Vulvar Intraepithelial Neoplasia

New concepts and strategy

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nieuwe inzichten en behandelstrategie

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List of abbreviations

ALA	aminolevulinic acid
APC	antigen-presenting cell
CIN	cervical intraepithelial neoplasia
CONSORT	Consolidated Standards for the Reporting of Trials
CTL	cytotoxic T-cell
DNCB	dinitrochlorobenzene
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbant assay
ELISPOT	enzyme-linked immunospot
EORTC	European Organisation for Research and Treatment of Cancer
5-FU	5-fluorouracil
CR	complete response
DC	dendritic cell
mDC	myeloid dendritic cell
pDC	plasmacytoid dendritic cell
EGWs	external genital warts
HPV	human papillomavirus
HRQL	health-related quality of life
IFN	interferon
IL	interleukin
ISGYP	International Society for Gynecological Pathologists
ISSVD	International Society for the Study of Vulvovaginal Diseases
LEEP	loop electrosurgical excision procedure
LS	lichen sclerosus
MRM	memory response mix
NGS	normal goat serum
NHS	normal human serum
NK	natural killer
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PDT	photodynamic therapy
PR	partial response

QoL	quality of life
QLQ	quality of life questionnaire
RCT	randomised controlled trial
RLB	reverse line blot
SCC	squamous cell carcinoma
Th	T-helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	T-regulatory
VIN	vulvar intraepithelial neoplasia
VLP	virus-like particles

1 General introduction

Part of this data has been published in:

- Preti M, van Seters M, Sideri M, van Beurden M. Squamous vulvar intraepithelial neoplasia. Clin Obstet Gynecol 2005;48:845-61.
- van Beurden M, van Seters M, Helmerhorst ThJM. Vulvaire intra-epitheliale neoplasie. In: van der Meijden WI, ter Harmsel WA, eds. Vulvapathologie. 1st ed. Assen: Koninklijke Van Gorcum 2007; 117-29.

1. Vulvar intraepithelial neoplasia

Vulvar intraepithelial neoplasia (VIN) is a rare condition which can develop into an invasive carcinoma.[1] This skin-disease affects mainly young women, and causes many severe and long-lasting symptoms such as pruritus, vulvodynia and psychosexual dysfunction. Over 80% of VIN-affected women present with multifocal vulvar disease, and often neoplastic changes can be found in the entire lower genital tract.[2] Clinically, it is important to distinguish unifocal from multifocal lesions, since unifocal VIN tends to progress to invasive carcinoma ten times more often than multifocal VIN does.[3]

1.1 Epidemiology

Since the early seventies, the incidence of VIN has increased.[4] This trend continued during the following two decades. Nevertheless, the incidence of vulvar cancer remained unchanged.[5] Recently, however, first case reports and then cohort studies documented an increasing incidence of VIN-associated carcinoma in younger women.[6-8] Spontaneous regression of VIN has been reported in only a few cases. Forty-one patients (13 studies), all younger than 35 years, showed spontaneous complete regression of their VIN-lesions.[1,9-20] This was related to pregnancy in 41%.

1.2 Nomenclature

Historically, various terms have been used to define VIN: morbus Bowen, Queyrat's erythroplasia, carcinoma simplex, bowenoid papulosis, early vulvar cancer, vulvar atypia, hyperplastic dystrophy, carcinoma in situ, dysplasia. In 1976, the International Society for the Study of Vulvovaginal Diseases (ISSVD) simplified terminology into carcinoma in situ and vulvar atypia.[21] Ten years later, in 1986, the ISSVD adopted a single term, VIN, discouraging any other terminology including carcinoma in situ and vulvar atypia.[22] In that ISSVD report the term VIN, as a general category, included three subdivisions: VIN 1 (mild dysplasia), VIN 2 (moderate dysplasia) and VIN 3 (severe dysplasia) (Table 1). In addition, the report described a separate lesion - differentiated VIN - and recommended this lesion to be also classified as VIN 3. The three-grade system of VIN was set up equivalent to the classification of cervical intraepithelial neoplasia (CIN), although there is no evidence that the morphologic spectrum of VIN 1 to 3 reflects a biologic continuum or that VIN behaves similarly to CIN. In 2004, this was reason for the ISSVD to modify VIN terminology again, this time into a two-tier classification: VIN, usual type (warty, basaloid and mixed) and VIN, differentiated type. The two types differ in morphology, biology and clinical features.[23] VIN, usual type, is human papillomavirus (HPV)-associated, occurs predominantly in younger patients and tends to be a multifocal and multicentric disease. It is seen adjacent to approximately 30% of squamous cell carcinomas (SCC) of the vulva (basaloid and warty type). VIN, differentiated type, on the other hand, is less common, not related to

Table 1. Squamous Vulvar Intraepithelial Neoplasia (VIN) terminology

ISSVD, 1986	ISSVD, 2005
- VIN 1, mild atypia	- VIN, usual type
- VIN 2, moderate dysplasia	(*warty, basaloid, mixed)*
- VIN 3, severe dysplasia, CIS	
- VIN 3, differentiated type	- VIN, differentiated type

HPV, usually found in older women and often observed in association with keratinizing SCC. It is commonly thought that differentiated VIN is associated with lichen sclerosus (LS),[24,25] although argumentation for this is limited to a small number of studies describing epithelial alterations adjacent to vulvar SCC.[26-29] In this currently used classification, the term VIN 1 no longer exists. VIN should apply only to histologically 'high grade' squamous lesions. Therefore, it is recommended that the former terms VIN 2 and 3 are combined as a single diagnostic category, and referred to as high grade VIN, usual or differentiated type (Table 1).

1.3 Etiology

In 1982 it became apparent that HPV might be involved in the etiology of VIN.[30] Since then, several studies demonstrated a high prevalence of HPV DNA in high grade VIN lesions, usual type (between 78-92%).[2,31-34] In most cases HPV-16 DNA was detected. It is shown that HPV DNA is significantly more present in multifocal VIN than in unifocal VIN and more often in VIN coexisting with other multicentric intraepithelial lesions in the lower genital tract.[2]

HPV is a sexually transmitted virus. The estimated life-time risk of infection with HPV is 80%.[35] Most infections proceed asymptomatically, and cure spontaneously as the immune system is capable of eliminating the virus.[36] Persistence, on the other hand, can result in neoplastic changes of the anogenital tract.[37,38] So far, more than 100 types of HPV have been identified that can be grouped into high-risk (oncogenic) types and low-risk (non-oncogenic) types. High-risk HPV, of which HPV-16 is the most prevalent type, is associated with cervical carcinoma and high grade CIN or VIN, whereas low-risk HPV is mainly seen in genital warts or low grade cervical or vulvar lesions. HPV encodes for several viral proteins, of which 'early' oncoproteins E6 and E7 are the most important. E6 and E7 bind and inactivate gene products of tumor suppressor genes p53 and Rb, respectively.[39,40] These complexes cause disruption of cell cycle control in the proliferative cell and disable the cell to repair DNA damage. This can result in genetic instability, leading to mutations that are involved in (pre-) malignancy.[41]

2. Histology

VIN is characterized by loss of epithelial cell maturation with associated nuclear hyperchromasia, pleomorphism, cellular crowding and abnormal mitotic figures. VIN can be subclassified into different histologic subtypes – warty, basaloid and differentiated VIN.[24]

Warty VIN (Figure 1) is characterized by a condylomatous appearance, parakeratosis, hyperkeratosis and striking cellular pleomorphism. There is evidence of abnormal cell maturation. Multinucleation, corps rounds, acanthosis and koilocytosis are common, as are (abnormal) mitotic figures. The rete ridges are typically wide and deep, often reaching close to the surface.

Basaloid VIN (Figure 2) is characterized by thickened epithelium, with a relatively flat and non-papillomatous surface. The epidermis consists of a monotonous proliferation of relatively uniform undifferentiated cells with a basaloid appearance. Koilocytotic cells and corps ronds may be present, but less frequently than in warty VIN. Mitotic figures are numerous. As with warty VIN, the intraepithelial process may involve the underlying skin appendages.[42-45] Warty and basaloid VIN often coexist in one lesion, which is referred to as *mixed VIN*. Both types are related to the presence of HPV.[2,46]

Differentiated VIN (Figure 3) is characterized by prominent eosinophilic cells in the basal and parabasal area, often with keratin formation or 'pearl-like' changes within the rete ridges. These prematurely differentiated keratinocytes usually have large vesicular

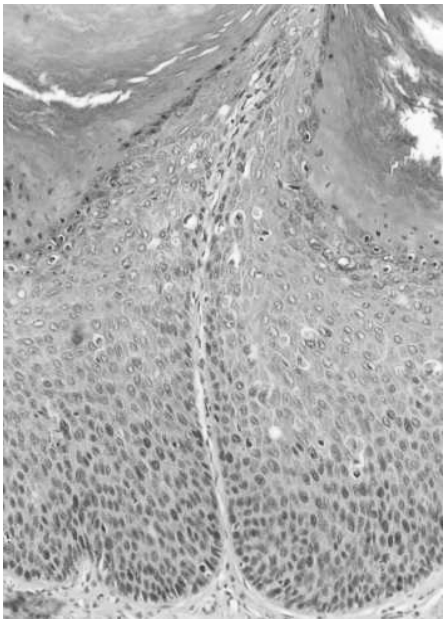

Figure 1. VIN usual type, warty. H&E staining x100.

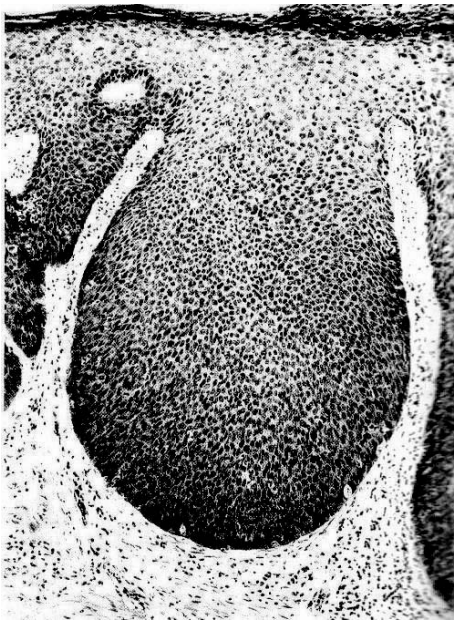

Figure 2. VIN usual type, basaloid. H&E staining x100

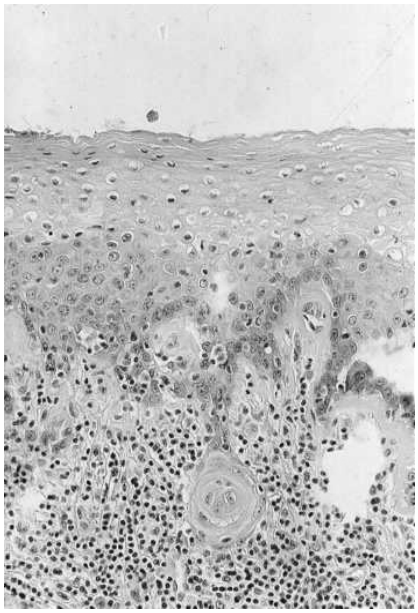

Figure 3. VIN differentiated type, H&E staining x100

nuclei and prominent nucleoli. A high degree of cellular differentiation and an absence of widespread architectural disarray make it difficult to recognize this type of VIN. Since histopathological changes are subtle, differentiated VIN is easily mistaken for benign lesions.[47,48] Immunostaining for p53 protein might be helpful in this situation. In 10 of 12 patients with differentiated VIN, overexpression of the p53 tumor suppressor gene has been demonstrated.[48]

3. Therapy

Until now, the choice of therapy for high grade VIN has been dominated by the pre-malignant nature of the disease. Although extensive surgery, such as vulvectomy, is not the advised treatment anymore, standard therapy for patients with VIN still comprises surgical removal of all visible lesions to relieve symptoms and to prevent the development of invasive disease. In 1995, Kaufman underlined the importance of individualization of treatment. Treatment should be directed towards preservation of the normal anatomy and function of the vulva.[49] Shortly after, van Beurden *et al* demonstrated vulvoscopically directed biopsies to be a safe method to exclude invasive disease, and restricted surgery to be effective in relieving symptoms in multifocal VIN 3.[50] In the Dutch consensus guidelines (1999), it is advised to radically excise unifocal VIN with a margin of 5mm, and to treat multifocal VIN as conservative as possible.[51]

3.1 Surgical treatment

Surgical treatment can be performed with different techniques. Cold knife surgery or CO_2-laser vaporization are used as a single technique or in combination. When representative biopsies have been taken beforehand, vaporization can be an effective treatment especially in non-hair bearing areas. Unfortunately, irrespective of the type and extent of operation performed, surgical margins are often positive and high recurrence rates are common.[52-54] Besides, one has to be aware of the correlation between (the extent of) surgical treatment and mutilation of the vulva, possibly resulting in psychosexual distress.[55-58]

3.2 Medical treatment

Because VIN is being diagnosed more often in younger patients, effective treatment is needed that does not mutilate or functionally incapacitate the patient. Therefore several medical treatment options in the management of VIN have been investigated in the past, varying from local chemotherapy to immunotherapy. Topical treatment is attractive because it can be applied directly by the patient and is easily monitored for efficacy. Unfortunately, study results have been disappointing thus far, with only a few responses and high complication and recurrence rates. For medical therapy, diagnosis has to rely on the biopsy only, with the risk that an early invasive lesion is overlooked.

3.2.1 Chemotherapy

The use of topical 5-fluorouracil (5-FU) in VIN 3 was first described in 1967.[59] 5-FU is a chemotherapeutic agent inhibiting DNA synthesis in the "S" phase of cell division. In addition, it works as a cell marker being incorporated into the neoplastic cell, where it can be recognized and destroyed by the patients' immune system.[60] A review summarized the results obtained by treatment with topical 5-FU in 68 patients with VIN 3 (15 studies).[61] Overall, remission was seen in 34%, improvement in 7%, while 59% failed therapy. As a result of severe side-effects, including painful ulcerations, duration of treatment with 5-FU was frequently limited by the patient.

The use of topical and intradermal bleomycin in the treatment of VIN resulted in poor response rates. Additionally, in five out of 12 patients, progression to an invasive lesion was seen.[62]

3.2.2 Immunotherapy

In studies with dinitrochlorobenzene (DNCB), inducing a type of delayed hypersensitivity reaction on topical application, generally a successful treatment of recurrent VIN 3 has been shown. However, recurrences still developed after treatment with DNCB, and side-effects were extensive, if not intolerable.[63]

Better results have been accomplished with interferon-α (IFN-α), an attractive therapeutic agent in HPV-related diseases, because of its inhibitory effect on viral replication and

cell growth. IFN-α can be administered systemically, intralesionally or topically, resulting in high response rates (biopsy-proven) with low morbidity.[64-66] In 1998, a high failure rate of interferon in combination with isotretinoin in HPV-related VIN 3 was reported. Despite clinical regression, histologic features of VIN 3 were still present.[67]

3.3 New treatment modalities

As no curative treatment of VIN has yet been identified, continuous efforts are being made to investigate new treatment strategies.

3.3.1 Photodynamic therapy

Photodynamic therapy (PDT) is a relatively new technique that uses a tumor-localizing photosensitizer, 5-aminolevulinic acid (ALA), in combination with non-thermal light of an appropriate wavelength to generate oxygen-induced cell death. Because PDT has been shown to be very effective in the treatment of nonmelanoma skin carcinomas, it was expected to be useful in the management of VIN. A clearance rate of 37% in 8 patients with high-grade VIN was found.[68] Similar results (31-46%) were reported in two other studies.[69,70] Both studies showed that unifocal lesions are more responsive to ALA-PDT than multifocal high-grade VIN, and that increased pigmentation and hyperkeratosis of the lesions are associated with low response rates. Fehr *et al* reported promising results in 11 of 15 patients (73%) being free of VIN 3 after treatment with ALA-PDT.[71] During follow-up, recurrence rate was not significantly different from patients treated with laser evaporation or local excision. PDT has the advantage of minimal tissue destruction with a short healing time and only few side-effects.[68-71]

3.3.2 Imiquimod

The first promising results on treatment of VIN with imiquimod were reported in 2000 in four patients.[72] Imiquimod is an immune response modifier with antiviral and antitumor properties, that has been shown safe and efficacious in the treatment of external genital warts caused by HPV.[73] Imiquimod binds to Toll-like receptor 7, a cell surface receptor on the immature plasmacytoid dendritic cell (DC). Binding initiates an intracellular signaling cascade that finally results in induction of an innate and cell-mediated immune response. It is hypothesized that topical treatment with imiquimod may be effective in stimulating cell-mediated immunity against different types of HPV and thus encourage regression of HPV-related preneoplastic vulvar lesions. Small observational, non-controlled series of patients with high response rates to imiquimod have been described since then.[74-76] A potential effect of treatment with imiquimod in the entire lower genital tract was also demonstrated.[74] One study reported clinical improvement in only 27% (n=15). Local side-effects limited the frequency of application, which might be an explanation for this low response rate.[77]

4. Immunology

The immune response to invading HPV is regulated by cells of both the innate and adaptive immune system. Innate immune cells, including monocytes, granulocytes, macrophages, mast cells, natural killer (NK) cells and DCs, recognize, internalize and/or phagocytose the invading virus or viral antigens. They release soluble effector molecules, e.g. complement components and cytokines, which regulate and coordinate many of their activities. DCs, important antigen-presenting cells (APCs), bind viral antigens by a set of specific receptors (Toll-like receptors), internalize and process bound antigens and transport them, under the influence of immune mediators such as chemokines, to secondary lymphoid organs. There, naïve T-cells are primed to mature into antigen-specific $CD4^+$ T-helper cells, (Th-cells), $CD8^+$ cytotoxic T-cells (CTLs), or regulatory T-cells (Treg cells), which are effector cells of the adaptive immune system.[78,79]

The adaptive immune system consists of cell-mediated and humoral immune responses. The cellular immune response targets the intracellular virus or viral antigens presented by APCs as described above. $CD4^+$ T-helper cells play a central role in regulating immune responses and are essential in antitumor immunity.[80,81] They activate and stimulate innate effector cells and $CD8^+$ cytotoxic cells through the release of immuno-stimulating Th1-type cytokines, such as IFN-γ, TNF-β and IL-2. They also produce immuno-inhibitory Th2-type cytokines, such as IL-4, IL-5, IL-10 and TGF-β. Th2-type cytokines predominantly induce humoral immune responses.

The effector cells for humoral immune responses, B-lymfocytes, produce antibodies that specifically recognize and bind to the extracellular virus that now can be eliminated by various mechanisms.

4.1 Local immune response

HPV-infection begins with binding of virions to the basal cells of the epithelium. In the upper layers where viral replication takes place, HPV DNA is encapsidated, and virions are released at the epithelial surface.[82] Since persistence of HPV-infection is necessary to cause anogenital disease, it is of interest to see how HPV effects the distribution of immunocompetent (effector) cells in the skin of patients with VIN. Only a few studies reported on the number of immunocompetent cells in VIN-affected skin, mostly dealing with $CD4^+$ and $CD8^+$ T-cells, and/or $CD1a^+$ DCs.[69,83,84] Overall, these studies demonstrated ambiguous results on the distribution of $CD1a^+$ DCs, and an increasing number of $CD4^+$ and $CD8^+$ T-cells in dermis or upper dermis of VIN patients. The distribution of a broader range of immunocompetent cells in both VIN-affected skin and normal vulvar skin is not yet fully investigated. More information is needed in order to understand the possible effect of the immune modifier imiquimod on immmunocompetent cells in vulvar dysplasia.

4.2 Systemic immune response

Little is known about the effect of HPV-infection on the systemic immune response. The importance of cell-mediated immune responses of the host in the course of infection is illustrated by an increased incidence of HPV-induced diseases in T-cell immuno-deficient individuals (Petry, 1996).[85] It was also demonstrated that type 1 (IFNγ) T-cell immunity against HPV 16 early antigens E2, E6 and E7 can be detected in the circulation of the majority of healthy sexually active individuals, but is weak or absent in patients with HPV 16-induced cervical neoplasia.[86-88] These data argue that the CD4+ type 1 T-cell response against the early antigens of HPV 16 may play an important role in the protection against progressive HPV-16 induced disease.

5. Outline of this thesis

In **Chapter 2** ninety-seven studies, published between 1943 and 2003, are systematically reviewed to establish the true natural history of high grade VIN from literature data. The aim was to assess both the risk of progression of VIN in untreated patients, and the effect of surgical treatment in relation to recurrences and progression of VIN.

In **Chapter 3** the coexistence between VIN and LS is further analyzed, since the presumption that differentiated VIN is related to LS is not based on much evidence.

Chapter 4 describes the results of a pilot study investigating imiquimod 5% cream in the treatment of high grade VIN.

Chapter 5 describes the results of a randomized controlled trial (RCT) investigating the effectiveness of imiquimod 5% cream in patients with multifocal high grade VIN. Outcome measures are reduction in lesion size, histological regression, clearance of HPV, changes in immunocompetent cells in (epi-)dermis, relief of symptoms, improvement of quality of life and durability of clinical response.

In **Chapter 6** the distribution of immunocompetent cells in the epidermis and dermis of HPV-related VIN-affected skin is characterized, and compared with HPV-negative vulvar skin from healthy controls.

Chapter 7 describes the role of HPV-16 specific $CD4^+$ T-cell immunity in the success or failure of treatment with imiquimod in 29 patients with high grade VIN.

The results presented in the previous chapters are discussed in **chapter 8**.

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2 Is the assumed natural history of vulvar intraepithelial neoplasia 3 based on enough evidence? A systematic review of 3322 published patients

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Abstract

Objective To establish the true natural history of VIN 3 from literature data.
Methods In a systematic review, data of women with VIN 3 indexed in several computer databases were pooled. The effect of treatment was correlated with recurrences and progression of VIN 3.
Results Ninety-seven articles met the inclusion criteria. Data of 3322 patients were available. The mean age at diagnosis of VIN 3 was 46. This decreased over time, although not significantly (P=0.08). Recurrences were seen as often after local excision as after vulvectomy. The percentage of recurrences was lower, but not absent, after free surgical margins than after involved surgical margins (P<0.001). 6.5% of the 3322 patients progressed to an invasive vulvar carcinoma. Occult carcinomas were diagnosed in 3.2% of patients and 3.3% carcinomas were diagnosed during follow-up. The mean age at diagnosis of invasive vulvar carcinoma was 52 years. Nine percent of 88 untreated patients progressed in 12 to 96 months to invasive vulvar carcinoma. Only 1.2% of the 3322 patients showed complete regression, mostly during the first 10 months after diagnosis, 41% of which was related to pregnancy.
Conclusion Evidence exists that VIN 3 may progress to invasive vulvar carcinoma. However, the available literature suggests that the progression rate to invasive vulvar carcinoma is low. The incidence of invasion as found in this systematic review is probably even too high, because overreporting of (micro)invasive cases cannot be excluded. Only a prospective registration using a standardized pathology examination will provide information about the real natural history of VIN 3.

Introduction

The natural history of untreated VIN 3 is unclear. Untreated patients who hardly ever progress to an invasive vulvar carcinoma have been published.[1-3] Others have seen progression in nearly all untreated patients.[4] Since data on the follow-up of untreated VIN 3 are scarce, the natural history of VIN 3 is mainly based on the follow-up after surgical treatment of VIN 3 and is considered low.[2] Although extensive surgery, such as vulvectomy, is not the advised standard treatment anymore, removal of all visible lesions to exclude the presence of an occult invasive squamous cell carcinoma is still recommended.[5]

The aim of this study was, by means of a systematic review, to assess both the risk of progression of VIN 3 in untreated patients and the effect of surgical treatment in relation to recurrences and progression of VIN 3.

Material and Methods

Data Identification and Extraction

Articles were located in November 2004 using various strategies. Firstly, computer searches of MEDLINE (from 1964), CANCERLIT (from 1980), EMBASE (from 1974), BIOSIS PREVIEWS AB (from 1970) and SCIENCE CITATION INDEX (from 1970) were performed. The following key words were used: vulvar neoplasms in combination with intraepithelial neoplasia, Bowen, bowenoid, Queyrat, carcinoma simplex, early vulvar cancer, hyperplastic dystrophy, condylomatous dysplasia, intraepithelial carcinoma, carcinoma in situ, vulvar atypia and precancerous conditions. Secondly, references from chapters on VIN 3 in several handbooks were checked. The titles and, if available, the abstracts of all citations were checked. Any citation that did not obviously fail the inclusion criteria on the basis of the title or the abstract was retrieved and reviewed in greater detail. Of the citations that met the inclusion criteria, all references were checked. Criteria for inclusion were (1) articles written in English, German or French and (2) data, clearly retrievable, on the surgical treatment and/or progression and/or regression of VIN 3. All relevant data for the evaluation were extracted from text, tables and figures. Articles in which data of VIN 3 were not distinguishable from data of VIN 1-2, (micro)invasive vulvar carcinoma or other vulvar diseases, were excluded. Case histories were excluded, except those concerning regression or progression of VIN 3. Surgical treatment was defined as cold knife surgery, laser excision, laser evaporization, LEEP excision or cryosurgery. The following items were recorded, if mentioned: year of publication, study period, minimum, maximum and mean age, presence of complaints (pain, pruritus) and visible lesions, previous radiotherapy, immunosuppression, unifocal or multifocal VIN 3, coexisting genital tract neoplasia (vagina, cervix), type of surgical treatment, free or involved surgical margins, occult invasion (i.e., an invasive vulvar carcinoma

in the surgical specimen, while only VIN 3 was assumed preoperatively), duration of follow-up, (time to) progression to invasive vulvar carcinoma, location of invasive vulvar carcinoma, depth of invasion and (time to) regression of VIN 3.

In the analysis radical vulvectomy, vulvectomy, complete vulvectomy, total vulvectomy, simple vulvectomy and skinning vulvectomy are defined as vulvectomy. Subtotal vulvectomy, hemivulvectomy and partial vulvectomy are defined as partial vulvectomy. Excision, local excision, wide excision, wide local excision, laser excision and LEEP are defined as local excision.

Statistical Analysis

Study and patient characteristics are presented as means or as proportions. Each study was weighted by the sum of the inverse of the within and between study variance (random effect model).[6] Random effects models incorporate potential heterogeneity of the mean or proportion between different studies by assuming that each study estimates a unique mean or proportion. In case of continuous data (e.g. mean age of patients in the study), each standard deviation was estimated from the range since this was usually not reported in the articles.[7] To assess whether mean age of diagnosis dropped over the years, linear regression analysis with mean age as dependent variable and the year of publication as independent variable was used. Each study was weighted by the inverse of its sampling variance. The year of publication was determined by calculating the average of the years the articles originated from. Whenever this was not reported, the year of publication was used in the analysis. Statistical analysis was performed with SAS, version 6.12.

Results

A total of 97 articles with data on 3322 patients met the inclusion criteria.[1-4,8-100] Seventy-nine studies dealt with treatment results, progression and regression of VIN 3. Twelve case reports only gave data on progression of VIN 3 and 6 case reports only dealt with regression of VIN 3.

Patients

The mean age was 46 (48 studies, 2152 patients), the mean minimum age was 21 (59 studies, 2585 patients) and the mean maximum age was 80 (56 studies, 2467 patients). The mean age at diagnosis in the published articles had decreased since the first published series in 1943, although not significantly (P=0.08) (Figure 1). The percentage of patients with complaints (pain and/or pruritus) was 64 (1777 patients, 43 studies). The percentage of patients that sought medical help because of visible lesions was 30 (1116 patients, 25 studies). The percentage of multifocal VIN 3 was 49 (1878 patients, 45 studies). The

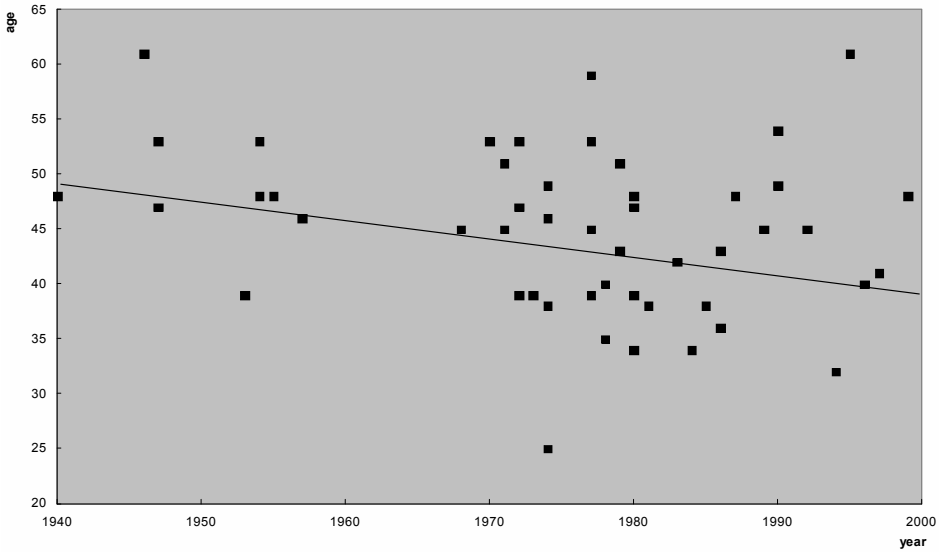

Figure 1. Regression analysis of the mean age of 2152 patients with VIN 3 at diagnosis, reported in 48 articles. Two articles gave 2 different study periods. The mean age decreased, although not significantly (P=0.08).

percentage of multicentric genital tract neoplasia was 32 (2067 patients, 46 studies). There was no change in time of complaints, visible lesions, multifocal VIN 3 or multicentric VIN 3.

Treatment and recurrences

The effect of 1921 surgically treated patients (68 studies) could be evaluated. The mean duration of follow-up was 39 months, mean range 12-75 months (15 studies, 597 patients). Recurrences could be found after vulvectomy (n=613) in 19%, after partial vulvectomy (n=62) in 18%, after local excision (n=808) in 22%, after laser-evaporization (n=253) in 23% and after cryocoagulation (n=16) in 56%. There was no statistical significant difference between recurrences after vulvectomy, partial vulvectomy, local excision and laser-evaporization. Recurrences were significantly lower after free surgical margins than after involved surgical margins (17% of 291 patients versus 47% of 189 patients, $P<0.001$).

Progression

A total of 215 invasive vulvar carcinomas (6.5%) were found. Nineteen cases of progression were reported as case histories. There were 107 occult carcinomas (3.2%) and 108 carcinomas (3.3%) were diagnosed during follow-up after treatment. The mean age at diagnosis of the carcinoma was 52, range 21-87 (118 patients). Eight patients were immunosuppressed and nine patients previously received radiotherapy in the lower genital tract. Previous type of surgery was known in 58 patients. Fifty-two percent progressed after a vulvectomy and 48% progressed after local excision. We could not find a difference

between progression of unifocal or multifocal VIN 3. In 11 out of the 31 women in whom the place of the carcinoma was known, it was located in the perianal area; in 15 out of 31 in the labial area. The mean time to progression was 55 months, range 4 to 216 months. In only 91 patients, the depth of invasion was mentioned. In 32 patients, invasion was described as superficial, early, initial or microinvasion. In 59 patients, invasion was measured. Of these 59 patients, 33 patients had invasion less than 1 mm and in 26 patients, it was more than 1 mm.

Spontaneous regression

Forty-one patients (13 studies) showed complete regression of all lesions. The mean age of these patients was 20 years, all were younger than 35 years. In 31 of the 33 patients in whom focality was known, multifocal VIN 3 regressed. In 17 patients, regression was related to pregnancy. In 68% of the patients, regression was found within 10 months.

Untreated patients

Ten studies reported on 88 untreated patients, who either received no treatment at all (n=61), or in whom gross macroscopic VIN 3 was left behind (n=27). Eight patients (9%) progressed in 12 to 96 months, four of whom had been treated previously with radiotherapy and one of whom was immunosuppressed. The mean follow-up of these 88 patients was 33 months (four studies, 43 patients, mean range 10-87 months).

Discussion

The dropping age (Figure 1) coincides with the increased incidence of VIN 3 between 1975 and 1981 [101]. However, one must realize that both these findings could be due to an increased awareness of this disease.

In this systematic review, the mean age at diagnosis of the invasive vulvar carcinoma was 52 years, while the mean age at diagnosis of VIN 3 was 46 years. The young age of the patients with invasive vulvar cancer in this study gives support to the idea that there are two different etiologies for vulvar carcinoma. One type is related to HPV and VIN 3 and occurs in younger women, such as in our study, and the other is related to the presence of lichen sclerosis and appears in older women.[102,103] However, one must keep in mind that VIN 3 can be seen in the adjacent skin in only 11-32% of all squamous cell carcinomas. Lichen sclerosis is seen in 47% of the cases in the adjacent skin.[104-109]

From our data, there is no indication that recurrences of VIN 3 depend on the type of surgery used, except for cryosurgery, which has a high failure rate. However, recurrences do occur significantly more often after involved surgical margins than after free surgical margins. The unknown extent of VIN 3 on the vulva in the majority of the articles may have

biased these results. This potential bias and the mean duration of follow-up of only 39 months should be taken into consideration while interpreting the value of these data.

If treatment of VIN 3 is aimed at avoiding an invasive vulvar carcinoma in the future several points should be remembered. Firstly, from our data, there is no difference in progression after different surgical procedures. Secondly, it could not be established whether free surgical margins diminish the change to progression in comparison to involved surgical margins. This means in our opinion that one should not enlarge the extent of excision hoping to obtain free surgical margins and thus diminishing the change of progression to invasive vulvar carcinoma. Thirdly, in 71% of the patients in whom depth of invasion is known, it is superficial. Besides this high percentage of superficially invasive carcinomas, one must be aware of the problems related to overdiagnosing early invasion. The finding of involved pilosebaceous units completely separated from the epidermis and tangential cuts may give an erroneous interpretation of invasive carcinoma.[110] Even when the depth of invasion was measured, it was not described in the articles reviewed herein how this was done exactly, that is, from the deepest rete pegs, from the most superficial dermal papillae or from the base of the epithelium. Since depth of infiltration was not mentioned in the majority of the articles, one can have doubts whether infiltration was not overdiagnosed in at least some of the reported cases of (micro)invasion. Finally, the mean time to progression was 55 months, range from 4 to 216 months. This means that patients with VIN 3 should be followed carefully for a very long period of time.

We could not find a difference between progression from unifocal or multifocal VIN 3, thus we could not support the suggestion that unifocal lesions are more likely to progress than multifocal lesions.[79] In one study the aspect of the lesion in relation to progression was examined. Lesions with a raised irregular surface more often contained an occult invasive vulvar carcinoma than flat, ulcerative and papular lesions.[64]

In this systematic review, untreated patients do have a 9% risk of progression to invasive vulvar carcinoma. Previous radiotherapy and immunosuppression may have played a role in the progression of those untreated patients.

Regression may be anticipated during several months in young women under 35 years of age with multifocal disease and without immunosuppression. Pregnancy was a substantial factor that may contribute to spontaneous regression.

It is found that more than 50% of women with VIN 3 have complaints (pain and/or pruritus). Up until now, management of complaints is still a matter of secondary concern, since treatment of VIN 3 is aimed at removal of all visible lesions to exclude the presence of an occult carcinoma.[5] The negative effect of vulvar surgery for the patient is great and irreversible. It has been shown that half of the women suffer from both a sexual dysfunction and psychological problems following radical or simple vulvectomy.[111] It has also been suggested that sexual functioning and somatopsychic reactions after treatment for VIN 3 correlate with the magnitude of the excision.[112] On the other hand, repeat local resec-

tions preserve the anatomy and functioning of the vulva better than primary extensive surgery[50] and symptomatic relief is best achieved by local excision in stead of a (skinning) vulvectomy.[71] Finally, it has been shown that after the presence of an invasive vulvar carcinoma has been excluded by way of multiple colposcopic-directed biopsies, long-term control of this disease in terms of complaints as well as progression is feasible by only removing the affected vulvar skin which is giving complaints.[3]

In conclusion, we underline that VIN 3 has a certain invasive potential both in untreated patients (9%) and in patients after treatment (3.3%). Invasion may occur many years after VIN 3 was diagnosed. Most of these invasive carcinomas are superficial and overdiagnosing early invasion is well known. Spontaneous regression may occur (1.2%). At this moment, there is not enough evidence from the available data to support the removal of all involved vulvar skin which would give many psychosexual sequelae. Only a prospective registration with standardized pathology examination will give information about the real natural history of VIN 3.

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3

In the absence of (early) invasive carcinoma, vulvar intraepithelial neoplasia associated with lichen sclerosus is mainly of undifferentiated type: new insights in histology and aetiology

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Abstract

Background Differentiated vulvar intraepithelial neoplasia (VIN) is presumed to be the precursor of invasive squamous cell carcinoma (SCC) of the vulva. It is commonly assumed that differentiated VIN is related to lichen sclerosus (LS). However, evidence for this is limited to a small number of studies describing epithelial alterations adjacent to vulvar SCC.
Aim To study the histology and human papillomavirus (HPV) status in patients with a history of both LS and VIN without coexistent SCC.
Methods Original biopsy specimens and surgical specimens of patients retrieved from the pathology files were revised for the presence of LS, VIN and (early) invasive SCC, specifically focused on the two different types of VIN: differentiated and undifferentiated. Thereafter, VIN lesions were tested for the presence of HPV DNA.
Results Twenty-seven patients fulfilled the criteria for LS and VIN without SCC. In all 27 patients, LS was found to be related to undifferentiated VIN. Grading yielded the following results: VIN 1 (n=10), VIN 2 (n=11) and VIN 3 (n=6). Additionally, VIN lesions from 26 patients could be tested for the presence of HPV DNA. HPV DNA, predominantly type 16, was present in eight (31%) of them. Seven of these eight patients had VIN 2 or 3. During follow-up, three patients progressed to (early) invasive carcinoma. In two of these patients, differentiated VIN was observed overlying early invasive SCC.
Conclusions VIN related to LS without coexisting SCC is likely to be undifferentiated, in contrast to what was previously thought. HPV DNA was demonstrated in 31% of the lesions, and was strongly related to high grade VIN.

Introduction

Invasive squamous cell carcinoma (SCC) of the vulva often arises in association with other vulvar abnormalities.[1-9] These abnormalities usually fall into two main categories, which can be considered as the main precursor states for invasive carcinoma of the vulva: vulvar intraepithelial neoplasia (VIN) and lichen sclerosus (LS).

According to the International Society for the Study of Vulvovaginal Diseases (ISSVD) and the International Society for Gynecological Pathologists (ISGYP), LS is a non-neoplastic disorder of the vulvar skin and mucosa.[10] Although the presence of LS in the adjacent skin of vulvar SCC is suggestive of a premalignant disease, longitudinal studies report only a slight tendency for LS to evolve into SCC (1-5%).[11-14]

VIN, on the other hand, is considered a pre-neoplastic disorder of the vulvar skin,[15] although progression to invasive carcinoma remains uncertain. Data on the follow-up of untreated VIN 3 are scarce, and the natural history is mainly based on follow-up after surgery. In the only systematic review on treatment of VIN, with data on 3322 patients, progression to invasive carcinoma was seen in 9% of the untreated patients and in 3% of patients after treatment.[16] VIN can be classified into undifferentiated (classic or bowenoid) and differentiated (simplex) VIN.[17] Undifferentiated VIN is associated with human papillomavirus (HPV), occurs predominantly in younger patients, and tends to be a multifocal and multicentric disease, whereas differentiated VIN is not related to HPV, is usually found in older women, and is commonly unifocal and unicentric. Differentiated VIN is rather uncommon. It is supposed to be associated with LS,[17,18] although evidence for this is limited to a small number of studies describing epithelial alterations adjacent to vulvar SCC.[4,7,19,20] Since differentiated VIN is often observed adjacent to or overlying superficially invasive SCC, it is presumed to be the precursor of most invasive SCCs of the vulva.[18]

Only four studies reported on the coexistence of LS and VIN, either differentiated or undifferentiated, *without* SCC.[19-22] A major disadvantage of all the four studies is that the coexistence of LS and VIN was not the main research question, but was a coincident finding. In a series of 86 patients with LS, differentiated VIN was observed twice, as was undifferentiated VIN.[20] Three other studies describing the histological features of VIN mentioned the presence of LS in 41 of 437 (9%) cases.[19,21,22] HPV DNA testing was performed in only one of them.[19] Since these three studies involved hardly any differentiated VIN, no conclusions can be drawn regarding the type of VIN and its relationship to LS.

As the relationship between differentiated or undifferentiated VIN and LS was never deliberately investigated, and since the role of HPV is not yet clarified, we studied the histology and HPV status in a large group of patients with a history of both LS and VIN *without* SCC.

Material and Methods

Patients

All cases with both histologically diagnosed LS and VIN were retrieved from the pathology files (1984–2004) of the Academic Medical Centre, the VU University Medical Centre and the Netherlands Cancer Institute in Amsterdam, and the Erasmus University Medical Centre in Rotterdam, The Netherlands. Firstly, a computer search for lichen sclero*sus* or sclero*sis* (et atrophicus) was performed (1807 specimens). Secondly, cases of women with anogenital LS were extracted from the list (1207 vulvar specimens). Thirdly, the pathology files of these patients with anogenital LS were searched for VIN *without* the initial presence of coexistent SCC (46 patients, 137 specimens).

Histology

Slides from the original biopsies as well as those from all subsequent surgical specimens of these 46 patients were collected and revised by an experienced pathologist (FJWtK) for the presence of LS, VIN and (early) invasive SCC.

The diagnosis of LS was based on the presence of dermal hyalinisation, vacuolar alterations of the dermal-epidermal junction and a variable dense lymphocyte infiltrate, whether or not accompanied by epidermal atrophy, progressive loss of rete ridges, hyperplasia and/or acanthosis.[23] VIN terminology was used according to the classification of the ISSVD.[15] We specifically looked for the two different types of VIN: undifferentiated and differentiated. *Undifferentiated VIN* is characterized by disorientation and loss of squamous epithelial architecture and maturation, together with a variable degree of cellular atypia. Depending on the level of cellular disarray, undifferentiated VIN was graded into VIN 1, 2 or 3. Subsequently, all revised VIN 1 cases were stained with MIB 1, a cell proliferation marker. *Differentiated VIN*, on the other hand, shows little or no atypia above the basal or parabasal layers, and is therefore a far more subtle lesion than undifferentiated VIN. Enlarged prematurely differentiated keratinocytes with prominent eosinophilic cytoplasm and abnormal nuclei are found deep within the epidermis, frequently near the tips of elongated and branching rete ridges. Deeply located squamous whorls with or without keratin pearls are sometimes seen.[15,17,24,25] Differentiated VIN should be regarded as VIN 3, owing to its supposed invasive potential.[15,24]

Depth of invasion, if present during follow-up, is described as early invasive or invasive carcinoma.

Human papillomavirus testing

All confirmed dysplastic lesions were tested for the presence of HPV. To this end, we extracted cellular DNA from corresponding formalin-fixed, paraffin-wax-embedded tissue. Testing for HPV was conducted by using a standard $GP5^{+}/6^{+}$ PCR enzyme immunoassay,

followed by reverse line blot analysis.[26] This test is clinically validated.[27] We used one assay for the 14 most prevalent high-risk types of HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68), and another for 22 low-risk HPVs (6, 11, 26, 34, 40, 42, 43, 44, 53, 54, 55, 57, 61, 70, 71, 72, 73, 81, 82 [both subtypes MM4 and IS39], 83, 84, and CP6108). In addition, PCR amplification products were analysed for individual types of HPV (reverse line blot). A β-globin test was performed as a control for the presence and quality of DNA in the paraffin-wax-embedded tissue.

Results

Patients

In 31 of the 46 patients who met our computer search criteria, the diagnosis of both LS and VIN could be confirmed after histological revision. Fifteen patients did not fulfil the criteria for VIN (n=11), LS (n=2), or both VIN and LS (n=2). Four more patients presented with lesions suspected to be malignant at first onset of either LS or VIN, and were therefore excluded. Twenty-seven patients (82 vulvar specimens) remained for evaluation of histology and HPV status. Their mean (range) age at first histological diagnosis was 64 (38-87) years.

Histology

In these 27 patients with both LS and VIN, VIN lesions were all classified as undifferentiated (100%). This was further graded into VIN 1 (n=10), VIN 2 (n=11) and VIN 3 (n=6) (Table 1).

To conform the diagnosis, MIB staining in VIN 1 showed increased mitotic activity in all cases. A warty histological pattern was seen in 24 (89%) lesions; basaloid-type VIN was

Table 1. Human papillomavirus DNA in 27 patients with lichen sclerosus and vulvar intraepithelial neoplasia without vulvar squamous cell carcinoma

HPV-DNA	VIN			total
	1	2	3	
HPV-positive	1*	4†	3‡	8
HPV-negative	8	7¶	3∫	18
unknown	1§	-	-	1

HPV, human papillomavirus;
*HPV16;
†HPV16 (n=3) and HPV59 (n=1);
‡HPV16 (n=3), one of these patients developed squamous cell carcinoma (SCC) surrounded by HPV-positive undifferentiated vulvar intraepithelial neoplasia (VIN) after 4 years;
¶One of these patients developed early invasive SCC surrounded by differentiated VIN not related to HPV after 4 years;
∫One of these patients developed early invasive SCC surrounded by differentiated VIN not related to HPV after 1 year;
§No material available.

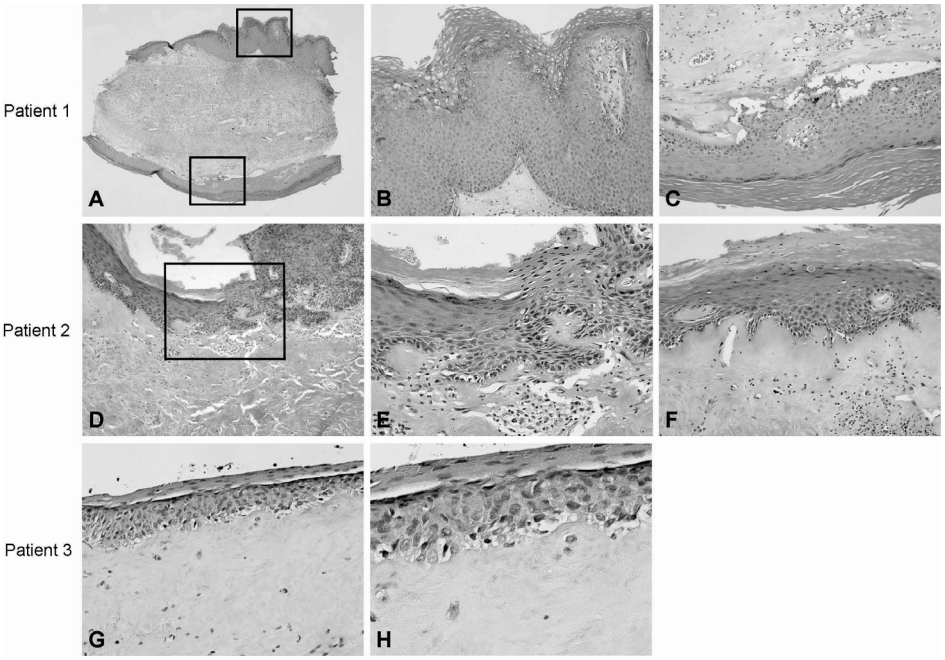

Figure 1. Patient 1: (A) vulvar biopsy with both condylomatous dysplasia and classic lichen sclerosus (LS; H&E, x20); (B) in more detail, verricuform dysplastic changes that tested positive for human papillomavirus (HPV)-59 DNA, showing mild-to-moderate disturbance of the epithelial architecture with acanthosis and koilocytosis, conform vulvar intraepithelial neoplasia (VIN) 2 (H&E, x100); (C) classic LS in more detail, showing oedema and hyalinisation of the dermis, vacuolisation of the dermal-epidermal junction, and hyperkeratosis, with normal architecture of the squamous epithelium (H&E, x100). Patient 2: (D) local excision of the vulva in a patient with a diagnosis of VIN 3, showing undifferentiated HPV16-positive VIN in the background of LS (H&E, x80); (E) a higher magnification of (D), showing the transition of normal to dysplastic epithelium (H&E, x200); (F) histological findings of LS in the same tissue with hyperkeratosis, thinning of the epidermis, and oedema with homogenisation of the dermal stroma (H&E, x125). Patient 3: (G) coexistence of LS and undifferentiated VIN 2 in the vulva (H&E, x200); (H) a higher magnification of (G), illustrating dysplastic changes that tested negative for HPV DNA (H&E, x400). See also Color Figures, page 145.

found in one (4%) lesion, and in two (7%) VIN 1 lesions it was not possible to distinguish warty and basaloid VIN. We diagnosed LS and VIN at the same time in the same lesion in 13 patients (Figure 1, patient 1-3), mostly contiguous, and at the same time but in a different lesion in three more patients (Figure 2, patient 4). In the other 11 cases, a period of 1-9 years was observed between first diagnosis of the two skin disorders (Figure 2, patients 5 and 6). VIN arose in pre-existent LS in nine of 11 (82%) cases, whereas LS was diagnosed in pre-existent VIN twice.

Three patients developed (early) invasive SCC 1-4 years after the initial diagnosis of undifferentiated high grade VIN in pre-existing LS. In one case the epithelium adjacent to invasive SCC showed undifferentiated VIN 3, whereas in the other two cases differentiated VIN was found in the epithelium surrounding early invasive SCC (Figure 3, patient 7).

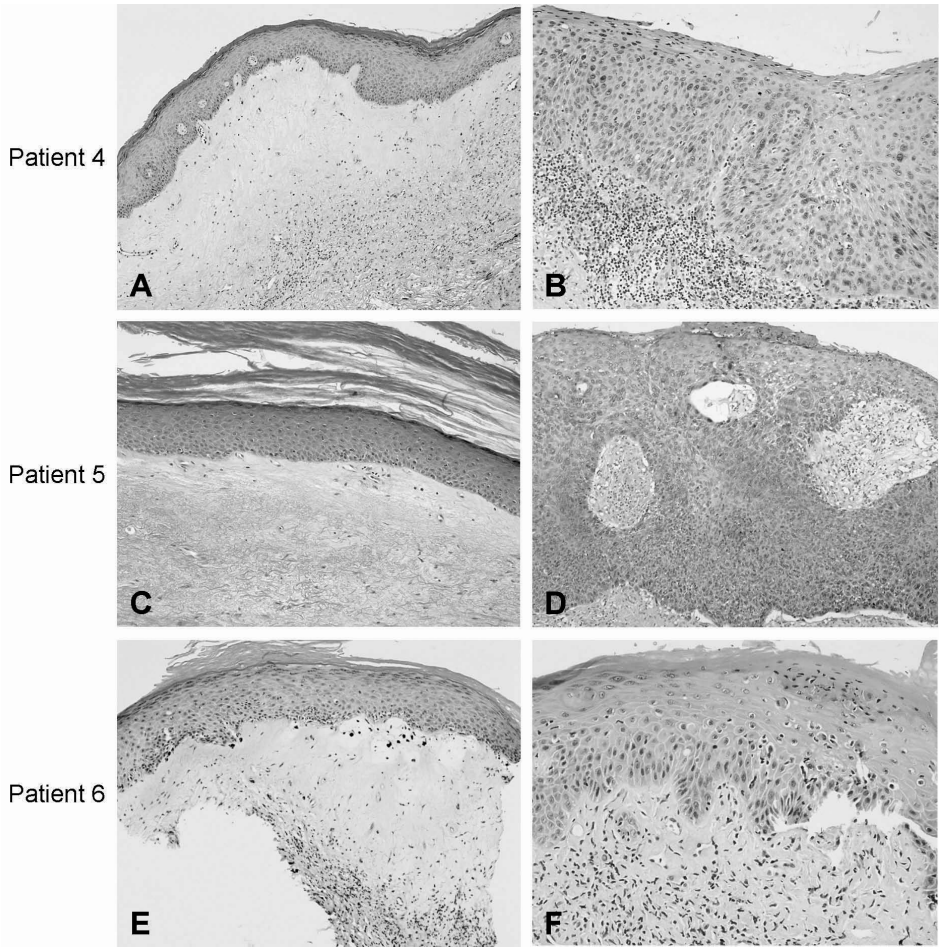

Figure 2. Patient 4: (A) lichen sclerosus (LS) showing a broad band of hyalinisation underlying an atrophic epidermis with loss of rete ridges (H&E, x50); (B) undifferentiated human papillomavirus (HPV) 16-positive vulvar intraepithelial neoplasia (VIN) 3 biopsied at the same time in the same patient, but in a different lesion (H&E, x100). Patient 5: (C) LS with normal epidermal maturation (H&E, x100); (D) undifferentiated HPV-negative VIN 3 in the same patient, which was diagnosed 6 years after the diagnosis of LS (H&E, x64). Patient 6: (E) classic LS showing extensive dermal hyalinisation with vacuolisation of the dermal-epidermal junction (H&E, x64); (F) undifferentiated HPV-negative VIN 1, with focal VIN 2 diagnosed 2 years after the diagnosis of LS (H&E, x160). See also Color Figures, page 146.

Human papillomavirus testing

Paraffin-wax-embedded tissue for HPV testing was available in 26 patients. HPV DNA was detected in eight of 26 (31%) tissue samples, whereas 18 lesions were found to be negative. Seven lesions contained HPV-16 DNA, and one contained HPV-59. There was a strong correlation between HPV-positive lesions and high grade VIN. Whereas almost all VIN 1 lesions were HPV negative (89%), a considerable number of VIN 2 and 3 lesions were

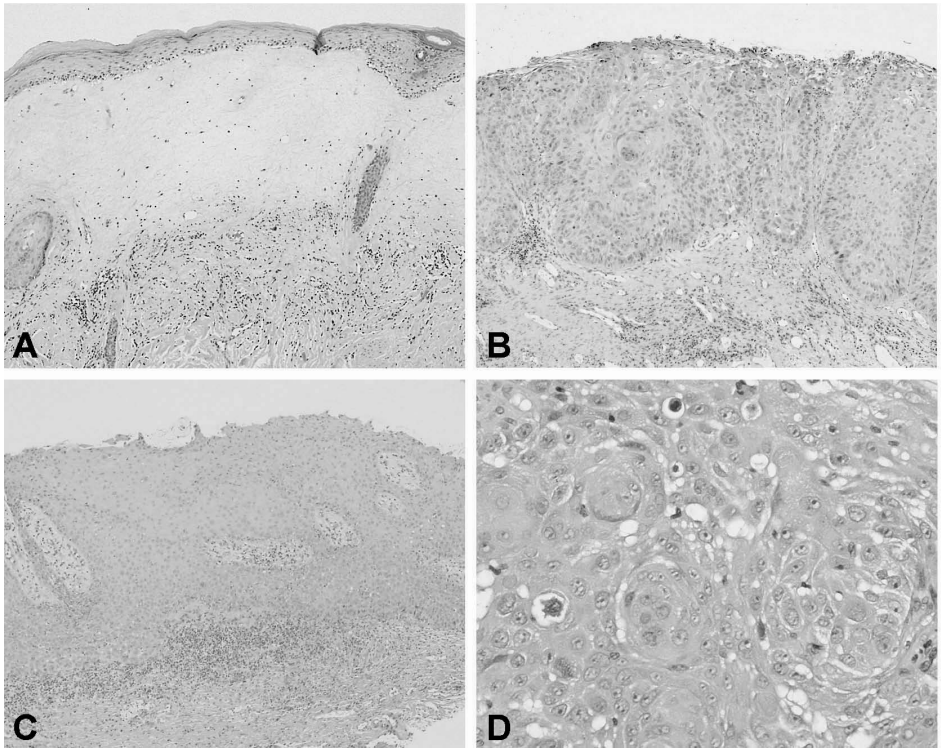

Figure 3. Patient 7: (A) lichen sclerosus (LS; H&E, x50); (B) undifferentiated vulvar intraepithelial neoplasia (VIN) 3 not related to human papillomavirus (HPV), which was diagnosed 9 years after the diagnosis of LS (H&E, x60); (C) one year later, this patient developed a lesion suspicious for squamous cell carcinoma, which was surrounded by HPV-negative differentiated VIN (H&E, x50). Little or no atypia is shown above the (para-) basal layer; (D) pearl-like changes can be distinguished (H&E, x250). See also Color Figures, page 147.

positive for HPV (41%). One of these patients with high grade HPV-related VIN developed SCC after four years, the epithelium being surrounded by HPV-positive undifferentiated VIN. The other two patients with progressive disease developed SCC with differentiated VIN not related to HPV in the adjacent skin (Table 1).

Discussion

This is the first study describing the histology and HPV status of a large series of patients with both LS and VIN *without* coexistent SCC. In this study, VIN associated with LS was always of undifferentiated type. HPV DNA was detected in eight of 26 (31%) patients, seven of whom had high grade VIN.

It is commonly thought that there is a relationship between differentiated VIN and LS.[17,18] There are, however, only few data available on this subject, and almost all these concern

Table 2. Histological findings of vulvar intraepithelial neoplasia and/or lichen sclerosus in the adjacent skin of vulvar squamous cell carcinoma

Study	population	N	skin adjacent to SCC					
			uVIN only	dVIN only	LS only	both VIN and LS		Other*
						uVIN (%)	dVIN (%)	
Leibowitch et al[4] *BJOG, 1990*	SCC	78	24†	-	20‡	-	**28 (36)**	6
Gómez Rueda et al[5] *J Reprod Med, 1994*	SCC	64	5	-	18	**11 (17)**	**3 (5)**	27
Haefner et al[19] *Hum Pathol, 1995*	SCC + VIN	15	8	3	-	**1§ (7)**	**3¶ (20)**	-
Scurry et al[20] *Int J Gynecol Cancer, 1997*	SCC	132	31	18	29	**1 (1)**	**33 (25)**	20
Carlson et al[28] *Hum Pathol, 1998*	SCC	60	16	-	14	**19 (32)**	**6 (10)**	5
Vilmer et al[7] *Eur J Gynaecol Oncol, 1998*	SCC	67	13	-	12	**-**	**39 (58)**	3
Carlson et al[22] *Am J Pathol, 2000*	SCC	16	1	-	13	**1 (6)**	**-**	1
Derrick et al[29] *Br J Dermatol, 2000*	SCC	21	3	-	13	**-**	**1 (5)**	4
Poulsen et al[9] *APMIS, 2003*	SCC	14	7	-	-	**7 (50)**	-	-
Total		467				**40 (9)**	**113 (24)**	

dVIN, differentiated VIN; LS, lichen sclerosus; SCC, squamous cell carcinoma; uVIN, undifferentiated VIN; VIN, vulvar intraepithelial neoplasia;
*Normal (n=22), squamous cell hyperplasia (n=31), lichen simplex chronicus (n=5), granulomatous vulvitis (n=1), lichen planus (n=4), or not associated with LS or VIN (n=3); †7/11 samples tested were HPV-positive by in situ hybridisation (ISH): HPV16 (n=6), HPV31 (n=1); ‡No samples tested were HPV-positive (ISH); §HPV16 DNA positive (PCR and ISH); ¶3 of 3 samples tested were HPV DNA negative (PCR).

the adjacent skin of vulvar SCC (Table 2).[4,5,7,9,19,20,22,28,29] In 153 of 467 (33%) patients whose skin lesions in the surrounding tissue of vulvar SCC were analysed, the existence of both VIN and LS has been observed (range 5-58%). Predominantly, this VIN was of differentiated type (74%). Sporadically, the coexistence of VIN and LS has been described in vulvar skin *without* invasive carcinoma (Table 3).[19-22] Since these studies were on either LS or VIN, mostly of undifferentiated type, selection bias might have affected the results.

To date, the presence of HPV DNA in VIN related to LS has been described only once.[19] Haefner *et al* reported on three patients with HPV-negative differentiated VIN, all in the adjacent skin of vulvar SCC, and on three patients with HPV-positive undifferentiated VIN 3, in one of whom VIN was adjacent to SCC and in the other two it was not. The detection of HPV DNA in 41% of our high grade VIN lesions supports the diagnosis of undifferentiated VIN, although, compared with the prevalence of HPV DNA in VIN not related to LS (78-92%), this percentage is rather low.[19,30-32] This lack of similarity between undifferenti-

Table 3. Coexistence of both vulvar intraepithelial neoplasia and lichen sclerosus without vulvar squamous cell carcinoma

Study	population	N	both VIN and LS		
			uVIN	dVIN	uVIN /dVIN
Haefner et al[19] *Hum Pathol, 1995*	VIN*	50	**2**†	-	-
Italian Study Group[21] *J Reprod Med, 1996*	VIN‡	370	**36**	-	-
Carlson et al[22] *Am J Pathol, 2000*	VIN§	17	-	**2**	**1**
		437	**38**	**2**	**1**
Scurry et al[20] *Int J Gynecol Cancer, 1997*	LS	86	**2**	**2**	-

dVIN, differentiated VIN; LS, lichen sclerosus; SCC, squamous cell carcinoma; uVIN, undifferentiated VIN; VIN, vulvar intraepithelial neoplasia;
*48 uVIN 3, 2 dVIN;
†Both human papillomavirus 16 DNA positive (PCR and in situ hybridization);
‡370 uVIN: VIN 1 (n=148), VIN 2 (n=53), VIN 3 (n=169);
§14 uVIN 3, 3 dVIN.

ated VIN with and without associated LS might point to a different pathogenesis, at least in half of the cases.

It is known that there are some difficulties in the histological diagnosis of VIN. Firstly, it can be very difficult to distinguish VIN 1 from atypical inflammatory reactive changes or (damaged) normal skin, resulting in a high inter-observer variability.[33-35] In our study, we excluded 10 of 46 patients, in whom low grade VIN diagnosed in the original histology report was not confirmed at revision following the definition given by the ISSVD.[15] The high prevalence of VIN 1 lesions that tested negative for HPV after revision (89%) gives the impression that we still overdiagnosed VIN. However, MIB 1 staining, which has been suggested to be useful in accurate grading of VIN,[35] supported the diagnosis of VIN 1 in all cases. Today, this VIN 1-3 classification is the subject of discussion, and a new classification in which the term VIN 1 will no longer be used has been proposed by the ISSVD. Similarly, Medeiros *et al* recently recommended a distinction between low grade VIN (VIN 1) and high grade VIN (VIN 2 or 3 and differentiated VIN) to identify those lesions at risk for vulvar carcinoma.[36] In our study, such a new classification would affect the number of patients considerably, causing a significant increase in the number of HPV-related high grade VIN associated with LS (43%). Secondly, the presence of differentiated VIN has to be considered when there seems to be only a slight degree of squamous atypia or it is limited to the lower epidermis. Because of its highly differentiated features and absence of widespread architectural disarray, the diagnosis can be easily missed. We specifically looked into the subtle but characteristic features of differentiated VIN, as described in Material and Methods. However, we did not observe any of these features, except during follow-up, surrounding early invasive SCC. Furthermore, it may be difficult to agree upon

whether early invasive growth has already occurred in differentiated VIN.[25] Conversely, differentiated VIN is easily diagnosed when there is an invasive SCC. The fact that none of our revised VIN 1 cases progressed to invasion strengthens our claim of not having missed any differentiated VIN.

As we studied only those cases with both LS and VIN from the start, a selection bias might have been caused by the design of our study. Revising all LS cases over several years could result in a higher prevalence of LS with coexisting VIN, and therefore possibly in a higher number of differentiated VIN. Revising all VIN cases, on the other hand, might result in a higher number of coexistent diseases as well, with VIN most likely to be of undifferentiated type.

In conclusion, it seems from an overview of the literature that the assumed relation between differentiated VIN and LS can be supported in the case of coexistent vulvar SCC,[4,7,19,20,29] but has been described in only five patients *without* SCC.[20,22] In this study, we found that VIN related to LS *without* SCC was always of the undifferentiated type. These data suggest that VIN in the background of LS without SCC is a different type of VIN than that was thought previously, with a different aetiology and perhaps a different prognosis. In that case, we might question ourselves whether or not we tend to overtreat patients if we radically excise VIN in the background of LS. In our opinion, treatment of VIN associated with LS should be individualised, based on type and localization of the lesion, in order to achieve a better conservation of the anatomy and function of the vulva.

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4 Imiquimod in the treatment of multifocal vulvar intraepithelial neoplasia 2/3 Results of a pilot study

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Abstract

Objective To investigate the efficacy of topical treatment with imiquimod 5% cream, an immune response modifier, in patients with vulvar intraepithelial neoplasia (VIN) 2/3.
Study design Fifteen women (aged 35-51) with histologically proven multifocal VIN 2/3 without invasion, were entered into a prospective, observational, pilot study. Imiquimod 5% cream was applied by the patient to the vulvar lesions one to three times a week at night. Clinical response was analyzed by macroscopic examination and categorized as complete (CR) or partial (PR).
Results Four patients achieved CR (27%) and nine patients PR (60%) after 6-34 weeks of treatment. Two patients discontinued medication. CR was reached after 6, 7, 11 and 30 weeks of treatment.
Conclusion This pilot study showed the potential beneficial effect of imiquimod 5% cream in multifocal VIN 2/3. In contrast to current surgical treatment, imiquimod focuses on the cause of VIN and preserves the anatomy and function of the vulva. Therefore, imiquimod may prove to be the treatment of choice in multifocal, high grade VIN.

Introduction

The incidence of vulvar intraepithelial neoplasia (VIN) 2/3 has increased in recent decades,[1,2] and patients are affected at younger ages than before.[3] The disease is often multifocal on the vulva,[4] and neoplastic changes can be found in a high proportion on the cervix and less frequently in the vagina.[5] High grade VIN is a skin disease causing many severe and long-lasting symptoms, such as pruritus, vulvar pain and sexual dysfunction.[6,7]

Human papillomavirus (HPV) plays a key role in the etiology of VIN. The prevalence of HPV DNA in VIN 3 has been studied by several groups using polymerase chain reaction. All of them showed the same high prevalence of HPV-16 DNA (around 90%).[8-12] Moreover, HPV RNA E6/E7 transcripts could be detected in almost 100% of the HPV-16 DNA-positive VIN lesions that were tested.[8,13,14] That underlines the oncogenic activity of HPV-16 in these lesions.

Management of patients with VIN remains a challenge for the gynecologist. Although extensive surgery, such as vulvectomy, has been abandoned, standard therapy still comprises radical removal of all visible lesions to prevent invasive disease.[15] However, surgical margins are often positive, irrespective of the type of operation performed.[3,16-19] This means that recurrences of VIN are still common, even after these extensive surgical procedures.[3,7,17,20] Moreover, a surgical approach often results in mutilation of the vulva and consequently leads to major psychosexual distress.[21,22]

As imiquimod 5% cream, an immune response modifier with indirect antiviral and antitumor properties, has been shown to be effective and safe in the treatment of HPV-associated external genital warts (EGWs),[23,24] it was hypothesized that this topical treatment may also be effective in stimulating cell-mediated immunity against cells infected with different HPV types and thus induce regression of dysplastic vulvar lesions.

The objective of this pilot study was to evaluate the effect of imiquimod 5% cream on VIN 2/3 lesions and related symptoms.

Material and Methods

Fifteen women with histologically proven multifocal VIN 2/3 without invasion, were entered into a prospective, observational, pilot study at the Academic Medical Center in Amsterdam, The Netherlands. Unifocal VIN 3 lesions were excluded from the study. For each patient the following items were recorded: age; previous surgical treatment; presence of symptoms; coexisting genital tract neoplasia (cervix, vagina, perianal region); use of immunosuppressives; history of smoking; frequency, length and discontinuation of treatment; response of lesions and symptoms to treatment; and side effects.

Imiquimod 5% cream (3M Medica, Borken, Germany) was applied by the patient to the vulvar lesions restricted to one side one to three times a week at night, depending on the side effects. Sulfur precipitate was applied the day after application of imiquimod to reduce the chance of a secondary bacterial infection. In the event of severe side effects, the medication was to be discontinued indefinitely.

At each visit a history was taken to evaluate the effect on the symptoms. Clinical response was analyzed by macroscopic examination with photodocumentation and categorized as complete response (CR) when all visible lesions had disappeared and partial response (PR) when more than 25% regression of the original VIN lesion had been reached.

Results

The mean age of our study population was 42.7 years (range, 35-51). Eleven of the 15 patients had been treated previously with surgical procedures (local excision, laser evaporation, partial vulvectomy), and four had never been treated before. Most of the women were experiencing vulvar pain and pruritus at baseline; two were without vulvar complaints. Nine women had a prior history of cervical intraepithelial neoplasia. Two of them had concomitant anal intraepithelial neoplasia. One patient had a history of previous severe immunosuppression: she had achieved complete remission of chronic, recurrent, low grade non-Hodgkin's lymphoma after allogen stem cell transplantation and total body irradiation in November 1998. Tobacco was used by 12 women in our study population; most (67%) smoked ≥20 cigarettes per day.

The frequency of study drug application varied from one to three times a week, according to clinical response and side effects. Most of the patients tolerated treatment well at a frequency of two times a week. If treatment produced severe side effects, a drug-free period was allowed. Eight patients were able to continue therapy without any rest periods; six patients stopped treatment for a variable period (one to eight weeks), after which they continued treatment with imiquimod. One patient discontinued treatment after four weeks.

After a treatment period ranging from 6 to 34 weeks, CR was achieved in four patients (27%) and PR achieved in nine patients (60%). Eleven of these 13 (85%) patients noted a significant reduction of symptoms. Two patients discontinued therapy; one was the patient with the history of non-Hodgkin's lymphoma, and she stopped after 11 weeks of treatment as she did not respond to the therapy and had severe side effects. The other patient stopped after four weeks of treatment because of side effects. CRs were reached after 6, 7, 11 and 30 weeks of treatment (Figures 1A-C). Two of the patients (one CR and one PR) showed regression of the lesions on the side where no imiquimod was applied. No correlation was found between response and smoking behavior.

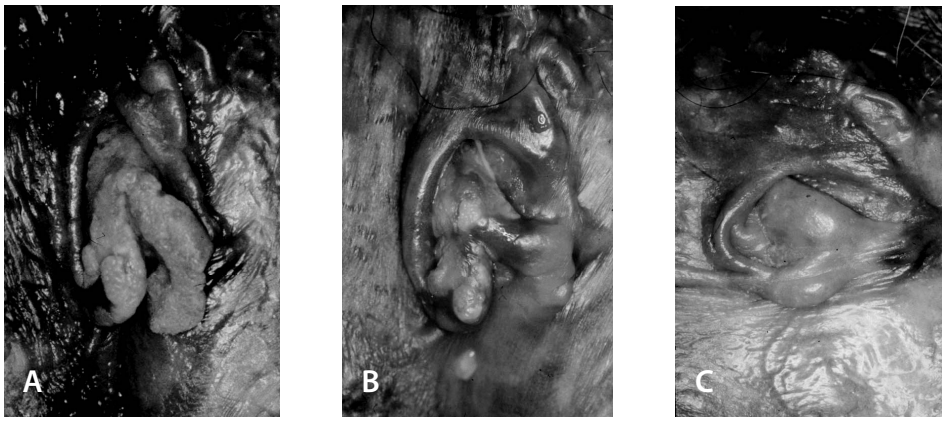

Figure 1. A, VIN 3 lesion of the clitoris in a 42-year-old woman who was diagnosed with VIN in 1996. She had been treated previously with local excision with the exception of the clitoral lesion. In January 2000 the patient started treatment with imiquimod as the clitoral lesion was still present and pruritus worsened. B, Same patient as in Figure 1A. The patient started treatment three times a week. After five weeks of treatment, vulvoscopic examination showed a clear response. However, side effects, such as a burning sensation, pruritus and vulvar irritation, were noted. Treatment was continued with a frequency of once a week. C, Same patient as in Figures 1A and B. After 17 weeks of treatment the clitoral lesion has completely disappeared, and the patient did not have any more symptoms. One year after she finished treatment, the patient still had a complete response.

Discussion

Until now, the premalignant character of high grade VIN has dominated the choice of therapy, leading to extensive surgery.[15] Although surgical margins are often positive[3,16-19] and high recurrence rates of VIN 2/3 are common,[3,7,17,20] irrespective of the type of operation performed, local excision, laser vaporization and skinning vulvectomy are still the standard treatments used today.[15] Therefore, the malignant potential of untreated VIN 3 has seldom been described and is unclear. In the only available systematic review in the literature, with data on 2,864 patients, 8 of the 88 (9%) untreated patients showed progression to invasive carcinoma. In treated patients this was the case in only 2-3%.[25]

HPV-16 is the cause of VIN 3.[8-14] Therefore, research on the potential benefit of antiviral and immune-stimulating medication has emerged. Imiquimod 5% cream is such an immune response modifier and has been shown to be safe and efficacious in the treatment of EGWs caused by HPV.[23,24] The activity of imiquimod is mediated through stimulation of cells involved in the innate immune response, such as monocytes, macrophages and dendritic cells. These immune cells secrete a number of cytokines (IFN-α, TNF-α, IL-12) and chemokines (IL-6, IL-8 and IL-10). Some of the cytokines induced by imiquimod, including IFN-α and IL-12, can also enhance acquired immune responses, in particular by stimulating T-helper cells type 1 and other cell-mediated immune responses that are important in the control of viruses, tumors and intracellular pathogens.[24] The clinical response is

accompanied by a decrease in the amount of HPV DNA and of mRNA for HPV proteins L1 and E7.[26] Therefore, it is hypothesized that this topical treatment may also be effective in stimulating cell-mediated immunity against different HPV types and thus inducing regression of dysplastic vulvar lesions.

Davis *et al* published the first results of imiquimod treatment in VIN 3.[27] In four cases imiquimod cream was applied three times a week for a maximum treatment period of 16 weeks. Treatment resulted in complete clearing of all lesions, and post treatment biopsies were negative. One patient developed a recurrence one year after treatment. In our pilot study, 15 patients with histologically proven multifocal VIN 2/3 without invasion were included. Of these 15 women, 13 (87%) achieved CR or PR (four CR, nine PR) after various treatment times (6-34 weeks). Eleven of the 13 (85%) patients with CR or PR noted a reduction in symptoms. One of the patients who had been severely immunosuppressed failed therapy. One stopped treatment because of side effects. Recently, Todd *et al* published results on 15 patients similar to our group. In contrast to the positive results of our pilot study, Todd found a response rate in only four of 13 patients treated with imiquimod.[28]

In conclusion, this pilot study showed the potential beneficial effect of imiquimod 5% cream in patients with multifocal VIN 2/3 in whom invasion has been ruled out by mapping. In contrast to current surgical treatments, imiquimod permits more specific targeting of the cause of VIN. This nonsurgical approach leaves the anatomy of the vulva intact. Therefore, imiquimod may prove to be the treatment of choice in patients with multifocal, high grade VIN in whom risk factors for micro-invasion, such as unifocal diseases,[29] raised lesions,[6] older age[6,30] and previous radiotherapy,[30] have been ruled out. To further study this potential effect, we have started a prospective, randomized, double-blind, placebo-controlled clinical trial.

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5

Treatment of vulvar intraepithelial neoplasia with topical imiquimod

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Abstract

Background Alternatives to surgery are needed for the treatment of vulvar intraepithelial neoplasia. We investigated the effectiveness of imiquimod 5% cream, a topical immune-response modulator, for the treatment of this condition.

Methods Fifty-two patients with grade 2 or 3 vulvar intraepithelial neoplasia were randomly assigned to receive either imiquimod or placebo, applied twice weekly for 16 weeks. The primary outcome was a reduction of more than 25% in lesion size at 20 weeks. Secondary outcomes were histologic regression, clearance of human papillomavirus (HPV) from the lesion, changes in immune cells in the epidermis and dermis of the vulva, relief of symptoms, improvement of quality of life, and durability of response. Reduction in lesion size was classified as complete response (elimination), strong partial response (76 to 99% reduction), weak partial response (26 to 75% reduction), or no response (≤25% reduction). The follow-up period was 12 months.

Results Lesion size was reduced by more than 25% at 20 weeks in 21 of the 26 patients (81%) treated with imiquimod and in none of those treated with placebo (P<0.001). Histologic regression was significantly greater in the imiquimod group than in the placebo group (P<0.001). At baseline, 50 patients (96%) tested positive for HPV DNA. HPV cleared from the lesion in 15 patients in the imiquimod group (58%), as compared with two in the placebo group (8%) (P<0.001). The number of immune epidermal cells increased significantly and the number of immune dermal cells decreased significantly with imiquimod as compared with placebo. Imiquimod reduced pruritus and pain at 20 weeks (P=0.008 and P=0.004, respectively) and at 12 months (P=0.04 and P=0.02, respectively). The lesion progressed to invasion (to a depth of <1 mm) in three of 49 patients (6%) followed for 12 months (two in the placebo group and one in the imiquimod group). Nine patients, all treated with imiquimod, had a complete response at 20 weeks and remained free from disease at 12 months.

Conclusions Imiquimod is effective in the treatment of vulvar intraepithelial neoplasia.

Surgery, the treatment of choice for vulvar intraepithelial neoplasia, removes all visible lesions, with the aim of relieving symptoms and preventing vulvar cancer.[1] However, there are limitations to surgery. The percentage of lesions with positive surgical margins ranges from 24 to 68%.[2,3] Recurrences are common, because surgery does not eliminate human papillomavirus (HPV), the cause of most vulvar intraepithelial neoplasia.[4,5] Progression is not influenced by radical excision,[4,6] and surgery can mutilate the vulva, thereby causing psychosexual distress.[7-10] Thus, alternative treatments are needed.

Vulvar intraepithelial neoplasia is caused by HPV,[11] which has prompted the use of imiquimod 5% cream (Aldara, 3M Pharmaceuticals), a topical immune-response modifier,[12] for treatment of the disease. Efficacy has been reported, although only in small, uncontrolled studies.[13-16] The aim of this study was to assess the effectiveness of imiquimod 5% cream in patients with multifocal grade 2 or 3 vulvar intraepithelial neoplasia in a placebo-controlled, double-blind, randomized clinical trial.

Materials and Methods

Patients

All patients 18 years of age or older with grade 2 or 3 vulvar intraepithelial neoplasia who were seen at the Academic Medical Center of the University of Amsterdam or the Erasmus University Medical Center of Rotterdam between April 2001 and July 2003 were asked to participate. The inclusion criteria were histologically proven, multifocal grade 2 or 3 vulvar intraepithelial neoplasia without microinvasion and contraceptive use for sexually active, premenopausal women (to avoid any possible teratogenic effects of imiquimod). The exclusion criteria were a history of cancer or inflammatory dermatosis of the vulva, pregnancy, immunodeficiency, any treatment for vulvar intraepithelial neoplasia or warts within the previous month, hypersensitivity to the cream, or an inability to understand Dutch or English. A Consolidated Standards for the Reporting of Trials (CONSORT) diagram appears in the Supplementary Appendix.

Study Design

A formalin-fixed biopsy specimen was obtained for histologic analysis within 3 months before enrollment. A second specimen from the same lesion was frozen in liquid nitrogen and stored at –80°C for HPV DNA testing and immunohistochemical analysis. Patients with extensive vulvar intraepithelial neoplasia underwent surgical mapping before enrollment to establish the extent and grade of neoplasia and to rule out invasive disease. If a lesion was suspicious for invasion (i.e., was raised, erosive, ulcerative, or indurated), a wide local excision was performed.

During the first visit, a medical history was taken and a physical examination was performed. Blood samples were drawn for pregnancy testing and for hematologic and serum chemical analysis at the first visit and at 4 weeks after treatment (20 weeks after the first visit). A cervical smear was taken at the first and last study visits.

Eligible patients were randomly assigned to receive 250 mg of imiquimod 5% or placebo cream, a complete vehicle control. Neither the patients nor the examining physicians were aware of the treatment assignments. Randomization was carried out by 3M Pharmaceuticals in blocks of four (with a two-by-two design) without stratification. The patients applied a thin layer of study medication to the lesions and let it remain overnight without a cover twice a week for a period of 16 weeks. In case of severe side effects, application could be reduced to once a week, or a treatment-free period of 1 week was permitted. The patients were advised to use sulfur precipitate 5% in zinc oxide ointment the day after application of the cream to avoid superinfection.

The patients used a diary to report concomitant medication and side effects. Every 4 weeks, the patients were monitored for the efficacy of treatment, symptoms, and side effects. At 20 weeks, a post-treatment biopsy specimen was obtained for histologic analysis, and a sample was stored at –80°C for detection of HPV DNA and immunohistochemical analysis. Photographs were used to ensure that the post-treatment biopsy specimen was taken from the same site.

To investigate long-term effects and to evaluate possible recurrences of vulvar intraepithelial neoplasia, we performed post-treatment assessments at 7 months and at 12 months. If a recurrence was suspected at 12 months, a biopsy specimen was obtained. In cases of persistent or residual disease after one year, treatment with imiquimod or surgery was recommended. If lesions suspicious for invasion developed during the study, wide local excision was performed. Except for cases of serious side effects, the randomization code was not broken until all women had been seen at 12 months. The ethics committees of the Academic Medical Center of the University of Amsterdam and the Erasmus University Medical Center of Rotterdam approved the study protocol. All women voluntarily provided written informed consent; they were informed that surgery was the treatment of choice for vulvar intraepithelial neoplasia.

All vulvar intraepithelial neoplasia lesions were measured with calipers and photographed at baseline, every 4 weeks during treatment, and at follow-up visits. A computer program (ImageJ) was used to calculate the total lesion size in square centimeters by adding the measurements for each separate lesion together. To avoid bias caused by side effects, one of the investigators and an independent gynecologist with expertise in vulvar pathology evaluated the clinical response with the use of photographs taken at the first study visit and at 20 weeks. Clinical response was defined as a reduction in total lesion size and was classified as a complete response, a strong partial response (76 to 99% reduction in lesion size), a weak partial response (26 to 75% reduction in lesion size), or no response

(reduction in lesion size of 25% or less). Skin reactions during treatment were recorded. To evaluate the long-term response, photographs taken at 12 months were compared with those taken at baseline.

All biopsy evaluations were reviewed independently by two experienced gynecologic pathologists who were unaware of the clinical data. Biopsy specimens were classified as grade 1, 2, or 3 vulvar intraepithelial neoplasia.[17] A consensus meeting was arranged when the pathologists did not agree. Histologic regression was defined as regression from grade 2 or 3 vulvar intraepithelial neoplasia to a lower grade. If infiltration was present, the depth of infiltration was measured by Wilkinson's method.[18]

Frozen biopsy specimens were analyzed for the presence of HPV DNA with the use of a standard GP5+/6+ polymerase-chain-reaction (PCR) enzyme immunoassay, followed by reverse line-blot analysis.[19,20] High-risk and low-risk probe cocktails were used to identify the 14 most prevalent high-risk and the 22 most prevalent low-risk types of HPV. In addition, PCR amplification products were analyzed to identify individual HPV types by reverse line-blot analysis. A PCR assay for the β-globin gene was performed to ascertain the presence and quality of target DNA. The histochemical analysis of immune cells and the statistical analysis of immunologic data are described in the Supplementary Appendix.

Pruritus and pain were rated by the patients every 4 weeks during treatment and at follow-up visits on a visual analogue scale from 0 (no symptoms) to 10 (severe symptoms). The mental health scale of the Medical Outcomes Study 36-Item Short-Form General Health Survey (ranging from 0 to 100, with higher numbers indicating a better health-related quality of life) and the overall quality-of-life scale of the European Organization for Research and Treatment of Cancer (EORTC) quality-of-life questionnaire (QLQ-C30) were used to assess generic and cancer-specific health-related quality of life, respectively.[21-23] Body image and sexuality were assessed with the EORTC QLQ-BR23.[24] These questionnaires were administered at baseline, at 20 weeks, and at 12 months.

Primary and Secondary End Points

The primary outcome was a reduction in lesion size of more than 25% 4 weeks after the end of treatment (20 weeks after the beginning of treatment). The secondary outcomes were histologic regression from grade 2 or 3 vulvar intraepithelial neoplasia to a lower grade, clearance of HPV, and changes in immune cells in the epidermis and dermis at 20 weeks; relief of clinical symptoms and improvement of quality of life at 20 weeks and at 12 months; and durability of the clinical response at 12 months.

Statistical Analysis

In the placebo group, we expected no reduction in lesion size in 95% of the patients. For the purpose of calculating sample size, we considered treatment with imiquimod adequate if 50% of the patients showed at least a weak partial response. To detect such a

difference with a power of 80% (α=0.05 and β=0.20), a sample size of 36 patients would be needed. Taking into account the possibility of withdrawal by some patients, we chose to include 52 patients.

Analyses were performed according to the intention-to-treat principle. For a comparison of responses between the two groups, Fisher's exact test was used. The Pearson chi-square test was used to compare clinical, histologic, and viral outcomes between the groups. To assess the correlation between post-treatment histologic findings and viral clearance, a test for trend was performed. Repeated-measures analysis of variance was used to test for between-group differences over time in self-reported symptoms, health-related quality of life, body image, and sexuality. Analysis of covariance was used to compare group scores on these outcomes at 20 weeks and at 12 months, with adjustment for baseline scores. All reported P values are two-sided and are not adjusted for multiple testing. No interim analyses of efficacy were performed. 3M Pharmaceuticals was not involved in the study except to provide study medication and to perform randomization.

Results

Study population

Table 1 shows the baseline characteristics of the 52 patients assigned to study groups. The two groups were well balanced. The patients had received a diagnosis of vulvar intraepithelial neoplasia at a mean of 5.4 years before enrollment (range, one month to 20 years). The most recent surgery was performed more than three months before enrollment in all patients. Mapping (which involved more than three biopsies) was performed in 12 patients (six in the imiquimod group and six in the placebo group). To rule out invasion, wide local excision was performed in three patients (all in the placebo group). One patient in the imiquimod group with a positive test result for HPV DNA had coexisting lichen sclerosus. Two patients were using local corticosteroids at enrollment and discontinued corticosteroid use before starting the study.

One patient in the imiquimod group discontinued study medication at 4 weeks because her lesions had spontaneously disappeared after the initial biopsy. One patient in the placebo group stopped at 14 weeks because of a lack of response. Two other patients, one in each group, needed a treatment-free period of more than 1 week for personal reasons. The median number of sachets of cream used was 32 (range, 27 to 33) in the placebo group and 30 (range, 6 to 32) in the imiquimod group. The frequency of imiquimod application was reduced to once a week in five patients because of severe local inflammation. Other side effects were itching or burning immediately after application of imiquimod or on the next day, flulike symptoms, headache, apathy, weariness, and muscular ache (Table 2). Skin reactions noted by the investigator included erythema, erosion, vesiculation, and edema.

*Table 1. Baseline characteristics of the patients**

Characteristic	Imiquimod (n=26)	Placebo (n=26)	P-value†
Age (yr)			0.08
Median	39	44	
Range	22-56	31-71	
Previous surgical treatment (no. of patients)			1.00
Any treatment	18	19	
1 treatment	6	7	
2 or 3 treatments	7	7	
4 or 5 treatments	2	4	
>5 treatments	3	1	
No treatment	8	7	
Symptoms (no. of patients)			0.50
Any symptom	22	19	
Itchiness	2	8	
Pain	3	1	
Itchiness and pain	17	10	
No symptoms	4	7	
Smoking status			1.00
Smoking (no. of patients)	23	23	
1-9 cigarettes/day	3	5	
10-20 cigarettes/day	12	7	
>20 cigarettes/day	8	11	
No smoking (no. of patients)	3	3	
Previous cervical neoplasia (no. of patients)			0.78
Yes	15	17	
No	11	9	
VIN grade (no. of patients)¶			0.60
1	1	0	
2	2	2	
3	23	24	
HPV DNA (no. of patients)			1.00
Positive	25	25	
Negative	1	1	
HPV type (no. of patients)			0.47
16	20	21	
18	0	1	
33	5	3	
Lesion size (cm^2)∫			0.37
Median	4.7	5.4	
Range	(0.0-20.8)	(0.7-45.0)	

*VIN denotes vulvair intraepithelial neoplasia; HPV denotes human papillomavirus

†P values were calculated by Fisher's exact test, except for age and lesion size, for which the Mann-Whitney U test was used, and VIN grade and HPV type, for which the Pearson chi-square test was used.

¶The grades were reviewed by two independent gynecologic pathologists.

∫VIN lesions spontaneously disappeared after the initial biopsy in one patient in the imiquimod group. No lesions could be measured at the time of initiation of treatment.

Hematologic and serum biochemical test results remained within the normal range in both study groups.

Table 2. Side-effects

Side effect		Imiquimod (n=26)	Placebo (n=26)	P-value[§]
Reported by the patient[¶]				
Vulvar pain or pruritus		24	7	<0.001
Headache		7	5	0.52
Apathy		5	0	0.03
Weariness		8	4	0.20
Muscular ache		3	1	0.35
Flulike symptoms		5	3	0.47
Other side effects[‡]		4	4	1.00
No side effects		1	13	<0.001
Reported by the investigator				
Erythema	mild/ moderate	14	2	<0.001
	severe	6	0	0.02
Erosion	mild/ moderate	17	5	0.001
	severe	0	0	1.00
Vesiculation		4	0	0.06
Edema		11	0	<0.001
Ulceration		0	0	1.00

[§] P values were calculated by the Pearson chi-square test;

[¶]In the imiquimod group, one patient reported no side effects during treatment, nine patients reported one side effect, five patient s reported two side effects, eight patients reported three side effects, and three patients reported four side-effects. In the placebo group, 13 patients reported no side effects, seven patients reported one side effect, two patients reported two side effects, three patients reported three side effects, and one patient reported four side effects. The number of patients reporting no side effects differed significantly between the two groups (P<0.001).

[‡]The other side effects were loss of hair, excessive vulvar perspiration, loss of blood from treated skin, watery eyes, reactive lymph nodes.

Clinical outcome

Figure 1 summarizes the change in total lesion size after treatment with imiquimod or placebo at 20 weeks and at 12 months. At 20 weeks, lesion size was reduced by more than 25% in 21 of 26 patients treated with imiquimod (81%) and in no patients in the placebo group (P<0.001). The lesions had completely disappeared in nine imiquimod-treated patients and were reduced by more than 75% in five (Table 3, and Fig. 1 of the Supplementary Appendix). There were no significant differences between the findings of the two investigators who evaluated the clinical response.

Histological, viral and immunological outcome

Pretreatment biopsies showed that 47 patients had grade 3 vulvar intraepithelial neoplasia, four had grade 2, and one had grade 1. Table 4 summarizes the histologic results after treatment. There was disagreement between the observers in the grading of 19 of 104 biopsies (18%), mainly concerning grades 2 and 3 (13 of 19), and consensus was reached in all 19 cases. Histologic regression from grade 2 or 3 to a lower grade was seen in 18 patients treated with imiquimod (69%) and in one patient treated with placebo (4%)

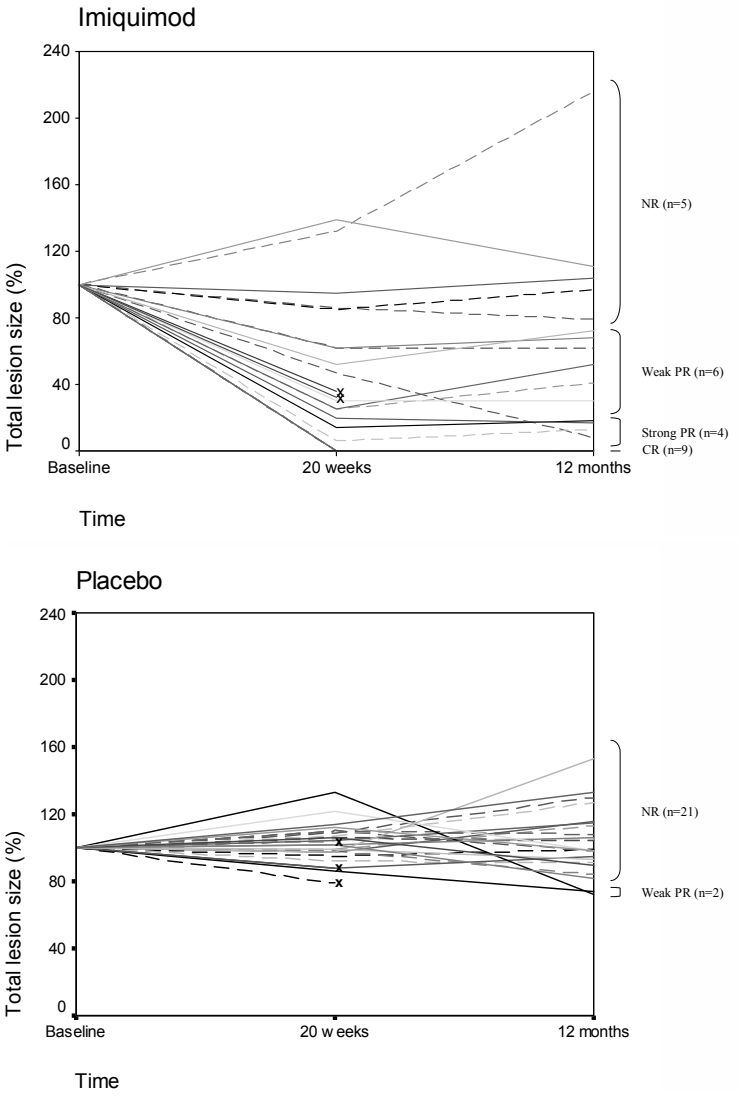

Figure 1. Effects of imiquimod and placebo on total lesion size at 20 weeks and at 12 months. Total lesion size as a percentage of baseline is shown at 20 weeks and at 12 months after the beginning of treatment with imiquimod (Panel A) or placebo (Panel B). The solid red line in Panel A represents the nine patients who had a complete response; one patient had no measurable disease but was treated anyway. Data were missing for five patients at 12 months. See also Color Figures, page 148.

(P<0.001). Eight patients no longer had vulvar intraepithelial neoplasia; before treatment, six of these patients had grade 3 vulvar intraepithelial neoplasia, one had grade 2, and one had grade 1. One patient with grade 2 vulvar intraepithelial neoplasia at baseline had grade 3 after treatment with imiquimod.

*Table 3. Clinical response at 20 weeks and at 12 months after the beginning of treatment**

Response	20 weeks†		12 months	
	Imiquimod (n=26)	Placebo (n=26)	Imiquimod (n=26)	Placebo (n=26)
None	5	26‡	5	21
Weak partial	7§	0	6	2
Strong partial	5	0	4	0
Complete	9¶	0	9	0
No data	0	0	2	3

*Clinical response was classified as no response (reduction in lesion size of 25% or less), weak partial response (26 to 75% reduction), strong partial response (76 to 99% reduction), or complete response (100% reduction).

†The difference between the treatment groups at 20 weeks was significant (P<0.001 by Pearson chi-square test for trend).

‡Progression to invasive disease occurred in two of these patients during the study. One of the patients underwent surgery before the end of the study, so the total lesion size was not measured at 12 months. A third patient did not have a response to the study medication and withdrew from the study at 20 weeks. A fourth patient, who had severe pain, withdrew from the study at 8 months to undergo surgery.

§Progression to invasive disease occurred in one of these patients during the study. She underwent surgery before the end of the study, so the total lesion size was not measured at 12 months. Another patient was lost to follow-up at 20 weeks because of unrelated medical problems.

¶In one patient, vulvar intraepithelial neoplasia lesions spontaneously disappeared after the initial biopsy. Another patient, with an original diagnosis of grade 2 vulvar intraepithelial neoplasia, received a diagnosis of grade 1 disease after independent review.

At baseline, 25 patients in each group had lesions positive for HPV DNA (Table 1). At 20 weeks, HPV was cleared in 17 lesions: 15 after treatment with imiquimod and 2 after treatment with placebo (P<0.001) (Table 4). Fifteen lesions had HPV type 16, and two lesions (one treated with imiquimod and one treated with placebo) had HPV type 33. There was no significant association between HPV type and viral outcome. There was a strong association between viral clearance and histologic regression (P<0.001). Of 14 lesions that regressed to grade 1 vulvar intraepithelial neoplasia or to no neoplasia, 13 were cleared of HPV after treatment with imiquimod and the other lesion was HPV-negative at baseline.

Treatment with imiquimod increased the numbers of $CD1a^+$ dendritic cells, CD8+ T cells, and $CD94^+$ natural killer cells in the epidermis 4 weeks after the end of therapy. The increase was significant only for patients whose lesions regressed by more than 75%. In the dermis of imiquimod-treated patients whose lesions regressed by more than 75%, the numbers of $CD207^+$ dendritic cells, $CD208^+$ dendritic cells, and regulatory T cells were reduced; the reduction was significant for $CD207^+$ dendritic cells and regulatory T cells (see the Supplementary Appendix for details).

Self-reported symptoms and quality of life

As compared with placebo, treatment with imiquimod reduced pruritus and pain at 20 weeks (P=0.008 and P=0.004, respectively) and 12 months (P=0.04 and 0.02, respectively),

*Table 4. Histologic and virologic results 20 weeks after beginning of treatment with imiquimod or placebo**

VIN grade†	Imiquimod (n=26)		Placebo (n=26)	
	no. of patients	*no. HPV-negative*	*no. of patients*	*no. HPV-negative*
No disease	8‡	8	0	0
Grade 1	7	6§	1	1
Grade 2	3	2	0	0
Grade 3	8¶	0	24∫	2§
Invasive disease (<1mm)	0		1	0

*HPV denotes human papillomavirus;

†The grades were reviewed by two independent gynecologic pathologists;

‡Two patients received a diagnosis of grade 2 vulvar intraepithelial neoplasia at baseline; the diagnosis was changed to grade 1 in one of these patients after revision;

§One patient was already HPV-negative at baseline;

¶One patient received a diagnosis of grade 2 vulvar intraepithelial neoplasia at baseline;

∫Two patients received a diagnosis of grade 2 vulvar intraepithelial neoplasia at baseline.

according to analysis of covariance with adjustment for baseline scores. In a repeated-measures analysis, no significant differences at baseline, at 20 weeks, or at 12 months were observed between the imiquimod group and the placebo group in self-reported health-related quality of life, body image, or sexuality.

Follow-up

All but three patients were followed for 12 months (Table 3). One patient did not have a response to the study medication and withdrew at 20 weeks. Another patient was lost to follow-up at 20 weeks because of an unrelated medical problem. A third patient with grade 3 vulvar intraepithelial neoplasia stopped treatment at 8 months when she underwent surgery (skinning vulvectomy) for severe pain. Complete follow-up information was available for 49 patients. All patients with a complete response after treatment with imiquimod at 20 weeks remained free of disease at 12 months. Two of 12 patients (17%) with a partial response after imiquimod treatment had enlargement of their lesions.

In one patient treated with imiquimod, newly developed vulvar intraepithelial neoplasia progressed to invasion at 7 months. A radical local excision confirmed the presence of invasion to a depth of less than 1 mm. Invasion also occurred in two other patients treated with placebo. Invasion to a depth of less than 1 mm developed at 12 months in one patient with preexisting vulvar intraepithelial neoplasia. A subsequent radical local excision showed no further invasion. In the other patient, progression of newly developed vulvar intraepithelial neoplasia was found at 20 weeks. A radical local excision confirmed the presence of invasion to a depth of less than 1 mm.

Discussion

This trial demonstrates the effectiveness of imiquimod in the treatment of vulvar intraepithelial neoplasia during an observation period of one year. Complete response was achieved in nine (35%) and partial response in 12 (46%) of 26 patients treated with the cream. Regression from grade 2 or 3 vulvar intraepithelial neoplasia to a lower grade was seen in 18 of 26 lesions (69%), 15 of which tested negative for HPV DNA after treatment. None of the nine patients with a complete response showed any evidence of vulvar intraepithelial neoplasia at 12 months. Four of the nine patients who had a complete response had undergone surgery two or three times before receiving imiquimod. Three lesions (6%) progressed to invasion to a depth of less than 1 mm, two after treatment with placebo and one after treatment with imiquimod. These results are similar to those of other studies showing progression of vulvar intraepithelial neoplasia in 9% of untreated patients and 3% of surgically treated patients.[4,5] In our study, two of three patients with progression to invasive disease (one treated with imiquimod and two with placebo) had newly developed vulvar intraepithelial neoplasia.

The strength of our study lies in the randomized, placebo-controlled comparison. To avoid any bias caused by side effects of study medication, two independent observers using photographs obtained before and after treatment evaluated the reduction in lesion size. No significant differences between the results of the two observers were found. Histologic evidence of regression was evaluated independently by two pathologists, and consensus was reached when they disagreed as to whether lesions should be classified as grade 2 or grade 3. Difficulty in grading vulvar intraepithelial neoplasia[25,26] led to a new classification in which grades 2 and 3 are combined as vulvar intraepithelial neoplasia (usual type or differentiated type).[27] Grade 1 vulvar intraepithelial neoplasia as an entity was abandoned, since the minimal changes associated with this grade are usually a result of transient HPV infection. According to the new classification, all patients would have been classified as having vulvar intraepithelial neoplasia (usual type), and 15 patients (58%) instead of 8 (31%) would have shown complete histologic regression after treatment with imiquimod.

It is not known why some patients had a response to imiquimod and others did not. Imiquimod binds to toll-like receptor 7, a cell-surface receptor on the immature plasmacytoid dendritic cell. Binding initiates an intracellular signaling cascade that results in innate and cell-mediated immune responses. Imiquimod promotes maturation of antigen-presenting cells and secretion of proinflammatory cytokines and initiates a shift to type 1 T-cell–mediated immunity.[28] Imiquimod also has direct proapoptotic activity against tumor cells.[29] We found that a preexisting type 1 T-cell response specific to HPV type 16 is associated with an improved outcome after imiquimod treatment.[30] Perhaps induction of this specific T-cell response before imiquimod treatment would be useful.

HPV infection suppresses chemokine expression, resulting in the inhibition of infiltration and activation of T cells and natural killer cells. Moreover, the number of Langerhans' cells is significantly reduced at HPV-infected sites.[31] The increase in the number of immune cells in the epidermis of patients with a clinical response of more than 75% after imiquimod treatment may reflect reactivation of the resident epidermal cells. The decrease in the number of immune cells in the dermis of these patients may reflect a return to normal conditions after a successful immune response against HPV.

In conclusion, imiquimod 5% cream is a promising agent for the treatment of vulvar intraepithelial neoplasia. Regression of lesions is strongly associated with clearance of HPV. As a convenient, self-administered treatment, imiquimod is well tolerated, is less invasive than surgery, relieves itching and pain, and does not influence health-related quality of life, body image, or sexuality. Therefore, we consider imiquimod the first-choice treatment for vulvar intraepithelial neoplasia.

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No potential conflict of interest relevant to this article was reported.

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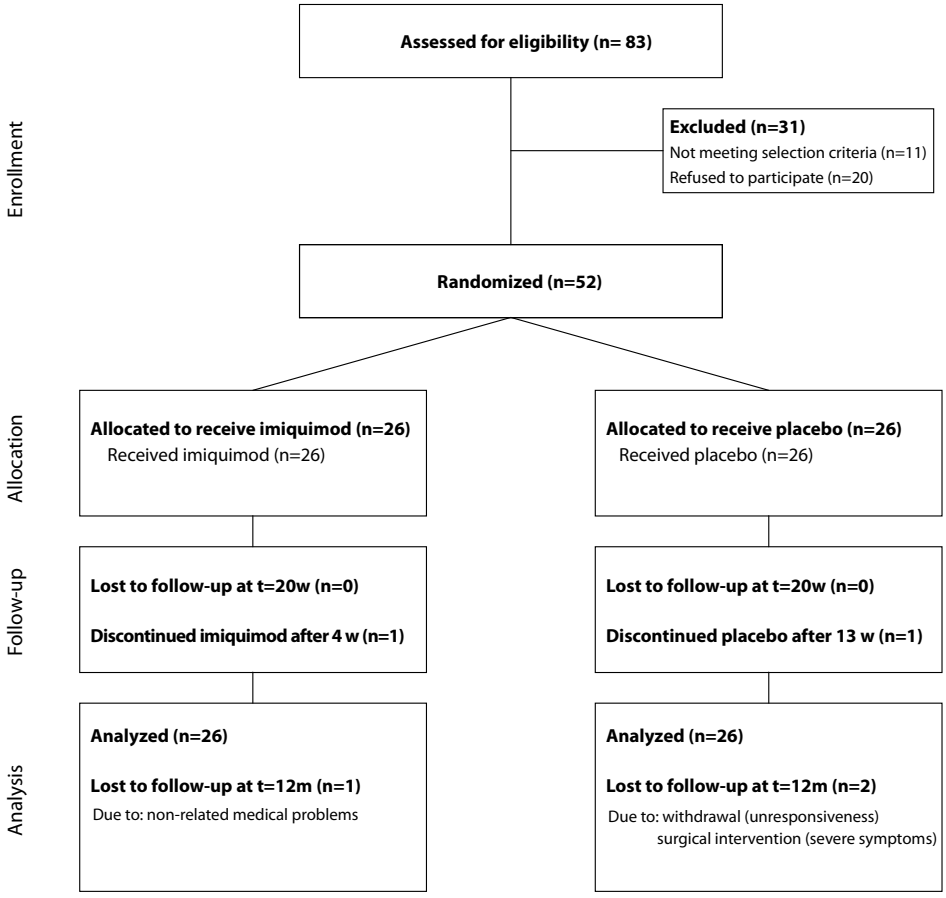
Supplementary Appendix

Supplement to: van Seters M, van Beurden M, ten Kate FJW, et al. Treatment of vulvar intraepithelial neoplasia with topical imiquimod. N Engl J Med 2008;358:1465-73.

Appendix I

CONSORT Flow Diagram



Appendix II

(Analysis of immunocompetent cells in epidermis and dermis of vulvar skin before and after treatment with imiquimod or placebo)

Materials and Methods

Immunohistochemical staining

Pre-staining, frozen tissue specimen were cut into serial 6 µm thick sections on a Micronic, Adamas cryostat, transferred to poly-L-lysine-coated microscope slides (Menzel-Glaser, Omnilabo, Breda, the Netherlands), dried and restored at -80°C. The following markers and their primary antibodies were selected for immunohistochemical staining: CD1a, classic marker for Langerhans cells (Okt-6 Orthoclone, Orthobiotech, Bridgewater, NJ, U.S.A.); CD207, marker for immature dendritic cells (DCs) expressing Langerin (DCGM4 Beckman Coulter, Mijdrecht, The Netherlands); CD208, DC-lamp marker for mature DCs (104.G4 Beckman Coulter); CD94, marker for natural killer cells (NKs) (HP.3b1 Beckman Coulter); CD4 marker for T-helper cells (MT.310 Dako, Glostrup, Denmark); CD8, marker for cytotoxic T-cells (DK25 Dako); and CD25/HLA-DR, marker for regulatory T-cells (Treg cells) (AKT-1 Dako/ 1E5 Sanquin). For plasmacytoid DCs (pDCs), characterized by the presence of CD123 and absence of CD11c, antibodies for both markers were used (anti CD123 (9F5 Becton Dickinson) and anti CD11c (SHCL-3; Becton Dickinson)). Antibodies in staining procedures were applied in optimal concentrations varying from 0.1µg/ml to 1.0 µg/ml.

Single-staining (CD4, CD8, CD1a, CD207, CD208 and CD94)

Sections were defrozen, fixed in acetone for 10 minutes, and rinsed with phosphate buffer saline (PBS, pH 7.8) for 5 minutes. The staining procedure was then continued in a half automatic stainer (Sequenza, Shandon Scientific, Zeist, the Netherlands), where the slides were incubated with 10% normal goat serum (NGS) (Sanquin, Amsterdam, The Netherlands) for 10 minutes, and subsequently for 60 minutes with mouse anti-human antibodies against CD4, CD8, CD1a, CD207, CD208, and CD94, respectively. All antibodies had been diluted in 1% block buffer (Blocking Reagent in PBS, Roche Diagnostics GmbH, Mannheim, Germany). During the whole staining process, incubation steps were always followed by rinsing with PBS for 5 minutes. After incubation with primary antibodies, sections were rinsed and incubated with biotinylated goat anti-mouse antibodies (BioGenex HK325-UM, Klinipath, Duiven, the Netherlands) and 10% normal human serum (NHS) (Sanquin) for 30 minutes. This was followed by incubation with alkaline phosphatase conjugated

streptavidin (BioGenex HK321-UK, Klinipath, Duiven, The Netherlands) and 10% NHS for another 30 minutes. Slides were rinsed with both PBS and substrate TRIS buffer (TRIS HCl 0.1 mol/L, pH 8.5), and then incubated for 30 minutes with a new fuchsine substrate (Chroma, Kongen, Germany). Finally, the sections were washed again, counterstained with Gill's haematoxilin (Merck, Amsterdam, The Netherlands) for 30 seconds, rinsed with tap water, dried, and embedded in VectaMount (Vector, Burlingame, CA). Control staining was performed according to the same procedure, using isotype controls.

Double-staining CD25/HLA-DR and CD123/CD11c

After fixation in acetone and washing with PBS, endogenous peroxidase was blocked with 0.1% sodium azide and 0.03% hydrogen peroxide in PBS for 30 minutes. Sections were rinsed and incubated with 10% NGS and 10% normal rabbit serum (Sanquin), followed by incubation with mouse anti-human antibodies against CD25 for 60 minutes at room temperature. The sections were then rinsed, incubated with biotinylated goat anti-mouse antibodies and 10% NHS for 30 minutes, rinsed, incubated with alkaline phosphatase conjugated streptavidine and 10% NHS for 30 minutes, and rinsed again. Thereafter, the slides were incubated with 10% normal mouse serum (Sanquin) for 10 minutes, followed by FITC conjugated mouse anti-human antibodies against HLA-DR for 60 minutes. Rinsed again, incubation with HRP conjugated rabbit anti-FITC antibodies followed. After rinsing with PBS and substrate TRIS buffer, slides were incubated for 30 minutes in fast blue substrate (Sigma, St.Louis, Mo, U.S.A.). Finally, sections were rinsed and incubated with peroxidase nova red substrate (Vector, Burlingame, CA, U.S.A.) for 10 minutes, rinsed with PBS and embedded in VectaMount.

In a similar procedure as described above for double-staining CD25/HLA-DR, sections were incubated with 10% NGS. Primary antibodies were substituted with mouse anti-human CD11c antibodies and with phycoerythrin-labeled mouse anti-human CD123 antibodies. Secondary antibodies were biotinylated goat anti-mouse antibodies with alkaline phosphatase conjugated streptavidine for CD11c and rabbit anti-phycoerythrin (AbD Serotec, Duesseldorf, Germany) and alkaline phosphatase conjugated goat anti-rabbit antibodies (Sigma) for CD123. Staining was performed with fast blue substrate for CD123 and with amino-ethylcarbazol substrate (Sigma) for CD11c.

Light microscopic evaluation

Light microscopic evaluation was performed in a blinded session. Stained cells were counted throughout the entire epidermal surface and 100 µm deep into the dermis of each biopsy specimen following at least 2 mm of basal membrane length (range 2-5 mm). After estimating the total area of both the epidermis and dermis by using the Leica Image Analysis System, the number of cells per square millimeter was calculated for each layer separately.

Statistical analysis

Statistical analysis was performed with the SPSS 15.0 software for Windows.

Preliminary Kolmogorov-Smirnov tests showed a non-normal distribution for some cell types. Accordingly, differences in cell counts before and after treatment with study medication were evaluated with the non-parametric Wilcoxon test for paired samples. A two-tailed P-value of 0.05 was chosen to represent statistical significance.

a. Changes in immunocompetent cells in epidermis of VIN-lesions after treatment with imiquimod

Cells[†]	Imiquimod									Placebo		
	all pts (n=25)[‡]			patients (n=13) with clinical response > 75%			patients (n=12) with clinical response ≤ 75%			all patients (n=26)		
	before	after	P-value[§]	before	after	P-value	before	after	P-value	before	after	P-value
$CD1a^+$	177[¶] (65-478)	255 (103-465)	0.009	211 (65-418)	293 (174-465)	0.02	144 (82-478)	223 (103-371)	0.16	153 (23-347)	173 (71-453)	0.24
$CD207^+$	135 (2-407)	181 (17-300)	0.20	187 (28-407)	213 (86-300)	0.28	104 (2-251)	107 (17-298)	0.53	96 (2-236)	102 (11-283)	0.29
$CD208^+$	27 (2-148)	31 (3-70)	0.43	17 (2-63)	19 (3-44)	0.86	28 (10-148)	42 (20-70)	0.31	20 (2-47)	25 (4-112)	0.06
$CD123^+/11c^-$	14 (0-59)	17 (0-147)	0.78	10 (0-59)	21 (0-116)	0.34	20 (4-58)	13 (0-147)	0.24	14 (0-78)	19 (0-59)	0.89
$CD8^+$	86 (9-357)	119 (28-385)	0.12	70 (9-305)	157 (79-256)	0.02	122 (17-357)	94 (28-385)	0.81	83 (2-214)	84 (16-416)	0.21
$CD4^+$	300 (38-925)	312 (123-923)	0.74	300 (38-925)	336 (154-923)	0.35	303 (39-786)	272 (123-632)	0.70	242 (31-656)	243 (18-987)	0.60
CD25/HLA-DR^+	0 (0-7)	0 (0-9)	0.05	0 (0-7)	0 (0-7)	0.24	0 (0-5)	0 (0-9)	0.14	0 (0-6)	0 (0-10)	0.47
$CD94^+$	23 (0-153)	36 (6-174)	0.13	23 (0-140)	36 (6-174)	0.03	30 (4-153)	36 (13-58)	0.75	21 (0-261)	22 (0-303)	0.41

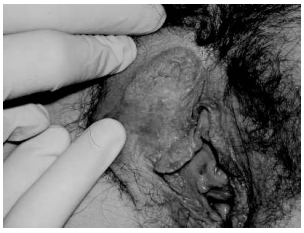
Appendix III

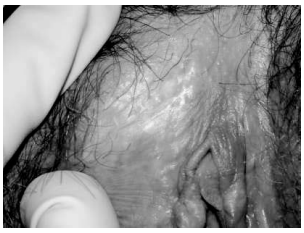
b. Changes in immunocompetent cells in dermis of VIN-lesions after treatment with imiquimod

Cells[†]	Imiquimod									Placebo		
	all pts (n=25)[‡]			patients (n=13) with clinical response > 75%			patients (n=12) with clinical response ≤ 75%			all patients (n=26)		
	before	after	P-value[§]	before	after	P-value	before	after	P-value	before	after	P-value
CD1a$^+$	145[¶] (33-498)	165 (32-593)	0.95	142 (33-498)	165 (32-593)	0.60	176 (47-374)	151 (38-310)	0.51	139 (17-461)	141 (75-324)	0.72
CD207$^+$	52 (3-278)	37 (7-180)	0.05	61 (12-278)	52 (11-102)	0.02	52 (3-142)	26 (7-180)	0.70	57 (0-188)	57 (9-145)	0.50
CD208$^+$	155 (15-539)	136 (32-424)	0.19	124 (15-539)	98 (32-193)	0.06	170 (48-537)	160 (53-424)	0.88	115 (3-534)	172 (13-389)	0.17
CD123$^+$/11c$^-$	234 (56-1140)	212 (56-770)	0.88	250 (96-525)	159 (56-770)	0.86	213 (56-1140)	226 (82-547)	0.94	242 (93-733)	244 (48-555)	0.68
CD8$^+$	336 (99-1580)	367 (94-1074)	0.51	327 (99-1580)	367 (94-728)	0.70	417 (206-817)	347 (95-1074)	0.64	378 (45-2430)	393 (50-1307)	0.32
CD4$^+$	1264 (236-2909)	1079 (428-3011)	0.10	1264 (236-2617)	887 (489-1924)	0.22	1291 (631-2909)	1151 (428-3011)	0.35	1155 (242-2807)	1226 (420-3246)	0.71
CD25/HLA-DR$^+$	54 (0-231)	34 (5-183)	0.09	52 (0-231)	29 (14-46)	0.02	57 (0-140)	47 (5-183)	1.00	46 (0-209)	62 (0-271)	0.55
CD94$^+$	65 (3-438)	55 (17-283)	0.76	42 (3-438)	36 (17-248)	0.75	68 (30-141)	83 (19-283)	0.94	48 (0-225)	53 (3-502)	0.26

[†]CD1a$^+$: Langerhans cells; CD207$^+$: immature dendritic cells (DCs); CD208$^+$: mature DCs; CD123$^+$11c$^-$: plasmacytoid DCs; CD4$^+$: T-helper cells; CD8$^+$: cytotoxic T-cells; CD25/HLA-DR$^+$: T-regulatory cells; CD94$^+$: natural killer cells; [‡]One patient with a pre-treatment diagnosis of VIN 1 (after revision) was excluded from analysis; [¶]Values are presented as medians (range); [§]Statistical significance was analyzed with the non-parametric Wilcoxon test for paired samples.

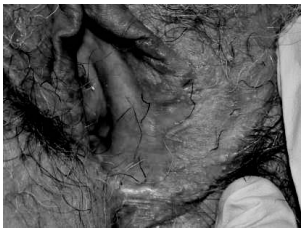
Supplementary figure

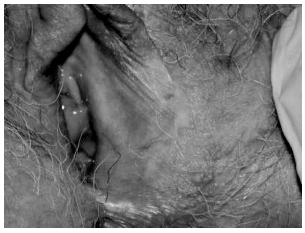




Patient 1

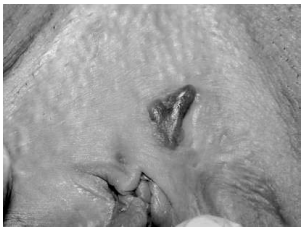
A B

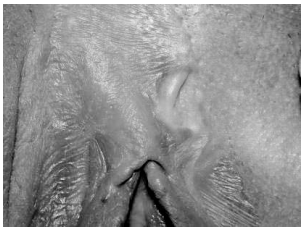




Patient 2

C D





Patient 3

E F

Supplementary figure 1. Clinical results before and after treatment with imiquimod.
Clinical pictures of three patients with HPV DNA-positive VIN 3 showing the results after treatment with imiquimod. Picture A, C, E: before treatment; B, D, F: after treatment. All three patients showed complete regression of the shown lesion, histological regression to VIN 1 (patient 1 and 2) or no dysplasia (patient 3), and clearance of HPV. See also Color Figures, page 149.

6 Disturbed patterns of immunocompetent cells in usual type vulvar intraepithelial neoplasia

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Freek J Zijlstra
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Cancer Res 2008;68 (in press)

Abstract

Genital infection with human papillomavirus (HPV) is usually transient, as the immune system is capable of eliminating the virus. When immunity 'fails' and the infection persists, vulvar intraepithelial neoplasia (VIN) may develop. In this study, we examined the distribution of inflammatory cells in 51 patients with HPV-associated usual type VIN and in 19 healthy controls. Frozen vulvar tissue samples were tested for the presence of HPV DNA, and immunohistochemical staining for the markers CD1a, CD207, CD208, CD123/CD11c, CD94, CD4, CD8 and CD25/HLA-DR was performed. Cells were counted in both the epidermis and dermis over at least 2 mm of basal membrane length. In the epidermis of VIN patients, $CD1a^+$ and $CD207^+$ (Langerin) dendritic cells (DCs), and $CD8^+$ T-cells were significantly lower than in controls, whereas the number of $CD123^+/CD11c^-$ plasmacytoid (p)DCs was significantly increased. No significant changes were observed for $CD208^+$ DCs, $CD94^+$ natural killer (NK) cells, $CD4^+$ T-cells and $CD25^+/HLA\text{-}DR^+$ T-regulatory (Treg) cells. In the dermis of VIN patients, elevated numbers of $CD208^+$, $CD123^+/CD11c^-$, $CD94^+$, $CD4^+$, $CD8^+$ and $CD25^+/HLA\text{-}DR^+$ cells were observed when compared to healthy controls. The numbers of $CD1a^+$ and $CD207^+$ DCs were not different between groups. In summary, hrHPV-related usual type VIN lesions are characterized by an immunosuppressive state in the epidermis, showing a reduction of immature myeloid (m)DCs and $CD8^+$ T-cells. In the dermis, inflammatory activation is reflected by the influx of mature mDCs and pDCs, NK-cells and T-cells, suggesting that the cellular immune response on viral HPV infection occurs in the dermis of VIN patients.

Introduction

Genital infection with human papillomavirus (HPV) is very common, especially among sexually active young adults. The lifetime risk of becoming infected with HPV is estimated at 80-85%.[1] Most infections proceed asymptomatically, and cure spontaneously as the immune system is capable of eliminating the virus.[2] Persistence of HPV infection, on the other hand, can result in neoplastic changes of the anogenital tract, presented in this study as vulvar intraepithelial neoplasia (VIN).

There is evidence that cell-mediated immune responses of the host are important determinants in the course of infection, illustrated by an increased incidence of HPV-induced diseases in T-cell immuno-deficient individuals.[3] The immune response to invading HPV is regulated by cells of both the innate and adaptive immune systems. During the innate immune response, viral antigens are recognized, bound and processed by antigen-presenting cells (APCs) with dendritic cells (DCs) as important representatives. DCs can be divided into immature or mature myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). mDCs play a major role in the regulation of anti-bacterial and anti-fungal responses, whereas pDCs are receptive for viral interactions. pDCs produce in response large amounts of IFN-α that has direct anti-viral effects and stimulates cytotoxicity of macrophages and natural killer (NK) cells. Influenced by NK-cells, DCs maturate and transport "processed" antigens to secondary lymphoid organs, where naïve T-cells are primed to mature into $CD4^+$ T-helper cells, $CD8^+$ cytotoxic T-cells, or regulatory T-cells (Treg cells), all functional members of the adaptive immune response.[4,5]

Studies describing the distribution of immunocompetent cells in VIN lesions are scarce.[6-11] Limitations of these studies include absence of or inappropriate control groups, an undefined HPV status of samples, or incompletely described qualitative or semi-quantitative cell counting. Thus far, only a few cell markers have been analyzed. Detailed insight into the influence of VIN on the distribution of immunocompetent cells in vulvar skin might be helpful to explain clinical and immunological changes observed after treatment of VIN with topical imiquimod.[12]

To examine the role of the innate and cellular local immune responses in patients with hrHPV-associated VIN, we investigated in this study the presence of selected DCs, NK-cells and T-cells by immunohistochemical staining in the vulvar skin of 51 patients with usual type VIN, and compared results with data obtained from 19 healthy controls. Our results suggest a disturbed distribution pattern of immunocompetent cells in VIN-affected skin.

Materials and Methods

Study-population

Fifty-one immunocompetent women (median age, 43 yrs; range, 22-71 yrs) with clinically and histologically proven multifocal usual type VIN participated in this study. On the average, patients were diagnosed as having VIN 5.3 yrs before enrolment in our study (range 1 month - 20 yrs). In all cases the histological diagnosis was confirmed at study entrance. Thirty-seven of these 51 patients had undergone previous treatment at least three months before enrolment (surgical excision, laser therapy, local chemotherapy), whereas 14 had not been treated before. Nineteen healthy women undergoing elective vulvar cosmetic surgery (reduction of labia minora) served as healthy controls (median age, 40 yrs; range, 19-56 yrs). Histological examination of the removed vulvar tissue revealed no abnormalities. The Medical Ethical Committees approved our study design and all women voluntarily gave written informed consent.

Clinical material

4 mm punch biopsies taken from patients with usual type VIN and from excised vulvar tissue in healthy controls were immediately frozen in liquid nitrogen and stored at -80°C until further analysis. Anatomically, biopsies were taken from the same tissue as used for histological diagnosis.

HPV-DNA testing

Frozen tissue samples were analyzed for the presence of HPV DNA by using a standard GP5+/6+ PCR enzyme immunoassay followed by reverse line blot analysis, as described previously .[13]

Immunohistochemical staining

Frozen tissue specimens were cut into serial 6 µm thick sections on a Micronic, Adamas cryostat, transferred to poly-L-lysine-coated microscope slides (Menzel-Glaser, Omnilabo, Breda, the Netherlands), dried and restored at -80°C. The following markers and their primary antibodies were selected for immunohistochemical staining: CD1a, classic marker for immature mDCs, in the skin known as Langerhans cells (Okt-6 Orthoclone, Orthobiotech, Bridgewater, NJ, U.S.A.); CD207, marker for immature mDCs expressing Langerin (DCGM4 Beckman Coulter, Mijdrecht, The Netherlands); CD208, DC-lamp, marker for mature mDCs (104.G4 Beckman Coulter); CD94, marker for NK-cells (HP.3b1 Beckman Coulter); CD4, marker for T-helper cells (MT.310 Dako, Glostrup, Denmark); CD8, marker for cytotoxic T-cells (DK25 Dako); CD25/HLA-DR, marker for Treg cells (AKT-1 Dako/ 1E5 Sanquin) and Foxp3, marker for Treg cells (PHC 101 Bioscience, Halle, Belgium). For pDCs, characterized by the presence of CD123 and absence of CD11c, antibodies for both markers were used

(anti CD123 (9F5 Becton Dickinson) and anti CD11c (SHCL-3; Becton Dickinson)). Antibodies in staining procedures were applied in optimal concentrations varying from 0.1 µg/ml to 1.0 µg/ml.

Single-staining (CD1a, CD207, CD208, CD94, CD4, CD8 and Foxp3)

Sections were defrozen, fixed in acetone for 10 minutes, and rinsed with phosphate buffer saline (PBS, pH 7.8) for 5 minutes. The staining procedure was then continued in a half automatic stainer (Sequenza, Shandon Scientific, Zeist, the Netherlands), where the slides were incubated with 10% normal goat serum (NGS) (Sanquin, Amsterdam, The Netherlands) for 10 minutes, and subsequently for 60 minutes with mouse anti-human antibodies against CD1a, CD207, CD208, CD94, CD4, CD8, respectively, and rat anti-human antibody against Foxp3. All antibodies had been diluted in 1% block buffer (Blocking Reagent in PBS, Roche Diagnostics GmbH, Mannheim, Germany). During the whole staining process, incubation steps were always followed by rinsing with PBS for 5 minutes. All following incubations with antibodies were in the presence of 10% normal human serum (NHS) (Sanquin). After incubation with primary antibodies, sections were rinsed and, with the exception of Foxp3, incubated with biotinylated goat anti-mouse antibodies as secondary antibodies (BioGenex HK325-UM, Klinipath, Duiven, the Netherlands) for 30 minutes, followed by incubation with alkaline phosphatase conjugated streptavidin (BioGenex HK321-UK, Klinipath, Duiven, The Netherlands) for another 30 minutes. In case of Foxp3, the secondary antibody was alkaline phosphatase conjugated goat anti-rat antibody; this incubation was followed by incubation with rat APAAP (Alkaline phosphatase anti-alkaline phosphatase (Dako)). Slides were rinsed with both PBS and substrate TRIS buffer (TRIS HCl 0.1 mol/L, pH 8.5), and then incubated for 30 minutes with a new fuchsine substrate (Chroma, Kongen, Germany). Finally, the sections were washed again, counterstained with Gill's haematoxilin (Merck, Amsterdam, The Netherlands) for 30 seconds, rinsed with tap water, dried, and embedded in VectaMount (Vector, Burlingame, CA). Control staining was performed according to the same procedure, using isotype controls.

Double-staining CD25/HLA-DR and CD123/CD11c

After fixation in acetone and washing with PBS, endogenous peroxidase was blocked with 0.1% sodium azide and 0.03% hydrogen peroxide in PBS for 30 minutes. Sections were rinsed and incubated with 10% NGS and 10% normal rabbit serum (Sanquin), followed by incubation with mouse anti-human antibodies against CD25 for 60 minutes at room temperature. The sections were then rinsed, incubated with biotinylated goat anti-mouse antibodies and 10% NHS for 30 minutes, rinsed, incubated with alkaline phosphatase conjugated streptavidine and 10% NHS for 30 minutes, and rinsed again. Thereafter, the slides were incubated with 10% normal mouse serum (Sanquin) for 10 minutes, followed by FITC conjugated mouse anti-human antibodies against HLA-DR for 60 minutes. Rinsed again,

incubation with HRP conjugated rabbit anti-FITC antibodies followed. After rinsing with PBS and substrate TRIS buffer, slides were incubated for 30 minutes in fast blue substrate (Sigma, St.Louis, Mo, U.S.A.). Finally, sections were rinsed and incubated with peroxidase nova red substrate (Vector, Burlingame, CA, U.S.A.) for 10 minutes, rinsed with PBS and embedded in VectaMount.

In a similar procedure as described above for double-staining CD25/HLA-DR, sections were incubated with 10% NGS. Primary antibodies were substituted with mouse anti-human CD11c antibodies and with phycoerythrin-labeled mouse anti-human CD123 antibodies. Secondary antibodies were biotinylated goat anti-mouse antibodies with alkaline phosphatase conjugated streptavidine for CD11c and rabbit anti-phycoerythrin (AbD Serotec, Duesseldorf, Germany) and alkaline phosphatase conjugated goat anti-rabbit antibodies (Sigma) for CD123. Staining was performed with fast blue substrate for CD123 and with amino-ethylcarbazol substrate (Sigma) for CD11c.

Light microscopic evaluation

Light microscopic evaluation was performed in a blinded session. Stained cells were counted throughout the entire epidermal thickness and 100 µm deep into the dermis of each biopsy specimen following at least 2 mm of basal membrane length (range 2-5 mm). After measuring the total area of both the epidermis and dermis by using the Leica Image Analysis System, the number of cells per square millimeter was calculated for each layer separately.

Statistical analysis

Statistical analysis was performed with the SPSS 15.0 software for Windows. Preliminary, Kolmogorov-Smirnov tests showed a non-normal distribution for some cell types. Accordingly, the non-parametric Mann-Whitney test was used for evaluation of differences in cell counts between two independent groups (VIN patients versus healthy controls). The possible influence of different previous treatments on cell counts in VIN patients was investigated by means of the non-parametric Kruskal-Wallis test. Spearman's correlations were used to investigate possible relations between cell counts and duration of the disease or age of the patients. A two-tailed P-value of 0.05 was chosen to represent statistical significance.

Results

Patients

Spearman's correlations between cell counts for all investigated cell types and the duration of VIN or age of the patients at study entrance were not significant (data not shown). There was also no statistically significant difference in cell counts between previously untreated

patients (n=14), patients treated with surgical excision (n=18) or patients undergoing laser treatment (n=12). Groups for other treatment modalities (n=7) were too small for statistical evaluation.

HPV-DNA testing

Forty-nine of 51 patients tested positive for hrHPV DNA. HPV types detected were HPV-16 (n=40), HPV-33 (n=8) and HVP-18 (n=1). All healthy controls were HPV DNA-negative.

Analysis of inflammatory cells in VIN lesions and normal vulvar skin (Table 1)

Dendritic cells

Immunohistochemical analysis of epidermis and dermis showed that the majority of immature mDCs were located in the epidermis. Staining for CD1a and CD207 identified immature DCs spread over the whole epidermis, including the basal layer. There were no DCs in the superficial layers of the epidermis. In the dermis, the majority of DCs were situated in focal infiltrates. Compared to healthy controls a significant decrease in $CD1a^+$ and $CD207^+$ cells in the epidermis of VIN patients was observed, but in the dermis numbers of these mDCs were not different for the two groups. Data are summarized in Figure 1a and b.

Table 1. Immunocompetent cells in epidermis and dermis of VIN-lesions compared with samples from healthy women

	Epidermis			**Dermis**		
	all patients n=51	**Controls n=19**	**P-value**	**all patients n=51**	**Controls n=19**	**P-value**
	median (range)	**median** (range)		**median** (range)	**median** (range)	
$CD1a^+$	**157** (23-478)	**212** (104-311)	**0.042**	**142** (17-498)	**133** (51-277)	0.2
$CD207^+$	**106** (2-407)	**166** (14-319)	**0.024**	**54** (0-278)	**41** (0-103)	0.1
$CD208^+$	**21** (2-148)	**19** (1-43)	0.3	**124** (3-539)	**45** (15-172)	**<0.001**
$CD123^+/11c^-$	**14** (0-78)	**8**[¶] (0-35)	**0.023**	**234** (56-1140)	**160**[¶] (57-302)	**0.004**
$CD94^+$	**22** (0-261)	**28** (0-144)	0.3	**52** (0-438)	**30** (0-209)	**0.048**
$CD4^+$	**279** (31-925)	**269** (89-591)	0.6	**1162** (237-2909)	**748** (406-1060)	**<0.001**
$CD8^+$	**83** (2-357)	**130** (51-526)	**0.011**	**354** (45-2430)	**181** (76-491)	**0.001**
$CD25/HLA\text{-}DR^+$	**0** (0-7)	**0**[‡] (0-3)	0.5	**53** (0-231)	**26**[‡] (5-118)	**0.007**

[¶] controls n=17; [‡] controls n=16; CD1a, classic marker for Langerhans cells; CD207, marker for immature dendritic cells (DCs) expressing Langerin; CD208, DC-Lamp, marker for mature DCs; CD123/CD11c, marker for plasmacytoid DCs; CD94, marker for natural killer cells; CD4, marker for T-helper cells; CD8, marker for cytotoxic T-cells; CD25/HLA-DR, marker for T-regulatory cells.

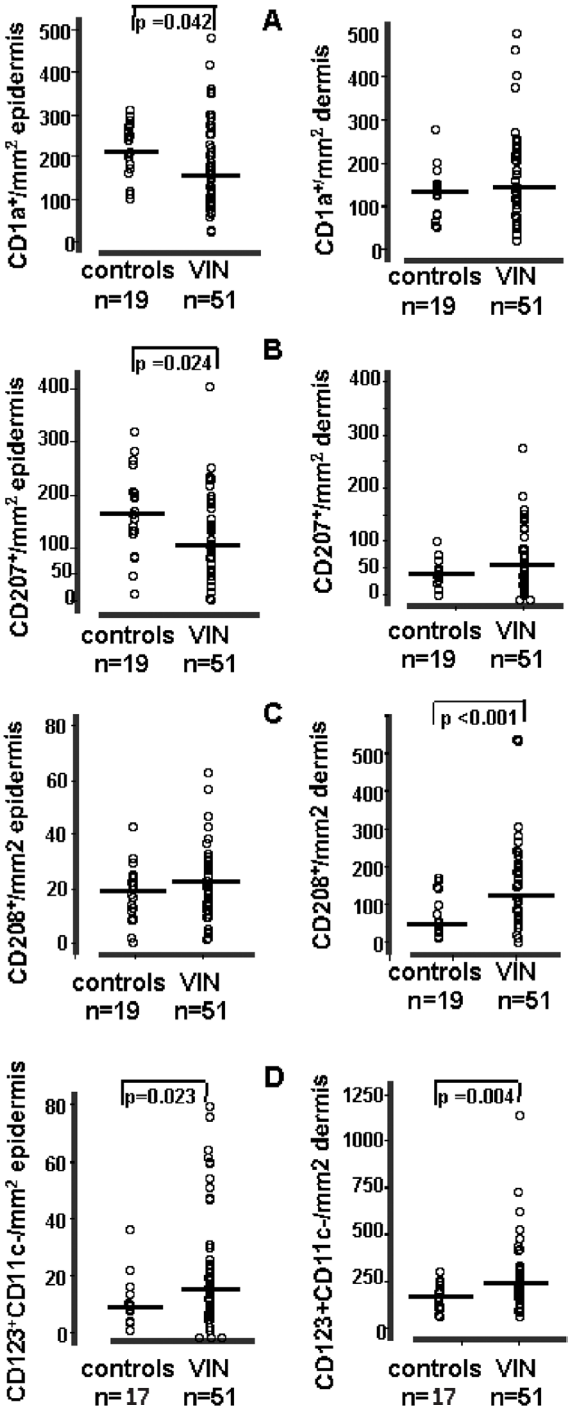

Figure 1. CD1a$^+$, CD207$^+$, CD208$^+$ and CD123$^+$/CD11c$^-$ DCs in the epidermis and lamina propria of VIN lesions and normal vulvar skin. Median values are indicated by horizontal lines.

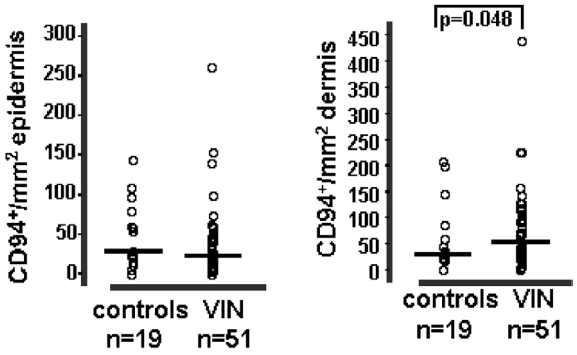

Figure 2. CD94$^+$ NK-cells in the epidermis and lamina propria of VIN lesions and normal vulvar skin. Median values are indicated by horizontal lines.

The number of mature CD208$^+$ cells in epidermis was low compared to the numbers of immature mDCs. In the epidermis there was no difference in numbers between healthy controls and VIN-affected skin, but at least twice as many cells were observed in the dermis of VIN patients when compared to controls (Figure 1c).

CD123$^+$ pDCs were found evenly distributed through the whole thickness of both epidermis and dermis. The numbers were significantly increased in VIN-affected skin when compared with controls (Figure 1d).

NK-cells

CD94$^+$ NK-cells were sporadically found in the epidermis, preferentially in the basal layer just above the basal membrane. In the dermis NK-cells were situated mainly in infiltrates. The number of CD94$^+$ NK-cells was not different in the epidermis, but was more than doubled in the dermis of VIN-patients when compared with healthy women (Figure 2).

T-cells

Predominantly, CD4$^+$ T-cells were located in the dermis just beneath the basal membrane. In the epidermis numbers did not differ between VIN patients and healthy controls, but in VIN-affected dermis numbers were significantly increased when compared to healthy skin (Figure 3a). There were significantly less CD8$^+$ cells in the epidermis and more CD8$^+$ cells in the dermis of VIN-affected skin than in healthy controls (Figure 3b).

Treg cells were analyzed by a staining procedure targeting CD25 and HLA-DR expression. The number of dermal CD25$^+$/HLA-DR$^+$ cells was higher in VIN patients than in controls. In the epidermis no differences between patients and healthy controls were observed (Figure 3c). The results for CD25$^+$/HLA-DR$^+$ Treg cells were controlled in 20 biopsies by staining sequential sections with Treg cell markers Foxp3 and CD25/HLA-DR, respectively. Staining results with both markers were fully comparable (data not shown).
Cell distribution for different inflammatory cells is shown in Figure 4.

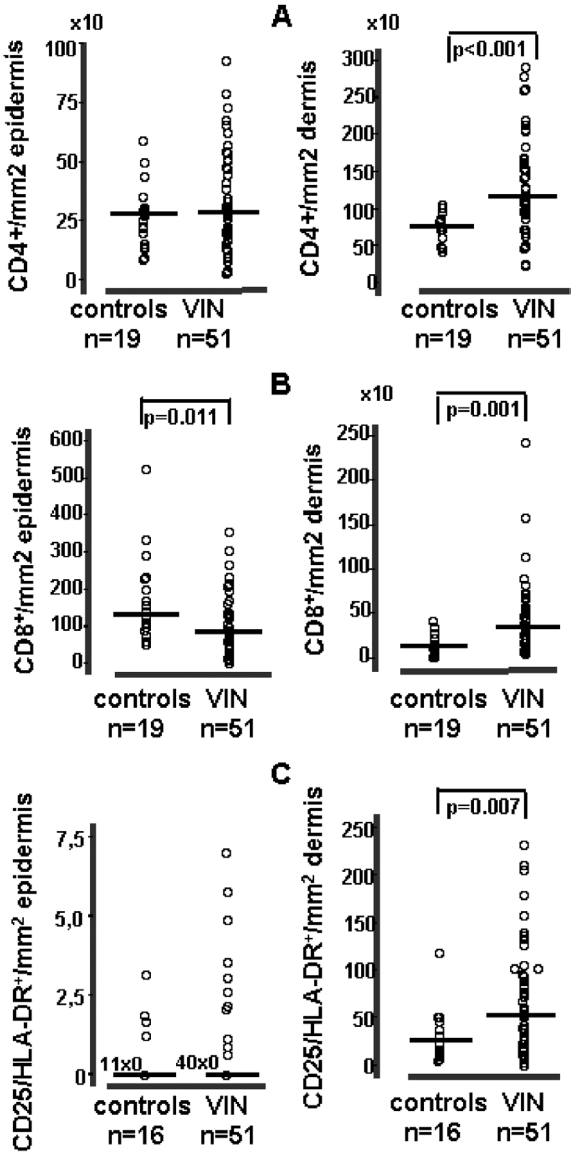

Figure 3. CD4+ and CD8+ T-cells, and CD25/HLA-DR+ Treg-cells in the epidermis and lamina propria of VIN lesions and normal vulvar skin. Median values are indicated by horizontal lines.

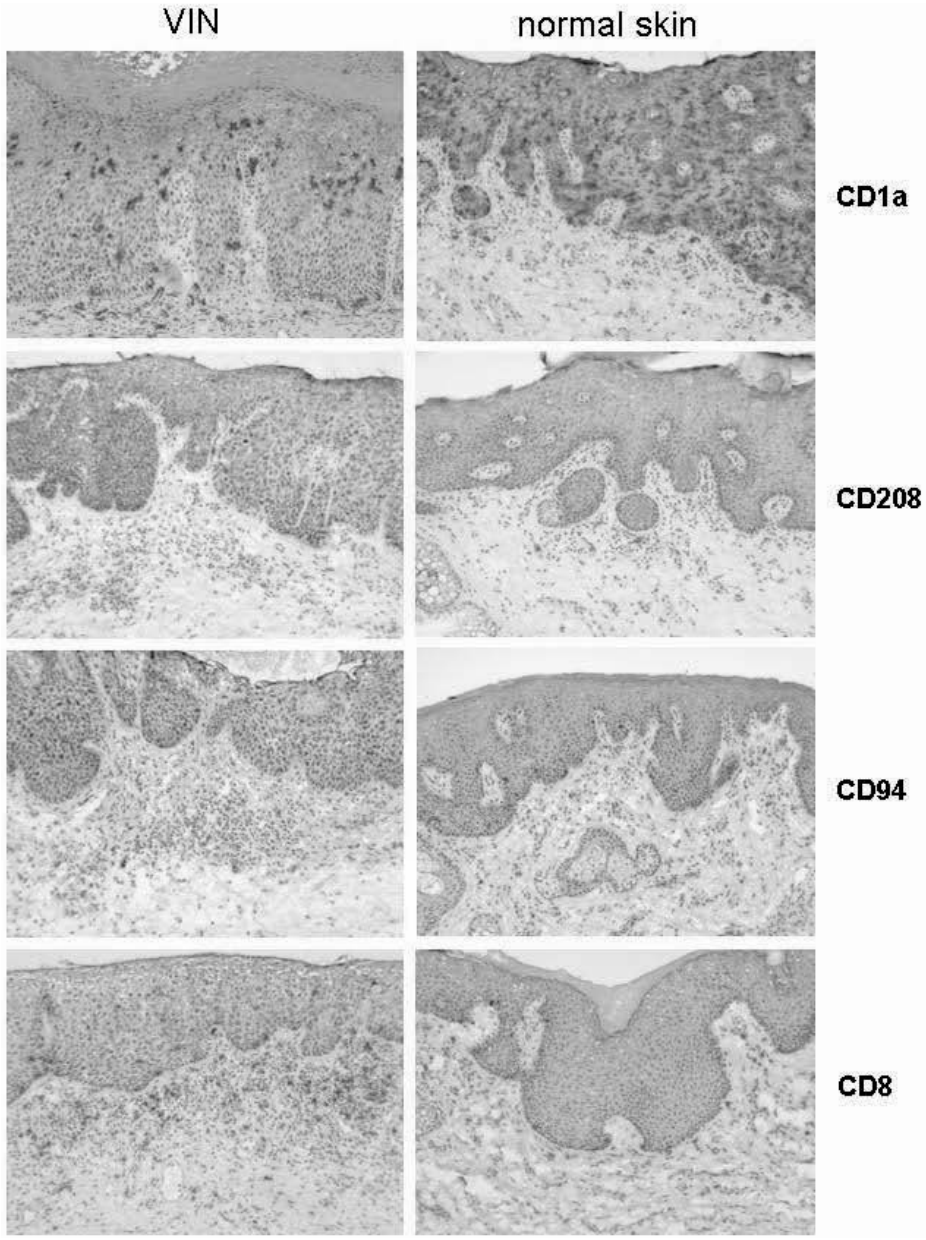

Figure 4 (photographs A-H, 10x). Representative photographs of positive (red stained) immunocompetent cells in VIN lesions and normal vulvar skin.

Compared to healthy controls VIN-affected skin showed a strong decrease of mDCs stained with CD1a in the epidermis (photograph A and B). Twice as many CD208$^+$ (mature) DCs were observed in the dermis of VIN compared to healthy controls (photograph C and D). NK cells stained with antibodies directed against CD94 are more numeric in the dermis of VIN-affected skin than in normal skin (photograph E and F). Less CD8$^+$ cells were observed in the epidermis, whereas significantly more CD8$^+$ cells were seen in the dermis of VIN-affected skin when compared to normal skin (photograph G and H). See also Color Figures, page 150.

Discussion

To our knowledge this is the first study characterizing the distribution of a broad spectrum of immunocompetent cells in epidermis and dermis of vulvar skin from patients with hrHPV-associated usual type VIN and from HPV-negative healthy controls. There are only a few studies investigating immunocompetent cells in VIN-affected skin, mostly dealing with $CD4^+$ and $CD8^+$ T-cells and/or $CD1a^+$ DCs.[6,10,11] Study designs vary significantly, especially when it comes to the presence of hrHPV, but also in the choice of VIN-inflicted vulvar layers, in cell counting procedures and, most important, in the choice of control groups. In our study, vulvar material from healthy HPV-negative women was used, this in contrast to other studies where normal tissue was isolated from resection margins of vulvar specimens taken from patients undergoing surgery for carcinoma or for benign vulvar diseases.[6,10,11]

In the epidermis of hrHPV-positive usual type VIN significantly less $CD1a^+$ and $CD207^+$ DCs were observed than in epidermal vulvar tissue from control women. These results are supported by Singh *et al* who describe an inverse correlation between the numbers of intraepithelial $CD1a^+$ DCs and the stage of VIN.[9] The significant reduction of $CD1a^+$ and $CD207^+$ immature mDCs in epidermal hrHPV-positive VIN tissue could be the result of migration into the dermis under the influence of pro-inflammatory cytokines such as TNF-α and IL-1β.[14] These cytokines are produced during antigen-induced DC activation and down-regulate the expression of the adhesion molecule E-cadherin on Langerhans cells.[15,16] E-cadherin mediates contact between keratinocytes and Langerhans cells. Down-regulation of the adhesion molecule not only prevents stimulation of DCs by HPV-infected keratinocytes[17] but also facilitates migration of the DCs. A defect in re-population of epidermal DCs through suppression of migration of immature Langerhans precursor-like cells by hrHPV-16 E6 and E7 proteins could also contribute to the observed decrease in mDCs.[18]

Interestingly, Mulvany and Allen observed increased numbers of $CD1a^+$ cells compared to the surrounding normal epithelium in differentiated type VIN which is not related to HPV and behaves biologically different.[19]

The significant reduction of immature mDCs in the epidermis of our patients seems to be compensated by a significant increase in the number of pDCs, suggesting involvement of pDCs in the immune response. Our results are supported by a study of Lee *et al*, who report decreased mDCs and increased pDCs in peripheral blood of patients with cervical squamous intraepithelial lesions.[20] The results are also in agreement with the suspected role of pDCs in viral infections. Lenz *et al* showed in an in vitro study that HPV-16 is bound by freshly isolated immature pDCs and CpG maturated pDCs, that internalization of the virus preferentially occurs in immature pDCs, and that it induces the production of IFN-α and IL-6, important factors for the production of antibodies.[21] Bontkes *et al* showed that

pDCs are present in cervical cancer lesions and that HPV-16 virus-like particles are able to activate pDCs.[22] In the dermal layer of VIN-afflicted skin no differences for cell numbers of immature mDCs were observed, but the more mature CD208$^+$ (CD-Lamp) DCs were significantly increased. This seems to indicate that persistent HPV-infection may indeed lead to maturation and to accumulation of these antigen-presenting DCs, possibly caused by a disturbed migration out of the dermis.

The observed increase of NK-cells in the dermis of usual type VIN lesions is in accordance with activation of the innate immune response and may contribute to initiation of the CD8$^+$ T-cell response against viral infection, as suggested by Robbins *et al*.[23]

Dermal influx of CD4$^+$ and CD8$^+$ T-cells in VIN-affected skin has been described previously.[10,11] The observed significant increase of dermal CD4$^+$ T-helper cells, CD8$^+$ CTLs and CD25/HLA-DR$^+$ T-reg cells in our study indicates local activation of the adaptive immune system in HPV-related usual type VIN. This is in accordance with observations by van Poelgeest *et al* and Todd *et al* about systemic activation of cell-mediated immunity by hrHPV infection.[24,25] Van Poelgeest detected HPV-16 specific CD4$^+$ T-cell immunity in the circulation of patients with persistent HPV-16 induced VIN. Todd demonstrated CD8$^+$ T-cell reactivity to one or more proteins of the HPV-16 oncopeptides E6 and E7 in the peripheral circulation of patients with high grade VIN.

It appears that Ag-presenting DCs are the key regulators of immune responses. DCs migrate from the epidermis into draining lymph nodes where they activate naïve T-cells and initiate cellular immunity. Different studies have demonstrated that this trafficking of DCs is controlled by soluble chemotactic factors known as chemokines.[26-28] Recent evidence has shown that chemokines not only direct the trafficking of DCs but also can regulate their maturation status.[29] Further studies of DC trafficking and the responsible chemokines in hrHPV-based vulvar lesions will be necessary to provide more insight into the immunological basis of hrHPV-related usual type VIN.

Acknowledgements

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7

Detection of human papillomavirus (HPV) 16-specific CD4+ T-cell immunity in patients with persistent HPV16-induced vulvar intraepithelial neoplasia in relation to clinical impact of imiquimod treatment

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Abstract

Purpose Topical application of the immune response modifier imiquimod is an alternative approach for the treatment of human papillomavirus (HPV)-positive vulvar intraepithelial neoplasia (VIN) and aims at the immunologic eradication of HPV-infected cells. We have charted HPV16-specific immunity in 29 patients with high grade VIN and examined its role in the clinical effect of imiquimod treatment.

Experimental design The magnitude and cytokine polarization of the HPV16 E2-, E6- and E7-specific $CD4^+$ T-cell response was charted in 20 of 29 patients by proliferation and cytokine bead array. The relation between HPV16-specific type 1 T-cell immunity and imiquimod treatment was examined in a group of 17 of 29 patients.

Results HPV16-specific proliferative responses were found in 11 of the 20 patients. In eight of these patients, T-cell reactivity was associated with IFNγ production. Fifteen of the women treated with imiquimod were $HPV16^+$, of whom eight displayed HPV16 E2- and E6-specific T-cell immunity before treatment. Imiquimod neither enhanced nor induced such immunity in any of the subjects. Objective clinical responses (complete remission or >75% regression) were observed in 11 of the 15 patients. Of these 11 responders, eight patients displayed HPV16-specific type 1 $CD4^+$ T-cell immunity, whereas three lacked reactivity. Notably, the four patients without an objective clinical response also lacked HPV16-specific type 1 T-cell immunity.

Conclusions HPV16-specific IFNγ-associated $CD4^+$ T-cell immunity, although not essential for imiquimod-induced regression of VIN lesions, may increase the likelihood of a strong clinical response (P=0.03).

Introduction

Genital infections with high-risk human papillomaviruses (HPV) are very common.[1-3] Fortunately, the majority of infected subjects clear the infection.[4,5] A persistent infection with a high-risk HPV, mostly HPV16, can lead to neoplasia of the anogenital tract, of which cervical intraepithelial neoplasia and cervical carcinoma are the most well-known.[6,7] HPV16 infection may also cause a chronic skin disorder of the vulva known as vulvar intraepithelial neoplasia (VIN).[8-10] In contrast to cervical intraepithelial neoplasia, which in general is effectively treated by eradication of the area involved, VIN is a chronic disease with high relapse rates after standard treatments.[11-13]

Imiquimod therapy has been put forward as an alternative approach for the treatment of VIN. This immune response modifier acts through Toll-like receptor 7 of the innate immune system resulting in the secretion of a multitude of proinflammatory cytokines. There is recent evidence that imiquimod also possesses direct proapoptotic activity against tumor cells.[14-16] Topical application preserves the anatomy and function of the vulva, whereas surgical excision or ablation of affected skin may be extensive and disfiguring and can carry considerable psychosexual morbidity. Clinical success rates differ and are estimated on 30% to 87%.[17-21]

The HPV16 early antigens E2, E6 and E7 are among the first of proteins that are expressed in HPV-infected epithelia. Our previous studies on HPV-specific T-cell immunity against these early antigens showed that type 1 (IFNγ) T-cell memory against the early antigens can be detected in the majority of healthy sexually active individuals but is weak or absent in patients with HPV16-induced cervical neoplasia.[22-24] In combination with earlier reports that point at a role for $CD4^+$ T-cells in the protection against progressive HPV infection (reviewed in ref. 25), our data argue that the $CD4^+$ type 1 T-cell response against the early antigens of HPV16 plays an important role in the protection against progressive HPV16-induced disease.

To examine the role of HPV16-specific $CD4^+$ T-cell immunity in the success or failure of treatment with imiquimod, we have done a detailed analysis with respect to the magnitude and cytokine polarization of the HPV16-specific $CD4^+$ T-cell response in patients with high grade VIN. Furthermore, HPV16-specific type 1 immunity was analyzed before, during, and after topical treatment with imiquimod. Our data indicate that chronic exposure of the immune system to the HPV16 viral proteins results in the induction of type 1 T-cell immunity in about half of the patients. Importantly, the presence of these type 1 T-cell responses is likely to be associated with a more favorable clinical response to imiquimod treatment.

Material and Methods

Patients

Twenty-nine women with high grade VIN (age range, 24-73 years; median age, 47 years) were recruited from the departments of gynecology of the Academic Medical Center and Leiden and Erasmus University Medical Center, The Netherlands. On the average, these patients had been diagnosed with VIN 3 5.4 years before enrollment in the study (range, 6 months to 15 years). Eighteen women had undergone previous treatments for VIN 3 (surgical excision, laser therapy, or imiquimod treatment (patients 20, 21, 24, 27)) before study entry.

Seventeen of these 29 subjects (age, 29-60 years; median, 43 years) were experimentally treated with a 5% imiquimod cream. The patients were asked to apply the cream to the affected areas on the vulva twice weekly overnight for a maximum period of 16 weeks. To analyze the effect of imiquimod treatment on the HPV16-specific immune response, we collected serial blood and serum samples before the start of imiquimod treatment (T=0), after 8 weeks of treatment (T=8), and at the end of treatment (T=16). Vulvar lesions were assessed by direct measurement and photographic records at entry and after 8 and 16 weeks of treatment. Clinical responses were defined as a complete response; a partial response type 1, as defined by a reduction in lesion diameter from 76% to 99%; a partial response type 2, as defined by a reduction in lesion diameter from 26% to 75%; or no clinical response.

From 20 of 29 women peripheral blood mononuclear cells (PBMC) were isolated and directly used to analyze HPV16-specific proliferative T-cell reactivity. Of these 20 women, eight patients had also participated in the imiquimod study. In six cases blood was taken 3 months (patient 1), 4 months (patient 10), 10 months (patient 5) to over 1 year (patients 12, 13 and 15) after the end of the imiquimod study, in the other 2 cases (patients 2 and 4) blood was taken within 4 weeks after the start of treatment. Serum was collected to study the presence of virus-like particle L1 (VLP)-specific antibodies.

All subjects were typed for HPV by GP5+/6+ PCR followed by reverse line blot analysis as described previously.[26] The study design was approved by the Medical Ethical Committees and all women gave written informed consent.

Antigens

A set of peptides spanning the whole HPV16 E2, E6, and E7 protein were used for the T-cell proliferation assays. The E2 peptides consisted of twenty-two 30-mer peptides with a 15-amino-acid overlap and the COOH-terminal peptide with a length of 35 amino acids. For the T-cell proliferation assays, the E2 peptides, 32-mer peptides of the E6 protein, and the 35-mer peptides of the E7 protein with an overlap of 14 amino acids were used in pools of two peptides per pool. For the IFNγ enzyme-linked immunospot (ELISPOT) assays,

the peptides used spanned the HPV16 E2, E6, and E7 protein and consisted of the most immunogenic regions of the E2 30-mer peptides[22] and 15 E6 and nine E7 overlapping 22-mer peptides. The peptides were synthesized and dissolved as described previously.[27] The peptide pools are indicated by the first and last amino acid of the region in the protein covered by the two peptides (e.g., $E2_{1-45}$, residues 1-30 and 16-45). Memory response mix (MRM), consisting of a mixture of tetanus toxoid (0.75 *Limus flocculentius*/mL final concentration; National Institute of Public Health and Environment, Bilthoven, The Netherlands), *Mycobacterium tuberculosis* sonicate (2.5µg/mL; generously donated by Dr. P. Klatser, Royal Tropical Institute, Amsterdam, The Netherlands), and *Candida albicans* (0.005%, HAL Allergenen Lab., Haarlem, The Netherlands), was used as a positive control.

Short-term T-cell proliferation assay

Freshly isolated PBMCs were incubated with 12 pools of HPV16 E2-derived 30-mer peptides, four pools of E6 32-mer peptides, and two pools of E7 35-mer peptides (each pool consisted of two overlapping peptides). PBMCs were seeded at a density of 1.5 x 10^5 cells per well in a 96-well U-bottomed plate (Costar, Cambridge, MA) in 125 µL of Iscove's medium (Bio Whittaker, Verviers, Belgium) supplemented with 10% autologous serum. HPV16 E2-, E6-, and E7-derived peptides were added at a concentration of 10 µg/mL/peptide. Medium alone was taken along as a negative control, and MRM (dilution, 1:50) served as a positive control. For each peptide pool, eight parallel microcultures were incubated. Fifty microliters of supernatant from the microcultures were taken at day 6 after incubation and stored at -20°C until cytokine analysis. Peptide-specific proliferation was measured at day 7 by [^{3}H]-thymidine incorporation. Cultures were scored positive when the proliferation of ≥ 75% of the test wells exceeded the mean proliferation + 3x SD of the control wells containing medium only, and the stimulation index, defined as the mean of all test wells divided by the mean of the control wells, was ≥ 3.[22]

Analysis of cytokines associated with HPV16-specific proliferative responses

The detection of cytokines in the supernatants of the short-term proliferation assays was done using the cytometric bead array (Becton Dickinson, Erebodegem-Aalst, Belgium). This technique allows the simultaneous detection of six different Th1 and Th2 cytokines IFNγ, tumor necrosis factor α (TNFα), interleukin 2 (IL-2), IL-4, IL-5, and IL-10. The cytometric bead array was done according to the manufacturer's instructions. Cut-off values were based on the standard curves of the different cytokines (50 pg/mL for IFNγ and 10 pg/mL for the remaining cytokines). Antigen-specific cytokine production was defined as a cytokine concentration above cut-off level and >2x the concentration of the medium control.[23,28]

Analysis of HPV16-specific T-cell reactivity by IFNγ enzyme-linked immunospot

The number of IFNγ producing HPV-specific T-cells, present in the peripheral blood of the 17 patients treated with imiquimod, was quantified using ELISPOT that was done as described previously.[29,30] Briefly, PBMC were thawed, washed, and seeded at a density of 2 x 10^6 cells per well of a 24-well plate (Costar) in 1mL of Iscove's modified Dulbecco's medium (Bio Whittaker) enriched with 10% human AB serum, in the presence or absence of indicated HPV16 E2, E6, and E7 peptide pools. Peptides were used in pools of four to five peptides at a concentration of 5µg/mL/peptide. The peptides, as indicated by their first and last amino acid in the protein, were used in the following pools: *E2-I*: 1-30, 16-45, 31-60, 46-75; *E2-II*: 61-90, 76-105, 91-120, 106-135; *E2-III*: 121-150, 136-165, 151-180, 166-195; *E2-IV*: 271-300, 286-315, 301-330, 316-345, 331-365; *E6-I*: 1-22, 11-32, 21-42, 31-52; *E6-II*: 41-62, 51-72, 61-82, 71-92; *E6-III*: 81-102, 91-112, 101-122, 111-132; *E6-IV*: 111-132, 121-142, 131-152, 137-158; *E7-I*: 1-22, 11-32, 21-42, 31-52; *E7-II*: 41-62, 51-72, 61-82, 71-92, 77-98. Following 4 days of incubation at 37ºC, PBMC were harvested, washed, and seeded in four replicate wells at a density of 10^5 cells per well in 100µL Iscove's modified Dulbecco's medium enriched with 10% FCS in a Multiscreen 96-well plate (Millipore, Etten-Leur, The Netherlands) coated with an IFNγ-catching antibody (Mabtech AB, Nacha, Sweden). Further antibody incubations and development of the ELISPOT was done according to the manufacturer's instructions (Mabtech). Spots were counted with a fully automated computer-assisted video-imaging analysis system (Bio Sys, Frankfurt, Germany). Specific spots were calculated by subtracting the mean number of spots + 2x SD of the medium control from the mean number of spots in experimental wells provided that the mean number of spots of the medium control wells were either <10 or >10 with a SD <20% of the mean. Antigen-specific T-cell frequencies were considered to be increased when specific T-cell frequencies were ≥1 in 10,000 and at least ≥2x background.[30] The background number of spots was 2.6 ± 2.2 (mean ± SD), with one exception (patient 23, 51 ± 10 spots).

HPV16 VLP ELISA

For the detection of HPV16-specific antibodies in serum we used an ELISA method previously described by Kirnbauer et al.[31] Each serum sample was tested for reactivity against HPV16 VLPs (baculovirus-expressed capsids comprising the L1 protein) and against bovine papillomavirus capsids, the latter disrupted by treatment with 0.1 mol/L carbonate buffer to serve as a negative control. Both VLP and bovine papillomavirus were kindly provided by Prof. Dr. J. Dillner (LUNDS University, Sweden). The patients were tested for both HPV16-specific IgG and IgA. A set of sera of healthy children (n=8; mean age, 7.3 years; range, 4.3-14.1 years) was tested to determine background reactivity. For HPV16 L1-VLP IgG type responses a cut-off absorbance value of 0.230 was used (mean A=0.060; range, –0.056 to 0.150; mean + 2x SD =0.230). For IgA type responses a cut-off of A=0.215 was used (mean A=0.189; range, 0.171 to 0.205).

Statistical analysis

Statistical analysis of the HPV16-specific proliferative responses associated with cytokine production was done using Fisher's exact test. Fisher's Exact test (two tailed) was used to analyze HPV-specific immunity to clinical response upon treatment with imiquimod. Statistical analyzes were performed using Graphpad Instat Software (version 3.0).

Results

HPV16-specific cellular and humoral responses in patients with high grade VIN

VIN forms a unique aspect of HPV-induced disease because patients are frequently treated, but the infection often persists. HPV16 is found most often. To gain a more profound insight in the $CD4^+$ T-cell response against HPV16 in VIN, we charted the magnitude, specificity,

A

	E2												E6				E7		
	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	1	2	MRM
P1			5,5															5,2	61
P2		7,5	6,9																62
P3					29							40					36		256
P4																			110
P5			12																112
P6																			7,5
P7				4,6															190
P8																			15
P9																			281
P10																			69
P11																		3,0	8,8
P12		7,6											7,0					11	49
P13		6,1	7,8			3,4	5,3		3,1					5,7		8,5		6,9	37
P14																			9,8
P15				5,4		13									9,2				30
P16																			12
P17				6,0															203
P18																			64
P19																			12
P20		8,4												5,6	5,4	4,8			25

Figure 1a. HPV16-specific proliferative T-cell responses in VIN

Freshly isolated peripheral blood mononuclear cells from 20 patients with high-grade HPV16-associated VIN were tested in short-term proliferation assays using a complete set of HPV16 E2-, E6-, and E7-derived peptide pools. Responses were scored positive when the proliferation (cpm) of ≥6 of eight test wells exceeded the mean proliferation ± 3x SD of the control (medium only) wells, and the mean stimulation index of all test wells over control wells was ≥ 3. Memory response mix, consisting of a mixture of recall antigens, was used as a positive control. The stimulation indices of responses scored positive are indicated. Abbreviation: MRM, memory response mix.

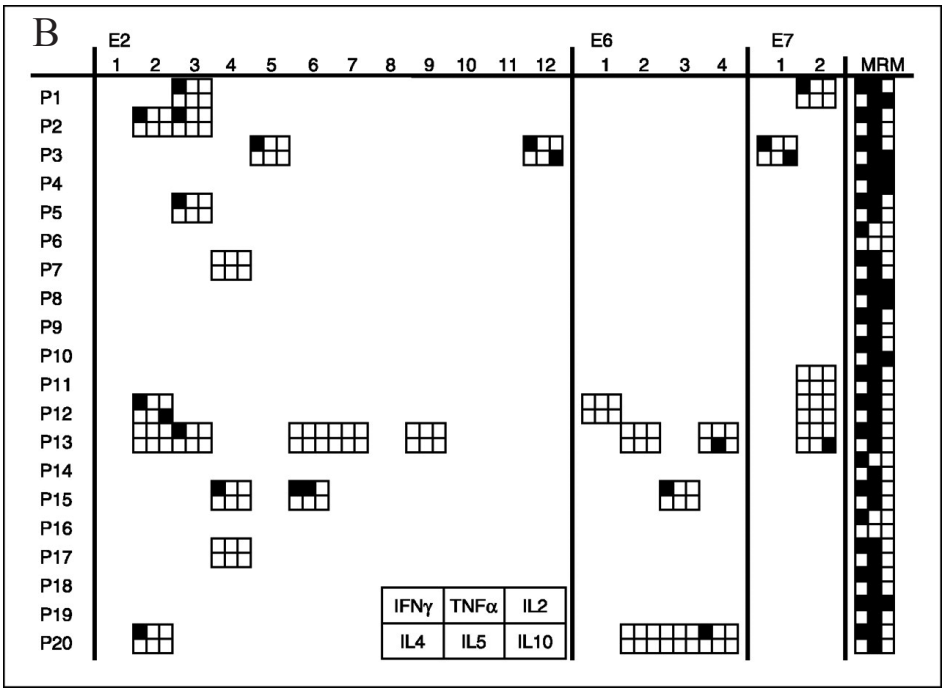

Figure 1b. Supernatants of the positive proliferative responses indicated in Table 1 were analyzed for the presence of IFNγ, tumor necrosis factor-α, IL-2, IL-4, IL-5, and IL-10 by cytometric bead array. The indicated layout is used for the six measured cytokines. Antigen-specific cytokine production (■). Cutoff values were based on the standard curves of the different cytokines (50 pg/mL for IFN and 10 pg/mL for the remaining cytokines). Antigen-specific cytokine production was defined as a cytokine concentration above cutoff level and >2x the concentration of the medium control.

and functionality of HPV16 E2, E6, and E7-specific proliferative T-cell responses in a group of 20 women with HPV16-associated high grade VIN.

PBMC isolated from VIN patients were stimulated with peptides derived from HPV16 proteins E2, E6, and E7 as well as with a mix of common recall antigens (MRM), in a short-term proliferation assay. We have previously shown that this assay is geared towards the detection of $CD4^+$ T-cell responses.[23] HPV16-specific proliferative T-cell responses against E2 and/or E6 were detected in 10 of 20 patients (Figure 1A). E7-specific responses were detected in 5 of 20 subjects. Analysis of the supernatants of these T-cell cultures for the presence of type 1 and type 2 cytokines revealed the secretion of the Th1 cytokine IFNγ in 8 of 20 patients. In some of the patients, the production of TNFα, IL-5 and IL-10 was occasionally detected (Figure 1B). Although the overall frequency of proliferative responses is similar when compared with that previously found for cervical cancer patients, the number of patients with IFNγ-associated HPV-specific T-cell responses in these VIN patients was higher (8 of 20 versus 4 of 17, respectively[23]).

Table 1. Distribution of absolute absorbance values among IgG- and IgA-seropositive samples

Immunoglobulin type ($n_{positive}$/28)	$\Delta A_{415\ nm}$	
	Mean (SD)	**Median (range)**
IgG seropositive (n=25)	1.10 (0.61)	1.22 (0-1.93)
IgA seropositive (n=13)	0.34 (0.26)	0.31 (0-0.88)

HPV16 L1 IgG and IgA antibodies detected in the sera of 28 VIN 3 patients. Serum antibody responses were measured by VLP-ELISA. Depicted are the absolute absorbance values at 415 nm. The absorbance values were calculated by subtraction of the background response value and the mean absorbance value of the young children's sera.

In addition to T-cell immunity, the humoral response to HPV16 was measured in 28 VIN patients by ELISA using HPV16 L1-VLP as antigen. Overall, HPV16 L1-VLP IgG and IgA antibodies were detected in 25 of 28 (89%) and 13 of 28 (46%) subjects, respectively (Table 1). Based on the absorbance values, the HPV16 L1-VLP-specific IgG response exceeded that of IgA (Table 1). In general, HPV16-specific IgA responses were detected when patients displayed relatively high levels of HPV16-specific IgG. If IgG absorbance values were ≥0.5, 11 of 19 (58%) of the samples contained HPV16 L1-specific IgA, whereas at IgG levels <0.5 only two of nine samples were IgA seropositive.

In conclusion, HPV16 L1-specific humoral immunity was detected in the great majority of patients, whereas HPV16 E2-, E6-, and/or E7-specific IFNγ-associated type 1 T-cell reactivity was detected in about half of the patients tested.

HPV16-specific immunity is associated with a more favorable clinical response on immunomodulatory treatment with imiquimod

Our analysis of HPV16-specific proliferation indicates that a high number of the proliferative T-cell responses is associated with IFNγ production. To examine the role of these HPV16-specific type 1 T-cell responses in the success or failure of treatment with the immunomodulator imiquimod, we studied this immune response in a group of patients with high grade HPV16-positive VIN. PBMC were isolated before (T=0), during (T=8), and after (T=16) treatment and stored in liquid nitrogen. HPV-specific T-cell reactivity against HPV16 peptides E2, E6, and E7 was analyzed by IFNγ ELISPOT. This is a sensitive method for the analysis of antigen-specific type 1 T-cell reactivity on frozen material.[32,33] Three of these patients had been treated with imiquimod in the year before inclusion in our study (Table 2; patients 21, 24, and 27). Of these 17 patients, 15 were HPV16-positive. Preexisting IFNγ-associated T-cell responses (T=0) were detected in 8 of 15 patients by IFNγ ELISPOT. In 5 of 15 patients, HPV16-specific T-cell reactivity against E2 was detected, whereas 4 of 15 patients displayed a response against E6 (Table 2). None of these patients showed preexisting T-cell responses against HPV16 E7. In two cases the T=0 sample was not available and the reaction in PBMC from T=8 are shown (Table 2; patients 1 and 22).

Despite that for some patients one of the two follow-up samples was not available (patients 5, 13, 27, and 28), it was clear that we could not detect a direct influence of

Table 2. HPV16-specific T-cell responses in patients treated with imiquimod

Patient*	HPV type	clinical response†	VLP‡	T	E2-peptides				E6-peptides				E7-peptides		MRM
					1§-75	61-135	121-195	271-365	1-52	41-92	81-132	111-158	1-52	41-98	
1	16	no	++	8	-	-	-	-	-	-	-	-	-	-	**179**
2	16	PR1	+	0	**52**	**54**	**15**	**31**	-	-	*3*	-	*9*	-	**295**
4	16	CR	++	0	-	-	-	-	-	*1*	-	-	-	-	**124**
5	16	CR	++	0	-	**32**	*9*	*3*	-	-	-	-	-	-	**32**
10	16	PR1	-	0	-	*4*	-	-	-	-	**84**	-	*5*	-	**54**
12	16	PR1	++	0	**15**	-	-	-	-	-	-	-	-	-	**32**
13	16	CR	++	0	-**	-	-	-	-	-	**104**	-	-	-	**105**
15	16	PR1	+	0	-	-	-	-	-	-	-	-	-	-	**179**
21	neg	PR1	++	0	-	-	-	-	-	-	-	-	-	-	**364**
22	16	PR2	++	8	*8*	-	-	*4*	-	-	-	-	-	-	**25**
23	16	PR1	++	0	-	*1*	**59**	*5*	-	**74**	-	-	*21*§§	*11*	**52**
24	16	no	++	0	-	-	-	-	-	-	-	-	-	-	**172**
25	neg	CR	+	0	-	-	-	*4*	-	-	-	-	-	-	**444**
26	16	CR	++	0	-	-	-	-	-	-	-	-	-	-	**21**
27	16	no	++	0	-	*4*	-	-	-	-	-	-	-	-	**70**
28	16	CR	++	0	*6*	-	**45**	**18**	-	*7*	*3*	*2*	*3*	-	**20**
29	16	CR	-	0	-	*2*	*7*	-	-	**40**	-	-	-	*2*	**157**

Abbreviations: CR, complete response; PR, partial response; MRM, memory response mix.

* PBMC from 17 VIN3 patients were tested for type 1 T-cell reactivity against HPV16 peptides. PBMC were stimulated with different pools of HPV16 E2, E6, and E7 peptides and tested for antigen-specific IFNγ production by ELISPOT.

† Clinical responses were defined as no clinical response; a partial response type1, as defined by a reduction in lesion diameter from 76-99%; a partial response type2 (PR2), as defined by a reduction in lesion diameter from 26-75%; and a complete response (CR).

‡ Sera of the patients were tested for the presence of HPV16 L1-VLP specific IgG antibodies. Indicated is the presence (+) or absence (-) of antibodies.

§ The first and last amino acid in the indicated protein of the peptide pool used are indicated.

** Per patient, T-cell responses on T=0 are shown. In case of a missing T=0 sample, data from T=8 are shown. Specific responses were calculated by subtracting the mean number of spots ± 2 x SD of the medium control from the mean number of spots of experimental wells. The number of specific spots per 100,000 PBMCs are given. Responses were considered positive if peptide pool specific T-cell frequencies were ≥ 10 in 100,000 PBMCs. These values are indicated in bold. Values below this threshold are shown in italics. (-), no specific response to E6 or L1. MRM was used as a positive control.

§§ Responses considered negative because values did not exceed ≥ 2 times the medium control.

imiquimod on the numbers of HPV-specific T-cells. In none of the patients was a clear-cut increase of HPV16-specific T-cells detected upon imiquimod treatment (Figure 2A-B). In some cases, patients had already been treated with a course of imiquimod before this study, but even this repeated treatment did not result in an increase of HPV 16 specific T-cells (Table 3; patients 21 and 24). In addition, the HPV16 VLP-specific IgG and IgA response did not overly change when patients were treated with imiquimod (Figure 3).

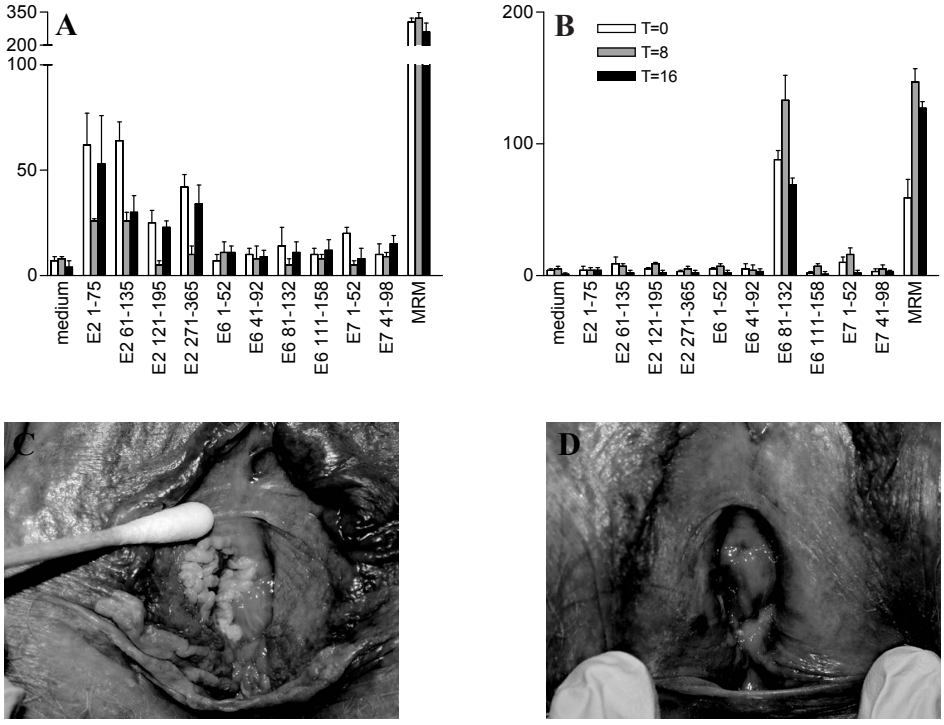

Figure 2. A and B, HPV16-specific IFNγ-producing T-cell responses in two representative patients with high grade VIN (patient 2, left and patient 10, right). T-cell responses are shown at week 0 (before imiquimod treatment), week 8 (during imiquimod treatment), and at week 16 (after imiquimod treatment). Local application of 5% imiquimod containing cream does not result in enhanced systemic HPV16-specific T-cell responses. Note that the magnitude of the T-cell responses varies slightly over the different time points. The mean number of spots and SE induced by the medium control or the peptides present in the E2, E6, and E7 pools per 100,000 PBMCs are depicted. As positive control, the memory recall mix (MRM) was used. C and D, patients with preexisting HPV16-specific T-helper type 1 responses show objective clinical responses after imiquimod treatment. A typical example is shown. C, biopsy-proven VIN 3 lesion of patient 5 before imiquimod treatment. D, the same vulvar area of patient 5 after 16 weeks of treatment. See also Color Figures, page 151.

Thirteen of the 17 women treated (76%) displayed an overt clinical response upon treatment with imiquimod as indicated by 76% to 100% reduction in the size of their lesion (complete response or partial response 1; Table 2; Figure 2C-D). Three patients showed no reduction in size of the affected area of vulvar disease and one woman showed only minimal improvement upon treatment.

Importantly, when the group of HPV16$^+$ patients (n=15) was divided in patients either with or without an HPV-specific Th1 immune response, all eight patients with an HPV-specific immune response displayed a complete or near complete clinical response (complete response or partial response 1) upon imiquimod treatment (Table 2). In contrast, patients without an HPV-specific immune response were less likely to show such a clinical improvement (P=0.03, two-sided Fisher's exact test).

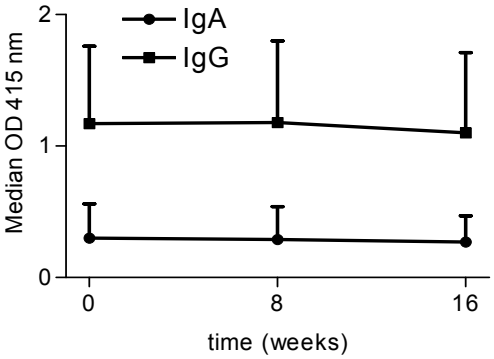

Figure 3. IgG and IgA reactivity to HPV16 VLPs over time in 17 VIN 3 patients treated with imiquimod. At least two serum specimens were tested in every patient. Serologic responses are shown at week 0 (before imiquimod treatment), week 8 (during imiquimod treatment), and at week 16 (after imiquimod treatment). The absorbance (OD) values are depicted as median ± SD of positive responses. The absorbance values were calculated by subtraction of the background response value and the mean absorbance value of the young children's sera.

Taken together, chronic viral antigen exposure can induce type 1 $CD4^+$ T-cell immunity against the HPV16 early antigens E2, E6, or E7 in patients with VIN 3. The presence of these HPV16-specific Th1-cells as detected by IFNγ ELISPOT, although not essential for imiquimod-induced regression of VIN lesions, does increase the likelihood of a strong clinical response. The presence of L1-specific humoral reactivity was not correlated with imiquimod-induced regressions.

Discussion

We have analyzed the HPV16 E2-, E6-, and E7-specific $CD4^+$ T-cell responses in a group of 29 patients with high grade VIN, 17 of whom were treated with the immunomodulator imiquimod. HPV16-specific type1 (IFNγ) $CD4^+$ T-cell proliferative immunity is present in about half of patients with VIN 3 (8 of 20). Virus-specific $CD4^+$ Th1-type T-cells have emerged as an essential component in the immune response to chronic viral infection, fulfilling a multifactorial role, including the activation of antigen-presenting cell maturation for efficient $CD8^+$ priming, the release of cytokines important in $CD8^+$ T-cell proliferation and differentiation, and in the recruitment of other effector cells such as eosinophils and macrophages. Indeed, a substantial number of patients with VIN 3 were reported to display high frequencies of HPV16-specific $CD8^+$ T-cells.[34-36] In contrast, only in a few occasions HPV16-specific $CD8^+$ T-cell reactivity was detected in patients with cervical intraepithelial neoplasia 3 and cervical carcinoma.[37-40] However, these latter types of patients display an impaired HPV16-specific $CD4^+$ T-cell response.[23]

Topical application of imiquimod neither enhanced the preexistent HPV16-specific $CD4^+$ T-cell responses nor resulted in the induction of such responses in any of the other subjects. Todd *et al* made a similar observation with respect to HPV16-specific $CD8^+$ T-cells.[36] Notably, we found that a preexisting HPV-specific type 1 T-cell response was associated with a more favorable clinical outcome upon topical imiquimod treatment of VIN 3. This indicates that a combination therapy, in which the HPV16-specific T-cell response is induced or boosted by vaccination and the affected skin is treated with imiquimod, may increase the number of patients that benefit from treatment.

Compared with normal vulvar skin, a number of VIN lesions display increased infiltration of $CD4^+$ and $CD8^+$ T-cells.[41-43] The clinical consequences of the infiltration of immune cells in these VIN lesions are poorly understood, but the immunological make-up of the vulvar microenvironment may determine the clinical outcome.[43] The local cytokine microenvironment in high grade cervical neoplasia is associated with a decreased expression of the pro-inflammatory Th1 cytokines, TNFα and IFNγ.[44-46] It is conceivable that similar to cervical intraepithelial neoplasia, the vulvar microenvironment also lacks pro-inflammatory cytokines. Imiquimod is known to directly stimulate Langerhans cells and macrophages,[17,47] of which the latter are increased in VIN lesions.[42] Furthermore, it stimulates natural killer cells and T-helper type 1 cells via indirect mechanisms.[17,47] Upon stimulation, the antigen-presenting cells release pro-inflammatory cytokines, predominantly IFNα, TNFα, and IL-12.[14,15,17,47] This may restore an inducive environment in which the innate effector cells, macrophages and natural killer cells, as well as activated HPV16-specific T-cells may act in concert to form an effective immune response. The requirement for these additional signals to activate T-cells is sustained by recent observations in animal models. In the HPV16 E7-transgenic skin transplantation model, Matsumoto *et al* showed that despite the presence of large numbers of E7-specific memory T-cells $E7^+$ skin transplants were not rejected, except when these E7-specific memory T-cells were activated through vaccination.[48] This suggested that the presence of the HPV16 E7 antigen itself is not sufficient to evoke a strong skin-destroying immune response but that additional activating signals were required. Similarly, Van Mierlo *et al* showed that adenovirus-specific $CD8^+$ T-cells developed in the draining lymph nodes of mice bearing adenovirus-positive tumors, indicating that tumor-antigen was detected by T-cells of the immune system.[49] The tumor was rejected only when strong pro-inflammatory signals were provided. Likewise, HPV16-induced VIN 3 lesions may fail to endow the immune system with strong inflammatory signals and exogenously provided signals will be required to provide a state of inflammation. These signals can be delivered by imiquimod, electro coagulation,[50] or by vaccines.[32-34]

Currently, it is not clear whether immune activation causes the HPV16-specific IFNγ-producing $CD4^+$ T-cells to migrate into the HPV-infected tissue or whether these T-cells should simply provide help to activate effector cells in the draining lymph nodes. Therefore,

we are currently examining both local and systemic immune response in patients with high grade VIN.

Acknowledgments

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8 General discussion

Vulvar intraepithelial neoplasia (VIN) is a difficult disease to treat. Symptoms can be long-lasting and severe and progression to invasive vulvar carcinoma is seen, although the available literature suggests that the progression rate without treatment is low (9%).[1] To date, treatment is aimed at the surgical removal of all visible lesions.[2] Unfortunately, surgery does not prevent progression to invasive disease and recurrences of VIN are common, the latter presumably because persistent infection with HPV, the viral cause of usual type VIN, is not affected by surgical treatment. In contrast to what was previously thought, VIN associated with lichen sclerosus is also of the usual type and related to HPV in 31%.[3]

From our systematic review it appeared that no difference in progression is seen after different surgical procedures. Iversen *et al* showed that free surgical margins will not prevent progression.[4] Whether free surgical margins diminish the change to progression in comparison to involved margins could not be established in our review. Another observation was that untreated patients are more likely to progress to invasive vulvar carcinoma than patients treated for VIN (9% vs 3.3%, respectively). According to Jones *et al*, this figure is higher. In a large study of 405 women with VIN, 10 (18.5%) of 63 untreated patients (i.e. biopsy alone or grossly incomplete excision) progressed to invasion in 13 months to 7.3 years (mean 3.9 years).[5] Importantly, invasion may occur many years after VIN 3 is diagnosed. In our review, the mean time to progression was 4.6 years (range 4 months to 18 years), indicating the importance of a long and careful follow-up. Another problem highlighted in our review was the high recurrence rate after surgical treatment. Recurrences were seen as often after local excision as after (partial) vulvectomy (22% vs 18%, respectively), showing a lower percentage after free surgical margins than after involved surgical margins ($p<0.001$). In 2005, Hillemans *et al* also reported high recurrence rates for different treatment modalities (>40%), showing a significantly increased risk with VIN grade ($P=0.02$), multifocality ($P=0.01$), multicentricity ($P=0.05$) and presence of hr HPV infection ($P=0.001$).[6]

VIN is being diagnosed with increasing frequency in relatively young women.[4] This rise in incidence could be the result of a higher awareness and knowledge of VIN, but is more likely due to the overall increase in sexually transmitted diseases, and especially the rise in HPV infections. For this reason and the above mentioned limitations of surgery, a new treatment strategy for VIN by means of imiquimod, a local immune response modifier with antiviral and antitumour activity, was examined and described in this thesis. After the first positive results from our pilot study in which 13 of 15 patients with VIN showed a partial or complete response,[7] a placebo-controlled, double-blind, randomized clinical trial (RCT) was designed to investigate the effectiveness of imiquimod in VIN ($n=52$) during an observational period of 12 months.[8] Twenty-one (81%) of 26 patients treated with imiquimod showed reduction in lesion size, whereas none of the placebo subjects experienced a response of more than 25% ($p<0.001$). Complete response was achieved in 9 (35%) and partial response in 12 (46%) patients treated with the cream. Compared to

placebo, imiquimod was significantly associated with histological regression, viral clearance and relief of itchiness and pain. All complete responders (n=9) remained free from disease after 12 months of follow-up. About the same time, Mathiesen *et al* published similarly positive results for the only other RCT so far evaluating the use of imiquimod in VIN.[9] In his study thirty-one patients, most of them presenting with unifocal VIN (n=22), were treated with imiquimod or placebo in a 2:1 ratio using an escalating dose regimen during 16 weeks. Seventeen (81%) of 21 patients treated with imiquimod showed a complete response (defined by complete disappearance of dysplastic changes), whereas a partial response was seen in two patients (defined by reduction from VIN 3 to maximally VIN 1 in biopsies taken two months after last treatment). Long term follow-up was not mentioned. As in our study, local side effects were a common feature, but tolerable after dose-reduction. The use of immunotherapy is also encouraged by Le *et al* who investigated the clinical response in 39 VIN-patients treated with imiquimod with a median follow-up of 16 months.[10] An overall response rate of 77% was shown. Recurrence data within this group were compared with data from a historical cohort of surgically treated VIN patients, showing recurrence after treatment with imiquimod in 20.5% compared to 53.5% in surgically treated patients (P=0.013). Neither Mathiesen nor Le *et al* found progression of VIN or cancer during follow-up. In our RCT, three (6%) of 49 patients progressed to invasion (<1mm); two of them after placebo, one after imiquimod. These results are not different from the progression rate in VIN after surgical treatment.

So far, 17 studies published from 2000 to 2007 reported on the effectiveness and safety of imiquimod cream in the treatment of VIN. Summarized recently in a review article by Iavazzo *et al*, data from these 17 studies show complete regression in 26 to 100% of patients, and partial regression in 0 to 60%.[11] Recurrence was observed in 0-37%. A lack of response to imiquimod treatment did not seem to be associated with a higher risk of lesion progression.

It is not yet clear why some patients showed a better response to imiquimod than others. Since involvement of HPV in the aetiology of VIN suggests an immunological defect in the diseased, we investigated the influence of imiquimod on the presence of immunocompetent cells in the epidermis and dermis of VIN-affected skin. Treatment with

Values as median (range), logarithmic scale	
1: controls (n=19)	
2: imiquimod group before medication (all patients, n=25)	**6:** imiquimid group before medication (patients with clinical response < 75%, n=12)
3: imiquimod group after medication	**7: imiquimod group after medication**
4: imiquimod group before medication (patients with clinical response > 75%, n=13)	**8:** placebo group before medication (n=26)
5: imiquimod group after medication	**9: placebo group after medication**
	* **p<0.05**

Legends for Figure 1 and 2

imiquimod resulted in a significant increase of immature CD1a$^+$ myeloid dendritic cells (mDCs), CD8$^+$ T-cells and CD94$^+$ natural killer (NK) cells in the epidermis of VIN-patients with a clinical response of more than 75%.[5] In the dermis of these patients, immature CD207$^+$ and mature CD208$^+$ mDCs, and T-regulatory (Treg) cells decreased after treatment with imiquimod.[8] Combination of these results with data obtained from healthy vulvar

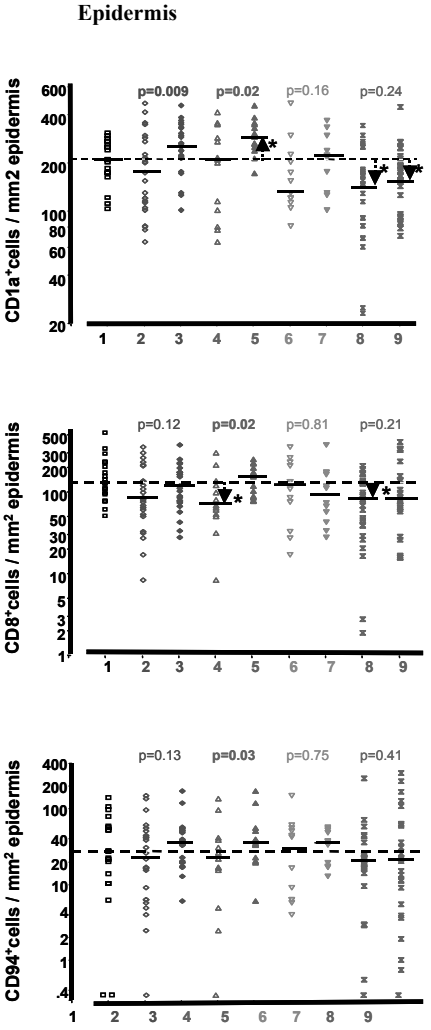

Figure 1. Results of immunohistochemical staining for CD1a$^+$ DCs, CD8$^+$ T-cells, and CD94$^+$ NK-cells in the epidermis of healthy normal skin (controls), and in VIN-affected skin before and after treatment with imiquimod or placebo. See also Color Figures, page 152.

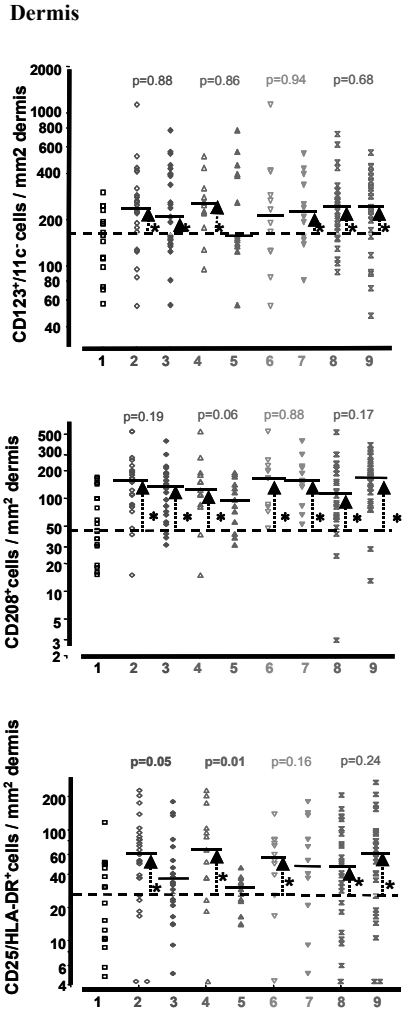

Figure 2. Results of immunohistochemical staining for CD123$^+$/11c$^-$ and CD208$^+$ DCs, and CD25/HLA-DR$^+$ Treg-cells in the dermis of healthy normal skin (controls), and in VIN-affected skin before and after treatment with imiquimod or placebo.

skin gave us more insight into the immunological effect of imiquimod in VIN-lesions. Compared to normal vulvar skin, we found VIN to be characterized by an immunosuppressive state in the epidermis, showing a reduction of immature myeloid DCs and $CD8^+$ T-cells.[12] In the dermis, inflammatory activation is reflected by the influx of mature myeloid and plasmacytoid DCs, NK-cells and T-cells. It seems that for certain cell types the disturbed pattern in VIN-affected skin is normalized by treatment with imiquimod as presented in Figures 1 and 2. This repair towards normal cell levels is only observed in patients with a clinical response of more than 75%, suggesting that the immunological make-up in VIN-affected skin is directive for the clinical outcome. In addition, we found that chronic exposure of the immune system to the HPV16 viral proteins resulted in the induction of systemic type 1 T-cell immunity in half of patients, which is likely to be associated with a more favorable clinical response to imiquimod treatment.[13]

Similar to our data, Santegoets *et al* reported increased levels of mature $CD208^+$ DCs in the dermis when HPV-related VIN tissue was compared to HPV-negative control vulvar tissue from the same patient, suggesting that DCs, after recognition of viral antigens, are stimulated to move into the dermis and to access secondary lymphoid organs.[14] According to Santegoets *et al*, however, this migration process seems to be disturbed because of a lack of accurate chemokine signalling. Most DCs will therefore stay in the dermis, not able to present the viral antigen to naïve T-cells in the lymph node. This may be one of the reasons for an inaccurate initiation of the adaptive immune response. During cervical carcinogenesis, Caberg *et al* observed a virus-induced altered expression of immune mediators necessary for Langerhans cell chemoattraction, suggesting a similar inability of the local immune system to mount a protective cell-mediated immune response against HPV-infected keratinocytes.[15] Further studies are needed to fully understand the role of the immune system in HPV-infected disease.

Recommendations and future prospects

Although VIN is a premalignant disease, it already displays several hallmarks of cancer as shown by our research group.[16] For this reason, we recommend that VIN should be treated pro-actively. In our opinion, imiquimod deserves a prominent role in this treatment. Showing a clinical response similar to or even better than after surgical treatment, in a way that is less invasive, well tolerated, and does not influence health-related quality of life, body image or sexuality, we would like to propose imiquimod first choice treatment in VIN. To achieve an even better clinical response rate to imiquimod treatment, it is hypothesized that boosting different aspects of cellular immune responses, for example by means of vaccination, will further enhance cellular immunity, and stimulate disease clearance in patients with VIN. Study protocols combining imiquimod with vaccination against HPV

are currently under development. In addition, therapeutic HPV-vaccine studies - that until now have not shown high efficacy in clinical trials[17,18] - are also ongoing.

In the meantime, it is planned for prophylactic HPV vaccination to be implemented in the general population, hopefully resulting in reduced rates of HPV-related vulvar (pre-) malignancies. Recently, Joura *et al* showed evidence that prophylactic administration of a quadrivalent HPV6/11/16/18 L1 virus-like-particle (VLP) vaccine, developed to prevent cervical cancer, also prevents HPV-related vulval and vaginal pre-cancers in 16 to 26-year-old women.[19] Yet, even if prophylactic vaccination effectively prevents HPV-infection in the majority of the human population, it is not to be expected that such a vaccine will abandon HPV-related disease within the next few decades. The fact that prophylactic HPV vaccination is targeted against a limited number of HPV types might contribute to ongoing disease. Until then, effective treatment is needed and imiquimod should be considered first-choice treatment for patients with usual type VIN.

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S Summary/Samenvatting

Summary

Vulvar intraepithelial neoplasia (VIN) is a rare condition from which an invasive carcinoma can develop. Most women suffer from severe and long-lasting symptoms, such as pruritus and vulvar pain. The incidence of VIN has increased over time, and nowadays patients are being affected at a younger age. As there is hardly any data on the follow-up of untreated patients, it is difficult to predict the outcome of disease for the individual patient. Studies with untreated patients who hardly ever progress to an invasive vulvar carcinoma have been published, whereas others have seen progression in nearly all untreated patients. Currently, standard therapy for high grade VIN comprises surgical removal of all visible lesions to relieve symptoms and to prevent the development of invasive disease. However, there are limitations to surgery. Despite extensive treatment, surgical margins are often positive. Recurrences are common, presumably because persistent infection with human papillomavirus (HPV), the viral cause of usual type VIN, is not affected by surgical treatment. Progression is seen as often after vulvectomy as after local excision. Moreover, one should realize that surgery can mutilate the vulva, thereby causing psychosexual distress.

The aim of this thesis was to investigate a new treatment strategy for VIN by means of an immunotherapy; a treatment that is effective by focusing on the viral cause of disease, without being mutilative to the patient. In the different chapters, attention is paid to clinical, histological, viral and immunological aspects of (this immunotherapy in) VIN.

A general introduction on VIN is presented in **Chapter 1**.

In **Chapter 2** we tried to establish the true natural history of VIN 3 from literature data. In a systematic review, data of 3322 women with VIN 3 (97 studies) were analyzed to assess both the risk of progression of VIN in untreated patients and the effect of surgical treatment in relation to recurrences and progression of VIN. From this data, there is no indication that recurrences of VIN depend on the type of surgery used, except for cryosurgery, which has a high failure rate (56%). Recurrences were seen as often after laserevaporization (23%) as after local excision (22%) or vulvectomy (19%). Recurrences were significantly lower, but not absent, after free surgical margins than after involved surgical margins ($P<0.001$). It could not be established whether free surgical margins diminish the change to progression. Untreated patients were more likely to progress to invasive vulvar carcinoma than patients treated for VIN (9% versus 3.3%). Spontaneous regression was seen in 41 (1.2%) of the 3322 patients; in 17 (41%) of them regression was related to pregnancy. From this

data, there is not enough evidence to support the removal of all involved vulvar skin. It is evident that only a prospective registration using a standardized pathology examination will provide information about the real natural history of VIN.

Chapter 3 describes the histology and HPV status in patients with a history of lichen sclerosus (LS) and VIN. It is commonly assumed that differentiated type VIN is related to LS, although evidence for this is limited to a small number of studies describing epithelial alterations adjacent to vulvar squamous cell carcinoma (SCC). In this study, we revised original biopsy and surgical specimens from patients with a history of both LS and VIN *without* coexistent SCC. In all 27 patients that met our inclusion criteria, LS was found to be related to undifferentiated VIN. HPV DNA was demonstrated in 31% of the lesions, and was strongly related to high grade VIN. During follow-up, three patients progressed to (early) invasive carcinoma. In two of these patients, differentiated VIN was observed overlying early invasive SCC.

In **Chapters 4 and 5** we investigated the effectiveness of imiquimod, a topical immune-response modifier with antiviral and antitumor activity, as a new treatment for patients with VIN. Imiquimod has been shown to be safe and effective in the treatment of genital warts. A pilot study, presented in **Chapter 4**, showed promising results, and was followed by a placebo-controlled, double-blind, randomized clinical trial (RCT). **Chapter 5** describes this RCT, in which 52 patients with usual type VIN were randomly assigned to receive either imiquimod or placebo twice a week for a period of 16 weeks. The follow-up period was 12 months. The primary outcome was a reduction of more than 25% in lesion size at 20 weeks. Secondary outcomes were histologic regression, clearance of HPV from the lesion, changes in immune cells in the epidermis and dermis of the vulva, relief of symptoms, improvement of quality of life, and durability of response. Lesion size was reduced by more than 25% at 20 weeks in 21 of the 26 patients (81%) treated with imiquimod and in none of those treated with placebo ($P<0.001$). Histologic regression was significantly greater in the imiquimod group than in the placebo group ($P<0.001$), and was strongly related to clearance of the underlying causative HPV infection, as was clinical regression. The number of immune epidermal cells increased significantly and the number of immune dermal cells decreased significantly with imiquimod as compared with placebo. Imiquimod significantly reduced pruritis and pain at 20 weeks and at 12 months. The lesion progressed to invasion (<1 mm) in three of 49 patients (6%) followed for 12 months (two in the placebo group and one in the imiquimod group). All nine patients (35%) showing a complete response at 20 weeks, remained free from disease at 12 months. Following the results of our studies, we concluded that imiquimod is effective in the treatment of VIN.

Genital infection with HPV is usually transient. However, when immunity 'fails' and the infection persists, VIN or other HPV-related diseases may develop. In **Chapter 6** we examined the epidermal and dermal distribution of different immunocompetent cells (dendritic cells, natural killer cells, T-cells) in patients with usual type VIN and compared this with data from healthy controls. Our results demonstrated that high-risk HPV-related usual type VIN lesions are characterized by an immunosuppressive state in the epidermis, showing a reduction of immature myeloid dendritic cells and $CD8^+$ T-cells. In the dermis, inflammatory activation is reflected by the influx of mature myeloid and plasmacytoid dendritic cells, natural killer cells and T-cells, suggesting that a cellular immune response on viral HPV infection occurs in the dermis of VIN patients.

Chapter 7 describes the impact of type 1 T-cell immunity on the clinical outcome of treatment with imiquimod in patients with usual type VIN. A detailed analysis with respect to the magnitude and cytokine polarization of the HPV16 E2-, E6- and E7-specific $CD4^+$ T-cell response was done by proliferation and cytokine bead array in patients with usual type VIN. Furthermore, HPV16-specific type 1 T-cell immunity was analyzed in patients before, during and after topical treatment with imiquimod. HPV16-specific proliferative responses were found in half of the patients, mainly associated with IFNγ-production. Although imiquimod did not enhance or induce T-cell immunity in any of the subjects, the presence of these type 1 T-cell responses is likely to be associated with a more favorable clinical response to imiquimod treatment (P=0.03).

Chapter 8 provides a general discussion based on the main findings.

Samenvatting

Vulvaire intraepitheliale neoplasie (VIN) is een zeldzame aandoening waaruit een invasief carcinoom kan ontstaan. Het merendeel van de vrouwen ondervindt ernstige en langdurige klachten in de vorm van jeuk en pijn. De incidentie van VIN is de afgelopen jaren toegenomen, en de diagnose wordt op steeds jongere leeftijd gesteld. Er is weinig bekend over het natuurlijk beloop van onbehandelde VIN. Het is daarom moeilijk een uitspraak te doen over de uitkomst van ziekte voor de individuele patiënt. Er zijn studies over patiënten beschreven met onbehandelde VIN die vrijwel nooit ontaardt in maligniteit, terwijl andere studies berichten over progressie in vrijwel alle onbehandelde patiënten. De huidige behandeling van VIN bestaat uit het chirurgisch verwijderen van alle zichtbare laesies, om klachten te verminderen en de kans op maligne ontaarding te voorkomen. Helaas heeft deze chirurgische benadering nadelige aspecten. Ondanks uitgebreide behandeling zijn chirurgische snijvlakken vaak positief. Daarnaast is de recidiefkans hoog, waarschijnlijk omdat de chirurgische ingreep weinig tot geen invloed heeft op de onderliggende causale infectie met humaan papillomavirus (HPV). Progressie na behandeling komt even vaak voor na vulvectomie als na lokale excisie. Het is van belang zich te realiseren dat chirurgische interventie een mutilerend effect kan hebben, hetgeen gepaard kan gaan met seksueel dysfunctioneren en psychosomatische stress.

Het doel van dit proefschrift was het onderzoeken van een nieuwe effectievere behandeling voor usual type VIN in de vorm van een immunotherapie. Hierbij ligt de nadruk niet langer op het verwijderen van de afwijking, maar op het uitschakelen van de oorzaak, namelijk infectie met HPV. In de verschillende hoofdstukken is aandacht besteed aan klinische, histologische, virale en immunologische aspecten van (deze immunotherapie bij) VIN.

Hoofdstuk 1 geeft een algemene introductie over VIN .

In **Hoofdstuk 2** is de beschikbare literatuur bestudeerd om het natuurlijke beloop van VIN te achterhalen. In een systematisch review zijn data van 3322 vrouwen met VIN 3 (97 studies) geanalyseerd, waarbij is gekeken naar de kans op progressie bij onbehandelde VIN en naar het effect van chirurgische behandeling bij VIN in relatie tot recidiefkans en progressie. Uit deze data blijkt dat de kans op een recidief niet afhangt van het type chirurgie dat is gebruikt, behalve bij cryochirurgie, waarbij sprake is van een hoge recidiefkans (56%). Recidieven komen even vaak voor na laserevaporisatie (23%) als na lokale excisie

(22%) of vulvectomie (19%). De kans op een recidief is lager, maar niet helemaal afwezig, wanneer de chirurgische snijvlakken vrij zijn ($P<0.001$). Of vrije snijvlakken de kans op progressie ook verlagen, was in deze studie niet aantoonbaar. Wel is duidelijk dat de kans op maligne ontaarding groter is bij onbehandelde VIN dan bij behandelde VIN (9% versus 3.3%). Spontane regressie werd gezien bij 41 (1.2%) van de 3322 patiënten; bij 17 (41%) van hen was regressie gerelateerd aan zwangerschap. Concluderend kunnen we zeggen dat er onvoldoende aanwijzingen zijn om het radicaal verwijderen van alle zichtbare laesies te ondersteunen. Alleen een prospectieve registratie waarbij gebruik wordt gemaakt van gestandaardiseerd pathologisch onderzoek zal duidelijkheid geven over het natuurlijke beloop van VIN.

Hoofdstuk 3 beschrijft de histologie en HPV status van patiënten met een voorgeschiedenis van lichen sclerosus (LS) en VIN. Algemeen wordt aangenomen dat gedifferentieerde VIN gerelateerd is aan LS, ook al is het bewijs hiervan beperkt tot een klein aantal studies die de epitheliale veranderingen naast een plaveiselcel carcinoom van de vulva beschrijven. In deze studie werd histologisch materiaal gereviseerd van patiënten met een voorgeschiedenis van zowel LS als VIN *zonder* carcinoom. Bij alle 27 vrouwen die aan onze inclusie criteria voldeden, kwam LS voor in combinatie met ongedifferentieerde VIN. HPV DNA kon worden aangetoond in 31% van de laesies, waarbij duidelijk een relatie werd gezien met hooggradige VIN. Tijdens follow-up ontwikkelden drie patiënten een invasief carcinoom. Bij twee van hen werd naast het carcinoom gedifferentieerde VIN aangetroffen.

In **Hoofdstukken 4 en 5** is de effectiviteit van imiquimod, een lokale immuun modulator met antivirale en antitumor activiteit, als behandeling bij patiënten met VIN bestudeerd. Imiquimod is veilig en effectief gebleken in de behandeling van condylomata acuminata. Na veelbelovende resultaten uit een pilot studie, beschreven in **Hoofdstuk 4**, werd een placebogecontroleerde, dubbelblinde, gerandomiseerde klinische studie opgezet. **Hoofdstuk 5** beschrijft deze studie, waarin 52 vrouwen met usual type VIN zijn gerandomiseerd voor een behandeling met imiquimod of placebo twee keer per week gedurende 16 weken. De totale follow-up periode was 12 maanden. Primaire uitkomstmaat was een reductie in laesiegrootte van >25% bij 20 weken. Secondaire uitkomstmaten waren histologische regressie, klaring van HPV in de laesie, veranderingen van immuuncompetente cellen in de epidermis en dermis van de vulva, vermindering van klachten, verbetering van kwaliteit van leven, en duurzaamheid van de klinische respons. Een reductie in laesiegrootte van >25% bij 20 weken werd gezien bij 21 (81%) van de 26 met imiquimod behandelde vrouwen, maar bij geen van de met placebo behandelde vrouwen ($P<0.001$). Histologische regressie trad significant vaker op in de imiquimod groep dan in de placebo groep ($P<0.001$), en was net als klinische respons sterk gecorreleerd met klaring van HPV. Het aantal immuuncompetente cellen in de epidermis nam significant toe na

behandeling met imiquimod, terwijl het aantal immuuncompetente cellen in de dermis significant daalde. In vergelijking met placebo waren klachten van jeuk en pijn significant verminderd na behandeling met imiquimod. Na 12 maanden follow-up waren alle negen patiënten met een complete respons bij 20 weken nog steeds vrij van ziekte. Progressie van de laesie naar invasie (<1mm) werd gezien bij drie van de 49 patiënten (6%) waarvan follow-up bekend was; twee daarvan zaten in de placebogroep, één in de imiquimod groep. Uit deze studieresultaten kunnen we concluderen dat imiquimod effectief is in de behandeling van VIN.

Genitale infectie met HPV is meestal van voorbijgaande aard. Echter, wanneer het immuunsysteem 'faalt' en er sprake is van een persisterende infectie, kan VIN of een andere HPV-gerelateerde afwijking ontstaan. In **Hoofdstuk 6** hebben we de epidermale en dermale verdeling van immuuncompetente cellen (dendritische cellen, natural killer cellen en T-cellen) in patiënten met usual type VIN onderzocht en vergeleken met data van gezonde controles. Onze resultaten lieten zien dat hoog-risico HPV-gerelateerde usual type VIN gekenmerkt wordt door een immuunsuppressieve status in de epidermis, hetgeen wordt geïllustreerd door een afname in het aantal myeloide dendritische cellen en het aantal $CD8^+$ T-cellen. In de dermis wordt VIN juist gekenmerkt door een influx van mature myeloide en plasmacytoide dendritische cellen, natural killer cellen en T-cellen. Dit wijst erop dat een cellulaire immuunrespons bij een virale infectie met HPV plaats vindt in de dermis van patiënten met VIN.

Hoofdstuk 7 beschrijft de impact van type 1 T-cel immuniteit op de klinische respons op behandeling met imiquimod bij patiënten met usual type VIN. Een uitgebreide analyse van de HPV16 E2-, E6- en E7 specifieke $CD4^+$ T-cel respons werd verricht middels een proliferatie assay met cytokine-profiel bij patiënten met VIN. Verder is gekeken naar HPV16-specifieke type 1 T-cel immuniteit voor, tijdens en na lokale behandeling met imiquimod. Bij de helft van de patiënten met VIN werd een HPV16-specifieke proliferatieve respons gezien, voornamelijk gepaard gaand met de productie van IFN-γ. Alhoewel behandeling met imiquimod geen invloed heeft op de omvang van de T-cel respons, lijkt aanwezigheid van deze type 1 T-cel respons te resulteren in een beter klinisch resultaat na behandeling met imiquimod (P=0.03).

Hoofdstuk 8 geeft een algemene beschouwing gebaseerd op de resultaten van de beschreven studies.

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Dankwoord

Het begon allemaal in Mozambique.

Februari, 2000. Ik had m'n coschappen net afgerond, en ging voor een paar maanden het avontuur tegemoet in Mozambique. Eerst een maand reizen, en daarna drie maanden als vrijwilliger aan de slag in een klein hospitaaltje in Namapa. Een dorpje zonder stromend water en zonder elektriciteit. Bericht dat het land geteisterd werd door overstromingen kwam bij pas bij ons aan, toen het thuisfront al drie dagen in rep en roer was. Dus wat schetste mijn verbazing toen er ineens een fax binnenkwam uit Nederland:
"…. Laat even weten of je geïnteresseerd bent in een baan als arts-onderzoeker. Er komt een onderzoeksplek vrij bij de gynaecologie en het is in eerste instantie voor een jaar…."
Afzender: Aagje Bais

U begrijpt, het is niet bij dat ene jaar gebleven.

Professor Helmerhorst, deze fax kwam natuurlijk eigenlijk van u. Ik heb even over het antwoord na moeten denken, maar eenmaal uit de Afrikaanse droom ontwaakt ben ik vol enthousiast het onderzoek ingestapt. Na de eerste kleine tegenvallers (het project waar ik instapte lag na 3 maanden volledig op z'n gat), hebben we de VIN-studie opgezet en zie wat er van is geworden. Zelfs het tweede proefschrift is bijna in aantocht! U heeft me geleerd altijd net dat beetje verder te kijken. En, als ik vond dat het daarna nog steeds beter en mooier kon, liet u me inzien dat het ook weleens goed was zoals het was. Bedankt voor alle vrijheid, en voor het vertrouwen dat er een einde aan zou komen, en dat er een goed einde aan zou komen!

Marc van Beurden, beste Marc, je bent een co-promotor uit duizenden. Eigenlijk was jij de motor achter de VIN-studie. Ik vind het bewonderenswaardig hoe jij in de beginperiode van mijn onderzoek de energie en de tijd kon vinden om mij op pad te helpen. Ik kon je altijd bellen, en als ik je weer eens op de valreep een nieuwe versie van het een of ander had toegestuurd met het verzoek dat svp zo snel mogelijk gecorrigeerd terug te sturen, kreeg je dat ook bijna altijd voor elkaar. Zelfs als je ergens op een vakantie adresje was… bedankt!

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Professor ten Kate, bedankt voor uw beoordeling van het manuscript en voor uw bereidheid deel te nemen in de kleine commissie. Ik zal onze revisiesessies 's ochtends vroeg om zeven uur niet snel vergeten.

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P.S. Neemt een van jullie Tipp-Ex mee, want er is bijna geen plaats meer op de muur van het zweetkamertje…

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Vera, Roy, Martine, Ed, Femke, Hans, Anne en Guy: al meer dan 15 jaar vriendschap! Ik ben hartstikke blij met jullie, en trots op wat we allemaal hebben bereikt! Dat er nog maar heel

veel etentjes, ski-vakanties, weekendjes Frankrijk en middagen gewoon-omdat-we-zin-hebben-om-te-kletsen mogen volgen.

Jaarclub Blixem, vanaf morgen ga ik de schade inhalen. Waar moet ik beginnen met kraambezoek?

M'n tennismaatjes Ellard, Nienka, Ad, Kaj en Carola, bij jullie kon ik letterlijk alles van me afslaan! De (champagne-)ontbijtjes houden we erin. Volgend jaar kampioen??

Lieve (broer) Marc, het is geweldig om jou als papa te zien! Doet je beseffen dat er veel belangrijkere dingen op de wereld zijn! Ik ben heel blij met jou, en Mandy en Robin.

Lieve papa en mama, zonder jullie onvoorwaardelijke steun was dit nooit gelukt. Bedankt voor al het vertrouwen dat jullie in me hebben. Dit boekje is voor jullie!

Lieve Mark, I can't believe that you're still there! Moving over from Brighton, living here with me in a time that I was in a constant hurry and did not have time for anything, but work. You helped me through the final bit, and made your sandwiches famous!
Morgen 1000 leuke dingen doen in Nederland???

Curriculum Vitae

Manon van Seters was born on May 27, 1973 in Goes, The Netherlands. She finished secondary school at the Sint Laurens College in Rotterdam in 1991. In the same year she started medical school at the University of Antwerp, Belgium, which she continued after one year at the Erasmus University in Rotterdam, The Netherlands. She obtained her medical degree in 1999. During her study she worked as a nurse assistant in the gynaecology department at the Erasmus Medical Center in Rotterdam (1994-1998). After graduating, she worked as a volunteer for four months in a rural hospital in Namapa, Mozambique. In 2000 she started her research activities as a PhD student at the Department of Obstetrics and Gynaecology in the Erasmus Medical Center, Rotterdam (Prof.dr. Th.J.M. Helmerhorst) and in the Academic Medical Center, Amsterdam (Prof.dr. M.P.M. Burger), resulting in the work described in this thesis. In September 2005 she started her residency in obstetrics and gynaecology in the Medical Center Rijnmond Zuid (MCRZ) in Rotterdam (dr. A.M. van Heusden), continuing in September 2007 at the Erasmus Medical Center Rotterdam (Prof. dr. C.W. Burger). She won a prize for the best oral presentation at the 3rd European Congress for Colposcopy and Cervical Pathology in Paris (2004); the Karel Verschoof prize for the best abstract (Werkgroep Cervix Uteri, 2004); and the Prof.dr. J.C. Birkenhäger award for her PhD project (Erasmus University Medical Center, 2007).

Color figures

Chapter 3

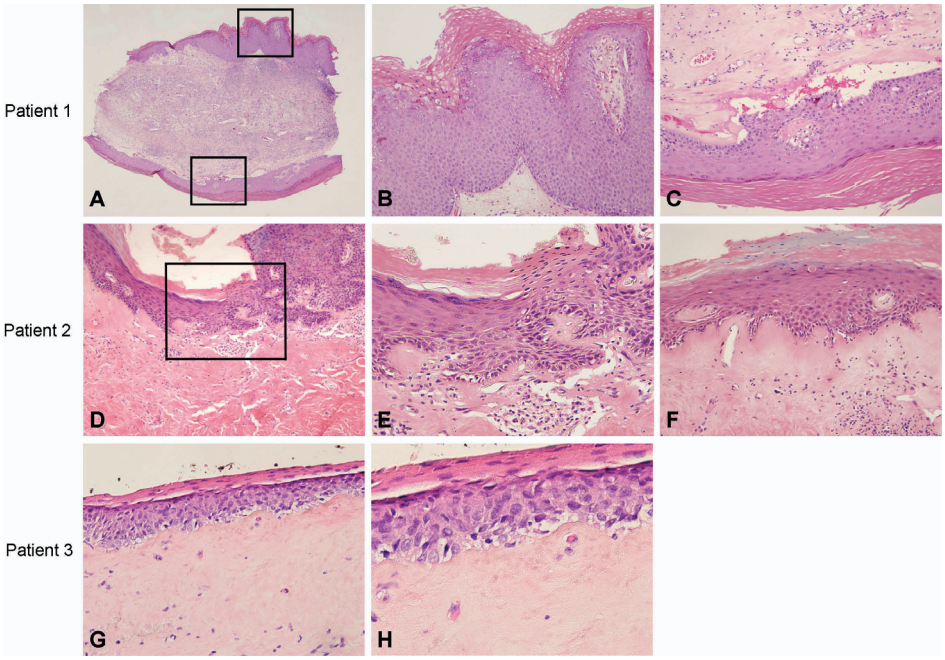


Figure 1. Patient 1: (A) vulvar biopsy with both condylomatous dysplasia and classic lichen sclerosus (LS; H&E, x20); (B) in more detail, verricuform dysplastic changes that tested positive for human papillomavirus (HPV)-59 DNA, showing mild-to-moderate disturbance of the epithelial architecture with acanthosis and koilocytosis, conform vulvar intraepithelial neoplasia (VIN) 2 (H&E, x100); (C) classic LS in more detail, showing oedema and hyalinisation of the dermis, vacuolisation of the dermal-epidermal junction, and hyperkeratosis, with normal architecture of the squamous epithelium (H&E, x100). Patient 2: (D) local excision of the vulva in a patient with a diagnosis of VIN 3, showing undifferentiated HPV16-positive VIN in the background of LS (H&E, x80); (E) a higher magnification of (D), showing the transition of normal to dysplastic epithelium (H&E, x200); (F) histological findings of LS in the same tissue with hyperkeratosis, thinning of the epidermis, and oedema with homogenisation of the dermal stroma (H&E, x125). Patient 3: (G) coexistence of LS and undifferentiated VIN 2 in the vulva (H&E, x200); (H) a higher magnification of (G), illustrating dysplastic changes that tested negative for HPV DNA (H&E, x400).

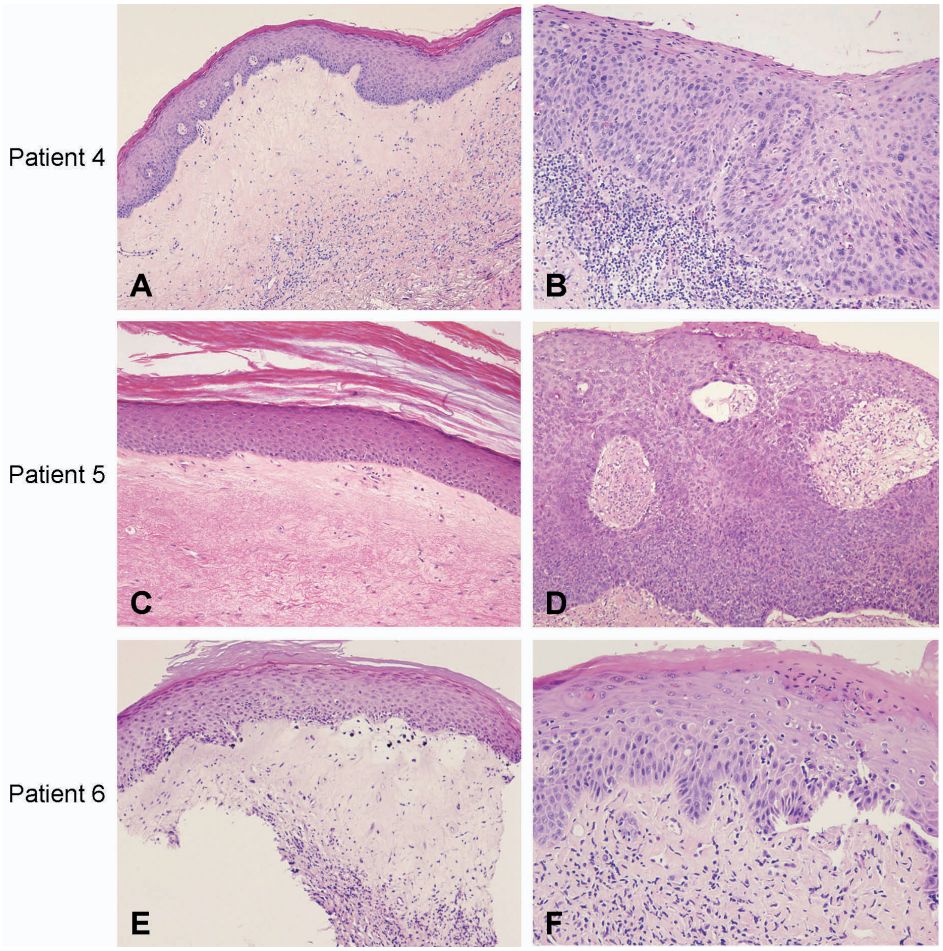


Figure 2. Patient 4: (A) lichen sclerosus (LS) showing a broad band of hyalinisation underlying an atrophic epidermis with loss of rete ridges (H&E, x50); (B) undifferentiated human papillomavirus (HPV) 16-positive vulvar intraepithelial neoplasia (VIN) 3 biopsied at the same time in the same patient, but in a different lesion (H&E, x100). Patient 5: (C) LS with normal epidermal maturation (H&E, x100); (D) undifferentiated HPV-negative VIN 3 in the same patient, which was diagnosed 6 years after the diagnosis of LS (H&E, x64). Patient 6: (E) classic LS showing extensive dermal hyalinisation with vacuolisation of the dermal-epidermal junction (H&E, x64); (F) undifferentiated HPV-negative VIN 1, with focal VIN 2 diagnosed 2 years after the diagnosis of LS (H&E, x160).

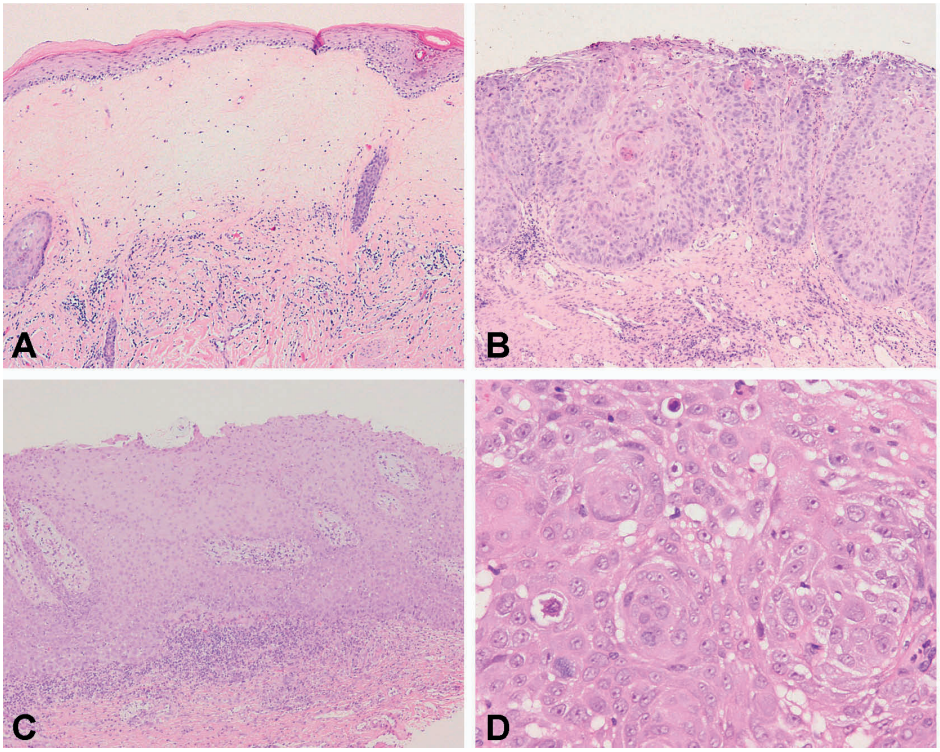


Figure 3. Patient 7: (A) lichen sclerosus (LS; H&E, x50); (B) undifferentiated vulvar intraepithelial neoplasia (VIN) 3 not related to human papillomavirus (HPV), which was diagnosed 9 years after the diagnosis of LS (H&E, x60); (C) one year later, this patient developed a lesion suspicious for squamous cell carcinoma, which was surrounded by HPV-negative differentiated VIN (H&E, x50). Little or no atypia is shown above the (para-) basal layer; (D) pearl-like changes can be distinguished (H&E, x250).

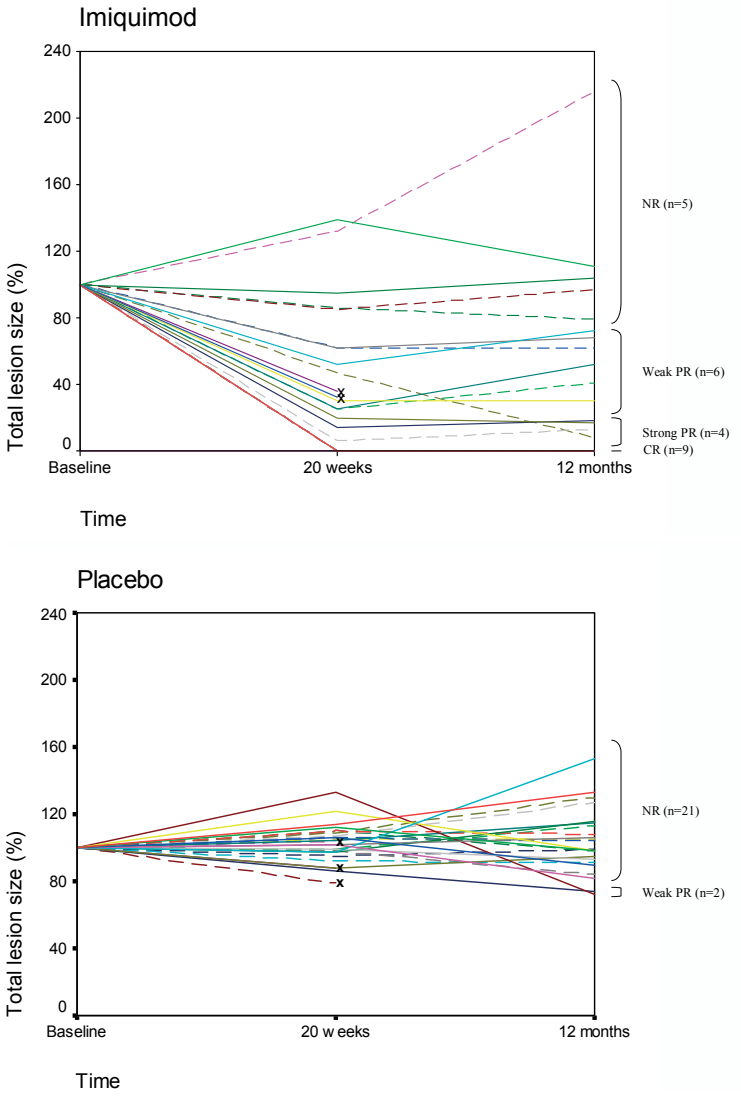
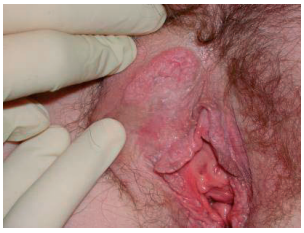
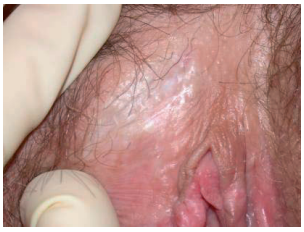


Figure 1. Effects of imiquimod and placebo on total lesion size at 20 weeks and at 12 months. Total lesion size as a percentage of baseline is shown at 20 weeks and at 12 months after the beginning of treatment with imiquimod (Panel A) or placebo (Panel B). The solid red line in Panel A represents the nine patients who had a complete response; one patient had no measurable disease but was treated anyway. Data were missing for five patients at 12 months.

Supplementary figure

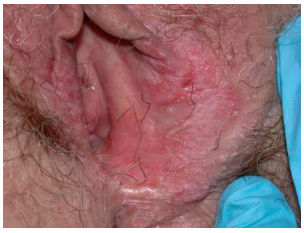


A



B

Patient 1



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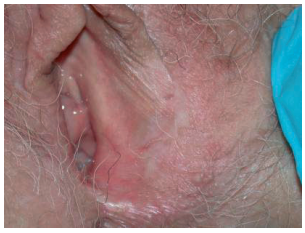
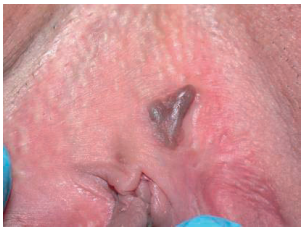


D

Patient 2



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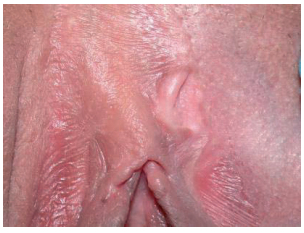

F

Patient 3

Supplementary figure 1. Clinical results before and after treatment with imiquimod.
Clinical pictures of three patients with HPV DNA-positive VIN 3 showing the results after treatment with imiquimod. Picture A, C, E: before treatment; B, D, F: after treatment. All three patients showed complete regression of the shown lesion, histological regression to VIN 1 (patient 1 and 2) or no dysplasia (patient 3), and clearance of HPV.

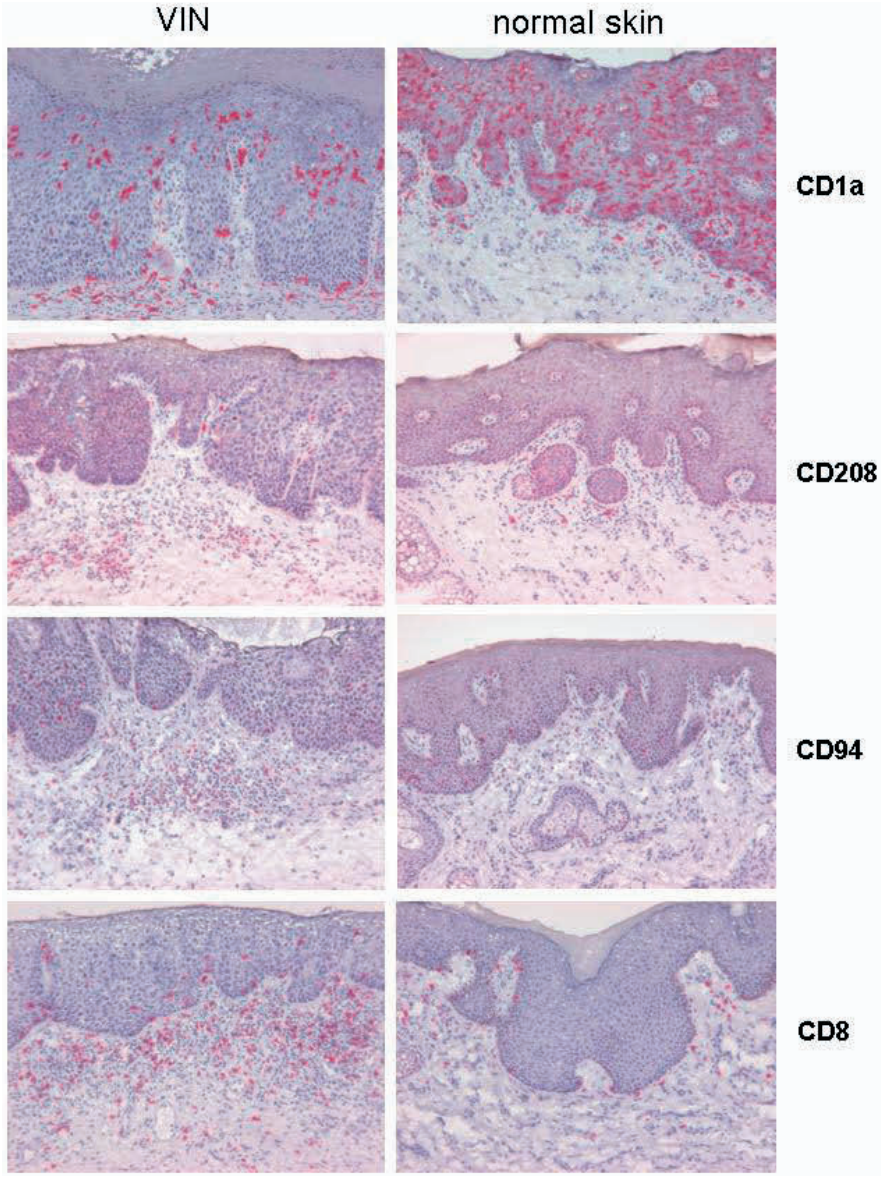

Figure 4 (photographs A-H, 10x). Representative photographs of positive (red stained) immunocompetent cells in VIN lesions and normal vulvar skin.
Compared to healthy controls VIN-affected skin showed a strong decrease of mDCs stained with CD1a in the epidermis (photograph A and B). Twice as many CD208$^+$ (mature) DCs were observed in the dermis of VIN compared to healthy controls (photograph C and D). NK cells stained with antibodies directed against CD94 are more numeric in the dermis of VIN-affected skin than in normal skin (photograph E and F). Less CD8$^+$ cells were observed in the epidermis, whereas significantly more CD8$^+$ cells were seen in the dermis of VIN-affected skin when compared to normal skin (photograph G and H).

Chapter 7

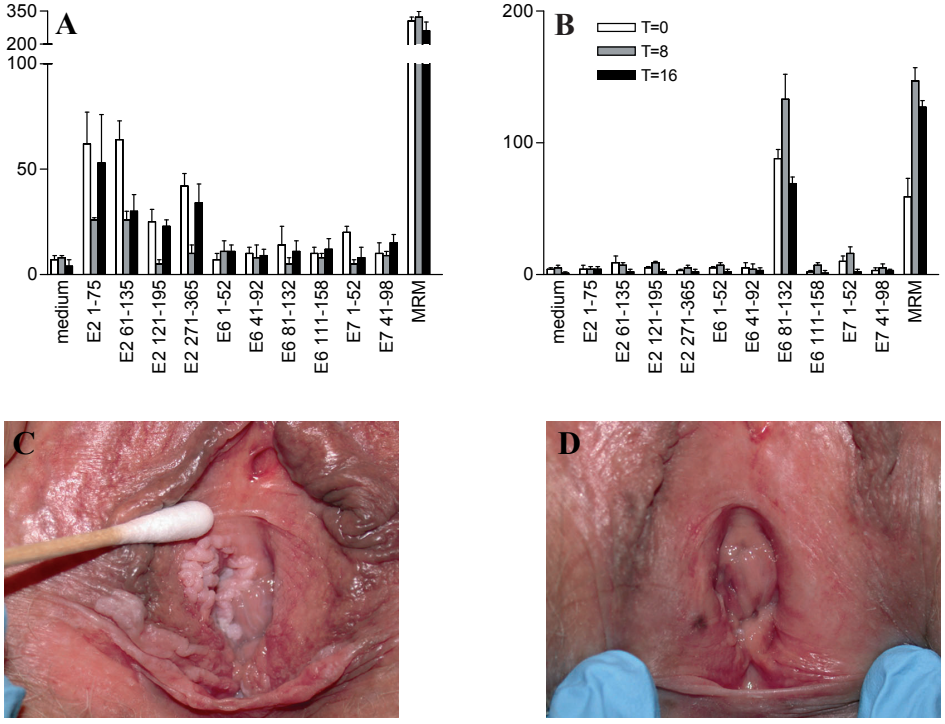

Figure 2. A and B, HPV16-specific IFNγ-producing T-cell responses in two representative patients with high grade VIN (patient 2, left and patient 10, right). T-cell responses are shown at week 0 (before imiquimod treatment), week 8 (during imiquimod treatment), and at week 16 (after imiquimod treatment). Local application of 5% imiquimod containing cream does not result in enhanced systemic HPV16-specific T-cell responses. Note that the magnitude of the T-cell responses varies slightly over the different time points. The mean number of spots and SE induced by the medium control or the peptides present in the E2, E6, and E7 pools per 100,000 PBMCs are depicted. As positive control, the memory recall mix (MRM) was used. C and D, patients with preexisting HPV16-specific T-helper type 1 responses show objective clinical responses after imiquimod treatment. A typical example is shown. C, biopsy-proven VIN 3 lesion of patient 5 before imiquimod treatment. D, the same vulvar area of patient 5 after 16 weeks of treatment.

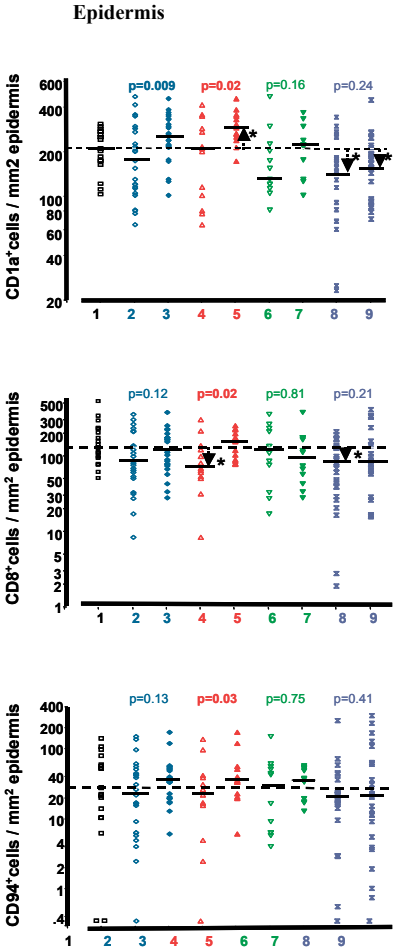

Figure 1. Results of immunohistochemical staining for CD1a$^+$ DCs, CD8$^+$ T-cells, and CD94$^+$ NK-cells in the epidermis of healthy normal skin (controls), and in VIN-affected skin before and after treatment with imiquimod or placebo.

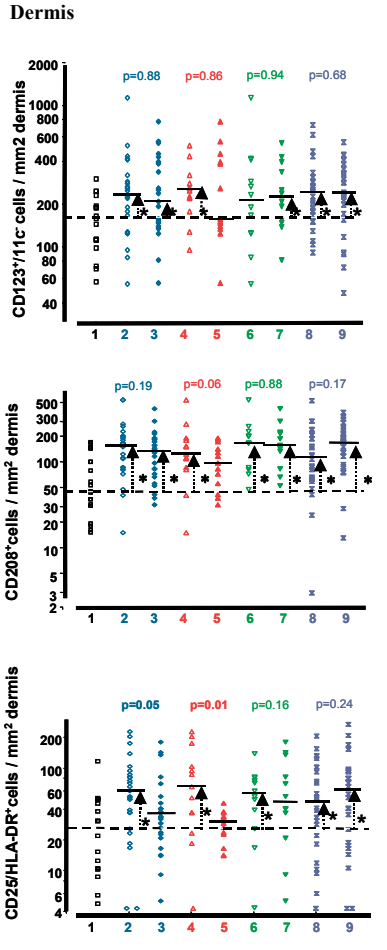

Figure 2. Results of immunohistochemical staining for CD123$^+$/11c$^-$ and CD208$^+$ DCs, and CD25/HLA-DR$^+$ Treg-cells in the dermis of healthy normal skin (controls), and in VIN-affected skin before and after treatment with imiquimod or placebo.

Values as median (range), logarithmic scale

1: controls (n=19)

2: imiquimod group before medication (all patients, n=25)

3: imiquimod group after medication

4: imiquimod group before medication (patients with clinical response > 75%, n=13)

5: imiquimod group after medication

6: imiquimid group before medication (patients with clinical response < 75%, n=12)

7: imiquimod group after medication

8: placebo group before medication (n=26)

9: placebo group after medication

* **p<0.05**