 28.03% ± 34.93 respectively) than the promoter region, which was almost universally unmethylated or methylated to very low levels (3.14% ± 13.44). These findings are supported by similar patterns found in a study of VIN investigating the same regions previously conducted by the same laboratory (Bryant, Onions *et al*. 2014). These differences could be explained by selective growth advantages/ disadvantages that methylation confers at each region.

4.7.5.2.5 Patient Age, Smoking, VIN Disease Episode and HPV DNA Methylation

No statistically significant correlation was identified between HPV DNA methylation and patient age, smoking status or disease episode. It was hypothesised that older patients would have higher levels of HPV DNA methylation. This association is thought to result from the longer duration of infection making disease progression more likely (Clarke et al. 2012).This was not found to be the case; no relationship between age and methylation was identified. This study was not designed to identify such associations but in view of similar findings reported elsewhere in the literature (Mirabello *et al*. 2013 Mirabello *et al*. 2012), it is increasingly likely that no relationship exists. Similarly, patients with recurrent VIN disease were also hypothesised to have higher levels of methylation due to putative longer duration of infection but, again, no relationship was identified. Finally as described previously, smoking has been associated with diminished local immunity and is well established as a co-factor for the development of CIN and cervical cancer (Barton *et al*. 1988; Giuliano *et al*. 2002; Holly *et al*. 1986; Castle *et al*. 2002; McIntyre-Seltman *et al*. 2005). Although smoking was common in this studied cohort (58.8% current smokers and 23.8% previous smokers) suggesting a possible aetiological role, no association was found between smoking and HPV DNA methylation.

4.7.5.2.6 Strengths

Pyrosequencing measures DNA methylation in a sequencing-by-synthesis manner that quantitatively monitors the real time incorporation of nucleotides through the enzymatic conversion of released pyrophosphate into a proportional light signal. This quantitative measurement is more informative than other techniques available. The relatively simple protocol limits the influence of human error and enables efficient workflow with high throughput, testing multiple CpG's in a single assay. Pyrosequencing is widely accepted as the technique of preference for methylation studies avoiding the biases inherent to the cloning procedure (Siqueira *et al*. 2012).

Stringent quality control measures were applied to the data. The pyrosequencing software incorporates internal quality control checks identifying those samples, which have failed bisulphite conversion of sequencing, and therefore should be excluded. Any samples identified as 'check' indicating that manual assessment for inclusion was required were thoroughly reviewed. Additionally, all samples were repeated in duplicate within a run and only included if the variation in the results was less than three standard deviations of the mean standard deviation calculated for all CpG site for each region. Results of quality control analyses are included in methods chapter.

4.7.5.2.7 Limitations

The quality assurance checks applied to this data impacted on the available data for analysis. The promoter region withstood the checks better than the *E2* and *L1/L2* regions. High levels of viral integration were found in those cases that failed the initial pyrosequencing (84.4% for *E2*, 65.6% for *L1/L2* and 62.5% for the promoter). Viral integration was tested for using a tiling PCR targeting several overlapping fragments of the *E1* and *E2* genes, which could explain why higher rates were detected in those cases of *E2* failure. It is possible that the addition of a *L1/L2* region-tiling assay would identify more cases of integration. One possible explanation for the high failure rate would be the high levels of disrupted virus in the cohort of high-grade disease. The promoter region may be less affected by viral disruption due to integration. Additional factors contributing to the high failure rate include poor DNA integrity of the sample in general, human error in running the assay and overly stringent parameters being applied with regard to variation between duplicate repeats.

4.7.5.3 HPV Gene Expression

Expression levels of the *E6* and *E7* but particularly the *E2* gene varied in the cohort of VIN 3 compared to expression of two stably expression human reference genes *TBP* and *HPRT*. This supports the findings of a small published study conducted by Bryant *et al.* who also

found varied *E2/E6/E7* expression in a cohort of 25 cases of high anogenital neoplasia (Bryant, Onions *et al*. 2014).

Exploratory analysis of *E2* expression relative to *E6* expression for each sample was undertaken to further investigate the biological and pathological heterogeneity in the cohort that was indicated in the data generated for viral integration and methylation. Cases with high *E2* expression relative to *E6* expression were classified as 'regulated' oncogene expression, in view of the controlling presence of *E2*. Cases with high *E6* expression relative to *E2* expression were classified as 'deregulated' oncogene expression potentially associated with loss of *E2*. The majority of HPV 16 postive cases in the cohort were found to have high E6 expression relative to E2 expression (74.1% vs. 25.9%) indicating that in the majority of cases of VIN 3, deregulated oncogene expression was present. This aspect of the work was purely exploratory and is not a proven method of identifying regulated and deregulated oncogene expression. There is no established method for accurately determine regulated and deregulated gene expression and for this reason, comparison with data in the literature is not possible.

The final important finding was that gene expression did not appear to be a proxy marker for any of the tested patient characteristics. Although older patients expressed lower levels of *E2* than younger patients and the mean age of patients with *E6>E2* was higher than patients expressing *E2>E6* (48 years vs. 43 years) neither of these findings were statistically significant. Patients with recurrent disease were significantly more likely to have higher levels of *E6* expression relative to *E2* than patients with first episode disease ($p = 0.004$). However, *E6>E2* expression was not a proxy marker for recurrent disease because in 37/59 cases of first episode disease, the pattern of expression was also *E6>E2*. There were no correlations identified between gene expression and smoking status.

4.7.5.3.1 Strengths

This study constitutes the largest cohort of VIN 3 cases in which HPV gene expression has been assessed. The comparison of expression levels of individual genes within the cohort, as well as the comparisons of expression levels between genes within each sample represents a very thorough analysis of gene expression. In addition, the use of Biogazelle Qbase+ software facilitated robust and rigorous analysis of the data. This software uses expression levels of human reference genes to 'normalise' the expression level of each GOI, which helps to reduce the impact that differing levels of template DNA in each sample have on expression level. The software also generates expression levels for each GOI that are relative to the other samples in the cohort. This means that the expression level of a gene in a cohort can accurately be compared between the samples. The qBase+ software

also incorporates internal quality controls, which were strictly adhered to. These are outlined in section 4.5.1. To further improve data reliability all reverse transcription (RT) positive reactions were repeated in duplicate within a run. All samples that generated data indicative of inadequate RT reaction were excluded from analysis.

4.7.5.3.2 Limitations

The mean RNA concentration from samples used in this study was 115 ng/μl and the mean 260/280 nm ratio of absorbance generated using Nanodrop spectrophotometry was 2.1 indicating high purity (the NanoDrop manufacturers suggest that a ratio of ~2.0 is regarded as 'pure'). However, assessment of RNA integrity using an Agilent Bioanalyser in a random selection of 10% of the samples indicated a mean RIN of 3.34 (Appendix 1). This suggests RNA of poor integrity and this could reduce the accuracy of mRNA quantification.

The use of corrected, normalised relative quantification of RNA expression assumes that for every cell present containing the reference genes to which the normalisation takes place, HPV is also present. This is not necessarily the case and consequently, represents a limitation of the method.

4.7.5.3.3 Broader implications

The E2 protein regulates the expression of the *E6* and *E7* oncogenes. Loss of this control is thought to result from viral integration (as well as epigenetic mechanisms such as DNA methylation) and is fundamental in the development of a transforming infection (Stanley 2002; Kessis *et al*. 1993; Pierry *et al*. 2012). Expression of all HPV genes tested in this cohort (*E2*, *E6* and *E7*) varied, but the expression of *E2* seemed to be particularly variable. Using *E2:E6* ratio as an indicator, there were cases demonstrating the presence of both regulated (*E2>E6*) and deregulated (*E6>E2*) gene expression in the cohort; 74.1% expressed E6>E2 and 25.9% expressed *E2>E6*. The varied gene expression found in this cohort further supports the concept that VIN 3 represents a biologically heterogeneous disease. Cases with relatively low *E2* expression may represent those cases in which HPV is driving disease. Cases with relatively high *E2* expression may be cases in which HPV is present as a bystander to disease caused by alternative pathology or may be cases of HPV associated VIN 3 that have not yet reached the stage of cellular transformation and are therefore less close to malignancy.

Although not statistically significant, trends were found between increased age and decreased *E2* expression, and between recurrent disease and decreased *E2* expression. It is possible, that this finding indicates that a transforming infection is more likely to have developed with the longer duration of HPV infection older patients and patients with recurrent disease may have experienced. Alternatively, age is an independent risk factor

for the development of most cancers (Anisimov 2007; Anisimov & Petrov 1987; Anisimov 2003); it may be the case that should an older patient contract HPV infection the likelihood of transformation is higher independent of duration of infection.

4.7.5.4 Relationships Between Viral Characteristics

Correlations were investigated between three characteristics of transforming HPV infection to further develop understanding of HPV pathogenesis. HPV integration was assessed using an *E1/E2* tiling PCR data, high HPV DNA methylation was defined as mean methylation >50% of the *E2* and *L1/L2* CpG sites and deregulated oncogene expression was represented by *E6* expression >*E2* expression. Ninety-two cases had valid results for all assays. At least one transforming viral characteristic was present in 73/92 (79.3%) and these may be cases in which HPV is driving disease. None of the transforming characteristics were detected in 19/92 (20.7%) of cases and these may be cases in which HPV is an incidental finding.

4.7.5.4.1 Mechanisms Leading to Deregulated Oncogene Expression

HPV integration (indicated by the presence of HPV disruption) strongly correlated with cases of lower *E2* expression (p = <0.0001) and with cases of deregulated oncogene expression (represented by the expression profile *E6>E2*), (p = 0.009). However, no relationships were identified between HPV integration and *E6/E7* gene expression. Similar findings were reported in a recent study of the HPV biology in 25 cases of VIN (Bryant, Onions *et al*. 2014). This indicates that viral integration is associated with reduced *E2* expression but apparently not with significantly altered *E6/E7* expression. It is possible that in non-integrated cases, alternative mechanisms de-regulating E6/E7 expression exist. This idea is supported by research in cervical disease where evidence also exists that expression levels of *E6* and *E7* are consistently maintained and what changes is the expression of *E2* (Doorbar 2006; Baldwin & Münger 2010; Münger & Howley 2002; Baker *et al*. 1987). Loss of E2 expression has been linked to increased stability of HPV 16 E6/E7 mRNA (Jeon, Lambert 1995; Jeon *et al*. 1995). It is therefore possible that the effect of loss of *E2* expression is increased stability of E6/E7 mRNA, which in turn is what drives disease rather than increased expression levels per se. This finding is important when considering the role of HPV gene expression as a biomarker. The varied expression of *E2*, compared to the relatively consistent expression of *E6* and *E7* (as well as the biological justification) makes *E2* a better biomarker candidate.

Viral integration and high HPV DNA methylation were strongly correlated (p = <0.0001). Two smaller studies have reported similar correlations (Bryant, Onions *et al*. 2014; Kalantari *et al*. 2010). The reason for this correlation is unclear and whether these

characteristics are independent of one another or not is difficult to determine. Three reasons why HPV DNA methylation arises have been proposed:

- Methylation of HPV DNA in VIN HPV DNA may arise via *de novo* methylation-mediated silencing of foreign DNA upon host recognition of viral integration into the host genome (Heller *et al*. 1995)
- Methylation may arise as a result of activation of an instructive program of methylation imposed by HPV gene expression that occurs as a result of viral integration disrupting the *E2* ORF (Leonard *et al*. 2012)
- Methylation may arise as an HPV self-regulatory mechanism designed to prevent excessive oncogene expression that again, results from HPV integration disrupting the *E2* ORF (De-Castro Arce *et al*. 2012)

All three reasons above indicate that HPV DNA methylation occurs as a result of HPV integration and could therefore explain the correlation seen in this work and others. It has however, also been hypothesised that HPV DNA methylation may represent an alternative mechanism of deregulated oncogene expression in the absence of HPV integration. High levels of HPV DNA methylation also strongly correlated with lower *E2* expression ($p = <0.0001$) and with deregulated oncogene expression ($p = 0.008$ and $p = 0.004$ respectively). It is therefore feasible that high HPV DNA methylation is a mechanism leading to deregulated oncogene expression. Of the 92 cases with data available for all three viral characteristics 66 had deregulated oncogene expression (*E6>E2*), of which only 6 cases (9.1%) had high HPV DNA methylation without HPV integration and only 11/66 (16.7%) had HPV integration without high HPV DNA methylation. The presence of both these characteristics was found in a significant number of cases 25/66 (37.9%). This data suggests the possibility that deregulated oncogene expression can be caused by both HPV DNA methylation and HPV integration independently but is more likely to happen in samples with both characteristics. Most surprisingly however, was that in 24/66 (36.4%) cases of deregulated oncogene expression, neither of the proposed mechanisms was identified. Research in cervical disease offers a potential explanation for these cases. Although viral integration is present in the majority of cases of cervical cancer, it is not present in all. Alternative mechanisms leading to deregulated oncogene expression have been identified (Doorbar 2006). For example, studies have shown that HPV associated cellular transformation can result from exposure to glucocorticoids (Pater *et al*. 1988; Chan *et al*. 1989) as well as chronic oestrogen exposure (Arbeit, Howley 1996). It is possible that alternative mechanisms, such as these, are relatively more important in VIN. Additionally, although aberrant oncogene expression can predispose to the development

of cervical cancer, their expression alone is not considered sufficient, and *E6* and *E7* expression alone cannot fully transform human keratinocytes in culture. It is generally accepted that HPV mediated carcinogenesis requires the accumulation of additional genetic changes caused by alternative means such as age and cigarette smoking (Collins *et al*. 2010; Doorbar 2006) and possibly in the case of VIN, other disease processes such as lichen sclerosus. It is however, important to remember that the nature of this work is exploratory; *E2:E6* expression ratio is not a validated method of determining deregulated oncogene expression and inferences drawn from this data must be interpreted with caution.

4.8 Conclusion

HPV prevalence in this cohort of VIN 3 was 98.2% and 100% prevalence could not be excluded due to inherent limitations of the methods used to detect HPV. Despite potential cohort recruitment bias towards HPV associated VIN, the median age of the cohort was close to that of VIN in general, and this suggests significant bias was unlikely. The sequential use of multiple HPV detection assays targeting different viral regions indicated that PCR target disruption (likely as a result of viral integration) is an important cause of false negative results in VIN, similar to the seminal study in cervical cancer conducted by Walboomers et al. 1999. Based on these findings, it could be hypothesised that, as is the case with CIN and cervical cancer, all VIN (and possibly vulval cancer also) is too, associated with HPV. This hypothesis is supported by the fact that the majority of studies investigating HPV prevalence in VIN use detection assays that target the *L1* region of the virus: a region frequently found to be disrupted. However, this hypothesis is opposed by the absence of viral characteristics associated with HPV carcinogenesis in a significant number of cases, indicating that HPV positivity does not necessarily equate with HPV driven disease. Research exists demonstrating that the absence of HPV characteristics typically associated with HPV carcinogenesis does not necessarily exclude the aetiological role of HPV in disease (Parfenov *et al*. 2014; Bosch *et al*. 2002). It could be speculated that the heterogeneous nature of the biology of HPV observed in this study represents one of, or a combination of, three things:

- That while VIN 3 comprises a single histological entity, there exists a spectrum of molecular abnormality within it.
- That HPV carcinogenesis in VIN is biologically diverse and that HPV integration (+/- HPV DNA methylation) leading to deregulated oncogene expression leading to cellular transformation is an over-simplification of a much more complex situation.
- That HPV positivity does not necessarily equate with HPV driven disease

This study was not able to determine with any certainty which of the above explanations is most likely, it is quite possible that in reality, a combination of all three exists. However, the possibility cannot be ignored that, as is the case in cervical disease, all VIN is associated with HPV infection but, in VIN, other factors contributing to carcinogenesis are more important e.g. chronic skin disease (Collins *et al*. 2010; Doorbar 2006; Pater *et al*. 1988; Sun *et al*. 2011; Arbeit, Howley 1996). It may well be the case that lichen sclerosus represents an alternative aetiology of VIN independent of HPV. It could also be the case however that lichen sclerosus provides the deficiencies in epithelial integrity required by HPV in order to reach its target basement membrane. Rather than driving VIN independently of HPV, lichen sclerosus perhaps promotes HPV driven disease. This is supported by the fact that HPV is not infrequently detected in cases dVIN (de Sanjosé *et al*. 2013) and also by the fact that false negative HPV detection is more likely in case of integrated virus. If lichen sclerosus aids HPV pathogenesis, then viral integration is perhaps more likely to be present and therefore HPV more likely to go undetected.

Chapter 5

Results

HPV characteristics as biomarkers to predict response to treatment of VIN

5 HPV Characteristics as Biomarkers to Predict Response to Treatment of VIN

5.1 Introduction

This chapter reports the results of investigation into the suitability of viral characteristics, as potential biomarkers to predict response to treatment of VIN with two medications: cidofovir and imiquimod. The RT3 VIN clinical trial was a phase II clinical trial that investigated the efficacy and safety of treating patients with VIN 3 with cidofovir and imiquimod. The full design and results of this study are discussed in chapter 3. The principal findings of the study however, were that 41/72 (57%) VIN 3 patients treated with cidofovir responded and that 42/69 (61%) VIN 3 patients treated with imiquimod responded. These response rates make both cidofovir and imiquimod appealing treatment alternatives to surgery, and worthy of investigation in a phase III clinical trial. The response rates do however highlight the potential benefits of a biomarker (BM) that could identify women who are more likely to respond to treatment, avoiding unnecessary side effects and prolonged treatment with medication destined not to work. With the rationale for biomarker development established four candidate viral biomarkers were identified:

1. HPV positivity
2. HPV disruption status
3. HPV DNA methylation
4. HPV gene expression

Guidelines for predictive biomarker development published by CRUK (Figure 5.1) were adhered to. The first section of this chapter investigates the distribution of each biomarker in the RT3 VIN baseline cohort working towards BIDD BM discovery –stage 1. The second section of this chapter describes the retrospective correlation with response to treatment of patients in the RT3 VIN clinical trial, working towards BM discovery – stage 2. All viral characteristic work was conducted using cases that tested positive for HPV 16; it is therefore only these cases that are discussed from this point on. It is also of note that all data analysed comes from patients included in the intention to treat analysis arm of the main clinical trial. This means the analysis all patients enrolled in the trial who provided a pre and post treatment biopsy despite whether or not they strictly adhered to the treatment regime assigned to them.

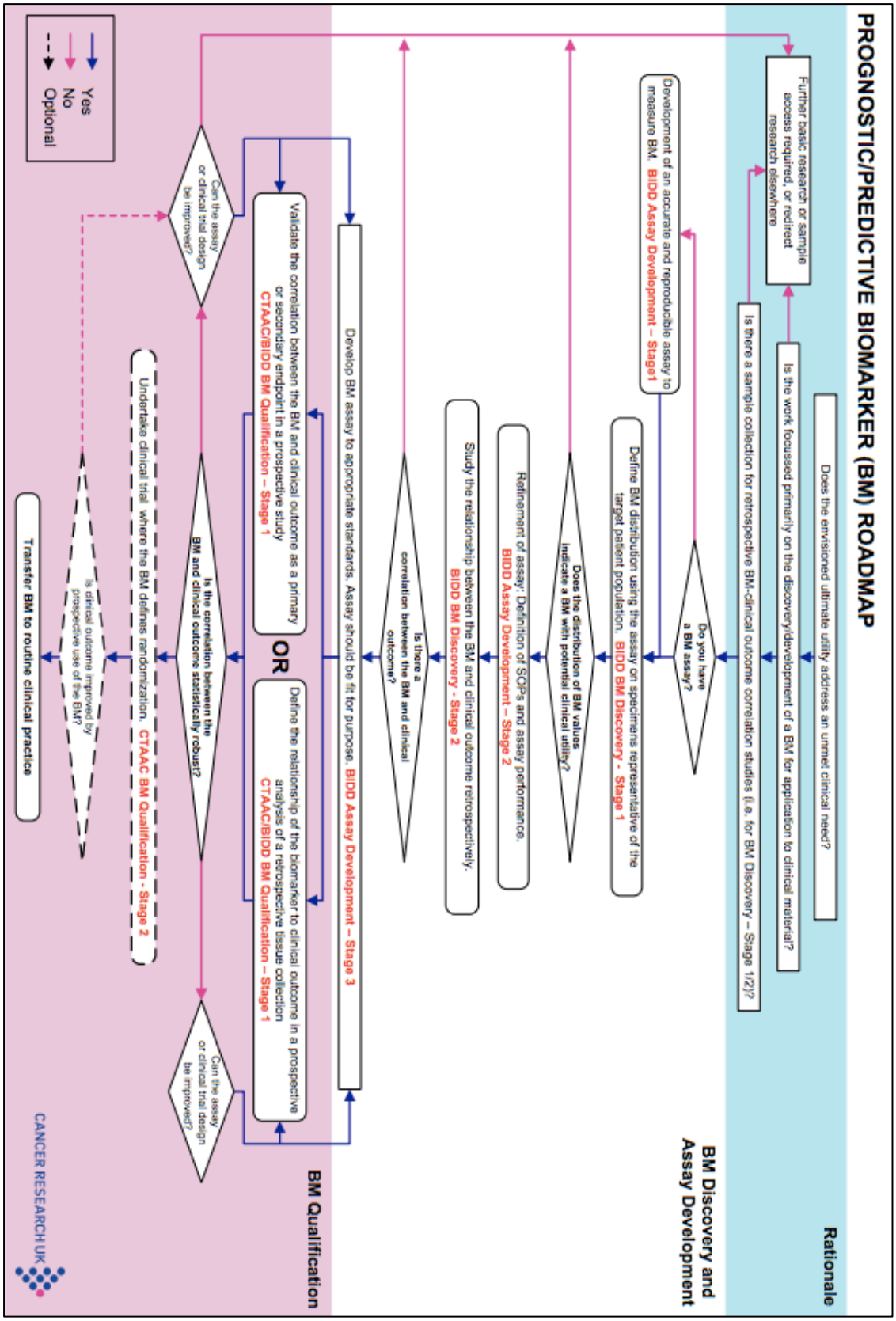


Figure 5.1 – CRUK BIDD Biomarker Roadmap

5.2 CRUK Biomarker Discovery – Stage 1

To achieve BM development to discovery stage 1 according to the CRUK guidelines, the distribution of a candidate biomarker must vary in a cohort representative of the target population. The baseline cohort from the RT3 VIN clinical trial represented a suitable study cohort with all patients having been diagnosed with histologically confirmed VIN 3. There were tissue biopsies from 167 patients enrolled in the study at baseline, available for analysis. HPV positivity was investigated in all 167 samples using four HPV detection assays (PapilloCheck®, HPV 16 *E6* PCR, DiaMex and type-specific *E7* PCR) (Section 4.2). Analyses of the distribution of HPV integration, HPV DNA methylation and HPV gene expression were restricted to the 136 HPV 16 positive cases identified through a combination of PapilloCheck® data and HPV 16 *E6* PCR data. This was because the assays were designed to investigate the most prevalent genotype and because data was only available from these two assays at the time of this analysis.

5.2.1 Variability in HPV Status

HPV was detected in 164/167 (98.2%) cases. Full HPV genotyping results are detailed in section 4.2. HPV 16 was detected in 140/167 (81.4%) cases and represented the most prevalent genotype followed by HPV 33, which was detected in 14/167 (8.5%). With the majority of cases testing positive for HPV, HPV positivity alone was not deemed suitable for further BM development. Although high (140/167, 81.4%), HPV 16 positivity did demonstrate varied distribution in the cohort and was therefore assessed further.

5.2.2 Variability in HPV Disruption

HPV disruption, defined by the failure to amplify ≥ one *E1/E2* tiling PCR fragments, was used detected in 71/136 (52.2%) cases, whereas 65/136 (47.8%) contained intact HPV. HPV disruption was therefore deemed suitable for further BM development. Full analysis of HPV disruption in VIN 3 is available in chapter 4, section4.3.

5.2.3 Variability in HPV DNA Methylation

The methylation level of three HPV regions (*E2*, *L1/L2* and the promoter region) was tested using pyrosequencing. Detailed analysis of HPV DNA methylation in the cohort can be found in chapter 4 section 4.4. For the *E2* region (n = 82) mean methylation was 27.2% ± 35.8% (p = <0.000), for the *L1/L2* region (n = 93), mean methylation was 37.5% ± 34.9% (p = <0.000) and for the promoter region (n = 122), mean methylation was 3.9% ± 13.44% (p = <0.002) (Figure 4.7). Based on these findings, HPV DNA methylation of all regions was considered suitable for further biomarker development.

5.2.4 Variability in HPV Gene Expression

Investigation of HPV gene expression in 136 HPV 16 positive patients in the RT3 VIN baseline cohort is fully detailed in section 4.5. The range of *E2* gene expression was CNRQ 0.00 – 3.68, with a mean CNRQ of 1.88 ± 1.30. The range of E6 expression was 0.00 - 2.19 with a mean CNRQ of 1.01 ± 0.48 and the range of E7 expression was 0.00 – 2.32 with a mean CNRQ of 1.2 ± 0.49 (Table 4.6). Figure 4.13shows the variation in expression of each gene in the cohort and enables visual comparisons to be made between expression levels of each gene. The range of both HPRT and TBP expression was CNRQ 0.00 – 0.83, the mean HPRT and TBP CNRQ were 0.43 ± 0.23 and 0.40 ± 0.23 respectively. Expression of each HPV gene was more variable than the expression of the human reference genes, and therefore deemed suitable for further BM development. *E2* expression demonstrated the most variation.

5.2.4.1 HPV *E2:E6* Gene Expression in VIN 3

This exploratory analysis found that in the majority of cases (83/112, 74.1%) *E6* expression was greater than *E2* indicating the presence of deregulated gene expression and in 29/112 (25.9%) *E2* expression was greater than *E6* indicating those cases with regulated oncogene expression. E2:E6 expression ratio to distinguish cases of deregulated oncogene expression from cases of regulated oncogene expression was therefore considered suitable for further biomarker development. For more details on gene expression data, see section 4.5.

5.2.4.1.1 Distribution of 'HPV Driven Disease'

With the broad acceptance that not all VIN 3 is caused by HPV (Sideri *et al*. 2005) and that HPV may be present as a bystander to alternative disease aetiology (de Sanjose *et al*. 2013), the presence of ≥ one HPV characteristics associated with transformation was used to distinguish cases of HPV driven disease from cases of HPV independent disease. Transforming HPV characteristics were defined as HPV integration (represented by failure to amplify ≥ 1 *E1/E2* tiling fragments), high HPV DNA methylation (represented by mean methylation >50% of all *E2* and *L1/L2* CpG sites) and deregulated oncogene expression (represented by an exploratory method identifying those cases with *E6>E2*expression). Investigation of this potential biomarker was only possible for HPV 16 positive cases (for which the assays were designed). Data for all three characteristics was available for 92/136 cases following quality control checks (Chapter 4, section 2.7.4). Using these definitions, HPV driven disease was present in 73/92 (79.3%) of cases, and HPV independent disease was present in the remaining 19/92 (20.7%). Although the majority

of cases of VIN 3 were defined as 'HPV driven', a fifth of cases (20.7%) were not, hence, this classification was considered suitable for further BM development.

5.3 BIDD Biomarker Discovery– Stage 2

Retrospective correlation between the potential BM and response to treatment are required to satisfy the criteria of biomarker discovery stage 2. This was undertaken for the following candidate viral characteristics:

- HPV 16 status
- HPV driven disease (determined by the presence of ≥1 viral characteristics associated with transformation)
- HPV disruption
- HPV DNA methylation of the *E2*, *L1/L2* and promoter regions
- *E2*, *E6* and *E7* gene expression
- *E2:E6* gene expression ratio to distinguish regulated oncogene expression from deregulated oncogene expression

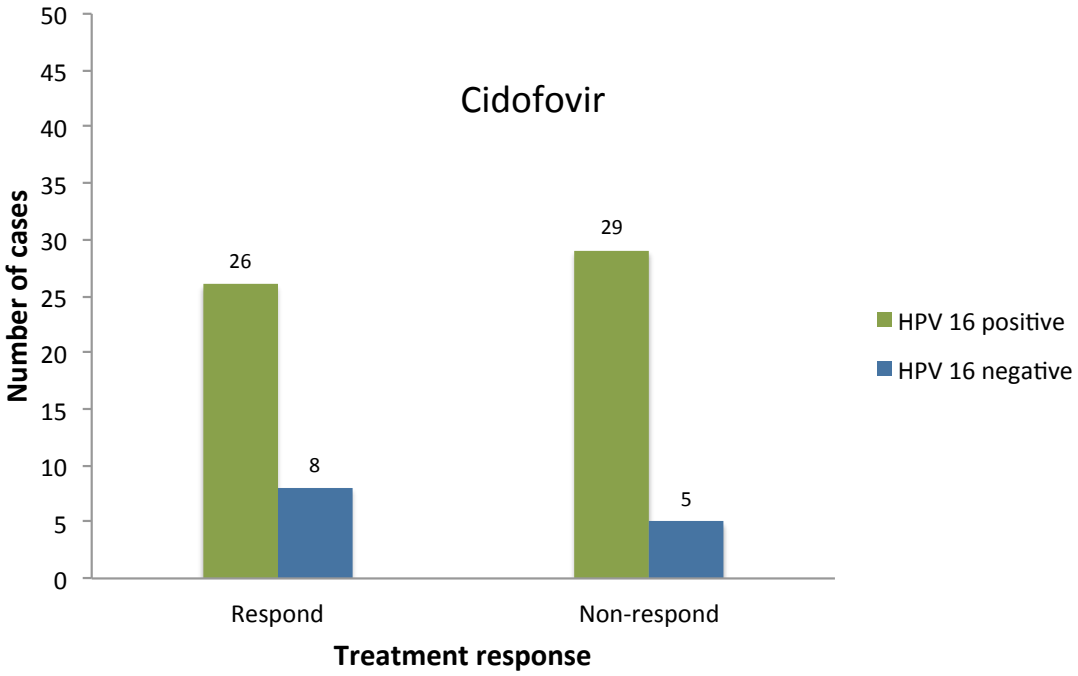
Response to treatment with either cidofovir or imiquimod was determined by the absence of VIN in a tissue biopsy taken from the previously affected area 6 weeks following the completion of treatment. The presence of VIN 1 or greater was considered persistent disease and failure to respond. Full data including the post treatment histology result and baseline HPV result was available for 135 patients.

5.3.1 HPV 16 Positivity and Response to Treatment

The proportions of HPV 16 positive patients who responded or failed to respond to treatment with cidofovir were similar (26/55, 47.3% vs. 29/55, 52.7%). This was also the case for treatment with imiquimod, for which 28/56 (50.0%) of patients responded and 28/56 (50.0%) failed to respond. On the other hand, patients who were HPV 16 negative were more likely to respond to (8/13, 61.5%), than not to respond to treatment with cidofovir (5/13, 38.5%). Similarly, with imiquimod 8/11 (72.7%) HPV 16 negative patients responded to treatment and 3/11 (27.3%) failed to respond. A chi-square test was run to determine whether the observed values differed significantly from those expected. A Bonferroni correction was applied to allow for multiple comparisons, a p value of 0.017 was considered significant. No statistically significant difference was found for treatment with cidofovir ($p = 0.335$) or treatment with imiquimod ($p = 0.167$), (Figure 5.2).

Further analysis of the 24 HPV 16 negative cases revealed that in those cases that failed to respond to treatment (n=8) an alternative high-risk HPV genotype had been detected. In those cases that responded to treatment (n=16), no HPV was detected in three cases and low-risk only HPV was detected in 2 cases; alternative high-risk genotypes were detected

in the other 10 cases. It is of note that there were only three cases in the whole study cohort in which no HPV was identified, all of whom responded to treatment (two with imiquimod and one with cidofovir). Additionally, there were only five cases in which low-risk only HPV was identified in the whole cohort, two of whom had data suitable for this section of analyses and both responded to treatment. Therefore, all patients in the cohort with no detectable high-risk HPV responded to treatment.



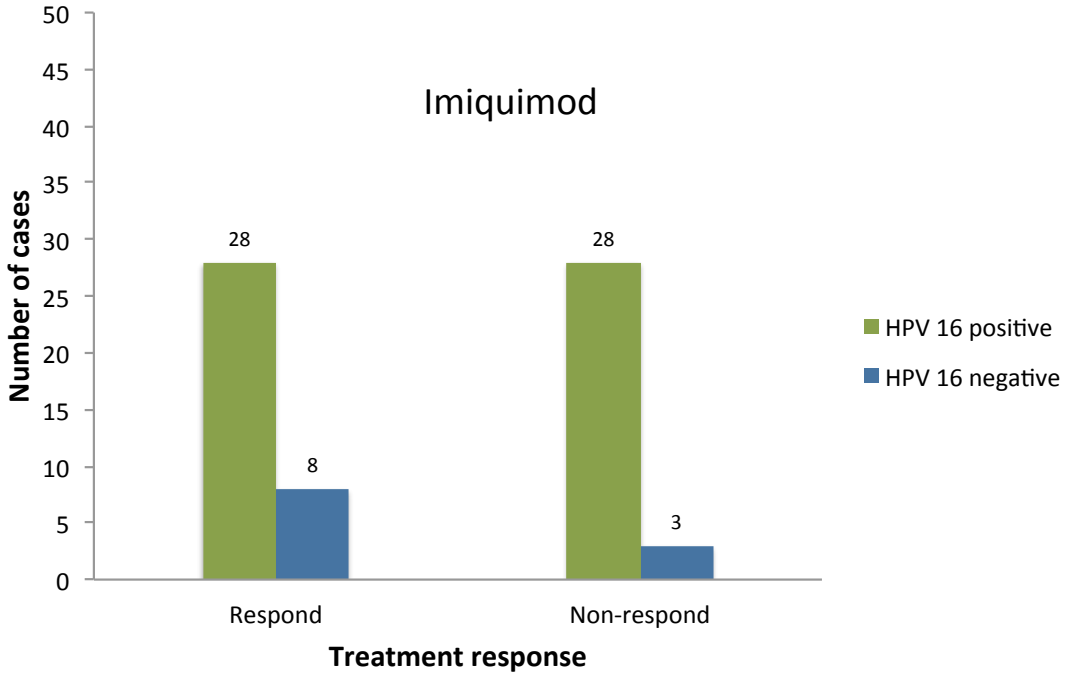


Figure 5.1 HPV 16 status and response to treatment. Response to treatment defined as histologically confirmed absence of VIN in tissue biopsy taken 6 weeks following treatment. The top graph shows cidofovir data and the bottom graph shows imiquimod data.

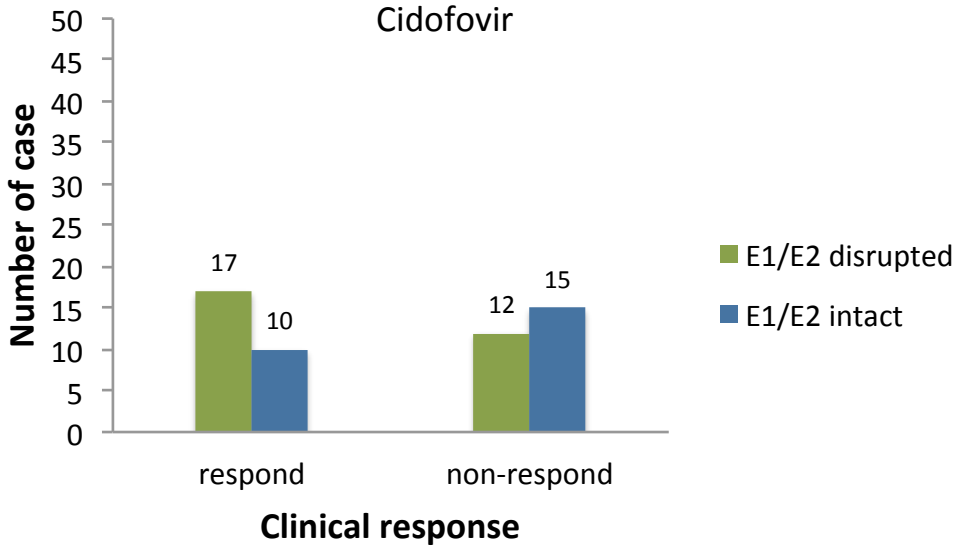
5.3.2 HPV Driven Disease and Response to Treatment

The presence of ≥ one of three viral characteristics (HPV disruption, HPV DNA methylation >50% and HPV gene expression *E6>E2*) was used as an exploratory method to identify cases in which HPV was driving disease. Ninety-two cases generated a full viral data set of which, 78 had clinical outcome data available from a post-treatment tissue biopsy.

Of those patients who responded to treatment with cidofovir, evidence for HPV driven disease was detected in 17/21 (81.0%) and HPV independent disease in 4/21 (19.0%). In patients who did not respond, evidence for HPV driven disease was detected in 16/19 (84.2%) and HPV independent disease in 3/19 (15.8%). No statistically significant association was identified using a Chi-square test incorporating a Bonferroni correction of p = 0.016 to allow for multiple comparisons (Chi-square = 0.073, p = 0.787). Similarly, for imiquimod treatment, of patients who responded to treatment, evidence for HPV driven disease was detected in 18/21 (85.7%) and HPV independent disease in 3/21 (14.3%). In patients who did not respond, evidence for HPV driven disease was detected in 12/17 (70.6%) and HPV independent disease in 5/17 (29.4%). No statistically significant association was identified using a Chi-square test (Chi-square = 1.293, p = 0.255).

5.3.3 HPV *E1/E2* Disruption and Response to Treatment

Valid data was available for 54 patients treated with cidofovir. Twenty-seven (50.0%) responded to treatment of whom, 17/27 (63.0%) had disrupted HPV and 10/27 (37.0%) had intact HPV. Of the twenty-seven patients who did not respond to treatment, intact HPV was found in the majority (15/27, 55.6%) and disrupted HPV in 12/27 (44.4%), (Figure 5.3). This association was not significant, (Fisher's exact test p = 0.275). For patients treated with imiquimod (valid data for 53 patients) the opposite trend was observed; of patients who responded to treatment, 15/28 (53.6%) had intact HPV and 13/28 (46.4%) had disrupted HPV. In patients who did not respond to treatment, 14/25 (56.0%) had disrupted HPV and 11/25 (44.0%) had intact HPV (figure 5.5). The association between *E1/E2* disruption and response to treatment with imiquimod was also not significant (Fisher's exact test, p = 0.586).



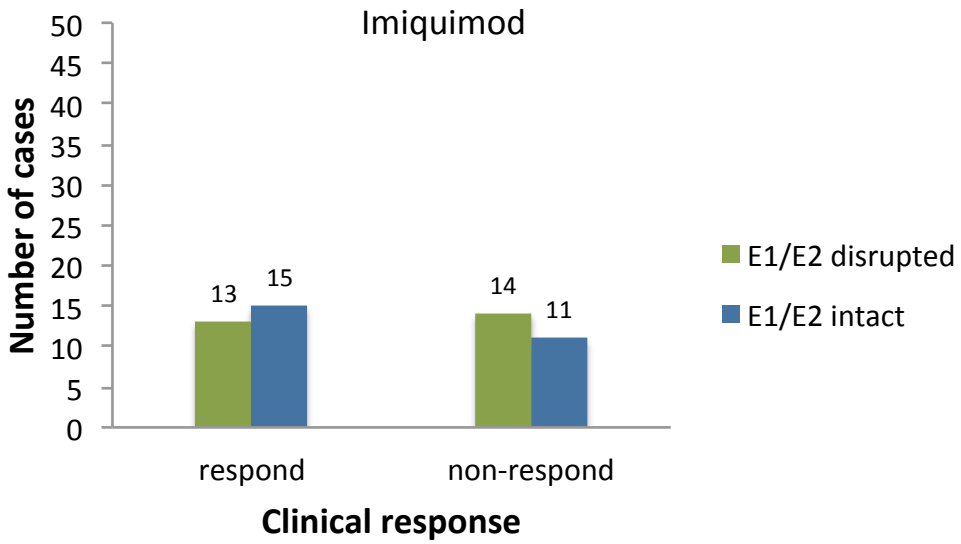


Figure 5.2 E1/E2 disruption and response to treatment. The top graph shows patients treated with cidofovir (n=54) and the bottom graph patients treated with imiquimod (n = 53). E1/E2 status was not statistically significantly associated with response to treatment with either treatment.

5.3.4 HPV DNA methylation and response to treatment

Of the 136 cases identified as HPV 16 positive, 29 did not have post-treatment clinical outcome data, therefore 107/136 were available for response to treatment analyses. Quality assurance checks applied to the methylation data further decreased the number of cases with analysable data; for the *E2* region 63/107 cases were available, for the *L1/L2* region 73/107 cases were available and for the promoter region 95/107 cases were available. Levels of E2, *L1/L2* and promoter region methylation were compared between patients who responded and did not respond to treatment. Firstly, median methylation was calculated for responders and non-responders in each treatment arm and for treatment overall, then, a Mann-Whitney U test was run to determine any statistically significant difference between mean levels. A Bonferroni correction to account for multiple comparisons was incorporated making a p value of p = 0.005 significant.

5.3.4.1 *E2* Methylation

For treatment overall (cidofovir or imiquimod), 34/63 (54.0%) responded and 29/63 (46.0%) did not. The mean level of *E2* methylation was similar for responders and non-responders (4.26% vs. 3.25%). For those patients treated with cidofovir, 17/30 (56.7%) responded and 13/30 (43.3%) did not. Median *E2* methylation was **higher** in patients who responded to treatment than in patients who did not (9.14% vs. 2.19% p = 0.003). For patients treated with imiquimod, 17/33 (51.5%) responded and 16/33 (48.5%) did not. Median *E2* methylation was **lower** in patients who responded to treatment than in patients who did not (2.57% vs. 24.22%), this finding did not reach the statistical significance required with a p value of 0.225 (Figure 5.4).

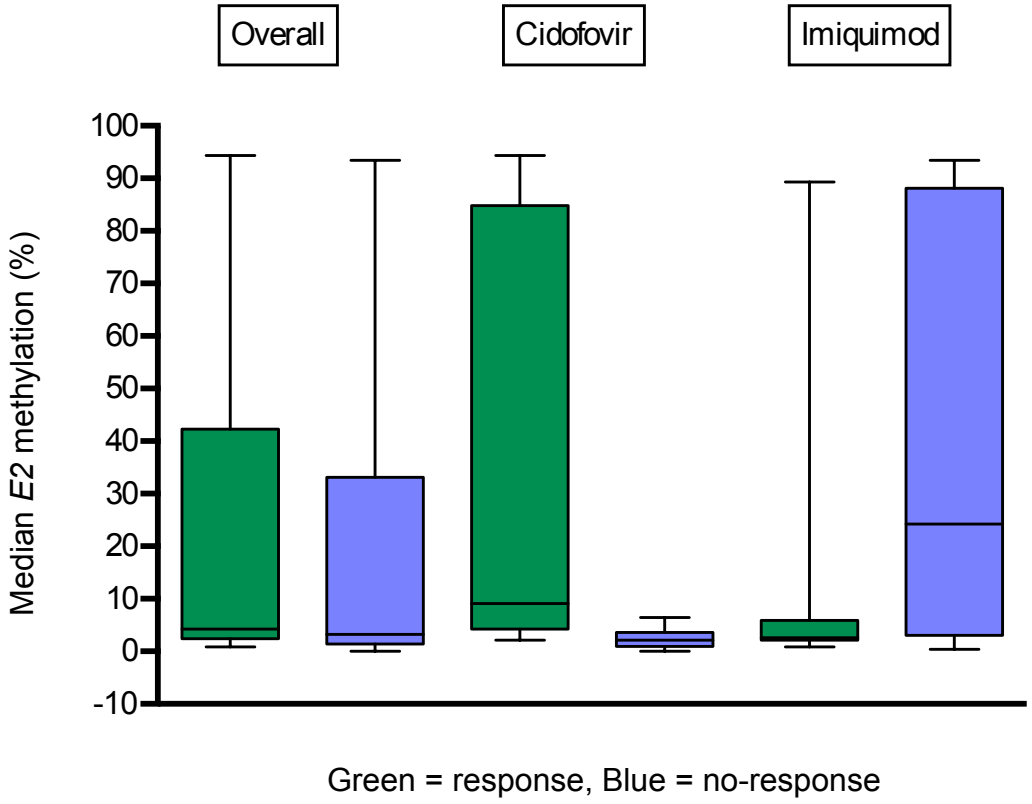


Figure 5.3 Median *E2* methylation in responders and non-responders to treatment. *E2* methylation quantified using pyrosequencing. Overall treatment represents combined data from both cidofovir and imiquimod treatment arms. Whiskers represent minimum and maximum values obtained.

5.3.4.2 *L1/L2* Methylation

For treatment overall (cidofovir or imiquimod), 36/73 (49.3%) responded to treatment and 37/63 (50.7%) did not. Median *L1/L2* methylation was similar between responders and non-responders (13.17% vs. 17.09%). For cidofovir treated patients, 17/39 (43.6%) responded to treatment and 22/39 (56.4%) did not. Median *L1/L2* methylation was found to be **higher** in patients who responded to treatment (59.03%) than in patients who did not (9.62%); this finding was not statistically significant (p = 0.431). For patients treated with imiquimod, 19/34 (55.9%) responded to treatment and 15/34 (44.1%) did not. Median *L1/L2* methylation was **lower** (11.72%) in patients who responded to treatment with imiquimod than in those patients who did not (42.91%). This finding however, did not reach statistical significance p = 0.621 (Figure 5.5).

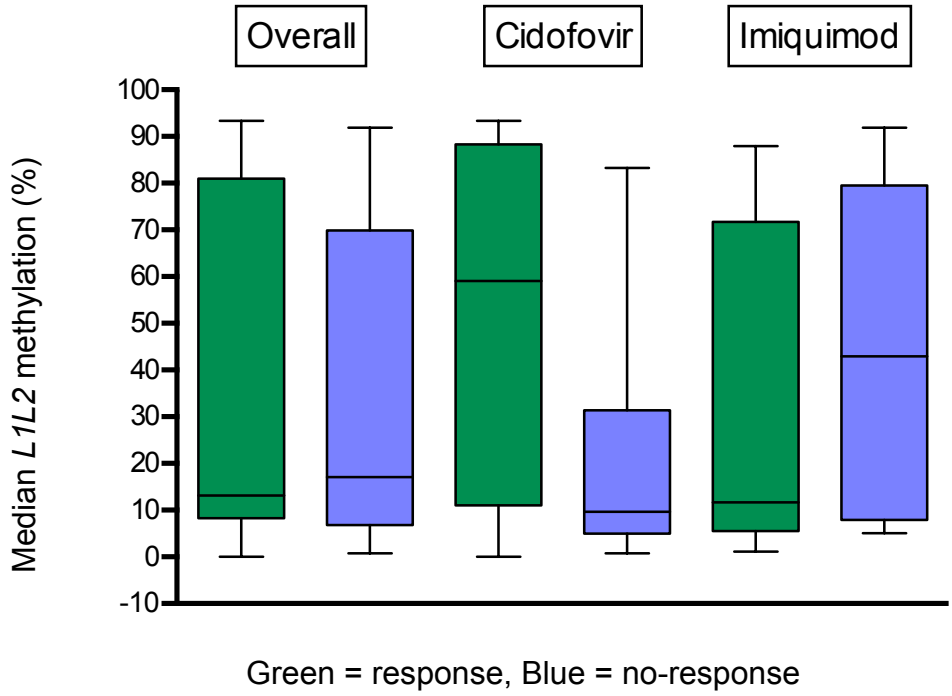


Figure 5.4 Median L1/L2 methylation in responders and non-responders to treatment. L1/L2 methylation quantified using pyrosequencing. Overall treatment represents combined data from both treatment arms. Whiskers represent maximum and minimum values. No statistically significant differences were identified.

5.3.4.3 Promoter Methylation

For treatment overall (cidofovir or imiquimod), 50/95 (52.6%) responded to treatment and 45/95 (47.4%) did not. Median promoter methylation was similar between responders and non-responders (0.19% vs. 0.26%). For cidofovir treated patients, 26/51 (51.0%) responded to treatment and 25/51 (49.0%) did not. Median promoter methylation was **similar** in patients who responded to treatment (0.20%) and in patients who did not (0.24%). This was not statistically significant p = 0.891. For patients treated with imiquimod, 24/44 (54.5%) responded to treatment and 20/44 (45.5%) did not. Median promoter methylation was **lower** (0.16%) in patients who responded to treatment with imiquimod than in those patients who did not (0.26%). This finding however, did not reach statistical significance p = 0.205 (Figure 5.6). Based on the finding of consistently low promoter region methylation in the cohort and the absence of correlation with treatment outcome, development as a biomarker was halted at this stage.

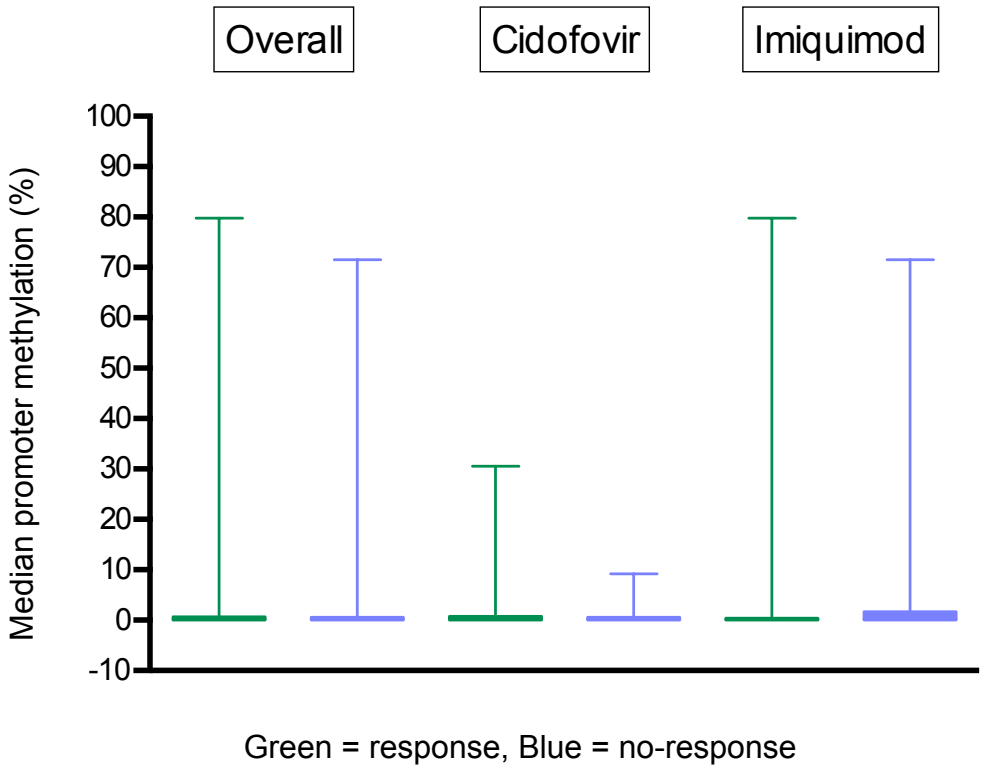


Figure 5.5 Promoter region methylation and response to treatment. Promoter methylation quantified using pyrosequencing. Overall treatment represents combined data from both treatment arms. Whiskers represent maximum and minimum values. No statistically significant differences between medians were identified.

5.3.5 Can HPV DNA Methylation Distinguish Responders from Non-Responders?

ROC curve analysis was performed to determine if methylation of the *E2* and *L1/L2* region could distinguish between patients who would respond to treatment and those who would not. Based on the data above (section 5.3.4), increasingly high levels of *E2* and *L1/L2* methylation were used to distinguish cidofovir responders from non-responders and increasingly low levels for imiquimod. The AUC value was calculated using SPSS and the result was classified as follows:

Increasingly high levels of *E2* methylation were found to be 'excellent' at distinguishing cidofovir responders from non-responders with an AUC value of 0.919 (95% CI 0.822-1.000), p = <0.0001. The ability of increasingly high levels of *L1/L2* methylation to distinguish cidofovir responders from non-responders was 'fair to poor' based on an AUC value of 0.698 (95% CI 0.525-0.870), p = 0.036 (Figure 5.7, Figure 5.8).

Increasingly low levels of *E2* methylation were found to be 'fair to good' at distinguishing imiquimod responders from non-responders with an AUC of 0.721 (95% CI = 0.538-0.903), p = 0.031. *L1/L2* methylation was not found to be useful in distinguishing imiquimod responders from non-responders with an AUC of 0.595 (95% CI 0.402–0.788), p = 0.337 (Figure 5.9, Figure 5.10).

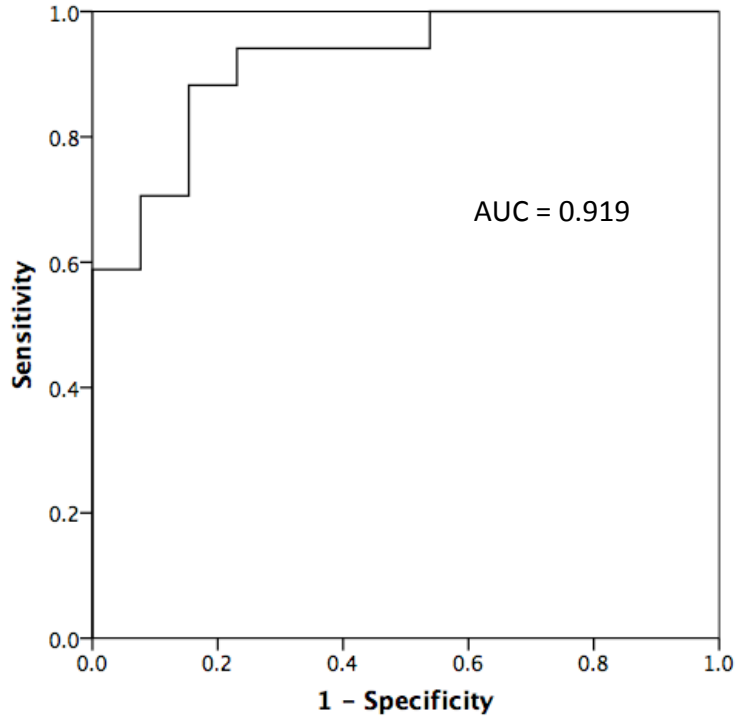


Figure 5.6 ROC curve analysis of increasing *E2* methylation to distinguish cidofovir responders from non-responders. N = 30. Increasing level of *E2* methylation demonstrates 'excellent' ability to distinguish cidofovir responders from non-responders with AUC 0.919 (95%CI 0.882–1.00), p = < 0.0001.

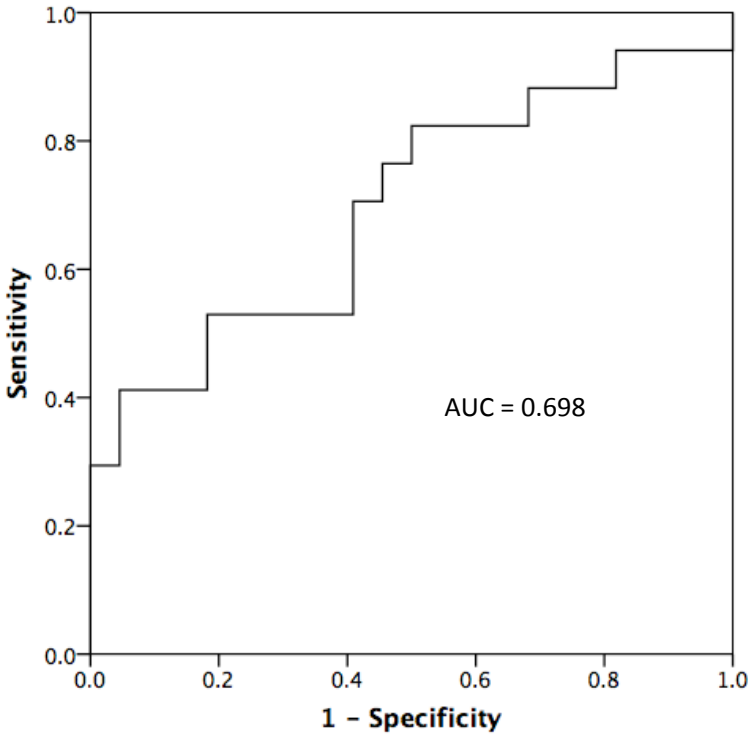


Figure 5.7 ROC curve analysis of increasing *L1/L2* methylation to distinguish cidofovir responders from non-responders. N = 39. Increasing level of *L1/L2* methylation demonstrated 'poor to fair' ability to distinguish cidofovir responders from non-responders with AUC 0.698 (95% CI 0.525–0.870), p = 0.036.

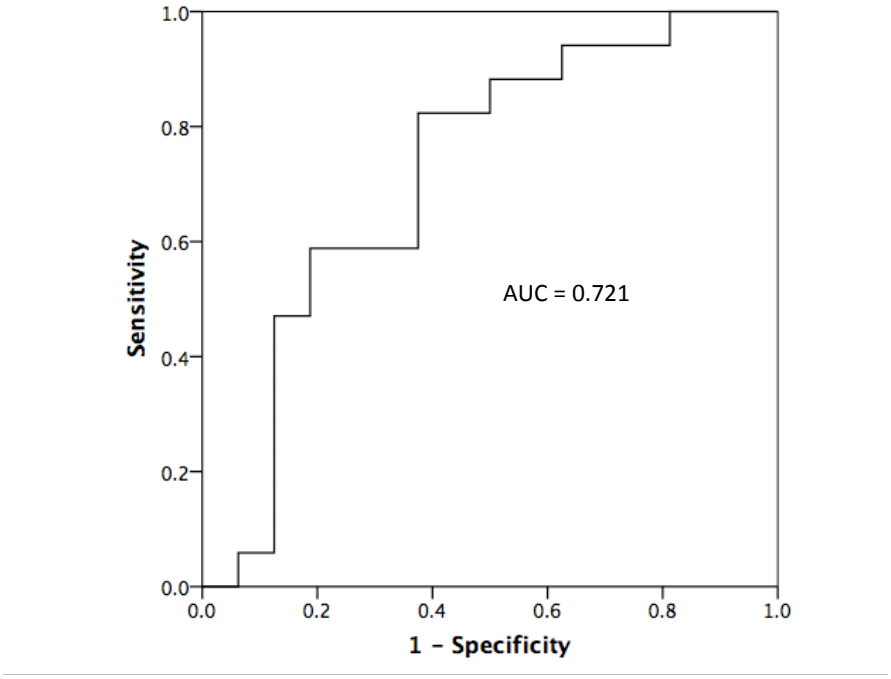


Figure 5.8 ROC curve analysis of decreasing *E2* methylation to distinguish imiquimod responders from non-responders. N = 33. Decreasing *E2* methylation demonstrated 'fair to good' ability to distinguish imiquimod responders from non-responders with an AUC of 0.721 (95%CI 0.538–0.903), p = 0.031.

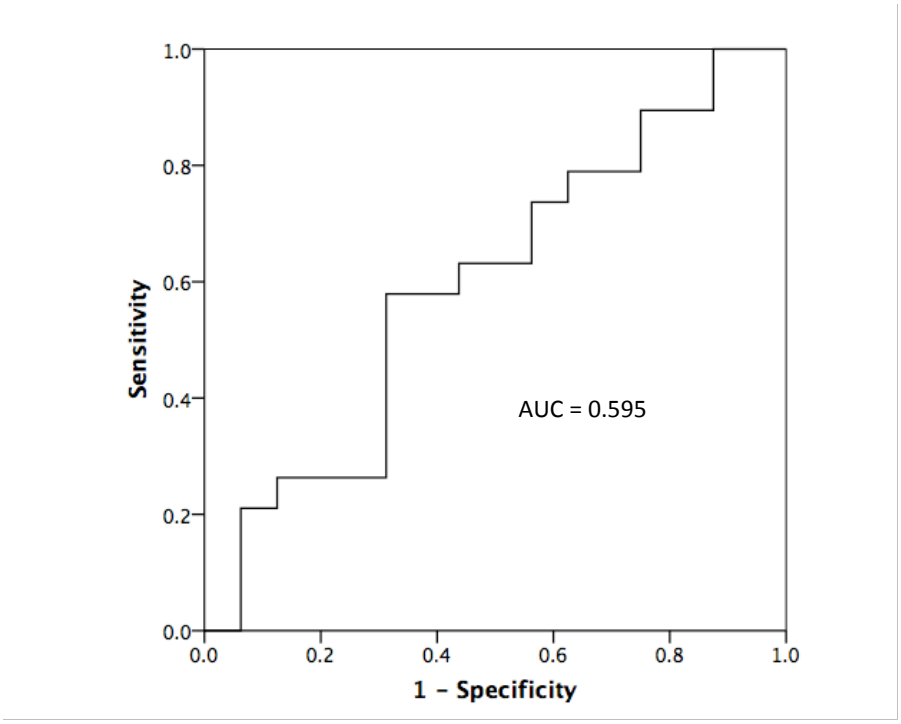


Figure 5.9 ROC curve analysis of decreasing *L1/L2* methylation to distinguish imiquimod responders from non-responders. N = 35. Decreasing *L1/L2* methylation demonstrated 'poor' ability to distinguish imiquimod responders from non-responders with an AUC of 0.595 (95% CI = 0.402-0.788), p = 0.337

5.3.6 Investigation into Appropriate Methylation 'Cut-Off' Level

The data shown in section 5.3.5 indicated that *E2* methylation had the best ability to distinguish responders from non-responders to either treatment. However, response to treatment with cidofovir was associated with higher levels of methylation and the opposite was true for response to imiquimod (low methylation levels). The ROC analysis was used to determine potential cut-off levels of methylation based on sensitivity and specificity that could be used.

5.3.6.1 Cidofovir

The ROC curve analysis of sensitivity and specificity of different *E2* methylation cut-off levels to distinguish cidofovir responders from non-responders are shown in Table 5.1. Three cut-off levels were selected to investigate further based on relative sensitivity and specificity: firstly *E2* methylation >2%, secondly, *E2* methylation >4% and finally *E2* methylation >5%. Sensitivity, specificity, PPV and NPV specific to these three cut off levels were calculated and are shown in Table 5.2. The potential clinical utility of these *E2* methylation cut off values are indicated by this analysis; *E2* methylation >4.0% appeared offer the best compromise between sensitivity, specificity, PPV and NPV. The benefits and limitations of each level are discussed in detail in the discussion section of this chapter.

5.3.6.2 Imiquimod

The ROC curve analysis of sensitivity and specificity of different *E2* methylation cut-off levels to distinguish imiquimod responders from non-responders are shown in Table 5.3. Two cut-off levels were selected to investigate further. A cut-off of *E2* methylation <10% appeared to offer the best compromise between sensitivity and specificity at this stage. A second cut-off of *E2* methylation <4.0% was also assessed as this would represent the cohort in which cidofovir treatment would not be offered based on the same analysis. Sensitivity, specificity, PPV and NPV specific to these cut off levels were calculated and are shown in Table 5.4. Potential clinical utility of these *E2* methylation cut off values are indicated by this analysis; *E2* methylation <10.0% appeared to offer the best compromise between sensitivity, specificity, PPV and NPV. The benefits and limitations of each level are discussed in detail in the discussion of this chapter.

Table 5–1 Sensitivity and specificity of different E2 methylation cut-off levels to discriminate between cidofovir responders and non-responders.

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

*Cut-off 1: E2 meth > 2.0%

Cut-off 2: E2 meth > 4.0%

Cut-off 3: E2 meth > 5.0%

*Data generated from ROC analysis. Three potentially clinical useful *E2* methylation level cut-off points (identified by the red lines). The smallest cut-off value represents the minimum E2 methylation value obtained -1 and the largest cut off point represents the maximum value +1. Cut-off values between these are the average of two consecutive ordered observed test values, generated by SPSS ROC analysis.

Table 5–2 Analysis of potential clinical utility of three E2 methylation cut-off levels to predict response to treatment with cidofovir.

E2 methylation cut-off level						
			Cidofovir response	**Cidofovir non-response**	**Total**	
	E2 meth ≥ 2.0%	**E2 meth > 2.0%**	17	7	24	
		E2 meth <2.0%	0	6	6	
		Total	17	13	30	
			Sensitivity	**Specificity**	**PPV**	**NPV**
		E2 meth >2.0%	100.0%	46.15%	70.8%	100.0%
			Cidofovir response	**Cidofovir non-response**	**Total**	
	E2 meth ≥ 4.0%	**E2 meth > 4.0%**	15	2	17	
		E2 meth <4.0%	2	11	13	
		Total	17	13	30	
			Sensitivity	**Specificity**	**PPV**	**NPV**
		E2 meth >4.0%	88.2%	84.6%	88.2%	84.6%
			Cidofovir response	**Cidofovir non-response**	**Total**	
	E2 meth ≥ 5.0%	**E2 meth > 5.0%**	12	1	13	
		E2 meth <5.0%	5	12	17	
		Total	17	13	30	
			Sensitivity	**Specificity**	**PPV**	**NPV**
		E2 meth >5.0%	70.6%	92.3%	92.3%	70.6%

Table 5–3 Sensitivity and specificity of different E2 methylation cut-off levels to distinguish imiquimod responders and non-responders

Responds to treatment if 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

*Cut-off 1: E2 meth <4.0%

Cut-off 1: E2 meth <10.0%

Data generated from ROC analysis. Two potentially clinical useful *E2* methylation level cut-off points (identified by the red lines). The smallest cut-off value represents the minimum E2 methylation value obtained -1 and the largest cut off point represents the maximum value +1. Cut-off values between these are the average of two consecutive ordered observed test values, generated by SPSS ROC analysis.

Table 5–4 Analysis of potential clinical utility of two different *E2* methylation cut-off levels to distinguish imiquimod responders from non-responders

E2 methylation cut-off level						
			Imiquimod response	**Imiquimod non-response**	**Total**	
	E2 meth ≤ or > 4.0%	**E2 meth <4 .0%**	12	6	18	
		E2 meth >4.0%	5	10	15	
		Total	17	16	33	
			Sensitivity	**Specificity**	**PPV**	**NPV**
		E2 meth <4.0%	70.6%	62.5%	66.7%	66.7%
			Imiquimod response	**Imiquimod non-response**	**Total**	
	E2 meth ≤ or >10.0%	**E2 meth <10.0%**	14	7	21	
		E2 meth >10.0%	3	9	12	
		Total	17	16	33	
			Sensitivity	**Specificity**	**PPV**	**NPV**
		E2 meth <10.0%	82.4%	56.3%	66.7%	75.0%

5.3.7 HPV gene expression and response to treatment

Mean CNRQ for each gene (*E2*, *E6* and *E7*) was calculated for patients who responded and patients who did not respond to: a. treatment overall (cidofovir or imiquimod), b. cidofovir and c. imiquimod. A Mann-Whitney U test was then used to determine if there was a statistically significant difference between gene expression in responders and non-responders. A Bonferroni correction to account for multiple comparisons was incorporated making a p value of p = 0.005 significant.

5.3.7.1 E2 gene expression

There were 96 cases with available *E2* gene expression data and clinical outcome data. Regarding treatment overall, mean *E2* gene expression was similar between responders and non-responders (CNRQ 1.8 ± 1.3SD vs. CNRQ 1.9 ±1.4SD).For cidofovir treatment mean E2 CNRQ was lower (1.5 ± 1.3SD) in responders than non-responders (1.9 ± 1.3SD); this difference was not statistically significant. For imiquimod treatment, mean E2 expression was higher in responders than non-responders (CNRQ 2.1 ±1.2SD vs. CNRQ 1.8 ± 1.4SD), but this difference was not statistically significant (p = 0.699). Similar *E2* gene expression levels were present in responders and non-responders for all treatments (overall, cidofovir or imiquimod) therefore, *E2* gene expression was deemed unsuitable for further biomarker development.

5.3.7.2 E6 gene expression

There were 98 cases with available *E6* gene expression data and clinical outcome data. Regarding treatment overall (cidofovir or imiquimod), mean *E6* gene expression was the same in both responders and non-responders (CNRQ 1.0 ± 0.5SD). For cidofovir, mean E6 CNRQ was the same between responders and non-responders (CNRQ 1.0 ±0.5SD). For imiquimod, mean E6 expression was similar between responders and non-responders (CNRQ 0.9 ± 0.5SD vs. CNRQ 1.0 ± 0.5SD). Similar *E6* gene expression levels were present in responders and non-responders for all treatments (overall, cidofovir or imiquimod) therefore,*E6* gene expression was deemed unsuitable for further biomarker development.

5.3.7.3 E7 gene expression

There were 97 cases with available E7 gene expression data and clinical outcome data. Regarding treatment overall (cidofovir or imiquimod), mean E7 gene expression was similar between responders and non-responders (CNRQ 1.1 ± 0.5SD vs. CNRQ 1.2 ± 0.5SD). For cidofovir, mean E7 expression was similar between responders and non-responders (CNRQ 1.1 ± 0.6SD vs. CNRQ 1.2 ± 0.4SD). For imiquimod, mean E7 expression was also similar between responders (CNRQ 1.1 ± 0.4SD) and non-responders (CNRQ 1.2 ± 0.5SD). Similar *E7* gene expression levels were present in responders and non-

responders for all treatments (overall, cidofovir or imiquimod) therefore; it was deemed unsuitable for further biomarker development.

5.3.8 *E2:E6* expression ratio and response to treatment

E2:E6 expression ratio was used in an exploratory capacity to identify cases of regulated oncogene expression (*E2>E6)* and cases of deregulated oncogene expression (*E2<E6)*. Rates of regulated vs. deregulated gene expression were compared between responders and non-responders to a. treatment overall (cidofovir or imiquimod), b. cidofovir and c. imiquimod.

5.3.8.1 Treatment overall

For treatment overall, rates of regulated and deregulated gene expression were similar between responders and non-responders. *E2>E6* expression was detected in 14/49 (28.6%) responders and 12/44 (27.3%) non-responders. *E2<E6* expression was detected in 35/49 (71.4%) responders and 32/44 (72.7%) non-responders.

5.3.8.2 Cidofovir

The proportions of cases with *E2>E6* expression and *E2<E6* expression were similar between patients who responded and did not respond to treatment with cidofovir. *E2>E6* was present in 6/25 (24.0%) patients who responded to treatment and 6/22 (27.3%) who did not. *E6>E2* expression was detected in 19/25 (76.0%) of patients who responded to treatment and in 16/22 (72.7%) who did. Based on this finding, *E2:E6* expression ratio was not deemed suitable for further development as a BM to predict response to treatment with cidofovir.

5.3.8.3 Imiquimod

Similar proportions of*E2>E6* and *E2<E6* cases were detected amongst imiquimod responders and non-responders. *E2 >E6* expression was detected in 8/24 (33.3%) of responders and in 6/22 (27.3%) of non-responders. *E6>E2* expression was detected in 16/24 (66.7%) of responders and 16/22 (72.7%) of non-responders. As was the case with cidofovir, further development of *E2:E6* expression as a BM to predict response to treatment with imiquimod was not deemed appropriate.

5.4 Discussion

5.4.1 Principal Findings

The principal finding of this work was that *E2* and*L1/L2* methylation significantly correlated with response to treatment with both cidofovir and imiquimod but at opposing levels. The level of *E2* methylation was found more successfully distinguish responders from non-responders for both treatments and demonstrated potential clinical utility as a predictive biomarker. Additional findings were:

- Associations (which did not reach statistical significance), were found between response to treatment with cidofovir and the presence of:
    - Disrupted virus
    - Relatively low levels of *E2* expression
- Associations (which did not reach statistical significance), were found between response to treatment with imiquimod and the presence of:
    - Intact virus
    - Relatively high levels of *E2* expression
- All non-HR positive cases of VIN responded to treatment

The pharmacology of cidofovir and imiquimod is different. The proposed mechanism of action of these drugs in HPV related VIN is also therefore different and they are therefore discussed separately below.

5.4.1.1 E2 Methylation and Response to Treatment with Cidofovir

Mean *E2* methylation was significantly higher in patients who responded to treatment (38.4%) with cidofovir than patients who did not (2.4%). ROC analysis of the data demonstrated the ability of *E2* methylation to distinguish responders from non-responders with an AUC of 0.919. Further analysis of the data revealed the potential utility of *E2* methylation as a predictive biomarker in the treatment of VIN 3 with cidofovir. Several potential 'cut-off' methylation levels were investigated and a level of 4% conferred a good balance between sensitivity (88.2%) and specificity (84.6%).

A biological explanation for the correlation between high levels of *E2* methylation and response to treatment is difficult to determine. It is also impossible to be certain if it is the level of methylation per se, that is important to the action of cidofovir or, if methylation is a surrogate marker of another relevant process. It is possible that the action of cidofovir in this context is as a de-methylating agent. Decitabine is a drug used to treat myelodysplatic blood conditions and exerts its effect through its incorporation into DNA and blocking the

activity of DNA methyltransferases (DNMTs) leading to de-methylation. Decitabine is a cytidine deoxynucleoside analogue, similar to cidofovir and therefore it is possible that cidofovir acts similarly in this setting (Gros *et al*. 2012). This theory is supported by a small study of five cases of failed cidofovir treatment in recurrent respiratory Papillomatosis (caused by HPV 11), which found that all cases were uniformly unmethylated (Gall *et al*. 2011).

It is also possible that *E2* methylation is a surrogate marker of another relevant process. This is consistent with the hypothesis that response to cidofovir may be dependent on the absence of functioning p53/pRb. This hypothesis was developed based on a growing body of evidence in the literature supporting the concept that the selectivity of action of cidofovir for transformed cells (both HPV positive and negative) compared to normal cells is due to the absence, or altered function, of normal DNA repair pathways (Andrei *et al*. 2015). It has been hypothesised that these abnormal DNA repair pathways are a result of aberrant p53/pRb signalling (Andrei *et al*. 2015). It has been shown that cidofovir can generate double-stranded breaks in cellular DNA that can be repaired by normal cells, but not in tumour cells (de Schutter *et al*. 2013). The same study demonstrated that higher amounts of cidofovir were incorporated into the genomic DNA of transformed cells compared to normal cells, despite the fact that the intracellular levels of cidofovir metabolites were similar. This suggests that the increased levels of incorporated cidofovir are not a result of altered metabolism of the drug.

It was not possible to measure levels or function of either p53 or pRb in this study. However HPV characteristics that could be associated with aberrant p53/pRb were investigated. It is broadly accepted that the level of p53/pRb is reduced through ubiquitination via the HPV E6 and E7 oncoproteins, which can become deregulated as a result of HPV integration and/or HPV DNA methylation (Doorbar 2006). HPV integration and increased methylation could therefore identify cases, which are more likely to contain 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 as a surrogate marker of absent/low level p53/pRb. This is supported by the fact that patients who responded to treatment were more likely to have disrupted HPV and lower levels of *E2* expression than patients who did not respond (although, unlike *E2* methylation, these finding did not reach statistical significance). However, it was not supported by the absence of correlation between deregulated oncogene expression and response to treatment. Both the lack of significance in the associations found between viral integration, low *E2* expression and response to treatment and the absence of an association between deregulated oncogene expression

and response to treatment could be explained by limitations in the methodology (see below).

5.4.2 E2 Methylation and Imiquimod

Contrary to the case with cidofovir, mean *E2* methylation was statistically significantly **lower** in patients who responded to imiquimod (11.6% vs. 40.0%). ROC analysis of the data demonstrated the ability of *E2* methylation to distinguish responders from non-responders with an AUC of 0.721. Further analysis of the data revealed the potential utility of E2 methylation as a predictive biomarker in the treatment of VIN 3 with imiquimod. Several potential 'cut-off' methylation levels were investigated and a level of 10% conferred a good balance between sensitivity (82.4%) and specificity (56.3%).

It is again difficult to be certain whether it is the level of *E2* methylation per se that is important in the activity of imiquimod, or whether *E2* methylation is a surrogate marker for another important process or viral state. The pharmacology of imiquimod and the pathology of HPV indicate that it is more likely to be the latter. 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. INFα is able to enhance cell-mediated cytoloytic activity against viral targets (Stanley 2002; Diaz-Arrastia *et al*. 2001; Edwards 1998). However, the enhanced host immune response needs direction in order to be effective. It is plausible that a proliferative HPV infection provides this direction. It has been demonstrated that treating VIN with imiquimod when HPV exists in a mixed physical state (i.e. episomal and integrated virus) results in clearance of the episomal HPV but not the integrated (Herdman *et al*. 2006; Pett *et al*. 2006). The success of HPV is often attributed to its ability to hide from normal host defence mechanisms permitting persistent infection (Doorbar 2006). Persistent infection is associated with the development of high-grade intraepithelial neoplasia and certain viral characteristics such as HPV integration and increased HPV DNA methylation. It is therefore plausible that these HPV characteristics confer successful evasion of host immunity. It is possible that cases of episomal HPV infection (in which a proliferative HPV infection is present) are more likely to generate pathogen associated molecular patterns (PAMPs) on the cell surface. Based on this, it was hypothesised that response to treatment with imiquimod would be more likely in cases with evidence of episomal HPV infection. Specific identification of episomal HPV was not possible in this project. However, HPV integration frequently results in disruption of the *E2* and/or *E1* regions of the virus, therefore intact *E1/E2* was used to identify cases of episomal HPV. Additionally, studies (including this one) have shown that low levels of HPV DNA methylation strongly

correlate with the presence of intact (episomal) HPV (Oka *et al*. 2013): relationships between HPV DNA methylation and response to treatment were also explored. Likewise, the work of this thesis, also demonstrated that higher levels of *E2* expression correlated with intact HPV (see section 4.6.1), the biomarker potential of E2 expression was also investigated.

As was hypothesised, intact *E1/E2*, low HPV DNA methylation and high *E2* gene expression were all associated with response to treatment with imiquimod. Statistical significance however, was only present for the association between *E2* and *L1/L2* DNA methylation and response. Potential reasons for the lack of statistical significance in the associations between intact *E1/E2* and high *E2* expression are explored in the limitations section of this discussion (below).

5.4.3 Strengths

The bio-resources used were of high quality, originating from tissue biopsies, and the application of stringent quality assurance ensured only high quality data was assessed (see methods chapter 4).

5.4.4 Limitations

The biomarker potential of the HPV characteristics was investigated using clinical outcome data from all patients enrolled in the trial who provided a pre and post treatment biopsy, even if they did not strictly adhere to the treatment regime. This has advantages and disadvantages. The advantage is that the estimation of real-world clinical utility is potentially more accurate. The disadvantage is that, the performance of the biomarkers in the optimum setting may be underestimated. In the cidofovir arm of the study, 78/89 patients adhered to the treatment regime; in the imiquimod arm, 78/91 patients adhered to the treatment regime. Patients who did not adhere to the treatment regime, typically reduced dosing due to side effects.

The assay designed to detect HPV disruption was a tiling PCR that targeting multiple fragments of the *E1* and *E2* genes. False positive test results could be generated by this assay as a result of DNA with poor integrity. Additionally, a positive result would also be generated in the event of intact *E2* but disrupted *E1*; the biological significance of this may not be relevant in this context. However, intact *E2* with disrupted *E1* was only detected in four cases in the section of analyses and is unlikely to have had a significant impact on the data.

Among the gene expression data, there were a number of cases that generated a non-specific PCR product during RT-qPCR. All these samples were found to have disruption in

the E2 region. It was desirable to include these cases in the analysis to ensure that the sample was not skewed towards investigating cases with intact virus. These samples were considered to contain no E2 mRNA and were given a CNRQ value of 0, representing no expression. This aspect of the methodology could also affect the accuracy of the data and could also hide statistically significant correlations. Finally, the use of *E2:E6* expression ratio to identify cases of regulated (*E2>E6*) expression and deregulated (*E6>E2*) was exploratory and was not well-validated. It is quite feasible that deregulated gene expression is not accurately represented by an *E6>E2* expression ratio.

5.4.5 Broader Implications

Reported complete response rates in VIN treated with cidofovir range from 15% - 47% (see background, table 1.3). A review of the literature suggests that complete response to imiquimod ranged from 20.0% - 100.0% (see background, table 1.4). The most recent clinical trial with which this work is associated (RT3 VIN, chapter 3), demonstrated complete response in 41/89 (46.0%) patients treated with cidofovir and 42/91 (46.0%) of patients treated with imiquimod. A biomarker that could identify patients more likely to respond to treatment is an attractive prospect. This is the first study investigating the potential role of viral characteristics as biomarkers in the treatment of VIN. HPV E2 DNA methylation meets all of the criteria laid out in the CRUK biomarker development roadmap (Figure 5.1) up to the point of BM qualification stage 2. E2 methylation varied in the RT3 VIN cohort, which is representative of the cohort to which the BM would apply. The assay was accurate and reproducible (see methods, section 2.5.2). 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. Finally, the relationship between *E2* methylation and clinical outcome indicated potential clinical utility as a predictive BM in a prospective analysis of a retrospective tissue collection. However, prior to further qualification in the context of a clinical trial utilising the *E2* methylation as a BM in the randomisation process, its 'fitness for purpose' needs to be addressed. The criteria determining whether or not a BM is fit for purpose have not been formally defined. Such criteria may include cost efficiency, ease of incorporation into the clinical setting, efficiency of the assay testing the BM and patient coverage. Cost efficiency analyses are beyond the scope of this PhD. Incorporating the assay into the clinical setting is feasible. Testing could be carried out on remaining biopsy material following histological assessment. Consideration would have to be given to the quality of the DNA being extracted if this were the case. Alternatively, a slightly larger diagnostic biopsy than is currently standard (3-4 mm) could be taken and divided into sections for histology and for methylation analysis. The equipment required is relatively compact

within a laboratory setting, the assay is subjectively, moderately labour intensive requiring a total of approximately 4 hours of single operator work split over the course of 2 days and the assay itself is high-throughput (in its current form, 47 cases can be run at a time in duplicate). The efficiency of the *E2* methylation pyrosequencing assay in its current form means that the patient coverage is quite low. In the cohort of VIN 3 available to this study (likely to be broadly representative of VIN 3 overall) percentage *E2* methylation could not be analysed for 72/135 (53.3%) patients(Figure 5.11). In the majority of cases (28/135, 22.4%), it was because HPV 16 was not detected in the biopsy and the assay is currently designed to test *E2* methylation in the HPV 16 genotype only. Among HPV16-negative cases, 14/28 (50.5%) were positive for HPV 33, which was the second most prevalent genotype detected. It could therefore be worthwhile, to modify the assay to include cases of HPV 33 *E2* methylation. It was noted that all cases testing negative for a high-risk genotype (n=5) responded to treatment; four of these five patients were treated with imiquimod and one with cidofovir. It would be worth investigating the outcome of HR HPV negative cases in larger study cohort to determine how best to manage these patients. Twenty-six cases were identified as fails by the pyrosequencing software due to 'insufficient DNA'; this is normally because there is a problem with the integrity of the template DNA. Of these 26 cases, 23/26 (88.5%) were found to have disrupted *E1/E2* regions, this is a difficult issue to overcome. One option might be to treat these cases as highly methylated in view of the strong correlation between viral integration and high methylation that was identified in chapter 5, section 4.7.5.4,this would not provide the same sensitivity and specificity profile and advantages of increased patients coverage would need to be considered against the disadvantages of reduced sensitivity and specificity. The remaining eighteen cases failed internal quality control steps of the pyrosequencing software due to varying degrees of sequence variation in the DNA. Several measure could be taken to improve this including optimisation of the assay (i.e. PCR conditions, primer quality, reagent quality) as well as taking care to optimise the quality of the DNA obtained from tissue biopsies as much as is possible.

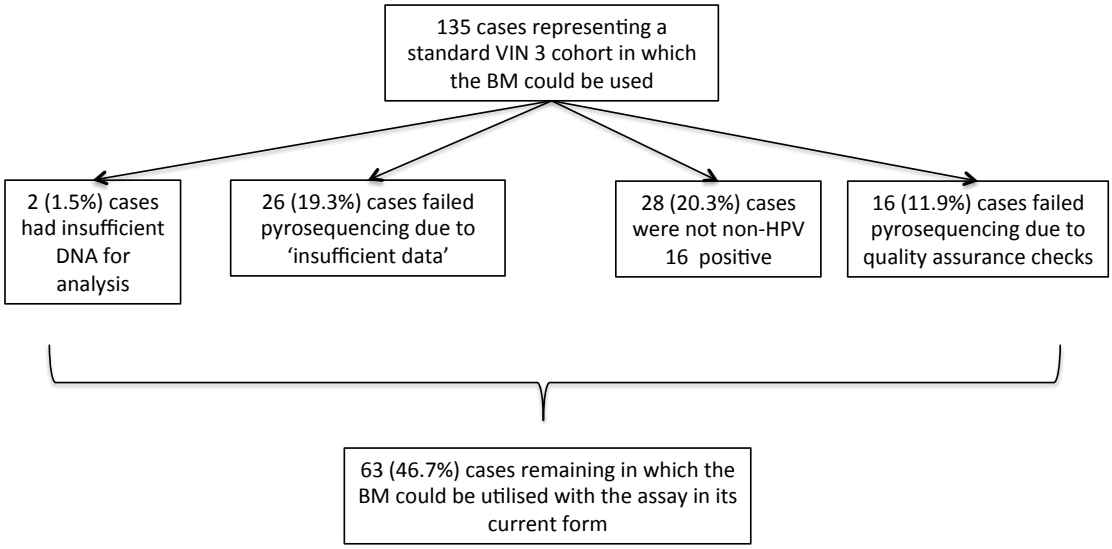


Figure 5.10 Analysis of cases generated a 'failed' pyrosequencing result.

The incorporation of a biomarker such as*E2* methylation in to the clinical management of patients with VIN could have significant value. VIN has malignant potential; therefore time to efficient treatment is important. A biomarker such as this could significantly reduce time wasted trying medications destined to fail, resulting in better outcomes for women (due to earlier treatment) and more cost effective approaches for the healthcare team (fewer failed approaches, less risk of progression to malignant disease). This would also avoid unnecessarily subjecting women to treatments with significant side effects profiles. The RT3 VIN clinical trial demonstrated that 72.6% of women reported adverse events of grade 2 or above (NCI CTCAE v 3.0). This emphasises the advantage of targeting therapy to those patients in whom it is more likely to work.

5.4.5.1 Future Work

Two overall approaches could and should be taken by future work in this area. Firstly, further development of *E2* methylation as a predictive BM in the treatment of VIN with cidofovir and imiquimod could be undertaken in the form of a prospective clinical trial utilising the biomarker in randomisation (in accordance with the CRUK BIDD road map). Prior to this, measures would need to be taken to address the issues described above relating to the pyrosequencing assays 'fitness for purpose'. It would be clinically and statistically valuable to include neoplasia of additional anogenital sites e.g. anus, in this study. Secondly, with the knowledge that cidofovir and imiquimod appear to be generating a clinical response in two biologically defined sub-groups that combined comprise the majority of cases of VIN 3, a treatment combining these two medications in one application is potentially, a very attractive prospect.

Chapter 6

Final Conclusion

6 Final Conclusion

The RT3 VIN clinical trial found that approximately 50% of cases of VIN 3 treated with cidofovir completely responded and likewise, approximately 50% of cases treated with imiquimod responded. A biomarker that could identify patients more likely to respond to treatment would be advantageous in several ways including: avoidance of unnecessary side effects; offering patient centred care; enabling effective treatment and avoiding the need for surgery. With the knowledge that HPV was implicated in the pathogenesis of the majority of cases of VIN 3, this study investigated the biology of HPV in the disease and HPV physical characteristics as potential predictive biomarkers to treatment response.

This study found high HPV prevalence (98.2%) in 167 cases of VIN 3. Further to this, it found that disruption of the HPV genome was a common event, present in 52.2% cases of HPV 16 positive disease and that viral disruption correlated with false negative HPV test results with a commercially available HPV detection assay (PapilloCheck®). Application of multiple HPV detection assays targeting different HPV genotypes and genomic regions increased detection of HPV from 81.4% to 98.2%. This finding has three important implications. Firstly, that the choice of HPV detection assay used in both the research setting and clinical setting should be carefully considered to minimise the impact that HPV disruption has on false negative HPV rates; assays targeting multiple regions of the HPV genome should be utilised when possible. Secondly, this study demonstrates the possibility that, as for high-grade CIN, all VIN is associated with the presence of HPV infection; HPV could be implicated in the pathogenesis of all cases of VIN. This hypothesis is worthy of further investigation. Finally, HPV positivity overall was not a suitable candidate biomarker in this disease; failing to fulfil the required criterion of variability in a cohort representative of the cohort to which the biomarker would apply. HPV 16 was the most prevalent genotype detected, found in 140/167 (83.8%) of cases, supporting findings from previous studies. HPV 16 positivity was therefore variable in the cohort but no correlation was identified with response to treatment with either medication following retrospective correlation with clinical outcome.

Several physical characteristics of HPV were investigated in detail in this study: viral disruption, DNA methylation and gene expression. These characteristics were chosen for study based on the knowledge that viral integration, high DNA methylation and deregulated oncogene expression are physical characteristics associated with higher grade intraepithelial disease (Clarke *et al*. 2012; Wiley *et al*. 2005; Kalantari *et al*. 2008; Park *et al*. 1991; Klaes *et al*. 1999; Hudelist *et al*. 2004; Cricca *et al*. 2009a; Bryant, Tristram *et al*. 2014) and a sound biological explanation indicating their role in the transforming potential of HPV (Doorbar *et al*. 2012b). Despite the uniform histological

grade of disease (VIN 3) in the studied cohort there was heterogeneous HPV biology detected in all three physical characteristics. As observed in some previous investigations, this study found strong correlations between these viral characteristics. Median *E2* expression was significantly lower in cases of viral disruption (p=<0.0001); increasing levels of HPV *E2* DNA methylation significantly correlated with decreasing levels of *E2* expression (*r*=-0.679, p=<0.0001) and median *E2* methylation was significantly higher in cases of viral disruption that in cases of intact virus (80.6% vs. 3.2%, p=<0.0001). This is the largest study demonstrating the relationships between these viral characteristics and contributes significantly to the understanding of how HPV drives disease. It supports findings that, in some cases of HPV infection, the virus becomes integrated into the host genome (and therefore disrupted) and that this is associated with high levels of HPV DNA methylation. These high levels of HPV DNA methylation may, or may not be a direct result of integration into the host genome. It also shows that both viral integration and high DNA methylation are associated with reduced *E2* gene expression. It is broadly acknowledged that the E2 protein plays a role in the regulation of the expression of the *E6* and *E7* oncogenes and its loss of expression leads to deregulated oncogene expression and potentially transformation of the cell into a malignant state. However, this study also found that there were a significant number of HPV positive cases in the cohort in which none of these viral characteristics were detected. VIN 3 therefore would appear to represent a spectrum of HPV biology, which is an important finding that has two possible explanations. Firstly, although VIN 3 indicates those cases of VIN, which have a higher risk of developing into a vulval malignancy, the majority of cases do not progress to cancer. This could be due to the varying HPV biology within the disease grade and it could be, that cases of VIN 3 with the presence of HPV characteristics associated with a transforming potential are actually at increased risk of developing a malignancy. It is possible therefore that detection of viral characteristics associated with a transforming potential (HPV integration/disruption, increased DNA methylation and reduced *E2* expression) in the specimen is a more accurate way of identifying cases at increased risk of progression. HPV DNA methylation has been extensively researched in this capacity, and does appear to be able to distinguish cases of high grade CIN from cases with low-grade disease (Clark *et al*. 2012). A small amount of work also exists demonstrating a similar of role of HPV DNA methylation in other anogenital and oropharyngeal disease (Wiley *et al*. 2005; Kalantari *et al*. 2010; Lorincz *et al*. 2013; Sun *et al*. 2011; Ding *et al*. 2009; Mirabello *et al*. 2013). The findings of this study indicate that HPV DNA methylation (as well potentially HPV disruption and decreased *E2* expression) may serve a similar purpose in vulval disease. An alternative possibility is that cases without any detectable transforming physical characteristics are cases in which, HPV is not responsible for driving disease and is an

incidental finding in disease caused by alternative aetiology e.g. lichen sclerosus. If this were the case, then it could be argued that these cases may have the highest risk of malignancy in view of the fact that lichen sclerosus has been more commonly found in association with vulval malignancy than HPV (Eva *et al*. 2009; McCluggage 2009). More research is required in this area to accurately determine the VIN disease process and to answer these questions. In view of the distinct possibility that all VIN in this cohort could have been HPV positive, it may be that as for CIN in the cervix, HPV is also necessary for the development of VIN and that lichen sclerosus and other similar chronic skin conditions augment the process.

Finally, the most important finding of this research was the potential role of HPV *E2* DNA methylation in guiding topical treatment choice (cidofovir or imiquimod) in VIN. All viral physical characteristics tested were variable in the cohort and therefore fulfilled that criterion of biomarker development. Further to this, a positive result for any of the three physical characteristics associated with HPV transforming potential was associated with response to treatment with cidofovir; the opposite being true for treatment with imiquimod. However, following retrospective correlations in the prospectively collected cohort, HPV *E2* DNA methylation represented the most powerful predictor of response for both treatments. *E2* methylation >4% was able to predict response to treatment with cidofovir with a sensitivity of 88.2% and a specificity of 84.6%. Conversely, *E2* methylation <4% was able to predict response to treatment with imiquimod with a sensitivity of 70.6% and a specificity of 62.5%. This finding indicates that these drugs are active in opposing HPV biological states and that their biologically guided use could result in much higher response rates overall. This could potentially reduce dependence on surgical excision in the management of this disease and offer a more satisfactory treatment approach for patients, healthcare professionals, and for the NHS overall. Further research is required to take this into clinical practice and a large prospective study using *E2* methylation to allocate patients to treatment arms would be the obvious next step. Another possibility also exists; a formulation combining the two drugs may have the same efficacy and obviate the need for a biomarker. This is an attractive option and would potentially speed up access to treatment, negating the need to wait for a biomarker result, as well as being a potentially more cost effective option. A potential challenge with a double therapy approach might be increased levels of side effects that may be intolerable to patients.

It is clear that HPV E2 methylation represents a biological state in intraepithelial disease that has potential utility as a predictive biomarker of treatment response. It is also likely that *E2* methylation is a marker of disease grade. Incorporation of *E2* HPV DNA methylation testing into clinical practice in the future would appear to be advantageous in

the management of intraepithelial neoplasia of the cervix, the vulval, the vagina, the anus and the penis which, combined represent a significant disease burden in the UK. Not only could it serve to identify those patients with higher-grade disease with an increased risk of malignant progression but also to determine mode of management. Both imiquimod and cidofovir could be suitable for use in the treatment of all these conditions, all of which are currently managed by surgical excision. With the new found knowledge that ~50% efficacy rates reported for each drug are due to their activity in opposing HPV biological states, their use in intraepithelial neoplasia more broadly should be investigated.

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8 Appendix 1 – RNA integrity number (RIN) values for a random selection of baseline sample generated by the Agilent Bioanalyzer of RNA. (Scale = 0-10 (10 = best quality)).

Sample ID	Date of extraction	Nanodrop RNA Conc.	260/280	260/230	RIN number
RT1	06/11/09	69	2.2	2.6	7.5
RT11	21/12/09	238	2.1	0.9	2.2
RT21	28/01/10	201	2.1	2.2	1.2
RT31	12/02/10	184	2	2	7.8
RT41	20/08/10	27	2.2	1.2	n/a
RT51	28/08/10	82	2.0	1.9	4.3
RT61	20/08/10	55	2	1.7	n/a
RT71	12/01/11	71	2.1	2	1
RT81	28/01/11	37	2	1.4	n/a
RT91	08/02/11	105	2	1.9	1.5
RT101	10/03/11	279	2.1	2.3	2.4
RT111	30/03/11	126	2.1	1.17	1.5
RT121	05/04/11	46	2	0.9	1
RT131	12/04/11	62	2.1	1.9	1
RT141	10/05/11	79	2	1.4	4.2
RT151	24/05/11	299	2.	2.1	4.3
RT161	31/05/11	78.1	2	1	1
RT171	09/06/11	137	2.1	2	3.3
RT181	09/06/11	280	2	2.1	n/a
RT191	21/06/11	49	2	1.8	1.2
RT200	22/06/11	180	2	2.1	2.4
RT211	17/08/11	21	2.1	1.1	1
RT221	01/12/11	267	2.1	2.1	5.7
RT231	13/12/11	164	2	1.3	3.3

9 Appendix 2 – RT3 VIN Paper