PHASE II TRIAL OF IMIQUIMOD AND HPV THERAPEUTIC VACCINATION IN PATIENTS WITH VULVAL INTRAEPITHELIAL NEOPLASIA

A thesis submitted to The University of Manchester for the degree of Doctor of Medicine in the Faculty of Medical and Human sciences, School of Medicine

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Sai L Daayana

Cancer Research UK Paterson Institute of Cancer Research
The University of Manchester
Wilmslow Road, Withington
Manchester M20 4BX
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Imiquimod, an immune response modifier can stimulate local innate immunity and also drive an adaptive immune response. Therapeutic HPV vaccines are designed to generate cell-mediated immunity against HPV infected cells. The rationale of this study was that local imiquimod treatment, in addition to having a direct effect on VIN could also provide an immunological platform for the therapeutic HPV vaccination to achieve an enhanced and durable response. In this phase II trial, we used a combination of imiquimod and vaccination with TA-CIN (HPV16 E6E7L2 fusion protein).

Women with biopsy proven high-grade VIN were recruited. Imiquimod treatment for 8 weeks was followed by three i.m. injections of TA-CIN. The objectives were to measure lesion size, histology, lesion HPV status, symptoms, immune responses before and after treatment as well as safety, toxicity, and tolerability. Lymphoproliferation to HPV antigens was used to analyse immunity to HPV before and after treatment. Local immune factors (CD4, CD8 and T regulatory cells) were assessed by immunofluorescence.

74, 85 and 79% of women had a \( \geq 50\% \) reduction in the size of lesion and 32, 58 and 63% had regression of VIN on histology at weeks 10, 20 and 52 respectively. At baseline, 79% had moderate to severe symptoms compared to 21% at week 52 (\( P=0.01 \)). Of the women who showed histological responses at week 52, 5/10 also cleared their HPV 16 infection. Follow-up for an average of 15 months showed 84% of patients with a \( \geq 50\% \) reduction in lesion size. Treatment was well tolerated.

A significant post vaccination increase in proliferation to TA-CIN and its components was associated with histological responders (\( P=0.008 \)) but not the non-responders (\( P=0.7 \)). In the group as a whole, significant increases in the number of CD4, CD8 and T regulatory cells (Treg) were evident by week 20 compared to baseline (\( P=0.03, 0.01, 0.04 \) respectively); At week 20, the increased CD4 and CD8 density was significantly associated only with the histological responders (\( P=0.03; P= 0.03 \)) while increased Treg density was associated with only non-responders (\( P=0.05 \)). Intralesional Treg density was significantly higher in non-responders compared to responders pre and post treatment (\( P=0.01, 0.05 \) respectively).

This study demonstrated that imiquimod followed by vaccination achieved histological clearance of VIN at 52 weeks in almost 60%. Higher pre-existing and post-treatment levels of Treg cells were associated with a lack of treatment response. Lymphoproliferation of PBMC established that the vaccination was immunogenic and HPV 16 antigen specific. Importantly, these systemic immune responses to HPV 16 antigens were significantly associated with treatment responders.
DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or any other institute of learning.

Sai L Daayana
MBBS MRCOG
The author would like to clarify that all the trial participants were recruited from St Mary’s hospital, Manchester. The author primarily carried out the clinical examination for all the patients, at all time-points in the colposcopy clinic, St Mary’s hospital. The blood and biopsy samples were stored at the Paterson Institute for Cancer Research for further analysis. The author carried out all the immunofluorescence studies on biopsy samples and all the proliferation assays on blood samples and is responsible for the immunofluorescence and proliferation assay data.

The author did not carry out HPV typing and neutralisation assays, the methodology and results of which are mentioned in this thesis. HPV typing was carried out in the virology lab, St Mary’s hospital by Dr Andrew Bailey. Dr Richard Roden at the John Hopkins Institute, USA, carried out neutralisation assays on serum samples.
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THE AUTHOR

The author studied Medicine at Osmania Medical College, Hyderabad, India from 1991-1996. After graduation (MBBS), a career in Obstetrics and Gynaecology in the United Kingdom was pursued.

Basic training at Senior House Officer level in obstetrics and gynaecology at the Countess of Chester Hospital, Chester and Blackpool Victoria Hospital, Blackpool was undertaken. Three years were spent as a registrar on the Northwest training programme leading to membership examination of the Royal College of Obstetricians and Gynaecologists (MRCOG - runner up for the prize medal).

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DEDICATION

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LIST OF ABBREVIATIONS

Ab - Antibody
AIN – Anal intraepithelial neoplasia
ALL – Aminolevulinic acid
APC – Antigen presenting cell
APES - Aminopropyltriethoxysilane
CIN – Cervical intraepithelial neoplasia
CR – Complete Response
CTL – Cytotoxic T lymphocyte
DC – Dendritic cell
DMSO – Dimethyl sulfoxide
DNA – Deoxyribonucleic acid
ELISA – Enzyme linked immunosorbent assay
FDA – Food and drug administration
FOXP3CD4 – Forkhead box P3
5-FU – 5 Fluorouracil
G-CSF – Granulocyte colony stimulating factor
GM-CSF Granulocyte macrophage colony stimulating factor
GSK - Glaxo Smith Kline
HIV - Human immunodeficiency virus
HLA - Human leukocyte antigen
HPV - Human papilloma virus
HSP - Heat shock protein
HSV - Herpes simplex virus
IFN - Interferon
IL – Interleukins
i.m. – intramuscular
IPEX- Immunedysregulation, polyendocrinopathy, enteropathy, X linked syndrome

ISSVD- International Society for Study of Vulval Disease

LPA – Lymphocyte proliferation assay

MHC - Major histocompatibility complex

mRNA - messenger ribonucleic acid

MWU - Mann Whitney U test

NK - Natural killer

OCT – Optimum cutting temperature

ORF - Open reading frame

Pap - Papanicoloau

PBMC - Peripheral blood mononuclear cell

PCR - Polymerase chain reaction

PDT - Photodynamic therapy

PHA – Phytohaemagglutinin

PR – Partial Response

RCT – Randomised controlled trial

RPM - Rotations per minute

RPMI - Roswell Park Memorial Institute

RB - Retinoblastoma

RLBA - Reverse line blot analysis

SI – Stimulation index

SD – Stable Disease

TGF - Transforming growth factor

Th T – T-helper

TLR - Toll like receptor

TNF - Tumour necrosis factor
T reg - T regulatory cell

VAIN – Vaginal intraepithelial neoplasia

VIN - Vulval intraepithelial neoplasia

VLP - Virus like particle

WSR - Wilcoxon’s signed ranks test
CHAPTER 1
INTRODUCTION
1.1 VULVAL (VULVAR) INTRAEPITHELIAL NEOPLASIA
The term vulval (vulvar) intraepithelial neoplasia (VIN) was introduced in 1986 to define squamous intraepithelial lesions of the vulva. The term VIN was coined by the International Society for the Study of Vulvovaginal Disease (ISVVD) and since adopted by the International Society of Gynaecological Pathology and the World Health Organisation. On histology VIN is described as disorder of cell maturation and nuclear atypia detected at various levels in the vulval epithelium [Buckley et al., 1984]. It is a non-invasive condition wherein the epithelial basement membrane is intact. Nevertheless, the condition is associated with an invasive potential.

It has become increasingly clear that there are two distinct types of VIN, which differ from each other with respect to aetiology, pathogenesis and clinical picture. Currently there are two different classifications of VIN, the WHO classification and the ISSVD classification.

1.1.1 WHO CLASSIFICATION OF VULVAL INTRAEPITHELIAL NEOPLASIA

WHO CLASSIFICATION [Wilkinson, 2003]
VIN (warty/basaloid) with grades VIN1, VIN2 and VIN3
VIN (simplex type)

ISVVD CLASSIFICATION OF VULVAL INTRAEPITHELIAL NEOPLASIA
Classification of VIN has evolved over several decades. International Society for the Study of Vulvovaginal Disease (ISSVD), have recently (2003) reclassified VIN into 3 categories:
1. VIN, usual type (undifferentiated type), which can be further subdivided into
   a) VIN, warty type
   b) VIN, basaloid type
   c) VIN, mixed (warty / basaloid) type
2. VIN, differentiated type
3. VIN, unclassified type
The occasional example of VIN that cannot be classified into either the above two VIN categories (usual type and the differentiated type) may be classified as VIN unclassified type. e.g. the rare VIN of pagetoid type.

In contrast to the WHO classification the ISVVD committee recommended revision of the traditional three-tiered grading system of VIN. As there is no evidence that the ‘VIN1-3 morphologic spectrum reflects a biologic continuum nor that VIN1 is a cancer precursor’, ISSVD recommended that ‘the term VIN should apply only to histologically high-grade squamous lesions (former terms VIN 2and VIN3 and differentiated VIN3)’. Further ISSVD proposed that ‘histological changes previously described as VIN1 should be described either as flat condyloma acuminatum or HPV effect’.

As per WHO recommended grading of HPV associated VIN, VIN grade 1 is defined as ‘maturation present in the upper two-thirds of the epithelium and the superficial cells contain variable but usually mild atypia which may include viral cytopathic effect’. If the full thickness of the epithelium has abnormal cells, VIN III, also called vulvar carcinoma in situ, is diagnosed.

There is some debate as to which classification to follow and some histopathologists prefer to utilise the WHO classification in their diagnostic work.

1.1.2 INCIDENCE

VIN although not a very common condition, the incidence is increasing, more so in younger women. VIN has been identified as one of the twelve neoplasias whose incidence has increased in the developed countries [Akerman et al., 2007b]. Studies have shown a nearly three-fold overall increase in the incidence of VIN across the world over the last twenty years [Herod et al., 1996;Iversen and Tretli, 1998;Jones et al., 1997;Joura et al., 2000;Sturgeon et al., 1992]. The SEER (Surveillance, Epidemiology, End results) American cancer registry database reveals a 411% increase in the incidence of VIN from year 1973 to 2000, whilst the corresponding increase in vulval cancer in
the same time period has only been 20% [Akerman et al., 2007b]. The increase in the incidence of VIN, a precursor lesion in the majority of the cases of squamous cell carcinoma (SCC) of the vulva is expected to be followed by an increase in the incidence of invasive vulval cancer, but this has not been the case. As suggested by Akerman et al the unknowns of natural history of VIN and the effects of treatment may make any extrapolation hazardous. The disproportionate increase in the incidence of VIN in comparison with vulval cancer is thought to be due to an increase in the incidence of genital human papilloma virus (HPV) infections.

Also, a study conducted in four hundred and five women diagnosed with VIN between the years 1962 and 2003 showed the mean age to decrease from fifty in the sixties to a mean age of thirty-nine in the subsequent years [Jones et al., 2005;Joura et al., 2000]. Lanneau et al identified fifty-six women under the age of forty-five years diagnosed with SCC of the vulva and observed that the incidence of both VIN and invasive SCC of the vulval had increased at the rate of 2.4% per annum; particularly in young women [Lanneau et al., 2009]. Increasing awareness of this condition among medical practitioners and increasing willingness by women to present with symptoms associated with VIN could also to a small extent have contributed to the rise in the detection of this condition.

1.1.3 AETIOLOGY

Aetiology of VIN is multifactorial and heterogeneous. The usual or undifferentiated type of VIN is more common than the differentiated and unclassified types of VIN. The usual or undifferentiated type is strongly associated with HPV infection of the female lower genital tract. More than one hundred and thirty HPV genotypes have thus far been cloned from clinical lesions in the human beings. About thirty-four HPV types are known to infect the genital tract, of which, about fifteen types are considered to be oncogenic and therefore high-risk. Usual or undifferentiated type of VIN was found to be associated with high-risk infection, HPV16 in about 78-90% of cases and less commonly with HPV 18 infection [Buscema et al., 1988;Ikenberg et al.,
Meta-analyses of ninety-three studies of prevalence and type distribution of HPV in VIN using polymerase chain reaction (PCR) assays conducted across four continents demonstrated 85.3% HPV positivity in VIN 2 and VIN 3 lesions [De Vuyst et al., 2009]. A systematic review by Insinga et al of prevalence and attribution of HPV types among genital tract pre-cancers and cancers in the United States estimated that following multitype adjustment, which is adjustment for the presence of multiple HPV types in a single specimen, (n=503, 2 studies) HPV 16 was estimated to contribute to more than three quarters (77.7%) of VIN3 lesions. HPV 16, 33, 6, 11 and 31 were the five most frequent HPV types detected, in order of prevalence, and multiple infections were observed in 5.8% of cases [Insinga et al., 2008]. A retrospective case-note study of long-term follow-up over a fifteen-year period of hundred and thirty-three women with biopsy confirmed VIN demonstrated that the number of women diagnosed with VIN had increased throughout the study period. Women who were HPV positive on biopsy sample taken from the VIN lesions were significantly younger than women who were HPV negative in the biopsy taken from the VIN lesions [Herod et al., 1996]. Skapa et al subjected two hundred and sixty nine vulval excision samples representing lichen sclerosus, lichen simplex chronicus, differentiated VIN, usual VIN, condyloma acuminata and squamous cell carcinoma to HPV typing. Different HPV profiles and differing frequency of multiple-type HPV infections were noted in women with high-grade usual or undifferentiated VIN compared to women with other types of vulval disorders [Skapa et al., 2007]. Presence of HPV infection appears to be the most influencing factor in the development of VIN. Infection with HPV on its own may not however cause VIN. It is presumed that associated factors that interfere with body’s immune system i.e. smoking, immunosuppression, and inherited immunity problems may also contribute to the disease, which will be discussed in the subsequent sections of the thesis. VIN thus appears to be a component of multifocal HPV associated lower genital tract neoplasia as it is often multifocal and exhibits an obvious association with vulval warts, vaginal intraepithelial neoplasia (VAIN) and anal intraepithelial neoplasia (AIN).
The second and less common type of VIN, referred to as differentiated VIN (also known simplex VIN) is not associated with HPV infection but commonly arises from a vulval dystrophy, either lichen sclerosus or squamous hyperplasia. Although Abel and colleagues first described features of this condition in the 1960’s, it is only in the recent years that the morphological features of differentiated VIN are made clear and the existence of this type of VIN stressed [Hart, 2001]. In a large study of five hundred and eighty women diagnosed with premalignant vulval conditions, only one hundred and seventy one were diagnosed with the usual (undifferentiated) type of VIN, nearly half the women were diagnosed with differentiated type of VIN and lichen sclerosis (two hundred and sixty one) and the remaining hundred and forty five women were diagnosed with squamous hyperplasia. The risk of squamous cell carcinoma of the vulva was also found to be significantly higher in the differentiated VIN group (85.7%) in comparison with the usual VIN group (25.7%) [Eva et al., 2009].

1.1.4 CLINICAL FEATURES

VIN lesions are polymorphic both in appearance and size. On inspection of the vulva, the lesions can be appearing to be unifocal, multifocal or diffusely scattered, with hypo or hyper-pigmented appearance (figure 1). On palpation, the lesions can be felt to be raised, rough surfaced or flat. Majority of the lesions although are present on the inner aspects of the labia majora and minora, they can present anywhere on the vulva including the clitoral, periurethral and perianal aspects of the vulva (figure 2). Most lesions are symptomatic and cause the distressing symptoms of itch, soreness, irritation or discomfort. Asymptomatic lesions are uncommon and usually are detected incidentally when the woman is undergoing a gynaecological examination.
Figure 1 a – Unifocal VIN, 1b – multifocal VIN, 1c – multipigmented VIN, 1d – hyperpigmented VIN
1.1.5 NATURAL HISTORY OF VIN

Studying the natural history of VIN is difficult, as most women diagnosed with VIN undergo treatment either for symptom relief or because malignancy is suspected. The more common HPV associated usual or undifferentiated type of VIN and the differentiated type of VIN which is not associated with HPV infection, are both likely to progress to pre-invasive early stromal invasion (ESI) of the vulva and invasive SCC of the vulva. The rate of progression of VIN to invasive carcinoma is thought to be variable and about a tenth of the
proportion of cases are thought to progress to invasive carcinoma. Spontaneous regression of VIN is however uncommon and is thought to occur in less than 1.5% of patients [van Seters et al., 2005].

1.1.6 MALIGNANT POTENTIAL

The progression of grade 1 VIN to invasive vulval cancer is considered to be very low [Bancher-Todesca et al., 1997] in contrast to the clearly established progression of VIN3 lesions in 3-19% of cases to SCC of the vulva [Crum, 1992; Herod et al., 1996]. In a retrospective study by Leibowitch et al, when seventy-eight specimens of SCC of the vulva were reviewed retrospectively, in most cases of SCC of the vulva, adjacent epithelium showed morphological changes of VIN or HPV changes in 22-67% of cases, VIN3 in 20-30% of cases, and lichen sclerosis in the nearby epithelium in 10-62% of cases indicating that in the majority of cases, a common factor plays a role in the development of pre-malignant and malignant lesions on the vulva [Leibowitch et al., 1990]. In some cases there is found to be a clinicopathological overlap between HPV associated and non-HPV associated SCC of the vulva. The majority of SCC of the vulval is found to be associated with vulval lichen sclerosis (differentiated VIN) but VIN is also adjacently found in a third of vulval carcinomas [MacLean, 2006]. Some studies have confirmed a 15-22% rate of occult carcinoma in histologically diagnosed VIN3 [Chafe et al., 1988; Herod et al., 1996; Husseinzadeh and Recinto, 1999; Modesitt et al., 1998a]. In a recent study by Eva et al greater than a thousand biopsy samples (n=1309) from five hundred and eighty women diagnosed with various pre-malignant vulval conditions were studied and matched with prior, synchronous or subsequent diagnosis of SCC of the vulva. Among women diagnosed with the HPV associated usual or undifferentiated type of VIN, the rate of progression to SCC was found to be 25.7% in comparison to a markedly high 85.7% risk of progression to SCC of the vulva, linked with differentiated type of VIN. A large study based on the Netherlands Nationwide database of histological and cytopathology conducted over a period of fourteen years concluded that overall percentage of the usual or undifferentiated type of VIN that were later diagnosed with SCC of the vulva was 5.7%, which was significantly lower than
the 32.8% risk of progression noted with the differentiated VIN. In addition, the time of progression to SCC of the vulva for differentiated VIN was significantly shorter than the usual or undifferentiated VIN [van de Nieuwenhof et al., 2009]. There is thus enough evidence to suggest that differentiated type of VIN is significantly more associated with vulval SCC than the usual type of VIN [Eva et al., 2009].

In a retrospective case-note study of hundred and thirty-three women diagnosed with VIN, all of who underwent treatment for VIN, progression to invasive disease occurred in 7% of the cases [Herod et al., 1996]. Similarly, in a study of hundred and thirteen women diagnosed with VIN by Jones et al among the hundred and five women who received treatment for VIN, four women developed cancer (4/105), whilst among eight women who did not receive any treatment for VIN but were only clinically observed, seven women (7/8) developed cancer within eight years of clinical observation [Jones and Rowan, 1994]. Jones et al in their natural history and outcome study of four hundred and five women diagnosed with VIN concluded that untreated VIN in women over 30 years of age has an appreciable invasive potential [Jones et al., 2005].

In a large review article on three thousand three hundred and twenty two women with VIN, only eighty eight women were found to not have undergone any treatment for VIN, of who, eight women (9 %) developed disease progression to invasion [van Seters et al., 2005]. The rate of progression of VIN to vulval SCC thus appears to vary from less than 5% to up to 20% as quoted in the above mentioned different studies and it appears that the untreated group carry a higher risk of progression to invasion.

1.2 RISK FACTORS FOR DISEASE PROGRESSION IN WOMEN DIAGNOSED WITH VIN

Many factors contribute to an increased risk of SCC of the vulva. Age has been shown to be one of these risk factors. It has been show that women aged forty or more are at an increased risk of HPV associated SCC of the
vulva compared to younger women [MacLean et al., 1993]. The incidence of HPV associated cancer is known to be much higher in the immunosuppressed, [Al Ghamdi et al., 2002; Brown et al., 2005; Buscema et al., 1980] further discussed in this manuscript. History of previous lower genital tract neoplasia i.e. pre-invasive and invasive disease of the cervix, vagina, vulva and anus, more so if the previous condition was treated with radiotherapy has also been shown to be a risk factor for VIN [van Seters et al., 2005]. Proximity of the lesion to the anal margin and squamo-columnar junction (SCJ) has also been shown to be one of the risk factors [Buscema et al., 1980; MacLean et al., 1993]. p53 over expression has also been identified as one of the risk factors for progression of VIN to SCC of the vulva.

**p53 OVEREXPRESSION & VEGF EXPRESSION IN VIN**

The TP53 gene encodes the transcriptor factor p53, which plays a vital role in cell cycle regulation, tumour suppression and prevention of cancer. p53 gene mutation has been demonstrated in some cases of undifferentiated VIN [Almeida et al., 2004]. The immunoreactivity of mutated p53 is however non-specific, as it is also known to be over expressed in HPV positive vulval cancer and vulval lichen sclerosis where p53 over expression has been used as a marker for invasion [Rolfe et al., 2003]. In one study of seventy-three cases of VIN only three women over expressed the p53 gene who were diagnosed with concurrent or previous vulval cancer [Rosenthal et al., 2003]. In another study of twenty women who underwent surgical treatment for VIN and were under regular clinical follow-up, p53 over expression was observed in ten out of twenty women. Interestingly, nine of these women subsequently developed disease recurrence and one woman developed SCC of the vulva [Almeida et al., 2004]. Vascular endothelial growth factor (VEGF) expression was found in 6% of cases of VIN and 92% of cases of SCC of the vulva and perhaps VIN expressing VEGF exhibits a greater risk of turning invasive [Lewy-Trenda et al., 2005].
1.2.1 SPONTANEOUS REGRESSION

Literature about spontaneous regression of VIN is limited, mostly case reports and a retrospective case note review [Friedrich, Jr., 1972; Jones and Rowan, 2000; Skinner et al., 1973]. In the literature thus far, the demographic and clinical traits of women in whom spontaneous regression occurred were very dissimilar to the routine. The patient group was much younger (mean age of 19.5 years), non-white ethnicity that typically presented with symptoms in pregnancy or immediate post-natal period. So far, spontaneous regression has not been reported in any other patient groups. In a recent, small two-year prospective study, eight women who were newly diagnosis with VIN were kept under careful observation. Only women who consented to the ‘wait and watch’ policy were recruited into this study. Surgical treatment was exclusively considered when progression to invasive disease was suspected or for uncontrollable symptoms. Six of the eight women underwent surgical treatment very soon after recruitment and overall seven women underwent treatment within the two-year study period, majority for symptom control. No disease progression or spontaneous regression was observed in the remaining one participant demonstrating the chronic nature of the condition [McFadden et al., 2009].

1.3 ASSOCIATION OF CIGARETTE SMOKING WITH VIN

Clearly there are factors that drive the outcome of high risk HPV genotype infection towards a high-grade or a low-grade intraepithelial lesion. There is plenty of evidence for smoking to be one such factor. The number of cigarettes, years of smoking and early age of smoking initiation are all shown to be associated with increased risk of genital tract neoplasia. Cigarette smoking has been implicated both in the acquisition of high-risk HPV genotypes and the development of both high-grade intra-epithelial neoplasia and cancer of the lower genital tract [Sherman et al., 2008]. History of smoking at diagnosis was found to be an important risk factor in the progression of intra-epithelial neoplasia to cancer [Daling et al., 1992]. No such association between smoking and low-risk HPV genotypes was however
noted [Madsen et al., 2008]. In a recent study by Sherman et al women who smoked although were not at increased risk of becoming infected with high-risk HPV genotypes, but were at a significantly increased risk of developing high-grade lower genital tract intra-epithelial neoplasia in comparison to non-smokers [Sherman et al., 2008]. In a study of eighty women with histologically confirmed VIN3, women who smoked were not only more likely to be diagnosed with multicentric genital tract neoplasia but also were found to be thirty times more likely to have persistent disease in comparison to women who never smoked or women who quit smoking following treatment for VIN [Khan et al., 2009]. Harris et al proposed that in cases of CIN, cigarette smoking may act by increasing cell turnover in the transformation zone of the cervix and supported their hypothesis by their findings of increased proliferation marker Ki-67 in heavy smokers [Harris et al., 2004]. A study by Goffin et al revealed an 8.7 fold (CI 2.0-38.4) increased association between smoking, HPV and active VIN status [Goffin et al., 2006]. Although there is no transformation zone in the vulva, the effects of tobacco smoking on intra-epithelial lesions of the vulva may be due to the systemic effects of smoking.

1.4 ASSOCIATION OF IMMUNOSUPPRESSION WITH VIN

Immunosuppression has long been associated with an increased incidence of HPV associated intraepithelial neoplasia and its subsequent progression to malignancy. Higher incidence of persistent HPV infections and related HPV associated dysplasia in both the immunosuppressed transplant recipients and human immunodeficiency virus (HIV) infected individuals has been clearly shown [Bouwes Bavinck and Berkhout, 1997;Palefsky and Holly, 2003]. Strickler et al. studied the association between HIV-positivity, plasma HIV RNA level, CD4+ T lymphocyte count and the natural history of HPV infection or HPV reactivation in eighteen hundred and forty-eight HIV positive women and compared the findings with five hundred and fourteen HIV negative women. Both the groups were assessed at six-monthly intervals for the presence of HPV in the cervico-vaginal fluid by PCR and in the squamous intraepithelial lesions by Pap (Papanicoloau) smears. In HIV positive women,
plasma HIV RNA levels and CD4+ T lymphocyte count in combination appeared to have a strong and significant association with the presence of incidental detection of HPV infection, some of which might represent reactivated HPV. Although an association between HIV co-infection and persistent HPV infection was also noted, this association was less strong in comparison to an incidental infection [Strickler et al., 2005]. Dedes et al studied thirty-one HIV infected women on retroviral therapy who were also diagnosed with vulval, vaginal and perianal intraepithelial neoplasia. A higher than average 65% developed disease recurrence and a third developed invasive cancer [Dedes et al., 2008]. Similarly, Brown et al in a retrospective case-note review of women diagnosed with vulval carcinoma before the age of forty noted an association between VIN progression to cancer, other HPV related diseases and the presence of an antecedent acquired immune deficiency syndrome (AIDS) defining illnesses. Intraepithelial neoplasia in HIV positive women was to be associated with high rates of recurrence and invasion despite treatment [Brown et al., 2005]. Koshiol et al. studied five hundred and twenty-two women who were HIV-seropositive and two hundred and seventy-nine women who were HIV-seronegative in their HIV Epidemiology Research Study (HERS, 1993-2000). They noted that high-risk HPV types had lower estimated clearance rates in comparison to low-risk HPV types, with no significant effect by the HIV serostatus on the clearance rate of the HPV infection [Koshiol et al., 2006].

Meeuwis et al. in their study of two hundred and twenty four female renal transplant recipients on immunosuppressive therapy observed an increased propensity for development of pre-malignant and malignant lesions involving the skin and the female lower genital tract. In this group of women the increase in the incidence of CIN was two to six-fold, the increase in the incidence of carcinoma of the cervix was three-fold, but disturbingly the increase in the incidence of SCC of the vulva was fifty-fold [Meeuwis et al., 2009].

Aynaud et al conducted a retrospective study in hundred and six women who underwent laser treatment for ano-genital condylomatous lesions or ano-
genital intraepithelial neoplasia and were followed-up for up to six months after treatment. Within this cohort, they compared immunocompetent women with those who were rendered immunosuppressed for therapeutic reasons and also with women who were immunosuppressed with HIV infection. Ano-genital intraepithelial neoplastic lesions, in women, comprising CIN, VAIN, VIN and AIN were significantly more common in the HIV positive group (47.4%) in comparison to the immunocompetent group (20.2%) and this difference was statistically significant. As well, immunocompetent women and women who were immunosuppressed for therapeutic reasons more commonly presented with disease recurrence at follow-up in contrast to women who were immunosuppressed with HIV infection who presented with disease persistence [Aynaud et al., 2008].

Immunosuppression, irrespective of the cause, in association with ano-genital HPV infection is characterised by persistent dysplasia and an increased risk of progression to malignancy [Ferenczy et al., 2003;Sillman et al., 1997].

1.5 ASSOCIATION OF VIN WITH HUMAN PAPILLOMA VIRUS

The lifetime risk of acquiring a genital infection on at least one occasion with an oncogenic strain of HPV is thought to be above 80% [Koutsky et al., 1988]. More than hundred and thirty HPV genotypes have thus far been cloned from clinical lesions of which, about thirty to forty types infect the epithelial and mucosal lining of the female ano-genital tract and other areas [Steben and Duarte-Franco, 2007]. The low risk HPV types, predominantly types 6 and 11, cause genital warts and about fifteen high-risk (oncogenic) types, predominantly types 16 and 18, infect the genital tract and contribute to a significant proportion of cases of CIN and VIN [Parkin and Bray, 2006].

Incidental infection with HPV 16 is thought to occur in about 6% of the general population. Low risk HPV 6 or 11 infection was detected in 64.5% of VIN 1 lesions and 29% of VIN 2 or 3; whereas, high-risk HPV 16 was detected in 6.5% of VIN1 and 64.5 % of VIN2/3 [Garland et al., 2009]. HPV infection of the genital tract is often multicentric [Beckmann et al., 1991;van Beurden et
al., 1998]. Nearly one-third to a half of women with HPV positive high-grade VIN were shown to also possess CIN or other lower genital tract neoplasia [Hording, 1995; Jones and Rowan, 1994]. It has also been shown that up to 76-94% of women with active VIN3 lesions harbour high-risk HPV types concurrently both on the cervix and on the vulva and the HPV types are usually concordant [Beckmann et al., 1991; Lijnen and Blindeman, 1994; van Beurden et al., 1998]. In another retrospective study of fifty-two women who were either previously treated for high grade VIN or were diagnosed with active VIN during the study period, high risk HPV DNA was detected at multiple sites on the vulva and also on the cervix. Past or current status of the disease was found not to have any effect on the presence of high risk HPV [Goffin et al., 2006].

Insinga et al. conducted a systematic review on prevalence and attribution of HPV types among cervical, vaginal and vulval precancers and cancers [Insinga et al., 2008]. It was estimated that three thousand four hundred and ninety women in the USA would be diagnosed with vulval cancer in year 2007 resulting in eight hundred and eighty deaths. HPV 16 (n=503, 2 studies) was estimated to contribute to more than three quarters (77.7%) of VIN3 lesions. Among vulval cancers, (n=197, 4 studies) only data for squamous cell carcinoma of the vulva were available. In this group, HPV types 16, 33, 6, 1 and 31 were the five most frequent HPV types detected, in order of prevalence, and multiple HPV infections were observed in 5.8% of cases. Following adjustment for multiples types of HPV within the same lesion, HPV 16 was found in nearly 50% of all the cases and in more than 75% of HPV-positive cases. In another study of fifty-five women diagnosed with vulval carcinoma over a period of ten years by Monk et al, HPV prevalence within the lesions was described in relation to the patient’s age group. The HPV positivity varied from 100% in the forty-five or younger age group to 71% in the forty-five to sixty-nine age group to 47% in women aged seventy years or more [Monk et al., 1995].
1.5.1 THE LIFECYCLE OF HPV

Human papilloma viruses are epitheliotropic, non-enveloped, small, circular double-stranded DNA viruses that belong to the papilloma viridae family. HPV infections are not systemic; remaining at the site of initial infection, usually resulting in an asymptomatic infection, which often clears. Only a minority develop high-grade lesions [Kotloff et al., 1998].

1.5.2 FUNCTION OF VIRAL PROTEINS

The early (E) and late (L) proteins of the HPV have different functions in the life cycle of the virus. The following comprise the early and late viral proteins.

- E1 viral replication
- E2 viral replication and transcription
- E4 destabilization of cytokeratin network
- E5 mediated mitogenic signal of growth factors
- E6 cellular transformation
- E7 cellular transformation

- L1 major viral coat protein
- L2 minor viral coat protein

1.5.3 INFECTION

For epitheliotropic viruses such as the HPV, to cause an infection HPV requires access of infectious particles to cells in the basal layer of the epithelium. This requires a break in the stratified epithelium. It has been speculated that for the sustenance of infection the virus must infect an epithelial stem cell [Egawa, 2003]. On the vulva, stem cells are abundant within the hair follicles. HPV infection is thought to occur through micro trauma of the genital tract enabling access of the viral particles to target lymphocytes, probably the stem cells present in the basal layer of the epithelium.
1.5.4 GENOME MAINTENANCE

Following infection, it is believed that the virus maintains its genome in the basal layer of the epithelium. It is generally thought that viral E1 and E2 proteins are expressed whose function is to maintain the viral DNA as an episome and to facilitate correct segregation of genomes during cell division [You et al., 2004; Zhang et al., 1999]. The viral transforming proteins E6 and E7 are also expressed in the cells of the basal layer, though their role in genome maintenance is not as defined as the early viral proteins E1 and E2 [Crum, 1998]. It is believed that viral genome is maintained in the basal layer at around 10-200 copies per cell and that viral early proteins (E6, E7, E1 and E2) are expressed at lower levels [De Geest et al., 1993; Stanley et al., 1989].

1.5.5 PROLIFERATIVE PHASE

In the uninfected epithelium, the process of terminal differentiation allows the basal cells to exit the cell cycle soon after migrating into the suprabasal cell layers. Changes that lead to natural terminal differentiation include the physical cross linking of keratin intermediate filaments, formation of cornified envelopes and secretion of lipids which together allow the epithelial surface to form a physical barrier against the microenvironment [Madison, 2003]. During papilloma virus infection, E6 and E7 are expressed in these cells, which are thought to have a synergistic effect. As a result, the restraint on the cell cycle progression is abolished and normal terminal differentiation is retarded [Sherman et al., 1997]. It has been shown that both E6 and E7 have functions that stimulate cell cycle progression and both can associate with regulators of cell cycle [Munger et al., 2001]. In addition, the association of E7 with members of the pocket protein family such as pRb, a negative regulator of the cell cycle that normally prevents S phase entry and other proteins involved in cell proliferation is well characterised. Despite the ability of E7 to stimulate cell proliferation during productive infection, only subsets of cells in the parabasal layers are mitotically active. The expression of cyclin E is absolutely necessary for S phase entry and is expressed during natural infection. It appears that during natural infection the ability of E7 to stimulate S phase
progression is limited to the subset of differentiated cells, which express high enough levels of E7 to overcome the block to S phase entry. The viral E6 protein complements the role of E7 and is thought to prevent the induction of apoptosis in response to unscheduled S phase entry mediated by the E7. Although the association of E6 with p53 and the inactivation of p53 mediated growth suppression and/or apoptosis is well been documented, E6 can also associate with other pro-apoptotic proteins including BaK [Thomas and Banks, 1998] and BaX [Li and Dou, 2000]. The presence of E6 is considered as a predisposing factor in the development of HPV associated cancers allowing the accumulation of possible errors in host cell DNA to go unrestrained. The E6 protein of high-risk HPV types can also stimulate cell proliferation independently of E7. In addition to E6 and E7 it is thought that the other viral early proteins (i.e., E1, E2, E4, E5) are expressed prior to the onset of genome amplification in order to ensure maintenance of the viral episome at a low copy number [Middleton et al., 2003].

1.5.6 VIRUS SYNTHESIS

Once viral genome amplification has been completed, papillomaviruses encode two structural proteins in the upper layers of the infected tissue, L1 and L2. L2 is a minor coat protein but like L1 is produced in the subset of cells that express E4 [Doorbar et al., 1996]. The major capsid protein L1 is expressed after L2 allowing the assembly of infectious particles in the upper layers of the epithelium [Florin et al., 2002]. Papillomavirus particles comprise an approximate 8000 base pair genome within a capsid that contains 360 copies of the L1 protein and probably 12 copies of L2 organised into a 72 capsomere icosahedral shell [Modis et al., 2002]. The L2 protein accumulates at nuclear structures known as PML bodies during viral assembly and recruits L1 to these domains. It has been suggested that PML bodies may be the sites of papillomavirus DNA replication [Day et al., 1998;Swindle et al., 1999]. Although virus like particles can assemble in the absence of L2, the L2 protein is thought to enhance packaging and infectivity [Roden et al., 2001]. To be successful, the virus must eventually escape from the infected skin cell and survive extracellularly prior to reinfection. Papillomaviruses are non-lytic and
are not released until the infected cells reach the epithelial surface. Papillomaviruses are resistant to desiccation [Roden et al., 1997] and their extra cellular survival may be enhanced if they are shed from the epithelial surface within a cornified squame [Bryan and Brown, 2001]. The intracellular retention of papillomavirus antigens until the cell reaches the uppermost epithelial layers may compromise immune detection of the virus, particularly if the virus also has molecular mechanisms that limit the presentation of the viral epitopes to the immune system in the lower epithelial layers [Marchetti et al., 2002]. Although the expression of viral proteins can inhibit expression of differentiation markers preventing the formation of normal cornified squames, [Doorbar et al., 1997] it has also been suggested that it is the viral E4 protein that contributes directly to virus egress in the upper epithelial layer by disturbing keratin integrity [Doorbar et al., 1991] and effecting the assembly of cornified envelope [Bryan and Brown, 2001; Lehr et al., 2004].

1.6 IMMUNOEVASION, PERSISTENCE OF HPV INFECTION AND DEVELOPMENT OF NEOPLASIA

1.6.1 IMMUNE EVASIVE STRATEGIES ADOPTED BY HPV

Immune system plays an important role in controlling the spread of HPV associated infections. There is ample evidence to show that individuals with immune defects (i.e., transplant recipients, HIV infected individuals and in some individuals with genetic defects in immune cell function) are particularly susceptible to HPV infection and can develop widespread lesions that are refractory to treatment. HPV is perfectly adapted to its natural host tissue, the differentiating epithelial cells of the skin or mucosa. Several factors minimise or prevent exposure of the virus to the immune system. HPV do not infect and replicate in antigen presenting cells (APC) nor do they lyse the keratinocytes. HPV replication and release does not cause cell death, as the differentiating keratinocyte is already programmed to die, and this “death by natural causes” does not present as a danger signal to the immune system. In addition, HPV suppresses the expression of several proinflammatory proteins that are crucial
in clearing infection and activating the cytotoxic T lymphocytes (CTL) involved in killing virus-infected cells.

1.6.2 PERSISTENCE OF HPV INFECTION

The frequent detection of HPV 16 DNA in the cervical lesions in the absence of any obvious disease may be explained by its presence in the latent state with only very few cells supporting the productive cycle during the epithelial cell differentiation. Following immune regression, papilloma virus DNA is thought to remain in the basal epithelial cells and then be reactivated when levels of immune surveillance decline. It has been suggested that latent gene expression is restricted to E1 and E2 proteins and that during this phase of the virus life cycle the E6 and E7 genes are not required [Zhang et al., 1999].

E6 and E7 proteins between high and low-risk HPV types are shown to be different, more of a quantitative rather than a qualitative nature. High-risk HPV types show more tendencies towards genomic integration, where as low-risk types are preferentially maintained as episomes – portions of genetic material that can exist independent of the main body of the genetic material [Arends et al., 1998].

Genital infections with HPV are usually transient with the majority of individuals showing clearance of virus within a year of detection [Evander et al., 1995; Ho et al., 1998; Sellors et al., 2003; Woodman et al., 2001]. Only a minority of women appear to develop persistent infections with focally high levels of HPV DNA, a proportion of whom progress to developing high-grade disease and invasive carcinoma [Herrero et al., 2000].

1.6.3 PROGRESSION OF PERSISTENCE HPV INFECTED LESIONS

Considering the high prevalence of HPV infections in the human population, the number of lesions that progress to cancer is in fact very low. Key event in the progression of productive lesions to high-grade neoplasia may result from deregulation in the expression of the viral transforming proteins E6 and E7,
resulting in increased cell proliferation in the lower epithelial layers. Failure to restore the normal status and secondary mutations in the host cell DNA [von Knebel, 2002] retention of E6 and E7 genes and loss of E2 and E4 genes, which can exert a negative effect on cell growth, all these changes contribute and accompany the development of invasive cancer [Peh et al., 2004].

1.6.4 REGRESSION OF HPV INFECTED LESIONS

Despite the ability of the HPV to inhibit host defences, a successful immune response to genital HPV infections is established in most cases resulting in the regression of lesions. It has been suggested that in humans although about 80-90% of genital HPV infections resolve with time, in the remaining 10-20% of cases, the individual does not become HPV DNA negative and develops persistent infection [Beckmann et al., 1991].

1.7 HOST IMMUNITY

There are two main arms to the immune response, which play a role in the natural clearance of infection, the innate and the adaptive immune responses. Protective immunity results from the interaction of the non-specific innate immunity and antigen specific adaptive immunity. Without the antigen non-specific defence by the innate arm of the immune system, the adaptive arm of the immune response, which generates pathogen specific antibody and cellular effectors, is not activated and a pathogen can effectively hide from the host immunity. The nature of HPV life cycle, where the local infection does not result in necrosis and the production of virus particles occurs only in cells that are terminally differentiated enables the virus to surreptiously take advantage of not been recognised by the innate division of the immune response and thus avoid potential immune control [Stern et al., 2000].

1.7.1 INNATE IMMUNITY

The innate immunity system senses tissue damage via signals from molecules, which would normally not be found exogenously. This involves production of immunoregulatory molecules such as cytokines (alpha, beta and
gamma interferons (α β γ IFN), transforming growth factor beta (TGFβ), tumour necrosis factor-alpha (TNF-α) and interleukins (IL-1, IL-6, IL-10, IL-12, IL-15), monocytes, macrophages, natural killer (NK) and antigen presenting cells (APCs), all of which have direct controlling effect on virally infected cells. The defence mechanisms involving these immunoregulatory molecules represents the first line of defence [Dermime et al., 2002a; Matzinger, 2002a]. In the female genital tract, dendritic cells (DC) or Langerhan cells (LC) are the antigen presenting cells (APCs) with the unique ability to take up and process antigens in the peripheral blood or tissues. The information acquired by these cells can shape the subsequent T cell mediated immunity by the adaptive immune response arm of the immune system into T helper (Th) type 1 or Th type 2 or T regulatory response [Kalinski et al., 1999; Wang et al., 2006].

1.7.2 ADAPTIVE IMMUNITY

The generation of an adaptive immune response is important in the long-term control of infection or tumour. The APCs activate a range of T lymphocytes. Th type 1 response will favour the production of cytotoxic T cell effectors which would be important in clearing any virally infected cells whilst a Th type 2 response will facilitate the stimulation of B lymphocytes and their production of neutralising antibodies. With HPV infections, activated B-lymphocytes make antibodies against the viral capsid proteins or optimally activate cytotoxic T lymphocytes. The adaptive immune system is capable of generating antigen specific memory and can deliver long-term protection against subsequent HPV infection of the same type. Despite the ability of the HPV to inhibit host defences, a successful immune response to genital HPV infections is established in most cases. This is characterized by local cell mediated immunity that is associated with lesion regression and the generation of serum neutralizing antibodies directed against L1 protein displayed on the outer surface of the intact virus particle. However, serum neutralizing antibody levels following natural HPV infection, even at peak titres, are low [Carter et al., 2000]. Neutralising antibodies, although of uncertain significance in preventing infection or reinfection after natural exposure (prior infection) are
known to be highly protective after immunisation with HPV virus-like-particles-based-vaccines [Einstein et al., 2009].

Innate and adaptive immunity are not two separate entities because innate immunity controls both the development and nature of the adaptive immunity. APCs provide an important link between innate and adaptive immunity with dendritic cells acting as the most potent inducers of primary and secondary immune responses. Dendritic cells capture antigens, migrate to secondary lymphoid organs, and after maturation, they present the processed antigens to antigen-specific lymphocytes where clonal immunity is initiated. The innate activation of dendritic cells can trigger their differentiation into immunogenic APCs that are able to prime and expand naïve T lymphocytes. Therefore, activation or maturation of dendritic cells, as an essential component of the immune system, translates antigen-non-specific innate immune recognition into antigen-specific adaptive immunity.

1.8 MAJOR HISTOCOMPATIBILITY COMPLEX

One method of identification of the antigen is by B-lymphocytes with their membrane bound antibodies, also known as B cell receptors. Whereas B cell receptors of B-lymphocytes can bind to antigens without much outside help, T cell receptors of T lymphocytes require presentation of the antigen. This is the primary role of major histocompatibility complex (MHC) in the immune system. It is a very gene dense region, containing one hundred and forty genes and in the humans the MHC region is represented on chromosome number six. An individual is expected to have a maximum of eighteen MHCI or II alleles. There is however a high degree of MHC polymorphism in the general population.

MHC proteins act as indicators, presenting fragmented pieces of an antigen on the host's cell surface. Cancer cells and cells infected by a virus have a tendency to display unusual, non-self antigens on their surface. These non-
self antigens, regardless of which type of MHC molecule they are displayed on, will initiate the specific immunity of the host’s body.

MHC class I proteins are expressed on all the nucleated cells and present antigen fragments to the cytotoxic T lymphocytes and bind to the CD8 receptor on the cytotoxic T lymphocytes. MHC class I dependent pathway of antigen presentation is the primary way for a cell infected with virus to signal the influx of T lymphocytes. The subsequent fate of a virus-infected cell is almost always apoptosis, initiated by the cytotoxic T lymphocyte (CTL).

MHC class II is expressed selectively on the APCs including macrophages, dendritic cells and B-lymphocytes. These cells present antigen fragments to T helper cells by binding to the CD4 receptor on the T helper cells. The antigen peptides presented by class II molecules are derived from extracellular proteins, hence the MHC class II dependent pathway of antigen presentation is called endocytic or exogenous.

The sub-set of genes in the MHC region that encodes cell-surface antigen presenting proteins is referred to as human leukocyte antigen (HLA) genes. The nine classical MHC HLA genes are HLA-A, HLA-B, HLA-C, HLA-DPA1, HLADPB1, HLA-DQ-A1, HLA-DQB1, HLA-DRA and HLA-DRB1. The A, B and C genes belong to MHC class I, whereas the six D genes (HLA-DPA1, HLADPB1, HLA-DQ-A1, HLA-DQB1, HLA-DRA and HLA-DRB1) belong to class II. HLA has a critical role in the complex immunological processes that occurs between various cells in the body and the T lymphocytes.

HLA down regulation when studied in relation to VIN and SCC of the vulva, poor response of VIN lesions to treatment has been attributed to HLA class I down regulation and reduced number of intralesional langerhan cells and CD8 T lymphocytes. In 82% of cases of SCC of the vulval, total loss of HLA class I genes was observed [Abdel-Hady et al., 2001]. Abdel-Hady et al in their clinical study of photodynamic therapy for VIN observed that none of the women with HLA class I down regulation responded to the treatment in contrast to women who did not demonstrate HLA class I down regulation and
in 50% of women with HLA class I down regulation, VIN progressed to invasion [Abdel-Hady et al., 2001].

1.9 CYTOKINES

Cytokines are critical to the development and functioning of both innate and adaptive immune responses. They are secreted by immune cells in response to pathogens to cascade recruitment of more immune cells and increase the system’s response to the pathogen. The predominant producers of cytokines are helper T lymphocytes and macrophages.

1.9.1 CYTOKINES STRUCTURAL CLASSIFICATION

Based on their structure, cytokines have been classified into four types, the four α-helix family comprising IL2, IFN and IL10 subfamilies, the IL-1 family, which primarily includes IL1 and IL18, the IL17 family (yet to be completely characterized, member cytokines have a specific effect in promoting proliferation of T lymphocytes that cause cytotoxic effects) and the chemokines.

1.9.2 CYTOKINES FUNCTIONAL CLASSIFICATION

Based on their function, type 1 cytokines enhance cytokine response (IFN γ, TGF β etc.) whereas type II cytokines favour antibody responses (IL4, IL10, IL13 etc.). Cytokines in one of these two sub-sets tend to inhibit the effect of those in the other. This has been an area of intense interest as dysregulation of this tendency is under intensive study for its possible role in the pathogenesis of autoimmune disorders.

Other groups of cytokines include interferons (IFN) and chemokines. IFN α and IFN β inhibit viral replication in infected cells, where as IFN γ also stimulates APCs. Chemokines attract leukocytes to the sites of infection.
Some cytokines are predominantly inhibitory. For example, IL10 and IL13 inhibit inflammatory cytokine production by the macrophages.

**1.9.3 CYTOKINE PRODUCTION PATTERN IN LOWER GENITAL TRACT NEOPLASIA**

Cytokines that modulate immunological control have long been speculated to be critical in regulating tumour growth. The type I cytokines IL2 and IFN γ are immunostimulatory and capable of limiting tumour growth. The type 2 cytokines IL4 and IL10 are immunoinhibitory and capable of stimulating tumour growth. In a study which looked into production of cytokines by peripheral blood mononuclear cells (PBMCs) in women with CIN, associated with localised or extensively spread HPV infection, thirty women with CIN and ten age and sex matched healthy control subjects were recruited. IL-2 production by PBMCs in response to stimulation with soluble antigen or HLA all antigens was reduced in the group with extensive disease in comparison to the group with localised disease or healthy control subjects. With extensive HPV infection, the production of immunoinhibitory cytokines IL4 and IL10 was at its highest with a pronounced shift from type 1 immunostimulatory to type 2 immunoinhibitory cytokine production [Clerici et al., 1997].

**1.10 T LYMPHOCYTES**

T lymphocytes play a vital role in the cell-mediated immunity. The presence of T cell receptor on their cell surface differentiates them from other lymphocytes. T lymphocytes are categorised into different subsets based on their function. The major subsets of T lymphocytes are:

- **Helper T lymphocytes** (effector T lymphocytes or Th cells)
- **Cytotoxic T lymphocytes** (Tc or CTLs)
- **Regulatory T lymphocytes** (T regs)
1.10.1 HELPER T LYMPHOCYTES (effector T lymphocytes or Th cells)

These cells arise in the thymus and have two important functions; stimulation of cellular immunity and inflammation, and stimulation of B-lymphocytes to produce antibodies. When Th lymphocytes are presented with both antigen and appropriate cytokines, they begin to proliferate and become activated. It is the nature of stimulation that determines which path they enter, leading to Th1 or Th2 pathway. Th1 and Th2 cytokines are antagonistic in activity. The equilibrium between Th1 and Th2 activity may guide the immune response in the direction of cellular or humoral immunity. T lymphocytes are initially activated as Th 0 cells, which produce IL2, IL4 and IFNγ. The nearby cytokine environment then influences their differentiation into Th1 or Th2 cells. Th1 cells produce IL2, IFNγ and TNFβ, which activate CTLs and macrophages to stimulate cellular immunity and inflammation. Th1 cells also secrete IL-3 and GM-CSF (granulocyte macrophage-colony stimulating factor) to stimulate the bone marrow to produce more leukocytes. Th2 cells in turn secrete IL4, IL5, IL6 and IL10, which stimulates antibody production by the B-lymphocytes (Microvet).

Specific CD4+T lymphocyte help is crucial for the development of cellular effector and humoral mechanisms against viral infections. For progressive HPV induced neoplasia, impaired responses against the viral antigens will result in unfavourable disease prognosis.

1.10.2 CYTOTOXIC T LYMPHOCYTES

A number of different cells in the immune system possess cytolytic activity against a broad range of cells. The most thoroughly studied of these cells are the cytotoxic lymphocytes. Cytotoxic large granular lymphocytes that spontaneously lyse certain tumour cells are called natural killer cells, important mediators of the first line defence mechanism, the innate immunity. These cells are unique in that no previous sensitisation is required for them to kill. Another type of killer cells are the lymphokine activated killer cells which
are known to lyse any type of cell and such cells are used in cancer therapy. Mechanism of action of these cells is however not yet very well understood.

The last group of cytotoxic cells are the cytotoxic T lymphocytes, which kill infected, damaged or dysfunctional cells in an antigen-specific fashion and are believed to be essential for the elimination of virally or oncogenically altered cells. Although cytotoxic T lymphocytes are capable of lysing a wide range of target cells, an individual cytotoxic T lymphocyte is capable of lysing only those target cells that carry the appropriate antigen and therefore require prior sensitisation in order to function. These cells are thought to mediate graft rejection, mount responses against viral infections and intracellular bacterial infections, and play a major role in tumour destruction.

Cytotoxic T lymphocytes are activated when cells infected with the virus or other pathogens via the antigen processing undergo degradation and release foreign proteins. Following activation, the cytotoxic T lymphocytes undergo expansion with the help of IL2, thereby enhancing the quantitative lymphocyte response to the target antigen. The primary mechanism through which the cytotoxic T lymphocytes control infected cells is by killing them. The T lymphocyte receptor on the CD8 T lymphocytes possess a glycoprotein, CD8, which recognises a specific antigenic peptide bound to the MHC class I molecules. CD8 T lymphocytes can also suppress pathogens by other means such as release of chemokines and cytokines. Characteristically, an antigen-specific cytotoxic T lymphocyte response is in three stages. Stage one involves antigen specific proliferation of the T lymphocytes, stage two is acquisition of effector functions by the T lymphocytes resulting in elimination of the target cells and stage three is contraction of effector phase into maintenance phase, in which, the surviving antigen specific T cells develop into the long-lived memory CD8+ T cell population [Kaech et al., 2002; Ahmed and Gray, 1996; Sprent and Surh, 2002; Wong and Pamer, 2003].
1.10.3 REGULATORY T LYMPHOCYTES (suppressor T lymphocytes)

These cells are a specialised subpopulation of T lymphocytes that act to suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self-antigens. These cells are potent immune regulators. Regulatory T lymphocytes come in many forms, CD4, CD25, FoxP3CD4 (CD4+CD25+ regulatory T lymphocytes or T regs), CD8 and other T lymphocytes types that have suppressive function. CD4+FoxP3CD4+ regulatory T lymphocytes (T regs) have been referred to as naturally occurring regulatory T lymphocytes to distinguish them from suppressor T lymphocyte population generated in vitro.

T regs are capable of mediating peripheral tolerance to auto antigens and regulation of T lymphocyte responses directed to foreign antigens. A proposal for the existence of two general subsets of CD4+CD25+T regulatory cells has been made; naturally occurring and induced, that not only differ in their mechanism of action but also in their origin, development and activation requirements [Piccirillo and Shevach, 2004]. FoxP3CD4 is a member of the forkhead / winged helix family of transcription factors critically involved in the development and function of CD4+CD25+ T regulatory cells and is also a key regulator of immunosuppression. It is a nuclear product and not expressed in the cell cytoplasm or on the cell surface. Expression of this single transcription factor causes a developmental switch in naïve T lymphocytes to a suppressor cell phenotype, more commonly referred to as regulatory T lymphocyte. FoxP3CD4 expression was previously thought to be restricted to T lymphocytes. The discovery of FoxP3CD4 as a specific marker for T regulatory cells led to an outburst of research on the biological activity of regulatory cells.

The immunosuppressive cytokines TGF β and IL10 have also been implicated in enhancing the regulatory T lymphocyte function. A large number of FoxP3CD4 expressing T regulatory cells are found within the MHC class II restricted CD4 expressing CD4+ helper T lymphocyte population and express
high levels of IL 2 receptor alpha chain (CD25). In addition to the FoxP3CD4 expressing CD4+CD25+, there also appears to be a minor population of MHC class I CD8+ FoxP3CD4 expressing regulatory T lymphocytes. Prior to the identification of FoxP3CD4, these two cell surface markers CD4 and CD25 were used to identify and define this population and thus these cells are often referred to as CD4+CD25+ regulatory T lymphocytes. As defined by CD4+ and CD25+ expression, regulatory T lymphocytes comprise about 1-2% of CD4+ helper T lymphocytes in humans. T regulatory cells prevent migration of effector immunocytes to the target organs and inhibit their cooperation with APCs.

1.11 THE ROLE OF REGULATORY T LYMPHOCYTES IN IMMUNOSUPPRESSION, INFECTION & CANCER

Regulatory T lymphocytes having CD4+ CD25+ FoxP3CD4+ (T regs) or CD4+IL10+(Tr1) phenotype are found to be capable of inducing anergy toward self and allo-antigens thereby playing an important role in autoimmunity, tolerance of allografts, pregnancy and in the development of cancer. In human cells, alteration in the number of T regulatory cells, in particular, those expressing FoxP3CD4 has led to a variety of conditions. Humans with mutations in FoxP3CD4 gene suffer from a severe and rapidly fatal autoimmune disorder known as Immune dysregulation, polyendocrinopathy, enteropath X-linked syndrome (IPEX).

T regulatory cells are shown to make up for a large fraction of CD4+ T lymphocytes in the normal human skin [Hirahara et al., 2006;Sather et al., 2007]. Skin is a complex immunological organ subjected to constant exposure to microbial flora and antigens. In cases of deregulated immunity in the skin, many common inflammatory and allergic disorders could arise. Recent studies have confirmed FoxP3CD4 expression in the tumour cells indicating malignant transformation of healthy cells can induce FoxP3CD4 expression [Ebert and Groh, 2008]. Tumours induce or recruit T regs to disable immune priming and hinder the function of antitumour effectors.
1.12 T LYMPHOCYTES IN VIN

Alteration in the local immune environment is thought to play a role in the development of pre-malignant vulval lesions and persistent disease in chronic conditions like VIN is thought to be due to non-functional T lymphocytes [Tindle, 2002]. In the vulva, CD8 T lymphocytes are shown to be predominant in the epidermis, whilst in the dermis, both the CD4 and CD8 T lymphocytes are equally distributed [Abbas and Janeway, Jr., 2000]. Gul et al have suggested that increase in CD8 T lymphocyte count is the initial local immune response in low grade VIN and that this protective cytotoxic CD8 lymphocyte activity is lost as VIN progresses. It has also been speculated that CD8+ T lymphocytes present in high grade VIN, especially in non-responders could be functionally inactive [Gul et al., 2004]. Similarly, when prognostic significance of tumour infiltrating lymphocytes (TIL) in gynaecological and other cancers were evaluated, CD8+ T lymphocyte infiltration was associated with better prognosis [Curiel et al., 2004;Piersma et al., 2008;Wolf et al., 2007]. Other studies have suggested that inadequate generation of tumour specific CD4 T lymphocytes in the VIN lesions can result in an inability to reject a tumour by the host [Greenberg, 1991]. Studies on advanced cancer however have demonstrated that a decrease in the ratio of CD4:CD8 in the tumour infiltrating lymphocytes is a very likely indicator of disease progression [Igarashi et al., 1992;Sheu et al., 2001].

In high-grade VIN, even when cytotoxic T lymphocytes are generated, T regulatory cells with immunosuppressive properties are found to dominate the lesion environment and counteract the beneficial defence response of the cytotoxic T lymphocytes [Kobayashi et al., 2004;Stanley, 2008a].

In a study of thirty-eight women with HPV 16 positive high-grade squamous intraepithelial lesions of the lower genital tract, HPV 16 specific T lymphocyte responses were absent in the circulation of the majority (nearly 60%) of the patients. Any obvious HPV 16 specific T lymphocyte activity was in patients with persistent or recurrent high-grade disease, however, in the majority, the T lymphocyte response was not associated with secretion of pro-inflammatory
cytokines resulting in lack of cellular immunity [de Vos van Steenwijk PJ et al., 2008]. In fact, HPV derived lesions were found to be having significantly higher number of infiltrating lymphocytes and T regs in comparison to other tumours like colon cancer, melanoma of the skin and bronchial carcinoma [Loddenkemper et al., 2009].

In a study of fifty-one women with active high-grade VIN by van Seters et al tissue samples obtained from the VIN lesions were compared with vulval samples obtained from nineteen healthy controls for HPV DNA, T lymphocytes and various other immune markers. In the epidermis of the VIN tissue sample, the cytotoxic CD8+T lymphocytes were significantly lower than in the control group. No significant changes were observed for the helper CD4+ T lymphocytes or the regulatory T lymphocytes between both the groups. In the dermis, however, of VIN patients, elevated number of both helper and cytotoxic T lymphocytes were present in the VIN group in comparison to the control group. In summary, high risk HPV associated usual or undifferentiated type of VIN lesions are characterised by an immunosuppressive state in the epidermis. In the dermis, however, inflammatory activity is reflected by the influx of helper and cytotoxic T lymphocytes suggesting that the cellular immune response to viral HPV infection occurs in the dermis of VIN patients [van Seters et al., 2008a].

1.13 TREATMENT OPTIONS FOR VIN
Current treatment for ano-genital neoplasia fall into five main categories, surgical excision, laser ablation, cytotoxic agents, photodynamic therapy and immunomodulators. The latter three treatment options are currently experimental.

1.13.1 SURGICAL EXCISION AND LASER ABLATION
The traditional treatment options for treatment of VIN and in fact most HPV related disease rely primarily on surgical excision or laser ablation of the lesions. Surgical excision and laser ablation although have been the standard and traditional methods of treatment, both these options are unpopular with
patients and physicians alike. Consequently and not uncommonly, a number of women diagnosed with VIN do not undergo any treatment and opt for the policy of conservative (wait and watch) management with regular clinical follow-up. Surgical treatment for VIN is usually reserved for symptom control or to rule out malignant transformation.

The basis of surgical treatment is complete elimination of the lesion. Excisional surgery is more suitable for small, unifocal lesions, however results in scarring, positive margins if incompletely excised and therefore a risk of recurrence. Routine vulvectomy is no longer considered to be an acceptable treatment option for VIN. On the other hand, multiple excisions for multifocal disease can result in significant disfigurement of the vulva. Although methods have evolved for rebuilding the defects associated with extensive vulval surgery, surgical mutilation of the vulva is best avoided if possible. For VIN affecting the clitoris, treatment in the form of partial or total clitoral amputation affects quality of life and sexual function. Although some recent studies have advocated skinning clitorectomy with either a thin skin flap or split-thickness skin graft as a sound organ-sparing alternative for clitoral amputation in the treatment of clitoral VIN [Terlou et al., 2009]. Skinning vulvectomy in place of surgical excision has also been recommended for high grade VIN and in a small study of twenty one women with high-grade VIN who underwent this procedure with minimal post operative complications and good cosmetic outcome, the treatment was considered successful with no disease recurrences [Ayhan et al., 1998]. Although such surgical advances show promise, are technically demanding and require surgical expertise with multidisciplinary involvement.

Laser treatment of VIN lesions in the form of carbon di oxide (CO2) surgical laser ablation can be easily and precisely performed in an outpatient clinical setting with good functional and cosmetic outcome. Following laser ablation however the area takes a long time to heal and does not provide a tissue sample for histo-pathological analysis. Laser ablation treatment is more suitable for superficial lesions in the non-hair bearing areas of the vulva due to the limited depth of CO2 laser beam penetration.
1.13.1.1 RECURRENCE OF VIN

Both surgical excision and laser ablation are associated with high rates of disease recurrence. In a study of one hundred and thirty three women diagnosed with VIN who were followed up for a period of fifteen years, by Herod JJ et al histological or symptom recurrence occurred in 48% of women within four years of treatment. The rate of recurrence was significantly higher following laser ablation (75%) compared to following surgical excision (45%) [Herod et al., 1996]. In a retrospective study by Ahr et al of sixty-eight women diagnosed with VIN 3, the aim of which was to study the risk factors for recurrence of VIN, by univariate analysis, HPV positivity and immunosuppression due to positive HIV status correlated with VIN recurrence. In addition, a resection margin of less than five millimetres (mm) was also significantly associated with disease recurrence. On multivariate analyses HPV positivity, immunosuppression due to positive HIV status and resection margins less than five mm were all found to be independent risk factors for recurrence of VIN. No significant association however was noted with age of the woman or multifocality of the disease [Ahr et al., 2006]. Jones et al in their study of natural history of VIN in four hundred and five cases of high grade VIN from 1962 to 2003 observed that with both surgical excision and laser ablation treatment options, recurrence rate was high with no less than half the treated group needing at least one further treatment, more so in those cases where the surgical excision margins were positive [Jones et al., 2005]. Li et al. studied twenty-four women diagnosed with VIN3 between 1992 and 2002, all of who underwent surgical treatment. One-third underwent extended local excision whilst the rest underwent simple vulvectomy. Positive surgical margins correlated with disease recurrence in three women (12.5%) who developed disease recurrence [Li et al., 2005]. Herod et al. with a view to study the long-term outcome of VIN conducted a retrospective case-note study on hundred and thirty-nine women with a primary diagnosis of VIN over a fifteen-year period. 86% underwent surgical treatment, 48% of who developed disease recurrence (histological, symptom or both) within four years of undergoing primary treatment. Treatment with laser ablation was associated with much higher rate of disease recurrence at 70% [Herod et al.,
1996]. In another long-term outcome study of sixty-five women diagnosed with high grade VIN over a period of ten years from 1989 to 1999 by Sykes et al, 84% of women underwent surgical treatment, a staggering 65% of who were found to have positive surgical margins and 51% warranted further surgical treatment for persistent or recurrent disease [Sykes et al., 2002]. Similar results were noted in another retrospective study of hundred and one women diagnosed with VIN3 by McNally et al. over a period of sixteen years from 1981 to 1997, 78% of who underwent surgical excisional treatment. Over a period of a mean follow-up period of thirty-six months, 38% of women required at least one further surgical treatment for recurrent disease. Smoking, multifocality, presence of HPV and positive surgical margins although were all linked with disease recurrence, although the association was not statistically significant [McNally et al., 2002]. In a study by of sixty-five women diagnosed with high-grade VIN over a period of ten years from 1989-1999, 65% of women who underwent surgical treatment (84%) had positive surgical excision margins. Similar to the findings in other studies, 51% required further treatment [Rodolakis et al., 2003].

The recurrence rates with both surgical excision and laser ablation are probably high because these therapies target the visible lesion and the latent HPV infected keratinocytes may persist. Targeted anti-viral therapy may actually be of more value.

1.13.2 PSYCHOLOGICAL MORBIDITY ASSOCIATED WITH VIN

Surgical treatment is mutilating and even after extensive surgery, recurrences are common. Considerable emotional trauma with extensive surgery, especially in younger woman is very likely, increasing the risk of developing psychosexual disease. Surgical treatment of VIN affects quality of life [Zhang et al., 2009]. Thuesen et al in their study of selected eighteen women diagnosed with VIN who were aged sixty or less, all of whom underwent surgical excisional treatment for VIN, found that older age and more extensive vulval surgery were more likely to be associated with poorer sexual function [Thuesen et al., 1992]. Other studies have confirmed an accentuated
psychosexual morbidity associated with VIN but however, could not relate it to the extent of the surgical treatment. Shylasree et al recently reported the results of a questionnaire study of eighty-two women diagnosed with VIN, of whom forty-four were sexually active at the time of the study. The design of the questionnaire enabled quantification of demographic, psychological and disease related factors and related their effect on quality of life outcomes. In this study, duration of disease, presence of symptoms and the number of treatments did not have a measurable impact on the quality of life [Shylasree et al., 2008]. In another study of forty-three women diagnosed with VIN all of whom underwent excisional surgical treatment for VIN, neither VIN grade, location of surgical excisional scar nor time since surgical excision correlated with sexual function or quality of life [Likes et al., 2007]. Both the studies however clearly showed an increased psychological morbidity in women diagnosed with VIN compared to women with no VIN. In an attempt to assess women’s perception of vulval appearance and sexual function following surgical excision for VIN, seventy women responded to a questionnaire study by Fong et al and only 3% expressed dissatisfaction [Fong et al., 2008]. A similar study by Mc Fadden et al in eight women diagnosed with VIN scored poorly on quality of life and sexual functioning, although this did not appear to be due to depression or anxiety [McFadden et al., 2009]. Although the evidence for psychosexual consequences of HPV-related genital warts and intraepithelial lesions is limited, it is convincing.

### 1.13.3 MEDICAL MANAGEMENT OF VIN

The chronic natural history associated with difficult symptoms, unacceptable treatment options with a constant fear of disease recurrence and impending malignancy beg for effective strategies in the management of VIN. Surgical morbidity, loss of body image and sexual dysfunction associated with these procedures have clearly made the conventional treatment sub optimal and unacceptable driving the need for medical management [Likes et al., 2007; Shylasree et al., 2008]. Increasing incidence in younger women as well justifies a more conservative approach in the management of VIN. It is also certain that surgical treatment for VIN is associated with high rates of disease
recurrence. In fact, treatment for VIN is known to be associated with higher rates of recurrence in comparison to CIN or VAIN [Hillemanns et al., 2006]. Anatomical location of the disease and multifocality of the lesions probably result in incomplete surgical excision and positive excision margins contributing to a high risk of disease recurrence. On the other hand, disease recurrence does not seem to be influenced by positive surgical margins alone as extensive surgery with clear surgical margins does not necessarily appear to evade the development of recurrent VIN. Failure to eradicate the HPV infection and not the radicalism of treatment adopted appears to be contributing to persisting infection and recurrence of genital tract neoplasia.

Agents that perhaps enhance or induce strong cell mediated immune responses to HPV related disease hold promise for treatment and reduction of future recurrences [Gastrell and McConnell, 2001;Modesitt et al., 1998b]. Choosing the best therapeutic approach is however challenging.

1.13.4 INVESTIGATIONAL TREATMENT OPTIONS FOR VIN

VIN is one condition where the need to develop non-invasive, durable treatment option is pressing. In addition to various cytotoxic agents, some of which have a proven role in the management of dermatological conditions and condylomatous lesions, there is a role for toll like receptor agonists, photodynamic therapy and therapeutic HPV vaccination, either as solitary treatment or in combination.

1.13.4.1 CIDOFOVIR

Cidofovir is an acyclic nucleoside analogue with a broad-spectrum anti-viral activity against broad range of DNA viruses, including HPV. The mechanism of action is thought to be apoptosis of infected cells. Due to its antiviral and anti-proliferative activity topical cidofovir has been used in viral infections like resistant herpes simples, ano-genital condyloma and to a limited extent, in ano-genital intraepithelial neoplasia. Tristram et al. conducted a pilot study in
twelve women of whom ten were evaluable to assess the usefulness of topical cidofovir in women diagnosed with ano-genital neoplasia. Four women showed histological and viral disease clearance whereas three women made a partial clinical response [Tristram and Fiander, 2005]. Topical cidofovir may have a place in the management of VIN. Cidofovir has increasingly been advocated in the management of anogenital condylomata acuminata [Coremans and Snoeck, 2009] and further studies (unpublished) are underway evaluating its efficacy in VIN.

1.13.4.2 INDOLE-3-CARBINOL (I3C)
Indole-3-carbinol (I3C) a phytochemical (naturally derived product) derived from cruciferous vegetables has been shown to be anti-carcinogenic and anti-estrogenic. Oral treatment with I3C has been shown to increase the anti-proliferative metabolite 2-hydroxyestrone and decrease the potentially carcinogenic metabolite 16-alpha-hydroxyestrone in the serum. I3C has shown efficacy in the causing regression of CIN lesions in a small randomised controlled trial in thirty women who received oral I3C [Bell et al., 2000]. There was significant regression of CIN in women who received I3C compared to women treated with a placebo. One other study has been reported where I3C was used in twelve women with histologically proven high-grade VIN. Significant improvement in symptomatology and lesion size was noted. Histological response was however absent at six months [Naik et al., 2006].

1.13.4.3 RETINOIDS & INTERFERON
Anti-neoplastic lesions such as retinoids (RA) and interferon (IFN) have been reported to be active against a variety of tumours and HPV related lesions [Slotman et al., 1988;Vilmer et al., 1998]. Retinoids (RA) are important for regulation of cell growth, differentiation and apoptosis and have been successfully used in the treatment of various dermatological disorders. In a preliminary study of two women with high-grade VIN, following application of retinoid cream, an improvement in their symptoms and the appearance of the lesion was observed, however, biopsy of the lesion did not confirm histological disease resolution. In another small-randomised double-blind crossover trial, IFN α was compared with IFN α with nonoxyl-9 and showed 67% response in
all patients, independent of the addition of nonoyxl-9. Although the results from these studies appear promising, the efficacy cannot be determined until large preferably placebo controlled studies with more participants are conducted.

1.13.4.4 5-FLUOROURACIL

5-Fluorouracil (5-FU) is a pyrimidine analogue and although has many mechanisms of action, mainly blocks the transport of extracellular thymidine, thereby inhibiting the thymidine salvage pathway. 5-FU is available as a topical preparation but there is some hesitation in advocating this preparation for VIN due to the severe inflammation it causes. In a small case-series where topical 5-FU was administered for VIN, although a 50-60% clinical response was observed, treatment was associated with significant desquamation, inflammation and painful ulcerations and treatment was poorly tolerated [Sillman et al., 1985].

1.13.5 IMMUNOTHERAPY

In the management of any chronic condition, to enhance the cellular immunity and eradicate persistent infection, single or combination immune modulating therapies that incline the balance in favour of host effector response and away from immune regulatory and immune evasive mechanisms may be the key. The aim of immunotherapy for VIN is to enhance the natural immune response against HPV. There is a significant role for the development of immunotherapeutic modalities as treatment options for HPV associated intraepithelial neoplasia. It has been postulated that the induction of a local inflammatory environment, which stimulates immature tolerising dendritic cells to become activated dendritic cells in combination with enhanced cytotoxic T lymphocyte responses against HPV proteins, might provide a superior approach to treating established HPV infection.

HPV suppresses the expression of several proinflammatory proteins that are crucial in clearing infection and activating the cytotoxic T lymphocytes involved in killing virus-infected cells. Immunosuppression has been shown to
be a risk factor for increased incidence and progression of HPV associated lower genital tract neoplasias [Bouwes Bavinck and Berkhout, 1997; Palefsky and Holly, 2003]. Immune equilibrium permits the persistence of HPV infection due to immunoevasive nature of the HPV virus. Despite HPV's ability to inhibit host defences, however, a successful immune response to genital HPV infections is established in most cases. A certain proportion of HPV related intraepithelial neoplasia does undergo immune-mediated regression.

Immunotherapy or immunotherapies (combination treatments) that tip the balance of this equilibrium in favour of the host effector response and away from the regulatory T lymphocyte and viral evasion strategies may be the key to enhancing the cell mediated immunity required to eradicate persistent HPV infection and established disease. There has been increasing focus on immunotherapy as alternative treatment option for VIN.

In this unit, previously, solo PDT and therapeutic HPV vaccination trials were conducted when some clinical response was observed [Davidson et al., 2003a; Davidson et al., 2004; Martin-Hirsch et al., 1998]. Since, encouraging data for toll like receptor agonists like imiquimod in the management of VIN has emerged (table 1). One of the potential combination therapies that meet the criteria of ideal immunotherapy is to combine a topical immune response modifier with a therapeutic HPV vaccination. This is the approach that we propose to follow in the clinical trial discussed in this thesis. Immunotherapy, if successful, should effectively be a suitable alternative option by addressing the local lesion, causal HPV infection and all the lesions associated with HPV infection irrespective of their location, inducing long lasting immunity and thus preventing recurrences.

1.13.5.1 TOLL LIKE RECEPTORS

Toll like receptors (TLRs) are a class of surface molecular proteins that play an important role in the innate immune system. TLRs are considered to be the key molecules that alert the immune system to the presence of microbial infections. Once the microorganisms have breached the physical barrier such
as skin or mucosa, they are recognised by the TLRs that function as primary sensors for the innate immune system to recognise pathogen associated molecular patterns that are expressed on the microorganisms and mediate the production of cytokines necessary for the development of effective immunity.

Thirteen TLRs have thus far been identified in the human population (TLR1 to TLR13) [Chuang and Ulevitch, 2000; Du et al., 2000; Tabeta et al., 2004]. TLRs are very specific in their function and for most of the TLRs, ligand recognition specificity has been established. TLRs bind and become activated by different ligands, which are located on different organisms or structures. Since research has identified the TLRs, it has sparked interest in the therapeutic manipulation of the innate immune system. TLR agonists are being developed as treatment options for infectious diseases, viral infections, allergies, cancer and also as adjuvants for vaccines in various preventive and therapeutic treatment strategies. It is believed that with increasingly impressive safety record and efficacy of the TLRs, their applications are likely to increase in future [Meyer and Stockfleth, 2008].

It has been shown that HPV type 16, the most carcinogenic type among the high risk HPV interferes with the innate immunity by affecting the expression of TLR. TLR7 lies in close proximity to another family member, TLR8 on the human X chromosome. TLR7 and TLR8 agonists are known to activate dendritic cells and thereby elicit T helper 1 and CD8 positive T lymphocyte responses. Activation of TLR7 and/or TLR8 agonists is therefore a prospective therapeutic opportunity for the treatment of various viral infections. And also, this ability of the TLR7 and TLR8 agonists to elicit T helper responses has been exploited to enhance the efficacy of vaccines by including them as adjuvants in some cancer trials [Craft et al., 2005]. TLR7 specific agonists have been shown to activate plasmacytoid dendritic cells, B-lymphocytes and induce mainly IFNα and other IFN-regulated cytokines. TLR8 specific agonists have been shown to activate myeloid dendritic cells, monocytes, and monocyte derived dendritic cells leading primarily to the
production of proinflammatory cytokines, such as TNFα and IL-12 [Gorden et al., 2005].

Using TLR7 deficient mice and HEK293 cells transfected with human TLR7, Hemmi et al. demonstrated that TLR7 is involved in the recognition of the imidazoquinoline compounds imiquimod (R837) and the related compound resiquimod (R848) [Hemmi et al., 2002]. Both human TLR 7 and human TLR 8 proteins sense imidazoquinolines. Infection of the human primary keratinocytes with HPV16 E6 and E7 recombinant retroviruses inhibits TLR 9 transcription and hence the functional loss of TLR 9 regulated pathways and this effect of HPV 16 on TLR 9 was also demonstrated in vivo in cervical carcinoma patients. E6 and E7 proteins from low risk HPV type 6 were unable to down-regulate the TLR 9 promoter. On the contrary, E6 and E7 proteins of the high risk HPV 18 exhibited reduced efficiency when compared to HPV16 in inhibiting TLR 9 transcription. This is presumed to be a novel mechanism used by the HPV 16 to suppress the host immune response. Deregulation of the TLR 9 transcription provides evidence that abolishing the innate responses may be a crucial step involved in the carcinogenic events mediated by the HPV [Hasan et al., 2007]. Trials are underway to further assess the function of TLRs in various infectious and neoplastic conditions.

1.13.5.1.1 IMIDAZOQUINOLINES

Imidazoquinolines are double cyclic organic molecules that are able to activate TLRs and therefore induce immune modulatory cytokines. Imidazoquinoline compounds and derivatives are often used as the active components of antiviral and anti allergic creams [Hemmi et al., 2002;Peet et al., 1985]. The two imidazoquinoline compounds, imiquimod and resiquimod are low molecular weight immune response modifying drugs that have demonstrated effective anti-viral and anti-tumour properties.

1.13.5.1.2 IMIQUIMOD & RESIQUIMOD

The mechanism of action of imiquimod although appears to be multimodal, it mainly acts by amplifying the anti-tumour immune responses through the
TLRs 7 and 8 proteins on the dendritic cells [Hemmi et al., 2002]. Imiquimod and its homologues are shown to activate macrophages and other immune cells via binding to cell surface receptors like TLR7, thereby inducing secretion of pro-inflammatory cytokines, predominantly IFN-α, TNF and IL-12. This locally generated cytokine surge encourages a Th1 cell mediated immune response with generation of cytotoxic T cells [McInturff et al., 2005;Stanley, 2002]. Histological examination of the inflammatory cell infiltrate of different superficial malignant tumours when treated with the imiquimod cream revealed the prevalence of T helper lymphocytes with significant infiltration of CD8 + cytotoxic lymphocytes [Wolf et al., 2007].

Natural killer (NK) cells are notorious for their preferential ability to lyse virally infected cells and spare uninfected cells. People deficient in NK cells are known to suffer from recurrent viral infections [Biron et al., 1989;Orange, 2002]. Hart et al following their in vitro studies on NK leukaemia cell lines, discuss the role for TLR agonists in the activation of NK cells and demonstrate that the relative contribution of both direct and indirect mechanisms of activation of human NK cells varies for specific TLRs. TLR3 has been shown to be directly activated on the resting NK cells, while the activation of TLR7/8 is mediated primarily through accessory mechanisms such as release of IL-12 and IFN-γ [Hart et al., 2005].

Dumitru et al in their in vivo mice studies and in vitro human studies reported enhancement of natural killer cytotoxicity by the TLR7/8 agonist and also concluded that IFN-γ dependent functions of natural killer cell populations appear essential for TLR7/8 agonist associated cancer immunotherapy. The long lasting activation of natural killer cells was determined by the sustained expression CD69, an activation marker for natural killer cells [Dumitru et al., 2009].

A novel mechanism of action of imidazoquinolines has recently been described which has important physiological relevance and allows strategies to be developed for use of these agents. In invitro cultures using pancreatic and colorectal cancer cells as well as squamous cancer cells from head and
neck, where the immune system and TLRs were eliminated, the imidazoquinolines were found to up regulate the opioid growth factor receptor OGFr. This results in retardation of cell proliferation at the G1-S phase of the cell cycle. This is novel and exciting knowledge of the mechanism of action of imidazoquinolines [Zagon et al., 2008].

Resiquimod is thought to be a more potent analogue of imiquimod. It is known to be having a mixed TLR7 and 8 activity and has been evaluated in the clinical trials for topical use in herpes simplex viral infections and hepatitis C infections with some success [Miller, 2008].

1.13.5.1.3 IMIQUIMOD IN HUMAN STUDIES
The US Food and Drug administration (FDA) first approved topical imiquimod treatment in 1997 for genital warts. Imiquimod is currently an FDA approved treatment option for HPV induced warts, actinic keratosis and superficial basal cell carcinoma (BCC). It is the first TLR agonist approved for these dermatological conditions [Miller, 2008]. Imiquimod is generically marketed as 5% Aldara cream for topical usage. To supplement the clinical applications of topical imiquimod, as a topical immune response modifier attracting the local immune markers to the site of application and inducing an immunological memory, numerous uses outside the licensed indications of warts, actinic keratosis and BCC have been proposed and practised.

1.13.5.1.4 IMIQUIMOD FOR THE TREATMENT OF VIN
Amongst the investigational medical treatment options for VIN, imiquimod is the most studied drug. The initial case-reports of imiquimod for VIN were published in the year 2000. The literature on the clinical efficacy of imiquimod in the treatment of VIN consists mainly of case reports and observational studies. Recently, two randomised trials of imiquimod for VIN have been published [Mathiesen et al., 2007;van Seters et al., 2008b].

The first publication describing the use of imiquimod in VIN was in the year 2000 by Davis et al who reported a case series of four women diagnosed with VIN3 all of whom responded to imiquimod. The maximum allowed dose was
up to three applications per week until the lesions clinically disappeared or for a maximum of sixteen weeks. Two women developed recurrence at one year follow-up [Davis et al., 2000]. In another retrospective case-note review by Diaz-Arrasti et al. eight women with recurrent high-grade vulval intraepithelial neoplasia were treated with topical imiquimod, three times per week for six to sixteen weeks. Of the seven women evaluable for treatment response, three of who were immunosuppressed (HIV positive), four women achieved a complete clinical and histological response; two women attained partial clinical response and in one woman VIN progressed to invasive disease. This group was clinically followed for a mean of thirty-three months, when two women, both of who were HIV positive developed disease recurrence. Women with recurrent VIN were again treated with imiquimod, resulting in a complete clinical response. In this group, pre and post treatment RNA levels of interferons when assessed, an increase in 2,5 oligoadenylate synthetase RNA expression was seen after therapy. This is an IFN induced enzyme activated by the double stranded RNA which is believed to lead to inhibition of protein synthesis, which may be part of the antiviral function of the interferons [Diaz-Arrastia et al., 2001].

Case-reports of individual cases of successful treatment with imiquimod of Bowenoid papulosis (VIN3) of the vulva [Petrow et al., 2001], VIN3 in a woman on immunosuppressant treatment following a lung transplant [Travis et al., 2002] and another case of high grade VIN which vulvar-pemphigus as a side effect of imiquimod treatment have been reported in the literature [Campagne et al., 2003]. Todd et al published the first prospective trial of imiquimod for VIN in 2002. They reported on an uncontrolled, observational study of fifteen women with VIN3 who underwent treatment for sixteen weeks with a thrice-weekly imiquimod cream application regime. In four out of fifteen women clinical improvement was detected and in three women, post-treatment biopsies were negative for VIN. Thrice weekly drug application was limited by the side effects of imiquimod. At five months post treatment visit, all the four women who had demonstrated a clinical response relapsed with biopsy proven recurrent VIN [Todd et al., 2002]. In 2003, van Seters et al published the results of their pilot study of topical imiquimod treatment of
fifteen women with multifocal VIN2 or 3. In this study the length of treatment varied between six and thirty-four weeks. Based on the complete response defined as complete disappearance of all the visible lesions and a partial response as 25% reduction in the lesion size criteria, four women (27%) made a complete response and nine women (60%) made a partial response to treatment. Eleven women (85%) benefited from symptom relief. Two out of fifteen women discontinued imiquimod treatment because of severe side effects associated with the imiquimod treatment [van Seters et al., 2008b].

In 2002 Jayne et al published a retrospective review of thirteen women with VIN 2 or 3 treated with imiquimod. They reported eight (62%) complete responses and four partial responses (31%); in this study partial response was defined as at least 75% reduction in the lesion size. Two of the partial responders were found to have invasive disease in their residual lesions. This group therefore concluded that any residual disease three months after completion of imiquimod trial treatment should be surgically excised and the excision sample subjected to histopathological analysis [Jayne and Kaufman, 2002]. In 2004 Wendling et al published a prospective uncontrolled study of twelve women with VIN treated with imiquimod for up to seven months. They accounted three histologically confirmed complete responses and four partial responses, defined as at least a 50% reduction in the lesion size. Three out of twelve women withdrew because of the severe side effects associated with imiquimod cream [Wendling et al., 2004]. More recently Le et al reported a prospective uncontrolled study of imiquimod for VIN2 and 3. Twenty-three women were recruited and responses were evaluable in seventeen of them. The duration of treatment was for sixteen weeks. Complete response defined as VIN1 or less on histology was reported in nine women (53%) and partial response in five women (29%) at completion of treatment with no available long-term follow-up data [Le et al., 2007]. Mathiesen et al. in 2007 used a prospective, randomised, double blind, placebo controlled study model of topical imiquimod treatment for VIN. Twenty-one and ten women were randomised to the treatment and the placebo arms respectively. Treatment entailed thrice weekly imiquimod applications for sixteen weeks. 81% of women in the treatment group demonstrated a complete response and 10%
showed a partial response to treatment compared to no responses in the placebo group. All the responses were evaluated by histo-pathological analysis of biopsies taken two months after completion of trial treatment. Long-term results are not available for this trial, which has demonstrated an overall excellent response to imiquimod treatment [Mathiesen et al., 2007]. van Seters et al. conducted a randomised, double blind placebo controlled trial for VIN with imiquimod in fifty-two patients. Treatment regime comprised twice-weekly imiquimod applications for sixteen weeks. 81% of women showed a ≥ 25% response to treatment in the imiquimod arm of the trial whereas none in the placebo arm of the trial showed a response. On further analysis, the rates of histological regression of VIN, HPV clearance and density of intralesional immune cell infiltrates (CD4, CD8) was significantly different in the imiquimod group when compared to the placebo group. A significant improvement in the symptoms was also seen in the imiquimod group. Twelve month follow-up data is available when one in the imiquimod arm and two women in the placebo arm were diagnosed with invasive disease [van Seters et al., 2008b]. Iavazzo et al published a review article assessing the safety and effectiveness of 5% imiquimod cream in the treatment of VIN and VAIN. Drawing a conclusion from the seventeen identified articles, (one randomised controlled trial, ten case series, six case reports), complete lesion regression was observed in 26% to 100% of patients, partial lesion regression in 0% to 60% of patients and disease recurrence was confirmed in 0% to 37% of patients. The most common side effects remarked were local burning and soreness, but not severe enough for patients to warrant treatment discontinuation [Iavazzo et al., 2008]. There is thus abundant clear evidence in the literature for imiquimod treatment of VIN.

Making direct comparison between different studies is complex due to the inconsistency in the definition of ‘treatment response’. Frequency of imiquimod cream application varied from once a week to up to three times week and the total treatment duration also varied from eight weeks to six months in different studies. In most trials, with the background knowledge of the side effects associated with imiquimod use, to improve treatment
compliance, a dose-escalating regime was practised, whereby; application frequency is gradually increased from once a week to a maximum of three times a week over a three-week period. In spite of the wide variation in the reported response rates (27%-82%) between the published studies with lack of follow-up data for most studies, imiquimod as a treatment option for VIN is emerging.

In terms of cost of topical imiquimod cream for treatment of VIN, it is a more economical treatment option compared to surgical treatment options of surgical excision or laser ablation. Imiquimod is marketed as Aldara cream in 5gm sachets, is easily available in most hospital pharmacies due to its already FDA approved licensing status and the cost of imiquimod 5% cream when applied three times a week over an eight week period amounts to less than two hundred Great British pounds (imiquimod summary sheet). Imiquimod cream is also suitable for self-application and the treatment does not warrant hospitalisation at any point. Compliance is however an issue with imiquimod treatment due to treatment related side effects.

1.13.5.1.5 SIDE EFFECTS ASSOCIATED WITH IMIQUIMOD

Topical imiquimod application stimulates local immunity and induces the release of interferon α and IL-1, resulting in a pseudo-influenza like syndrome. Most patients experience localised oedema, erythema, skin peeling, ulceration, crusting consequently resulting in soreness and burning. Some patients also experience systemic side effects such as flu like illness, fever, lethargy and feeling weary. The side effects are known to subside within a short period of discontinuation of the drug. Interestingly, in immunocompetent patients, the intensity of local reaction to topical imiquimod treatment has been found to be directly proportional to the degree of clinical response when imiquimod was used for the treatment of FDA approved conditions such as actinic keratosis, warts and basal cell carcinoma. In the literature, there is one adverse event reported in a sixty-year-old woman who developed multiple
pemphigus like lesions during imiquimod treatment for BCC. Systemic absorption of the drug and/or enhanced cytokine synthesis was thought to be the likely reason for this type of reaction. To avoid such a reaction, the authors recommend using low doses of imiquimod when treating large or multiple lesions [Bauza et al., 2009].
| STUDY                                                                 | HISTOLOGY AT Wk 0    | No: of patients | LENGTH OF TREATMENT | SIDE-EFFECTS                      | RESULTS | FOLLOW-UP                      |
|----------------------------------------------------------------------|---------------------|-----------------|---------------------|-----------------------------------|---------|-------------------------------|
| Retrospective case-series [Diaz-Arrastia et al., 2001]               | High-grade VIN      | N=8             | 6-16 weeks          | No VIN – 2 PR - 1                 |         | 22 to 42 months; 1 recurrence at 15 months |
| Case-report [Petrow et al., 2001]                                    | Bowenoid papulosis  | N=1             | 8 weeks             | No VIN                           |         | 18 months                      |
| Prospective uncontrolled [Todd et al., 2002]                         | VIN3                | N=15            | 16 weeks            | 11 reduced freq of application   | No VIN – 3 PR - 1 | 1 Month; 3 recurrences       |
| Case-series [Davis et al., 2000]                                     | VIN3                | N=4             | 16 weeks            | No VIN - 4                       |         | 12 months; 2 recurrences      |
| Retrospective case-series [Jayne and Kaufman, 2002]                  | VIN2/3              | N=13            | Mean 33 months      | 1 withdrawn due to side-effects  | 8 – CR 4 PR | 55 months                     |
| Prospective uncontrolled [Travis et al., 2002]                       | VIN3                | N=1             | 8 months            |                                   |         |                               |
| Study Type                              | VIN Category | N  | Duration | Co-Morbidities | Follow-up |
|----------------------------------------|--------------|----|----------|----------------|-----------|
| Case-report [Campagne et al., 2003]    | High-grade VIN | 1  | 8 weeks  | Vulval pemphigus | 18 months |
| Prospective uncontrolled [Buck and Guth, 2003] | VAIN 2/3 | 42 | 1 week to 6 months |  |
| Prospective uncontrolled [Wendling et al., 2004] | Undiff VIN | 12 | Up to 7 months mean 5 months | 3 withdrawals due to SE | 16 months |
| Prospective uncontrolled [Marchitelli et al., 2004] | Bowenoid basaloid VIN2/3 | 8  | Up to 16 weeks |  |
| Retrospective case review [Haidopoulos et al., 2005] | VAIN 2/3 | 7  | 8 weeks  |  |
| Prospective uncontrolled [van Seters et al., 2002] | VIN2/3 | 15 | 6-34 months | 2 withdrawals due to SE | 4-CR 9-PR |
| Prospective uncontrolled [Le et al., 2005] | VIN2/3 | 17 | 16 weeks | 8 dose | 9 CR 5- |
| Study Description                                                                 | VIN   | N  | Duration | PR                  |
|----------------------------------------------------------------------------------|-------|-----|----------|---------------------|
| Prospective uncontrolled [Le et al., 2007]                                       | VIN3  | 33  | 16 weeks |                     |
| Randomised double-blind placebo controlled trial [Mathiesen et al., 2007]        | VIN2/3| 31  | 16 weeks | 81% histology response | 12 months |
| Randomised double-blind placebo controlled [van Seters et al., 2008b]            | VIN2/3| 52  | 16 weeks | 81% ≥ 25% reduction in lesion size | Median 12 months |

Table 1: Studies of imiquimod treatment for vulval intraepithelial neoplasia. PR – partial response; CR – complete response; SE – side-effects.
1.13.6 PHOTODYNAMIC THERAPY

Photodynamic therapy (PDT) is based on the principle of combination of light at a certain wavelength with the photosensitising drugs with the intention of damaging the tumour tissue. In response to the tissue damage and cell death induced by the PDT, acute inflammation results by various mechanisms. The APCs that produce IL12, crucial for the release of IFN secreting effector T lymphocytes, are activated. The resulting effects on the cytokine expression incorporate an increase in IL-6 production and a decrease in IL-10 production. In addition to the enhanced invasion and infiltration of the tumour by the leukocytes and expression of heat shock proteins, which aid in protein-to-protein interactions. Singlet oxygen (Reactive oxygen species-ROS), are generated after the activation of the photosensitiser with the PDT light at an appropriate wavelength. The singlet oxygen released is thought to either modify the cellular function or induce cell death by necrosis or apoptosis [Banchereau and Steinman, 1998;Canti et al., 2002;Castano et al., 2006;Manetti et al., 1993;Matzinger, 2002a;Matzinger, 2002b].

1.13.6.1 THE SCIENCE OF PDT

PDT involves the preferential accumulation of the photosensitiser in the target tissue with precise illumination. The light penetrates the tissue and causes excitation of the photosensitiser. Activation of the photosensitisers is a prerequisite to successful PDT. A particular wavelength of light is needed for each photosensitiser to maximise penetration through the tumour and cause excitation of the photosensitiser.

Photosensitisers have a stable electronic configuration, which is in a singlet stage in their lowest or ground state energy level. Following absorption of a photon of light of specific wavelength, a molecule is promoted to an excited state, which is also a singlet state, and which is short lived. Photosensitiser returns to the ground state by emitting a photon (fluorescence) or by internal conversion with energy loss as heat.
There are two mechanisms by which the triple state photosensitiser can react with bio molecules. These are known as type 1 and type 2 reactions. Type 1 reaction involves electron / hydrogen transfer directly from the photosensitiser, producing ions / electrons from the substrate molecules to form free radicals. These radicals then react rapidly usually with oxygen resulting in production of highly reactive oxygen species (the super oxide and peroxide anions). These radicals thus released attack cellular targets.

Type 2 reactions produce the electronically excited and highly reactive sate of oxygen known as singlet oxygen. Direct interaction of the excited triple state photosensitiser with molecular oxygen (which unusually has a triplet ground state) results in the photosensitiser returning to its singlet ground state and the formation of singlet oxygen.

In PDT, it is difficult to distinguish between the two reaction mechanisms. It is possible that the mechanism of damage is dependent on both the oxygen tension and the concentration of the photosensitiser involving both the type 1 and type 2 reaction mechanisms. PDT is thought to achieve cytotoxic effects through photo damage to sub-cellular organelles and bio molecules.

1.13.6.2 PHOTODYNAMIC THERAPY FOR VIN
The distinctive properties of PDT make it an attractive treatment option for superficial epithelial disorders including VIN. Martin-Hirsch et al. in 1998 first reported a study of eighteen women with high grade VIN treated with PDT with 5- amino-laevulnic acid (5-ALA) as the photosensitiser. In the first ten women who received 50J/cm2 of PDT, two clinical responses were observed. In an attempt to improve the clinical responses, the PDT dose was increased to 100J/cm2 in the subsequent eight recruits, of whom three women (37%) showed a clinical response. Overall, 89% of women reported symptom relief [Martin-Hirsch et al., 1998]. Hillemanns et al. in 2000 reported a study of twenty-five women (total one hundred and eleven lesions) diagnosed with VIN of different grades. 5-ALA was used as the photosensitising agent and 100J/cm2 of laser light PDT was used. In this study, complete clinical
response was seen in 52% of women. Pigmented and hyperkeratotic lesions were found to be associated with a poorer response to PDT. Treatment was poorly tolerated under local anaesthesia warranting general or regional anaesthesia in the majority (80%). This group reported excellent cosmetic outcome with minimal side effects and tissue destruction with PDT [Hillemanns et al., 2000]. Kurwa et al. in 2000, in a study of six women with high grade VIN resistant to standard surgical treatment reported a poor response to single episode ALA PDT [Kurwa et al., 2000]. Fehr et al. in 2001 reported a complete response rate of 73% in fifteen women diagnosed with high grade VIN who underwent treatment with 120J/cm2 laser light PDT. Unexpectedly the procedure was well tolerated in an outpatient setting and only three disease recurrences were observed over a twelve-month follow-up period [Fehr et al., 2001]. Campbell et al. in 2004 evaluated the role of systemic photosensitiser Foscan, as a substitute to topical photosensitiser in six women with high grade VIN with some success. As systemic photosensitisers warrant a much shorter light exposure, it is sited as an advantage over PDT using topical ALA. The drawback however with systemic photosensitiser is the prolonged photosensitivity lasting for up to one to two weeks after treatment completion requiring a strict light protection regime [Campbell et al., 2004]. Olejek et al. in 2008 who conducted a PDT trial with topical ALA in twenty women diagnosed with VIN reported a higher reduction in subjective symptoms in comparison to histological improvement following PDT [Olejek et al., 2008].

Abdel-Hady et al. considered exploration of immunological and viral factors associated with responsiveness of VIN to PDT. Treatment response was found to be associated with a post-treatment surge in the intra lesional cytotoxic T lymphocyte density and this was significantly higher in the clinical responders compared to the non-responders. None of the women who demonstrated HLA class I down regulation responded to PDT treatment and in fact in 50% of them VIN progressed to invasive disease [Abdel-Hady et al., 2001].
1.13.7 THERAPEUTIC HPV VACCINATION

Therapeutic vaccination programmes independently or in combination with other treatment modalities are expected to have the potential to prevent progression of low-grade disease to high-grade disease, regress the existing lesions, prevent the spread of metastatic cancer and also prevent recurrences following treatment [Chu, 2003; Stanley, 2003].

Understanding HPV biology has helped researchers to use the virus as a model for the development of not only prophylactic vaccines, but also therapeutic vaccines. The preventative HPV vaccines rely upon generation of the neutralising antibodies, which prevent or neutralise the biologic activity of the virus. On the other hand therapeutic HPV vaccines aim to eliminate the HPV infection by inducing a virus-specific cellular immune response. The close association of VIN with high-risk HPV such as the oncogenic HPV types 16 and 18 provides a role for therapeutic HPV vaccines in the management of VIN. Therapeutic HPV vaccines if successful, are expected to offer women already diagnosed with pre-invasive HPV lesions, who obviously have by now acquired the HPV infection, a therapeutic response of the lesions to the vaccination and expectantly this applies to more advanced disease also. Therapeutic vaccines also represent an attractive treatment alternative for many pre-invasive and invasive diseases primarily because of their ability and specificity to generate immunologic memory, which is important for controlling recurrences. The key objectives with therapeutic vaccination programme are controlling the progress of established disease and prevention of disease recurrence.

1.13.8 DEVELOPMENT OF THERAPEUTIC HPV VACCINES

The advantages of HPV as a model system stems from the fact that a limited number of proteins are encoded by the HPV genome that can be potentially targeted by the vaccines, and also for the reason that the expression of certain viral proteins is restricted during different stages of the HPV infection.
Immunologically, in an outline, the role of therapeutic HPV vaccines is to generate cell mediated immunity against HPV infected cells that express early viral proteins such as E6 and E7, reverse the immune suppressive microenvironment of the local HPV infection and allow the cytotoxic killer T lymphocytes to access the infected, neoplastic cells. The efficacy of therapeutic vaccines may thus depend on formulations that not only generate effective immune responses but also overcome the systemic and local immune evasion mechanisms adopted by the HPV infection and the progressing infection. Development of a therapeutic HPV vaccine can therefore be technically challenging.

In the design of therapeutic vaccines, the choice of target antigen(s) is a very important consideration. HPV vaccines directed against tumour specific antigens of the HPV, in general, focus on the E6 and E7 proteins; the main oncogenes necessary for the growth and progression of HPV infected cells. E6 and E7 oncogenes are particularly appealing in the development of therapeutic HPV vaccines not only because of their functional role in the neoplastic process but also because both these proteins are co-expressed in HPV lesions. Natural immune responses to both these antigens, although limited, have been identified in the presence of HPV positive lesions.

1.13.8.1 THERAPEUTIC HPV VACCINES
The development of therapeutic HPV vaccines has been brisk in the last two decades. A variety of therapeutic HPV vaccines have been tested in clinical studies. Therapeutic HPV vaccines are currently administered under close observation as investigations products and are not as yet approved or licensed for regular clinical use.

An ideal therapeutic HPV vaccine, in addition to being safe and effective, must also possess numerous other properties. It must be multivalent, offer long lasting protection, preferably not require booster immunisations, and be cheap and easily available.
Therapeutic HPV vaccines thus far have included fusion proteins used alone or with the addition of an adjuvant, encapsulated polynucleotides, protein with adjuvant, recombinant viruses, DNA constructs, dendritic cells and chimeric virus like particle (VLP) constructs as shown in the table 2. Some of these vaccines have been tested in healthy individuals, and some in a spectrum of HPV associated disease cohorts like patients with lower genital tract neoplasia (CIN, VAIN, VIN, AIN) or end-stage cervical cancer. Early clinical trials were conducted in patients with late-stage HPV 16 associated cervical cancer. Despite obtaining positive pre-clinical data, evidence of benefit measured in clinical and immunological terms, in women with late stage cervical cancer was limited. It was then concluded that women in late stages of cervical cancer, however, could anyhow be immunocompromised due to the terminal cancer and also due to the treatment received for the cancer [Borysiewicz et al., 1996; Ferrara et al., 2003; Frazer, 2004; Kenter et al., 2008; van Driel et al., 1999]. In addition, the fact that end stage cervical cancers commonly have mutations in genes involved in antigen processing and presentation was thought to be interfering with development of an immune response to therapeutic HPV vaccination [Brady et al., 2000; Evans et al., 2001]. More recently, the therapeutic HPV vaccination studies have been conducted in patients with pre-invasive HPV associated conditions as shown in the table 2.

1.13.8.2 PRE-CLINICAL DATA

Studies of canine oral papillomavirus infections, which are usually benign and self-limiting have shown that immune responses to two early viral proteins, E2 and E6 correlate with lesion regression [Jain et al., 2006]. Rabbit oral papillomavirus infections and cottontail rabbit papillomavirus infections are self-limiting in most strains. Chronic infection, however, can lead to localised skin cancers which have been shown to respond to immunotherapy with therapeutic HPV DNA vaccination [Brandsma et al., 2007]. Similarly, several HPV positive murine tumours have shown susceptibility to a range of HPV antigen specific vaccinations [Frazer, 2004; Kanodia et al., 2008].
The following types of therapeutic HPV vaccines have been evaluated successfully in clinical studies in humans.

1.13.9 TYPES OF THERAPEUTIC HPV VACCINES

1.13.9.1 TA-HPV
TA-HPV is a recombinant vaccinia virus expressing HPV 16 and 18 E6 and E7 proteins. This vaccine was studied in eighteen women diagnosed with HPV 16 positive high-grade VIN. A single dose of the vaccine was administered intramuscular (i.m). Overall, post vaccination, thirteen women demonstrated HPV 16 specific systemic immune responses. Partial clinical response was observed in eight out of thirteen women (62%) and reduction of HPV viral load in six out of eight women who made a clinical response and six out of ten women who did not make a clinical response. Women who made a response to the treatment were found to have significantly higher levels of lesion associated helper, cytotoxic and CD1a immune cells in comparison to treatment non-responders. This group hypothesized that enhanced local immune infiltration may play a critical role in the potential responsiveness to therapeutic vaccine therapy in HPV associated neoplasia [Davidson et al., 2003a].

1.13.9.2 ZYC-101a
ZYC-101a is a DNA vaccine synthesised from some proteins containing cytotoxic T lymphocyte epitopes against E6 and E7 oncoproteins of HPV types 16 and 18. This vaccine was tested in a multicentre randomised double-blind placebo controlled trial in women with confirmed high-grade CIN. Women were randomised to either a placebo or 100mcg dosage of ZYC-101a or 200-microgram dosage of ZYC-101a. The aim of the study was to histologically confirm resolution of CIN on a cone biopsy of the cervix performed six months after the scheduled three-i.m doses of the vaccine. Of the one hundred and twenty-seven evaluable study participants, the proportion of women whose CIN resolved on histological analysis was higher in the vaccine group, not influenced by the dose of the vaccine, compared to
the placebo group (43% vs. 27%), however, this was not statistically significant. Interestingly, in the vaccination arm of the trial, women under the age of twenty-five years were found to have a greater lesion resolution than the older counterparts. ZYC101a vaccine was well tolerated by the patients and its activity was not restricted to HPV 16 or HPV 18 positive lesions and cross-protection against other HPV types was also observed [Garcia et al., 2004].

1.13.9.3 TA-CIN

TA-CIN is a protein-based vaccine, developed by Xenova. It is a single fusion protein comprising HPV 16 L2, E6 and E7 proteins. The choice of these antigens has been based on therapeutic vaccination studies in animal models. van der Burg et al. in 2000 studied the effect of then a newly designed TA-CIN vaccine in a pre-clinical mouse model. TA-CIN was shown to elicit HPV 16 specific cytotoxic T lymphocytes, helper T lymphocytes and antibodies in the mouse HPV16 TC-1 tumour xenograft model. Proof-of-principle experiments demonstrated that treatment with TA-CIN prevented tumour outgrowth and also extended symptom-free survival in mice. In fact, one vaccination with TA-CIN on the same day of challenge with TC-1 tumour cells protected mice against tumour growth demonstrating the capacity of TA-CIN in inducing effective anti-tumour immunity [van der Burg et al., 2001]. More recent primate in vivo data have shown that administration of HPV 16 L2, E6 and E7 fusion protein can effectively neutralise not only HPV16 but also other high-risk HPV genotypes including HPV types 6, 11, 18, 31, 45 and 58. Vaccination with L2, E6 and E7 fusion protein completely prevented tumour growth after challenge with HPV16 transformed TC-1 tumour cells [Karanam et al., 2009].

HPV oncogenes E6 and E7 are known to be expressed throughout the HPV-associated disease process and offer to be attractive targets for immunotherapy, as they are exclusively expressed in virally infected neoplastic cells. The addition of L2 capsid protein to the fusion protein was of therapeutic interest, as L2 protein has been shown to induce neutralising antibodies and offer cross-protection against the other HPV types. In addition, it was also found to be highly effective in promoting tumour rejection in
animals when compared to L1 protein, with massive infiltration of lymphocytes into the tumours [Jarrett et al., 1991].

In a phase I double blind, placebo-controlled, randomised, dose escalating trial of three dose levels of TA-CIN, at the doses of 26, 128, 533 μg of TA-CIN per dose, TA-CIN was found to be safe and immunogenic in a dose dependent manner. Forty healthy volunteers tolerated the i.m.administered TA-CIN well with no side effects. No serious adverse events were noted during the study period. Vaccination with TA-CIN induced proliferative T lymphocyte responses in the majority (25 out of 32) of the healthy volunteers following three doses of the vaccine. Six out of sixteen volunteers who received the highest dose of TA-CIN (533 μg) revealed a lymphocyte proliferative response to TA-CIN after only one dose of the scheduled three-dose vaccination regime with TA-CIN, unlike the volunteers who received lower doses of the vaccine or the placebo. L2, E6 and E7 specific antibody response and positive T lymphocyte responses were nevertheless seen in all the volunteers who received the active vaccine and not the placebo. Antibodies specific to the E6 and E7 components of the TA-CIN were however found to dwindle after the last dose of the vaccination. It was hence suggested that sustained boosting of the immunity with the TA-CIN may be sub-optimal [de Jong et al., 2002].

1.13.9.4 TA-CIN / TA-HPV IN A PRIME BOOST REGIMEN

Pre-clinical studies in the mice showed that vaccination with TA-CIN followed by vaccination with TA-HPV stimulated greater T lymphocyte response than any other combination of these two vaccines [van der Burg et al., 2001]. The context of this notion led to a study in humans where TA-CIN was administered together with TA-HPV in a heterologous prime-boost regimen.

Davidson et al conducted a study in ten women with HPV 16 positive high grade VIN, who were initially primed with TA-HPV and then received three booster immunisations with TA-CIN. 90% of women demonstrated HPV 16 specific proliferative T lymphocyte and/or serological responses following
vaccination. In addition, three women also demonstrated lesion shrinkage with some symptom relief, but no correlation between clinical and immunological responses was observed. TA-HPV boosting with TA-CIN also failed to demonstrate any significant additional CD8 T lymphocyte responses or clinical improvement [Davidson et al., 2004].

Smyth et al. studied TA-CIN followed by the TA-HPV vaccine, in a prime boost regime fashion in twenty-nine women with high grade AGIN. Three i.m doses of TA-CIN at four weekly intervals were administered followed by a single dermal scarification with the TA-HPV vaccine. The prime-boost regimen was significantly immunogenic in all the women. This was confirmed by checking immunogenicity against the test and control antigens pre-vaccination and at different post-vaccination time-points by the lymphoproliferation assays. Almost a third showed a three-fold change in the TA-CIN specific proliferation response at one or more time-points following the vaccination. In addition vaccination also was found to result in induction of antibodies against individual HPV 16 E6 and E7 oncoproteins. Clinical response was slightly short of immunological response at 21%. In nineteen out of twenty-nine women (65%), no disease progression or regression was observed during the study period of twelve weeks. Disease progression was however noted in four women (14%) during this period. The prime boost regimen, accordingly although positively immunogenic for both cellular and humoral immunity in women with AGIN, this study showed no clear-cut association between induction of HPV 16 specific systemic immunity and clinical outcome [Fiander et al., 2006; Smyth et al., 2004].

1.13.9.5 CHIMERA VLP
HPV 16 L1E7 chimeric virus-like particles (CVLP) consist of a carboxy-terminally truncated HPV16L1 protein fused to the amino-terminal part of the HPV 16 E7 protein and self-assemble by recombinant expression of the fusion protein. The CVLP are able to induce L1 and E7 specific cytotoxic T lymphocytes. The first therapeutic trial to determine the therapeutic potential of this vaccine was by Kaufmann et al. in a randomised, double-blind placebo controlled trial in thirty-nine women diagnosed with mono-HPV 16 infected
high-grade CIN. Volunteers were randomised to receive either a 75µg dosage of the vaccine or 250µg dosage of the vaccine and four doses in total were scheduled. The vaccine was well tolerated and no major side effects were observed. In addition to cellular immune responses, antibodies with high titres against HPV 16L1 and low titres against HPV 16E7 proteins were also detected. The immune responses between lower and the higher concentration of the vaccine were comparable. Histological regression to CIN or no CIN was seen in 39% of vaccine recipients and 25% of the women who received placebo treatment. In addition, 37.5% of women in the vaccine group were HPV16 DNA negative compared to 14% in the placebo group, at week 48. It was observed that between the groups receiving vaccine or placebo treatment, although the trend towards clinical and histological response was only clearly obvious at week 48, the disparity in the HPV 16 DNA status was evident from week 24 onwards, signifying that histological responses probably follow HPV DNA clearance [Kaufmann et al., 2007].

1.13.9.6 HEAT SHOCK PROTEIN (SGN-00101)
Einstein et al. studied this novel therapeutic vaccine consisting of a fusion protein containing an *M.bovis* BCG heat shock protein (Hsp 65) covalently linked to the entire sequence of HPV 16 E7 protein. Fifty-eight women with biopsy proven high grade CIN were recruited into two cohorts, with thirty-one and twenty-seven women in each of the cohorts. The purpose of the study was to establish the efficacy of the vaccine and study clinical response rates at long term follow up. Following three doses of the vaccination at a dose of 500µg per vaccine at monthly intervals, a large loop excision of the transformation zone of the cervix or a cone biopsy of the cervix was then performed in all the women. On histological analysis of the resected cervical specimen, 22% were found to have achieved complete resolution of CIN on histological analysis, 55% achieved partial resolution to a lower CIN grade, 19% were found to have stable disease and in 4%, disease progression was confirmed. At a cellular level, there was significant association between induction of local inflammation, immune cell infiltration and histological response. The Hsp component of this vaccine which targets HPV 16 E7
oncoprotein has also been shown to be efficacious against HPV types other than HPV 16 alone suggestive of a possible cross-reactive function [Einstein et al., 2007].

Roman et al. studied the role of this same vaccine Hsp (SGN-00101) in women with high grade CIN. Twenty-one women received four injections of HPV 16 Hsp E7 fusion protein at a dose of 500 μg per dose, three weeks apart, following which a large loop excision of the transformation zone of the cervix was carried out for histological analysis. 35% were found to have completely cleared the CIN on the loop biopsy specimen. 55% had stable disease with no disease progression or regression. Immune responses were measurable in nine out of seventeen women (53%) tested. Five out of seven women who made a complete histological response also demonstrated immune responses. The vaccine at the given dose and schedule resulted in lesion regression with corresponding immune responses. This group concluded that enhancing the immunogenicity of the vaccine in any way might enhance clinical and histological responses [Roman et al., 2007].

1.13.9.7 VACCINIA VIRUS – MVA – E2
This is a recombinant vaccinia virus expressing bovine papilloma virus (BPV) E2. Gracia-Hernandez al. conducted a phase II clinical trial in women with HPV positive high grade CIN. Thirty four women received this therapeutic vaccine at the concentration of $10^7$ virus particles per dose injected directly into their uterus once every week for six-weeks. Twenty women who underwent cone biopsy of the cervix for high grade CIN were recruited to the control arm of this trial. In the group who received the vaccination, complete histological clearance of CIN was demonstrated in 59% of women and a greater than 50% reduction in the size of the lesions was noted in another 32%. All the women who received the vaccine developed antibodies against the MVA E2 vaccine and were also shown to generate specific cytotoxic cellular immune response. In addition, HPV DNA viral load was significantly reduced in the vaccinated group. In comparison, in the group who underwent cone biopsy of the cervix, on histological analysis, CIN was found to be
completely excised in only 80% of women. Specific cytotoxic cellular immune activity was also absent and predictably, there was no effect of the cone biopsy on the HPV viral load. At twelve-month follow-up, three out of twenty women treated with cone biopsy of the cervix developed lesion recurrence in comparison to none in the vaccinated group [Garcia-Hernandez et al., 2006].

1.13.9.8 LONG PEPTIDE VACCINE

van Driel et al. conducted a phase I / II trial with HPV 16 E7 peptide vaccination in nineteen patients diagnosed with HPV 16 positive cervical carcinoma who were also refractory to the conventional treatment. This study proved that HPV 16 E7 peptide vaccination was feasible with no major side effects or serious adverse events in women who were immunocompromised and terminally ill with cervical cancer [Ressing et al., 2000; van Driel et al., 1999].

In another phase I toxicity assessment study, Kenter et al. studied the therapeutic HPV vaccine consisting of the complete set of long overlapping peptides of the oncogenic proteins E6 and E7 of HPV16 virus in formulation with Montanide ISA-51 adjuvant. A similar patient cohort to van Driel et al, comprising forty-three women with histologically proven advanced or recurrent carcinoma of the lower genital tract who ran out of any feasible options for further therapeutic treatment were recruited into this trial. Vaccines were administered subcutaneously four times at three weekly intervals. In this study the vaccine proved safe and highly immunogenic. Unsurprisingly, no therapeutic responses were observed [Kenter et al., 2008].

1.13.10 LONG PEPTIDE VACCINE IN THE MANAGEMENT OF VIN

Kenter et al. further studied the immunogenicity and efficacy of a synthetic long peptide therapeutic HPV vaccine in women diagnosed with VIN who were otherwise healthy, different to their previous cohort of terminally ill women diagnosed with end-stage cervical cancer. The vaccine consisted of nine HPV 16 E6 and four HPV 16 E7 synthetic peptides dissolved in
dimethylsulfoxide and emulsified with incomplete Freund’s adjuvant. Twenty women with high grade VIN were vaccinated on three or four occasions at three weekly intervals. At twelve months of follow-up, 79% demonstrated objective clinical response (47% complete clinical response and 32% partial clinical response). Symptom relief was noted in 63% of women. All the women who made a complete clinical response continued to be in remission at twenty-four month follow-up visit as well. Systemic HPV 16 specific T lymphocyte proliferative responses to HPV 16 E6, E7 or both proteins were detectable in 85% of women after the last dose of the vaccination schedule. Also, lymphocyte proliferation responses were accompanied by the production of both T-helper type 1 and type 2 cytokines. Post hoc analysis suggested that women who made a complete response at three months had a significantly stronger IFN$\gamma$ associated proliferative CD4 positive T lymphocyte responses and a broad CD8 positive T lymphocyte response in comparison to women who did not make a clinical response. In the majority of the women assessed (twelve out of fourteen), proliferative responses were even detectable one to two years after completion of the vaccination programme [Kenter et al., 2009].

DNA therapeutic HPV vaccines have been shown to generate effective cytotoxic T lymphocyte activity and also antibody responses by delivering foreign antigens to antigen presenting cells that stimulate helper and cytotoxic T lymphocytes. Naked DNA plasmid vaccines are shown to be relatively safer with ease of administration compared to live viral or bacterial vectors [Kim et al., 2008]. In addition, DNA vaccines are easy to prepare on a large scale with high purity and stability. There however appears to be a need to develop strategies to enhance the potency of therapeutic HPV DNA vaccines. Clinical efficacy and HPV specific immunogenicity have both been shown to be sub-optimal in clinical trials that have advocated DNA vaccines in comparison to long peptide therapeutic HPV vaccines (table 2).
| STUDY                                           | DELIVERY SYSTEM           | ANTIGENS                      | DISEASE GROUP                      | IMMUNOGENICITY       | CLINICAL OUTCOME                        |
|------------------------------------------------|---------------------------|-------------------------------|-----------------------------------|---------------------|----------------------------------------|
| Open-label phase I/II uncontrolled trial       | Vaccinia virus (TA-HPV)   | E6, E7 fusion protein         | Late stage cervical cancer (n=8)  | CTLs (1/18) Ab (3/18) | Outcome not demonstrated               |
| [Borysiewicz et al., 1996]                      |                           |                               |                                   |                     |                                        |
| Open-label uncontrolled trial [van Driel et al., 1999] | Peptide oil/plus water adjuvant | E7 peptides                   | Refractory cervical cancer (HPV-16+, HLA-A201 (n=19) | CTLs               | 2/19 stable disease                     |
| Open-label uncontrolled trial [Frazer, 2004]    | Protein/algammlin adjuvant | E7-GST fusion protein         | Cervical cancer (n=24)            | Ab, DTH             | No alteration in natural h/o disease    |
| Open-label uncontrolled trial HPV-16+ [Muderspach et al., 2000] | Peptide plus IFA          | E7 A0201 peptide              | VIN, CIN HPV 16+ HLA A2 (n=18)    | CTLs 10/16 no DTH   | 3/18 – CR 6/18 - PR                     |
| Open-label uncontrolled trial [Zhang et al., 2000] | VLPs                      | L1                            | Genital warts (n=33)              | Ab, DTH             | 25/33 - CR                              |
| Double-blind placebo controlled trial [de Jong et al., 2002] | Fusion protein TA-CIN (no adjuvant) | HPV 16 L2, E6, E7 fusion-protein | Healthy volunteers (n=40) | Ab, T lymphocyte, IFNγ, ELISPOT | No HPV infections                     |
| Study Description                                                                 | Vaccine/Adjuvant                                         | Disease Model                          | Primary Endpoint                                                                 | Secondary Endpoints                                                                 |
|---------------------------------------------------------------------------------|----------------------------------------------------------|----------------------------------------|----------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|
| Open-label uncontrolled trial [Goldstone et al., 2002]                          | HSP fusion protein (HSP-E7)                              | HPV 16 E7 peptide                      | Genital warts (n=22)                                                            | Not done                                                                            |
|                                                                                 |                                                          |                                        |                                                                                  | 3- CR, 10 - PR                                                                      |
| Open-label uncontrolled trials [Garcia et al., 2004; Klencke et al., 2002;     | Encapsulated polynucleotides ZYC 101                      | HPV 16 E7                              | Anal, cervical dysplasia; HPV 16+, HLA-A2 (anal n=12, cervical n=15)            | ELISPOT; E2 specific immunity                                                      |
| Sheets et al., 2003]                                                            |                                                          |                                        |                                                                                  | Regression of AIN: 3/12-PR; CIN: 5/15 CR                                            |
| Multi-centre double-blind randomised placebo-controlled trial [Garcia et al.,   | Encapsulated polynucleotide ZYC 101                      | HPV 16 E7                              | CIN2/3 any HPV type (n=127)                                                      | ELISPOT, induction of E2 specific immunity                                           |
| 2004; Frazer, 2002]                                                             |                                                          |                                        |                                                                                  | Lesion regression higher in <25yr; not restricted to HPV 16/18 lesions              |
| Randomised placebo controlled trial [Frazer, 2004] Frazer 2002                  | Protein/Iscomatrix adjuvant (E6, E7-IMX)                 | HPV 16 E6, E7 fusion protein           | CIN (n=31)                                                                       | Ab, DTH, CTLs                                                                      |
|                                                                                |                                                          |                                        |                                                                                  | 7/14 – CR 7/14 - PR                                                                 |
| Open-label uncontrolled trial [Davidson et al., 2003a]                          | Vaccinia virus (TA-HPV)                                  | E6, E7 fusion protein                  | VIN (n=18)                                                                       | Ab, CMI                                                                            |
|                                                                                |                                                          |                                        |                                                                                  | PR- 8/18; loss of viral load in 12/18                                               |
| Open-label uncontrolled trial [Baldwin et al., 2003]                            | Vaccinia virus (TA-HPV)                                  | E6, E7 fusion protein                  | VIN (n=12)                                                                       | CTL, ELISPOT                                                                        |
|                                                                                |                                                          |                                        |                                                                                  | CR-1/12; PR-5/12; T                                                                 |
| Study Description                                      | Immunostimulatory Agent | Antigen(s)                  | Disease Stage | Biomarkers Studied | Clinical Response |
|--------------------------------------------------------|-------------------------|-----------------------------|---------------|--------------------|-------------------|
| Open-label uncontrolled trial [Ferrara et al., 2003]   | Dendritic cells         | HPV 16 E7, HPV 18 E7        | Cervical cancer stage IV (n=15) | Ab, ELISPOT       | No objective clinical response |
| Non-randomised phase II prime boost vaccine trial [Fiander et al., 2006] | L2, E6, E7             | Fusion protein L2, E6, E7 (TA-CIN) | VIN (n=27); VAIN (n=2) | | CR-1/29; PR – 5/29; 15/29 – symptom response |
| Open-label phase II uncontrolled clinical trial [Garcia-Hernandez et al., 2006] | BPV E2                 | Vaccinia virus (MVA-E2)      | CIN, vaccine arm (n=34) CIN, cone biopsy arm (n=20) | Ab against MVA E2, CTL, viral load | Histological response 20/34 in the vaccine arm |
| Open-label phase II uncontrolled clinical trial [Roman et al., 2007] | Hsp-7 (SGN-00101)       | E7                          | CIN (n=21) | CTL, HPV testing | CR-35%; PR-5%, SD-55%; disease progression-5% |
| Randomised placebo-controlled trial [Kaufmann et al., 2007] | Chimeric virus like particle (CVLP) | HPV 16 L1E7 protein | CIN2/3; HPV 16 only (n=39) | Ab, CTL | 39% no VIN in vaccinated vs. 25% no VIN in placebo, 59% of responders became |
| Study Type                        | Vaccine Type                        | Antigen Description                                      | Comparator          | Outcome Measure | Additional Details                                                                 |
|----------------------------------|-------------------------------------|-----------------------------------------------------------|---------------------|-----------------|-------------------------------------------------------------------------------------|
| Open-label uncontrolled trial    | Peptide Montanide ISA-51 adjuvant   | HPV 16 E6 combined or separated from HPV 16 E7 overlapping long peptides | End stage cervical cancer (n=35) | ELISPOT | Immunity against E6 in pt vaccinated with E6, E7 at the same site; > response to E7 in pt vaccinated with E6 in one limb and with E7 in a diff limb |
| Open-label uncontrolled trial    | DNA vaccine                         | Sig-E7 (detox)-HSP70 fusion protein                      | CIN2/3 HPV 16+ (n=15) | ELISPOT | No VIN in 33% in the highest dose cohort, new responses                              |
| Open-label phase II uncontrolled clinical trial | Long peptide vaccine | HPV-16 E6,E7 in Freund’s adjuvant | VIN3 (n=20) | HPV specific T lymphocyte responses | CR-20%; PR-40; all pt has vaccine induced T cell responses |

**Table 2 – Studies of therapeutic HPV vaccination for lower genital tract neoplasia**

- CTLs – cytotoxic T lymphocytes; DTH – delayed type hypersensitivity; CIN – cervical intraepithelial neoplasia; VIN – vulval intraepithelial neoplasia; VAIN – vaginal intraepithelial neoplasia; AIN – anal intraepithelial neoplasia; HPV – human papilloma virus; Ab – antibody; CMI – cell mediated immunity; IFNγ - interferon gamma; BPV – Bovine papilloma virus; VLP – virus like particles; CR – complete response; PR – partial response
1.13.11 IMMUNOTHERAPY – COMBINATION TREATMENT

Combination immunotherapies in theory might prove to be superior to solo medical management by modulating the immune responses in more than one way. For instance, in some clinical trials for certain chronic conditions, imiquimod treatment induced T helper 1 response and the imiquimod primed effector cells were then boosted with other immunotherapeutic modalities or in some cases, the treatment at this stage was completed with the established method of treatment for that specific condition as described below.

1.13.11.1 IMIQUIMOD IN COMBINATION THERAPIES

Imiquimod is clearly shown to potentiate innate immune mechanism and then drive the adaptive immune response towards the T helper 1 or cell-mediated pathway and inhibit the T helper 2 pathways. To take advantage of this aspect of imiquimod treatment, many trials have been conducted where, for clinical conditions that are usually resistant to the standard treatment, topical imiquimod was recommended as a compassionate treatment.

A case of complete remission of nodular basal cell carcinoma was reported following treatment with topical imiquimod 5% cream and PDT [Devirgiliis et al., 2008]. Martinez Gonzalez et al remarked a rapid total response achieved in a patient with chronic, resistant mycosis fungoides to imiquimod and systemic interferon α 2a as spectacular [Martinez-Gonzalez et al., 2008]. Bassukas et al. attempted a trial combination of imiquimod and cryotherapy for the treatment of lentigo maligna and observed an enhanced treatment response. According to the authors, the rationale to use imiquimod, as an adjunct to cryotherapy was to reinforce apoptosis of the tumour cells and build up an imiquimod induced cascade of potent tumour-destructive immune responses [Bassukas et al., 2008]. The efficacy of imiquimod in combination with 5-Fluorouracil (5FU) when considered in forty-eight women with actinic keratosis, clearance of lesions was found to be relatively rapid and the
combination treatment was also convenient in comparison to solo treatment with imiquimod or 5-FU [Price, 2007].

1.13.11.2 IMIQUIMOD IN COMBINATION THERAPY FOR VIN

1.13.11.2.1 IMIQUIMOD & PDT
The first published study of combination immunotherapy for VIN was by Winters et al. In this study, twenty women diagnosed with high grade VIN were subjected to a treatment combination of topical imiquimod and 5 ALA-PDT. Topical imiquimod was used as a neo-adjuvant treatment for eight weeks in a dose escalating fashion. This was followed by two sessions of 5 ALA-PDT, the PDT sessions spaced a month apart. 80% of the women found the combination of imiquimod and PDT treatment bearable. A clinical response rate of 55% was observed at week 52, the study primary end-point. Also, 65% of the women were asymptomatic following treatment in comparison to 5% who were asymptomatic when the trial commenced. Nineteen out of the twenty participants remained under clinical follow up for more than two years after the trial completion. Reassuringly, all the participants who made a response to the study treatment remained in clinical remission at follow-up. In fact, one woman who was considered to be a partial clinical responder during the trial was in fact found to have completely cleared the lesion at follow-up. This study exemplifies the longevity of response achieved with combination treatment of imiquimod and PDT. The authors consider that imiquimod establishes a favourable microenvironment enhancing the immunological effect of PDT. Women who completed the scheduled treatment experienced better treatment outcome and compromised imiquimod and / or PDT treatment resulted in less positive results. Clinical responses accomplished in this trial were comparable to the results achieved in other clinical trials of solo imiquimod treatment where treatment schedule was as prolonged as sixteen weeks to six months. In fact, enhanced clinical response rates were observed in this trial in comparison to PDT alone trials for VIN [Winters et al., 2008].
CHAPTER 2
STUDY PROTOCOL AND METHODOLOGY
2.1 STUDY PROTOCOL

2.1.1 IMIQUIMOD IN COMBINATION WITH THERAPEUTIC HPV VACCINATION FOR THE MANAGEMENT OF VULVAL INTRAEPITHELIAL NEOPLASIA – STUDY PROTOCOL

This thesis portrays an open, uncontrolled phase II trial of twenty women diagnosed with high grade VIN. The study scheme was to consider the combination of topical imiquimod cream and intramuscular administration of TA-CIN therapeutic HPV vaccination in a sequential fashion in women diagnosed with VIN grades 2 or 3. The aim of the study was to detect an effective and non-invasive treatment option for VIN, which also contemplates preservation of vulval architecture, establishes longevity of response and reduces recurrence rate.

2.1.2 HYPOTHESIS

Immune response modifier imiquimod and therapeutic HPV vaccination have both a recognised role in the management of VIN in a clinical trial setting. With imiquimod treatment, compliance with the treatment schedule is an issue due to prolonged recommended periods of treatment (sixteen weeks to six months) coupled with severe local and systemic side effects. In the previous TA-HPV or TA-CIN therapeutic HPV vaccination clinical trials of VIN or AIN, majority of patients’ demonstrated symptom response with a reasonable proportion also displaying objective clinical responses. In most of these studies, it was frequently observed that enhanced local infiltration of helper and cytotoxic T lymphocytes often corresponded with clinical response to treatment.

In this study, the rationale behind using a combination of imiquimod and TA-CIN was that a short course of imiquimod in addition to having a direct effect on VIN by stimulating a local immune response would also provide an immunological platform for the therapeutic HPV vaccination to achieve an enhanced response rate than that achieved in the previous solo vaccination trials.
2.1.3 PRIMARY STUDY OBJECTIVE

The primary objective of the study was to evaluate the objective (clinical) response of VIN to treatment with imiquimod followed by vaccination with TA-CIN.

To assess the primary objective, the lesions were mapped and the marker lesions were identified at the baseline. The extent of the disease was assessed visually supported by the direct measurement of the marker lesion(s) with the aid of the colposcope on the standard vulvoscopy chart at the time of baseline assessment and then repeated at every clinic visit for the duration of the study. For each marker lesion, the longest perpendicular dimensions were measured. In addition, medical photographers from the medical illustration department took photographs of the lesions at baseline (week0), after imiquimod (week10), after the third and final dose of the TA-CIN vaccination (week20) and at follow-up clinic visits (weeks 26 and 52).

2.1.3.1 DEFINITION OF CLINICAL RESPONSE

Clinical response was defined as reduction equivalent to or greater than 50% in the size of the lesion(s). All the measurements were made during vulvoscopy examination by the same clinician, to avoid inconsistency. Complete clinical response (CR) was defined as total clearance of the lesion(s). Partial clinical response (PR) was defined as reduction in the size of the lesion(s) equivalent to or greater than 50% but less than 100%. Stable Disease (SD) was defined as less than 50% reduction in the size of the lesion(s) or less than 25% progression in the size of the lesion(s). Disease Progression was defined as 25% or more increase in the size of the lesion(s).

2.1.4 SECONDARY STUDY OBJECTIVES

The secondary study objectives were to evaluate histological response, symptom response, HPV response, safety, toxicity and tolerability of the treatment regime and also to evaluate, characterise and quantify immune response to the treatment.
2.1.4.1 **Histological response** to treatment was assessed by taking 4 mm punch biopsy(s) of the marker lesion(s) with Keyes punch biopsy forceps under local anaesthesia (5 ml of bupivicaine 0.25%). Biopsy was taken following imiquimod treatment at week 10, after the three doses of TA-CIN vaccination at week 20 and repeated at the week 52, the study primary endpoint. The biopsy sites were sealed with Monsel’s (ferric subsulfate) haemostatic solution, which is our normal practice. In cases of excessive bleeding uncontrolled with the Monsel’s solution, an absorbable suture was placed taking sterile precautions. The biopsy sample was sent in formalin for histopathological analysis by a dedicated histopathologist with special interest in gynaecology cancer. The same team of histopathologists consistently reviewed all the biopsy specimens taken from the trial participants.

**Definition of Histological Response**
Complete regression of high grade VIN (VIN 2 or 3) to no VIN on histopathology of the punch biopsy of the index lesion was defined as **histological response**.

2.1.4.2 **Symptom responses** were evaluated from patient’s symptom diary that was given to them at the beginning of the trial, where women were expected to document the symptoms that occurred during the treatment in a visual analogue scale structure. Women were instructed to take this diary to each of the clinic visits for closer monitoring of symptoms.

At each clinic visit, trial participants were also systematically questioned if they have felt any pain, itching, redness, discharge, and/or any other symptoms over the last seven days. Those who experienced any of these symptoms were asked to grade the symptoms into none, mild, moderate and severe categories. Symptoms which did not interfere with everyday life were classed into none or mild categories whereas symptoms which got in the way of everyday life were classed into the moderate or severe categories.
**Definition of Symptom Response**
Regression of symptoms from the ‘moderate’ or ‘severe’ category to the ‘mild’ or ‘none’ categories was defined as *symptom response*.

**2.1.4.3 HPV response** was evaluated by exploration of the different HPV genotypes in the biopsy sample of the index lesion at different time-points. This was facilitated by Reverse Line Blot Assay (RLBA) analysis of a small section of the punch biopsy taken from the index lesion at baseline (week 0), post imiquimod (week 10), post vaccination (week 20) and the primary endpoint (week 52).

**Definition of HPV Response**
Clearance of HPV 16 from the biopsy site in somebody who was previously positive for the presence of HPV 16 was considered to be HPV response.

The emphasis for measurement of *local immune responses* was on helper (CD4), cytotoxic (CD8) and regulatory T lymphocytes (Tregs), by using the immunofluorescence technique on frozen tissue sections acquired from the punch biopsy of the index lesion. *HPV specific systemic T lymphocyte immune responses* were assessed by lympho-proliferation assays and neutralisation antibody assays.

**2.1.5 SAFETY AND TOLERABILITY**
To evaluate the *safety, toxicity and tolerability* of both imiquimod and TA-CIN treatment, in addition to the regular clinical assessment, to confirm general well-being during the course of the trial, urinalysis (to check for the presence of any protein, blood, leucocytes, glucose, ketones, nitrites and urobilinogen) and blood samples for full blood count (FBC), renal function tests (U&E) and liver function tests (LFT) were carried out serially at baseline (week 0), post imiquimod (week 10) and following the final dose of the vaccination at week 20. All the samples were processed at the biochemistry laboratory, St Marys hospital. The intention was to follow-up clinically significant values until the end of the study period.
During every immunisation, adrenaline (1mg), chlorpheneramine (2.5-5.0mg) and hydrocortisone (100mg), in pre-filled syringes, and cardio-pulmonary resuscitation equipment was made immediately available for management of anaphylaxis.

Local reaction to TA-CIN was described as an extensive area of redness and swelling which becomes indurated and involves most of the antero-lateral surface or a major part of the circumference of the upper arm. General reaction to TA-CIN was described as fever equal to or more than 39.5°C within 48 hours of dosing, anaphylaxis, bronchospasm, laryngeal oedema, generalised collapse or convulsions.

At all clinic visits following vaccination with TA-CIN, women were asked to give details of all injection site reactions that occurred since the last visit in the symptom diaries provided to them. The policy was also to monitor any local or systemic adverse events by keeping a full record of each adverse event and intercurrent illness, whether believed to be related or unrelated to the treatment. This record would include date and time of appearance and resolution of any adverse event, intensity, seriousness, attributability to trial medication, and action taken (appendix 1).

2.2 METHODS

2.2.1 IMIQUIMOD TA-CIN TRIAL PROTOCOL
Ethical approval was obtained from the University of Manchester Ethics committee on the 8.10.2004 and the Central Manchester Local Research Ethics Committee – reference number – 05/Q1404 / 172 on 19.10.2005. The study was registered with the European Clinical Trials Database EudraCT No 2005-001824-35. The Medicines and Healthcare Products Regulatory Agency (MHRA) approved the trial and issued the clinical trials authorization number (CTA) 01501/0003/001-000.
Volunteers were recruited from the gynaecology, colposcopy and the vulva clinics at St Marys Hospital, Manchester. St Marys hospital is a tertiary referral centre for women diagnosed with vaginal or vulval intraepithelial neoplasia. Some volunteers were referred from other district general hospitals. Women with newly diagnosed or recurrent VIN were invited to participate in the trial. The following inclusion and exclusion criteria applied:

2.2.1.1 INCLUSION CRITERIA
- Female aged 18-70 years
- Non-child bearing or using effective contraception
- Patients with VIN3 or AIN3, histologically confirmed by biopsy within the previous three months
- Given written informed consent
- Patients must have at least one lesion of sufficient size to allow biopsies to be taken on three occasions and for disease evaluation to be performed. (It is estimated that a lesion of approximately 1 cm diameter would be sufficient.)

2.2.1.2 EXCLUSION CRITERIA
- Any patient who has received active treatment for VIN3 or AIN3 in the previous four weeks prior to enrolment
- Any patient with VAIN3 requiring treatment
- Any patient who has received HPV vaccine in the past
- Any patient with known immunodeficiency
- Any patient who is planning pregnancy, pregnant or breast feeding
- Any patient receiving immunosuppressive therapy
- Any patient with clinically significant abnormal serum biochemistry, haematology
- Any patient with evidence of ongoing infection
- History of severe allergy
- History of severe adverse reaction to any vaccine
- Receipt of blood or blood products in the last three months
Women whose last cervical smear was abnormal. If their last smear was more than three years ago, it must be repeated at screening.

Any patient who either has ano-genital carcinoma or who, in the investigator’s opinion, is at high risk of developing ano-genital carcinoma over the next six months.

2.2.2 TEST AGENTS

Imiquimod (Aldara™) is provided in 10 mg sachets containing 12.5 mg of 1-(2-methylpropyl)-1H-imidazo[4,5-c] quinolin-4-amine. Each gram of the 5% cream contains 50 mg of imiquimod in an off-white oil in water vanishing cream base consisting of isostearic acid, acetyl alcohol, stearyl alcohol, while petrolatum, polysorbate 60, sorbitan monostearate, glycerine, xanthum gum, purified water, benzyl alcohol, methyl paraben and propyl paraben.

TA-CIN is an HPV16 L2E7E6 fusion protein formulated in 5mM glycine buffer containing 0.9mM cysteine. Each vial consisted of 0.5 ml of TA-CIN at the dose of 128 μg/ml, stored at –20 °C.

2.2.3 PROTOCOL

2.2.3.1 SCREENING VISIT

The trial was discussed with potentially eligible patients in the clinic and they were given the patient information sheet to read at home. If willing to participate, they were then invited to the trial clinic for a screening appointment where the eligibility to participate was verified by a clinical examination, urinalysis and blood tests. Women were asked to provide a urine sample in a sterile container. This was tested for the presence of any blood, leukocytes, protein, glucose, ketones, nitrite, bilirubin and urobilinogen (dipstix urinalysis). The sample was sent for urine microscopy and culture sensitivity to the microbiology department at St Marys hospital if the dipstix urinalysis was indicative of possible infection. Venous blood was drawn for full blood count (5ml) and serum biochemistry (5ml). A punch biopsy of the lesion was
taken at this stage if it was not already carried out in the last three months, to establish the diagnosis of high grade VIN. A detailed medical history was obtained at this stage to make certain all the inclusion and exclusion criteria were met.

2.2.3.2 RECRUITMENT - WEEK 0 – COMMENCE IMIQIMOD TREATMENT

Twenty-five women met the inclusion criteria and agreed to participate in the trial. Five women subsequently changed their mind regarding trial participation. At the recruitment visit women were asked to sign two copies of the written consent form, one for them to keep and one to be kept with their medical notes. A separate consent form was used for the photographs whenever required. Women were identified by a study code number that began with IT (abbreviation for Imiquimod TA-CIN). Vulvoscopy was then carried out and the longest dimensions of the index lesion(s) measured followed by a photograph of the lesion. One 4mm punch biopsy of the index lesion was obtained a small fragment of which was sent in a dry pot to the virology department for HPV typing. The rest of the biopsy was snap frozen in liquid nitrogen and transported to the Paterson Institute for Cancer Research, Manchester, either in liquid nitrogen or dry ice to be stored at -80°C prior to obtaining sections. Forty ml of blood was drawn into a universal container with heparin (6000 iu heparin / 20 ml blood) and transported to the Paterson Institute for Cancer Research for further processing.

The colposcope in the clinic is equipped with live image capturing facility, which enabled women to visualise the lesions on the screen. The areas that needed imiquimod application were shown to the patient on the screen and the method of application demonstrated. Women were advised to apply imiquimod for eight weeks in total, in a dose-escalating manner, one application in the first week, two applications in the second week followed by three applications a week, for the next six weeks. This regime was used to minimise the side effects and improve tolerance to the imiquimod treatment. In addition to the manufacturers information leaflet about Aldara (imiquimod)
cream, an additional information sheet specifically designed for the trial purposes was provided to the patient. Women were instructed to use the required number of sachets to cover all the lesions. In most cases, one sachet sufficed for one application. Each woman was provided with a box of twelve sachets. Women were advised to apply the imiquimod cream at bedtime on clean affected areas, wash hands after application and if necessary, to wear only cotton loose fitting underwear. They were instructed to wash the cream off the next morning and carry on with their routine daily activities. They were instructed to refrain from sexual intercourse on the days of imiquimod application. In addition, they were advised to keep the symptom diary whilst on treatment. It was advised not to commence the imiquimod application until ten days after the recruitment visit to allow the biopsy site to heal. The general practitioner and the referring clinician were informed of the patient’s participation in the trial.
Figure 3 a, b: Method of assessing the size of the lesion on vulvoscopy
|                      | Screening | Wk 0 | Wk 1 | Wk 4 | Wk 10 | Wk 14 | Wk 18 | Wk 20 | Wk 26 | Wk 52 |
|----------------------|-----------|------|------|------|-------|-------|-------|-------|-------|-------|
| Consent              | X         |      |      |      |       |       |       |       |       |       |
| Medical History      | X         |      |      |      |       |       |       |       |       |       |
| Photograph           | X         | X    | X    | X    | X     | X     | X     |       |       |       |
| Vulval biopsy        | X         | X    | X    | X    | X     | X     |       |       |       |       |
| FBC, serum biochemistry, urinalysis | X |      | X    | X    |       |       |       |       |       |       |
| Blood for immunological assay | X | X | X | X | | | | | | |
| Commence Imiquimod   | X         |      |      |      |       |       |       |       |       |       |
| Vaccination          |           | X    | X    | X    | X     |       |       |       |       |       |

**Table 3 Trial Schedule Chart** The participant in total made nine visits to the hospital over a period of twelve months. Biopsies were taken on four occasions and bloods on three occasions in total.
2.2.3.3 WEEK 4 – REVIEW IMIQUIMOD TREATMENT

Women were advised to attend the clinic four weeks into the imiquimod treatment primarily to ascertain tolerance to the imiquimod treatment, to discuss an altered application regime if necessary and to suggest coping mechanisms for any intolerable side effects. Any requirement for analgesia was discussed, if necessary, prescriptions were arranged for oral analgesics, anti-histamines to relieve local itch related to VIN, and bedtime sedatives to those who requested. Vaseline gauze dressings (gelonet), local anaesthetic creams and gels were also provided. Every possible attempt was made to help women persevere with the treatment. An assessment of the dosage of imiquimod used per application was made and a further prescription provided if necessary.

At this stage, most of the participants had an inkling of what to expect over the next four weeks. Some women preferred to use the cream during daytime for various reasons and some participants felt they could not cope with three applications per week due to the side effects. A further appointment to attend the clinic two weeks after completing the imiquimod treatment (week 10) was scheduled.

2.2.3.4 WEEKS 10, 14 and 18; ASSESSMENT OF RESPONSE TO IMIQUIMOD TREATMENT, ADMINISTRATION OF TA-CIN VACCINATION

A complete clinical assessment with accurate measurement of the lesion(s) took place at week 10, with the clinical findings supplemented by photographs. Symptom response was assessed by asking the patient and also by reviewing their symptom diaries. Two punch biopsies were obtained to assess histological, HPV and local immune responses. It was attempted to take the biopsies close to the original biopsy site to allow comparison. Urinalysis and blood tests were repeated. Forty ml of venous blood was again drawn for immunological assays.
The TA-CIN vaccines were stored in the pharmacy at -20°C and were obtained on a named/patient based prescription and dispensed only to the clinic staff. The vaccine following defrosting at room temperature for up to thirty minutes, the vial was gently inverted about five times before drawing up the dose. Using a 25G (orange) needle and 1ml syringe, 0.5 ml (128 μg/ml) of the clear defrosted solution was drawn and administered intramuscularly into the deltoid muscle within two hours, under aseptic precautions. Women were advised to wait in the clinic area for about twenty minutes to make sure they suffered no immediate adverse effects due to the vaccination.

TA-CIN vaccination was similarly repeated at weeks 14 and 18.

2.2.3.5 WEEK 20
A complete clinical assessment with accurate measurement of the lesion(s) for a third time took place again at week 20, with the clinical findings supplemented by photographs. Symptom response was assessed by asking the patient and also by reviewing their symptom diaries. Two punch biopsies were again obtained to assess histological, HPV and local immune responses. It was attempted to take the biopsies close to the original biopsy site to allow comparison. Urinalysis and blood tests were repeated. Forty ml of venous blood was again drawn for immunological assays. This week marked completion of trial treatment and an appointment was made for week 26, to assess the overall clinical response to treatment.

2.2.3.6 WEEK 26
A complete clinical assessment with accurate measurement of the lesion(s) for the fourth time took place again at week 26, with the clinical findings supplemented by photographs. Symptom response was assessed by asking the patient and also by reviewing their symptom diaries.

2.2.3.7 WEEK 52
The final assessment was made at twelve months from the start of treatment, the primary end-point. A further assessment of clinical and symptom response
was made at this stage. In the absence of any apparent VIN on vulvoscopy, one punch biopsy was taken from the site of the original biopsy at recruitment. If however VIN was suspected on vulvoscopy, a punch biopsy was taken for histological diagnosis. A fragment of the punch biopsy was forwarded to the virology department for HPV typing.
Figure 4 – Appearance of the vulva in patient 10 at wk 0 (a), wk 10 (b), wk 20 (c) and wk 52 (d). A clinical record of the same lesions based on vulvoscopy appearance is shown in figures 5 (a, b, c, d)
Figure 5(a) – Appearance of vulva patient 10, wk 20. (corresponding to figure 4a)
Figure 5(b) – Appearance of vulva patient 10, wk 20. (corresponding to figure 4b)
Figure 5(c) – Appearance of vulva patient 10, wk 20. (corresponding to figure 4c)
Figure 5(d) – Appearance of vulva patient 10, wk 52. (corresponding to figure 4d)
2.2.4 STUDY SAMPLES
2.2.4.1 PATIENT BLOOD SAMPLES

Forty millilitres of peripheral venous blood drawn from each patient at weeks 0, 10 and 20 in a pre-heparinised tube (6000 iu of heparin / 20ml blood) were further processed at the Paterson Institute for Cancer Research. Lymphocytes from the blood were separated in the following manner.

2.2.4.2 LYMPHOCYTE SEPARATION

Heparinised blood was layered onto lymphoprep lymphocyte separation medium (LSM, Nycomed, Amersham) and centrifuged at 800g (units of times gravity - g) at 20°C for twenty minutes in Mistral 30001 centrifuge (acceleration at 5 and brake at 0). Peripheral blood mononuclear cells (PBMC) were collected from the LSM interface and washed with phosphate buffered saline (PBS) twice and then centrifuged for ten minutes, twice at the speed of 350g and 200g respectively. Aliquots of plasma and red blood cell pellets were labelled and stored at -20°C. Plasma was spun at 3000 rotations per minute (rpm) for 10 minutes to sediment any contaminated red blood cells and the supernatant was aliquoted. PBMC were counted in a Neubauer’s chamber and were frozen at the concentration of 10^7 cells / ml in 10% dimethyl sulfoxide (DMSO) in foetal calf serum (FCS, TCS biologicals) and stored in liquid nitrogen at -80°C until required.

2.2.4.3 PATIENT BIOPSY SAMPLES

Snap frozen biopsies were stored at -80°C prior to sectioning. They were embedded in optimum cutting temperature embedding compound (O.C.T), and sectioned by a cryostat to seven-micron sections and placed on aminopropyltriethoxysilane (APES)-coated slides, two-sections per slide. Slides were labelled and stored at -20°C until required.

2.2.4.3.1 DNA EXTRACTION FOR HPV TESTING

Two dedicated virologists at the virology department, St Marys Hospital, carried HPV detection and genotyping. DNA extraction was carried out as
follows; biopsy samples were first digested in proteinase K lysis buffer containing 10mM Tris/HCl pH 8.3; 1mM EDTA; 0.5% Triton X 100, 0.001% SDS and 250µgms/ml of proteinase K (the volume of lysis buffer was decided empirically depending on the size of the biopsy). Following digestion at 37°C overnight with constant rotation the DNA was extracted using the MagnaPure Total Nucleic Acid Extraction protocol. Extracted DNA was stored at -70°C prior to testing.

2.2.4.3.2 HPV GENOTYPING (ROCHE REVERSE LINE BLOT ASSAY)

HPV detection and genotyping was carried out using the prototype reverse line blot assay kindly supplied by Roche. This assay is designed to amplify and detect 27 HPV genotypes. In order to assess DNA integrity, this assay also amplifies and detects a fragment of the human β-Globin gene. Testing was carried out according to the manufacturer’s instructions. A 50µl volume of extracted DNA was amplified using the PGMY primer PCR reagents provided by Roche on a 9700 thermocycler. Following amplification, biotinylated PCR product was denatured and captured onto nylon strips coated with the HPV type specific oligonucleotides. By the reverse blot strip analysis; genotype discrimination of the multiple HPV types can be accomplished in a single hybridisation and wash cycle. Twenty-seven HPV probe mixes, two control probe concentrations, and a single reference line were immobilised to 75- by 6- mm nylon strips. Each individual probe line contained a mixture of two bovine serum albumin-conjugated oligonucleotide probes specific to a unique HPV genotype. The genotype spectrum discriminated to this strip includes the high-risk, or cancer-associated, HPV genotypes 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 55, 56, 58, 59, 68 (ME180), MM4 (W13B), MM7 (P291), and MM9 (P238A) and the low risk, or non-cancer associated genotypes 6, 11, 40, 42, 53, 54, 57, 66, and MM8 (P155). The primary advantage of the strip-based detection system is the ability to rapidly genotype the HPVs present in the genital samples with high sensitivity and specificity, minimizing the likelihood of misclassification. Immobilised product was visualised using streptavidin-horseradish peroxidase-mediated colour precipitation as described previously [Gravitt et al., 1998].
2.2.5 DOUBLE IMMUNOFLUORESCENCE STAINING OF SPECIMENS

The characterisation of the local immune environment in the punch biopsies was by double immunofluorescence staining of the tumour infiltrating lymphocytes using a combination of antibodies against CD4, CD8 and FoxP3CD4. The reagents and methods used to identify CD4, CD8 and Tregs required optimisation. This part of this chapter describes the optimisation procedures for these reagents.

Antibodies were tested initially on sections of the human tonsil, acknowledged for their abundance of various types of T lymphocytes at different concentrations for optimisation. For the detection of CD4 lymphocytes, two different antibodies were tested, Mouse Monoclonal primary (EDU-2) to CD4 (Ab 1246) and Abcam and Mouse Monoclonal Primary (B379) to CD4 (Ab 8476). The background was felt to be high with three washes in the methodology and this led us to increase the washes at all stages of the protocol to five times.

Following identification of the optimal antibody, the appropriate concentration and the best method, the antibodies alone and in combination were then tested and optimised on the VIN sections.

The following antibodies at the following concentrations were used in this study for the immunofluorescence labelling of the CD4, CD8 and FoxP3CD4 lymphocytes.

Double fluorescence labelling of CD4+FoxP3CD4+ lymphocytes was carried out with mouse IgG1 anti-FoxP3CD4 (eBioscience) antibody, at a concentration of 1:50 and mouse IgG2a anti-CD4 (Abcam) antibody at a concentration of 1:100 and detected respectively using goat antimouse IgG1 Alexa Fluor 488 at a concentration of 3 µl/1000µl and goat antimouse IgG2a Alexa Fluor 546 at a concentration of 3 µl/1000µl (Invitrogen) respectively. CD8+ lymphocytes were detected using Rabbit anti-CD8 (Abcam) antibody at
a concentration of 1:100 and detected using Goat anti Rabbit-IgG Alex Fluor 488 at the concentration of 3 µl/1000µl.

2.2.5.1 CD4 ANTIBODY - Mouse Monoclonal Primary (EDU-2) to CD4 (ab1246) - Abcam
Immunogen – Human T lymphocytes
Species Reactivity – Reacts with human, not yet tested in other species
Cellular localization – Type 1 membrane protein
Store at +4 degrees C, Clonality – monoclonal, Clone number – EDU-2
Isotype – IgG2a, Purity – IgG fraction
Storage Buffer – PBS, 0.5% BSA, 0.2% Sodium Azide
Form – liquid, Concentration – 0.20mg/ml

2.2.5.2 CD4 ANTIBODY - Mouse Monoclonal Primary (B379) to CD4 (ab8476) – Abcam (tested but sub-optimal)
Immunogen:  CEM cells, reacts with human
Specificity: human CD4 antigen 55kD glycoprotein
Tested applications – flow cytometry, ICC/IF, IHC-frozen
Recommended dilution – for IF 5-20 µg/ml
Cellular localization – type1 membrane protein, raised in mouse
Clonality – monoclonal, clone number –B379, Isotype – IgM
Concentration 0.4mg/ml

2.2.5.3 CD8 ANTIBODY - Rabbit polyclonal to CD8 (ab4055) – Abcam
Immunogen – Synthetic peptide comprising the 13-C terminal amino acids of the cytoplasmic domain of CD8 alpha chain.
Species reactivity – Reacts with human and Sheep, Not yet tested in other species
Recommended dilutions: Immunohistochemistry – 1/50 – 1/100 for 30min at RT
Positive Control: Tonsil
Cellular localization: Type 1 membrane protein (isoform 1) and secreted (isoform2)
Store at +4 degrees C, Concentration - 0.100 mg/ml.
2.2.5.4 FoxP3CD4 ANTIBODY - Affinity purified anti-human FoxP3CD4, catalogue number; 14-4777 - E-bioscience

Formulation: Phosphate buffer pH 7.2, 150mM NaCl, 0.09% NaN3 (3 subscript), Store at 4 degrees C, Clone: 236A/E7, Isotype: Mouse IgG1

This antibody has been reported for use in intracellular staining followed by flow cytometric analysis, immunoblotting (WB), and immunohistochemical staining of frozen and paraffin embedded sections.

Storage buffer – 50mM borate, 150 mM NaCl, 20% glycerol and 0.05% sodium azide Ph-8.0, liquid form, concentration 0.400mg/ml, storage +4 degrees

Clonality – polyclonal, Isotype – IgG, Purity – IgG fraction

Storage Buffer – PBS with sodium azide

2.2.5.5 Control Antibodies

Antibody for negative control for CD4 – goat anti-mouse IgG2a at a concentration of 200mg/l

Antibody for negative control for CD8 – goat anti-rabbit IgG at a concentration of 100mg/l

Antibody for negative control for FoxP3CD4 – goat anti-mouse IgG1 at a concentration of 200mg/l

2.2.5.6 Secondary ANTIBODIES - Molecular Probes - Labelled Goat Anti-Mouse Isotype-Specific Antibodies

Storage – 2-6°C, Protect from light
Working concentration – 1-10 microgram/ml

Molecular Probes’ fluorescent goat anti-mouse isotype-specific antibodies are prepared from affinity-purified antibodies that react with the Fc portion of the heavy chain of mouse IgG of the appropriate isotype. The Alexa Fluor dyes to which these antibodies are conjugated provide for extraordinarily bright antibody conjugates.
2.2.6 DOUBLE IMMUNOFLUORESCENCE LABELLING TECHNIQUE

Double immunofluorescence staining allows concurrent staining of two different antibodies. The following combination of antibodies was attempted in this study; CD4 + FoxP3CD4; CD8 + FoxP3CD4 and CD4 + CD8. The procedure of double immunofluorescence labelling includes slide fixation, blocking the non-specific binding of the antibodies, permeabilization of the cell membrane to target any intracellular antigens (FoxP3CD4 in this study), incubation with the primary antibodies followed by incubation with the secondary antibodies.

2.2.6.1 FIXATION

Slides stored at -20 °C were allowed to come to room temperature, which usually took about fifteen minutes. Slides were labelled with the patient number, date and the antibodies tested. In the meantime, acetone used as the fixative, was pre-cooled at -20°C for thirty minutes. Slides were fixed with the pre-cooled acetone fixative for ten minutes at room temperature followed by air-drying for an additional ten minutes. The slides were then rinsed and hydrated in phosphate buffered saline (PBS) for twenty minutes. As the sections were never exposed to formalin, the process of antigen retrieval was not required.

2.2.6.2 BLOCKING

Sections were then incubated in PBS +10% Horse serum for thirty minutes followed by PBS+1% Horse serum for another thirty minutes, at room temperature to block unspecific binding of antibodies. The sections were outlined with PAP pen (special marking pen that provides a thin film of hydrophobic barrier when a circle is drawn around a specimen on the slide) and the section incubated (1ml of antibody solution for each section) with the first surface / membrane primary antibody (CD4, CD8 or the control antibodies in this study) in PBS + 1% horse serum in a humidified chamber for one-hour at room temperature.
2.2.6.3 PERMEABILIZATION

The first primary antibody solution (CD4 or CD8) at this stage was decanted and the sections were washed in PBS + 0.1% Triton-X-100. This step was carried out as the subsequent target protein (FoxP3CD4 or the control antibody) is expressed intracellularly. Triton-X-100 is considered to be a very popular detergent for improving the intracellular penetration of the antibodies.

The sections at this stage were incubated with the intracellular primary antibody (FoxP3CD4) in the medium of PBS + 1% horse serum + 0.1% Triton X-100 at 4°C overnight in a humidified chamber.

2.2.6.4 WASHES FOLLOWING INCUBATION WITH THE PRIMARY ANTIBODIES

The following morning, antibody was decanted and the slides were washed in PBS + 0.1% Triton-X-100 at room temperature five times, each wash lasting for five minutes.

2.2.6.5 SECONDARY ANTIBODIES

Fluorescent secondary antibodies were used in the medium of PBS + 1% horse serum at the concentration of 3 μl/ ml. The slides were incubated at room temperature very stringently in the dark for an hour.

2.2.6.6 WASHES FOLLOWING INCUBATION WITH THE SECONDARY ANTIBODIES

Following decanting of the secondary antibodies, slides were washed again for five times in PBS + 0.1% Triton-X-100 in dark, each wash lasting for five minutes.

2.2.6.7 SLIDE PREPARATION

About 20 μl of DAPI (4′6-diamidino-2-phenylindole – fluorescent stain that binds strongly to the DNA. DAPI has the ability to pass through an intact T lymphocyte membrane, stains the nucleus and emits blue emissions) was
poured onto each of the sections and incubated in the dark for fifteen to twenty minutes. The slides were then dipped in 1% TBS (Tris buffered saline) briefly followed by quick gentle air-drying in the dark. A drop of the anti-fade and the mounting medium (ProLong Antifade Kit) was then placed on the section and the slides were fixed with a coverslip. Once dry, the slides were stored in the dark at 4 °C until microscopy of the immunofluorescence labelled sections was carried out.
Figure 6 – Immunofluorescence labelling of cell nuclei with DAPI, helper T cells with CD4, cytotoxic T cells with CD8 and regulatory T lymphocytes with FoxP3. (Pt IT 1, wk 20)
Double Immunofluorescence labelling of cell nuclei with DAPI (blue), helper T cells with CD4 (red) and regulatory T lymphocytes with FoxP3 (green) (Pt IT 9, wk 0). This is a composited image of double immunofluorescence labelling of regulatory T lymphocytes where the green stain of the cytoplasm is surrounded by the red CD4 stained cell membrane.
Figure 8 – Double Immunofluorescence labelling of cell nuclei with DAPI (blue), helper T cells with CD4 (red) and regulatory T lymphocytes with FoxP3 (green) (Pt IT 11, wk 10). This is a composited image of double immunofluorescence labelling of regulatory T lymphocytes where the green stain of the cytoplasm is surrounded by the red CD4 stained cell membrane.
Figure 9 – Immunofluorescence labelling of cell nuclei with DAPI (blue), helper T cells with CD4 (red) and regulatory T lymphocytes with FoxP3 (green) (Pt IT 5, wk 0). The extreme right lower quadrant depicts a composited image of the DAPI (blue), CD4 (red) and FoxP3 (green) where the double immunofluorescence labelling of FoxP3 is shown.
Figure 10 – Immunofluorescence labelling of cell nuclei with DAPI (blue), helper T cells with CD4 (red) and regulatory T lymphocytes with FoxP3 (green) (Pt IT 9, wk 0). The cell counting feature of the Image J Analysis software was used to count the numbers of the representative cells. In this figure, the red dot represents a FoxP3 positively stained cell and the yellow dot represents a CD4 positive cell.
Figure 11 – Immunofluorescence labelling of cell nuclei with DAPI (blue), helper T cells with CD4 (red) and regulatory T lymphocytes with FoxP3 (green) (Pt IT 11, wk 10). The cell counting feature of the Image J Analysis software was used to count the numbers of the representative cells. In this figure, the red dot represents a FoxP3 positively stained cell and the blue dot represents a CD4 positive cell.
2.2.7 IMMUNOFLOUORESCENCE – SLIDE ANALYSIS AND CELL COUNTING

2.2.7.1 SLIDE ANALYSIS

Slide analysis was manually performed on a microscope attached to a computerised digital camera. The 40x objective was selected and the section to be analysed examined to gain an impression of the geography of the section. An area of the section representative of immune infiltrating cells was initially chosen and the image captured using the digital camera. For each of the visualised section, by shifting the correct filter into position on the microscope, it was possible to visualise the different stains individually. Nuclei were consistently stained blue with DAPI. The primary antibodies of CD4, CD8 and FoxP3CD4 were either stained green or red as shown in the figures 6 to 9.

After obtaining all the relevant images for one particular section, another field of the section was chosen. Moving to another field of the section was carried out in a systematic fashion in a stepwise manner. In total, tumour-infiltrating lymphocytes from five representative fields of image were counted for each section. In fact, in most cases this almost represented the entire section, as the punch biopsies were small in size. Overall, five images for every section and for every antibody were captured. The images were saved on the computer attached to the digital camera in TIFF format. These images were then electronically transferred to a secure computer and saved using adobe photo shop 6.0. Images on the computer were saved with the study number, antibodies tested and the date to allow future verification of counts.

2.2.7.2 CELL COUNTING

Subsequently, using adobe photo shop software 6.0, the individual images of the differently stained antibodies, obtained from each field of the section visualised on the microscope, were composited to obtain a single hybrid image of either FoxP3CD4, CD4 and DAPI or FoxP3CD4, CD8 and DAPI, or
CD4, CD8 and DAPI as shown in the figure 7. This made it possible to visualise and distinguish the different lymphocytes present in one particular filed of the section simultaneously in a snap shot. The compositied images were saved in the TIFF format.

Following image composition, by means of the 'Image J analysis software' version 1.36b (http://rsb.info.nih.gov/ij/) specking the differently stained nuclei and cell membranes was straightforward as shown in the figures 10 and 11. The cell counting feature of the software enables easy calculation of the differently coloured cells on the image. In this process the cursor is moved over each cell to be counted and a mouse-click places a coloured dot over them. One is expected to opt for a different coloured dot for different cell types but maintain uniformity for the same cell type as shown in the figures 10 and 11. Once all the cells of interest were dotted, total counts for the different coloured dots were displayed, which were documented both by hand and also on a computer data record sheet. The image now complete with the overlying dots was also saved in a TIFF format. Five sets of images were available for each of the antibodies tested. An average count was then obtained for each cell type.

An independent observer (TS) reviewed a range of slides to verify the counts and the counts differed by no more than 5%. Results were reported as density per unit area of CD4, CD8 and double stained FoxP3CD4+ CD4+ Treg cells.
2.2.8 PERIPHERAL IMMUNE RESPONSES

2.2.8.1 LYMPHOCYTE PROLIFERATION ASSAY

Full functionality of CD4+ T helper lymphocytes can be measured by assessing their proliferation. PBMC isolated from heparinised blood by density gradient centrifugation are cultured in vitro with mitogens and specific antigens for 3–6 days, after which cells are pulsed with 3H-thymidine and 8–16 hours later harvested onto glass fibre filter mats. Proliferation as measured by incorporation of a radiolabelled nucleotide (3H-thymidine) into the DNA of dividing cells is evaluated by liquid scintillation spectroscopy with a beta-plate counter. The total amount of radiolabel/activity measured represents DNA synthesis by the whole cultured PBMC population, which may include both CD4, and CD8 T lymphocytes. Results are expressed as the mean counts per minute (cpm) (usually for triplicate cultures) with an error of the mean, and / or as a stimulation index (SI). The SI is the ratio of the experimental cpm divided by cpm of the negative / background control (cells with tissue culture medium alone).

2.2.8.2 LYMPHO PROLIFERATION ASSAYS – METHOD

Peripheral immune response to HPV antigens was assessed by lympho proliferation assays. Proliferative T lymphocyte responses to HPV16 peptides E61, E6II, E6III, E6IV, E7I, E7II and the HPV 16 proteins TA-CIN (fusion protein of HPV 16 E6, E7, L2), HPV 16 L2, GST-E6, GST-E7 and TA-GW (HPV 6 L2E7) for negative control and PHA for positive control were measured by tritiated thymidine incorporation following a five day incubation of the patient PBMC with the respective peptides / antigens. PBMC collected from the three time-points of week 0 (pre-treatment), week 10 (post imiquimod) and week 20 (post vaccination) were available for eighteen women. For one patient (IT9) week 10 blood sample was not available due to inadvertent collection of the sample into an incorrect tube.

PBMC were seeded in the wells of a 96-well round-bottomed microtiter plate (Alpha Laboratories Ltd.) in quadruples at the concentration of $2 \times 10^5$ cells / well in RPMI 1640 medium supplemented with 10% human AB serum (Quest
Biomedical), 100 µg/ml streptomycin, 100 IU/ml penicillin (Life Technologies, Inc.) and glutamine. PBMCs alone (medium control) or PBMCs with 25 µg/ml each of recombinant HPV-16 L2E6E7 protein (TA-CIN), HPV-16 L2, GST-E6, GST-E7, TA-GW and pools of E6I, E6II, E6III, E6IV, E7I peptides at 20µg/ml and E7II peptide at the concentration of 25 µg/ml were seeded. In addition, PHA at two different concentrations of 2 µg/well and 5µg/well were incubated for five days at 37°C.

During the final eighteen hours of the culture, 1 µCi/well of [3H] thymidine (NEN Life Science Products) was added. The cells were harvested using a Packard 96-well vacuum cell harvester onto Unifilter plates (Packard Biosciences) and were left to dry overnight. 30 µl/well of Microscint 20 scintillation fluid (Packard) was then added. [3H] Thymidine incorporation was measured using a Top count scintillation counter (Packard). Replicates were usually within 10%. Replicates outside the 10% range were not considered for analysis. Results are presented as stimulation index (SI) = the mean number of counts incorporated by antigen-stimulated PBMCs divided by the mean number of counts for PBMCs in the medium alone (negative control); A pre-existing proliferative T lymphocyte response was defined as SI ≥2. A post vaccination proliferative T-cell response was defined as a two-fold increase in the SI compared with the pre vaccination value.

2.2.8.3 REAGENTS USED FOR LYMPHOPROLIFERATION ASSAYS

The reagents and methods used in this study were previously optimised in this and the affiliated units for other studies [Davidson et al., 2003a;Davidson et al., 2004;de Jong et al., 2002;Smyth et al., 2004;Winters et al., 2008].

2.2.8.4 PEPTIDE POOLS

The configuration and concentration of the peptide pools utilised in the proliferation assay were previously optimised. The peptides spanned the HPV 16 E6 and E7 protein and consisted of fifteen E6 and nine E7 overlapping 22-mer peptides. Each peptide was identified by their first and last amino acid in
the protein. Four to five peptides were grouped into a pool at a concentration of 5 µl / per peptide. The following pools were made.

| Peptide pool | Peptide Identity | Amino acids in the protein |
|--------------|------------------|---------------------------|
| E6 I         | 1-22             | mhqkrtamfq dpqerprklp ql  |
|              | 11-32            | dpqerprklp qlctelqtti hd |
|              | 21-42            | qlctelqtti diilecvyc kq  |
|              | 31-52            | hdiiilecvyc kqqlrrrevy df |
| E6II         | 41-62            | kqqlrrrevy dfafrdlciv yr |
|              | 51-72            | dfafrdlciv yrdgnpyavc dk |
|              | 61-82            | yrdgnpyavc dkclkfyski se |
|              | 71-92            | dkclkfyski seyrhcysv yg  |
| E6III        | 81-102           | seyrhcysv ygttleqy ky   |
|              | 91-112           | ygttleqyn kplcdllirc in |
|              | 101-122          | kplcdllirc incqkplcpe ek|
|              | 111-132          | incqkplcpe ekqrrhldkkq rf|
| E6 IV        | 111-132          | incqkplcpe ekqrrhldkkq rf|
|              | 121-142          | ekqrrhldkkq rfhnrgrwt gr |
|              | 131-152          | rfhnrgrwt grcmsccrss rt  |
|              | 137-158          | grwtgrcmsc crssrtret ql  |
| E7I          | 1-22             | mhgdptlhe ymlidqpett dl  |
|              | 11-32            | ymlidqpett dlycyeqlns ss |
|              | 21-42            | dlycyeqlnsd sseedeidg pa |
|              | 31-52            | sseedeidg pagqaepdra hy  |
| E7II         | 41-62            | pagqaepdra hynivtfck cd  |
|              | 51-72            | hynivtfck cdstlrcvq st   |
|              | 61-82            | cdstlrcvq sthvdirtle dl  |
|              | 71-92            | sthvdirtle dlmggtlqiv cp |
|              | 77-98            | rtlldllmgpgivcpicsq kp   |

Table 4 Peptide pools for the Lymphoproliferation Assay – the peptides spanned the HPV 16 E6 and E7 protein and consisted of 15 E6 and 9E7 overlapping 22-mer peptides
The following peptide pools, available in fine particle form were made into a suspension in DMSO at a stock concentration of 50 mg/ml

2.2.8.4.1 GST TAGS
The HPV E6 and E7 proteins were available as GST Tag proteins. Epitope tags such as glutathione-S-transferase (GST) are often used as tags for proteins for expression and purification applications. Glutathione transferases are abundant enzymes involved in cellular defence against electrophilic chemical compounds, which bind glutathione with high affinity and specificity. The strength and selectivity of this interaction enables glutathione-based affinity resins to effectively purify GST-tagged proteins. The glutathione resin selectively binds the GST-tagged protein under normal conditions, allowing the specific protein of interest to be separated from whole cell extracts rapidly and efficiently allowing a high degree of purification.

GST is a 35-kDa protein that can be assayed biochemically as well as immunologically. This characteristic sets it apart from many epitope tags that are simply short peptides. However, the large size of GST results in a higher potential for degradation by proteases than other smaller tags. Therefore, performing GST-protein purification as quickly as possible under non-degrading conditions is necessary in order to minimize sample loss.

2.2.8.4.2 TA-GW
TAGW is a recombinant HPV6 L2E7 protein and also a therapeutic vaccine for the treatment of genital warts. The function of this protein in the assays was as a negative control to HPV 16 proteins. This protein was kindly supplied by the Xenova Research Ltd.

2.2.8.4.3 TA-CIN
The HPV 16 E6, E7, L2 vaccine is produced in Escherechia Coli and purified using a proprietary downstream GMP-compatible process. TA-CIN is formulated in 5mM glycine buffer containing 0.9mM cysteine. TA-CIN is provided in 1 ml ampoules containing 0.6 ml HPV 16 E6E7L2 fusion protein in a total volume of 0.5ml. The ampoules were stored at -20°C and allowed to
thaw for up to thirty minutes at room temperature. TA-CIN was also supplied by Xenova Research Ltd.

**HPV 16 GST E6 & HPV 16 GST E7** were kindly donated by Pawlita et al [Smyth et al., 2004].

**HPV 16 L2 protein** (full length HPV16L2 tagged with 6His at N-terminus) was a donation from Roden et al [Karanam et al., 2009].

### 2.2.8.5 NEUTRALISATION ANTIBODIES

Neutralising antibodies to HPV 16 pseudovirions were tested as by the SEAP pseudo virus neutralisation based assay at the virology research laboratories, John Hopkins University, Baltimore USA by Dr Roden and his team [Karanam et al., 2009]. The serum obtained from the blood samples of the trial participants, at weeks 0, 10 and 20 were aliquoted and shipped to USA for the SEAP (secreted alkaline phosphatase) pseudo virus neutralisation based assay.

### 2.2.9 STATISTICAL ANALYSIS

Analyses were performed using ‘Stats Direct Statistical Software’. Differences between responder and non-responder categories (baseline disease duration; HPV 16 positivity; smoking; symptom response at week 52 compared to baseline) were analysed using Fisher’s exact test. The non-parametric Mann-Whitney U test was used to test for differences between lesion associated T lymphocyte densities, proliferation assays and neutralising antibodies between groups (histological responder and non-responder). The non-parametric Wilcoxon’s signed rank test was used to analyse the differences in the lesion associated T lymphocyte densities, proliferation assays and neutralising antibodies within groups at different time-points. All reported P values are two-sided and have not been adjusted for multiple comparisons. A P value of <0.05 was considered to be statistically significant.
CHAPTER 3
CLINICAL RESULTS
This chapter describes the clinical results of this phase II study of sequential imiquimod and therapeutic HPV vaccination (TA-CIN) for high grade VIN. This study represents an attempt to develop an alternative non-surgical treatment option for VIN that would enable preserving the vulval anatomy and architecture. The objective of this study was to develop an effective treatment for VIN, which results in a sustainable response. We believe that a short course of imiquimod in addition to having a direct effect on VIN by stimulating a local immune response would also provide an immunological platform for the therapeutic HPV vaccination to achieve an enhanced response rate than that achieved in solo imiquimod or vaccination trials.

The following variables were assessed to characterise any response to treatment. The size of the lesion, histopathological analysis of the punch biopsy of the lesion, symptoms and presence or absence of HPV-16 DNA was evaluated before any treatment and after imiquimod and vaccination treatments.

3.1STUDY POPULATION

3.1.1 TRIAL PARTICIPATION

Twenty-five women with biopsy confirmed high-grade VIN were initially willing to participate in the study. Five women defaulted after the initial screening visit, who when contacted by telephone said they had changed their minds regarding participation in the trial for the following reasons: uncertainty as regards the efficacy of the trial treatment, inconvenience connected with frequent clinic visits and also lack of belief in any treatment for VIN as they have had developed recurrences on many occasions in the past.

Twenty women commenced imiquimod treatment. One woman (IT22) was withdrawn four weeks into imiquimod treatment. This participant was twenty-two years of age and was diagnosed with symptomatic high-grade VIN barely
few months prior to the trial enrolment. She did not undergo any treatment previously for VIN. This woman noted a new symptomatic white patch on her vulva four weeks into imiquimod treatment, which warranted an unscheduled visit to the hospital. On examination, a new VIN lesion with suspicious features was detected, which on biopsy was confirmed to be high-grade VIN with a focus (1mm) of micro invasive SCC of the vulva. At this stage she was withdrawn from the trial and underwent surgical excision of the lesion.

3.1.2 TREATMENT SCHEDULE & COMPLETION OF TREATMENT

This left a study population of nineteen women (table 5). All the nineteen women complied with the treatment schedule and follow-up appointments. One woman (IT22) received only two doses of the TA-CIN vaccination instead of the routine three, at weeks 10, 14 and missed her third vaccination scheduled for week 18. This was because the vaccine expired few days prior to her scheduled appointment and protein stability tests to check further validity of the vaccine were not carried out at this stage by the manufacturers. The rest eighteen women received all three doses of the TA-CIN vaccination as per protocol.

3.1.3 DEMOGRAPHICS

Age: The study cohort mainly comprised young and middle aged women as shown in figure 12. The mean age of women in the trial was 45yr (range: 22-66 yr). 25% were less than 39 years of age (22, 27, 28, 34 and 35yr respectively), 65% were aged between 40 and 59 years (40,40,43,43,45,47,48, 51, 51 51,55 56, 58yr respectively) and 10% were 60 years or more (65, 66yr respectively).
Figure 12 - Age distribution of the patients - The youngest pt was 22 yr and the oldest 66 yr, with the mean age of 45 yr

3.1.4 CIGARETTE SMOKING & PREVIOUS TREATMENT FOR VIN

As shown in the table 5, thirteen women (65%) were current smokers, three (15%) were ex-smokers and four (20%) never smoked. The study population was heavily pre-treated with thirteen women (65%) having had previous treatment for VIN. Seven women (35%) had not undergone any previous treatment for VIN. The variety of previous treatments included wide local excision, partial vulvectomy, vulvectomy, laser ablation, topical imiquimod treatment or diathermy to the vulva as shown in figure 13 and 14. On the whole, 60% of women underwent previous surgical treatment. Six women had undergone two or more modalities of treatment; three of who aged 35, 40 and 66 yr in fact underwent treatment on ten, seven and nine previous occasions. In the majority of the women who underwent previous vulvectomy and in three women who underwent five or more previous surgical excisions, vulval anatomy was severely disfigured and compromised. Two women already had experience-using imiquimod for VIN prescribed by their local gynaecologist, which resulted in a partial clinical response and significant symptom response. At the time of recruitment, VIN had recurred in these women.
Figure 13 – Method and number of previous treatments – Method and number of previous treatment(s) undergone by the trial participants prior to recruitment. 13 women had undergone 2 vulvectomies, 27 surgical excisions, 18 laser ablations, 2 courses of imiquimod and 1 diathermy session in total.

Figure 14 - Mode(s) of previous treatment for individual trial participant - prior to their recruitment to the trial. For example, IT1 had undergone vulvectomy, laser ablation and imiquimod treatment on separate occasions whereas IT9 had undergone vulvectomy and three surgical excisions on separate occasions.
3.1.5 PREVIOUS CERVICAL DISEASE

Given that VIN often co-exists with CIN, we looked at the incidence of CIN in this cohort. Three of the participants underwent total hysterectomy, which left seventeen evaluable women, of who ten (59%) had a previous diagnosis of CIN as shown in the figure 15. None had active CIN at recruitment.

3.1.6 DISEASE DURATION

In thirteen women (65%), VIN at recruitment was of recurrent type with mean disease duration of seven years (range 1-20 yr). In the remaining 35%, VIN was either newly diagnosed or was diagnosed for a while but kept under clinical observation without undergoing any treatment. In twelve women (60%), the disease was diagnosed in the last five years, in two women (10%), disease was known to be present for the last ten years and in six women (30%) disease was diagnosed more than ten years back as shown in the figure 16.

3.1.7 Focality

VIN was multifocal in seventeen women (85%) and unifocal in three women (15%) as shown in figure 16.
**Figure 15 – Incidence of smoking and CIN** - 65% actively smoked; 59% gave a positive h/o CIN in the past
Figure 16 – Duration of VIN and disease focality - 60% of the participants were given a diagnosis of VIN less than 5 yr ago, 10% between 5-10 years and 30% more than 10 yr ago; 85% of women had multifocal VIN and 15% had unifocal VIN
| AGE | MULTI/UNIFOCAL | SMOKING | H/O CIN | PREV TREATMENT | DISEASE DURATION |
|-----|----------------|---------|---------|----------------|------------------|
| IT1 | Multifocal     | No      | Yes     | Laser, Vulvectomy, Imiquimod | 4 yr             |
| IT2 | Multifocal     | Yes-20/day | Yes     | Laser x 4      | 9 yr             |
| IT3 | Multifocal     | Ex-smoker | Yes     | Excision       | 3yr              |
| IT4 | Multifocal     | Yes-20/day | Yes     | Excision x 3   | 10 yr            |
| IT5 | Multifocal     | Yes-20/day | No      | Excision x 7   | 20yr             |
| IT8 | Multifocal     | Ex-smoker | No      | None           | 4yr              |
| IT9 | Multifocal     | Yes-8/day | Yes     | Excision x 3, Vulvectomy | 15yr            |
| IT10| Multifocal     | Yes-6/day | Yes     | None           | 1yr              |
| IT11| Multifocal     | Ex-smoker | TAH     | Diathermy      | 1yr              |
| IT13| Multifocal     | Yes-20/day | Yes     | Excision       | 6 yr             |
| IT14| Unifocal       | Yes-20/day | No      | Laser x 2      | 16yr             |
| IT15| Unifocal       | No      | Yes     | None           | 1yr              |
| ID  | Age | Multifocal | Smoking Status | Previous Treatment | Duration |
|-----|-----|------------|----------------|--------------------|----------|
| IT16 | 56  | Multifocal | Yes - 12/day    | Yes                | None     | 2yr      |
| IT17 | 43  | Multifocal | No             | TAH Laser x 2, Excision | 4yr      |
| IT18 | 48  | Unifocal   | No             | No                 | None     | 1yr      |
| IT19 | 34  | Multifocal | Yes - 20/day    | No                 | Laser, Imiquimod | 3yr      |
| IT20 | 22  | Multifocal | Yes - 20/day    | No                 | None     | 1yr      |
| IT21 | 28  | Multifocal | Yes - 10/day    | No                 | None     | 1yr      |
| IT22 | 66  | Multifocal | Yes - 20/day    | TAH Excision x 5, Laser x 4 | 16yr     |
| IT23 | 35  | Multifocal | Yes - 15/day    | Yes                | Excision x 6 Laser x 4 | 10yr     |

Table 5 – Patient demographics - This table shows the age of the participant, if VIN was unifocal or multifocal, smoking status, whether there was a history of CIN in the past, previous treatment for VIN and the duration of years since the diagnosis of VIN was made along with the patient’s trial identification number.
3.2 RESPONSE TO THE IMIQUIMOD TREATMENT AT WEEK 10

The preliminary assessment of any response to the treatment was made at week 10, two weeks after completion of the eight-week course of the imiquimod treatment. Week 10 also marked the beginning of the TA-CIN vaccination schedule.

3.2.1 CLINICAL RESPONSE (POST IMIQUIMOD)

At week 10, fourteen out of the nineteen participants (74%) made a clinical response (defined as 50% or more reduction in the overall size of the lesions). This included three complete (16%) (total clearance of VIN on vulvoscopy) and eleven partial (58%) clinical responders (reduction in the size of the lesion(s) equivalent to or greater than 50% but less than 100%). The remaining five women maintained stable disease status (less than 50% reduction in the size of the lesion(s) or less than 25% progression in the size of the lesion(s)). In fact, no disease progression was noted at week 10.

3.2.2 HISTOLOGICAL RESPONSE

Six women (32%) had completely cleared VIN on the punch biopsy of the index lesion at week 10. Complete regression of high grade VIN (VIN 2 or 3) to no VIN on the histopathology of the punch biopsy of the index lesion was defined as histological response. This was rather unpredicted as three of the four women who demonstrated histological response in fact were classed as partial responders on vulvoscopy.

3.2.3 SYMPTOM RESPONSE

Upon questioning at week 10, seven out of nineteen women (37%) happened to perceive a change for the better in their symptoms. Symptom response was defined as regression of symptoms from the ‘moderate’ or ‘severe’ category to the ‘mild’ or ‘none’ categories. Although these women were experiencing
severe side effects linked with the application of the imiquimod cream, generally they were able to make a distinction between the symptoms associated with VIN and the side effects of the imiquimod cream.

3.2.4 HPV RESPONSE

At week 10, eleven women were HPV 16 negative on the biopsy. Six of these eleven women were positive for HPV 16 on the previous pre-treatment biopsy taken at the baseline and thus were considered to have made a HPV response (43%), defined as clearance of HPV 16 from the biopsy site in somebody who was previously positive for the presence of HPV 16. Eight women who were previously positive for HPV 16 on the baseline biopsy continued to remain so.

Correlating the HPV response with VIN resolution, of the six women who happened to clear HPV16 from their biopsy site, one woman made a complete clinical response and the remaining five women made a partial clinical response. Interestingly, four of the six women (67%) who cleared HPV 16 also established VIN clearance on histopathology of the index lesion. All the responses at week 10 are shown in figure 17.
Figure 17 – Responses at week 10 - This figure summarises the responses at week 10. 74% clinical response (≥50% reduction in the size of the lesions); 32% biopsy proven resolution of high grade VIN; 37% reduction in symptoms from moderate/severe category to mild/none category and 43% biopsy proven HPV 16 negativity in women who were previously positive.

3.3 RESPONSES TO THE TA-CIN VACCINATION AT WEEK 20

Two weeks following the third and final dose of the TA-CIN vaccination and twelve weeks following the completion of the imiquimod treatment, a comprehensive assessment took place when all the parameters were re-evaluated.

3.3.1 CLINICAL RESPONSE

At week 20, sixteen women (84%) showed a clinical response (50% or more reduction in the size of the lesions). Six women (32%) demonstrated a complete clinical response (total clearance of VIN on vulvoscopy) and ten women (52%) a partial clinical response (reduction in the size of the lesion(s) equivalent to or greater than 50% but less than 100%). Three women (16%) had stable disease (less than 50% reduction in the size of the lesion(s) or less than 25% progression in the size of the lesion(s)). No disease progression was observed.
3.3.2 HISTOLOGICAL RESPONSE

Eleven women (58%) were negative for VIN on histopathological analysis of a punch biopsy at week 20. On vulvoscopy however, again only six of the histological responders were located in the complete clinical response category and the remaining five women were in the partial clinical response group.

3.3.3 SYMPTOM RESPONSE

At recruitment, fifteen women reported moderate to severe symptoms whereas at week 20, only five of these women continued to experience moderate to severe symptoms. A 67% symptom response (resolution of symptoms from moderate to severe category to mild or none category) was thus observed at week 20.

Overall, at week 20, fourteen women accounted for none or mild symptoms (74%) and five women (26%) for moderate to severe symptoms.

3.3.4 HPV RESPONSE

Twelve women were HPV 16 positive at baseline, eight of who continued to demonstrate HPV 16 positivity at week 20 on the punch biopsy. A 33% HPV response (clearance of HPV 16 from the biopsy site in somebody who was previously positive for the presence of HPV 16) was observed at week 20.

Correlating HPV 16 clearance with disease resolution, of the four women who cleared HPV in their lesions, two women made a complete clinical response, the other two women made a partial clinical response; and in all the four women histological regression of VIN was also established. Additionally, three of the four women who cleared HPV were also symptom free at week 20. All the responses at week 20 are shown below in the figure 18.
Figure 18 – Responses at week 20- This figure summarises the responses at week 20, two weeks after completion of the vaccination schedule. 84% clinical response (≥50% reduction in the size of the lesions); 58% biopsy proven resolution of high grade VIN to no VIN on histopathology; 67% reduction in symptoms from moderate/severe category to mild/none category and 33% biopsy proven HPV 16 negativity in women who were previously positive is shown in this column chart.

3.4 RESPONSES AT THE PRIMARY END-POINT OF WEEK 52

At the study primary end-point of week 52, in addition to a thorough clinical assessment, histological analysis and HPV typing also took place.

3.4.1 CLINICAL RESPONSE

On the whole, fifteen women (79%) showed a clinical response. Ten women (53%) made a complete clinical response with no detectable lesion(s) on vulvoscopy. Partial clinical response (reduction in the size of the lesion(s) equivalent to or greater than 50% but less than 100%) was detected in five women (26%). In four women (21%) the disease was considered to be ‘stable’ (less than 50% reduction in the size of the lesion(s) or less than 25% progression in the size of the lesion(s)). One woman (IT9) by this stage developed a microinvasive focus that was completely excised on a punch biopsy. No further invasion was diagnosed at this stage.
3.4.2 HISTOLOGICAL RESPONSE

Twelve women (63%) were negative for VIN on histopathological analysis of the punch biopsy. On vulvoscopy, no VIN was visible in nine out of these twelve women and some residual VIN measuring about 3-4 mm was visible in three women, although the representative punch biopsy was negative for VIN on histopathology.

3.4.3 SYMPTOM RESPONSE

At the study finishing point, fifteen women agreed they were either asymptomatic or had only mild symptoms. Of these fifteen women, twelve of them reported moderate to severe symptoms at recruitment. An 80% symptom response (resolution of symptoms from moderate to severe category to mild or none category) was thus observed.

Overall, fifteen women had none or mild symptoms (79%) and four women (21%) reported moderate to severe symptoms.

3.4.4 HPV RESPONSE

Nine women were HPV 16 negative in the punch biopsy at week 52. Six of these women were previously positive for HPV 16. Ten women were HPV 16 positive even at week 52. Overall, out of fifteen women who were previously HPV 16 positive at any given time point, six of them were HPV 16 negative at week 52, resulting in a response rate of 40%.

Correlating HPV 16 clearance with treatment response, five of the six women who demonstrated HPV 16 negativity at week 52 also concurrently proved to have fulfilled clinical, histological and symptom response criterion too. All the responses at week 52 are shown below in figure 19.
Figure 19 – Responses at week 52- This figure summarises the responses at week 52, the primary end-point of the study. 79% clinical response (≥50% reduction in the size of the lesions); 63% biopsy proven resolution of high grade VIN to no VIN on histopathology; 80% reduction in symptoms from moderate/severe category to mild/none category and 40% biopsy proven HPV 16 negativity in women who were previously positive is shown in this column chart.

3.5 TRENDS IN THE RESPONSE OVER 52 WEEKS

3.5.1 TREND IN THE CLINICAL RESPONSES

As shown in figure 20, the majority of the clinical response was already established by week 10 (74%). This rate of clinical response marginally improved to 84% by week 20 with a slight drop to 79% by week 52. Clinical response includes complete and partial response. Complete clinical response improved from 16% at week 10 to 32% at week 20. This further improved to 53% at week 52. On the other hand, the rate of partial clinical response went down from 58% at week 10 to 52% at week 20 and 26% at week 52. The reduction in the partial response rate compensates the increase in the complete response rate. The increase in the rate of complete response, after completion of imiquimod and TA-CIN treatment, from 32% to 53% between the weeks 20 and 52 is noticeable.
Figure 20 – Trend in the clinical response Complete (CR) and partial (PR) clinical response rates as observed at weeks 10, 20 and 52. 16% CR and 58% PR at week 10; 32% CR and 52% PR at week 20; and 53% CR and 26% PR at week 52 was observed.
3.5.2 TREND IN THE HISTOLOGICAL RESPONSES

A gradual increase in histological response was noticed until week 20, as of 21% at week 10 to 58% at week 20 with a further slight rise to 63% at week 52 as shown below in figure 19. Unlike the clinical response majority of which was obvious by week 10, only 21% of women were proved to be negative for VIN on histopathology of the punch biopsy from the lesion site. This however improved to 58% by week 20 as shown in figure 21.

Figure 21 – Trend in the histological response - An improvement in histological response was observed from 32% at week 10 to 58% at week 20 and 63% at week 52.
3.5.3 CLINICAL RESPONSE VS. HISTOLOGICAL RESPONSE

There was a discrepancy between clinical response rates determined on vulvoscopy and histological response rates determined following the histological analysis of the punch biopsy. In comparison to the complete clinical response rates of 16%, 32% and 53% at weeks 10, 20 and 52, the histological response rates were 32%, 58% and 63% as shown in figure 22 and tables 6 and 7.

![Figure 22 – Discrepancy between clinical and histological response](image)

Comparison of complete (CR) and partial (PR) clinical response rates with histological response rates at different time points. At week 10, histological, CR, PR rates were 32%, 58% and 21%; at week 20 - 58%, 32%, and 52% and at week 52 – 63%, 53%, 26% respectively.
|           | CLINICAL RESPONSE | HISTOLOGICAL RESPONSE |          |
|-----------|------------------|-----------------------|----------|
|           | Post imiquimod   | Post vaccination      | Week 52  |
| IT1       | PR               | PR                    | No       |
| IT2       | SD               | SD                    | No       |
| IT3       | SD               | PR                    | No       |
| IT4       | SD               | SD                    | No       |
| IT5       | PR               | CR                    | Yes      |
| IT8       | PR               | PR                    | Yes      |
| IT9       | PR               | CR                    | Yes      |
| IT10      | PR               | PR                    | No       |
| IT11      | PR               | PR                    | No       |
| IT13      | PR               | CR                    | No       |
| IT14      | CR               | CR                    | Yes      |
| IT15      | CR               | CR                    | No       |
| IT16      | SD               | PR                    | No       |
| IT17      | PR               | PR                    | No       |
| IT18      | PR               | CR                    | Yes      |
| IT19      | SD               | SD                    | No       |
| IT20      | PR               | CR                    | No       |
| IT21      | SD               | SD                    | No       |
| IT23      | CR               | CR                    | Yes      |

**Table 6 – Clinical Response vs. Histological Response** - This table illustrates the unexpected inconsistency between complete, partial clinical response and histological response assessed from the representative punch biopsy. Partial clinical responders who actually made a histological response are highlighted in grey; complete clinical responders who did not make a histological response are highlighted in blue. CR – complete clinical response; PR – partial clinical response; SD – stable disease; No – no histological response; Yes – histological response.
| SIZE OF THE LESION in mm | HISTOLOGY |
|-------------------------|-----------|
|                         | Wk 0 | Wk 10 | Wk 20 | Wk 52 | Wk 0 | Wk 10 | Wk 20 | Wk 52 |
| IT1                     | 80   | 35    | 30    | 40    | VIN1/2/3 | VIN1/2/3 | VIN2/3 | VIN2/3 |
| IT2                     | 75   | 60    | 70    | 70    | VIN3     | VIN1/2/3 | VIN2/3 | VIN2/3 |
| IT3                     | 90   | 65    | 40    | 30    | VIN2/3   | VIN2/3   | VIN2/3 | VIN1/2 |
| IT4                     | 50   | 40    | 30    | 45    | VIN2/3   | VIN2/3   | VIN2/3 | VIN2/3 |
| IT5                     | 60   | 10    | 0     | 0     | VIN1/2/3 | no VIN   | no VIN | no VIN |
| IT8                     | 65   | 25    | 5     | 0     | VIN2/3   | no VIN   | no VIN | no VIN |
| IT9                     | 100  | 50    | 50    | 0     | VIN3     | no VIN   | no VIN | no VIN |
| IT10                    | 60   | 25    | 25    | 25    | VIN2/3   | VIN2/3   | VIN2   | no VIN |
| IT11                    | >100 | 25    | 20    | 10    | VIN3     | VIN2/3   | VIN2/3 | VIN2/3 |
| IT13                    | 25   | 10    | 0     | 0     | VIN2/3   | VIN1/2/3 | no VIN | no VIN |
| IT14                    | 25   | 0     | 0     | 0     | VIN3     | no VIN   | no VIN | no VIN |
| IT15                    | 20   | 0     | 0     | 0     | VIN3     | **VIN1/2** | no VIN | no VIN |
| IT16                    | 50   | 40    | 20    | 0     | VIN3     | VIN1/2   | no VIN | no VIN |
| IT17                    | 90   | 15    | 15    | 0     | VIN2/3   | VIN1/2   | no VIN | no VIN |
| IT18                    | 25   | 3     | 0     | 0     | VIN3     | no VIN   | no VIN | no VIN |
| IT19                    | 45   | 20    | 30    | 40    | VIN2/3   | VIN2/3   | VIN2/3 | VIN2/3 |
| IT20                    | 40   | 3     | 4     | 0     | VIN2     | VIN1/2   | no VIN | no VIN |
| IT21                    | 30   | 20    | 10    | 20    | VIN2/3   | VIN2/3   | VIN2/3 | VIN2/3 |
| IT23                    | >100 | 0     | 0     | 0     | VIN2/3   | no VIN   | no VIN | no VIN |

**Table 7- Clinical Response vs. Histological Response - Lesion size in mm at weeks 0, 10, 20 and 52 vs. histological analysis of the representative biopsy at weeks 0, 10, 20 and 52.** This table shows the overall size of the lesions decided on vulvoscopy at different time points and the respective histology of the representative biopsy of the lesions. Women who were positive for VIN on vulvoscopy but negative on histology are highlighted in grey; women who were negative for VIN on vulvoscopy but positive on histology for VIN are highlighted in blue.
In one case (IT15), clinical examination was indicative of complete clinical response with no detectable lesion, biopsy however revealed VIN 1 and 2 on histological analysis. Rather similarly, on ten occasions, clinical examination was indicative of partial clinical response with some residual VIN, but the comprehensive histological analysis of the representational biopsy indicated resolution of VIN, as shown in (tables 6 and 7).

### 3.5.4 TREND IN THE SYMPTOM RESPONSES

At baseline, fifteen women (79%) were experiencing moderate to severe symptoms, in comparison to eleven women (58%) at week 10, five women (26%) at week 20 and four women (21%) at week 52 as shown in table 8. The fall in the number of women who were experiencing severe symptoms was statistically significant between weeks 0 and 52 (P=0.01, Fisher’s exact test). Consequently, an improvement in symptoms from the moderate or severe category to the none or mild category (symptom response) was observed in 37% at week 10, 67% at week 20 and 80% at week 52 as shown in figures 23 and 24.

![Figure 23 – Trend in the Symptom Response](image)

**Figure 23 – Trend in the Symptom Response**  An increase in symptom response from 37% at week 10 to 67% at week 20 and 80% at week 52 was evident.
Figure 24-None/Mild symptoms vs. Moderate/Severe symptoms - This figure shows an increase in the percentage of ‘none/mild’ symptom category from 21% at recruitment (baseline) to 42% at week 10, 74% at week 20 and 79% at week 52 and a corresponding fall in the ‘moderate/severe’ category of symptoms from 79% at baseline to 58% at week 10, 26% at week 20 and 21% at week 52 (P=0.01 at week 52).
### INDIVIDUAL SYMPTOM RESPONSE

| Recruitment | After imiquimod | After vaccine | Week 52 |
|-------------|----------------|---------------|---------|
| IT1α        | Mild           | Moderate      | Mild    |
| IT2α        | Severe         | Moderate      | Moderate |
| IT3α        | Moderate       | Severe        | Mild    |
| IT4α        | Severe         | Moderate      | Mild    |
| IT5β        | Mild           | Moderate      | None    |
| IT8β        | Severe         | Mild          | None    |
| IT9β        | Severe         | Mild          | Mild    |
| IT10β       | Severe         | Severe        | Severe  |
| IT11α       | Severe         | Moderate      | Mild    |
| IT13β       | Severe         | None          | None    |
| IT14β       | Moderate       | Moderate      | None    |
| IT15β       | Moderate       | Moderate      | None    |
| IT16β       | None           | Mild          | None    |
| IT17β       | Moderate       | None          | Moderate|
| IT18β       | Severe         | None          | None    |
| IT19α       | Severe         | Severe        | Severe  |
| IT20β       | Moderate       | Moderate      | Moderate|
| IT21α       | Mild           | Mild          | Mild    |
| IT23β       | Severe         | Mild          | None    |

**Table 8 – Individual Symptom Response** – this table shows the different categories of symptoms experienced by women at different time points. α - women who did not make a histological response at week 52; β - women who made a histological response
3.5.5 TREND IN THE HPV RESPONSES

3.5.5.1 PRESENCE OF HPV 16 GENOTYPE

At weeks 0, 10, 20 and 52 - 14, 8, 9 and 10 of the nineteen women were HPV16 positive respectively.

3.5.5.2 PRESENCE OF OTHER HIGH-RISK HPV GENOTYPES

One woman (IT9) was also HPV 33 and 84 positive in addition to HPV16. This participant although made a complete clinical, histological and symptom response at week 52 was found to have developed a focus of early stromal invasion at the site of the previous punch biopsy between weeks 26 and 52. A 4 mm punch biopsy of the suspicious area was taken which served as both diagnostic and therapeutic in this case and no further treatment was warranted.

One other participant (IT20) on HPV genotyping was also positive for types 33,42,53,61 in addition to HPV16. This woman was subsequently withdrawn from the trial at week 4, half way through the imiquimod treatment, for having developed a new suspicious lesion which on biopsy was confirmed to be an early stromal invasion, warranting close clinical follow-up.

3.5.5.3 ABSENCE OF HPV 16 GENOTYPE

Four women (IT1, 13, 14, 15) were negative for any HPV type at baseline. This did not disqualify them from participating in the trial, as HPV16 positivity at baseline was not an essential criterion for trial enrolment. In fact, on subsequent biopsies at different time-points, three of these participants were HPV 16 (IT1, IT15), HPV 84 (IT14) and HPV 33 (IT15) positive. One woman (IT3) was only HPV 42 positive. One woman (IT13) was exclusively and consistently negative for any of the HPV genotypes at all the time points. This participant demonstrated a complete clinical, histological and symptom response at both weeks 20 and 52 respectively.
3.5.6 HPV CLEARANCE VS. CLINICAL, HISTOLOGICAL & SYMPTOM RESPONSE

As shown in figure 25 and table 9, at week 10, six women appeared to have cleared HPV 16 from the biopsy site. Of these six women, one woman demonstrated a complete clinical response and five women, partial clinical response. In addition, in four of the six women histological response was also confirmed.

At week 20, the outcome of HPV typing was not evaluable in two women due to technical difficulties with the test method. Four women who were previously HPV 16 positive were now HPV 16 negative on the biopsy. Complete clinical response was observed in two of these women and partial clinical response in the remaining two. All four women demonstrated a histological response. Symptom response was observed in three participants.

At week 52, nine women were HPV 16 negative. Six of these participants were previously HPV 16 positive. Of these six participants who showed HPV 16 clearance from the biopsy site, five women demonstrated clinical, histological and symptom responses and one woman did not.

Also at week 52, five out of ten (50%) histological responders and one out of six (17%) histological non-responders who were initially HPV 16 positive cleared HPV. This however did not reach statistical significance. Additionally there were no differences between women who made a histological response and who did not with respect to HPV detection at baseline (P=0.66, Fisher’s exact test).
Figure 25 – Trend in the HPV Response - This figure shows the HPV 16 response rates at weeks 10, 20 and 52. 43% who were previously HPV 16 positive were clear of HPV 16 on the biopsy at week 10, compared to 33% at week 20 (only 17 evaluable patients compared to 19 at weeks 0, 10 and 20) and 40% at week 52. No statistical significant differences were observed at any of the time points.
### Table 9 – Individual HPV Response

- **Primary endpoint week 52**

|     | Recruitment | Post imiquimod | Post vaccination | Primary endpoint week 52 |
|-----|-------------|----------------|----------------|--------------------------|
| IT1α | Neg         | Neg            | 16             | 16                       |
| IT2α | 16          | 16             | 16             | 16                       |
| IT3α | 42          | neg            | neg            | neg                      |
| IT4α | 16          | 16             | 16             | neg                      |
| IT5β | 16          | neg            | neg            | neg                      |
| IT8β | 16          | 16             | neg            | 16,53                    |
| IT9β | 16,33,84    | neg            | n/e            | 33                       |
| IT10β| 16          | 16,33,81       | n/e            | 16                       |
| IT11α| 16          | 16             | 16             | 16                       |
| IT13β| neg         | neg            | neg            | neg                      |
| IT14β| neg         | neg            | 84             | 84                       |
| IT15β| neg         | 33             | neg            | 16,33                    |
| IT16β| 16          | 16             | 16             | 16                       |
| IT17β| 16          | neg            | neg            | neg                      |
| IT18β| 16          | neg            | 16             | neg                      |
| IT19α| 16          | 16             | 16             | 16                       |
| IT20β| 16          | neg            | 16             | 16                       |
| IT21α| 16          | 16             | 16             | 16                       |
| IT23β| 16          | neg            | 6              | 6                        |

This table shows the different HPV genotypes detected on the biopsy at different time points. α - women who did not make a histological response at week 52; β - women who made a histological response, n/e, non evaluable;
Figure 26 – Clinical, histological, symptom and HPV responses at weeks 10, 20 and 52. This figure compares the response rates between different time points of week 10 – post imiquimod, wk 20 – post vaccination and wk 52 – primary end-point for the clinical, histological, symptom and HPV response categories. At weeks 10, 20 and 52, clinical response was 74%, 84% and 79%; histological response was 32%, 58% and 63%; symptom response was 37%, 67% and 80%; HPV response was 43%, 33% and 40% respectively.
3.5.7 SAFETY, TOXICITY & TOLERABILITY OF THE TREATMENT REGIME

One of the secondary study objectives was to evaluate the safety, toxicity and tolerability of the treatment regime. Overall the treatment schedule was safe. Only one adverse event was detected, which was not serious. No serious adverse-events were observed. Tolerance to imiquimod, however, was an issue. This section details the tolerability to imiquimod treatment.

3.5.7.1 TOLERABILITY TO IMIQUIMOD TREATMENT

3.5.7.1.1 IMIQUIMOD COMPLIANCE, DOSE REDUCTIONS, AND TREATMENT BREAKS

In this study, imiquimod application schedule was for eight weeks, in a dose escalating manner, building up to three applications each week for the final six weeks. Seventeen women (90%) completed the prescribed eight-week course of the imiquimod treatment. One participant (IT3) did not comply with the treatment schedule and one other participant (IT21) was unexpectedly advised to discontinue imiquimod treatment due to an adverse event. Of the remaining seventeen women who completed the course of imiquimod treatment, sixteen women required a break from the treatment due to the side effects. ‘Treatment break’ was defined as a ‘interruption from the treatment schedule for duration of few days but never more than a period of one week’, the purpose of which was to allow the side effects to calm down and improve treatment compliance. Women were advised not to have more than two ‘treatment breaks’ in total. Nine out of seventeen women (50%) completed the full course of the imiquimod treatment but with ‘treatment breaks’. The remaining eight women (50%) who completed the full eight-week schedule of the imiquimod treatment required ‘dose reduction’. ‘Dose reduction’ was defined as ‘reduction in the imiquimod dosing frequency from three applications per week to no less than two applications per week’. As shown in
figure 27, dose reduction varied from one dose to up to a maximum of four imiquimod doses.

Only one woman (IT18) could tolerate the imiquimod treatment regime entirely without any treatment breaks or dose reductions. At the time of recruitment, this woman was diagnosed with a unifocal lesion on the lateral aspect of the left labia majora.

![IMIQUIMOD DOSING](image)

**Figure 27 – Imiquimod dose per participant** - this figure shows the number of imiquimod doses used by the individual trial participants. 9 out of 19 women completed the full-prescribed 21-dose imiquimod regime. All women except IT18 required a break from the treatment schedule.

### 3.5.7.1.2 SIDE EFFECTS ASSOCIATED WITH THE IMIQUIMOD TREATMENT

Every one of trial participants reported side effects associated with the imiquimod treatment. The most common side effects experienced were localised burning, soreness, aching, smarting during micturition and lumpiness or swelling. Skin peeling at the site of imiquimod application and ulceration...
was also apparent in some women on examination. In fact two women necessitated oral antibiotics for the treatment of superimposed bacterial infection of the ulcerated areas. This was further confirmed as excessive growth of mixed skin flora on swabs taken for bacterial culture and sensitivity. Additionally, majority of the women also mentioned flu like symptoms i.e. symptoms of fatigue, myalgia, arthralgia, headaches, apathy and weariness associated with the imiquimod application. Side effects were first evident in the majority of women at week three with gradual build up in severity with continuing use of the imiquimod cream application.

Clearly as shown in the table 10, the side effects although varied in intensity from person to person, were extreme and categorised as ‘severe’ (severely interfering with lifestyle) in 79% and ‘moderate’ (moderately interfering with lifestyle) in intensity in the remaining 24% of women.

Upon further close questioning, the problems women faced with the application of imiquimod cream on the labia majora, labia minora, introitus, peri-urethra and peri-anal areas were relatively site-specific. In addition to finding it difficult to sit down or walk without experiencing discomfort, women with periurethral lesions found micturition extremely painful, whereas women with peri-anal lesions found defecating, wiping and keeping the area clean just as agonizing. Also, using sanitary protection at the time of menstrual periods was pointed out to be incredibly cumbersome by some of the participants.

Figures 28 a and b elicit the vulval inflammation and excoriation commonly observed with imiquimod treatment.
3.5.7.1.3 PATIENT DRIVEN APPROACH TO COPE WITH THE IMIQUIMOD TREATMENT

Most women believed they obtained relief from cold-water baths. Some women altered the timing of imiquimod application to daytime, when they were more likely to be distracted from the treatment related side effects as opposed to the suggested night time applications, when their sleep was disturbed due to the treatment related side effects. During the final weeks of the imiquimod treatment, some of the participants who were fairly sore and inflamed on the
vulva took help in applying the imiquimod cream from either close family members or their family doctor.

3.5.7.1.4 CLINICAL STRATEGIES TO IMPROVE COMPLIANCE WITH IMIQUIMOD TREATMENT

Women were advised to take oral analgesia as and when required and prescriptions were provided. A small proportion of women required potent oral opioid analgesia. Five women (26%) required a prescription for anxiolytics, sedatives and/or antihistamines to help them sleep better and to relieve vulval itching. A regularly supply of Vaseline coated dressings (gelonet) to create a non-adhesive barrier between the raw surfaces on the vulva and patient’s clothing was also made available. With the participant’s consent, their general practitioner was informed. Upon request, a brief letter to the participant’s employer explaining the clinical situation at that point of time was presented. Topical local anaesthetic cream or gel (Emla cream, istillagel pre-filled syringes) for application on the skin or mucosal surfaces was also offered having received positive feedback from the initial few participants who found it beneficial. Mostly women needed regular encouragement over phone.

3.5.7.1.5 ADVERSE EVENT ASSOCIATED WITH IMIQUIMOD TREATMENT

One out of the nineteen women (IT21) experienced unusual side effects with the imiquimod treatment. She initially remarked on severe headache and dizziness through the fourth week of the imiquimod treatment. There was uncertainty as regards the cause, warranting a thorough clinical examination, urinalysis and blood tests. No abnormality was detected and the woman felt better within few days of taking a break from the imiquimod treatment. Two weeks later imiquimod treatment was resumed. No further headaches or dizziness occurred except during week six of imiquimod treatment, she developed flu like illness with myalgia, arthralgia and generalised unwell feeling. She also complained of a rash. She was on a holiday when this happened and noticed the rash to be photosensitive. Steven-Johnson syndrome was suspected. Examination revealed a generalised papular rash,
more severe on the dorsal aspects of her upper limbs. Although the conjunctiva and oral mucosa appeared inflamed, no obvious ulcerations were visible. All the standard tests were within normal limits. She was commenced on a low dose steroid cream and was advised to stop using the imiquimod cream and felt normal within two weeks. This adverse event did not exceed grade two of the ‘Common Terminology Criteria for Adverse Events of the National Cancer Institute’ and therefore no further action related to reporting the adverse event to higher authorities was taken.

3.5.7.1.6 THE EFFECT OF IMIQUIMOD TREATMENT ON THE SKIN
During the course of the imiquimod treatment, three women observed a dramatic remission in facial acne. An improvement in their generalised skin condition with smoother skin and fewer spots was also obvious. While assessing the effect of imiquimod on the skin was not one of the trial objectives, it was nevertheless interesting and worthy of note.
|   | Side effects | Adverse Effects | Treatment Breaks | Dose Reductions Number | Symptom Improvement |
|---|--------------|----------------|------------------|------------------------|---------------------|
| IT1 | Severe       | No             | Yes              | Yes-4                  | No                  |
| IT2 | Severe       | No             | Yes              | Yes-3                  | Yes                 |
| IT3 | Severe       | No             | Yes              | Yes-8                  | No                  |
| IT4 | Severe       | No             | Yes              | Yes-4                  | Yes                 |
| IT5 | Severe       | No             | Yes              | Yes-2                  | No                  |
| IT8 | Severe       | No             | Yes              | Yes-2                  | No                  |
| IT9 | Moderate     | No             | Yes              | No                     | Yes                 |
| IT10| Severe       | No             | Yes              | No                     | No, Acne improved   |
| IT11| Severe       | No             | Yes              | Yes-3                  | Yes                 |
| IT13| Moderate     | No             | Yes              | No                     | No                  |
| IT14| Moderate     | No             | Yes              | No                     | No                  |
| IT15| Severe       | No             | Yes              | No                     | No, Acne improved   |
| IT16| Severe       | No             | Yes              | No                     | No                  |
| IT17| Severe       | No             | Yes              | Yes-1                  | Yes, Acne improved  |
| IT18| Moderate     | No             | No               | No                     | Yes                 |
| IT19| Severe       | No             | Yes              | No                     | No                  |
| IT20| Severe       | No             | Yes              | Yes-2                  | No                  |
| IT21| Severe       | Yes            | Yes              | Yes-3                  | No                  |
| IT23| Severe       | No             | Yes              | No                     | Yes                 |

*Table 10 - Tolerability of imiquimod treatment* - Details of side effects, treatment breaks, dose reductions and symptom improvement
3.5.7.2 TOLERABILITY TO TA-CIN VACCINATION

Eighteen out of nineteen women received all the three doses of the TA-CIN vaccination. One woman (IT21) received only two doses of the vaccination and missed out the third dose due to the vaccine expiration. TA-CIN vaccination was well tolerated with no evident side effects or adverse events. The only side effect experienced by all the women was a sharp and smarting sensation followed by a dull ache or discomfort lasting for few hours at the injection site.
Figure 29 Complete clinical response in patient 8 (a) pre-treatment (b) post imiquimod wk 10 (c) post vaccination wk 20 (d) week 52
Figure 30 Partial clinical response in patient 11 (a) pre-treatment (b) post imiquimod wk 10 (c) post vaccination wk 20 (d) week 52
CHAPTER 4
IMMUNOLOGICAL RESULTS
4 IMMUNOLOGICAL RESULTS

In addition to evaluating the clinical responses, representative tissue biopsies from the VIN lesions and the blood samples from the nineteen women who participated in the trial were also considered to improve our understanding of the immunological phenomena, both within the lesions and systemically, in response to the treatment at various stages. It is already established that VIN lesions contain increased number of helper (CD4) and cytotoxic (CD8) T lymphocytes when compared to the normal vulval epithelium [Abdel-Hady et al., 2001; Davidson et al., 2003c; Gul et al., 2004]. Effect of treatment on the local immune environment is likely to determine and influence the clinical outcome of treatment [Davidson et al., 2003c]. Imiquimod acts at least partly through inducing a local inflammatory response whilst TA-CIN induces virus specific T lymphocyte responses. In this context, the local immunological monitoring of the lesion was carried out using immunofluorescence with emphasis on T regulatory cells on sections of frozen tissue samples collected from women who participated in the trial. The emphasis on examining the local immune environment was to decide whether the numbers of infiltrating lymphocytes altered in response to the treatment with imiquimod and TA-CIN.

HPV 16 specific systemic responses were determined using proliferation assays to the different HPV antigens. In addition, neutralising antibody assays were also carried out.

4.1 DEFINITION OF TREATMENT RESPONDERS AND TREATMENT NON-RESPONDERS

In the clinical results chapter, discrepancy in the results between clinical responses, complete clinical responses and histological responses was shown. Considering histological response to any treatment is traditionally considered to be the gold standard, we defined ‘complete clearance of VIN on histology of the punch biopsy at week 52, the study primary end-point ’ as ‘treatment response’ and ‘failure to clear VIN on histology of the punch biopsy at week 52’ as ‘treatment non-response’. Women who made a treatment
response were called ‘responders’ (R) and women who did not make a treatment response were called ‘non-responders’ (NR).

All the results from this section onwards are compared between treatment responders (R) and treatment non-responders (NR). As shown in the table 11, twelve treatment responders (IT5, 8,9,10,13,14,15,16,17,18,20,23) and seven treatment non-responders (IT1, 2,3,4,11,19,21) were identified.

| IT   | Histology at Wk 52 | Response Category |
|------|-------------------|-------------------|
| IT1  | VIN2/3            | NR                |
| IT2  | VIN2/3            | NR                |
| IT3  | VIN1/2            | NR                |
| IT4  | VIN2/3            | NR                |
| IT5  | no VIN            | R                 |
| IT8  | no VIN            | R                 |
| IT9  | no VIN            | R                 |
| IT10 | no VIN            | R                 |
| IT11 | VIN2/3            | NR                |
| IT13 | no VIN            | R                 |
| IT14 | no VIN            | R                 |
| IT15 | no VIN            | R                 |
| IT16 | no VIN            | R                 |
| IT17 | no VIN            | R                 |
| IT18 | no VIN            | R                 |
| IT19 | VIN2/3            | NR                |
| IT20 | no VIN            | R                 |
| IT21 | VIN2/3            | NR                |
| IT23 | no VIN            | R                 |

Table 11 – Assignment of treatment response category- this table shows the individual histology results of the punch biopsy of the index lesion or the residual VIN on vulvoscopy at week 52 (study primary end-point) and the corresponding treatment response status. R – treatment responder; NR – treatment non-responder. 12 treatment responders and 7 treatment non-responders were identified.
4.2 TUMOUR INFILTRATING LYMPHOCYTES

Cryostat sectioning of the punch biopsies was planned and the slides were laid out and labelled in way to facilitate serial sections to be placed consecutively on the slides. When studying the proportion of different types of the T lymphocytes in the biopsy specimen, serially laying out the sections to some extent maintained the continuity of the tissue architecture.

The densities per unit area of CD4, CD8 and T regulatory (Tregs) lymphocytes (FoxP3CD4) were assessed. For the detection of Tregs and the ratio of CD4 and CD8 lymphocytes within a tissue section, double immunofluorescence labelling was carried out. This enabled simultaneous detection of FoxP3CD4 and CD4+ lymphocytes on one spot of the tissue section, and similarly FoxP3CD4 and CD8 + lymphocytes on one other spot and CD4 and CD8 T lymphocytes on one other spot (two slides with two spots each were used for the detection of the above mentioned lymphocytes. The fourth spot was used for control antibodies). This approach was more practical in overcoming any inter section variability in the densities of the T lymphocyte infiltration, more so when the aim was to assess the proportions of different types of lymphocytes in each of the tissue sections.

Tissue samples from the three clinically relevant time-points of week 0 (pre-treatment) post imiquimod but pre TA-CIN vaccination treatment (week 10) and post TA-CIN treatment (week 20) were available for all the nineteen women.
4.2.1 TUMOUR INFILTRATING LYMPHOCYTES

At recruitment the majority of the lymphocytes infiltrating the VIN lesions were CD4 expressing lymphocytes (median 104; range 63 to 188) with a smaller proportion of CD8 expressing lymphocytes (median 38; range 26 to 68). FoxP3CD4 expressing cells could also be detected in much smaller numbers (median 14; range 6 to 33). At recruitment, the median density per unit area of CD4 expressing cells in the responder group was 80 (range 58-130) compared to a median of 109 (range 82-190) in the non-responder group; The median density of CD8 expressing lymphocytes was 40 (range 27-67) in the responder group compared to a median of 37(range 22-97) in the non-responder group. The median density of FoxP3CD4 expressing T regulatory lymphocytes in the responder group was 11 (range 2-14) in comparison to a median of 25 (range 18-35) in the non-responder group as shown in table 12.

The trends in the median density of CD4, CD8 and FoxP3 lymphocytes following imiquimod and vaccination treatment in both the responder and non-responder patient groups are shown in figure 31. In the treatment responders, a significant increase in the CD4 cell count was noticed at week 20 compared to pre-treatment levels (P=0.03). A similar significant increase in the CD8 lymphocyte count was observed in the responder group, however, both at weeks 10 and 20 (P=0.05, 0.03 respectively). In contrast, a significant increase in FoxP3CD4 positive cells was only observed in the non-responder group, at week 20 (P=0.05) as shown in table 13.
|                        | Group (n=19) | Responders (n=12) | Non-Responders (n=7) |
|------------------------|--------------|-------------------|----------------------|
| **Recruitment**        |              |                   |                      |
| CD4                    | 104 (63-188) | 80 (58-130)       | 109 (82-190)         |
| CD8                    | 38 (26-68)   | 40 (27-67)        | 37 (22-97)           |
| FoxP3CD4               | 14 (6-33)    | 11 (2-14)         | 25 (18-35)           |
| **Week 10**            |              |                   |                      |
| CD4                    | 103 (65-155) | 93 (67-130)       | 139 (48-160)         |
| CD8                    | 61 (30-111)  | 51 (24-110)       | 66 (50-145)          |
| FoxP3CD4               | 25 (8-37)    | 26 (8-39)         | 22 (6-31)            |
| **Week 20**            |              |                   |                      |
| CD4                    | 170 (76-301) | 124 (65-214)      | 215 (76-350)         |
| CD8                    | 89 (30-142)  | 72 (20-106)       | 152 (37-170)         |
| FoxP3CD4               | 19 (5-50)    | 8 (4-37)          | 43 (19-95)           |

**Table 12** - Lesion associated immune cells in the group, responders, non-responders at recruitment, following Imiquimod (week 10) and TA-CIN (week 20). Median number +/- upper and lower quartiles of lesion associated immune cells in the brackets in patient biopsies.
Figure 31 – Lesion associated immune cells in the Responders (R) and Non-Responders (NR) groups pre-treatment (Wk 0), post-imiquimod (Wk 10), post-vaccination (Wk 20). Median number +/- upper and lower quartiles per unit area of the biopsy are shown in box-feather plots. Wilcoxon signed rank test was used to determine the significance of within group differences pre and post-treatment. † denotes a statistically significant value. P value of <0.05 was considered statistically significant.
4.2.2 TUMOUR INFILTRATING LYMPHOCYTES AT RECRUITMENT RESPONDERS VS. NON-RESPONDERS

Upon comparing the differences between tumour infiltrating CD4, CD8 and FoxP3CD4 cell densities between the responder and the non-responder groups at recruitment as shown in table 12, with the Mann-Whitney U test, the difference for CD4 and CD8 lymphocytes was not statistically significant (P=0.2; 0.5 respectively) but non-responders had significantly higher infiltration of FoxP3CD4 positive lymphocytes in comparison to the responders (P=0.05) as shown in table 13.

4.2.3 TUMOUR INFILTRATING LYMPHOCYTES AT WEEK 10 (post imiquimod) and at WEEK 20 (post vaccination) - RESPONDERS VS. NON-RESPONDERS

As shown in table 13 at recruitment, the median density per unit area of CD4 expressing cells in the responder group was 93 (range 67-130) compared to a median of 139 (range 48-160) in the non-responder group; The median density of CD8 expressing lymphocytes was 51 (range 24-110) in the responder group compared to a median of 66(range 50-145) in the non-responder group. The median density of FoxP3CD4 expressing T regulatory lymphocytes in the responder group was 26 (range 8-39) in comparison to a median of 22 (range 6-31) in the non-responder group.

Upon comparing the differences between tumour infiltrating CD4, CD8 and FoxP3CD4 cell densities between the responder and the non-responder groups at week 10 with the Mann-Whitney U test, the difference in the density of infiltration for CD4, CD8 and FoxP3CD4 lymphocytes was again not statistically significant (P=0.2; 0.2, 0.2 respectively).
Table 13 Lesion associated immune cells in whole group, responders, non-responders pre-treatment, following Imiquimod (week 10) and TA-CIN (week 20) with statistical significance. Median number +/- upper and lower quartiles of lesion associated immune cells in patient biopsies. The Wilcoxon’s signed ranks test was used to determine the significance of within group differences pre and post treatment.

4.2.4 TUMOUR INFILTRATING LYMPHOCYTES AT WEEK 20 (post vaccination) - RESPONDERS VS. NON-RESPONDERS

As shown in table 13, at week 20, the median density per unit area of CD4 expressing cells in the responder group was 124 (range 65-214) compared to a median of 215 (range 76-350) in the non-responder group; The median density of CD8 expressing lymphocytes was 72 (range 20-106) in the responder group compared to a median of 152(range 37-170) in the non-responder group. The median density of FoxP3CD4 expressing T regulatory
lymphocytes in the responder group was 8 (range 4-37) in comparison to a median of 43 (range 19-95) in the non-responder group.

Upon comparing the differences between tumour infiltrating CD4, CD8 and FoxP3CD4 cell densities between the responder and the non-responder groups at week 20 with the Mann-Whitney U test, the difference for CD4 and CD8 lymphocytes was again not statistically significant (P=0.1; 0.07 respectively) but similar to the findings on the pre-treatment biopsy at recruitment where the potential non-responders demonstrated higher infiltration of FoxP3CD4 cells and in contrast to the findings at week 10, at week 20, non-responders showed significantly higher infiltration of FoxP3CD4 positive lymphocytes in comparison to the responders and this was statistically significant (P=0.01).

4.3 THE CYTOTOXIC VS. HELPER T LYMPHOCYTE RATIO PRE-TREATMENT, WEEK 10 AND WEEK 20

In the vulval epidermis, CD8 T lymphocytes are shown to be more prevalent with equal distribution of CD4 and CD8 T lymphocytes in the vulval dermis [Abbas and Janeway, Jr., 2000]. We were interested in assessing the CD8:CD4 ratios following treatment and compare the results with pre-treatment values, expecting an enhancement in the CD8 T lymphocyte infiltration [Gul et al., 2004; Piersma et al., 2008; Curiel et al., 2004; Wolf et al., 2005a]. Table 14 shows the individual ratios of CD8 and CD4 density in the tumour at different time-points.
Table 14 Ratio of CD8:CD4 density in the individual participants at weeks 0, 10 and 20. This table shows the trend in the ratio of CD8:CD4 double immunofluorescence labelled lymphocytes pre-treatment (Wk0), post imiquimod (Wk10) and post vaccination (Wk 20). α represents - women who did not make a histological response at week 52; β represents - women who made a histological response.
When CD8:CD4 ratios are compared as shown in table 15, the CD8:CD4 ratio increased to 0.4 from 0.3 at both weeks 10 and 20, in the group as a whole and this increase was statistically significant (P=0.006 at week 10) and (P=0.03 at week 20) at both the time-points.

However, when the group was stratified into treatment responders and non-responders, the increase in CD8:CD4 ratio was significant only in the responder group at both weeks 10 and 20 (P=0.02 at week 10 and P=0.05 at week 20). A significant increase in the ratio of CD8:CD4 was evident in the non-responders at week 10, following imiquimod, but this enhancement was not significant any more at week 20.

| Group (n=19)       | CD8:CD4 Ratio |
|--------------------|---------------|
| Wk0                | 0.25 (0.1-0.4)|
| Wk10               | 0.42 (0.1-0.7)|
| Wk20               | 0.37 (0.1-0.7)|
| Wk0 vs. wk10       | P=0.006       |
| Wk0 vs. wk20       | P=0.03        |

| Responders (n=12) | CD8:CD4 Ratio |
|-------------------|---------------|
| Wk0               | 0.22 (0.1-0.5)|
| Wk10              | 0.40 (0.1-0.7)|
| Wk20              | 0.48 (0.1-0.8)|
| Wk0 vs. wk10      | P=0.02        |
| Wk0 vs. wk20      | P=0.03        |

| Non-Responders (n=7) | CD8:CD4 Ratio |
|----------------------|---------------|
| Wk0                  | 0.29 (0.1-0.7)|
| Wk10                 | 0.59 (0.1-0.5)|
| Wk20                 | 0.36 (0.1-0.5)|
| Wk0 vs. wk10         | P=0.02        |
| Wk0 vs. wk20         | P=0.2         |

Table 15 Comparison in the ratio of CD8:CD4 between wk 0 and 10; wk 0 and 20 in the group, responders and non-responders. The Wilcoxon’s signed ranks test was used to determine the significance of within group differences pre and post treatment.
4.4 SUMMARISING THE OVERALL INTRALESIONAL LYMPHOCYTE RESPONSES

In the group as a whole, significant increases in the number of CD4, CD8 and FoxP3CD4 positive T lymphocytes were evident by week 20 compared to pre-treatment levels (P=0.03, P=0.01, P=0.04 respectively); a significant increase after imiquimod treatment was only apparent for CD8 lymphocytes in the group (P=0.04) and treatment responders (P=0.05). Further stratification of the group into responders and non-responders led us to believe that the significant increase in CD4 and CD8 cells witnessed in the group was only significant in the treatment responders (p=0.03, 0.03 respectively). Conversely the significant increase in FoxP3CD4 cells noted in the group was actually only significant in the treatment non-responders (p=0.05). Although the non-responders at week 20 did demonstrate a trend towards denser infiltration of CD4 and CD8 lymphocytes, this did not reach statistical significance (p=0.2, 0.1 respectively).

There were no significant pre-treatment differences in the density of CD4 or CD8 T cells in the treatment responder and non-responders (P=0.2, P=0.5 respectively) but intralesional FoxP3CD4 density was significantly higher in non-responders compared to responders (P=0.05). By week 20, a significant reduction in FoxP3CD4 T cell population density was apparent in the treatment responders, which was significantly different from non-responder densities (P=0.01).
4.5 SYSTEMIC IMMUNE RESPONSES

4.5.1 LYMPHOCYTE PROLIFERATIVE RESPONSES TO HPV 16 ANTIGENS

Lymphoproliferation to HPV antigens was used to analyse patient systemic cellular immunity to HPV 16 before and after the imiquimod and vaccination steps in the protocol. PBMC alone or PBMC with HPV antigens: HPV16 E6E7L2 protein - TA-CIN (Xenova Research Ltd.), HPV16 GST E6, HPV16 GST E7[Smyth et al., 2004], HPV16L2 (full length HPV16L2 tagged with 6His at N-terminus), TA-GW (HPV 6 L2E7; Xenova Research Ltd. For negative control) and PHA (for positive control) were performed.

A pre-existing proliferative T lymphocyte response was defined as a stimulation index (SI) of two or greater than two. A post treatment proliferative response was defined as two fold increase in the SI compared with the pre treatment value. This section of this chapter described the lymphoproliferative responses of the trial participants at different time points, pre treatment, following imiquimod treatment and post TA-CIN vaccination. An attempt was also made to compare the lymphoproliferative responses between the responders (women who managed to clear VIN from the biopsy site taken at week 52) and the non-responders (women who failed to clear VIN on histology) pre and post imiquimod and vaccination treatment.

4.5.2 PRE-EXISTING PROLIFERATIVE RESPONSE TO TA-CIN, CORRELATION WITH TREATMENT RESPONSE

As illustrated in the table 16, sixteen of the nineteen women (84%) demonstrated a pre-existing proliferative response to the TA-CIN protein. Of these sixteen women, eleven women (61%) subsequently made a histological response to the treatment and five women (59%) failed to demonstrate a histological response. Of the remaining three women who did not show a pre-existing response to the TA-CIN antigen, one woman subsequently was a responder and the remaining two were non-responders.
The difference in the magnitude of the lymphoproliferative response (SI) between the responder and the non-responder groups was not statistically significant (P=0.4, Fisher’s exact test).

|   | Pre-existing response (SI) | Pre-existing response | Treatment Response |
|---|----------------------------|-----------------------|--------------------|
| IT1 | 7.2                       | Yes                   | No                 |
| IT2 | 1.0                       | No                    | No                 |
| IT3 | 7.0                       | Yes                   | No                 |
| IT4 | 1.9                       | No                    | No                 |
| IT5 | 4.4                       | Yes                   | Yes                |
| IT8 | 2.9                       | Yes                   | Yes                |
| IT9 | 0.9                       | No                    | Yes                |
| IT10 | 26.6                   | Yes                   | Yes                |
| IT11 | 5.1                      | Yes                   | No                 |
| IT13 | 3.0                      | Yes                   | No                 |
| IT14 | 10.9                   | Yes                   | Yes                |
| IT15 | 3.5                      | Yes                   | Yes                |
| IT16 | 3.3                      | Yes                   | Yes                |
| IT17 | 5.6                      | Yes                   | Yes                |
| IT18 | 2.5                      | Yes                   | Yes                |
| IT19 | 7.4                      | Yes                   | No                 |
| IT20 | 27.8                     | Yes                   | No                 |
| IT21 | 6.3                      | Yes                   | No                 |
| IT23 | 6.0                      | Yes                   | No                 |

Table 16 Pre-existing response (SI) and the final treatment response — no statistical significant difference between pre-existing response was noted between the subsequent treatment responders and non-responders (P=0.4 Fisher exact test).
4.5.3 PRE-EXISTING PROLIFERATIVE RESPONSE TO TA-CIN, CORRELATION WITH HPV POSITIVITY

Women who previously received any HPV vaccination were not eligible to participate in this study. Any demonstrated pre-existing lympho proliferative response to HPV 16 proteins ought to have resulted from the immunological memory associated with previous HPV infection. As shown in the table 17 of the sixteen women who demonstrated a pre-existing response to TA-CIN protein, fourteen women were HPV 16 positive in the biopsy specimen at any given time-point during the study period. Two women were positive for the other oncogenic HPV types (HPV 42 and 84, respectively). One woman who indeed expressed a pre-existing response to TA-CIN (IT15, SI – 3.5) at week 0 was actually negative for any HPV genotype in the biopsy at all the time-points. All the three women who failed to mount a pre-existing response to the TA-CIN fusion protein were all proved to be HPV 16 positive on the biopsy sample.
|   | Pre-existing Response (SI) | Pre-existing response | HPV genotypes |
|---|----------------------------|---------------------|---------------|
| IT1 | 7.2                        | Yes                 | 16            |
| IT2 | 1.0                        | No                  | 16            |
| IT3 | 7.0                        | Yes                 | 42            |
| IT4 | 1.9                        | No                  | 16            |
| IT5 | 4.4                        | Yes                 | 16            |
| IT8 | 2.9                        | Yes                 | 16            |
| IT9 | 0.9                        | No                  | 16            |
| IT10 | 26.6                      | Yes                 | 16            |
| IT11 | 5.1                        | Yes                 | 16, 53        |
| IT13 | 3.0                        | Yes                 | 16, 33, 84    |
| IT14 | 10.9                       | Yes                 | 16, 33, 81    |
| IT15 | 3.5                        | Yes                 | Neg           |
| IT16 | 3.3                        | Yes                 | 84            |
| IT17 | 5.6                        | Yes                 | 16, 33        |
| IT18 | 2.5                        | Yes                 | 16            |
| IT19 | 7.4                        | Yes                 | 16            |
| IT20 | 27.8                       | Yes                 | 16            |
| IT21 | 6.3                        | Yes                 | 16            |
| IT23 | 6.0                        | Yes                 | 6, 16         |

*Table 17 Pre-existing response (SI) and the presence of HPV oncogene type – three women who were negative for HPV 16 at any given time point demonstrated a pre-existing response*
4.6 LYMPHOPROLIFERATIVE RESPONSE TO TA-CIN

Treatment with imiquimod cream although was expected to encourage local immune infiltration, HPV antigen specific immune response was unpredicted. Accordingly, a two-fold increase in the SI following imiquimod treatment was only evident in two women from the ‘responder’ group and none from the ‘non-responder’ group. The difference in the magnitude of the SI pre-treatment and post imiquimod treatment in the group, responders or non-responders was not significant, $P = 0.5, 0.8$ and $0.2$ respectively. The difference in the magnitude of proliferative response between pre-treatment and post vaccination treatment was significantly different in the group as a whole and in the responders, $P=0.0002, 0.0002$ respectively, but not in the non-responders, $P=0.3$.

Upon comparison of the magnitude of the stimulation index (SI) between the responder and non-responders groups, statistical significant difference in SI was observed at both week 10, following imiquimod treatment ($P=0.05$) and at week 20, following vaccination treatment ($P=0.003$) but not at recruitment ($P=0.4$). The above data is shown in tables 18, 19 and 20.
| Responders | Pre-treatment | Post imiquimod (Wk 10) | Post vaccination (Wk 20) |
|------------|---------------|------------------------|------------------------|
| IT5        | 4.4           | 6.3                    | 9.4                    |
| IT8        | 2.9           | 4.7                    | 32.9                   |
| IT9        | 0.9           | N/A                    | 12.3                   |
| IT10       | 26.6          | 18.5                   | 84.2                   |
| IT13       | 3.0           | 3.1                    | 3.8                    |
| IT14       | 10.9          | 10.0                   | 31.6                   |
| IT15       | 3.5           | 6.4                    | 10.9                   |
| IT16       | 3.3           | 8.4                    | 15.6                   |
| IT17       | 5.6           | 4.0                    | 11.6                   |
| IT18       | 2.5           | 1.4                    | 5.4                    |
| IT20       | 27.8          | 1.3                    | 13.8                   |
| IT23       | 6.0           | 1.0                    | 17.4                   |
| Non-responders          |               |                        |                        |
| IT1        | 7.2           | 5.7                    | 25.0                   |
| IT2        | 1.0           | 1.2                    | 2.5                    |
| IT3        | 7.0           | 7.2                    | 2.2                    |
| IT4        | 1.9           | 2.0                    | 5.5                    |
| IT11       | 5.1           | 1.6                    | 1.1                    |
| IT19       | 7.4           | 2.2                    | 3.6                    |
| IT21       | 6.3           | 1.1                    | 1.2                    |

Table 18 Proliferative Responses to TA-CIN in the responders and non-responders at weeks 0, 10, 20 Two fold change in the stimulation index (SI) was considered as a response, highlighted in grey, N/A – not available.
|                  | Wk 0 | Wk 10 | Wk 20 | Wk 0 vs. Wk 10 (P, WSR) | Wk 0 vs. Wk 20 (P, WSR) |
|------------------|------|-------|-------|-------------------------|-------------------------|
| **Group (n=19)** | 5.1  | 3.6   | 10.9  | P=0.5                   | P=0.0002                 |
| **Responders**   |      |       |       |                         |                         |
| (n=12)           | 4.0  | 4.7   | 13.1  | P=0.8                   | P=0.0002                 |
| **Non-Responders** |      |       |       |                         |                         |
| (n=7)            | 6.3  | 2.0   | 2.5   | P=0.2                   | P=0.3                   |

Table 19 Change in the lymphoproliferative responses to TA-CIN in the group, responders, non-responders between weeks 0, 10, 20. A significant difference in the magnitude of response (median Stimulation Index SI) was observed in the group and responders following vaccination treatment; Wilcoxon signed rank test (WSR)

|                  | Responders vs. Non-Responders (P, MWU) |
|------------------|----------------------------------------|
| **Wk 0**         | P=0.4                                  |
| **Wk 10**        | P=0.05                                 |
| **Wk 20**        | P=0.003                                |

Table 20 Statistical significance of the difference in the lymphoproliferative response to TA-CIN between responders and nonresponders at weeks 0, 10 and 20, calculated using Mann-Whitney U test.
4.7 LYMPHOPROLIFERATIVE RESPONSE TO HPV 16 L2 PROTEIN

A significant difference in the magnitude of lympho-proliferative response to HPV 16 L2 protein was observed in the group as a whole and in the responders, P=0.01 and 0.05 respectively, but not in the non-responders, P=0.5 as shown in the tables 21 and 22. No significant difference in the magnitude of response was observed between the responders and non-responders at any give time-point as shown in the table 23.

LYMPHOPROLIFERATIVE RESPONSE TO HPV 16 L2 PROTEIN

| Responders | Pre-treatment | Post imiquimod (Wk 10) | Post vaccination (Wk 20) |
|------------|--------------|------------------------|-------------------------|
| IT5        | 2.8          | 6.4                    | 5.2                     |
| IT8        | 0.7          | 6.2                    | 14.7                    |
| IT9        | 1.6          | N/A                   | 7.5                     |
| IT10       | 3.0          | 8.2                    | 21.0                    |
| IT13       | 0.8          | 0.7                    | 0.8                     |
| IT14       | 1.6          | 2.1                    | 2.1                     |
| IT15       | 2.0          | 5.0                    | 3.0                     |
| IT16       | 2.9          | 7.8                    | 7.6                     |
| IT17       | 3.1          | 5.7                    | 7.9                     |
| IT18       | 2.8          | 1.7                    | 2.9                     |
| IT20       | 17.0         | 2.0                    | 9.0                     |
| IT23       | 1.0          | 1.3                    | 3.7                     |
| Non-responders |          |                        |                          |
| IT4        | 3.2          | 3.7                    | 3.0                     |
| IT11       | 3.3          | 1.6                    | 4.7                     |
| IT19       | 2.0          | 2.0                    | 9.4                     |
| IT21       | 17.0         | 2.0                    | 9.0                     |

Table 21 Proliferative Responses to HPV 16L2 in the responders and non-responders at weeks 0, 10, 20 Two fold change in the stimulation index (SI) was considered as a response, highlighted in grey, N/A – not available
Table 22 Change in the lymphoproliferative responses in the group, responders, non-responders between weeks 0, 10, 20. A significant difference in the magnitude of response (median Stimulation Index SI) was observed in the group and responders following vaccination treatment; Wilcoxon signed rank test (WSR).

|                  | Wk 0 | Wk 10 | Wk 20 | Wk 0 vs. Wk 10 (P, WSR) | Wk 0 vs. Wk 20 (P, WSR) |
|------------------|------|-------|-------|-------------------------|-------------------------|
| Group (n=16)     | 2.8  | 2.1   | 6.4   | P=0.4                   | P=0.01                  |
| Responders (n=12)| 2.4  | 5.0   | 6.4   | P=0.07                  | P=0.05                  |
| Non-Responders (n=4) | 3.3  | 2.0   | 6.8   | P=0.7                   | P=0.5                   |

Table 23 Statistical significance of the difference in the lymphoproliferative response to HPV16-L2 between responders and nonresponders at weeks 0, 10 and 20, calculated using Mann-Whitney U test.

|                  | Responders vs. Non-Responders (P, MWU) |
|------------------|---------------------------------------|
| Wk 0             | P=0.09                                |
| Wk 10            | P=0.5                                 |
| Wk 20            | P=0.9                                 |

Table 22 Change in the lymphoproliferative responses in the group, responders, non-responders between weeks 0, 10, 20. A significant difference in the magnitude of response (median Stimulation Index SI) was observed in the group and responders following vaccination treatment; Wilcoxon signed rank test (WSR).

|                  | Responders vs. Non-Responders (P, MWU) |
|------------------|---------------------------------------|
| Wk 0             | P=0.09                                |
| Wk 10            | P=0.5                                 |
| Wk 20            | P=0.9                                 |

Table 23 Statistical significance of the difference in the lymphoproliferative response to HPV16-L2 between responders and nonresponders at weeks 0, 10 and 20, calculated using Mann-Whitney U test.
4.8 LYMPHOPROLIFERATIVE RESPONSE TO GST E6 PROTEIN

4.8.1 RESPONSE TO GST-E6 PROTEIN

At week 10, four responders and one non-responder patient showed a response greater than two fold when compared to week 0. At week 20, eight responders and two non-responders showed a greater than two fold change in the SI when compared to baseline. In the whole group, the differences in the SI's (stimulation indices) between week 0 and week 10 was not significant; however, there was a significant difference between week 0 and 20 (P=0.002), as shown in tables 24 and 25. When the findings were assessed for both the responder and non-responder groups separately, in the responder group, the increase in the SI between weeks 0 and 20 was statistically significant (P=0.005). However, in the non-responder group, no significant difference in the SI's at any time-point was noted. When the responder group was compared to the non-responder group, there was a significant difference in the fold change both at week 10 (post imiquimod) and week 20 (post vaccination) between responders and non-responders (p=0.05) as shown in table 26.
## Proliferative Responses to GST-E6

| Responders | Pre-treatment | Post imiquimod (Wk 10) | Post vaccination (Wk 20) |
|------------|---------------|------------------------|-------------------------|
| IT5        | 5.1           | 11.8                   | 11.9                    |
| IT8        | 0.6           | 1.3                    | 8.3                     |
| IT9        | 1.3           | N/A                    | 9.1                     |
| IT10       | 6.6           | 5.7                    | 16.3                    |
| IT13       | 1.1           | 0.8                    | 0.9                     |
| IT14       | 5.1           | 5.1                    | 3.0                     |
| IT15       | 1.9           | 5.4                    | 5.5                     |
| IT16       | 3.4           | 7.7                    | 10.4                    |
| IT17       | 6.7           | 9.2                    | 11.2                    |
| IT18       | 2.8           | 3.9                    | 7.1                     |
| IT20       | 27.2          | 3.5                    | 9.7                     |
| IT23       | 5.6           | 1.3                    | 11.4                    |

| Non-responders | | |
|----------------|------------------------|
| IT1            | 1.8                    |
| IT2            | 0.6                    |
| IT3            | 0.9                    |
| IT4            | 3.1                    |
| IT11           | 7.1                    |
| IT19           | 7.6                    |
| IT21           | 7.4                    |

Table 24 Proliferative Responses to GST-E6 in the responders and non-responders at weeks 0, 10, 20. Two fold change in the stimulation index (SI) was considered as a response, highlighted in grey. N/A – not available.
|                 | Wk 0 | Wk 10 | Wk 20 | Wk 0 vs. Wk 10 (P, WSR) | Wk 0 vs. Wk 20 (P, WSR) |
|----------------|------|-------|-------|------------------------|------------------------|
| **Group (n=19)** | 3.4  | 2.6   | 7.7   | P=0.8                  | P=0.002                |
| **Responders (n=12)** | 4.3  | 5.1   | 9.4   | P=0.5                  | P=0.005                |
| **Non-Responders (n=7)** | 3.1  | 1.9   | 3.0   | P=0.2                  | P=0.2                  |

**Table 25** Change in the lymphoproliferative responses to GST-E6 in the group, responders, non-responders between weeks 0, 10, 20. A significant difference in the magnitude of response (median Stimulation Index SI) was observed in the group and responders following vaccination treatment; Wilcoxon signed rank test (WSR)

|                 | Responders vs. Non-Responders (P, MWU) |
|----------------|---------------------------------------|
| Wk 0           | P=0.9                                 |
| Wk 10          | P=0.05                                |
| Wk 20          | P=0.05                                |

**Table 26** Statistical significance of the difference in the lymphoproliferative response to GST-E6 between responders and non-responders at weeks 0, 10 and 20, calculated using Mann-Whitney U test.
4.9 LYMPHOPROLIFERATIVE RESPONSE TO GST E7 PROTEIN

As shown in the tables 27 and 28, post imiquimod, at week 10, three women demonstrated a greater than two fold change in the SI when compared to week 0. All of these women were responders. At week 20, eight women showed a greater than two fold change in the SI when compared to week 0. In the group as a whole, there is no significant increase in response between weeks 0 and 10 (P=0.8). However, there is a significant increase in proliferative response between weeks 0 and 20 (P=0.006). In the responder group, there is no significant difference between weeks 0 and 10 (P=0.7); however, there is a significant difference between weeks 0 and 20 (P=0.02).

In the non-responder group, unlike with the other HPV proteins, a significant increase in proliferative response between weeks 0 and 20 was noticed (P=0.03). When the responder group was compared to the non-responder group, a significant difference in the level of responses was seen both at weeks 10 (P=0.05) at week 20 (P=0.008), with greater significance at week 20 as shown in table 29.
## Lymphoproliferative Response to GST E7 Protein

|                | Pre-treatment | Post imiquimod (Wk 10) | Post vaccination (Wk 20) |
|----------------|---------------|------------------------|--------------------------|
| **Responders** |               |                        |                          |
| IT5            | 4.3           | 9.0                    | 14.1                     |
| IT8            | 0.8           | 1.2                    | 5.7                      |
| IT9            | 0.8           | N/A                    | 4.7                      |
| IT10           | 6.2           | 6.0                    | 20.4                     |
| IT13           | 0.8           | 0.3                    | 0.6                      |
| IT14           | 4.4           | 7.3                    | 3.9                      |
| IT15           | 1.1           | 3.1                    | 4.7                      |
| IT16           | 0.7           | 3.6                    | 2.0                      |
| IT17           | 1.3           | 5.0                    | 5.1                      |
| IT18           | 1.3           | 1.2                    | 1.6                      |
| IT20           | 13.8          | 1.1                    | 3.7                      |
| IT23           | 5.9           | 1.1                    | 13.1                     |
| **Non-responders** |             |                        |                          |
| IT1            | 1.8           | 1.3                    | 2.6                      |
| IT2            | 0.7           | 0.7                    | 0.9                      |
| IT3            | 1.5           | 1.0                    | 1.3                      |
| IT4            | 1.7           | 2.5                    | 3.2                      |
| IT11           | 3.0           | 4.0                    | 3.6                      |
| IT19           | 3.2           | 0.7                    | 1.6                      |
| IT21           | 2.4           | 0.8                    | 1.4                      |

Table 27 Proliferative Responses to GST E7 in the responders and non-responders at weeks 0, 10, 20. Two fold change in the stimulation index (SI) was considered as a response, highlighted in grey. N/A – not available.
Table 28 Change in the lymphoproliferative responses to GST-E7 in the group, responders, non-responders between weeks 0, 10, 20. A significant difference in the magnitude of response (median Stimulation Index SI) was observed following vaccination treatment; Wilcoxon signed rank test (WSR)

|                  | Wk 0 | Wk 10 | Wk 20 | Wk 0 vs. Wk 10 (P, WSR) | Wk 0 vs. Wk 20 (P, WSR) |
|------------------|------|-------|-------|-------------------------|-------------------------|
| **Group (n=19)** | 1.6  | 1.3   | 3.3   | P=0.8                   | P=0.006                 |
| **Responders**   | 1.3  | 3.3   | 4.7   | P=0.7                   | P=0.02                  |
| (n=12)           |      |       |       |                         |                         |
| **Non-Responders | 1.8  | 1.0   | 1.6   | P=0.5                   | P=0.03                  |
| (n=7)            |      |       |       |                         |                         |

Table 29 Statistical significance of the difference in the lymphoproliferative response to GST-E7 between responders and non-responders at weeks 0, 10 and 20, calculated using Mann-Whitney U test.

|                  | Responders vs. Non-Responders (P, MWU) |
|------------------|----------------------------------------|
| Wk 0             | P=0.9                                  |
| Wk 10            | P=0.05                                 |
| Wk 20            | P=0.008                                |

Table 28 Change in the lymphoproliferative responses to GST-E7 in the group, responders, non-responders between weeks 0, 10, 20. A significant difference in the magnitude of response (median Stimulation Index SI) was observed following vaccination treatment; Wilcoxon signed rank test (WSR)

|                  | Responders vs. Non-Responders (P, MWU) |
|------------------|----------------------------------------|
| Wk 0             | P=0.9                                  |
| Wk 10            | P=0.05                                 |
| Wk 20            | P=0.008                                |

Table 29 Statistical significance of the difference in the lymphoproliferative response to GST-E7 between responders and non-responders at weeks 0, 10 and 20, calculated using Mann-Whitney U test.
4.10 LYMPHOPROLIFERATIVE RESPONSE TO E6 & E7 PEPTIDES

No significant lymphoproliferative responses were observed for all the E6 peptides. A significant lymphoproliferative response to E7I peptide was demonstrated in the responder group at both weeks 10 and 20, however the significance of the change in response was greater at week 20. Stimulation indices with fold change for individual E6 and E7 peptides are shown in the appendix 4.

4.11 LYMPHOPROLIFERATIVE RESPONSE TO TA-GW PROTEIN

TA-GW protein was used as a negative control to the HPV 16 proteins. Although proliferative response to TA-GW protein was demonstrated, there was no significance in relation to treatment response or type of treatment as shown in tables 30 - 32.

| Proliferative Responses to TA-GW |
|----------------------------------|
| **Responders**                  | Pre-treatment | Post imiquimod (Wk 10) | Post vaccination (Wk 20) |
| IT8                             | 0.5           | 0.9                     | 1.5                       |
| IT9                             | 2.0           | N/A                     | 5.1                       |
| IT10                            | 10.8          | 7.3                     | 21.4                      |
| IT13                            | 0.6           | 1.5                     | 1.9                       |
| IT15                            | 1.2           | 5.3                     | 3.6                       |
| IT16                            | 1.1           | 4.2                     | 5.2                       |
| IT17                            | 2.3           | 3.5                     | 1.6                       |
| IT18                            | 3.6           | 1.1                     | 1.4                       |
| IT23                            | 4.2           | 1.2                     | 2.1                       |
| **Non-responders**              |               |                         |                           |
| IT11                            | 1.0           | 2.2                     | 1.2                       |
| IT19                            | 16.0          |                         | 7.5                       |
| IT21                            | 1.9           | 0.5                     |                           |

Table 30 Proliferative Responses to TA-GW protein in the responders and non-responders at weeks 0, 10, 20 Two fold change in the stimulation index (SI) was considered as a response, highlighted in grey, N/A – not available
|                     | Wk 0 | Wk 10 | Wk 20 | Wk 0 vs. Wk 10 (P, WSR) | Wk 0 vs. Wk 20 (P, WSR) |
|---------------------|------|-------|-------|-------------------------|-------------------------|
| Group (n=12)        | 2.0  | 2.2   | 2.1   | P=0.2                   | P=0.4                   |
| Responders (n=9)    | 2.3  | 3.5   | 2.1   | P=0.1                   | P=0.4                   |
| Non-Responders (n=3)| 1.9  | 1.4   | 4.4   | P=0.4                   | P=0.9                   |

Table 31 Change in the lymphoproliferative responses to TA-GW protein in the group, responders, non-responders between weeks 0, 10, 20. No significant difference in the magnitude of response (median Stimulation Index SI) was observed following vaccination treatment; Wilcoxon signed rank test (WSR)

|                     | Responders vs. Non-Responders (P, MWU) |
|---------------------|---------------------------------------|
| Wk 0                | P=0.9                   |
| Wk 10               | P=0.5                   |
| Wk 20               | P=0.9                   |

Table 32 Statistical significance of the difference in the lymphoproliferative response to TA-GW protein between responders and nonresponders at weeks 0, 10 and 20, calculated using Mann-Whitney U test
4.12 LYMPHOPROLIFERATIVE RESPONSE TO PHA

All women demonstrated a proliferative response to PHA. There was no difference in the responsiveness to PHA at different time-points or between patient groups as shown in tables 33-35.

| Responders | Pre-treatment | Post imiquimod (Wk 10) | Post vaccination (Wk 20) |
|------------|---------------|------------------------|-------------------------|
| IT5        | 4.4           | 32.6                   | 22.3                    |
| IT8        | 12.1          | 90.0                   | 35.0                    |
| IT9        | 6.6           | N/A                    | 19.0                    |
| IT10       | 23.3          | 33.7                   | 6.7                     |
| IT13       | 7.8           | 10.1                   | 2.6                     |
| IT14       | 62.9          | 88.9                   | 83.4                    |
| IT15       | 22.9          | 21.6                   | 4.0                     |
| IT16       | 33.6          | 10.3                   | 8.2                     |
| IT17       | 11.2          | 2.9                    | 18.3                    |
| IT18       | 13.6          | 6.1                    | 23.7                    |
| IT20       | 19.3          | 2.9                    | 8.2                     |
| IT23       | 12.0          | 6.5                    | 105.2                   |

Non-responders

| Responders | Pre-treatment | Post imiquimod (Wk 10) | Post vaccination (Wk 20) |
|------------|---------------|------------------------|-------------------------|
| IT1        | 11.2          | 17.9                   | 7.5                     |
| IT2        | 9.7           | 16.0                   | 16.9                    |
| IT3        | 6.1           | 10.5                   | 4.6                     |
| IT4        | 5.1           | 40.4                   | 20.2                    |
| IT11       | 16.6          | 10.1                   | 2.3                     |
| IT19       | 8.7           | 4.8                    | 14.2                    |
| IT21       | 12.6          | 10.6                   | 98.7                    |

Table 33 Proliferative Responses to PHA in the responders and non-responders at weeks 0, 10, 20 Two fold change in the stimulation index (SI) was considered as a response, highlighted in grey, N/A – not available
| Group (n=19) | Wk 0 | Wk 10 | Wk 20 | Wk 0 vs. Wk 10 | Wk 0 vs. Wk 20 |
|-------------|------|-------|-------|----------------|----------------|
|             |      |       |       | (P, WSR)       | (P, WSR)       |
| Responders  | 12.9 | 10.3  | 18.6  | P=0.7          | P=0.5          |
| (n=12)      |      |       |       |                |                |
| Non-Responders | 9.7 | 10.6  | 14.2  | P=0.3          | P=0.3          |
| (n=7)       |      |       |       |                |                |

Table 34 Change in the lymphoproliferative responses to PHA in the group, responders, non-responders between weeks 0, 10, 20. No significant difference in the magnitude of response (median Stimulation Index SI) was observed following vaccination treatment; Wilcoxon signed rank test (WSR).

| Wk 0 | Wk 10 | Wk 20 |
|------|-------|-------|
|      | P=0.6 |       |
|      | P=0.08|       |
|      | P=0.2 |       |

Table 35 Statistical significance of the difference in the lymphoproliferative response to PHA between responders and nonresponders at weeks 0, 10 and 20, calculated using Mann-Whitney U test.
In summary, sixteen women (84%) demonstrated pre-existing lymphoproliferative response to TA-CIN (SI ≥ 2), of whom 14 were HPV16 positive on vulval biopsy on at least one occasion. Of these, 11 were lesion responders and 5 non-responders with no significant difference in magnitude of pre-existing response (P=0.4). Following vaccination, 10/12 lesion responders compared to 3/7 non-responders showed ≥ 2-fold SI change compared with baseline. Strong PHA responses were demonstrated by all the women with median SIs of 12, 10.6, 16.9 at week 0, 10 and 20 respectively; these responses were not significantly different after imiquimod or vaccination (P=0.4, 0.2 in the group) or when patients were stratified by lesion response (P=0.7, 0.5 in responders and P=0.3, 0.3 in non-responders). By contrast, a significant increase in proliferation to TA-CIN was seen in the patients after vaccination compared to pre-treatment (P=0.01) and this was associated with lesion responders (P=0.008) but not the non-responders (P=0.7) Similar significant patient proliferative responses to each of the individual HPV16L2, E6 and E7 antigens after vaccination were seen (P=0.02, 0.01, 0.02 respectively), with this increased proliferation associated with lesion responders (P=0.01,0.03,0.03) and not non-responders (P=0.5,0.5,0.2). Specificity of the HPV 16 responses was supported by the absence of any significant increase in patient lymphoproliferation post-vaccination to TA-GW (HPV6 L2E7), nor in the responder (P=0.4) or non-responder (P=0.9) groups. No significant increase in proliferation to any of the antigens was noted post-imiquimod in any of the patient groups. A significant difference in the magnitude of proliferation response was evident between the responder and the non-responders groups following imiquimod treatment for some of the HPV 16 proteins, which enhanced following vaccination. Post-vaccination proliferative response to TA-CIN and its components appears to correlate with the clearance of VIN on histology.
Figures 32 Lymphoproliferation Responses to HPV 16 proteins in treatment responders. Patient 5 demonstrated a response to TA-CIN, GST-E6 and GST-E7. Patient 10 demonstrated a response to TA-CIN, HPV 16L2, GST-E6 and GST-E7;
Figures 33 Lymphoproliferation Responses to HPV 16 proteins in treatment non-responders. Patient 11 and patient 19 demonstrated lymphoproliferative response to TA-CIN, HPV 16-L2, GST-E6 and GST-E7 in a non-specific manner with no relation to treatment.

IT11-Proliferative Responses

IT19 Proliferative Responses
4.13 NEUTRALISING ANTIBODIES

Pre-existing HPV 16 antibodies correlated with documented HPV 16 exposure in twelve out of fifteen women (80%) as shown in table 36. Overall patient pre-existing neutralizing antibody levels did not differ or alter significantly after imiquimod or vaccination in either the responders who cleared VIN on histological analysis or in the histological non-responders who failed to clear VIN.

Individual patient neutralisation antibody titres are shown in the table 37 and no significant stimulation in the titres with treatment was observed, irrespective of the patient’s response to the treatment (P=0.2 in the responders and P=0.5 in the non-responders).

Trend in the neutralisation antibody titres with treatment is compared with the magnitude of the SI on lymphoproliferation assays to HPV 16 proteins and the negative control TA-GW and the positive control PHA is shown in the table 38. Again, no stimulation with treatment was observed in contrast to the responses seen to HPV 16 proteins on the lymphoproliferation assay.
| HPV positivity | NA titres |
|----------------|----------|
| 0, 10, 20, 52 wk | 0 10 20 |
| 1 - - ++ | 6400 6400 6400 |
| 2 + + ++ | 0 200 200 |
| 3 - - - - | 100 200 200 |
| 4 + + + - | 3200 800 800 |
| 11 + + + + | 12800 12800 2800 |
| 19 + + + + | nt nt nt |
| 22 + + + + | 200 400 400 |
| 5 + - - - | nt nt nt |
| 8 + + - + | 100 200 6400 |
| 9 + - ne + | 12800 3200 3200 |
| 10 + + - + | 400 1600 1600 |
| 13 - - - - | 100 0 0 |
| 14 - - - - | 1600 1600 1600 |
| 15 - - + + | 0 0 200 |
| 16 + + + + | 1600 1600 1600 |
| 17 + - - - | nt nt nt |
| 18 + - + - | 400 200 200 |
| 21 + - + + | nt nt nt |
| 23 + - - - | 800 1600 1600 |

**Table 36 Relation between HPV positivity and the neutralisation antibody titres** - correlation between pre-existing antibodies and HPV positivity was seen in twelve women. NA-neutralisation antibody; nt – not tested; + HPV positive; - HPV negative; ne – not evaluable
Table 37 Neutralization Assay Titres for individual patients at wk0 (pre treatment), wk10 (post imiquimod) and wk 20 (post vaccination) No significant difference in the titres was evident with treatment and no significant difference in the titres was obvious between treatment responders and non-responders.
### NEUTRALISING ANTIBODIES TABLE

| Pt | NA | 10 | 20 | TA-CIN | 6400 | 6400 | 6400 |
|----|----|----|----|--------|------|------|------|
| 1  | 6400 | 6400 | 6400 | 7.2 | 5.7 | 25.0 | 1.8 | 1.0 | 2.4 | 1.8 | 1.3 | 2.6 | nt | nt | nt | 1.1 | 1.4 | 2.1 | 11.2 | 17.9 | 7.5 |
| 2  | 0 | 200 | 200 | 1.0 | 1.2 | 2.5 | 0.6 | 0.3 | 1.3 | 0.7 | 0.7 | 0.9 | nt | nt | nt | 1 | 1.2 | 2.1 | 9.7 | 16.0 | 16.9 |
| 3  | 100 | 200 | 200 | 7.0 | 7.2 | 2.2 | 0.9 | 1.7 | 1.3 | 1.5 | 1.0 | 1.3 | nt | nt | nt | 3.4 | 1.2 | 1.6 | 6.1 | 10.5 | 4.6 |
| 4  | 3200 | 800 | 800 | 1.9 | 2.0 | 5.5 | 3.1 | 1.9 | 11.3 | 1.7 | 2.5 | 3.2 | 2.0 | 2.0 | 9.4 | 1.8 | 1.6 | 3.6 | 5.1 | 40.4 | 20.2 |
| 11 | 12800 | 12800 | 2800 | 5.1 | 1.6 | 11.1 | 7.1 | 7.6 | 5.0 | 3.0 | 4.0 | 3.6 | 3.2 | 3.7 | 3.0 | 1.0 | 2.2 | 1.2 | 16.6 | 10.1 | 2.3 |
| 19 | nt | nt | nt | 7.4 | 2.2 | 3.6 | 7.6 | 2.0 | 3.0 | 3.2 | 0.7 | 1.6 | 17.0 | 2.0 | 9.0 | 16.0 | 8.0 | 7.5 | 8.7 | 4.8 | 14.2 |
| 21 | 200 | 400 | 400 | 6.3 | 1.1 | 12 | 7.4 | 2.6 | 11.0 | 2.4 | 0.8 | 1.4 | 16.3 | 4.7 | 4.2 | 1.2 | 2.1 | 12.6 | 10.6 | 98.7 |
| 5  | nt | nt | nt | 4.4 | 6.3 | 9.4 | 5.1 | 11.8 | 11.9 | 4.3 | 9.0 | 14.1 | 2.8 | 6.4 | 5.2 | 1.8 | 2.1 | 4.2 | 4.4 | 32.6 | 22.3 |
| 8  | 100 | 200 | 6400 | 2.9 | 4.7 | 32.9 | 0.6 | 1.3 | 8.3 | 0.8 | 1.2 | 5.7 | 0.7 | 6.2 | 14.7 | 0.5 | 0.9 | 1.5 | 12.1 | 90.0 | 35.0 |
| 9  | 12800 | 3200 | 3200 | 0.9 | nt | 11.0 | 1.3 | nt | 9.1 | 0.8 | nt | 3.7 | 1.6 | nt | 7.5 | 2.0 | nt | 2.5 | 6.6 | 19.0 |
| 10 | 400 | 1600 | 1600 | 26.6 | 18.5 | 84.2 | 6.6 | 5.7 | 16.3 | 6.2 | 6.0 | 20.4 | 3.0 | 8.2 | 21.0 | 10.8 | 7.3 | 21.4 | 23.3 | 33.7 | 6.7 |
| 13 | 100 | 0 | 0 | 3.0 | 3.1 | 3.8 | 1.1 | 0.8 | 0.9 | 0.8 | 0.3 | 0.6 | 0.8 | 0.7 | 0.8 | 0.6 | 1.5 | 1.9 | 7.8 | 10.1 | 2.6 |
| 14 | 1600 | 1600 | 1600 | 10.9 | 10.0 | 31.6 | 5.1 | 5.1 | 3.0 | 4.4 | 7.3 | 3.9 | 1.6 | 2.1 | 2.1 | nt | nt | nt | 62.9 | 88.9 | 83.4 |
| 15 | 0 | 0 | 200 | 3.5 | 6.4 | 10.9 | 1.9 | 5.4 | 5.5 | 1.1 | 3.1 | 4.7 | 2.0 | 5.0 | 3.0 | 1.2 | 5.3 | 3.6 | 22.9 | 21.6 | 4.0 |
| 16 | 1600 | 1600 | 1600 | 3.3 | 8.4 | 15.6 | 3.4 | 7.7 | 10.4 | 0.7 | 3.6 | 2.0 | 2.9 | 7.8 | 7.6 | 1.1 | 4.2 | 5.2 | 33.6 | 10.3 | 8.2 |
| 17 | nt | nt | nt | 5.6 | 4.0 | 11.6 | 6.7 | 9.2 | 11.2 | 1.3 | 5.0 | 5.1 | 3.1 | 5.7 | 7.9 | 2.3 | 3.5 | 1.6 | 11.2 | 2.9 | 18.3 |
| 18 | 400 | 200 | 200 | 2.5 | 1.4 | 5.4 | 2.8 | 3.9 | 7.1 | 1.3 | 1.2 | 1.6 | 2.8 | 1.7 | 2.9 | 3.6 | 1.1 | 1.4 | 13.6 | 6.1 | 23.7 |
| 20 | nt | nt | nt | 27.8 | 1.3 | 13.8 | 27.2 | 3.5 | 9.7 | 13.8 | 1.1 | 3.7 | 17.0 | 2.0 | 9.0 | 1.9 | 0.5 | 1.1 | 19.3 | 2.9 | 8.2 |
| 23 | 800 | 1600 | 1600 | 6.0 | 1.0 | 17.4 | 5.6 | 1.3 | 11.4 | 5.9 | 1.1 | 13.1 | 1.0 | 1.3 | 3.7 | 2.3 | 3.5 | 4.5 | 12.0 | 6.5 | 105.2 |

**Table 38 Comparison of neutralising antibody titres with proliferative responses to HPV 16 proteins, negative control and positive control in treatment responder and non-responder patients** – this table shows the levels of response observed for each of the patients at different time-points from the neutralising antibody assay and the lymphoproliferation assays. In contrast to treatment linked lymphoproliferation response to HPV 16 proteins (TA-CIN, E6, E7, L2) in the treatment responders, no similar trend was observed with the positive control (PHA), negative control (TA-GW) or for the neutralising antibodies (NA).

Nt – not tested; Pt – patients 1,2,3,4,11,19, 21 – treatment non-responders; patients 5,8,9,10,13,14,15,16,17,18, 20, 23 – treatment responders.
4.14 RESULTS OF EXTENDED CLINICAL FOLLOW-UP

All the trial participants were followed up for an average of fifteen months past the primary endpoint of week 52. Extended follow-up of these women showed that 84% of women who previously achieved a greater than 50% reduction in the size of their lesions continued to stay in remission as shown in figure 34. Unfortunately, biopsy of any index lesion after week 52 was not performed unless clinically indicated. Overall, one woman developed microinvasive disease on two occasions, two women developed new lesions during the study period and one woman developed recurrence on two occasions six months following the primary end-point of week 52. Four women underwent additional treatment for symptom control.

4.14.1 NEW LESIONS DETECTED PRIOR TO STUDY COMPLETION

Two women (IT10, IT20) aged 27 and 22, smokers and HPV 16 positive in the biopsy, developed new lesions that were clearly absent at recruitment between weeks 26 and 52 of the study period. Both the women made clinical and histological response to the study treatment.

4.14.2 FURTHER TREATMENT AFTER TRIAL COMPLETION

Patients 2, 4 and 19, who did not demonstrate clinical or histological response during the study period, underwent laser treatment for symptom control. Patient 11 who made a partial clinical response and no histological response underwent laser treatment for completion of treatment.

4.14.3 DISEASE RECURRENCE AT FOLLOW-UP

Patient 17, forty-three years of age, non-smoker, who made a complete clinical and histological response to treatment, developed recurrences on two occasions six months following the study primary end-point.
4.14.4 DISEASE PROGRESSION

Patient 9, at week 30 complained of a non-healing symptomatic raw area on the vulva, which was the site of her previous punch biopsy. A diagnostic punch biopsy was taken, which confirmed a completely excised focus of early stromal invasion. Following the study primary end-point, this woman developed a recurrent malignancy, which was treated by surgical excision.

Figure 34 Reduction in Lesion Size at follow-up. Reduction in lesion size by 100%, >50% but less than 100% and <50% at baseline (Wk0), post-imiquimod (Wk10), post-vaccination (Wk20), primary end-point (Wk52) and follow-up (62 wk after primary end-point) in the group (n=19).
CHAPTER 5

DISCUSSION
5. DISCUSSION

5.1 MANAGEMENT OF VIN

Management of VIN is led by two main aims: symptom resolution and cancer prevention. Increasing incidence, especially in younger women [Akerman et al., 2007b], compounded by the characteristic features of distressing symptoms, persistent disease and recurrence following treatment begs for an irrefutable need to discover conservative management options for VIN. The existing treatment modalities such as surgery where the lesion is physically removed and laser ablation, often effective for short term can both be effective, however, do not address the issue of underlying HPV infection and are also limited by significant procedure related morbidity, psychosexual morbidity, high rates of incomplete treatment in turn resulting in high rates of recurrence [Likes et al., 2007; Shylasree et al., 2008].

HPV suppresses the expression of several proinflammatory proteins that are crucial in clearing infection and activating the cytotoxic T lymphocytes involved in killing virus-infected cells. Despite HPV's ability to inhibit host defences, a successful immune response to genital HPV infections is established in most cases as demonstrated by a certain proportion of HPV related intraepithelial neoplasia that undergo immune-mediated regression. As immunosuppression has been shown to be a risk factor for increased incidence and progression of HPV associated lower genital tract neoplasias [Bouwes Bavinck and Berkhout, 1997; Palefsky and Holly, 2003], there is a significant role for the development of immunotherapeutic modalities as treatment options for HPV associated intraepithelial neoplasia. The aim of immunotherapy is to enhance the natural immune response against HPV and to induce an immune environment that stimulates immature tolerising dendritic cells to become activated dendritic cells in combination with enhanced cytotoxic T lymphocyte responses against HPV proteins, thereby providing a superior approach to treating established HPV infection.

Majority of the non-surgical treatments for VIN such as topical 5-FU, cidofovir, IFN α have resulted in variable outcomes with response rates varying from
40% to 67% [Sillman et al., 1985; Tristram and Fiander, 2005]. Although the results from these small studies appear promising, the efficacy cannot be determined until large, preferably placebo controlled studies are conducted. At the time of imiquimod / PDT trial conducted in this unit [Winters et al., 2008] and when the current imiquimod / TA-CIN trial was envisaged, the only data available for imiquimod in the management of VIN was that from case-reports and small observational studies demonstrating some response to treatment (table 1). Since, two randomised trials of imiquimod alone treatment for VIN have been published quoting response rates of 60 to 90% [Mathiesen et al., 2007; van Seters et al., 2008b]. Information gained from the solo PDT [Martin-Hirsch et al., 1998] or therapeutic HPV vaccination trials [Davidson et al., 2004] also conducted in this unit led us to believe that a short course of imiquimod would encourage non-specific inflammation locally, which in addition to having a direct effect on the condition, would also create an enhanced immunological milieu for the PDT or TA-CIN treatment to take effect. Imiquimod in combination with PDT when used sequentially, the observed overall clinical response rate was 60% at week 52 [Winters et al., 2008]. As the majority of women with VIN have long-standing disease intractable to numerous previous treatments, in this study it was felt unreasonable to subject these women to placebo treatment. The study design was therefore prospective (uncontrolled) and observational. The objective of this study was thus to develop an effective treatment option for VIN, with the novel combination of the two relatively new modalities of treatment, imiquimod to induce non-specific inflammation and therapeutic HPV vaccination (TA-CIN) to induce HPV specific immunity. The emphasis was on accomplishment of sustainable and durable treatment response.

Although in the evaluation of response to treatment, an array of responses such as lesion size, histological grade of VIN, symptoms, and presence or absence of HPV-16 DNA was studied, resolution of VIN on histology was considered to be the gold standard against which the intralesional and systemic immune responses were compared. Histological diagnosis is objective, reproducible with a high sensitivity and specificity in contrast to clinical assessment.
5.2 STUDY COMPLETION

Twenty women were recruited and nineteen women completed the study. The mean age of the trial participants was forty-five at recruitment, with more than two-thirds going through a recurrent phase of VIN, with average disease duration of seven years. The cohort was also heavily pre-treated for VIN with more than two-thirds having undergone previous treatment(s) unsuccessfully. 80% of women gave a history of smoking cigarettes and 65% were actively smoking during the study period. No significant change in the smoking habits was noticed during the study period, despite education regarding the strong association between smoking and the risk of acquiring high-risk HPV infections [Madsen et al., 2008] and encouragement to quit. It has therefore not been possible to ascertain the effect of smoking on clinical response or disease recurrence at follow-up. Although women with a history of immunosuppression were excluded from the study, the diagnosis of immunosuppression was only established by taking a detailed medical history but not by any extra blood tests other than the routine FBC and serum biochemistry. One participant aged 22 years was withdrawn early in the imiquimod treatment phase of the trial for having developed a new lesion of VIN with a focus of micro-invasive carcinoma. This woman was subsequently (twelve months later) diagnosed with myelodysplastic syndrome, a haematological and immunological condition resulting in bone marrow failure. In retrospect, background immunosuppressive environment, which was not picked up on the routine blood tests, could have contributed to her very ‘unstable’ vulva.

5.3 CLINICAL RESULTS

5.3.1 TOLERABILITY OF IMIQUIMOD TREATMENT

In considering the imiquimod treatment, tolerability is a significant issue since the majority of women experience local and systemic side effects lasting for the duration of imiquimod treatment, which may impact on daily activities. In fact, in one of the first studies of imiquimod for VIN by Todd et al, it was
observed that the side effects with imiquimod treatment limited the frequency of application and that only twelve out of twenty women tolerated the regime that was intended with no dose reductions or treatment breaks [Todd et al., 2002]. Since, tolerance to imiquimod treatment has been a recurring theme in all the published studies of imiquimod for VIN (table 1).

Majority of the recent studies of imiquimod for VIN [Le et al., 2007; Mathiesen et al., 2007; van Seters et al., 2008b] recommend two or three applications per week for sixteen weeks, the strategy adopted from studies of imiquimod treatment for genital warts [Beutner and Ferenczy, 1997; Edwards et al., 1998]. In this study, women were advised to use imiquimod cream for only eight weeks, a significantly shorter duration, furthermore in a dose escalating fashion. Numerous supportive measures were in place and women were strongly encouraged from all fronts (study investigators, nurses, and general practitioners) to improve compliance to imiquimod treatment in this study. Completing the prescribed course of imiquimod however was a difficult task for the group as a whole. In this study, with significant hand holding, 84% of the participants applied imiquimod cream for the suggested eight weeks, with the all the participants reporting either moderate or severe degree of side effects. Also, in the imiquimod / PDT study, 20% of women reported intolerance to imiquimod treatment and discontinued the treatment [Winters et al., 2008]. Interestingly, when the response to imiquimod treatment was evaluated two weeks after completing the scheduled eight-week course of the imiquimod treatment, 74% of women in this study showed a clinical response and 32% demonstrated histological response in contrast to 50 % clinical response and 25% histological response in the imiquimod/PDT study [Winters et al., 2008]. The enhanced response in this study compared to the latter study is more than likely to be due to improved treatment compliance, as all the variables in both these studies were otherwise common and comparable. Two other recent studies by van Seters et al and Mathiesen et al of solo imiquimod treatment for VIN also reported intolerance to topical imiquimod treatment in 15% and 23% of cases respectively [Mathiesen et al., 2007; van Seters et al., 2008b].
Topical imiquimod treatment is expected to cause inflammation at the site of application and it has also been suggested that the degree of local inflammation matches the subsequent clinical response in most cases [Le et al., 2007; van Seters et al., 2008b]. The degree of local inflammation and the subsequent clinical response corresponded in most cases. Providing an obvious inflammatory response is evident in fact it might not even be necessary to strictly apply imiquimod cream as often as three times a week. This belief led to allowance of treatment free periods of no more than few days at a time to allow side effects to subside and thereby improve compliance to treatment.

5.3.2 TOLERABILITY OF TA-CIN

TA-CIN was well tolerated by all the study participants in this study, similar to the other previous TA-CIN vaccination studies [Davidson et al., 2004; de Jong et al., 2002; Karanam et al., 2009; Smyth et al., 2004].

Overall, our treatment regimen was feasible but as expected, was associated with considerable imiquimod induced discomfort necessitating breaks in treatment. Women with refractory VIN are, however, highly motivated to comply with the treatment protocol, and in the event, 85% persevered with treatment and finished the full course of imiquimod.

5.4 CLINICAL, HISTOLOGICAL, SYMPTOM & HPV RESPONSE TO TREATMENT

This study demonstrated that topical imiquimod followed by TA-CIN vaccination resulted in a 50% or more reduction in the overall size of the lesions (clinical response) in the majority of the participants from week 10 onwards. In fact, by week 10, clinical response was detected in 74% of women, in who the response was sustained and improved to some extent to 84% and 79% on successive assessments at weeks 20, 52 and to 84% at follow-up. Majority of the participants were diagnosed with multifocal disease.
at recruitment and no significant difference in the response rates was observed between the unifocal and the multifocal disease groups. It is believed that the local immunologic up regulation with imiquimod treatment is not just restricted to the area in direct contact with the cream but is widespread into the adjacent skin as well [Todd et al., 2002].

Other studies of imiquimod for VIN report varying response rates to imiquimod treatment (table 1) and differences in the treatment schedule, treatment duration and the demographics of the study participants makes it difficult to draw direct comparisons. Mathiesen et al in a randomised double-blinded study of imiquimod for VIN in twenty-one women who received active treatment with imiquimod for sixteen weeks observed clinical response in more than 90% of women who received the active treatment in comparison to none, who received placebo treatment [Mathiesen et al., 2007]. Le T et al in their study of imiquimod for VIN in thirty-three patients observed a 77% response rate by the intention to treat principle [Le et al., 2007]. van Seters et al in their randomised study of imiquimod for VIN scheduled twenty-six women to receive topical imiquimod treatment, twice weekly for sixteen weeks. This trial is an advance over the previous studies in the randomised trial design and reduction in the approved frequency of dosage to twice weekly applications. The primary study outcome was a reduction of more than 25% in the lesion size at week 20, which was in effect observed in 81% in the imiquimod arm and none in the placebo arm [van Seters et al., 2008b]. Response rates of such degree are clinically relevant and imiquimod treatment although not well tolerated is feasible. Our study differs from the above-mentioned studies by advocating imiquimod treatment for only eight weeks in contrast to the sixteen-week regime. Side effects with imiquimod treatment build up with time and appear to be most bothersome from week 4 onwards. In the solo imiquimod study by Le et al the need for dose reductions and treatment breaks in fact was most desperate at about week 9 (median) [Le et al., 2007]. In our study, in spite of halving the imiquimod treatment duration from the routine sixteen weeks to eight weeks, the response to treatment observed in this trial is comparable to other solo imiquimod studies, making a proposition for reducing the duration of imiquimod treatment for VIN.
The TA-CIN component of the study treatment has previously been tested as either the prime vaccine or in the form of a boost vaccine following priming with TA-HPV vaccine [Davidson et al., 2004; Fiander et al., 2006; Smyth et al., 2004] with minimal improvement in the clinical response rates. Davidson et al. in their study of ten women with high-grade VIN who received three booster vaccinations with TA-CIN observed objective clinical response in two women [Davidson et al., 2004]. Fiander et al. in their prime boost vaccination trial with TA-CIN for ano-genital intraepithelial neoplasia followed by a booster with TA-HPV noted one complete and five partial clinical responses out of twenty-nine women [Fiander et al., 2006]. Overall, in the vaccination trials with TA-HPV and TA-CIN, the clinical response rates appeared to be around 20%.

In our study, with the sequential combination regime, it is difficult to distinguish the contribution of the individual components of the treatment regimen. The contribution of the different treatment components could be additive or synergistic. Although some of the response noted at week 52 was evident from week 10 (post-imiquimod) onwards, response enhanced with time.

In addition to the clinical response, histological response (clearance of VIN on histology at week 52, primary end-point) enhanced steadily with time from 21% immediately following imiquimod treatment at week 10, to 58% at week 20 and 63% at week 52. Histological diagnosis is however considered to be the gold standard, because it is objective, reliable, and reproducible. In contrast to the clinical response that was immediately evident following imiquimod treatment, attainment of histological response was slow in comparison. It is however reasonable to argue that an immunologically challenged chronic condition which takes time to establish will equally take time to regress, explaining the continuing response noted long after treatment completion.

Histological responses were persistently lower than the combined clinical response rates (complete and partial) but higher than complete clinical response rates. This observation is worthy of note. In one case histological diagnosis of VIN1/2 was in disagreement with a clinical diagnosis of complete
clinical response. In some other cases, histological diagnosis confirmed absence of VIN within the biopsy, when in fact; partial clinical response was diagnosed on vulvoscopy. Discrepancy in the clinical and histological response rates could be due to the difficulty in the accurate assessment of the extent of the true lesion. Imiquimod treatment can result in a background of inflammation and pigmentation, confounding the true assessment of any lesion and thus emphasises the need for histological definition of VIN. One other likely reason for the discrepancy between clinical and histologic response rates is the possibility of the biopsy not been representative of the true clinical lesion, although every effort was made to overcome this risk.

The histologic response rates in this study compare well with other similar studies. In the other studies as well, the trend of clinical response rates subjugating the histologic response rates continued. In the imiquimod/PDT study, 25% showed evidence of complete histologic regression at both weeks 10 and 20 and 35% at week 52 compared to histologic response rates of 32%, 58% and 63% in this study at weeks 10, 20 and 52 [Winters et al., 2008]. In the other solo imiquimod studies for VIN, where imiquimod was used for sixteen weeks, Mathiesen et al reported outstanding histologic response rates, 81% complete histologic regression to no VIN and 10% partial regression from VIN2/3 to VIN1 in twenty-one women who had a biopsy taken two months after completion of imiquimod treatment [Mathiesen et al., 2007]. Le et al stated that a histologic response was seen in 64% of women and biopsies in this study were taken soon after completion of imiquimod treatment [Le et al., 2007]. van Seters et al quoted 69% histologic regression from biopsies taken 2 months after treatment completion [van Seters et al., 2008b]. The histological response rate of 63% noted in the current study was based on histopathology of the biopsy taken at week 52 emphasising the longevity of response achieved with imiquimod and TA-CIN treatment.

Achievement of clinical and histological response is an important element to any successful treatment fro VIN; however, the drive to undergo treatment, for many women diagnosed with VIN is to achieve symptom control. In a recent small prospective study, eight women with newly diagnosed VIN consented to
be observed at six monthly intervals for a two-year period rather than undergo any active treatment. Surgery would be on offer only to exclude disease progression or for uncontrollable symptoms. Nevertheless, six out of eight volunteers required immediate surgical treatment and of the remaining two, who were carefully observed for evidence of any malignancy, one required surgical treatment before the study completion period. Majority of the treatments were carried out for symptom control, clearly demonstrating that wait and watch policy is not particularly feasible for women with VIN as the majority appear to require treatment for symptom control [McFadden et al., 2009].

In the current study, 79% reported moderate or severe symptoms at recruitment compared to 58% at week 10, 26% at week 20 and 21% at week 52. Akin to clinical and histologic response, majority of the symptom response (regression of symptoms from moderate or severe category to none or mild category) was achieved by week 20. Of the four women who continued to experience symptoms of moderate or severe intensity at week 52, three women were non-responders to the trial treatment. Strangely, one woman, who in spite of making a clinical and histological response to treatment did not recover from the severe symptoms, was subsequently at follow-up diagnosed with a new lesion of VIN. It is plausible that symptoms precede the clinical manifestation of lesions.

Impressive symptom response rates were detected in other similar studies. In the imiquimod/PDT study, at recruitment 70% of the women declared moderate or severe symptoms in comparison to 25% at week 52 [Winters et al., 2008]. In the current and the imiquimod/PDT studies, symptom improvement was not necessarily associated with clinical or histological response. This could be due to the local effect of imiquimod or PDT on perineal sensory nerve fibres. A similar phenomenon was seen in the solo vaccination trials, where in, a higher degree of symptom response was evident compared to histological response [Baldwin et al., 2003; Davidson et al., 2003a], which is more difficult to explain. One could argue that symptom response could be due to placebo effect and this cannot be clarified when the
study is not randomised and placebo controlled. van Seters et al in their double-blind placebo controlled randomised study of imiquimod for VIN described a significant reduction in pain and pruritus both at week 20 and at twelve months in the treated group when compared to the placebo group [van Seters et al., 2008b]. Improvement in symptomatology for this group of women is an important outcome, and it cannot be easily explained other than as a response to treatment.

Unexpectedly, three out of nineteen women observed an improvement in their general skin condition and also acne remission, possibly due to the pro-inflammatory properties of imiquimod treatment. This overall improvement on the general condition of skin has thus far not been mentioned in the literature.

A weak HPV response (clearance of HPV 16 from the biopsy site in somebody who was previously positive for HPV 16) of 43% at week 10, 33% at week 20 and 40% at week 52 in this study in comparison with other variables such as clinical, histologic or symptom response is disappointing as this might be a prelude to persistent or recurrent disease. Three women who were negative for HPV 16 from the biopsy site did demonstrate a pre-treatment systemic immune response to HPV16. Such systemic antibody response in the absence of HPV16 on biopsy probably reflects latency of the virus rather than paucity of the HPV detection methodology, as this was the case in only a minority. At week 52, concomitant loss of HPV16 from the biopsy site was noted in five out of six histological responders and one out of six histological non-responders. Similarly, in the imiquimod/PDT study, 53% were HPV16 negative at week 10 compared to 16% at recruitment. Fourteen women were HPV typed at week 52, eight of who were HPV negative [Winters et al., 2008]. Although this did not reach statistical significance in either of the studies, a trend towards loss of HPV and clinical response was evident.

van Seters et al in the randomised imiquimod study for VIN reported just about 50% HPV clearance rate. 96% were HPV positive at recruitment compared to 42% and 92% HPV positivity in the imiquimod and placebo
treatment groups, four weeks after completing imiquimod treatment, respectively.

Wieland et al stated that imiquimod treatment for AIN lead to significant reduction in the levels of high-risk HPV DNA loads and also to a fall in the number of HPV types at the end of therapy [Wieland et al., 2006]. Kreuter et al conducted a follow-up study of these patients and observed that high-risk HPV-DNA loads continued to remain at low-levels at the end of the follow-up period (average 30.3months) and high-risk HPV-DNA loads continued to be significantly lower than pre-treatment levels [Kreuter et al., 2008]. These findings are not substantiated by any other studies of imiquimod for VIN.

Smyth et al in the TA-HPV vaccination study demonstrated a reduction in viral load after vaccination in twelve out of eighteen women with one women showing complete viral clearance who also happened to make a complete clinical response. This group of women who demonstrated a reduction in the viral load included six of eight women who made a clinical response [Smyth et al., 2004]. Although local HPV clearance with imiquimod or therapeutic HPV vaccination has not reached statistical significance in most of the studies for VIN, correlation with clinical response is apparent.

5.5 IMMUNOLOGICAL RESPONSES TO TREATMENT

5.5.1 LOCAL IMMUNOLOGICAL RESPONSES

In this study, following imiquimod and TA-CIN treatment, enhancement in the local infiltration of helper, cytotoxic and regulatory T lymphocytes was obvious in the majority. Upon stratification of the group into treatment responders and non-responders, the rise in helper and cytotoxic T lymphocytes was significant only in the responder group. Conversely, the rise in regulatory T lymphocytes was significant only in the non-responder group. It therefore appears that in some women with high-grade VIN, despite stimulation of an immune rich local environment, the activity of T regulatory cells appears to be dominant, suppressing the T helper 1 defence responses, subsequently resulting in failure to elicit a response to treatment.
Earlier studies suggested that the inability to reject a tumour by the host may be due to insufficient generation of tumour specific helper T-cells [Greenberg, 1991]. It has also been suggested that when aiming to target pre-invasive disease with immunotherapy, helper T-cell response should be an important consideration [Steele et al., 2005]. Similar to the findings in this study, imiquimod treatment resulted in significant increase in cytotoxic T lymphocytes in the imiquimod/PDT study and another randomised study of imiquimod for VIN [van Seters et al., 2008a;Winters et al., 2008]. Imiquimod treatment has been shown to polarise the immune responses towards T helper 1 pattern by Smyth et al. who reported an increased local cytotoxic T lymphocyte activity when imiquimod was used in patients with anal cell carcinoma [Smith et al., 2004]. Studies of naturally regressing genital warts and HPV infections have shown a T-helper 1 or cell-mediated immune response to be necessary and effective in the spontaneous regression of these viral conditions [Coleman et al., 1994;Sheu et al., 2001].

Gul et al have postulated that an increase in the cytotoxic/helper (CD8/CD4) T-cell density could re-establish local immune control which is lost or suppressed as VIN progresses [Gul et al., 2004]. Likewise, in this study, we noted a significant rise in CD8:CD4 ratio post treatment, at week 10 and 20, only in the responder group. The increase in helper and cytotoxic T lymphocyte density evident post treatment at week 20, but significant only in the responder group, may well be a useful prognostic biomarker.

**5.5.2 INTRALESIONAL T REGULATORY LYMPHOCYTES**

Of the many factors that play a role in dictating the final outcome of any treatment, one such factor that may impact negatively on the immune system is the T-regulatory lymphocyte activity. There is compelling evidence to show that cancers and pre-invasive cancers induce or recruit T regulatory cells to inhibit the immune system [Knutson et al., 2007]. Studies on CIN and cervical cancer have shown a large number of T-regulatory cells in HPV derived lesions suggesting their pivotal role in counteracting the host immune
response [Loddenkemper et al., 2009]. In cancer patients, an increased number of T-regulatory cells have been shown both locally within the tumour and also in the blood [Baecher-Allan and Anderson, 2006].

In this study, pre-treatment, women who subsequently did not make clinical or histologic response to treatment were found to be harbouring significantly more number of regulatory T lymphocytes when compared with women who later, following treatment, demonstrated a clinical and histologic response. However, following imiquimod treatment, by week 10, there was no apparent difference in the levels of T-regulatory cells between responders and non-responders, probably due to an increase in the density of both the helper and regulatory T lymphocytes. By week 20 however, a marked fall in the density of T-regulatory cells was apparent in the responder group and in sharp contrast, a remarkable spike in the infiltration of regulatory T lymphocytes in the non-responder group was obvious. Basically in treatment non-responders, there appears to be a higher intralesional density of T regulatory lymphocytes pre and post treatment. Although Foxp3 expressing T regulatory lymphocytes and their inhibitory role in the immune system has previously been described in studies of CIN [Kobayashi et al., 2004] and cervical cancer [Fattorossi et al., 2004], they were first studied in the context of VIN in the imiquimod/PDT study, in which treatment non-responders were found to harbour significantly higher number of T regulatory lymphocytes [Winters et al., 2008] and these findings were later confirmed in other imiquimod studies for VIN [van Seters et al., 2008a].

These observations reflect the balance of useful CD4 and CD8 effector T lymphocytes against T regulatory activity and the clinical impact of the resulting immune control. Thus, increasing the CD8/CD4 T lymphocyte density may be able to re-establish local immune control which is lost or suppressed in chronic VIN [Gul et al., 2004], where the T regulatory cells dominate the local immunological milieu and can continue to suppress the HPV antigen-specific cytotoxic T lymphocyte response facilitating persistent VIN [Kobayashi et al., 2004; Stanley, 2008a].
5.6 SYSTEMIC IMMUNOLOGICAL RESULTS

Therapeutic HPV vaccines are designed to generate cell-mediated immunity against HPV infected cells that express the early viral proteins E6, E7 which act as oncogenes and also tumour specific antigens. The TA-CIN vaccination component of the protocol was aimed at expanding E6 and E7 specific T lymphocyte effectors with the possibility that immediately following topical imiquimod treatment, their entry, and activity in the VIN lesions would not be limited by local immunosuppressive factors.

5.6.1 PERIPHERAL CHANGES IN HELPER AND CYTOTOXIC T LYMPHOCYTES

Studies have shown that HPV specific T lymphocyte responses are weak or lacking in patients with VIN [Baldwin et al., 2003;Davidson et al., 2003a;Smyth et al., 2004;Van Poelgeest et al., 2005]. Kenter et al in the long-peptide vaccination study for VIN noted a strong and significant correlation between post-treatment complete regression of the lesions and a high number of HPV specific CD4+ T lymphocytes producing large amounts of IFNγ [Kenter et al., 2009]. A definite direct link between enriched helper and cytotoxic T lymphocyte environment, both local and systemic, and treatment response thus exists.

5.6.2 PRE-EXISTING PROLIFERATION RESPONSE TO TA-CIN

Sixteen out of nineteen women in this study revealed pre-existing proliferative response to TA-CIN (fusion protein of HPV16 E6, E7, and L2). Of these sixteen women, fourteen women were HPV16 positive from the biopsy site and three women who were HPV 16 negative from the biopsy site demonstrated a pre-existing immune response to HPV 16. Carter et al. studied five hundred and eighty eight young women with incidental HPV infection women and demonstrated that seroconversion for HPV-16 infections occurred most frequently between six and twelve months after DNA detection
but the levels eventually diminish. Some women with persistent HPV DNA however, were found to never seroconvert [Carter et al., 2000]. Negative serology thus does not rule out previous infection or the possibility of the subject possessing HPV specific memory T lymphocytes.

A significant correlation between the presence of circulating, pre-existing HPV-16 specific, interferon-gamma-producing T lymphocytes and regression of HPV-16-positive lesions was suggested by Van Poelgeest et al in their study of imiquimod for VIN and Winters et al in the imiquimod/PDT study for VIN [Van Poelgeest et al., 2005;Winters et al., 2008]. In this study, a trend towards higher pre-existing responses to HPV16 early antigens in the lesion responder group was noted, which was significantly stimulated by the TA-CIN vaccination. By contrast, the lesion non-responders showed little pre-existing response and this was not boosted by the TA-CIN vaccine.

**5.6.3 PROLIFERATION ASSAY RESPONSES TO HPV 16**

It has long been predicted that therapeutic HPV vaccination might overcome the inertia of the immune system in patients with VIN, in whom the levels of IFN-γ producing virus specific CD4+ and CD8+ T lymphocytes directed against HPV16 E6 and E7 proteins are not detectable or present in low numbers [Baldwin et al., 2003;Davidson et al., 2003a;Smyth et al., 2004;Van Poelgeest et al., 2005].

In this study lymphoproliferation assay showed striking and significant lymphoproliferative response, but only in the in the histological responder group to the TA-CIN and the individual HPV 16 E6, E7 and L2 proteins, only following vaccination at week 20 but not before treatment or after imiquimod treatment at week 10 are suggestive of a vaccine response. The outstanding lymphoproliferative response in the responder group is probably related to TA-CIN vaccine induced HPV-16 specific T lymphocyte responses. It was however not possible to elicit similar lymphoproliferative responses for the respective peptides of the individual E6 and E7 oncoproteins in any of the
patients, irrespective of their treatment response category. This probably reflects an error in the methodology of preparing the peptides rather than a lack of lymphoproliferative response. Interestingly, although the response to HPV 16 proteins was not significant following imiquimod treatment in any of the patient categories, the magnitude of response between treatment responders and treatment non-responders was significant for some of the HPV 16 proteins. This further enhanced to significant proportion in the majority of the treatment responders. Either this is an indication that imiquimod treatment in fact does stimulate HPV 16 specific systemic immune response or that imiquimod stimulate a generalised immune activation which when reaches higher levels results in disease clearance.

The results of the lymphoproliferative assays clearly show that in addition to the differences in systemic immune response to HPV 16 between responder and non-responder patients, the proliferative responses vary for individual HPV 16 oncoproteins. In this study, the TA-CIN protein, followed by E6, HPV 16 L2 and the E7 oncoproteins, stimulated the best lymphoproliferative response. In other TA-CIN vaccination studies, vaccine induced T-cell responses were predominantly directed against HPV-16 oncoprotein E6. Smyth et al in the heterologous prime-boost HPV oncogene vaccination study similar to this study detected strong proliferative TA-CIN specific responses in patients with AGIN, and a higher level of proliferation was noted with respect to E6 oncoprotein than with the E7 oncoprotein [Smyth et al., 2004]. This finding is consistent with the association between spontaneous clearance of HPV infection and detection of CD8+T lymphocyte responses that are specific for HPV-16 oncoprotein E6 [Nakagawa et al., 1997;Nakagawa et al., 2000;Nimako et al., 1997].

In the imiquimod/PDT study, lymphoproliferative responses to TA-CIN were not significant post imiquimod or PDT treatment, clearly demonstrating that neither imiquimod nor PDT treatment are able to induce HPV specific immunity or delivery HPV specific immunotherapy [Winters et al., 2008]. Davidson et al in the TA-CIN booster vaccination study for VIN observed that the measured HPV specific immune responses were detectable only
transiently in the peripheral blood, peaking at four to eight weeks, and falling to their baseline levels by twelve weeks post vaccination [Davidson et al., 2004]. In a recent HPV-16 E6E7 ISCOMATRIX therapeutic HPV vaccine study for men with AIN, vaccination treatment induced strong and durable antibody and IFN-γ levels that fell to pre-vaccination levels by week 24 [Anderson et al., 2009]. In contrast, the synthetic long-peptide vaccination study in women with VIN demonstrated strong HPV 16 specific proliferative responses which were detectable even one to two years after the last vaccination in twelve out of fourteen patients [Kenter et al., 2009].

In this study, systemic immune responses were checked only once, two weeks following the triple dose vaccination schedule. Although sustained clinical responses at follow-up are indicative of disease control, it would be interesting to find out if the systemic immune responses were sustained after completion of treatment.

Thus following the imiquimod and TA-CIN vaccination schedule, while the responder patients have higher pre-existing responses to HPV 16 early antigens which can be significantly stimulated by vaccination, the non-responders show little pre-existing responses which are not stimulated by the vaccine.

5.6.4 NEUTRALISATION ASSAYS

There is an element of sequence homology between related HPV types, which prompted us to perform neutralisation antibody assays to detect cross-reactive responses to other genital HPV infections. Studies on HPV VLP vaccines have shown that although cross-reactive cross-neutralising antibodies are generated, the titres are reduced compared to vaccine-specific types and the titres required to protect against non-vaccine types are not yet known [Pastrana et al., 2004].
HPV 16 L2 protein has the ability to elicit cross-neutralizing antibodies and thereby its role as a component of the TA-CIN vaccine. In this study, however, the overall patient pre-existing neutralizing antibody levels did not differ or alter significantly after imiquimod or TA-CIN vaccination treatment in either the responders or non-responders.

5.7 EXTENDED CLINICAL FOLLOW-UP

Most VIN studies report short-term response rates when in fact the objective should be to achieve long-term disease control. For this study, data is available for an extended average follow-up period of fifteen-month following the completion of the study (week 52), when a continuing however enhanced clinical response (50% or more reduction in the size of the lesions) rate of 84% was detected implying continuing control of VIN. Response achieved due to any other treatment offered outside the trial did not influence the clinical response outcome. The quoted clinical response rate of 84% was purely owing to the trial treatment. Unfortunately, biopsy of any index lesion after week 52 was not performed unless clinically indicated. Containment of response for such length of time in a heavily pre-treated cohort of women with long standing disease is remarkable.

Similarly, in the imiquimod/PDT study, follow-up data is available for an average of thirty-four months past week 52, the study primary end-point, when 65% clinical response rate was observed (60% at week 52) [Winters et al., 2008]. van Seters et al in the randomised imiquimod study for VIN have follow-up data for an average of twelve months following study completion at week 20. They found at follow-up all the complete and partial clinical responders to be in remission [van Seters et al., 2008a]. Similarly, Kenter et al. in the long-peptide vaccine study of VIN described a 25% complete clinical and histological response and 35% partial clinical response three months following the vaccination schedule. Nevertheless, at twelve months of follow-up, the complete and partial clinical response rates increased spontaneously
to 47 and 32% respectively. At twenty-four month follow-up period, the complete clinical response was still maintained [Kenter et al., 2009].

This phenomenon of demonstrating a late response was also recognised in other vaccination studies and in fact Palefsky et al who studied therapeutic HPV vaccination in men with AIN upon detecting some late response to treatment suggested that longer follow-up in future vaccination studies is necessary to capture the late responses [Palefsky and Holly, 2003]. For chronic lesions that have been present for many years, the precise time course of immune responses relevant to HPV lesion clearance are completely unknown. There is thus no particular reason why immunotherapy would achieve its full potential at the arbitrary study endpoint, which explains the response observed long after completion of treatment.

In this study, treatment response in some women has been dramatic. For e.g. Patient 5 (40 yr of age at recruitment, VIN for twenty years, seven previous surgical excisions) and patient 23 (35yr of age at recruitment, multifocal VIN for ten years, ten previous surgical treatments) who have both achieved complete clinical and histological response to treatment and continue to be in remission, three years following the completion of the trial (unpublished data) illustrate particularly impactful treatment responses achieved by week 52 in this study but which have been maintained for a further three years thus far. In both these cases, intralesional pre-treatment T regulatory cell density was low, with no enhancement following treatment contrary to increased density of CD4 and CD8 T lymphocytes. Both these women also demonstrated a pre-existing proliferative response to HPV16 with significant increase in proliferation response post-vaccination.

5.8 RECURRENCES, MALIGNANT POTENTIAL AND NEW DISEASE

In this study, during the average fifteen-month follow-up period, one (IT17) out of nineteen participants, who demonstrated a complete clinical response and histological response to treatment, developed recurrence on two occasions. In the imiquimod/PDT study, one out of twenty participants, a clinical responder developed recurrence at week 52 [Winters et al., 2008]. Le et al observed
20.5% rate of disease recurrence following imiquimod alone treatment for VIN at a median follow-up of sixteen months, and found all the women with recurrence disease to be heavy smokers [Le et al., 2007]. van Seters et al reported enlargement of lesions in two out of twelve women, during the twelve month follow-up period following imiquimod treatment [van Seters et al., 2008a]. In a retrospective study by Hillemanns et al of ninety-three women with VIN who were followed up for a mean period of fifty-three months, the relapse rate was 40% following laser treatment, 48% following photodynamic therapy, 42% following local excision and 0% following vulvectomy [Hillemanns et al., 2006]. The recurrence rate observed in this trial (5%) and the other above-mentioned imiquimod trials appears to be lower than that following surgery or laser treatment. The possible rationale for recurrence of VIN could be compromised immune status, reinfection with new HPV types, reactivation of indolent HPV infection resulting in increase in HPV DNA loads or unknown host genomic mutations.

Two women (IT9, IT22) in this study developed microinvasive disease. Patient 22 was withdrawn from the trial early at week 4, whereas patient 9 developed microinvasive disease ten weeks following completion of the vaccination treatment and a recurrent malignant lesion at follow-up. No malignancy was reported in any of the participants in the imiquimod / PDT study [Winters et al., 2008]. In the peptide vaccination study of VIN by Kenter et al out of twenty participants, by twenty-four months of follow-up, one woman developed microinvasive carcinoma and two had developed invasive carcinoma [Kenter et al., 2009]. van seters et al in the imiquimod alone study for VIN reported 6% progression to invasive disease within twelve months of follow-up. This included two out of twenty-six women who received placebo treatment and one out of twenty-six women who received imiquimod treatment. The available long-term follow up data suggests that only 9% of untreated VIN patients will progress to invasive disease [van Seters et al., 2008b]. This assumption has however retrospectively been questioned by Jones et al who reported invasive disease in seven out of eight untreated VIN cases in their observation study of hundred and thirteen women diagnosed with VIN [Jones and Rowan, 1994]. Reassuringly, with long term follow up data available for
some of the investigational studies of VIN; the rate of disease progression appears to match that following the conventional treatment modalities.

New lesions were detected in two out of nineteen women (IT10, IT22) in this study, within the trial period, between weeks 26 and 52. Both the women were active smokers, aged 22, and 29 at recruitment. Both of them underwent laser treatment to the new lesions, and both coincidentally developed recurrences within six months of the initial laser treatment. On the contrary, four women in this study who failed to achieve a complete clinical response and underwent surgical excision or laser ablation for treatment completion did not develop disease recurrence at follow-up. A similar observable fact occurred following imiquimod /PDT treatment, when seven women who failed to achieve a complete treatment response, underwent additional surgical excision or laser ablation following imiquimod / PDT treatment for completion of treatment. No recurrences were noted during follow-up in this group either [Winters et al., 2008]. Similarly Le et al in the solo imiquimod study noted a trend towards lower local recurrence rate in women who required further surgical ablative treatment after finishing the study protocol compared to historical surgically treated patients [Le et al., 2007]. This observation has major clinical implications for women, who make partial clinical response to immunotherapy, as minimal surgery or laser ablation following immunotherapy might offer them a durable, recurrence free completion treatment. In fact, following an incomplete response to immunotherapy, surgery or laser could be offered as maintenance therapy.

In the treatment of VIN, duration of efficacy and prevention of recurrences is crucial. Immunotherapeutic approaches with imiquimod alone or in combination with PDT or therapeutic HPV vaccination appear promising. In those women who fail to achieve a complete response, immunotherapy followed by completion surgical excision and laser ablation appears more promising.
5.9 CONVENTIONAL AND EMERGING TREATMENT OPTIONS FOR VIN

Surgery and LASER remain the mainstay of treatment for VIN. Surgical excision can be both diagnostic and therapeutic. This is an advantage over laser or medical management options, as undetected foci of minimally invasive squamous cell carcinoma of the vulva remain a concern with VIN. The aim of surgical treatment is to obtain disease free biopsy margins. Removal of multi-focal disease might involve surgical removal of more or less the entire external female genitalia. It is equally important to avoid over treatment and permanent disfigurement of the vulva. With large surgical excisions, rotational skin flap procedures are increasingly used. For VIN involving the clitoris, organ sparing surgery in the form of skinning vulvectomy has also been described [Terlou et al., 2009]. In a recent study where hundred and eighty six patients with VIN were treated surgically with wide local excision or skinning vulvectomy, presence of multifocal VIN was the only factor that was associated with significant incomplete resection in univariate and multivariate analyses [Polterauer et al., 2009]. Fong et al reported only 3% patient dissatisfaction rate following surgical treatment for VIN [Fong et al., 2008]. Modified minimally invasive surgical procedures may have an important role in the surgical management of VIN. Surgery although remains the most common treatment modality for VIN, thus far, no systematic reviews or randomised controlled trials comparing surgery with other modalities of treatment are available.

LASER is one suitable treatment option for multiple superficial lesions in the non-hair bearing areas on the vulva. With LASER ablation, histological assessment of the entire specimen is not possible and unrecognised malignancy may be missed. There is always a risk of under diagnosis and missing occult cancers with pre-treatment diagnostic biopsies. A study of hundred and eighty six VIN patients who were treated with excisional surgery or skinning vulvectomy underwent preoperative biopsy to assess the accuracy by evaluating the rates of correct diagnosis, under diagnosis and occult cancer. A significant number of pre-operative biopsies were under diagnosed
when compared with excisional biopsies [Polterauer et al., 2009]. The risk of persistent or recurrent disease is also higher with ablative treatment compared with surgical treatment. Laser treatment is however, cosmetically more appealing.

**TLR 7 and 8 agonists** are emerging treatment options for VIN, now that TLR7 and 8 expressions on human langerhan cells (LC) are well established. TLR 7 and 8 agonists in the form of imiquimod and resiquimod, non-HPV specific immunomodulators, are expected to activate LC exposed to HPV16 leading to the induction of an HPV16 specific cell mediated immune response. Fahey et al compared the levels of immune responses induced on HPV16 infected cervical cells when treated with imiquimod (TLR7 agonist), 3 M-002 (TLR8 agonist), and resiquimod (TLR8/7 agonist). Unexpectedly, both phenotypic and functional hallmarks of immune activation were not observed when treated with imiquimod (TLR7 agonist) in contrast to treatment with resiquimod (TLR8/7 agonist) or 3M-002(TLR8 agonist), where high up-regulation of surface activation markers, proinflammatory cytokines and initiation of HPV-16-specific CD8+ T lymphocyte response was evident. This data strongly suggests that other Toll-like receptor agonists like resiquimod or TLR8 agonist (3M-002) might indeed be superior to imiquimod as therapeutic strategies for HPV infections and HPV induced intraepithelial neoplasia like VIN [Fahey et al., 2009].

For any therapy to be effective against multifocal disease, it must also promote strong systemic antitumour response in addition to local response that targets satellite foci in addition to the primary tumour. Preliminary data has shown that antitumour responses delivered locally by the TLR7 agonists such as imiquimod can be harnessed systemically by combining the treatment with surrogates for CD4 T lymphocyte help for treating the multifocal disease or satellite lesions or even distal tumour. Pending the results of these initial studies, modified TLR agonistic therapy might play a major role in the management of multifocal VIN [Broomfield et al., 2009].
Association of pre-existing proliferative response to HPV16 proteins and treatment response with imiquimod has been detected in the imiquimod studies of VIN indicating that the success of imiquimod treatment may depend on IFNγ producing HPV-16-specific T lymphocytes. If this is true, imiquimod might in fact be more beneficial if used after therapeutic HPV vaccination and generate better clinical response rates. Additionally, there might be a role for using imiquimod as a maintenance therapy after surgery or laser to prevent or reduce recurrences [Van Poelgeest et al., 2005].

The advantage of PDT is short healing time and preservation of vulval anatomy with minimal disfigurement. In a recent study, photodynamic therapy using a novel bio adhesive type patch loaded with 5-ALA designed to conform to the unevenness of vulval skin were tested. Among twenty-three women who in total underwent forty-nine cycles of PDT treatment, 52% reported a symptom response and 38% demonstrated histologic response [Zawislak et al., 2009]. Although imiquimod in combination with PDT has been used successfully in the management of other dermatological conditions [Devirgiliis et al., 2008], to our knowledge, only one study thus far in the management of VIN has been reported where sequential imiquimod and photodynamic therapy was shown to result in better clinical response rates than solo PDT trials and the results were comparable to those achieved with sixteen weeks of imiquimod alone treatment. Additionally, longevity of treatment response was established in this trial [Winters et al., 2008]. The imiquimod / PDT treatment is now being offered to a selected group of women diagnosed with multifocal disease not suitable for surgical excision or as a maintenance therapy following incomplete treatment with surgical excision or laser ablation.

**Prophylactic HPV vaccination** of young women against HPV 16 and 18 is anticipated to lessen the incidence of HPV16, 18 related diseases and with time may significantly avoid about 40% of vulval carcinoma and 75% of VIN [De Vuyst et al., 2009]. On the other hand, it is felt that the potential effect of HPV vaccines on vulval and vaginal cancers is unlikely to be as high as for cervical cancers [Smith et al., 2009]. Although **therapeutic HPV vaccines**
offer an alternative HPV specific immunotherapy, designing a successful therapeutic vaccine can be challenging due to the high genetic variability of the virus.

A number of candidate therapeutic vaccines have been developed and in the trials thus far have proven to be effective, safe, tolerable, and feasible but with limited immunogenicity. DNA vaccines are a potential alternative and patients vaccinated with TA-CIN alone have shown to develop weak HPV neutralising antibodies and E6/E7 specific T lymphocyte responses [Karanam et al., 2009]. Welters et al proposed subversion of the immune system, sub optimal design of therapeutic vaccines, intrinsic property of cancer cells and the regulatory networks imposed by the growing tumours to be the reasons for the vaccine induced T-cell mediated immunotherapy to fail to improve therapeutic results [Welters et al., 2008]. Adjuvant enhances the immunity to vaccine and new approaches to adjuvanted therapeutic HPV vaccines are in motion. By developing a nontransforming HPV16 E7 based vaccine supported by co-application of the adjuvanted gene containing DNA encoded cytokines and chemokines, an enhanced immune response was noted in women with cervical cancer [Ohlschlager et al., 2009].

Several other therapeutic HPV vaccines have been generated which have generated significant antitumour effects. Combining DNA vaccines with immunostimulatory molecules like calreticulin and methylating agents to enhance the level of expression of the antigen encoded in the vaccine have been shown more promise in controlling HPV associated malignancies. Lu et al demonstrate combining CRT/E7 HPV DNA therapeutic vaccination with 5-aza-2'-deoxycytidine (DCA), a demethylating agent, to be a potential promising approach in the management of HPV-associated neoplasia [Lu et al., 2009]. Another therapeutic HPV vaccine ISCOMARIX in randomised, placebo controlled, dose escalation study in HIV positive men for HPV infected AIN to determine the safety, tolerability, and immunogenicity revealed no dose limiting toxicities or serious adverse events. 96% of vaccine recipients had at least a four-fold increase in HPV16 antibody from pre-
vaccination levels and 71% had at least three-fold increase in interferon-gamma responses to E6E7 peptides [Anderson et al., 2009].

On the other hand, DNA vaccines are shown to be less immunogenic than protein or peptide based vaccines. Vaccination with long peptides appears to induce potent T lymphocyte response and this has been ascribed to the efficiency of the vaccine to target dendritic cells, absence of antigenic competition from viral vectors and the high-doses of the specific antigen [Melief and van der Burg, 2008]. The study by Kenter et al is the first therapeutic HPV vaccination (mix of long-peptides from the HPV-16 viral oncoproteins E6 and E7 in incomplete Freund’s adjuvant) study in women with VIN with remarkable results of 79% overall clinical response rate comprising 47% complete clinical response at twelve months, which was also maintained at twenty four months of follow-up. Complete clinical responders mounted a significantly stronger HPV-16 specific immunity and also showed significantly stronger IFNγ associated proliferative CD4+ T lymphocyte responses and a broad response of CD8+ interferon- gamma T lymphocyte in comparison to women who failed to demonstrate a treatment response [Kenter et al., 2009].

With the intention of improving the DNA vaccine efficiency, routes and methods of vaccine administration were also studied. Conventional intramuscular route of administration of a DNA vaccine was compared with electroporation mediated intramuscular delivery and epidermal gene gun-mediated particle delivery in an HPV-16 E7 expressing tumour line. The highest number of E7 specific CD8+ cytotoxic T lymphocytes were generated following vaccination via the electroporation mediated intramuscular delivery. Again, improvised route of vaccine delivery shows promise in human trials of DNA vaccines [Best et al., 2009].

Another challenge to the immunogenicity of vaccines that are specific for tumour antigens is the immune-suppressive effect of the tumour microenvironment and the modulation of T cell expansion by the inhibitory
cells such as the regulatory T cells. The down regulation of T regulatory cells might therefore be beneficial for the enhancement of the vaccine immunogenicity. In fact, T regulatory depletion prior to imiquimod or therapeutic vaccination, particularly in women with higher proportion of systemic or intralesional T regulatory cells pre-treatment might enhance the stimulation and efficacy of the useful HPV specific effector T cells. Thus, blocking T regulatory function could improve human cancer immunotherapy.

Indeed, there have been several attempts to improve the outcome of different vaccination regimes either by down regulating or blocking T regulatory cells with anti-CD25 antibodies. An anti-CD25 monoclonal antibody (PC61) when used prior to DNA vaccination which encoded E7 linked to heat shocked protein 70 (Hsp70), generated higher levels of E7-specific CD8+ T lymphocytes compared to the control antibody, leading to significantly improved therapeutic and long-term protective anti-tumour effects against E7-expressing tumour, TC-1[Chuang et al., 2009]. Elimination of T-regulatory cells using the recombinant IL-2 diphtheria toxin conjugate (DAB (389) IL-2) was studied in patients with metastatic renal cell carcinoma. In this study, DAB (389) IL-2-mediated elimination of T regulatory cells followed by vaccination with RNA-transfected dendritic cells significantly improved the stimulation of tumour-specific T lymphocyte responses in renal cell carcinoma patients when compared with vaccination alone, suggesting a role for T-regulatory cell depletion strategy to achieve potent anti-tumour immunity [Dannull et al., 2005].

5.10 FUTURE DIRECTION & CONCLUSIONS

VIN is not a common condition and research into this condition is limited. A number of treatment options have shown promise, but are yet to be proven in randomised controlled studies. Although progression of VIN to invasive carcinoma is assumed to be low, around 9% over a number of years of disease positivity, spontaneous regression is rare, around 1% [van Seters et al., 2005]. The rate of progression to invasive carcinoma has however been
questioned as majority of women diagnosed with VIN undergo treatment for one reason or the other [Jones et al., 2005; McFadden et al., 2009].

The treatment choice for VIN is based on several factors, including precise location of the lesions, the number of lesions and patient’s past medical, surgical and treatment history. Although the existing treatments of surgery and laser are effective, there are certain limitations, such as residual disease, high rates of recurrence and treatment associated physical and psychological morbidity causing an impact on the quality of life. Imiquimod and PDT, although associated with poor patient tolerability, show promise. Strategies are in place to improve the efficacy of therapeutic HPV vaccination. Monotherapies are unlikely to be effective and the way forward must be the combination of treatment that eliminate the lesion and prevent recurrences. Combination treatments of imiquimod with PDT or therapeutic HPV vaccination appear to deliver a greater response to treatment than PDT or vaccination alone.

Saraiya et al emphasised the need for reporting and submitting high quality data on VIN3 and more research on the data in the cancer registers to enable further assessment and understanding of the impact of HPV on the disease [Saraiya et al., 2008]. Not many studies thus far have recruited large enough subject numbers or long-enough follow-up to enable robust conclusions. For conditions like VIN, which pose therapeutic challenges, there is a strong case for establishing research collaboratives. A multi-centre trial has the advantages of quicker recruitment of the necessary number of patients with quality assurance related to recruitment, treatment, and follow-up. Consensus needs to be reached on how outcomes are measured and response is defined. The acceptance of results will then be higher as the patient sample of multicentre trials is supposed to be a true representation.

Developing means of pre-treatment identification of potential responders from the likely non-responders using local or systemic biomarkers will be valuable. Although immune correlates for a clinical response need determining, pre-treatment assessment of dominant immunological cell types in the lesion
microenvironment and levels of systemic cytokines might help differentiate potential responders from non-responders. Identifying assays that are likely to correspond to measurable immunological variables after treatment, like proliferation assays, interferon gamma ELISPOT assay, interferon-gamma ELISA assays [Baldwin et al., 2003; Davidson et al., 2003a; Kenter et al., 2009; Smyth et al., 2004; Van Poelgeest et al., 2005; Winters et al., 2008] are also likely to aid in the assessment of response to treatment. Smyth et al. have suggested that pre-selection of VIN patients with HLA class-I positive lesions displaying intralesional T lymphocyte infiltration before vaccination may identify a group of subjects who benefit the most from vaccination [Smyth et al., 2004].

At face value looking at pre-existing immunity to HPV could provide the basis for selecting patients most likely to benefit from imiquimod with or without vaccination regimes [Van Poelgeest et al., 2005; Winters et al., 2008]. Advance in the methods of identifying HPV specific T lymphocyte responses could enable monitoring of immune T lymphocytes in HPV associated clinical lesions, and of the correlation of tissue and systemic immune responses to HPV proteins. Equally, studying the lesion microenvironment some time after treatment can provide further insight into how the immunotherapy might work for persistent infection.

When attempting more than one form of medical treatment for VIN in a sequential fashion, similar to the current and previous studies from this unit [Winters et al., 2008], a crossover clinical trial design is more likely to distinguish the individual treatment contributions. In crossover trial design, there is a possibility of carrying over the benefit either through persistent action of the treatment or simply by an enhancement in immune response induced by the initial treatment. This might however make the second treatment in the crossover trial seem more effective than the first.

The response rates noted in this trial support further innovative trial, in particular the addition of an adjuvant to therapeutic HPV vaccines or a different type of HPV therapeutic vaccine.
A multicentre randomised study comparing upfront surgical excision/laser ablation with solo toll-like receptor agonists or therapeutic HPV vaccination, and a combination of toll-like receptors with therapeutic HPV vaccination in a crossover study design will be valuable to understand the individual role of each of these modalities and also to detect any synergistic action when administered sequentially.
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APPENDIX 1

Phase II trial of Imiquimod and HPV therapeutic vaccination in patients with Vulval Intraepithelial Neoplasia

Sai Daayana¹,², Eyad Elkord², Ursula Winters¹,², Michael Pawlita³, Richard Roden⁴, Peter L Stern²*, Henry C Kitchener¹.

¹Academic Unit of Obstetrics and Gynaecology, University of Manchester, St Mary’s Hospital, Whitworth Park, Manchester M13 0JH, UK;
²CR UK Immunology Group, Paterson Institute for Cancer Research, University of Manchester, Christie Hospital NHS Trust, Manchester M20 4BX, UK;
³Department of Genome Modifications and Carcinogenesis, German Cancer Research Centre (DKFZ), Im Neuenheimer Feld 280, Heidelberg, Germany
⁴Department of Pathology, Johns Hopkins University, Baltimore, MD 21231, USA.

*Correspondence: Professor Peter L Stern, CRUK Immunology Group, Paterson Institute for Cancer Research, University of Manchester, Manchester M20 4BX, UK
E-mail: PStern@picr.man.ac.uk. Phone: 44-161-4463127 or Professor Henry C Kitchener, Academic Unit of Obstetrics and Gynaecology, University of Manchester,
St Mary’s Hospital, Whitworth Park, Manchester M13 0JH, UK E-mail: Henry.c.Kitchener@manchester.ac.uk. Phone: 44-161-2766461

Running Title: Immunotherapy for Vulval Intraepithelial Neoplasia
ABSTRACT

BACKGROUND: Vulval intraepithelial neoplasia (VIN) is a premalignant condition, which is frequently associated with type HPV16 infection, and multifocal disease has high rates of surgical treatment failure.

METHODS: We report a phase II clinical trial of the topical immunomodulator, imiquimod for 8 weeks, followed by 3 doses (weeks 10,14,18) of therapeutic HPV vaccination (TA-CIN, fusion protein HPV16 E6E7L2) in nineteen women with VIN2/3. Histology and HPV testing of biopsies were performed at week 0, 10, 20 and 52. Intralesional infiltration of T cell subsets and lymphocyte proliferation for HPV systemic immune responses were also assessed.

RESULTS: Lesion response (complete regression of VIN on histology) was seen in 32% (6/19) of women at week 10, increasing to 58% (11/19) at week 20 and 63%(12/19) at week 52. At this time 36% (5/14) of lesions showed HPV 16 clearance and 79% (15/19) of women were symptom free. At week 20, after treatment with imiquimod and vaccination, there was significantly increased local infiltration of CD8 and CD4 T cells in lesion responders; by contrast non-responders (persistent VIN by histology) showed an increased density of T regulatory cells. Following vaccination, only lesion responders had significantly increased lympho-proliferation to the HPV vaccine antigens.

CONCLUSION: The therapeutic impact of treatment depends on the differential immune response of responders and non-responders impacting both locally and systemically.

Keywords: Vulval intraepithelial neoplasia (VIN), imiquimod, therapeutic HPV vaccination, T regulatory cells
INTRODUCTION

Vulval intraepithelial neoplasia (VIN) is increasing in incidence, more so in younger women. A greater than 400% increase in the incidence of VIN has been reported between 1973 to 2000, whilst the corresponding increase in incidence of vulval cancer has only been 20% [Akerman et al., 2007a]. This probably reflects an increasing incidence of human papillomavirus (HPV) high risk type genital tract infections, as the most common type of VIN (usual or undifferentiated) shows over 85% HPV positivity, with type16 DNA being detected in 75% of high grade VIN [De Vuyst et al., 2009]. HPV infection of the lower genital tract and its outcome are influenced by host immune response, virulence of the HPV, smoking and immunosuppression [Sherman et al., 2008; Duong and Flowers, 2007]. Successful adaptive immune responses which lead to clearance of genital HPV infection are believed to be mediated by local T cell mediated immunity [Farhat et al., 2009]. The virus life cycle and several immune evasion mechanisms limit virus-specific immunity facilitating persistent infection and the increased risk of carcinogenesis [Stern, 2005]. Indeed, it has been shown that patients with persistent HPV infection have depleted T cell responses against the viral early gene products E2 and E6 measured systemically [de Jong et al., 2004; Farhat et al., 2009]. In addition, T regulatory cells are negative regulators of otherwise useful anti-tumour T cell immunity and locally increased levels have been implicated in the persistence of both cervical and vulval HPV associated lesions [van der Burg et al., 2007; Winters et al., 2008].

VIN, which is often multifocal and extensive has been traditionally treated by surgical excision or laser ablation but such therapies are associated with high rates of recurrence [Jones et al., 2005]. Unfortunately, for patients with chronic VIN, younger
age and extent of surgery correlate directly with psychological morbidity and poorer sexual function [Likes et al., 2007; Shylasree et al., 2008; Thuesen et al., 1992]. The natural history of VIN can be viewed as a struggle between HPV driven premalignant intraepithelial neoplasia and immune control mechanisms. With time, the selection of cells with a malignant phenotype and ability to escape the immune control can result in vulval cancer. Non-surgical management of VIN with anti-viral and anti-neoplastic agents has had limited success [Tristram and Fiander, 2005; Vilmer et al., 1998] but a rational therapeutic strategy for chronic VIN could aim to alter the local immune response in favour of persistently HPV infected cells.

Several different clinical studies influencing local and/or systemic immunity to HPV using imiquimod, photodynamic therapy (PDT) and therapeutic HPV vaccination in the management of VIN have been reported. Imiquimod, an immune response modifier acting through a Toll like receptor (TLR), [Schon and Schon, 2007] can stimulate local innate immunity (non-specific) with potent anti-tumoral effects but also drives an adaptive immune response (e.g. specific T cell effectors) in secondary lymphoid tissues by activating tissue antigen presenting cells. In a recent randomised controlled trial, treatment of VIN with imiquimod twice weekly for 16 weeks resulted in a 25% or more lesion size reduction at 20 weeks in 81% of patients compared with none in the placebo group [van Seters et al., 2008b]. PDT uses a combination of light and photosensitiser drug to damage tumour tissue by modifying cellular functions, inducing cell death by necrosis or apoptosis, and encourages inflammation and anti-tumour immunity [Castano et al., 2006]. Studies using PDT have shown good symptom and clinical response in patients with VIN [Fehr et al., 2001; Martin-Hirsch et al., 1998; Olejek et al., 2008]. A recent trial of combined treatment with imiquimod
followed by PDT [Winters et al., 2008] showed an overall lesion response rate of 55% with 30% showing complete lesion resolution at 52 weeks. In this study, non-responders showed a significantly higher level of T regulatory cells in the lesions after imiquimod treatment [Winters et al., 2008]. Such response rates are clinically relevant, and the treatment regimen was feasible for the majority. Non-responders to imiquimod seem to be relatively refractory, and this may derive from their unfavourable local immune environment, in particular, increased proportions of T regulatory cells, limiting the action and development of any HPV T cell immunity. Therapeutic HPV vaccines are designed to generate cell-mediated immunity against HPV infected cells that express the early viral proteins E6, E7 which act as oncogenes and also tumour specific antigens. Clinical trials using either PDT or therapeutic HPV vaccination have demonstrated an association between clinical responses and tumour infiltrating lymphocytes (TILs) [Abdel-Hady et al., 2001;Davidson et al., 2003a].

Optimal therapeutic immunity results from the interaction of non-specific innate immunity and antigen specific adaptive immunity. Stimulation of the local innate immunity can have a direct controlling effect on virally infected cells as well as attracting local infiltration of TILs [Dermime et al., 2002b]. The challenge is to get the most useful balance of helper (CD4) and cytotoxic (CD8) T cell infiltration which are associated with positive prognosis and minimize the T regulatory cell infiltration which is known to be associated with poor outcome as shown in several studies of gynaecological cancer [Kondratiev et al., 2004;Sato et al., 2005;Wolf et al., 2005b;van der Burg et al., 2007]. The rationale of this study was that an initial local imiquimod treatment, in addition to having a direct effect on VIN can also provide an
immunological platform for the therapeutic HPV vaccination to achieve an enhanced and durable response.

In this phase II trial, we used a combination of imiquimod and vaccination with TA-CIN which is a subunit vaccine comprising a HPV16 E6E7L2 fusion protein, proven safe and immunogenic in previous phase I and II trials [Davidson et al., 2004; de Jong et al., 2002; Smyth et al., 2004].

PATIENTS AND METHODS
Women aged 18 - 70 years with biopsy proven VIN grades 2 and 3, were recruited between March 2006 and May 2007. Exclusion criteria were pregnancy, invasive disease, immunosuppression, history of severe allergy and previous HPV vaccination. Imiquimod 5% cream was self administered for 8 weeks escalating from one application in week 1 to two in week 2 and three applications weeks 3-8. This was followed by three intramuscular doses of TA-CIN (1ml of 128µg/ml) at weeks 10, 14 and 18. The primary objective was to measure treatment impact on VIN by lesion size and histology and the secondary objectives were to assess lesion HPV status, symptoms, immune responses as well as safety, toxicity, and tolerability. Women were reviewed at weeks 0, 10, 14, 18, 20, 26 and 52, the primary end-point. Punch biopsies were taken for histology and HPV typing at weeks 0, 10, 20 and 52. Heparinised blood (40ml) was obtained for immunological assays at weeks 0, 10 and 20.
Clinical response

Complete regression of VIN on histopathology of an index lesion(s) was designated the lesion response. Histological assessment was performed on formalin-fixed punch biopsies. Biopsies were analysed for HPV typing by Reverse Line Blot Assay which is able to amplify and detect 37 HPV genotypes [Gravitt et al., 1998]. Safety, tolerability and toxicity were assessed clinically, supplemented by FBC and serum biochemistry at weeks 0, 10 and 20. A full record of adverse events was kept. Patients were advised to maintain a symptom diary in a visual analogue scale form. This was reviewed at clinic visits when patient assessment of vulval pain, itch, swelling, discharge and/or any other symptoms were graded as none, mild, moderate or severe. Regression of symptoms from moderate or severe (interfering with life style) to the mild or none (not interfering with life style) categories was considered to be a symptom response.

Immunofluorescence

Local immune responses were quantified by assessing the TIL density on 7µm sections of the punch biopsy frozen in liquid nitrogen and stored at -80°C. TILs from five representative fields of image were counted for each section. T regulatory cells were identified by double immunofluorescence labelling for FoxP3 and CD4. This was performed with mouse IgG2a anti-CD4 (Abcam) concentration 1:100, mouse IgG1 anti-FOXP3 (eBioscience), concentration 1:50 and respectively detected using goat antimouse IgG2a Alexa Fluor 546 3µl/1000µl and goat antimouse IgG1 Alexa Fluor 488 at 3 µl/1000µl (Invitrogen). CD8 labelling was performed using rabbit IgG anti-CD8 (Abcam) concentration 1:100 and was detected using goat anti rabbit-IgG Alexa Fluor 488 at 3 µl/1000µl. Nuclei were stained with DAPI. Five microscopic
fields were imaged in a step-wise fashion for each cellular phenotype investigated in the VIN biopsies. In most cases, this covered almost the whole specimen, as the sections were small punch biopsies. The TIL counting was then performed using Image J image analysis software version 1.36b (http://rsb.info.nih.gov/ij/), using the cell counting feature of the software. An independent observer reviewed a range of slides and the counts differed by no more than 5%. Results were reported as density per unit area of CD4, CD8 and double stained FoxP3CD4 cells.

**Peripheral immune response**

Peripheral immune response to HPV antigens was assessed by lymphoproliferative assays. Peripheral blood mononuclear cells (PBMC) alone or PBMC with 25µg/ml recombinant HPV antigens: HPV16 E6E7L2 protein - TA-CIN (Xenova Research Ltd.), HPV16 GST E6, HPV16 GST E7[Smyth et al., 2004], HPV16L2 (full length HPV16L2 tagged with 6His at N-terminus), TA-GW (HPV 6 L2E7; Xenova Research Ltd.) and 2µg/well PHA were performed as previously described [Winters et al., 2008]. Responses were measured by tritiated thymidine incorporation following a 5-day incubation of patient PBMC with the different antigens. Results are presented as Stimulation Index (SI) defined as the mean number of counts incorporated by the antigen stimulated PBMCs divided by the mean number of counts for PBMCs in medium alone. A pre-existing proliferative T cell response was defined as SI ≥ 2. A post vaccination proliferative T cell response was defined as a 2-fold increase in the SI compared with the pre-vaccination value.

Neutralising antibodies to HPV 16 pseudovirions were tested as by the SEAP pseudo virus neutralisation based assay as previously described [Karanam et al., 2009].
**Statistical analysis**

Analyses were performed using ‘Stats Direct Statistical Software’. Differences between responder and non-responder categories (baseline disease duration; HPV 16 positivity; smoking; symptom response at week 52 compared to baseline) were analysed using Fisher’s exact test. The non-parametric Mann-Whitney U was used to test for differences in proliferative and lesion T cell density between groups (lesion responders and non-responders). The non-parametric Wilcoxon’s signed ranks test was used to analyse differences in the responses within groups at different time-points. All reported P values are two sided and have not been adjusted for multiple comparisons. A P value of $\leq 0.05$ was considered to indicate statistical significance.
RESULTS
Twenty women consented to the trial. One woman, found to have early stromal invasion on the first study biopsy, was withdrawn from the study and excluded from the analysis, which is based on 19 women. Patient demographics are presented in Table 1. The group had a mean age of 46 years with average disease duration of 7 years (range 1-20 yr). Thirteen patients had been previously treated and seven had at least three prior treatments. Only 4/19 (21%) never previously smoked cigarettes.

Tolerability
The majority of women experienced local inflammation, ulceration, malaise and flu like symptoms within the initial few weeks of imiquimod. Side effects were classed as severe in 14 and moderate in 5 women. In order to improve tolerance, treatment-free intervals of no more than a few days were allowed in order to achieve the total dose, but let side effects subside. 16/19 (84%) of women completed the prescribed course of imiquimod treatment. Three women noted a marked improvement in facial acne with fewer spots and smoother skin in general whilst on imiquimod treatment. TA-CIN, administered intramuscularly into the deltoid muscle was well tolerated with no side effects or adverse events.

Response Rates
Table 2 reports the VIN lesion size, histology, HPV status and any patient symptoms at baseline (week 0), after imiquimod (week 10), after vaccination (week 20) and at 52 weeks. Figure 1a stratifies lesion size reduction at weeks 10, 20 and 52 compared to week 0. For example, 74, 85 and 79% of women had a ≥ 50% reduction in the size of lesion at weeks 10, 20 and 52 respectively. However, patient 8 still had a visible
VIN like lesion at week 52 but the comprehensive histological analysis indicated resolution of VIN (Table 2). A background of inflammation and pigmentation can confound the true assessment of any lesion and emphasises the need for histological definition of VIN. Figure 1b summarizes the lesion response and HPV status as well as patient symptom responses with time. Complete regression of VIN on histology was 32, 58 and 63% at weeks, 10, 20 and 52 respectively. At baseline, 15/19 (79%) women had moderate to severe symptoms compared with 11/19 (58%) at week 10 with significant reduction at week 20, 5/19 (26%), sustained at week 52, 4/19 (21%), (P=0.01). At weeks 0, 10, 20 and 52; 14, 8, 9 and 10 of the nineteen women were HPV16 positive respectively; 16/19 were HPV 16 positive on at least one occasion. Three women were consistently HPV 16 negative but two showed other HPV type infection at some point. Of the women who showed lesion responses at week 52, 5/10 also cleared their HPV 16 infection compared to only 1/6 non-responders; this did not reach statistical significance. There were no statistical differences in the lesion responder and non-responder groups with respect to disease duration (Table 1) at trial entry (P=0.58) or HPV16 detection at baseline (P=0.66). 12 out of 19 women were active smokers during the trial period. Current smoking habit was not associated with lesion response (P=0.1).

Extended follow-up of these patients for an average of 15 months beyond the primary end point of week 52 showed 84% of patients with a ≥ 50% reduction in lesion size consistent with continuing control of VIN (Figure 1a). Unfortunately, biopsy of any index lesion after week 52 was not performed unless clinically indicated. Overall, one patient developed microinvasive disease (patient 7), two had new lesions after week
52 (patient 8 and 17), one had a recurrence 6 months after the trial endpoint (patient 14) (Table 2).

**Systemic immune response to HPV 16**

Pre-existing HPV 16 antibodies correlated with documented HPV 16 exposure in 12/15 cases. Overall patient pre-existing neutralizing antibody levels did not differ or alter significantly after imiquimod or vaccination in either the lesion responders who cleared VIN histologically nor the non-responders who failed to clear VIN (data not shown).

Lymphoproliferation to HPV antigens was used to analyse patient systemic cellular immunity to HPV before and after the imiquimod and vaccination steps in the protocol. Sixteen women (84%) demonstrated pre-existing lymphoproliferative response to TA-CIN (SI ≥ 2), of whom 14 were HPV16 positive on vulval biopsy on at least one occasion. Of these, 11 were lesion responders and 5 non-responders with no significant difference in magnitude of pre-existing response (P=0.4). Following vaccination, 10/12 lesion responders compared to 3/7 non-responders showed ≥ 2-fold SI change compared with baseline. Figure 2 shows box-plots of the median and quartile lymphoproliferative responses of all the patients as well as stratification into lesion responders or non-responders to the different antigens, TA-CIN vaccine (HPV16 E6E7L2 fusion protein), its component HPV 16 antigens L2, E6 and E7 as GST fusion proteins, TA-GW (HPV 6 L2E7 fusion protein) as a negative control and PHA as a positive control for lymphocyte proliferation. Strong PHA responses were demonstrated by all the women with median SIs of 12, 10.6, 16.9 at week 0, 10 and 20 respectively; these responses were not significantly different after imiquimod or
vaccination (P=0.4, 0.2 in the group) or when patients were stratified by lesion response (P=0.7, 0.5 in responders and P=0.3, 0.3 in non-responders) (Figure 2a). By contrast, a significant increase in proliferation to TA-CIN was seen in the patients after vaccination compared to pre-treatment (P=0.01) and this was associated with lesion responders (P=0.008) but not the non-responders (P=0.7) (Figure 2b). Similar significant patient proliferative responses to each of the individual HPV16L2, E6 and E7 antigens after vaccination were seen (P=0.02, 0.01, 0.02 respectively), with this increased proliferation associated with lesion responders (P=0.01,0.03,0.03) and not non-responders (P=0.5,0.5,0.2) (Figure 2c, d, e). Specificity of the HPV 16 responses was supported by the absence of any significant increase in patient lymphoproliferation post-vaccination to TA-GW (HPV6 L2E7), nor in the responder (P=0.4) or non-responder (P=0.9) groups (Figure 2f). No significant increase in proliferation to any of the antigens was noted post-imiquimod in any of the patient groups. Post-vaccination proliferative response to TA-CIN and its components appears to correlate with the clearance of VIN on histology.
**Tumour infiltrating lymphocytes**

To investigate local immune factors in the vulval biopsies pre-treatment, post-imiquimod and vaccination, the densities per unit area of CD4, CD8 and double stained FoxP3CD4 (T regulatory) cells were assessed. Imiquimod treatment was expected to enhance the local immune infiltration. The data are presented in Figure 3 as median/scatter plots for all the patients as a group or stratified by lesion response. In the group as a whole, significant increases in the number of CD4, CD8 and FoxP3CD4 T cells were evident by week 20 compared to baseline (P=0.03, P=0.01, P= 0.04 respectively); a significant increase in CD8 density was apparent after imiquimod (P=0.04). At week 20, the increased CD4 and CD8 density was significantly associated only with the lesion responders (P=0.03; P= 0.03) while increased FoxP3CD4 density was associated with the patients who do not show lesion response (P=0.05). There were no significant pre-treatment differences in the density of CD4 or CD8 T cells in lesion responder and non-responders (P=0.2, P=0.5 respectively) but intralesional FoxP3CD4 density was significantly higher in non-responders compared to responders (P=0.05). By week 20, a significant reduction in FoxP3CD4 T cell population density was apparent in lesion responders, which was significantly different from non-responder densities (P=0.01).
DISCUSSION

This study was designed on the premise that imiquimod and therapeutic HPV vaccination could combine to alter the balance of local immunity through inducing a local inflammatory environment and enhancing T cell responses to HPV E6 and E7 proteins. Immunotherapies that tip the balance of immune equilibrium in favour of the host effector response and away from regulatory and viral evasion strategies of the HPV may be the key to enhancing the cell mediated immunity required to eradicate persistent HPV infection and established disease. This study demonstrated that imiquimod followed by vaccination achieved histological clearance of VIN at 52 weeks in almost 60% of a heavily pre-treated cohort of women with high-grade, long-standing VIN.

With the proviso that the study size was small, the analyses of lesion associated T cells showed that higher pre-existing and post-treatment levels of regulatory T cells are associated with a lack of lesion response to treatment. As expected in the group as a whole, imiquimod treatment induced T cell infiltration, which was most apparent by week 20. However, the increased CD4 and CD8 T cell density was significant only in the lesion responder group while a significantly higher regulatory T cell density was only seen in the non-responder group. These observations are consistent with immune control and therapeutic impact reflecting the balance of useful CD4 and CD8 directed effectors against their control by T regulatory activity. Thus, increasing the CD8/CD4 T cell density may be able to re-establish local immune control which is lost or suppressed in chronic VIN [Gul et al., 2004]. Where the T regulatory cells dominate the local immunological milieu they can continue to suppress the HPV antigen-specific cytotoxic T cell response facilitating persistent VIN [Kobayashi et al.,]
T regulatory depletion prior to imiquimod or therapeutic vaccination, particularly in women with higher proportion of systemic or intralesional T regulatory cells pre-treatment might enhance the stimulation and efficacy of the useful HPV specific effector T cells. Indeed, there have been several attempts to improve the outcome of different vaccination regimes either by down regulating or blocking T regulatory cells with anti-CD25 antibodies [Chuang et al., 2009] or IL-2 diphtheria toxin conjugates [Dannull et al., 2005].

The TA-CIN vaccination component of the protocol was aimed at expanding E6 and E7 specific T effectors with the possibility that post imiquimod their entry and activity in the VIN lesions would not be limited by local immunosuppressive factors. Lymphoproliferation of PBMC established that all patients were immunocompetent and that the vaccination was immunogenic and HPV 16 antigen specific. Importantly, these systemic immune responses to HPV 16 antigens were significantly associated with lesion responders and not the non-responders. There was no influence on immunity to HPV as a result of the imiquimod treatment. However, a trend towards higher pre-existing responses to HPV16 early antigens in the lesion responder group was noted in this study, which was significantly stimulated by the vaccination. By contrast, the lesion non-responders showed little pre-existing response and this was not boosted by the vaccine. Previous studies have noted a significant correlation between pre-existing systemic HPV 16 specific T cells and regression of HPV 16 positive lesions [Van Poelgeest et al., 2005; Winters et al., 2008]. The immune response to the HPV vaccine used in this study might have additional impact if delivered with an adjuvant which can boost both serological and cellular immune responses to its HPV 16 antigens [Karanam et al., 2009].
Overall, it appears that the natural history of the VIN in the non-responders is related to modulation of both local and systemic immune responses allowing persistence of HPV infection. The mechanisms underlying this chronic VIN state are not known but might include genetic predisposition involving immune and other parameters [Davidson et al., 2003b]. In chronic VIN the sub optimal stimulation of HPV immunity probably also leads to anergy of HPV specific T effectors. The precise time course of immune responses relevant to HPV lesion clearance are completely unknown and there is no a priori reason why it would achieve its full potential at the arbitrary study endpoint especially for chronic lesions, which have been present for many years. Thus, although some of the histological and symptom response noted at week 52 was evident from week 10 (post-imiquimod), response enhanced with time. It is reasonable to argue that an immunologically challenged chronic condition which takes time to establish will equally take time to regress, explaining the continuing response noted long after treatment completion. In this study of a heavily pre-treated cohort of women with long standing disease, containment of response for two years is worthy of note. Patient 5 (40 yrs, VIN for 20 years, 7 surgical excisions) and patient 19 (35yrs, multifocal VIN for 10 years, 10 surgical treatments) illustrate particularly impactful lesion responses achieved by week 52 in this study but which have been maintained for a further two years thus far. In both these cases, intralesional pre-treatment T regulatory cell density was low, with no enhancement following treatment contrary to increased density of CD4 and CD8 cells. A pre-existing proliferative response to HPV16 with significant increase in proliferation response post-vaccination was also demonstrated. It would be beneficial if the likelihood of response to treatment could be determined using biomarkers either pre-treatment or in the case of combined therapy, following imiquimod, in order to avoid subjecting likely non-
responders to unnecessary treatment. At face value, pre-treatment assessment of dominant immunological cell types in the lesion microenvironment and looking at pre-existing immunity to HPV could provide the basis for selecting patients most likely to benefit from imiquimod with or without vaccination regimes.

In considering the imiquimod treatment, tolerability is a significant issue since the majority of women experience local and systemic side effects lasting for the duration of imiquimod treatment, which may impact on daily activities. Overall, our regimen was feasible but as expected, was associated with considerable imiquimod induced discomfort necessitating breaks in treatment. Women with refractory VIN are, however, highly motivated to comply with the treatment protocol, and in the event, 85% persevered with treatment and finished the full course of imiquimod. In this study, the lesion response was 63% at week 52 with 8 weeks of imiquimod followed by vaccination, which compares very favourably with imiquimod alone treatment of 16 weeks assessing histological regression of VIN as 64% assessed soon after treatment as VIN1 or better [Le et al., 2007], 81% [Mathiesen et al., 2007] and 69% [van Seters et al., 2008b] with no VIN at 2 months after treatment.

A recent study of VIN patients treated by 3 or 4 immunizations at 3 weekly intervals with a HPV vaccine composed of long HPV16 E6/E7 peptides in adjuvant (Montanide ISA-51, Seppic) showed 60% and 79% lesion size response rate (≥ 50% reduction) at 3 and 12 months of follow-up [Kenter et al., 2009]. As suggested by Kenter et al., imiquimod might be more beneficial if used after therapeutic HPV vaccination as studies indicate that imiquimod treatment may depend on IFNγ producing HPV specific T cells [Kenter et al., 2009].
Following another recently published study of imiquimod followed by photodynamic therapy [Winters et al., 2008], continued surveillance for up to 3 years (unpublished results) has shown a sustained clinical response rate of 65% at follow-up. This treatment is now being offered to women in our unit for whom surgical therapy is not suitable. Distinguishing the contribution of the individual components of such combination regimes and establishing proof of either additive or synergistic effects will require further innovative trials. All the recent studies of immunologically driven treatments of VIN provide momentum for further multicentre randomised trials with consistency in measurement of outcomes and definitions of response. Comparing upfront surgical treatment with imiquimod or other potential more potent Toll-like receptor agonists [Fahey et al., 2009] or therapeutic HPV vaccination [Karanam et al., 2009; Kenter et al., 2009], or a combination of Toll-like receptor agonists with therapeutic HPV vaccination in a crossover study design will be valuable.

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Disclosures

RBSR is an inventor on L2 patents licensed to Shantha Biotechnics, Ltd., PaxVax, Inc. and Acambis, Inc. The terms of these arrangements are being managed by Johns Hopkins University in accordance with its conflict of interest policies.

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Figure legends

**Figure 1a. Reduction in Lesion Size.** Reduction in lesion size by 100%, >50% but less than 100% and <50% at baseline (Wk0), post-imiquimod (Wk10), post-vaccination (Wk20), primary end-point (Wk52) and follow-up (62 wk after primary end-point) in the group (n=19).

**Figure 1b. Lesion and Symptom Responses.** Lesion response (complete disappearance of VIN on histology), symptom response (regression to mild/none symptoms), absence of HPV 16 on biopsy) at baseline (Wk0), post-Imiquimod (Wk10), post-vaccination (Wk20) and primary end-point (Wk52) in the group (n=19).

**Figure 2. Box-feather plots showing median and quartile lymphoproliferation responses.** The figures show stimulation indices of proliferation in response to PHA (a), TA-CIN (b), HPV-16L2 (c), GST-E6 (d), GST-E7 (e), TA-GW (f) in the whole group (n=19), lesion responders (R)(n=12), and lesion nonresponders (NR)(n=7) at baseline (Wk0), post-Imiquimod (Wk10), post-vaccination (Wk20).

P values which are statistically significant for stimulation indices (SI) at Wk20 compared to Wk0 are shown. The Wilcoxon’s signed ranks test was used to determine the significance of within group differences pre and post treatment. P value of \( \leq 0.05 \) was considered significant.

**Figure 3. Median/Scatter-dot plot of lesion associated immune cells in the group (G), lesion responders (R) and non-responders (NR).**

The figure shows median number (density per unit area) of CD4, CD8 and FoxP3CD4 cells pre-treatment (0), post-imiquimod (W10) and post-vaccination (W20) in the group (G), lesion responders (R) and non-responders (NR). P values for statistically
significant difference in the number of cells either at week 10 or week 20 compared to week 0 are shown. The Wilcoxon’s signed ranks test was used to determine the significance of within group differences pre and post treatment. P value of $\leq 0.05$ was considered significant.

Figure 1a.

| % Response | Week0 | Week10 | Week20 | Week52 | F/U - 62Week |
|------------|-------|--------|--------|--------|-------------|
| <50% Reduction | 100   | 26     | 15     | 21     | 16          |
| >50% Reduction  | 58    | 53     | 21     | 31     |
| 100% Reduction  | 16    | 32     | 58     | 53     |

Figure 1b.

| % Response | Week0 | Week10 | Week20 | Week52 |
|------------|-------|--------|--------|--------|
| Lesion Response | 0     | 74     | 84     | 79     |
| None/mild symptoms | 16    | 42     | 74     | 79     |
| HPV 16 negative     | 26    | 58     | 53     | 47     |
Figure 2

(a) Response to PHA

(b) Response to TA-CIN

P=0.008

P=0.01

(c) Response to HPV16-L2

P=0.02

P=0.01

(d) Response to GST-E6

P=0.01

P=0.03

(e) Response to GST-E7

P=0.02

P=0.03

(f) Response to TA-GW
Figure 3

CD4-G

CD4-R

CD4-NR

CD8-G

CD8-R

CD8-NR

FoxP3-G

FoxP3-R

FoxP3-NR

P=0.03

P=0.04 P=0.01

P=0.05 P=0.03

P=0.04

P=0.05

W0 W10 W20
Table 1. Patient Demographics. M, multifocal; U, unifocal; CIN, cervical intraepithelial neoplasia; Hyst, h/o hysterectomy; Ex, Ex-smoker.

| Pt no | Age (yr) | Focality | h/o CIN | Smoking | Prev Treatment | Disease duration (yr) |
|-------|----------|----------|---------|---------|----------------|----------------------|
| 1     | 51       | M        | Yes     | No      | LASER Vulvectomy | 4                    |
| 2     | 43       | M        | Yes     | Yes     | LASER x 4       | 9                    |
| 3     | 45       | M        | Yes     | Ex      | Excision        | 3                    |
| 4     | 40       | M        | Yes     | Yes     | Excision        | 10                   |
| 5     | 40       | M        | No      | Yes     | Excision x 7    | 20                   |
| 6     | 65       | M        | No      | Ex      | None            | 4                    |
| 7     | 58       | M        | Yes     | Yes     | Excision x 3    | 15                   |
| 8     | 27       | M        | Yes     | Yes     | None            | 1                    |
| 9     | 51       | Hyst     | Ex      | Diathermy|               | 1                    |
| 10    | 55       | U        | Yes     | Yes     | Excision        | 6                    |
| 11    | 51       | U        | No      | Yes     | LASER x 2       | 16                   |
| 12    | 47       | U        | Yes     | No      | None            | 1                    |
| 13    | 56       | M        | Yes     | Yes     | None            | 2                    |
| 14    | 43       | M        | Hyst    | No      | LASER x 2       | 4                    |
| 15    | 48       | U        | No      | No      | None            | 1                    |
| 16    | 34       | M        | No      | Yes     | LASER Imiquimod | 3                    |
| 17    | 22       | M        | No      | Yes     | None            | 1                    |
| 18    | 66       | M        | Hyst    | Yes     | Excision x 5    | 16                   |
| 19    | 35       | M        | Yes     | Yes     | Excisions x 6    | 9                    |

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Table 2. Clinical Responses in VIN patients treated by Imiquimod and TA-CIN. neg, negative; n/e, non evaluable. Pt 7 developed ESI (χ) at week 30; Pt 1,2,4, 10 underwent LASER treatment (δ) after trial completion, Pt 14 developed recurrence (β) 6 months after trial completion and underwent LASER and surgery. Pt 8 and 17 developed new lesions (α) after week 52 treated with LASER. Pt 16 repeated course of imiquimod (ϕ)after trial completion.

| Pt no | Lesion Size in mm | Histology | HPV type | Symptoms |
|-------|------------------|-----------|----------|----------|
| 18    | 80               | VIN1/2/3  | neg      | 16       | Mild     |
| 28    | 75               | VIN1/2/3  | VIN2/3   | 16       | Moderate |
| 3     | 90               | VIN2/3    | VIN2/3   | 42       | VIN1/2   |
| 46    | 50               | VIN2/3    | VIN2/3   | 16       | VIN2/3   |
| 9     | >100             | VIN3      | VIN2/3   | 16       | VIN2/3   |
| 16ϕ   | 45               | VIN2/3    | VIN2/3   | 16       | VIN2/3   |
| 18    | 30               | VIN2/3    | VIN2/3   | 16       | VIN2/3   |
| 5     | 60               | VIN1/2/3  | no VIN   | 16,33,84 | VIN2      |
| 6     | 65               | VIN2/3    | no VIN   | 16       | VIN2/3   |
| 7χ    | 100              | VIN3      | no VIN   | 16,16    | VIN2/3   |
| 8α    | 60               | VIN2/3    | no VIN   | 16,33,81 | VIN2      |
| 106   | 25               | VIN2/3    | no VIN   | 16       | VIN2/3   |
| 11    | 25               | VIN3      | no VIN   | 16,16    | VIN2/3   |
| 12    | 20               | VIN3      | no VIN   | 16       | VIN2/3   |
| 13    | 50               | VIN2/3    | no VIN   | 16       | VIN2/3   |
| 14β   | 90               | VIN2/3    | no VIN   | 16       | VIN2/3   |
| 15    | 25               | VIN3      | no VIN   | 16       | VIN2/3   |
| 17α   | 40               | VIN2      | VIN1/2   | 16       | VIN2/3   |
| 19    | >100             | VIN2/3    | no VIN   | 16       | VIN2/3   |
APPENDIX 2
Salford & Trafford Local Research Ethics Committee
Room 181
Gateway House
Piccadilly South
Manchester
M60 7LP

Telephone: 0161 237 2438
Facsimile: 0161 237 2383

19 October 2005

Professor Henry C Kitchener
Professor of Gynaecological Oncology
University of Manchester
Academic Unit of Obstetrics and Gynaecology
St. Mary’s Hospital
Manchester M13 9JH

Dear Professor Kitchener

Full title of study: Non-randomised phase II trial of Sequential Therapy of Imiquimod followed by Vaccination with TA CIN for High Grade Vulval Intraepithelial Neoplasia (VIN).

REC reference number: 05/Q1404/172
Protocol number: #4
EUraCT number: 2005-001824-35

Thank you for your letter dated 10th October 2005 and responding to the Committee’s request for further information on the above research and submitting revised documentation.

The further information was considered by the Chairman.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the research sites taking part in this study. The favourable opinion does not therefore apply to any site at present. I will write to you again as soon as one Local Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at sites requiring SSA.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

| Document | Version | Date          |
|----------|---------|---------------|
| Application | 4.1 | 02 August 2005 |
| Application | Revised | 10 October 2005 |
| Protocol | #4 | 10 October 2005 |
| Protocol | Draft 3 | 08 June 2005 |

An advisory committee to Greater Manchester Strategic Health Authority
Research governance approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

Statement of compliance

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

05/Q1404/172 Please quote this number on all correspondence

With the Committee’s best wishes for the success of this project

Yours sincerely

Dr Mike Addison
Chairman, Salford and Trafford LREC

Email: maggie.twiney@gmsha.nhs.uk

Enclosures: Standard approval

SF 1 list of approved sites

An advisory committee to Greater Manchester Strategic Health Authority
APPENDIX 3
**CD4 T lymphocyte density** – in the individual participants expressed as density – no: of cells /unit area at weeks 0, 10 and 20. $\alpha$ represents treatment non-responders and $\beta$ represents treatment responders.

|     | Week 0 | Week 10 | Week 20 |
|-----|--------|---------|---------|
| IT1$\alpha$ | 311    | 160     | 75      |
| IT2$\alpha$ | 109    | 81      | 215     |
| IT3$\alpha$ | 17     | 154     | 350     |
| IT4$\alpha$ | 188    | 139     | 76      |
| IT5$\beta$  | 63     | 74      | 132     |
| IT8$\beta$  | 121    | 65      | 110     |
| IT9$\beta$  | 56     | 186     | 310     |
| IT10$\beta$ | 68     | 45      | 57      |
| IT11$\alpha$| 104    | 48      | 301     |
| IT13$\beta$ | 124    | 136     | 215     |
| IT14$\beta$ | 133    | 83      | 208     |
| IT15$\beta$ | 192    | 211     | 42      |
| IT16$\beta$ | 44     | 26      | 26      |
| IT17$\beta$ | 237    | 107     | 407     |
| IT18$\beta$ | 37     | 110     | 88      |
| IT19$\alpha$| 190    | 43      | 367     |
| IT20$\beta$ | 66     | 76      | 174     |
| IT21$\alpha$| 82     | 360     | 170     |
| IT23$\beta$ | 92     | 103     | 116     |
**CD8 T lymphocyte density** – in the individual participants expressed as density – no: of cells /unit area at weeks 0, 10 and 20. \( \alpha \) represents treatment non-responders and \( \beta \) represents treatment non-responders

|     | Week 0 | Week 10 | Week 20 |
|-----|--------|---------|---------|
| IT1\( \alpha \) | 38     | 50      | 30      |
| IT2\( \alpha \) | 31     | 145     | 152     |
| IT3\( \alpha \) | 15     | 79      | 168     |
| IT4\( \alpha \) | 134    | 66      | 37      |
| IT5\( \beta \)  | 26     | 47      | 23      |
| IT8\( \beta \)  | 64     | 105     | 107     |
| IT9\( \beta \)  | 34     | 113     | 97      |
| IT10\( \beta \) | 26     | 22      | 48      |
| IT11\( \alpha \) | 60     | 61      | 47      |
| IT13\( \beta \) | 74     | 54      | 89      |
| IT14\( \beta \) | 55     | 30      | 104     |
| IT15\( \beta \) | 70     | 170     | 19      |
| IT16\( \beta \) | 8      | 11      | 19      |
| IT17\( \beta \) | 33     | 21      | 143     |
| IT18\( \beta \) | 47     | 100     | 14      |
| IT19\( \alpha \) | 97     | 29      | 170     |
| IT20\( \beta \) | 68     | 111     | 133     |
| IT21\( \alpha \) | 22     | 301     | 238     |
| IT23\( \beta \) | 32     | 42      | 55      |
**FoxP3 CD4 density** – in the individual participants expressed as density – no: of cells /unit area at weeks 0, 10 and 20. α represents treatment non-responders and β represents treatment responders

|       | Week 0 | Week 10 | Week 20 |
|-------|--------|---------|---------|
| IT1α  | 37     | 27      | 19      |
| IT2α  | 25     | 19      | 95      |
| IT3α  | 2      | 31      | 43      |
| IT4α  | 18     | 22      | 15      |
| IT5β  | 1      | 37      | 5       |
| IT8β  | 14     | 8       | 30      |
| IT9β  | 6      | 63      | 60      |
| IT10β | 13     | 8       | 1       |
| IT11α | 28     | 6       | 33      |
| IT13β | 2      | 50      | 2       |
| IT14β | 33     | 29      | 39      |
| IT15β | 14     | 27      | 4       |
| IT16β | 1      | 10      | 6       |
| IT17β | 66     | 8       | 90      |
| IT18β | 11     | 39      | 7       |
| IT19α | 35     | 3       | 120     |
| IT20β | 10     | 8       | 15      |
| IT21α | 18     | 173     | 50      |
| IT23β | 36     | 25      | 9       |
LYMPHOPROLIFERATIVE RESPONSE TO E6 & E7 PEPTIDES

No significant lymphoproliferative responses were observed for all the E6 peptides as shown in the tables below. A significant lymphoproliferative response to E7I peptide was demonstrated in the responders at both weeks 10 and 20, however the significance of the change in response was greater at week 20.

LYMPHOPROLIFERATIVE RESPONSE TO E6 I PEPTIDE

| Responders | Pre-treatment | Post imiquimod (Wk 10) | Post vaccination (Wk 20) |
|------------|--------------|------------------------|--------------------------|
| IT5        | 2.2          | 2.5                    | 1.4                      |
| IT8        | 0.5          | 1.7                    | 1.5                      |
| IT9        | 0.8          |                        | 1.9                      |
| IT10       | 1.3          | 1.1                    | 1.3                      |
| IT13       | 1.0          | 0.8                    | 1.3                      |
| IT14       | 0.6          | 1.0                    | 1.1                      |
| IT15       | 1.3          | 1.0                    | 0.8                      |
| IT16       | 1.1          | 0.8                    | 0.9                      |
| IT17       | 0.9          | 0.8                    | 0.9                      |
| IT18       | 0.9          | 0.6                    | 1.0                      |
| IT20       | 2.0          | 1.5                    | 1.0                      |
| IT23       | 2.0          | 1.0                    | 1.2                      |
| Non-responders |          |                        |                          |
| IT11       | 1.1          | 2.5                    | 0.9                      |
| IT19       | 0.4          | 0.9                    | 1.4                      |
| IT21       | 3.0          | 1.5                    |                          |

Proliferative Responses to E6 I peptide in the responders and non-responders at weeks 0, 10, 20 Two fold change in the stimulation index (SI) was considered as a response, highlighted in grey
Change in the lymphoproliferative responses to E6I peptide in the group, responders, non-responders between weeks 0, 10, 20. No significant difference in the magnitude of response (median Stimulation Index SI) was observed following vaccination treatment; Wilcoxon signed rank test (WSR)

LYMPHOPROLIFERATIVE RESPONSE TO E6 II PEPTIDE

|                  | Wk 0 | Wk 10 | Wk 20 | Wk 0 vs. Wk 10 (P, WSR) | Wk 0 vs. Wk 20 (P, WSR) |
|------------------|------|-------|-------|-------------------------|-------------------------|
| Group (n=15)     | 1.1  | 1.0   | 1.1   | P=0.9                   | P=0.5                   |
| Responders (n=12)| 1.1  | 0.9   | 1.0   | P=0.9                   | P=0.8                   |
| Non-Responders   | 1.1  | 1.5   | 1.2   | P=0.2                   | P=0.3                   |

Proliferative Responses to E6II

| Responders | Pre-treatment | Post imiquimod (Wk 10) | Post vaccination (Wk 20) |
|------------|---------------|-------------------------|--------------------------|
| IT5        | 1.6           | 1.3                     | 3.6                      |
| IT8        | 0.6           | 2.0                     | 1.5                      |
| IT9        | 1.1           |                         | 1.6                      |
| IT10       | 1.3           | 1.0                     | 1.9                      |
| IT13       | 2.1           | 0.5                     | 2.4                      |
| IT14       | 0.6           | 1.9                     | 1.5                      |
| IT15       | 1.0           | 1.1                     | 1.1                      |
| IT16       | 1.1           | 0.9                     | 1.3                      |
| IT17       | 1.3           | 1.3                     | 0.9                      |
| IT18       | 0.9           | 0.8                     | 1.3                      |
| IT20       | 2.6           | 1.7                     |                           |
| IT23       | 1.8           | 1.3                     | 1.0                      |
| Non-responders |        |                         |                          |
| IT11       | 2.0           | 3.1                     | 1.9                      |
| IT19       | 0.7           | 0.7                     | 0.7                      |
| IT21       | 2.6           | 1.7                     |                           |

Proliferative Responses to E6 II peptide in the responders and non-responders at weeks 0, 10, 20 Two fold change in the stimulation index (SI) was considered as a response, highlighted in grey
| Group (n=15) | Wk 0 | Wk 10 | Wk 20 | Wk 0 vs. Wk 10 (P, WSR) | Wk 0 vs. Wk 20 (P, WSR) |
|-------------|------|-------|-------|-------------------------|-------------------------|
| Responders (n=12) | 1.3  | 1.3   | 1.2   | P=0.5                   | P=0.2                   |
| Non-Responders (n=3) | 1.2  | 1.2   | 1.3   | P=0.4                   | P=0.5                   |

Change in the lymphoproliferative responses to E6II peptide in the group, responders, non-responders between weeks 0, 10, 20. No significant difference in the magnitude of response (median Stimulation Index SI) was observed following vaccination treatment; Wilcoxon signed rank test (WSR)

LYMPHOPROLIFERATIVE RESPONSE TO E6 III PEPTIDE

| Proliferative Responses to E6III | Pre-treatment | Post imiquimod (Wk 10) | Post vaccination (Wk 20) |
|----------------------------------|---------------|------------------------|--------------------------|
| Responders                       |               |                        |                          |
| IT5                              | 0.8           | 1.0                    | 3.3                      |
| IT8                              | 1.3           | 1.2                    | 1.2                      |
| IT9                              | 0.8           |                        | 1.8                      |
| IT10                             | 5.3           | 1.6                    | 2.3                      |
| IT13                             | 1.5           | 0.5                    | 2.1                      |
| IT14                             | 0.7           | 2.1                    | 1.6                      |
| IT15                             | 1.3           | 0.6                    | 1.2                      |
| IT16                             | 1.4           | 2.4                    | 0.7                      |
| IT17                             | 2.0           | 1.2                    | 1.5                      |
| IT18                             | 1.4           | 0.6                    | 1.1                      |
| IT20                             | 3.2           | 1.5                    | 0.9                      |
| IT23                             | 2.3           | 1.0                    | 1.4                      |
| Non-responders                   |               |                        |                          |
| IT11                             | 1.0           | 2.2                    | 0.7                      |
| IT19                             | 0.7           | 1.3                    | 1.4                      |
| IT21                             | 0.6           | 1.3                    |                          |

Proliferative Responses to E6 III peptide in the responders and non-responders at weeks 0, 10, 20. Two fold change in the stimulation index (SI) was considered as a response, highlighted in grey.
|                      | Wk 0 | Wk 10 | Wk 20 | Wk 0 vs. Wk 10 (P, WSR) | Wk 0 vs. Wk 20 (P, WSR) |
|----------------------|------|-------|-------|-------------------------|-------------------------|
| Group (n=15)         | 1.3  | 1.2   | 1.4   | P=0.5                   | P=0.4                   |
| Responders (n=12)    | 1.4  | 1.1   | 1.3   | P=0.6                   | P=0.5                   |
| Non-Responders (n=3) | 0.7  | 1.3   | 1.0   | P=0.5                   | P=0.8                   |

Change in the lymphoproliferative responses to E6III peptide in the group, responders, non-responders between weeks 0, 10, 20. No significant difference in the magnitude of response (median Stimulation Index SI) was observed following vaccination treatment; Wilcoxon signed rank test (WSR)

LYMPHOPROLIFERATIVE RESPONSE TO E6 IV PEPTIDE

| Proliferative Responses to E6IV | Pre-treatment | Post imiquimod (Wk 10) | Post vaccination (Wk 20) |
|---------------------------------|---------------|------------------------|-------------------------|
| Responders                      |               |                        |                         |
| IT5                             | 2.4           | 1.0                    | 1.4                     |
| IT8                             | 1.8           | 1.0                    | 1.6                     |
| IT9                             | 1.0           |                        | 4.5                     |
| IT10                            | 2.9           | 1.6                    | 2.5                     |
| IT13                            | 1.1           | 0.7                    | 2.1                     |
| IT14                            | 0.8           | 2.2                    | 1.1                     |
| IT15                            | 1.6           | 1.4                    | 1.8                     |
| IT16                            | 1.0           | 1.7                    | 0.8                     |
| IT17                            | 3.3           | 4.4                    | 2.0                     |
| IT18                            | 1.0           | 0.9                    | 1.0                     |
| IT20                            | 3.4           | 1.5                    | 1.7                     |
| IT23                            | 1.1           | 1.2                    | 1.6                     |
| Non-responders                  |               |                        |                         |
| IT11                            | 0.7           | 1.5                    | 0.6                     |
| IT19                            | 1.2           | 0.9                    | 1.2                     |
| IT21                            | 1.8           | 1.5                    |                          |

Proliferative Responses to E6 IV peptide in the responders and non-responders at weeks 0, 10, 20 Two fold change in the stimulation index (SI) was considered as a response, highlighted in grey
|                          | Wk 0 | Wk 10 | Wk 20 | Wk 0 vs. Wk 10 (P, WSR) | Wk 0 vs. Wk 20 (P, WSR) |
|--------------------------|------|-------|-------|-------------------------|-------------------------|
| **Group (n=15)**         | 1.2  | 1.4   | 1.6   | P=0.4                   | P=0.5                   |
| **Responders (n=12)**    | 1.4  | 1.4   | 1.7   | P=0.4                   | P=0.6                   |
| **Non-Responders (n=3)** | 1.2  | 1.5   | 0.9   | P=0.5                   | P=0.5                   |

Change in the lymphoproliferative responses to E6IV peptide in the group, responders, non-responders between weeks 0, 10, 20. No significant difference in the magnitude of response (median Stimulation Index SI) was observed following vaccination treatment; Wilcoxon signed rank test (WSR)

**LYMPHOPROLIFERATIVE RESPONSE TO E7 I PEPTIDE**

| Responders | Pre-treatment | Post imiquimod (Wk 10) | Post vaccination (Wk 20) |
|------------|---------------|------------------------|--------------------------|
| IT5        | 1.0           | 0.8                    | 1.3                      |
| IT8        | 0.9           | 1.7                    | 2.1                      |
| IT9        | 0.8           |                        | 1.3                      |
| IT10       | 1.7           | 0.7                    | 2.0                      |
| IT13       | 1.0           | 1.1                    | 1.6                      |
| IT14       | 2.5           | 1.1                    | 0.7                      |
| IT15       | 1.3           | 0.7                    | 1.0                      |
| IT16       | 1.3           | 0.8                    | 0.4                      |
| IT17       | 1.3           | 0.6                    | 1.5                      |
| IT18       | 1.1           | 0.9                    | 1.4                      |
| IT20       | 2.0           | 1.2                    | 3.3                      |
| IT23       | 1.4           | 1.0                    | 1.6                      |

**Non-responders**

| IT11       | 1.1           | 1.8                    | 1.0                      |
| IT19       | 0.6           | 0.8                    | 1.2                      |
| IT21       | 0.8           | 1.2                    |                          |

Proliferative Responses to E7 I peptide in the responders and non-responders at weeks 0, 10, 20. Two fold change in the stimulation index (SI) was considered as a response, highlighted in grey.
Change in the lymphoproliferative responses to E7I peptide in the group, responders, non-responders between weeks 0, 10, 20. A significant difference in the magnitude of response (median Stimulation Index SI) was observed in the group and responders following vaccination treatment; Wilcoxon signed rank test (WSR)

|                  | Wk 0 | Wk 10 | Wk 20 | Wk 0 vs. Wk 10 (P, WSR) | Wk 0 vs. Wk 20 (P, WSR) |
|------------------|------|-------|-------|-------------------------|-------------------------|
| Group (n=15)     | 1.1  | 1.0   | 1.5   | P=0.3                   | P=0.004                 |
| Responders (n=12)| 1.3  | 1.0   | 1.5   | P=0.03                  | P=0.008                 |
| Non-Responders (n=3) | 0.8 | 1.2   | 1.1   | P=0.3                   | P=0.1                   |

LYMPHOPROLIFERATIVE RESPONSE TO E7 II PEPTIDE

| Proliferative Responses to E7II | Responders | Pre-treatment | Post imiquimod (Wk 10) | Post vaccination (Wk 20) |
|---------------------------------|------------|---------------|------------------------|--------------------------|
| IT5                             | 0.6        | 0.5           | 0.5                    |                          |
| IT8                             | 0.9        | 1.7           | 2.1                    |                          |
| IT9                             | 1.3        |               | 2.1                    |                          |
| IT10                            | 1.3        | 0.7           | 1.7                    |                          |
| IT13                            | 1.1        | 0.5           | 0.8                    |                          |
| IT14                            | 1.5        | 1.6           | 1.1                    |                          |
| IT15                            | 1.0        | 0.5           | 1.1                    |                          |
| IT16                            | 1.3        | 1.2           | 0.4                    |                          |
| IT17                            | 2.3        | 0.4           | 0.8                    |                          |
| IT18                            | 0.8        | 0.7           | 1.1                    |                          |
| IT20                            | 2.9        | 0.9           | 1.9                    |                          |
| IT23                            | 1.1        | 1.6           | 1.4                    |                          |
| Non-responders                  |            |               |                        |                          |
| IT11                            | 1.1        | 1.8           | 1.0                    |                          |
| IT19                            | 0.6        | 0.8           | 1.2                    |                          |
| IT21                            | 0.8        | 1.2           |                        |                          |

Proliferative Responses to E7 II peptide in the responders and non-responders at weeks 0, 10, 20. Two fold change in the stimulation index (SI) was considered as a response, highlighted in grey
| Group (n=15) | Wk 0 | Wk 10 | Wk 20 | Wk 0 vs. Wk 10 (P, WSR) | Wk 0 vs. Wk 20 (P, WSR) |
|-------------|------|-------|-------|------------------------|------------------------|
| 1.1         | 0.9  | 1.1   | P=0.4 | P=0.05                 |

| Responders (n=12) | Wk 0 | Wk 10 | Wk 20 | Wk 0 vs. Wk 10 (P, WSR) | Wk 0 vs. Wk 20 (P, WSR) |
|-------------------|------|-------|-------|------------------------|------------------------|
| 1.2               | 0.7  | 1.1   | P=0.2 | P=0.2                  |

| Non-Responders (n=3) | Wk 0 | Wk 10 | Wk 20 | Wk 0 vs. Wk 10 (P, WSR) | Wk 0 vs. Wk 20 (P, WSR) |
|----------------------|------|-------|-------|------------------------|------------------------|
| 0.8                  | 1.2  | 1.1   | P=0.2 | P=0.5                  |

Change in the lymphoproliferative responses to E7II peptide in the group, responders, non-responders between weeks 0, 10, 20. A significant difference in the magnitude of response (median Stimulation Index SI) was observed in the group alone following vaccination treatment; Wilcoxon signed rank test (WSR)