IMMUNE REGULATION IN PREMALIGNANT VULVAR AND VAGINAL DISORDERS

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Immune regulation in premalignant vulvar and vaginal disorders

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1

GENERAL INTRODUCTION

1.1 THE VULVA

During embryogenesis, the external genitalia develop under the influence of hormones. The vulva originates from the genital tubercle (clitoris), urogenital groove (vestibulum), urethral folds (labia minora) and genital swellings (labia majora).[1] Structures belonging to the vulva are the mons pubis, labia majora and minora, the vestibule of the vagina, the hymen, the clitoris and the external urethral orifice. The outer aspects of the vulva are covered with normal cornified skin epithelium, which consists of three layers; the epidermis, dermis and subcutis. The epidermis of labia majora, labia minora and the frenulum of the clitoris is cornified stratified squamous epithelium and is developed from the embryonic ectoderm. It is histologically made up of four layers: the stratum germinativum or basal layer that forms the boundary with the dermis, the stratum spinosum, the stratum granulosum, and the stratum corneum (Fig. 1). The vestibule is lined with mucosal epithelium of endodermal origin, as is the vagina. The vaginal epithelium, however, is derived from the embryonic mesoderm.[2,3] The transition of cornified epithelium to non-cornified mucous epithelium can be seen in some patients as the Hart's line. The skin functions as a barrier to protect women from harmful external influences, which is particularly important in the genital area because of exposure to a wide range of bacteria, fungi and viruses. Cells that reside in the skin are keratinocytes, melanocytes, Merkel cells and Langerhans cells (LCs).[3,4] The latter is an important resident of the epidermis; it is a bone marrow-derived dendritic cell which plays an essential role in the immune surveillance of the skin.[5]

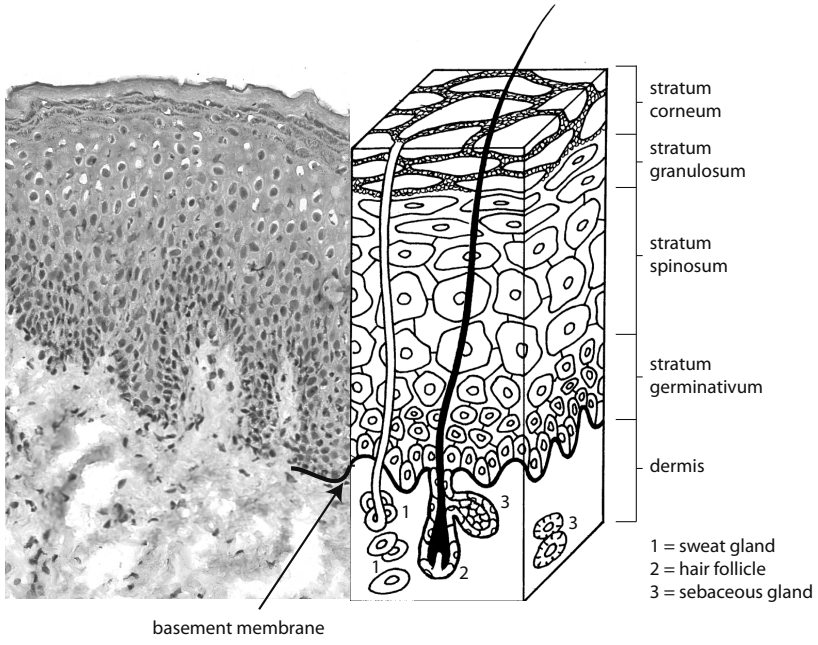

Figure 1. Section of vulvar skin.

1.2 IMMUNE RESPONSE

The role of the immune system is to protect us from potential dangerous microorganisms such as viruses and bacteria. The immune response is a complex interaction between cells, cytokines, chemokines and other soluble proteins. All these players have their own specific role, which is essential for an effective defense against infection.

1.2.1 Innate immune response

The innate immune response is the fast first line defense against an invading pathogen. It is a non-specific reaction initiated immediately after the first exposure to a microorganism. Soluble factors (e.g. complement molecules) and multiple immune cells are involved. These immune cells, like natural killer cells (NKs), mast cells, eosinophils, basophils, macrophages, neutrophils and dendritic cells (DCs) identify and eliminate pathogens that might cause infection. Additionally, they produce cytokines and chemokines that act together to recruit more immune cells to the site of infection.[6]

Of critical importance are the DCs, which are antigen-presenting cells (APCs) and the key regulators of the immune system. Different functional subsets exist; immature or mature myeloid DCs (mDCs) and plasmacytoid DCs (pDCs).[5,7] As mentioned before, Langerhans cells are the DCs that reside in the epidermis.[5]

Immature mDCs are responsible for the first recognition of antigens by toll-like receptors (TLRs) at their cell surface. TLRs recognize pathogen-associated molecular patterns (PAMPs), which are specific molecular structures conserved within a class of microbes.[8] After antigen-binding, the DC matures, expressing molecules that will lead to binding and stimulation of T cells, and migrates under the influence of chemokines to draining lymph nodes. Here they present antigens on their cell surface (bound to major histocompatibility complex (MHC)) to naive T cells. In this way, they can activate naive T cells, which is a crucial step in activating the adaptive immune response.[5] pDCs are less potent in their capacity to activate T cells than mDCs. They play a role in the defense against viral infections by producing large amounts of interferon-α (IFNα), which inhibits viral replication and stimulates cytotoxicity of macrophages and NK cells.[7,9]

In conclusion, the innate immune system responds to pathogens in a generic way and provides immediate defense against infection. Although it does not result in long-lasting immunity, it is able to induce the adaptive immune response.

1.2.2 Adaptive immune response

The adaptive immune response, in which T cells play a central role, regulates the destruction of infected cells and generates immune memory. It is a slow response, but highly specific.[10] As mentioned above, DCs can activate the adaptive immune response by presenting antigens to naive T cells in the draining lymph nodes. Binding to naive T cells will result in production of memory and effector T cells, such as T helper type 1 (Th1), Th2 and Th17 $CD4^+$ T cells, $CD8^+$ cytotoxic T cells, or regulatory

T cells (Treg cells). Activation of naive T cells by DCs is a complex interaction, and co-stimulatory signals are needed for an adequate activation. Cytokines produced by the DC provide the co-stimulatory signal that dictates whether the T-cell will take the Th1, Th2, Th17 or regulatory path (Fig. 2). Which kind of cytokines are produced, is in turn influenced by the response made by the innate immune system when it first recognizes the pathogen.[7,10-12]

The different effector T cells have a variety of functions and produce specific cytokines. $CD8^+$ cytotoxic T cells recognize virus-infected cells and kill them. Th1 $CD4^+$ T cells secrete IFNγ and promote the activation of macrophages, NK cells and cytotoxic $CD8^+$ T cells, hereby generating cell-mediated immunity. Th2 and Th1 $CD4^+$ cells coordinate the activation of B cells and production of different classes of antibodies, thus generating a humoral response. Th17 $CD4^+$ T cells enhance the acute inflammatory response by recruiting neutrophils to the site of infection. Finally, Treg cells are essential for controlling the immune response by production of inhibitory cytokines and are therefore important in preventing autoimmunity.[10]

In summary, the immune response is an intricate interplay between cells, cytokines, and chemokines, which is essential for protecting us from harmful pathogens. However, it can result in opposite effects when functions or interactions between players are disturbed, and in that case immune responses can potentially harm us. Hence, it is not surprising that disturbance of the immune response can result in a variety of diseases as discussed in the next paragraphs.

1.3 VULVAR INTRAEPITHELIAL NEOPLASIA

Vulvar intraepithelial neoplasia (VIN) is a vulvar skin disease with a malignant potential, which has increased in incidence over the last few decades affecting more and more

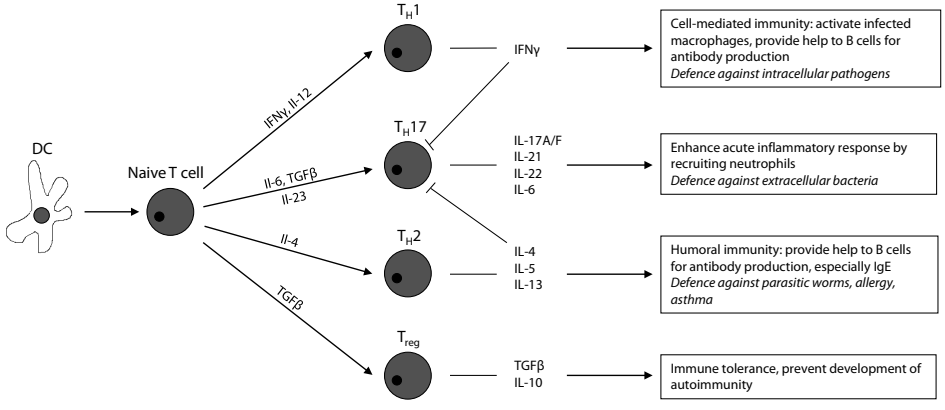

Figure 2. T-helper cell differentiation (modified from Tato and O'Shea, Nature 2006[11]). Driven by different types of cytokines produced by dendrite cells (DC), undifferentiated T helper cells can develop into Th1, Th2, Th17 or Treg cells, which all have their own specific cytokine profile and function.

young women.[13-17] In the past, various terms for VIN have been used, but in 2004 the terminology was revised. Since then, VIN can be divided in two subtypes: *usual type* VIN (uVIN), which is caused by a persistent infection with a high-risk type human papillomavirus (hrHPV), and *differentiated type* VIN (dVIN), which is associated with lichen sclerosus (LS) and lichen planus (LP).[18,19] Both subtypes differ in etiology, morphology, malignant potential, and consequently are treated differently.

1.3.1 Usual type vulvar intraepithelial neoplasia

uVIN is the most common subtype and it predominantly occurs in younger women with a peak incidence around 40 years of age.[16,20,21] Symptoms are pruritus and pain, but lesions can also be asymptomatic.[22,23] Typically, uVIN lesions are multifocal.[20,22,23] Accurate vulvar inspection during routine gynecologic examination is important for diagnosis, and to confirm diagnosis a biopsy of the most suspicious part of the lesion is required.[24]

HPV plays an important role in the etiology of uVIN, it can be found in 72–100% of VIN lesions, and in most cases HPV16 is detected.[21,25-29] Approximately 80% of all sexually active, female adolescents are at least once during their life-time infected with HPV.[30] When an hrHPV infection persists, a premalignant lesion, such as uVIN can develop.[31-33] If untreated, 9% of lesions will progress to invasive cancer within 1–8 years.[23]

Treatment of uVIN is a challenge, primarily because of its location, multifocality and tendency to recur.[34,35] Therefore, various treatments have been used in the past. For a long time, surgery was the treatment of choice.[35] However, since recurrence rates and psychosexual impact of mutilating surgery are high,[36-40] a trend towards more conservative treatments has been observed in recent years. These medical treatments, such as application with the immune response modifier imiquimod, or vaccination with therapeutic HPV vaccines, aim to enhance patients' immune response, and thereby treat the underlying cause of uVIN: a persistent infection with hrHPV.[41-47]

Topical treatments for uVIN are attractive because they can be applied by the patient and usually preserve the anatomy of the vulva. Imiquimod is an imidazoquinoline and its activity is mediated through stimulation of cells involved with the innate immune response including monocytes, macrophages and DCs.[48] Imiquimod binds to TLR 7 and 8 on DCs, which leads to activation of nuclear factor-kappa B (NF-κB). This activation results in secretion of multiple cytokines, including tumor necrosis factor alpha (TNFα), interleukin (IL)-12, IL-2, IL-6, G-CSF, GM-CSF, IFNγ, IFNα, IL-23, IL17A and IL-17F, as well as the chemokines IL-8 and monocyte-chemoattractant protein (MCP)-1 (Fig. 3).[48-50] Mouse studies showed that the IL-23/IL-17 axis is critical for imiquimod-induced skin inflammation.[50] The production of proinflammatory cytokines and chemokines results in an influx of immune cells to the site of application, in particular pDCs that produce high amount of IFNα, and in migration of LCs into the draining lymph nodes leading to increased antigen presentation.[48,49] Many of the cytokines induced by imiquimod can enhance adaptive

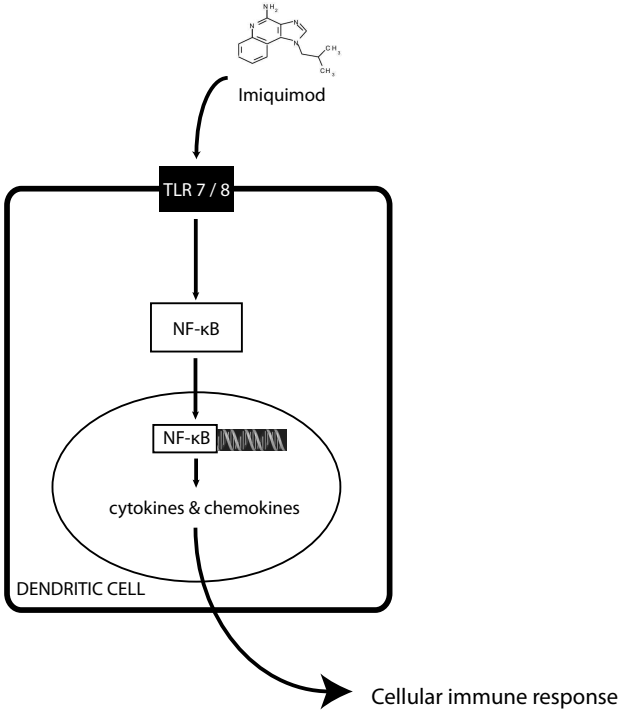

Figure 3. Imiquimod: mode of action. This figure is a simplified overview of the working mechanism of Imiquimod. It binds to Toll-like receptor (TLR) 7 and 8 on dendritic cells, which induces consecutive activation of nuclear factor-kappa B (NF-κB). This activation leads to secretion of multiple cytokines and chemokines, resulting in a profound cellular immune response.

immune responses, in particular Th1 production of IFNγ and other cell-mediated immune responses (e.g. Th17) that are important in the control of viruses, tumors, and intracellular pathogens. Besides TLR-dependent effects, imiquimod can also interfere with adenosine receptor signaling, hereby impairing an important negative feedback regulation that normally limits inflammatory responses, thus resulting in augmentation of the inflammatory response.[51]

Taken together, imiquimod is a potent immune activator and therefore the effectiveness of imiquimod was investigated in HPV-related disorders, as genital warts, uVIN and vaginal intraepithelial neoplasia (VAIN).[42,43,52-54] From the results of 17 studies on the efficacy of imiquimod treatment for VIN (210 patients in 1 randomized controlled trial (RCT), 10 case series and 6 case reports), 26–100% had a complete response to imiquimod, and 0–60% a partial response, while treatment duration ranged from 3 to 32 weeks.[43] Our department of Obstetrics and Gynecology also conducted an RCT and found a complete response to imiquimod therapy in 35% of patients and a partial response (a reduction in lesion size of >25%) in an additional 46% of patients. All complete responders were still disease-free at one year of follow-up.[42]

Recently, other immunomodifying treatments for uVIN as therapeutic vaccination against HPV or a combination of vaccination and imiquimod have been reported.[46,55] Complete response rate after vaccination with long-peptides of HPV16 viral oncoproteins E6 and E7 was 47% (9 of 19 women) and this response rate was maintained at 24 months of follow-up.[46] However, although results of immunomodifying treatments for uVIN are promising, long-term efficacy and recurrence rates are still unknown.[42,43,46,55]

1.3.2 Differentiated vulvar intraepithelial neoplasia

dVIN is very rare, <2–5% of all VIN lesions are of this type.[16,56] In contrast to uVIN, dVIN is not related to HPV, it is often found on a background of LS and is usually found in older women.[20] The clinical and histological diagnosis of dVIN is a challenge. Patients are often symptomatic with a long-lasting history of LS or LP-related symptoms of vulvar itching and/or burning.[57] In particular in patients with LS or LP, one has to be aware of dVIN in case of unifocal red lesions, areas with hyperkeratosis, ulceration or a rough and irregular surface.[20,57] However dVIN is mostly diagnosed in correlation with invasive squamous cell carcinoma (SCC), which is possibly due to the high malignant potential and the short intraepithelial phase of dVIN.[57,58] Because of its high malignant potential, the preferred treatment is radical surgical excision.[56]

1.4 LICHEN SCLEROSUS

LS is a chronic inflammatory skin disease that most frequently affects the anogenital area.[59] LS has a bimodal peak in incidence, it occurs mainly in elder women with a mean age of onset between the fifth and sixth decade, but it is also diagnosed in prepubertal girls.[60] Although various genetic-, autoimmune-, infectious- and local factors have been proposed to be involved in the etiology of LS, the exact cause is still unknown.[24,59-60] An autoimmunological basis of the disease is strongly suggested by studies describing the association of LS with HLA class II antigen DQ7.[61,62] An association was also observed between LS and autoimmune diseases, which showed that between 21.5 and 34% of all LS patients have an autoimmune disease and 21 to 74% of all patients have autoantibodies.[63-66] In addition, presence of autoantibodies against extra cellular matrix protein 1 and basement membrane zone (BMZ) components, and circulating autoreactive T cells against BMZ components have been demonstrated in LS patients.[67-69]

Patients with LS often present with severe pruritus and soreness of the vulvar and perianal area, but a few patients may be asymptomatic.[59,60,70] Lesions are white with textural changes as atrophy or hyperkeratosis, and appear as a figure-of-eight pattern around the vulva and anus. In advanced stages, there is destruction of vulvar anatomy characterized by resorption of labia, narrowing of the introitus and a buried clitoris. Histologically, LS is characterized by a thinned epidermis, vacuolar changes in

the basal layer, a band of homogenized collagen below the dermoepidermal junction and a band-like lymphocytic infiltrate.[59]

Since no cure exists for LS, therapies are primarily directed to reduce symptoms. Topical treatment with ultrapotent corticosteroids has proven to be the most effective treatment option.[71,72] In addition, as mentioned in section *1.3.2*, LS can progress to dVIN and subsequent vulvar SCC, although the exact pathogenesis is still unknown. Reported rate of progression to invasion is 4–5%.[59] Therefore, life-long follow-up is important.

1.5 LICHEN PLANUS

LP is another inflammatory dermatosis, which may involve the skin and mucosal surfaces. Most frequently the oral cavity and vulvovaginal area are involved.[73] The prevalence of LP is unknown, it accounts for about 1% of new cases seen in dermatology departments, and vulvar LP is diagnosed in about 3–6% of all patients seen in vulvar clinics.[73-75] Age of onset is generally between the fifth and sixth decade.[73,76,77] Similarly to LS, the etiology of LP is uncertain. There is evidence that it is immunologically mediated, since an association with HLA DRB1*0201 and autoimmune diseases as thyroid disease and vitiligo has been reported.[66,78] Autoreactive T cells and autoantibodies to BMZ components have also been demonstrated in LP.[67,79] However, the precise mechanisms leading to LP are unknown.

The clinical presentation of vulvar LP can be variable, and LP can affect the vulva in three different variants: a classical, a hypertrophic or an erosive form.[73] Erosive LP is most common, and in contrast to LS, vaginal involvement has been reported in up to 84% of patients with erosive LP.[73,75] Patients present with vulvar soreness, pruritus, burning, dyspareunia, and often vaginal discharge. Erosive lesions with denuded epithelium associated with typical white Wickham's striae can arise in vulvar or vaginal mucosa, and alteration of normal vulvar architecture may occur. In severe cases, adhesions and stenosis can lead to complete obliteration of the vagina. Histologically, LP is characterized by irregular acanthosis of the epidermis, basal cell liquefaction, and a band-like lymphocytic dermal infiltrate.[73,76,80]

As for LS, no curative therapy exists and topical treatment with ultrapotent corticosteroids is the first-line treatment option, although it does not abolish symptoms in the majority of patients.[76,77] Vaginal dilatators can be helpful in case of obliteration, but in severe cases surgery is needed to restore sexual function. Although the malignant potential of LP is unknown, malignant transformation to vulvar SCC has been reported in a few studies.[81,82] Therefore, likewise in patients with LS, life-long follow-up of LP patients is important.

1.6 OUTLINE OF THESIS

It is important that healthcare providers such as gynecologists and dermatologists are aware of the clinical features, behavior and management of different vulvar epithelial disorders, and therefore an extensive description about the relevant aspects of VIN, vulvar Paget's disease and melanoma in situ is given in **Chapter 2**.

Although imiquimod has proven to be an effective treatment for uVIN, long-term follow-up data about recurrence rates are still lacking. For that reason, the aim of the study described in **Chapter 3** was to evaluate long-term (>5 years) clinical response of VIN after imiquimod treatment.

Because previous observations demonstrated that success of imiquimod treatment in VIN patients is related to clearance of hrHPV, we assessed in **Chapter 4** immune cell counts and expression of $P16^{ink4a}$ in VIN tissue before and after imiquimod treatment, in relation to HPV clearance and clinical response.

Local inflammation and burning are common side effects of imiquimod treatment and patients often use nonsteroidal anti-inflammatory drugs (NSAIDs) to reduce these side effects. It is known that NSAID-use can potentially inhibit cell-mediated immune response, and therefore we investigated in **Chapter 5** whether NSAID-use interferes with the outcome of imiquimod treatment.

Treatment of VAIN is a challenge, with considerable morbidity and high recurrence rates. As for VIN, the underlying cause of VAIN is a persistent hrHPV infection. **Chapter 6** describes the results of a pilot study about the efficacy of imiquimod as a treatment for VAIN.

In **Chapter 7**, the immunological mechanisms involved in LS and LP were investigated in order to further clarify the pathogenesis of both diseases.

In **Chapter 8**, the main findings of this thesis are discussed and related to current and future perspectives about treatment of premalignant vulvar epithelial disorders.

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2

PREMALIGNANT EPITHELIAL DISORDERS OF THE VULVA: SQUAMOUS VULVAR INTRAEPITHELIAL NEOPLASIA, VULVAR PAGET'S DISEASE AND MELANOMA IN SITU

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ABSTRACT

No standard screening programs exist to detect vulvar carcinoma or its precursor lesions, and therefore gynecologists, dermatologists and other healthcare providers in this field should be aware of the clinical features, behavior and management of the different existing premalignant vulvar lesions, squamous vulvar intraepithelial neoplasia (VIN), vulvar Paget's disease and melanoma in situ. In 2004, a new classification for squamous VIN was introduced by the International Society for the Study of Vulvovaginal Disease (ISSVD), subdividing squamous VIN into the HPV-related *usual type*, and into *differentiated type*, which is associated with lichen sclerosus. This review describes the relevant aspects of squamous VIN, vulvar Paget's disease and melanoma in situ, its epidemiological characteristics, diagnosis, management, and malignant potential.

VULVAR INTRAEPITHELIAL NEOPLASIA

Terminology and classification

Squamous vulvar intraepithelial neoplasia (VIN) is a premalignant skin disorder that often causes severe and long-lasting pruritus, pain, and psychosexual dysfunction. It has a spectrum of clinical and histopathological appearances and can be divided into two subtypes: usual type VIN, which is caused by a persistent infection with high-risk human papillomavirus (HPV), and differentiated type VIN, which is associated with lichen sclerosus (LS). Squamous intraepithelial lesions were first described in 1912 by Bowen, and since then various terms have been used. In 1965, Kaufman grouped premalignant lesions into three categories: Queyrat's erythroplasia, Bowenoid carcinoma in situ, and carcinoma simplex.[1] A new simplified terminology was introduced by the International Society for the Study of Vulvovaginal Disease (ISSVD) in 1976, and all terms were replaced by carcinoma in situ and vulvar atypia.[2] Ten years later these terms were replaced by a single term, vulvar intraepithelial neoplasia (VIN).[3] In addition, VIN was graded, similar to cervical intraepithelial neoplasia (CIN), in three subtypes, VIN 1 (mild dysplasia), VIN 2 (moderate dysplasia) and VIN 3 (severe dysplasia).[3] This grading system suggests a biologic continuum of VIN lesions. However the presence of such a continuum is not supported by clinicopathological data. Therefore, the ISSVD abolished the grading system in 2004 and introduced a 2-tier classification for squamous VIN: *usual type* and *differentiated type* VIN. The two types differ in etiology, morphology, biology, clinical features and malignant potential.[4,5] However, the WHO-classification with the three subtypes VIN 1, 2 and 3 is still widely used.[6]

Usual type VIN (uVIN) has histologically been divided into warty, basaloid or mixed (warty/basaloid) VIN. uVIN is caused by a persistent infection with high-risk or oncogenic HPV (mostly HPV-type 16, 18 or 33).[7] It occurs predominantly in younger women and tends to be multifocal.[4]

Differentiated type VIN (dVIN) is less common, <2–5% of all VIN lesions are of this type, but it has the highest malignant potential.[1,8] It is not related to HPV but associated with LS and is usually found in older women.[9] dVIN is mostly unicentric and strongly associated with past or coincident invasive vulvar squamous cell carcinoma (SCC).[10,11]

Besides modifying the classification of VIN, the ISSVD also modified the grading system. Some studies demonstrated an overlap in the diagnosis of VIN 2 and VIN 3, while it was shown that VIN 1 only occurred in condylomata acuminata.[10,12] In addition, it was demonstrated that there is a lack of reproducibility of the pathologic diagnosis of VIN 1, 2 and 3, and that VIN 2 and 3 grouped together is better reproducible.[12,13] Nowadays, the term VIN is only applied to histologically 'high-grade' squamous lesions (VIN 2 and VIN 3). VIN 1 no longer exists.

Epidemiology

Over the last decades, the incidence of VIN has increased, most likely due to a rise in incidence of HPV infections. Overall incidence of vulvar SCC remained the same.[8,14-16] However, some studies reported an increase of vulvar SCC in younger women who tend to have a history of HPV and VIN.[16-18] A recent study observed the incidence of uVIN, dVIN and vulvar SCC in the Netherlands during a 14-year-period. The incidence of uVIN almost doubled from 1.2/100,000 patients in 1992 to 2.1/100,000 patients in 2005 and that of dVIN increased nine-fold from 0.013/100,000 patients to 0.121/100,000 patients, while the incidence of vulvar SCC remained stable.[8] The incidence of invasive vulvar SCC increases with age[8,19] and a higher rate of vulvar SCC has been observed in white women than in women of other races.[19]

Etiology

Usual type vulvar intraepithelial neoplasia. Lifetime risk to become infected with HPV in western societies is around 80% and approximately 40% of all sexually active, female adolescents are at least once infected with high-risk HPV (hrHPV).[20] When hrHPV persists (in less than 10% of cases), premalignant disorders of the lower anogenital tract, such as uVIN, can develop.[21-23]

The majority of VIN (90%) is of the usual type and persistent infection with hrHPV plays an important role in the etiology of uVIN. Reported prevalence of HPV in VIN range from 72 to 100% and in most cases HPV16 is detected.[7,24-29]

Immunosuppression and smoking (which reduces local immunity) are important risk factors for VIN[30-34] and it is known that immunosuppressed patients as HIV-positive women have an increased risk to develop uVIN.[31,35,36] Several immunological studies demonstrated that the host immune response is of critical importance in determining clearance or persistence of HPV-related VIN.[37-41] uVIN lesions are characterized by an immunosuppressive state in the epidermis,[40,41] while a reduced and insufficient immune response to the hrHPV infection occurs in the dermis of uVIN.[38,39,41] Furthermore, spontaneous regression of VIN has been observed with high detectable HPV-specific blood T-cell responses, while patients with persistent disease had no detectable anti-HPV T-cell responses.[37]

In the HPV-related pathways, a couple of molecular alterations occur due to infection and subsequent integration of hrHPV. HPV encodes for several viral proteins, of which the oncoproteins E6 and E7 are the most important. HPV E6 can interact with the tumor suppressor gene p53, leading to p53 dysfunction and consequently absence of cell cycle arrest.[42] HPV E7 can inactivate the retinoblastoma tumor suppressor gene pRb, which results in overexpression of the cell cycle related biomarkers $p16^{ink4a}$ and $p14^{arf}$, and hyperproliferation of infected cells.[43,44] As a result, most uVIN lesions are positive for $p16^{ink4a}$ and $p14^{arf}$, but p53 negative.[44-48] An increased expression of $p16^{ink4a}$ in combination with low p53 expression was observed in young women compared to older women with vulvar SCC.[49]

These markers are not conclusive in distinguishing uVIN from dVIN. It was observed that $p16^{ink4a}$ expression is high in all uVIN-associated tumors, but also in 10 of 105

dVIN-associated tumors. Almost all dVIN-associated tumors were HPV negative and none had integration of HPV, this is in contrast to the uVIN-related tumors of which 23 of 25 had integrated HPV.[50] Recently, it was shown that in all dVIN lesions gain of chromosome 3q26 was present, while this was only the case in 50% of uVIN lesions. Detection of 3q26 imbalance could be of additional diagnostic value in the diagnosis of VIN lesions together with staining for $p16^{ink4a}$ and p53.[51]

Differentiated type vulvar intraepithelial neoplasia. In contrast to uVIN, the presence of HPV in dVIN is very rare and the exact cause of dVIN is still unclear.[11,50,52,53] It is assumed that dVIN is related to LS[9] and several reports show a clear relationship between dVIN and LS in adjacent skin of vulvar SCC.[54-58] However, it seems that VIN associated with LS *without* coexisting vulvar SCC is more likely of the *undifferentiated* type.[59] Squamous hyperplasia is also commonly seen in adjacent epidermis of dVIN patients, and might be a step in carcinogenesis.[11,60] dVIN is often HPV-negative and p53 positive.[11,45,61,62]

Clinical features and diagnosis

The clinical presentation of VIN is diverse. Lesions can be red, white and pigmented, either flat or raised, erosions or ulcers may be present. Symptoms as pruritus or pain are observed in about 60% of patients.[63,64] Since patients can be asymptomatic, accurate vulvar inspection during routine gynecologic examination is important. Clinical features that can help in making the correct diagnosis are color, thickness, surface and focality.[1] To confirm the diagnosis, a biopsy of the most suspicious part of the lesion should be performed under local anesthesia.[65]

Commonest affected sites are labia majora and minora and the fourchette.[32,63] uVIN lesions are often multifocal.[9,63,64] In addition, multicentric disease (lesions of cervix, vagina or anus) is common in uVIN patients and is age-related, as it decreases from 59% in women aged 20–34 to 10% in women over 50 years of age.[9,29,32,66] Therefore, a careful examination of the lower anogenital tract (vulva, perineum and perianal areas) which also includes the cervix and vagina, is mandatory.

The diagnosis of dVIN is a challenge. dVIN lesions are almost always observed in areas of LS or lichen planus (LP), only one case report describes dVIN as a solitary lesion in a patient without a history of LS or LP.[67] Red lesions and areas with hyperkeratosis, ulceration or having a rough and irregular surface are suspicious for dVIN.[9,11] Patients are often symptomatic with a long-lasting history of LS or LP-related symptoms of vulvar itching and/or burning.[11]

Because of the highly malignant potential of dVIN, any suspicious area in patients affected by LS or LP should be biopsied or excised without delay to obtain a representative histopathological diagnosis.

Histology

Usual type vulvar intraepithelial neoplasia. Histopathologically, uVIN can be classified into different subtypes: *warty* and *basaloid*. Both can be easily recognized. Typically, the epidermis is thickened and is accompanied by a surface reaction of

hyperkeratosis and/or parakeratosis. There is loss of cell maturation with associated nuclear hyperchromasia, pleomorphism and numerous mitotic figures at all levels of the epidermis.[9] The intraepithelial process may also involve the underlying skin appendages.[68,69] The epidermis of *warty* VIN has wide and deep rete ridges, often reaching close to the surface, which gives a characteristic condylomatous appearance. There is striking cellular polymorphism and evidence for abnormal cell maturation. Koilocytosis, corps rounds, multinucleation, (abnormal) mitotic figures and acanthosis are common.[9] *Basaloid* VIN is characterized by a thickened epithelium with a relatively flat and non-papillomatous surface. Large numbers of relative uniform undifferentiated cells with a basaloid appearance are seen in the epidermis. Mitotic figures are numerous, but koilocytic cells and corps rounds are less frequently seen than in warty VIN. Patterns of warty and basaloid VIN are often found in the same lesion, which is referred to as *mixed* VIN.[9]

Differentiated type VIN can be easily mistaken for a benign dermatosis because of the high degree of cellular differentiation and absence of widespread architectural disarray.[5,9,11,62] Histological characteristics include a thickened epithelium with parakeratosis, elongated and anastomizing rete ridges, and enlarged abnormal keratinocytes with premature eosinophilic cytoplasmic differentiation.[9,11] These abnormal keratinocytes are confined to the basal and parabasal layers and are a hallmark of dVIN.[9] No studies have been performed to investigate the reproducibility of the histopathological diagnosis of dVIN. Staining with MIB1 (Ki-67), a marker that visualizes proliferating cells, can be helpful in distinguishing dVIN from normal vulvar epithelium. The basal layer of dVIN is positive for MIB1, while normal vulvar epithelium is characterized by an almost MIB1-negative basal layer.[70] Another tool that might be helpful is staining for p53 protein. Alteration in the p53 tumor suppressor gene appears to be involved in the development of dVIN and overexpression of p53 has been demonstrated in dVIN.[11]

Psychosexual impact

Since the incidence of VIN has increased dramatically – particularly in younger women[16] – attention should be paid to the psychosexual consequences of VIN and vulvar excision. However, the number of studies concerning psychosexual impact of VIN and vulvar surgery is limited. In general, surgical treatment of vulvar lesions may lead to disfigurement, postoperative loss of quality of life (QoL) and impaired sexual function.[71-73] In addition, sexual function is correlated to the extent of excision.[73,74] Therefore, treatments which preserve the anatomy of the vulva such as imiquimod or CO_2-laser vaporization can be important to prevent psychosexual sequelae. However, large studies comparing the effect on QoL of various treatments for VIN are lacking. A recent study evaluated the prevalence of psychological morbidity in women with VIN. Moderate-to-severe anxiety was observed in 32% and moderate-to-severe depression in 18% of women.[75] These psychological factors were strong determinants of QoL, while clinical variables such as duration of disease, presence of symptoms and number of treatments did not have a measurable impact on QoL.[75] In conclusion, clinicians

should give attention to the psychological and sexual consequences of VIN treatments. Future studies should focus on predictive factors that impair sexual function and QoL after excision for VIN and investigate whether outcomes on QoL and sexual function are better following anatomy- and function-preserving treatments.

Treatment modalities

Usual type vulvar intraepithelial neoplasia. For a long time, choice of therapy for uVIN has been dominated by the premalignant nature of the disease. In the past, extensive surgery such as vulvectomy has been performed to remove the disease. However, surgical margins are often positive, irrespective of the type of surgery, and high recurrence rates are common.[64,76,77] In 1995, Kaufman addressed the importance of individualization of treatment. Treatment should be directed towards preservation of the normal anatomy and function of the vulva.[78] Therefore, more limited surgery consisting of surgical removal of all visible lesions has been the surgical technique of choice since the last decades.[78] Surgical treatment can be performed with different techniques. Cold knife surgery or CO_2-laser vaporization have been used as a single technique or in combination. Laser vaporization can be an effective method in non-hair bearing areas. But because this technique destroys all tissue, it is recommended to take representative biopsies beforehand.[1,76] In conclusion, surgical treatment is effective in removal of premalignant lesions, but recurrence rates are high and one has to be aware of the effect of surgical treatment on quality of life and sexual function.

The main advantages of medical treatment are preservation of vulvar anatomy and function. Topical treatment is attractive because it can be applied directly by the patient and is easily monitored for efficacy. However, medical treatment does not provide a specimen for histological evaluation with the risk that early invasion is overlooked. Hence, taking accurate biopsies is important before starting medical treatment.

Imiquimod 5% cream is a topical immune response modifier that acts by binding on Toll-like receptor (TLR) 7 on the cell surface of dendritic cells, thereby inducing secretion of proinflammatory cytokines. This results in a profound tumor-directed cellular immune response.[79,80] The effectiveness of imiquimod has been assessed in several studies. A systematic review showed the results of 210 patients from 17 studies (1 RCT, 10 case series and 6 case reports). Treatment duration ranged from 3 to 32 weeks and follow-up from 1 week to over 30 months. Complete regression was observed in 26–100% of patients, 0–60% had partial regression and 0–37% experienced recurrence. Most common adverse events were local burning and soreness.[81] Recurrence data of imiquimod treatment compared with data from a historical cohort of surgically treated patients showed that the recurrence rate after 16 months follow-up was 20.5% for imiquimod treated patients and 53.5% for surgical treated patients.[82] Two double-blinded RCTs comparing placebo and imiquimod were performed.[83,84] In the first, 31 patients were treated with an escalating dose regimen during 16 weeks. Complete regression was observed in 81% and partial

regression in 10% of the imiquimod treated patients, while none of the patients treated with placebo showed a response. No progression to invasive disease was observed.[83] In the second RCT, a reduction in lesion size was observed in 81% of cases (35% complete responders, 46% partial responders), in comparison with 0% in the placebo group ($P<0.001$). In addition, no HPV DNA could be detected anymore in 58% of imiquimod treated patients. Two patients treated with placebo and one patient treated with imiquimod progressed to invasion (<1 mm). Reduction of lesion size was correlated with partial normalization of the numbers of immunocompetent cells.[84] It was shown that imiquimod increases the magnitude of the HPV16 specific $CD8^+$ T-cell activity in VIN patients, but magnitude and specificity of the response had no correlation with the clinical response.[85] Another study investigated the role of HPV16-specific interferon-γ (IFNγ) associated $CD4^+$ T-cell immunity in the clinical effect of imiquimod treatment.[86] No enhanced HPV16-specific $CD4^+$ T-cell response was seen upon imiquimod treatment, but, interestingly, a preexisting HPV-specific type 1 T-cell response was associated with a more favorable clinical outcome upon imiquimod treatment.[86] Imiquimod is now recommended as a first-line treatment for VIN. However, long term follow-up data after imiquimod treatment have not been reported so far, but are expected in the near future.

Topical photodynamic therapy (PDT) uses a tumor-localizing photo sensitizer, 5-aminolevulinic acid (ALA), in combination with non-thermal light of an appropriate wavelength to generate oxygen-induced cell death. Its efficacy has been proved in nonmelanoma skin cancer.[87] Several nonrandomized and uncontrolled studies were conducted to assess the efficacy in uVIN. Response rates vary from 0 to 71%.[88-93] Small unifocal lesions often respond to PDT, but multifocal, pigmented and high grade lesions are less likely to respond.[89,90] Furthermore, uVIN lesions that failed to respond were more likely to have detectable HPV levels than responsive uVIN lesions.[88,90] Recurrence rate (around 48%) does not significantly differ from surgery or laser vaporization.[94] Advantages of PDT are minimal tissue destruction, short healing time and limited side effects.[88-93] Recently, a study was published in which 20 uVIN patients were treated with sequential imiquimod combined with PDT. In this study, the overall response rate was 55% and at 52 weeks, 65% of patients were asymptomatic, compared to 5% at baseline.[95]

Therapeutic vaccines have been developed to enhance T-cell mediated immunity in uVIN lesions. Most vaccines elicit a specific immunity against the HPV E6 and E7 proteins. A reduction in lesion size of at least 50% was observed in 8 of 18 women vaccinated with TA-HPV, a recombinant vaccinia virus,[96] while others observed with the same vaccine a 50% reduction in lesion size in 5 of 12 patients. One patient showed complete regression.[97] Two studies investigated the effectiveness of three immunizations with HPVL2E6E7 protein combined with a single dose of vaccinia HPV 16/18 E6/E7 either before or after the immunizations.[98,99] Of 39 patients, nine showed reduction in lesion size, while 25 remained stable and five patients progressed. It was shown that this regimen is immunogenic, but no relation between induction of

HPV-16-specific immunity and clinical outcome was observed.[98,99] In a recent study, 20 women with HPV-16-positive high-grade VIN were vaccinated 3 or 4 times with a mix of long peptides from the HPV-16 viral oncoproteins E6 and E7. At 3 months after vaccination, five had a complete regression of the lesions and this number increased to nine at 12 months after last vaccination. Complete response rate was maintained at 24 months of follow-up. In addition, a partial response was seen in seven patients after 3 months and in six patients at follow-up after 12 months. Vaccine-induced T-cell responses were seen in all patients. Complete responders had a significantly stronger response of IFNγ-associated proliferative $CD4^+$ T cells and a broad response of $CD8^+$ IFNγ T cells than did non-responders, and therefore, complete responses appeared to be correlated with induction of HPV-16-specific immunity.[100]

In the past, several other medical therapies such as 5-fluorouracil, cidofovir, interferons and indole-3-carbinol have been investigated as a treatment for VIN.[101-105] However, studies are small and due to limited effectiveness or severe side effects, none is considered as a standard therapy for VIN.

As indicated by the number of treatment options for VIN, none is 100% effective and a lot of treatments are a burden for the patient. Consequently, some patients may not want to undergo treatment. A wait-and-see policy, aimed at controlling symptoms and prevention of malignancy, is an option.[106] Frequent follow-up visits are advised including careful examination and biopsies in case of suspected malignancy.

Since the host immune response plays an important role in clearance of HPV, prophylactic vaccination could be effective in prevention of HPV-related disease. In order to prevent infection and subsequent development of CIN and cervical cancer, several vaccines have been developed during recent years. There is a quadrivalent vaccine, which acts against HPV 16, 18, 6 and 11 and a bivalent vaccine, which acts against HPV 16 and 18. In a combined analysis of three randomized trials, vaccination with the quadrivalent vaccine was 97% effective in preventing uVIN associated with HPV 16 and 18 in a population that was naive to these viruses at time of first vaccination and 100% effective in a population that was naive throughout completion of the vaccination regimen. In the intention-to-treat population (which included women who, at day 1, could have been infected with HPV 16 or HPV 18), vaccine efficacy was 71%.[107] There are no data on the prevention of uVIN by the bivalent vaccine.

Differentiated type vulvar intraepithelial neoplasia. The preferred treatment for dVIN is radical surgical excision, as it occurs often in correlation with invasive SCC. Follow-up should take place at a specialized vulvar clinic or by a vulvar specialist who has had additional training in managing vulvar disease.[108]

Malignant potential

Usual type vulvar intraepithelial neoplasia. Although dVIN has a much higher malignant potential than uVIN,[8,11,57,67] the malignant potential of uVIN should not be underestimated. It has been shown that uVIN is highly proliferative.[109] A meta-analysis showed that 8/88 (9%) of untreated uVIN patients developed invasive SCC within 1–8 years.[64] Others observed a much higher percentage, 10/63 (15.8%) of untreated

patients progressed in 1.1–7.3 years.[76] In treated patients, rate of progression during follow-up after treatment was 3.3% (208/3322).[64] Progression rate was not affected by the surgical extent.[64] A population-based study from Norway showed that free resection margins did not prevent progression to invasive SCC. In this study, 50% of patients who developed vulvar SCC after treatment had free surgical margins.[15] Therefore, one should not enlarge the extent of resection to prevent progression.

The malignant potential of uVIN is also illustrated by the finding of occult carcinomas in VIN. A recent study showed an occult cancer rate in vulvar biopsies of 3.8% for VIN 2 and 11.9% for VIN 3 (the term uVIN was not used).[110] Other studies reported occult carcinoma rates of 3.2–18.8%.[64,111] Known risk factors for progression of uVIN are advanced age, raised lesions, immunosuppression and radiotherapy.[8,64,112] In addition, it has been suggested that basaloid type uVIN is of greater risk to progress than warty type.[5]

Spontaneous regression has also been described in patients with uVIN. It was observed in 1.2% of patients, all were younger than 35 years and often related to pregnancy,[64] presenting most often with multifocal pigmented lesions.[76]

Differentiated type vulvar intraepithelial neoplasia. A recent study showed that the overall percentage of dVIN lesions with subsequent diagnosis of SCC was 32.8% while this was 5.7% for uVIN lesions. Furthermore, median time from dVIN to SCC was 22.8 months, while median time from uVIN towards SCC was 41.4 months.[8] The relation between a prior, synchronous or subsequent vulvar carcinoma and dVIN is three times higher than uVIN (85.7% *vs.* 25.7%).[57] Furthermore, vulvar cancer arising on a background of dVIN appeared more likely to recur than cancers arising from uVIN.[58] Another study also noted that the prognosis in terms of 5-year disease-free and overall survival rate were worse in patients with vulvar SCC associated with LS and squamous cell hyperplasia 'with or without atypia' (the term dVIN was not used) than in patients with uVIN-associated SCC.[113]

Conclusion

The incidence of VIN has increased. It is of critical importance to distinguish uVIN from dVIN. Both subtypes have a different clinical appearance, pathology and – most important – malignant potential. Radical surgical excision is treatment of choice for dVIN, while a more conservative approach is recommended for uVIN lesions. uVIN occurs predominantly in younger women and an individual approach, including attention for the psychosexual sequelae, is important. Because of high recurrence rates after various treatments, a thorough follow-up regimen by trained vulvar specialists is recommended.

Recently, most western countries introduced prophylactic vaccination against HPV, which can potentially prevent most HPV-related premalignant lesions and about one third of all vulvar carcinomas. In the mean time, further studies are needed to improve treatment outcome.

VULVAR PAGET'S DISEASE

In 1986 the ISSVD classified vulvar extramammary Paget's disease (EMPD) as a non-squamous intraepithelial lesion of the vulva.[3] Vulvar EMPD is a relatively rare intraepithelial carcinoma. It affects mainly postmenopausal women, with a median age of 72 years. The most common signs and symptoms are itching, burning, moistening and bleeding, for up to 5 years.[114-118] Substantial delay between appearance of symptoms and diagnosis can occur in many patients, and this is significantly associated with larger lesions.[119] It is usually multifocal and may occur anywhere on the vulva, mons, perineum, perianal area, or inner thigh. There are often multiple extensive lesions presenting as moderately well demarcated, scale, moist, eczematoid, erythematous-white plaques often dotted with small, pale islands.

Vulvar EMPD predominantly is an intraepithelial lesion, but has the potential for dermal invasion and on occasion has been associated with an underlying adenocarcinoma. In one large study, 26% of patients had other primary tumors, such as breast, pancreas, endometrium, bladder, stomach and rectum malignancies. Associated vulvar adenocarcinoma (4%) and invasive vulvar EMPD (16%) may frequently coexists and recurrence rate of vulvar EMPD is high (35%).[114] Like in squamous cell carcinoma of the vulva, there is evidence to support the recognition of a category of minimally invasive vulvar EMPD (≤1 mm depth of invasion), that has a low risk of distant metastasis and death caused by disease.[120,121]

Histopathological examination shows epidermal acanthosis and elongated rete ridges. Paget's cells are large intraepidermal cells with a large nucleus that often has a prominent nucleolus and abundant usually clear, mucin positive, pale cytoplasm. The cells may occur singly in small clusters or large nests. The squamous epithelium is often hyperplastic with hyper- or parakeratosis. The Paget's cells may extend into the adnexal duct and pilosebaceous units[114,118,121] and they may be a proliferation of adnexal stem cells residing in the infundibulo-sebaceous unit of hair follicles and adnexal structures.[122] It has been suggested that at least a proportion of vulvar EMPD arises multicentrically within the epidermis from pluripotent stem cells.[123] There is evidence that vulvar EMPD represents a heterogeneous group of epithelial neoplasms that can be similar both clinically and histopathologically. Vulvar EMPD can be classified based on the origin of the neoplastic Paget's cells as either primary (of cutaneous origin) arising within the epithelium of the vulva, or secondary (of noncutaneous origin), resulting from the spread of an internal malignancy, most commonly from an anorectal adenocarcinoma or urothelial carcinoma of the bladder or urethra, to the vulvar epithelium. Primary EMPD can be further subdivided into a primary, intraepithelial cutaneous form with and without invasion and into an intraepithelial cutaneous Paget's disease as a manifestation of underlying skin appendage adenocarcinoma. Secondary EMPD has an anorectal, urothelial or other origin. These subtypes can present similarly on the skin and may appear similar on routine hematoxylin and eosin-stained slides. Immunohistochemical studies can be used to help differentiate them. Primary vulvar EMPD is immunoreactive for CK 7

and GCDFP-15, but uncommonly for CK 20. Vulvar EMPD secondary to anorectal carcinoma demonstrates CK 20 immunoreactivity but is usually nonreactive for CK 7 and consistently nonimmunoreactive for GCDFP-15. Vulvar EMPD secondary to urothelial carcinoma is immunoreactive for CK 7 and CK 20 but nonimmunoreactive for GCDFP-15. In addition, UP-III, which is specific for urothelium, is immunoreactive in secondary vulvar EMPD of urothelial origin. The distinction is important in that the specific diagnosis has a significant influence on current treatment.[124,125]

Wide local excision is the usual treatment of EMPD to a dept of 4–6 mm to include the pilosebaceous units and skin adnexal structures. Patients with an underlying adnexal adenocarcinoma or stromal invasion of EMPD over 1 mm should be treated more aggressively, with excision to the fascia in the involved area, and inguinofemoral lymphadenectomies bilaterally. Vulvoperineal reconstruction may be necessary by means of skin grafts, local skin flaps, muscle flaps and different fasciocutaneous flaps.[126] Based on small series, topical 5% imiquimod cream has been shown as a safe treatment and may induce complete responses in primary or recurrent vulvar EMPD. The therapeutic schedule used varies. A daily application for 3 weeks, followed by an every other day application for an additional 3 weeks seems to be effective.[127-129] The role of photodynamic therapy[130,131] in the multimodal approach to extensive or recurring vulvar EMPD is still unclear. Radiation therapy in selected cases may be feasible and effective.[132] Overexpression of the HER-2/neu protein has been found in about 30% of vulvar EMPD cases. Targeted therapy with trastuzumab may be considered as a possible new therapeutic strategy in selected cases of vulvar EMPD showing overexpression of HER-2/neu. Treatment can result in a significant regression of disease and resolution of symptoms.[133] Vulvar EMPD with Her-2/neu expression shows higher recurrence rates suggesting a more aggressive behavior.[134]

Recurrences are common[114,135] and may relate to the fact that the extent of histologically demonstrable disease is far greater than that of the visible lesion, the outline of the involved area is highly irregular and multiple foci of disease are present.[136] There is no correlation between disease recurrence and margin status, thus disease recurrence is common, regardless of surgical margin status.[118,132,137] Disease involving the perineum is suggested to be a significant risk factor for recurrences. Intra-operative frozen section analysis of the margins as well as radical surgery as initial treatment seems not to reduce recurrence rate.[132] Long-term monitoring is recommended, as recurrences are common and can be noted many years after the initial treatment and repeat surgical excision is often necessary.

MELANOMA IN SITU OF THE VULVA

Melanoma in situ (MIS) is rare on the vulva and appears to have a relatively slow but definite progression to invasive melanoma.[138] The ABCDE scheme for recognition of melanoma should be considered in pigmented lesions (**A**symmetry, **B**order irregularities, **C**olor variation, **D**iameter >6 mm, **E**nlargement or **E**volution of color

change, shape or symptoms).[139,140] All suspicious pigmented lesions in this region should be biopsied and a punch biopsy is the preferred method because establishing the depth of such lesions is critical. Destruction by cryosurgery, cautery or laser is contraindicated, and all such lesions must undergo histopathological evaluation. Small lesions often can be completely excised, and when sampling hyperpigmented areas, a biopsy of the thickest region is recommended.[138,141] If the diagnosis is considered within the differential diagnosis of pigmented vulvar lesions, it is easy to recognize and treat, with excellent prognosis.[142] An excisional biopsy of the entire clinically apparent lesion, with a narrow 1- to 2-mm margin of adjacent normal-appearing skin, is the biopsy technique of choice when melanoma is suspected, and shave biopsies should be avoided. An incisional biopsy may be acceptable for larger lesions, since studies have shown no worse prognosis if the initial biopsy does not remove the entire lesion, which is later excised.[143,144] In MIS the proliferating malignant melanocytes are confined to the epidermis. Although an in-situ phase exists for three of the four invasive forms of melanoma, superficial spreading melanoma, lentigo maligna melanoma, and acral lentiginous melanoma, for the clinician it is irrelevant, since it should be removed anyway. For patients with MIS, there are no data from randomized trials to define the optimal extent of surgical resection. However, retrospective data support the routine use of 0.5 cm margins.[145,146]

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3

TREATMENT OF VULVAR INTRAEPITHELIAL NEOPLASIA WITH TOPICAL IMIQUIMOD: SEVEN YEARS MEDIAN FOLLOW-UP OF A RANDOMIZED CLINICAL TRIAL

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ABSTRACT

Objective. Recently we reported on the efficacy of imiquimod for treating vulvar intraepithelial neoplasia (VIN) in a placebo-controlled, double-blinded randomized clinical trial (RCT). Four weeks after treatment, a complete response was observed in 35% of patients and a partial response in 46%. All complete responders remained disease-free at 12 months follow-up. In the current investigations, we assessed long-term follow-up at least 5 years after the initial RCT.

Methods. Twenty-four of 26 imiquimod-treated patients who had participated in the initial RCT were seen for follow-up. Primary endpoint was durability of clinical response to imiquimod assessed by naked eye vulvar examination and histology. Long-term clinical response was correlated to lesion size before start of the initial RCT. Secondary endpoints were mental health, global quality of life, body image and sexual function in relation with long-term clinical response.

Results. Median follow-up period was 7.2 years (range 5.6–8.3 years). VIN recurred in one of nine complete responders. Of the initial partial responders, two became disease-free after additional imiquimod treatment. In the other partial responders, VIN recurred at least once after the initial RCT. In long-term complete responders, lesion size at study entry was smaller and these patients had a significantly better global quality of life at follow-up than patients with residual disease and/or recurrence after imiquimod treatment.

Conclusions. In case of a complete response, imiquimod is effective in the long-term. Furthermore, patients with a long-term complete response had a significantly better global quality of life than patients who recurred after imiquimod treatment.

INTRODUCTION

Vulvar intraepithelial neoplasia (VIN) is a premalignant skin condition that often causes severe and long-lasting pruritus, pain and psychosexual dysfunction.[1] It can be classified into *differentiated type* VIN, which is associated with lichen sclerosus, and *usual type* VIN,[2] which is caused by a persistent infection of high-risk human papillomavirus (hrHPV).[3] Usual type VIN tends to be multifocal, and occurs mainly in younger women.[2] Furthermore, it has a certain invasive potential: 9% of untreated patients will progress to invasive vulvar cancer within 1–8 years and 3.3% will progress after treatment.[4]

For a long time, surgical removal of all visible lesions was the standard treatment for VIN.[5] However, surgical treatment often leads to disfigurement, postoperative loss of quality of life and impaired sexual function.[6-8] In addition, recurrences are common after surgery, since the underlying cause – a persistent hrHPV-infection – is not cleared.[4,9]

The immune response modifier imiquimod was recently introduced as an alternative for surgery. In a placebo-controlled, double-blinded randomized clinical trial (RCT), a complete response was observed in 35% of patients and a partial response in 46%, and all complete responders remained disease-free at 12 months follow-up.[10] Moreover, when the histologic diagnoses were defined in accordance with the latest International Society for the Study of Vulvovaginal Disease (ISSVD) VIN terminology[2] in which only VIN is used instead of VIN 1–3, histologic regression was complete in 58% of patients. Another RCT, with a follow-up period of 12 months, reported a complete response after imiquimod treatment in 81% of patients.[11] However, data on long-term follow-up after imiquimod treatment are still lacking. Published studies all suffer from small numbers or relatively short follow-up periods with median follow-up periods ranging from 5.5 to 30.5 months.[10,12] Therefore, in the current study we have evaluated durability of clinical response to imiquimod at least 5 years after the initial RCT[10] in which VIN patients were treated with imiquimod.

PATIENTS AND METHODS

Patients and study design

All imiquimod-treated patients who had participated in our initial placebo-controlled, double-blinded RCT[10] were invited for an additional follow-up assessment. During this follow-up visit, we used the same standard questionnaire as used in the initial RCT to review changes in medication or smoking habits and asked whether any treatment for VIN or other premalignant and malignant lesions in the anogenital tract had occurred since the last follow-up visit at 12 months. All these self reported data as well as detailed clinical and pathological data obtained from the medical files were evaluated.

To evaluate long-term response, a vulvar naked eye examination was performed during the follow-up visit. When vulvar lesions suspicious for VIN or malignancy were

noted, photographs were taken of the lesions as described earlier.[10] If no lesions were visible, photographs were taken of the site of the original lesions treated during the initial RCT. Photographs taken at the current follow-up visit were compared with those taken during the initial RCT.

If a recurrence was suspected in those patients who had a complete response in the initial RCT, a 4-mm punch biopsy was obtained. Biopsy specimens were used for histologic analysis according to the ISSVD VIN terminology[2] (reviewed by an experienced gynecologic pathologist (P.C.E)) and for HPV DNA testing. To compare the presence of HPV in biopsies taken at follow-up with biopsies taken earlier, the SPF_{10}-LiPA system (SPF_{10} HPV LiPA version 1, Labo Biomedical Products, Rijswijk, the Netherlands) was also performed on biopsy samples taken from these patients during the initial RCT.[13] Furthermore, long-term clinical response was correlated with lesion size before start of the initial RCT, measured as described earlier.[10]

Mental health was assessed with the use of the Medical Outcomes Study 36-Item Short-form General Health Survey and health related quality of life was assessed with the European Organization for Research and Treatment of Cancer (EORTC) quality-of-life questionnaire (QLQ-30).[14-16] Body image and sexuality were assessed with the QLQ-BR23.[17] These questionnaires had also been used during the initial RCT.

Primary endpoint was durability of clinical response to imiquimod assessed by naked eye vulvar examination and, in case a recurrence was suspected in patients with an initial complete response, by histologic examination. Secondary endpoints were mental health, global quality of life, body image and sexual function in long-term complete responders and in patients with residual disease and/or recurrence after imiquimod treatment.

All women provided written informed consent for this additional follow-up assessment and the study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

Statistical analysis

Data analysis was performed with the use of the SPSS 15.0 software package for Windows.

Patients were divided in two groups for analyses: patients with a long-term complete response and patients with residual disease and/or recurrence after the initial RCT. Data distribution of variables (lesion size, mental health, global quality of life, body image and sexual function) was assessed with the Kolmogorov-Smirnov test. Distribution was normal for all of these variables. Since both groups were similar for baseline characteristics (age, follow-up period, smoking status, and other HPV lesions, tested with the Mann-Whitney test), differences in mental health, global quality of life, body image and sexuality were measured with one-way analysis of variance. Differences in lesion size before study entry were measured with the Welch's corrected unpaired t-test. A two-tailed *P*-value of $P<0.05$ was chosen to represent statistical significance.

RESULTS

Patients

Twenty-four of the original 26 imiquimod-treated patients responded and came to our outpatient clinic for an additional follow-up assessment. Two patients were lost to follow-up assessment; for these patients data were retrieved from medical files. One of them had an initial reduction in lesion size of 26–75% and was treated twice with laser vaporization after the initial RCT. Recurrence of VIN after the second laser treatment was treated with vaccination by application of long peptides from the HPV 16 viral oncoproteins E6 and E7 in another hospital.[18] She had a partial response to vaccination, and therefore was treated with laser vaporization afterwards. The other patient lost to follow-up had an initial partial response of 76–99% and was treated elsewhere for basaloid anal carcinoma, stage T2N0. After treatment with radiotherapy, she had two recurrences of VIN without invasion and was treated with local excision. Of the 24 patients who were seen for follow-up, nine had a complete response to imiquimod in the initial RCT, four had a partial response of 76–99%, six had a partial response of 26–75%, and five patients had no response (Table 1). Median age of our study population at time of current follow-up was 48 years (range, 29–64 years). Median time between the current follow-up visit and start of the initial RCT was 7.2 years (range, 5.6–8.3 years).

Durability of clinical response in patients initially assessed as complete responders

Median follow-up period for the complete responders was 7.3 years (range, 5.6–8.3 years). Eight of nine complete responders had not received any treatment for VIN between the initial RCT and the current follow-up visit and were still disease-free (Table 1, Figs. 1 and 2). In one of the complete responders a recurrence of VIN was diagnosed 4 years after the initial RCT at the same location as the original lesion treated during the RCT, and she was treated with laser vaporization (Table 1, patient 11). At time of follow-up visit, she again had a lesion suspect for VIN at the same location and a biopsy was taken. Histologic diagnosis was VIN, and HPV 16 was detected. A vulvar biopsy was also taken in three other complete responders to rule out VIN. Histologically, no VIN was diagnosed in these three biopsies, and in two of three biopsies no HPV was detected (Table 1, patients 6 and 7). The third patient presented with condylomata acuminata, which was histologically confirmed, and an infection with HPV 6 and HPV 16 was detected (Table 1, patient 8).

Follow-up in patients initially assessed as partial or non-responders

Median time to follow-up of the initial partial and non-responders was 7.2 years (range, 5.7–8.3 years). All but two partial responders were treated for their residual lesions by local excision or laser vaporization. One patient with an initial partial response of 76–99% had two residual lesions which regressed after an additional imiquimod treatment for 12 weeks. At time of follow-up visit, she was still free of disease

Table 1. Long-term follow-up results in imiquimod treated patients

Patient	Age	Clinical response	HPV status	Duration follow-up	Treatments between 20 weeks and >5 years follow-up visit	VIN	HPV status	Vulvar cancer
	At >5 years follow-up	*At 20 weeks*	*At 20 weeks*	*In months*	*20 weeks and >5 years*	*At >5 years*	*At >5 years*	
1	53	CR	no HPV	100	-	no		no
2	40	CR	no HPV	95	-	no		no
3	44	CR	no HPV	87	-	no		no
4	56	CR	no HPV	91	-	no		no
5	62	CR	no HPV	88	-	no		no
6	53	CR	no HPV	67	-	no	no HPV	no
7	51	CR	no HPV	73	-	no	no HPV	no
8	39	CR	no HPV	81	condylomata	no	6 and 16	no
9	41	PR 76-99%	no HPV	93	residual lesion treated with imiquimod	no		no
10	41	PR 26-75%	no HPV	68	residual lesion treated with imiquimod	no		no
11	42	CR	no HPV	84	1 laser	yes	16	no
12	38	PR 76-99%	16	86	2 LE	no		no
13	29	PR 76-99%	16	78	1 LE+laser, 1 LE	yes		no
14	45	PR 76-99%	no HPV	73	1 LE[1], 3 imiquimod	yes		SCC at t=24 months
15	64	PR 26-75%	16	96	1 LE with pudendal flap recontruction	no		no
16	48	PR 26-75%	16	92	4 LE, 1 imiquimod (stopped due to severe side effects), 2 laser	no		microinvasive SCC at t=7 months

Table 1. Continued

Patient	Age	Clinical response	HPV status	Duration follow-up	Treatments between 20 weeks and >5 years follow-up visit	VIN	HPV status	Vulvar cancer
	At >5 years follow-up	*At 20 weeks*	*At 20 weeks*	*In months*	*20 weeks and >5 years*	*At >5 years*	*At >5 years*	
17	47	PR 26-75%	33	95	2 laser, 1 LE, 1 imiquimod (no response)	yes		no
18	53	PR 26-75%	no HPV	92	did not show up for follow-up visits	yes		no
19	42	PR 26-75%	no HPV	87	1 LE+laser	yes		no
20	45	no response	16	80	1 LE, 1 laser	yes		no
21	48	no response	no HPV	100	4 LE	yes		no
22	40	no response	33	80	4 laser, 1 imiquimod	yes		no
23	52	no response	33	95	3 LE, 1 laser+LE	yes		microinvasive SCC at t=38months
24	48	no response	16	85	1 laser, 1 LE, 1 imiquimod on DRY skin (periurethral lesion -> CR)	no		no
25 [2]	*54*	*PR 26-75%*	*16*	*77*	*3 laser, 1 vaccination with long peptides from HPV-16 viral oncoproteins*	*no data*		*no*
26 [2]	*60*	*PR 76-99%*	*no HPV*	*83*	*2 LE*	*no data*		*no*[3]

CR, complete response; HPV, human papillomavirus; LE, local excision; PR, partial response; SCC, squamous cell carcinoma; VIN, vulvar intraepithelial neoplasia.
[1] Patient was treated with wide local vulvar excision and lymphadenectomy for a squamous cell carcinoma of the left labium majus.
[2] Data retrieved from medical files.
[3] Patient was treated for basaloid anal carcinoma, stage T2N0, with radiotherapy three years after the initial RCT.

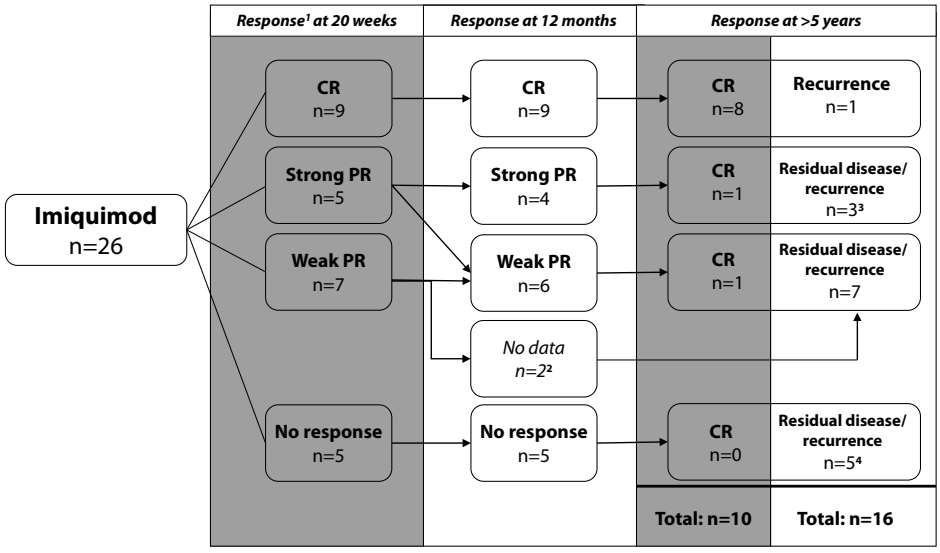

Figure 1. Clinical response at 20 weeks, 12 months and >5 years after start of imiquimod treatment. [1]Clinical response at 20 weeks and 12 months was classified as no response (reduction in lesion size of 25% or less), weak partial response (PR, 26-75% reduction), strong partial response (76-99% reduction), or complete response (CR, 100% reduction). [2]One patient with a weak partial response was lost to follow-up at 12 months because of unrelated medical problems, but this patient did show up for long-term follow-up. Another patient with a weak partial response progressed to invasion (<1 mm) at 7 months. She underwent surgery before follow-up at 12 months, so total lesion size was not measured at 12 months. [3]One patient developed squamous cell carcinoma at 24 months and was treated with local vulvar excision and lymphadenectomy. [4]One patient developed microinvasive squamous cell carcinoma at 38 months and was treated with local vulvar excision.

(Table 1, patient 9 and Fig. 1). Another patient with an initial partial response of 26–75% had a residual lesion on the clitoris after treatment in the RCT, and imiquimod was applied once a week for an additional period of 2 months. She became a complete responder after treatment and is still disease-free (Table 1, patient 10, and Fig. 1).

All other patients had additional treatments for their residual disease and had one to six treatments for recurrence of VIN between the initial RCT and the current follow-up visit (Table 1 and Fig. 1). Most patients were treated with local excision or laser vaporization. Five of them also received one or more imiquimod treatments during follow-up (Table 1, patients 14, 16, 17, 22 and 24). Of the patients with additional imiquimod treatments, one patient had a VIN lesion next to the urethra which did not respond during the initial RCT. After the RCT, she was treated with laser vaporization but had a recurrence after 1 year. Thereafter she was treated with a periurethral skinning local excision followed by pudendal transposition to restore the defect. One year later she suffered once more from a recurrence. She was again treated with imiquimod, but now she was instructed to apply the cream after thoroughly drying off the lesion. This resulted in a complete response (Table 1, patient 24). Of the other four patients who had additional imiquimod treatments,

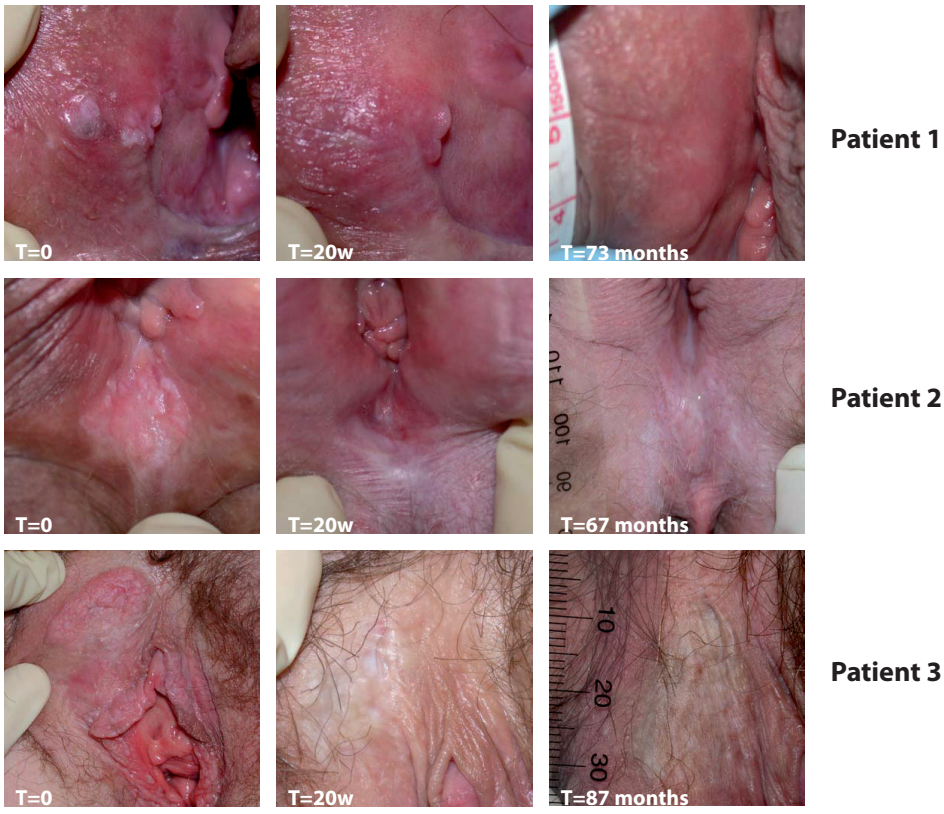

Figure 2. Clinical results before imiquimod treatment, 4 weeks after treatment and at >5 years follow-up. Clinical pictures of three patients with HPV DNA-positive VIN showing results before treatment (T=0), 4 weeks after treatment (T=20 weeks) and >5 years after treatment. All three patients showed complete regression of the lesion.

one patient with no response during the initial RCT showed a complete response after 2 months of imiquimod treatment but had again a recurrence 5 months later (Table 1, patient 22).

The lesion sizes of long-term complete imiquimod-responders (Table 1, patients 1–10) were significantly smaller than those of patients with residual disease and/or recurrence (Table 1, patients 11–26) (2.6±2.1 cm^2 *vs.* 9.0±6.7 cm^2, P=0.002).

Progression to invasion

One patient with a partial response of 26–75% progressed to invasion at 7 months as described previously.[10] Depth of invasion was <1 mm, and a radical excision was performed. She had five recurrences of VIN afterwards that did not progress to invasion (Table 1, patient 16).

One patient with no response during the initial RCT had a local excision for the residual lesions after the RCT, and had a recurrence of VIN 34 months after start of

the initial RCT. Four months later (38 months after start of the initial RCT), she again had perianal lesions and a biopsy was taken. She was diagnosed with squamous cell carcinoma (SCC) with an invasion depth of <1 mm. Additional excision of the lesion was performed and histologic evaluation showed VIN, without invasion. Afterwards she had a recurrence of VIN, for which she was treated with local excision. However, at time of long-term follow-up visit she again presented with a recurrence (Table 1, patient 23).

Another patient with a partial response of 76–99% was diagnosed with SCC located on the left labium majus with an invasion depth of 0.5 cm, 2 years after start of the initial RCT. Wide local vulvar excision of the tumor with lymphadenectomy was performed, one gland was positive without extracapsular growth. She is now 4 years post-treatment and has had no recurrence of the tumor, she did however have a recurrence of VIN, for which she was treated twice with imiquimod. She did not respond to the first treatment and underwent a second treatment with imiquimod at time of long-term follow-up visit. However, during this visit perianal and periclitoral VIN was diagnosed, and confirmed histologically (Table 1, patient 14).

Other hrHPV-related lesions of the anogenital tract

None of the complete responders has been diagnosed with cervical intraepithelial neoplasia (CIN) or other hrHPV-related lesions after completing the initial RCT. Six partial responders had abnormal smear results between the initial RCT and the current follow-up visit. Two of these had CIN 1, the other four patients had no CIN lesion.

Self-reported mental health, global quality of life, body image and sexual function

Patients with long-term complete response after imiquimod treatment (Table 1, patients 1–10) reported a significantly better global quality of life than patients with residual disease and/or recurrence (Table 1, patients 11– 4, P=0.025, Fig. 3). No statistically significant differences were observed between groups in scores on mental health, body image or sexuality.

DISCUSSION

To our knowledge, this is the first study reporting on long-term clinical response of VIN after imiquimod treatment.[12] In this study, eight of nine complete responders (89%) were still disease-free with a median follow-up of 7.3 years (range, 5.6–8.3 years). In addition, two patients with a partial response became complete responders during follow-up after a prolonged treatment with imiquimod. In contrast, all other partial responders had one or more recurrences after imiquimod treatment.

Several studies have indicated that imiquimod is an effective treatment for VIN. However, since these studies had short follow-up periods, it is unknown if imiquimod is a safe and effective treatment in the long term. Previous reported recurrence rates range from 0 to 37%, with median follow-up periods ranging from 5.5 to

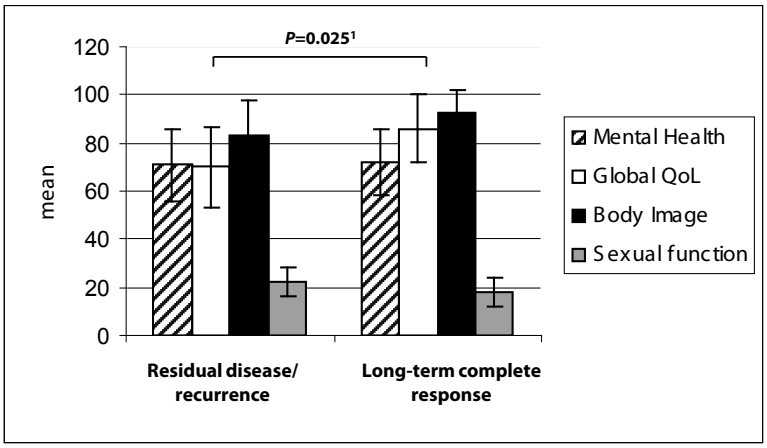

Figure 3. Mental health, global quality of life, body image and sexual functioning. Scores (ranging from 0 to 100, mean ± SD) on mental health, global quality of life, body image and sexual functioning in patients with (Table 1, patients 1 – 10) and without (Table 1, patients 11 – 24) long-term complete response after imiquimod treatment. [1]Patients with long-term complete response had a higher score on global quality of life (QoL) than patients with residual disease and/or recurrence (86±14 *vs.* 70±17, *P*=0.025, one-way analysis of variance).

30.5 months.[12] The strength of the current study is the long follow-up period, with a median follow-up time of 7.2 years (range, 5.6–8.3 years). Although we were able to retrieve long-term follow-up data of all imiquimod-treated patients in the initial RCT, a limitation of this study is the relatively small sample size. Data on recurrences after complete response are based on a small population, since we had only nine initial complete responders and two complete responders after prolonged imiquimod treatment.

Our long-term follow-up data demonstrate that 9% (1/11) of complete imiquimod responders developed a recurrence. This recurrence rate is lower than that observed for other treatments for VIN. Reported recurrence rates after surgical treatment are: 0–19% after vulvectomy,[4,19] 15–17% after local excision with free surgical margins, and 46–50% after local excision with involved surgical margins.[4,9,19,20] After laser vaporization there is a recurrence in 23–40% of patients,[4,19] for photodynamic therapy (PDT) this figure is 48%.[19]

A possible explanation for the low recurrence rate after imiquimod treatment could be that – in contrast with surgical treatments – there is clearance of hrHPV in complete responders, as we demonstrated previously.[10] Since HPV infection is the underlying cause of the disease, VIN is less likely to recur in the patients that clear HPV.

Although the GP5$^+$/6$^+$ PCR enzyme immunoassay[21] was used during the initial RCT to detect HPV DNA in biopsies taken at 0 weeks and at 20 weeks, we decided to use the SPF_{10}-LiPA system, because this is a more sensitive HPV test.[13] We also tested the biopsies taken before start of the initial RCT and at 20 weeks from patients who were biopsied at long-term follow-up with the SPF_{10}-LiPA system. One patient who

was tested HPV negative with the GP5+/6+ PCR enzyme immunoassay before start of the initial RCT, was tested HPV 33 positive with the SPF_{10}-LiPA system, all other results were similar to the GP5+/6+ PCR enzyme immunoassay. At long-term follow-up, hrHPV was detected in the complete responder with a recurrence (patient 11) and in the complete responder with perianal condylomata acuminata (patient 8), the same type (HPV 16) was detected as before start of the initial RCT. Since these patients had cleared HPV from the biopsied lesions at 20 weeks, it is likely that they were re-infected with hrHPV.

HPV exclusively infects epithelial keratinocytes and the virus does not elicit cell death, therefore infection is not accompanied by inflammation, which normally can activate the immune system. Consequently, it is difficult for the host immune system to recognize the virus during early stages of infection, which increases the risk for a persistent infection.[22,23] Imiquimod modulates the immune response by binding on Toll-like receptors 7 and 8 on the cell surface of dendritic cells, thereby inducing secretion of proinflammatory cytokines.[24,25] In this way, imiquimod alters the local immune response in favor of clearance of a persistent HPV infection. This therapeutic strategy is also used in vaccination trials.[18,26] Kenter recently reported on 20 women with HPV-16-positive VIN that were vaccinated three or four times with a mix of long peptides from the HPV 16 viral oncoproteins E6 and E7.[18] Three months after vaccination, 5 of 20 patients had a complete response, increasing to 9 of 19 at 12 months after last vaccination (47%). Complete response rate was maintained at 24 months of follow-up. Complete responders had a significantly stronger response of IFNγ-associated proliferative CD4+ T cells and a broad response of CD8+ IFNγ T cells than did non-responders. Therefore, complete response appeared to be correlated with induction of HPV-16-specific immunity. Furthermore, it has been demonstrated that HPV-16-positive VIN patients with a pre-existing HPV-specific type 1 T-cell response are more likely to have a strong response to imiquimod treatment.[27] Consequently, it has been suggested to combine imiquimod with HPV vaccination. In 19 VIN patients treated with imiquimod for 8 weeks followed by three doses of therapeutic HPV vaccination, complete histologic regression was observed in 32% of women at week 10, increasing to 58% at week 20 and 63% at week 52.[26] These results are promising, but results of long-term follow-up are still needed.

In summary, our data indicate that treatment with imiquimod is an effective long-term therapy for VIN. Small lesions are more likely to respond completely to imiquimod treatment; therefore treatment period in the initial RCT might have been too short to be effective for larger lesions. Consequently we advise prolonging the treatment period until regression has stopped. Finally, despite these positive results, yearly follow-up visits are still recommended until larger studies have definitively demonstrated the long-term effects and recurrence rate after imiquimod treatment of HPV-induced VIN.

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4

IMIQUIMOD-INDUCED CLEARANCE OF HPV IS ASSOCIATED WITH NORMALIZATION OF IMMUNE CELL COUNTS IN USUAL TYPE VULVAR INTRAEPITHELIAL NEOPLASIA

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ABSTRACT

Recently, we reported on the efficacy of imiquimod for treatment of usual type vulvar intraepithelial neoplasia (uVIN). A histologic regression of uVIN to normal tissue was observed in 58% of patients. As success of treatment is related to clearance of high-risk human papillomavirus (HPV), the aim of our study was to assess differences in immune cell counts and in the expression of $p16^{INK4a}$ in VIN tissue before and after imiquimod treatment, in relation to HPV clearance and clinical response.

Vulvar tissue samples taken prior to imiquimod treatment and 4 weeks after treatment were tested for the presence of HPV. Previously determined immune cell counts (CD1a, CD207, CD208, CD123/CD11c, CD94, CD4, CD8 and CD25/HLA-DR) in epidermis and dermis of 25 VIN patients and 19 healthy controls were completed with the counts for CD14 and CD68. The expression of $p16^{INK4a}$ was investigated by immunohistochemistry in 15 patients.

Before imiquimod treatment, both HPV cleared and HPV non-cleared patients showed mainly in the dermis significantly upregulated immune cell counts compared to healthy controls. However, in patients that cleared HPV and showed histologic regression already 4 weeks after imiquimod treatment, immune cell counts and $p16^{INK4a}$ expression were normalized.

In conclusion, our data indicate that imiquimod-induced clearance of HPV results in normalization of counts for certain immune cells and is strongly correlated with histologic regression of the disease.

INTRODUCTION

Vulvar Intraepithelial Neoplasia (VIN) is a premalignant disorder that is classified into *differentiated type* VIN, which is associated with lichen sclerosus, and *usual type* VIN (uVIN),[1] which is caused by a persistent infection with a high-risk or oncogenic HPV (hrHPV, usually HPV-type 16, 18 or 33).[2]

Over the last decades, the incidence of uVIN has increased, most likely due to a rise in the incidence of HPV infections.[3] Lifetime risk to become infected with HPV in western societies is around 80% and ~40% of all sexually active, female adolescents are at least once infected with hrHPV.[4-7] When hrHPV persists (in less than 10% of cases), premalignant disorders of the anogenital tract, such as uVIN, can develop.[8-10] uVIN has invasive potential (10% of untreated cases will progress in 1–8 years) and needs to be treated proactively.[11,12]

The host immune response is of critical importance in determining clearance or persistence of an HPV infection. In natural life, during the early stages of a viral infection, CpG-rich regions in the viral DNA are recognized by toll-like receptor (TLR) 7 and 9 on the cell surface of immature dendritic cells (DCs). Upon binding, TLRs activate kinase cascades eventually activating NF-κB, which will result in the production of cytokines, adhesions molecules and other effectors of the innate immune response.[13] Other receptor and adhesion molecules at the DC surface can bind viral antigen and will internalize and digest the antigen, which is followed by expression on the cell membrane in major histocompatibility complex (MHC) class I and in MHC class II. Class I MHC is recognized by CD8 and class II MHC by CD4. Upon binding antigen, the DC matures and migrates to the lymph nodes were it presents antigen bound to class II MHC, to naive T cells. Binding to naive T cells will result in production of memory and effector T cells, such as $CD4^+$ T-helper cells, $CD8^+$ cytotoxic T cells, or regulatory T cells (Treg cells).[14,15]

For a long time, surgical removal of all visible lesions was the standard treatment of uVIN.[16] However, the concept that the immune response in uVIN is insufficient[17-19] has lead to new, less invasive treatment options in which the immunomodulator imiquimod plays a role. Imiquimod is an immune response modulator that acts by activating the DC, thereby inducing secretion of proinflammatory cytokines and T-cell activation. This results in a profound tumor-directed cellular immune response.[20-23]

Recently, our group reported in a placebo-controlled, double-blinded, randomized trial (RCT) on the efficacy of imiquimod treatment for uVIN.[24] During this trial, patients were treated with imiquimod or placebo twice a week for a period of 16 weeks. An imiquimod-induced reduction in lesion size by ≥25% in 81% of patients was measured, while a complete response was observed in 35% of patients. Moreover, when we defined the histological diagnoses in accordance to the ISSVD guidelines[1] in uVIN or normal tissue instead of VIN 1, 2 or 3, complete histologic regression of uVIN was observed in 58% of patients.

Furthermore, our study put forward that reduction in lesion size was correlated to normalization of the numbers of immune cells.[25] In the present study, this concept

was investigated further. Therefore, we retrospectively assessed correlations between numbers of immature, mature and plasmacytoid DCs, natural killer (NK) cells, monocytes, macrophages and T cells in vulvar skin of 25 patients with uVIN before and 4 weeks after imiquimod treatment with the degree of histological response and clearance of HPV. Data from 19 healthy women were used as reference values. We also studied the presence of $p16^{INK4a}$ in relation to HPV status before and after imiquimod treatment. $P16^{INK4a}$ is a member of the INK4a family of cyclin dependent kinase inhibitors and during a persistent hrHPV infection, the viral oncoproteins E6 and E7 can interfere with the cell cycle regulatory pathway, which results in overexpression of $p16^{INK4a}$. Therefore, immunohistochemistry with $p16^{INK4a}$ is used as a diagnostic aid and positivity of $p16^{INK4a}$ is a surrogate marker for hrHPV infection.[26-29]

In addition to our previous article in which it was observed that VIN patients have disturbed immune cell counts,[30] we observed in the present study that already 4 weeks after treatment, HPV clearance and subsequent normalization of $p16^{INK4a}$ expression indicates a full or almost complete clinical response and is characterized by normalization of counts for certain immune cells in the epidermis and dermis.

MATERIAL AND METHODS

Patients

For the immunohistochemical staining frozen tissue sections of 4-mm punch biopsies were used. Biopsies from 25 women with multifocal uVIN who participated in a placebo-controlled RCT[25] were taken before treatment with imiquimod (at time = 0 weeks) and 4 weeks after treatment with imiquimod (at time = 20 weeks). During this trial, patients were treated with imiquimod or placebo twice a week for a period of 16 weeks. All biopsies were immediately frozen in liquid nitrogen and stored at –80°C until further analysis. Furthermore, 19 women who underwent elective vulvar surgery for cosmetic reasons served as healthy controls.

For staining with $p16^{INK4a}$, formalin-fixed paraffin-embedded biopsies were used,[25] taken before and 4 weeks after imiquimod treatment.

Histological diagnosis of all biopsy specimens was performed by two experienced gynecologic pathologists. In the previous study by van Seters *et al.*,[25] biopsy specimens were classified into VIN 1, 2 and 3. In the present study, biopsy specimens were classified uVIN according to the new ISSVD guidelines[1] (previously VIN 2 or 3). In this classification VIN 1 no longer exists and is considered normal.

All frozen tissue samples were analyzed for the presence of the 14 most prevalent hrHPV-types by using a standard GP5+/6+ PCR enzyme immunoassay followed by reverse line blot analysis, as described previously.[31] For reasons explained in the results section, two samples were also analyzed with the SPF10 DEIA/LiPA version 1 test, which is a more sensitive HPV test.[32] The HPV detection limit of the SPF10 DEIA/LiPa is 65 bp, and it detects 25 different HPV genotypes.

Retrospectively, patients were divided into two groups, namely patients who cleared HPV (HPV cleared) and patient who did not clear HPV (HPV non-cleared) after imiquimod treatment.

Medical Ethical Committees approved our study design and all women provided voluntarily written informed consent.

Immunohistochemical staining on frozen tissue sections

Immunohistochemical staining was performed on 6-µm-thick frozen tissue sections for the following markers: CD1a, classical marker for immature DCs/ Langerhans cells (Orthobiotech, Bridgewater, NJ); CD207, marker for immature DCs expressing Langerin (DCGM4; Beckman Coulter, Fullerton, CA); CD208, marker for mature DCs (104.G4; Beckman Coulter); CD94, marker for NK cells (HP.3b1; Beckman Coulter); CD14, marker for monocytes (M0825; Dako Denmark, Glostrup, Denmark); CD68, marker for macrophages (M0718; Dako); CD4, marker for T-helper cells (MT.310; Dako); CD8, marker for cytotoxic T cells (DK25; Dako); CD25/HLA-DR, marker for Treg cells (AKT-1; Dako/1E5; Sanquin); for plasmacytoid DCs ($CD123^+/CD11c^-$), a double staining with both markers was used [anti-CD123 (9F5; Becton Dickinson, Alphen aan den Rijn, the Netherlands) and anti-CD11c (SHCL-3; Becton Dickinson)]. Staining and light microscopic evaluation of the obtained data were performed as described earlier.[30]

$P16^{INK4a}$ staining

Staining for $p16^{INK4a}$ was performed on 4-µm-thick paraffin-embedded tissue sections. The tissue sections were first deparaffinised overnight and rehydrated through graded series of xylene and alcohol. Endogenous peroxidase was blocked in H_2O_2 for 5 min, followed by washing the sections in dH_2O. The slides were placed in a citrate buffer (pH 6) and heated in a 900 W microwave for 1 × 6.5 min, 1 × 3 min and – after adding dH_2O – again for 1 × 3 min. After cooling for 1 hr, tissues were washed in PBS for 3 × 5 min. Unspecific binding was blocked with 200 µl PBS with 0.3% bovine serum albumin (BSA) for 30 min. Sections were then incubated overnight at 4˚C in a dark humid chamber with the mouse monoclonal antibody $p16^{INK4a}$ (clone JC8, sc-56330, Santa Cruz biotechnology, Inc., Santa Cruz, CA) in a dilution of 1:100. After incubation with the primary antibody, sections were incubated with 200 µl biotin-conjugated goat antimouse immunoglobulin (Dako) diluted 1:400 in PBS with 0.3% BSA for 30 min. This was followed by incubation with 200 µl streptavidin-biotinylated-peroxidase complex (ABComplex) with horse radish peroxidase (HRP) conjugate (Dako) for 30 min in dark. Between all incubation steps, sections were washed with PBS for 3 × 5 min. Staining was developed using 0.133 g diaminobenzidine (DAB, Fluka, Sigma Aldrich, Buchs SG, Switzerland) in 200 ml PBS with 600 µl H_2O_2 and the reaction was stopped after 10 min by washing in dH_2O. Finally, sections were counterstained with haematoxylin (Klinipath, Duiven, the Netherlands) for 3 sec, washed with flushing water and dehydrated through graded series of alcohol and xylene.

Light microscopic evaluation was performed blinded. Nuclear and cytoplasmic staining was considered as a positive reaction. Sections were scored semiquantitative for the percentage of $p16^{INK4a}$ positive cells: 0–25% positive *(1)*, 25–50% positive *(2)*, 50–75% positive *(3)*, >75% positive *(4)*. Sections were also scored for the intensity of the staining: absent *(1)*, light *(2)*, moderate *(3)* and strong *(4)*.[33] The total score per sample was obtained by multiplying percentage with intensity.

Statistical analysis

Data analysis was performed with the use of the SPSS 15.0 software package for Windows. The Kolmogorov-Smirnov test was used to identify the distribution for the cell types. Because a non-normal distribution was observed for some cell types, the non-parametric Mann-Whitney test was used for evaluation of differences in cell counts between the following groups: HPV cleared *vs*. healthy controls, and HPV non-cleared *vs.* healthy controls, both before and after imiquimod treatment. Differences in $p16^{INK4a}$ expression before and after treatment within the HPV cleared and HPV non-cleared VIN patients were assessed with the use of the Wilcoxon signed rank test.

The Mann-Whitney test was used to assess differences between the HPV cleared and HPV non-cleared patients. A two-tailed *P*-value of $P<0.05$ was chosen to represent statistical significance.

RESULTS

Patients

The median age of the 25 VIN patients was 39 years (range, 22–56 years). In the healthy control group, the median age was 40 years (range, 19–56 years). Cryosections were obtained from al these patients, but paraffin-embedded samples were limited and only obtained from 15 of 25 VIN patients before as well as after treatment.

Twenty-three patients were smokers, two patients had a history of immunosuppressant use, and 14 patients had a history of CIN. Patients had not received any treatment for at least 3 months before treatment with imiquimod. Of all controls, six were smokers and six had a history of smoking, none of the controls had a history of immunosuppressant use and none of them had a history of CIN.

Histological response and presence of HPV DNA and $p16^{INK4a}$

Before imiquimod treatment, 19 of the 25 VIN patients were positive for HPV 16, five patients were positive for HPV 33, and one patient was HPV DNA-negative.[25] The HPV DNA-negative patient was excluded from further analysis. Four weeks after imiquimod treatment, seven patients were positive for HPV 16, three were positive for HPV 33 and 14 patients were HPV DNA-negative. All healthy controls were HPV DNA-negative.

Immunohistochemical analysis for $p16^{INK4a}$ expression was performed on tissue sections from 15 patients before and after imiquimod treatment. Before treatment,

12 of 15 patients were HPV 16 positive and three patients were HPV 33 positive. After treatment, four patients were HPV 16 positive, two were HPV 33 positive and nine patients were HPV DNA-negative.

P16^{INK4a} scores and staining before and after treatment in relation to histologic regression and viral clearance are shown in Table 1 and in Figure 1. Median score of p16^{INK4a} expression (percentage × intensity) before imiquimod treatment was 12 for the HPV cleared and 12 for the HPV non-cleared patients. After treatment, p16^{INK4a} expression was significantly decreased in HPV cleared patients (*P*=0.008) but not in HPV non-cleared patients (*P*=0.084). In addition, p16^{INK4a} score after treatment was significantly lower in HPV cleared patients compared to HPV non-cleared patients (*P*=0.006). Interestingly, two patients who cleared HPV were still diagnosed with uVIN after treatment and additional observations and experiments were performed to clarify this situation.

Patient one had a clinical response to imiquimod treatment of 76–99% and was negative for HPV and for p16^{INK4a} expression as measured in a biopsy obtained 4 weeks after treatment. The patient was followed for an additional period after which it was observed that at that point all lesions had disappeared. To date (>5 years), this patient is still disease-free.

Patient two had a clinical response to imiquimod treatment of less than 25%, and remained positive for p16^{INK4a}, although the HPV test indicated clearance of the virus. Follow-up of this patient showed several recurrences after imiquimod treatment. To rule out the possibility of having missed an HPV infection, a different more sensitive HPV test was performed (SPF10 DEIA/LiPA version 1[32]). Using this sensitive HPV test for both patients we could not detect the presence of HPV viral DNA, signifying the absence of hrHPV infection.

Table 1. Histologic diagnosis after imiquimod treatment and p16^{INK4A} scores in HPV cleared and HPV non-cleared patients before and after imiquimod treatment.

Diagnosis after treatment	HPV cleared			HPV non-cleared		
	n	P16^{INK4A} before median (range)	P16 ^{INK4A} after median (range)	n	P16 ^{INK4A} before median (range)	P16 ^{INK4A} after median (range)
uVIN	2	10.5 (9-12)	3.5 (1-6)	6	12 (12-16)	8 (2-16)
normal	7	12 (6-16)	2 (1-3)	0	-	-
All patients	9	12 (6-16)	2 (1-6)[1]	6	12 (12-16)	8 (2-16)[2]

[1]Significant different compared to before treatment, *P*=0.008
[2]Not significantly different compared to before treatment, *P*=0.084

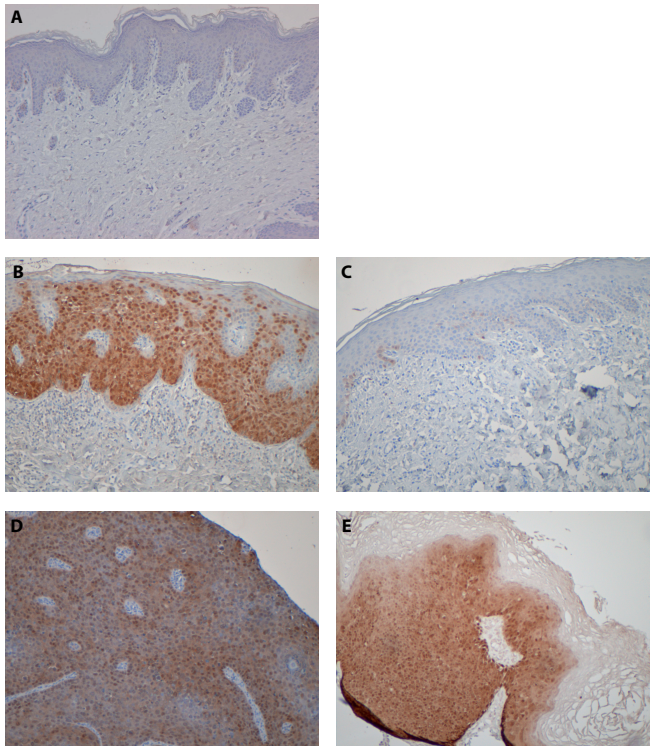

Figure 1. P16^{INK4a} expression in control tissue and in VIN lesions before and after imiquimod treatment. **A.** hrHPV negative control tissue does not show expression of p16^{INK4a}. **B, C.** p16^{INK4a} expression before (B) and after (C) imiquimod treatment in a patient that did show clearance of HPV. **D, E.** p16^{INK4a} expression before (D) and after (E) imiquimod treatment in a patient that did not clear HPV (photographs A-E, magnification: ×10).

Evaluation of immune cell numbers in VIN lesions before and after imiquimod treatment and in normal vulvar skin

Epidermis. Immunohistochemical analysis of the epidermis showed significant differences in immune cell counts between HPV cleared (n=14) or non-cleared (n=10) VIN patients and controls (n=19) (Table 2).

Before treatment, CD8$^+$ cytotoxic T cells were significantly lower in HPV cleared patients as compared to controls (Table 2, *P*-value 1 *vs.* 2), while CD14$^+$ monocytes were higher in HPV cleared patients and in HPV non-cleared patients compared to healthy controls (Table 2, *P*-value 1 *vs.* 2 and *P*-value 1 *vs.* 4). After treatment, counts for CD8$^+$ and CD14$^+$ cells returned to control levels in HPV cleared patients (Table 2, *P*-value 1 *vs.* 3, Figs. 2a, 2b, and 3), while in HPV non-cleared patients the elevated CD14$^+$ cell numbers did not return to control levels (Table 2, *P*-value 1 *vs.* 5). In addition, CD123$^+$/CD11c$^-$ pDCs, CD208$^+$ mDCs and CD68$^+$ macrophages were also significantly higher in HPV non-cleared patients after treatment compared to controls (Table 2, *P*-value 1 *vs.* 5).

CD1a$^+$ Langerhans cells in HPV cleared and non-cleared patients were not different from healthy controls before treatment, but were significantly higher in HPV cleared patients after treatment (Table 2, *P*-value 1 *vs.* 3).

Table 2. Immune cell counts in epidermis in controls, in patients who cleared HPV and patients who did not clear HPV upon imiquimod treatment.

Cells	Controls (n=19)	HPV cleared (n=14)				HPV non-cleared (n=10)					
	(1) median (range)	before (2) median (range)	after (3) median (range)	P-value 1 vs 2	P-value 1 vs 3	before (4) median (range)	after (5) median (range)	P-value 1 vs 4	P-value 1 vs 5	P-value 2 vs 4	P-value 3 vs 5
CD1a+	212 (104-310)	189 (65-478)	280 (103-388)	0.70	**0.032**	144 (106-418)	225 (128-465)	0.11	0.49	0.95	0.32
CD207+	166 (14-319)	161 (2-407)	214 (50-300)	0.61	0.34	112 (7-219)	111 (17-298)	0.099	0.25	0.29	0.079
CD208+	19 (1-43)	29 (2-63)	25 (3-69)	0.090	0.24	16 (10-148)	35 (20-70)	0.75	**0.001**	0.60	0.18
CD8+	130 (51-526)	73 (9-357)	139 (43-385)	**0.038**	0.83	129 (17-305)	94 (28-226)	0.65	0.12	0.38	0.16
CD4+	269 (89-591)	295 (38-786)	316 (123-613)	0.40	0.15	290 (39-925)	335 (195-923)	0.58	0.11	0.86	0.56
CD94+	28 (0-144)	22 (0-153)	35 (6-174)	0.73	0.72	20 (4-63)	39 (13-58)	0.31	0.82	0.82	0.82
CD25+/ HLA-DR+	0 (0-3)	0 (0-2)	0 (0-7)	0.13	0.39	0 (0-5)	0 (0-9)	0.82	0.39	0.16	0.69
CD123+/ CD11c-	8 (0-35)	8 (0-58)	16 (0-44)	0.53	0.19	20 (4-59)	20 (2-147)	**0.008**	**0.031**	0.084	0.38
CD14+	1 (0-50)	30 (1-316)	0 (0-16)	**0.001**	0.40	61 (1-287)	76 (1-190)	**0.000**	**0.003**	0.38	**0.001**
CD68+	19 (0-136)	36 (1-172)	41 (0-151)	0.55	0.48	57 (13-95)	134 (18-243)	0.22	**0.003**	0.68	**0.012**

CD1a^{+}, immature dendritic cells (DCs)/ Langerhans cells; **CD207**$^{+}$, immature DCs expressing Langerin; **CD208**$^{+}$, mature DCs; **CD8**$^{+}$, cytotoxic T cells; **CD4**$^{+}$, T-helper cells; **CD94**$^{+}$, natural killer cells; **CD25**$^{+}$**/HLA-DR**$^{+}$, Treg cells; **CD123**$^{+}$**/CD11c**$^{-}$, plasmacytoid DCs; **CD14**$^{+}$, monocytes; **CD68**$^{+}$, macrophages.

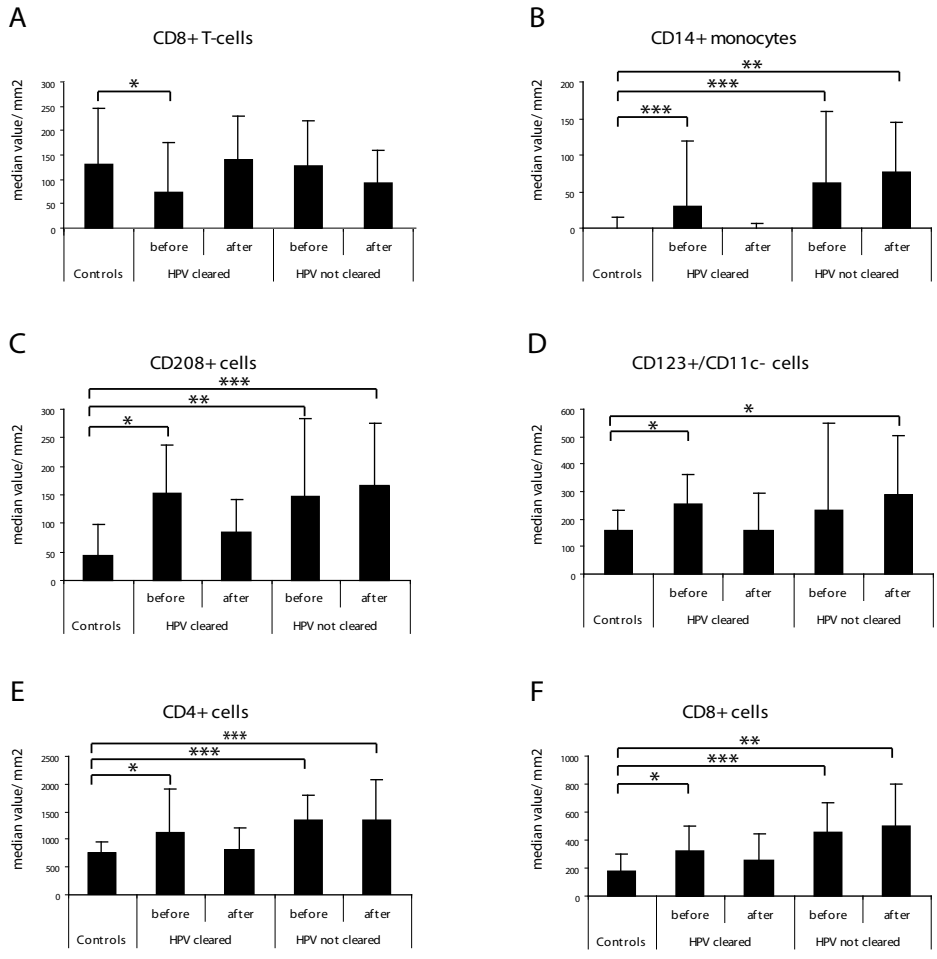

Figure 2. Numbers of immune cells in control and VIN tissues before and after imiquimod treatment in patients who cleared or did not clear HPV from the site of the lesion. **A, B.** $CD8^+$ and $CD14^+$ cells in epidermis of VIN patients before and after imiquimod treatment. **C – F.** $CD208^+$, $CD123^+/11c^-$, $CD4^+$ and $CD8^+$ cells in dermis of VIN patients before and after imiquimod treatment. Median ± SD. *****P<0.05, ******P<0.005, *******P<0.001, significant different from controls (Mann-Whitney test).

No significant differences before treatment were seen when we compared the HPV cleared patients with HPV non-cleared patients (Table 2, *P*-value 2 *vs.* 4). However, when we performed the same analysis after treatment, the number of $CD14^+$ and $CD68^+$ cells were significantly higher in the HPV non-cleared group (Table 2, *P*-value 3 *vs.* 5).

Dermis. In the dermis, differences in cell numbers were much more pronounced (Table 3). Before imiquimod treatment, there were significantly higher numbers of $CD4^+$ T-helper cells, $CD8^+$ cytotoxic T cells, CD25/HLA-DR^+ Treg cells, $CD208^+$ mDCs

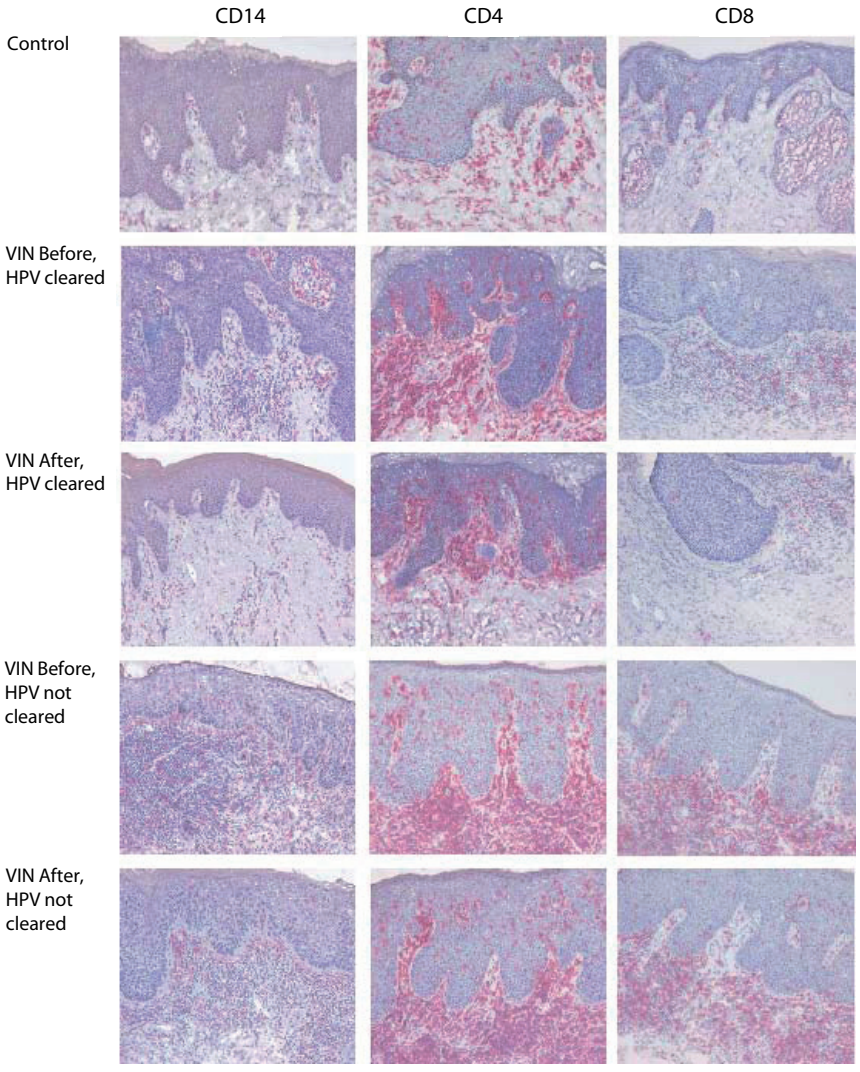

Figure 3. CD14, CD4 and CD8 positive cells in control and VIN tissues before and after imiquimod treatment in patients who cleared or did not clear HPV from the site of the lesion (magnification: ×10).

and $CD123^+/CD11c^-$ pDCs in HPV cleared patients *vs.* controls (Table 3, *P*-value 1 *vs.* 2). Also in the HPV non-cleared group, the numbers of these cells were higher, although this was not significant for $CD123^+/CD11c^-$ pDCs and $CD25/HLA\text{-}DR^+$ Treg cells (*P*=0.079, *P*=0.082 respectively, Table 3, *P*-value 1 *vs.* 4). In the dermis, similar to the epidermis, in HPV cleared patients cell numbers returned to normal after treatment (Table 3, *P*-value 1 *vs.* 3), this in contrast to the cell numbers in HPV non-

Table 3. Immune cell counts in dermis in healthy controls, patients who cleared HPV and patients who did not clear HPV upon imiquimod treatment.

Cells	Controls (n=19)	HPV cleared (n=14)				HPV non-cleared (n=10)					
	(1) median (range)	before (2) median (range)	after (3) median (range)	P-value 1 vs 2	P-value 1 vs 3	before (4) median (range)	after (5) median (range)	P-value 1 vs 4	P-value 1 vs 5	P-value 2 vs 4	P-value 3 vs 5
CD1a+	133 (51-277)	174 (33-498)	138 (32-339)	0.24	0.52	141 (47-221)	186 (48-593)	0.38	0.039	0.73	0.089
CD207+	41 (0-103)	53 (3-278)	36 (7-102)	0.19	0.59	52 (6-147)	51 (13-180)	0.41	0.55	0.82	0.48
CD208+	45 (15-172)	154 (15-283)	85 (32-193)	**0.007**	0.061	146 (74-537)	167 (53-424)	**0.002**	**0.001**	0.75	**0.030**
CD8+	181 (76-491)	319 (99-684)	258 (94-700)	**0.011**	0.12	454 (212-817)	504 (95-1074)	**0.001**	**0.002**	0.16	**0.035**
CD4+	748 (406-1060)	1120 (236-2909)	811 (428-1777)	**0.041**	0.33	1336 (631-2190)	1350 (475-3011)	**0.000**	**0.001**	0.41	**0.022**
CD94+	30 (0-209)	59 (3-15)	36 (17-248)	0.24	0.40	57 (29-141)	100 (23-283)	0.090	**0.019**	0.64	**0.035**
CD25+/ HLA-DR+	26 (5-118)	61 (0-231)	31 (5-54)	**0.009**	0.62	50 (0-140)	56 (9-183)	0.082	0.065	0.46	0.11
CD123+/ CD11c-	160 (57-302)	257 (85-444)	156 (56-459)	**0.021**	0.23	231 (56-1140)	291 (140-770)	0.079	**0.007**	0.94	0.089
CD14+	429 (107-949)	543 (132-1238)	372 (204-616)	0.50	0.27	563 (167-1644)	638 (126-1115)	0.069	0.094	0.41	**0.030**
CD68+	432 (40-707)	434 (211-1122)	444 (213-747)	0.34	0.64	817 (154-1700)	734 (330-1439)	**0.008**	**0.002**	0.089	**0.006**

CD1a^{+}, immature dendritic cells (DCs)/ Langerhans cells; **CD207**$^{+}$, immature DCs expressing Langerin; **CD208**$^{+}$, mature DCs; **CD8**$^{+}$, cytotoxic T cells; **CD4**$^{+}$, T-helper cells; **CD94**$^{+}$, natural killer cells; **CD25**$^{+}$**/HLA-DR**$^{+}$, Treg cells; **CD123**$^{+}$**/CD11c**$^{-}$, plasmacytoid DCs; **CD14**$^{+}$, monocytes; **CD68**$^{+}$, macrophages.

cleared patients that did not return to normal (Table 3, *P*-value 1 *vs.* 5, Figs. 2c-2f and 3).

When we compared HPV cleared and HPV non-cleared patients, no significant differences in cell counts were seen before treatment (Table 3, *P*-value 2 *vs.* 4), while after treatment, $CD208^+$, $CD8^+$, $CD4^+$, $CD94^+$, $CD14^+$ and $CD68^+$ cells were significantly lower in the HPV cleared patients in comparison to the HPV non-cleared patients (Table 3, *P*-value 3 *vs.* 5).

Individual values for immune cell counts per patient, combined with the data on viral clearance and $p16^{INK4a}$ expression, are shown in the supplementary Table 1 (http://www.erasmusmc.nl/47393/1584119/1603959/Terlou).

DISCUSSION

The observations in our study demonstrate that clearance of HPV infection already 4 weeks after treatment with imiquimod, results in normalization of immune cell counts and normalization of $p16^{INK4a}$ expression at the site of the lesion. Winters et al.[34] also studied immune cell counts and measured the HPV status of VIN patients before and after treatment with imiquimod combined with photodynamic therapy (PDT). Interestingly, Winters observed that besides all complete responders, 2 of 4 partial responders and one patient with stable disease had become HPV DNA negative. In our study, 12 of 14 patients that cleared HPV 4 weeks after imiquimod treatment showed complete histologic regression of uVIN and an additional patient showed a somewhat delayed complete regression. An explanation for the delayed regression could be that clearance of HPV precedes regression of uVIN. This has been shown previously in patients with abnormal cervical smears, in which hrHPV clearance preceded regression of cervical lesions by an average of 3 months.[35]

Another patient, who cleared HPV after treatment, however, showed a clinical response of less than 25% and continuing elevated expression of $p16^{INK4a}$. Our first thought was that the method used to detect viral DNA was not sensitive enough. However, even a more sensitive method on paraffin sections adjacent to those used for $p16^{INK4a}$ detection could not detect HPV. In other words, despite imiquimod-induced clearance of HPV the disease had only marginally improved and $p16^{INK4a}$ level remained elevated. These findings seem to indicate that in this particular case the disease may have progressed beyond HPV control. Elevation of $p16^{INK4a}$, normally a hallmark of hrHPV,[26-29] in this case can be considered a compensation for abnormal cell proliferation as is observed in many HPV-negative carcinomas.[28,36,37]

HPV clearance results in normalization of immune cell counts

Since persistent HPV infection is the underlying cause of VIN and the host immune response is primarily directed against this HPV infection, we focused our investigations on differences in local immunity between patients who were able to clear HPV and patients who were not able to clear HPV upon local treatment with imiquimod. Our

most important finding is that, already 4 weeks after the end of treatment, imiquimod-induced clearance of HPV results in normalization of the numbers of immune cells at the site of the lesion (Table 2 and 3). Although imiquimod treatment is generally well tolerated and for a number of patients a good alternative for otherwise mutilating treatments like vulvectomy or local excision, it is of interest to know beforehand which patients will respond to therapy and which patients will not. Winters *et al.*[34] reported that Treg cells in nonresponders before treatment were significantly increased compared to numbers in responders, a finding which we could not confirm. However, we did compare the numbers of immune cells before treatment between those patients that cleared HPV upon treatment *vs*. those patients that did not clear HPV to find other possible predictors of HPV clearance upon imiquimod treatment. Our results demonstrate lower $CD8^+$ cell numbers in the epidermis of patients that cleared HPV, but not in patients who did not clear HPV when we compared to healthy control levels (Table 2). Furthermore, $CD123^+$ pDCs in the epidermis and $CD68^+$ cells in the dermis of HPV non-cleared patients were significantly higher compared to controls (Table 2 and 3), which was not the case in the HPV cleared group. In theory, the above indicated CD-markers can be used to predict success or failure of imiquimod treatment to clear HPV. However, when we compared the HPV cleared group directly with the HPV non-cleared group (before treatment, Table 2 and 3, *P*-value 2 *vs.* 4), we did not measure any significant differences between the two groups. On the basis of our current findings we consider it too early to do any recommendations on the above markers to predict HPV clearance upon imiquimod treatment.

Previously, our study group reported on the immunosuppressive state of the epidermis of VIN patients, demonstrated by a decrease in $CD1a^+$ and $CD8^+$ cells.[30] Interestingly, the present study shows a higher number of $CD14^+$ monocytes in the epidermis of VIN patients compared to controls. It is known that $CD14^+$ cells in an environment rich of TGF-β1 (which is produced by most immune cells), and in response to CCL20 (for which $CD14^+$ cells express the receptor molecule CCR6) differentiate into immature Langerhans cells ($CD207^+$). Under conditions of cutaneous inflammation, when the proinflammatory cytokine GM-CSF is released along with TGF-β1, $CD14^+$ cells can differentiate into mature Langerhans cells ($CD1a^+$).[38-41] This is in accordance with our findings. After treatment, we observed a decreased number of $CD14^+$ monocytes (*P*=0.005, Wilcoxon signed rank test) in the HPV cleared epidermis, while the number of $CD1a^+$ Langerhans cells in this group seemed to be increased (*P*=0.074, Wilcoxon signed ranked test). During successful imiquimod treatment, $CD14^+$ monocytes in the epidermis might have differentiated into Langerhans cells.

An important limitation of the present study is the absence of biopsy specimens during treatment to assess the specific changes in immune cells induced by imiquimod that are associated with the clearance of lesions. Such data would be important in identifying the specific immune cells that must be targeted for successful treatment.

In conclusion, our data indicate that already 4 weeks after the end of treatment, imiquimod-induced clearance of HPV (confirmed by normalization of $p16^{INK4a}$ expression) results in normalization of immune cell counts and is strongly correlated

with histologic regression of the disease. Interestingly, van Seters *et al.*[24] could show that histologic regression is maintained till at least one year after the start of treatment and preliminary long-term follow-up data (>5 years) further confirm the success of imiquimod treatment.

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5

NONSTEROIDAL ANTI-INFLAMMATORY DRUGS DO NOT INTERFERE WITH IMIQUIMOD TREATMENT FOR USUAL TYPE VULVAR INTRAEPITHELIAL NEOPLASIA

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ABSTRACT

Imiquimod has been shown to be an effective treatment for usual type vulvar intraepithelial neoplasia (uVIN). Since local inflammation and burning are common side effects, patients often use nonsteroidal anti-inflammatory drugs (NSAIDs). Our study investigated whether NSAID-use, which has been documented to inhibit the cell-mediated immune response, interferes with the outcome of imiquimod treatment.

Monocyte-derived dendritic cells (moDCs) and Langerhans cells (moLCs) were cultured in the presence of NSAIDs. The expression of relevant surface markers (CD80, CD86, CD40, HLA-DR, CCR6 and CCR7), stimulatory function, and cytokine production were evaluated. Furthermore, we analyzed in uVIN patients whether frequent NSAID-use had an effect on the clinical response and on immunocompetent cell counts before and after imiquimod treatment.

Although an effect was observed on the expression of moDC and moLC maturation markers, NSAIDs did not affect the ability of moDCs and moLCs to stimulate allogeneic T-cell proliferation, or the production of cytokines in an allogeneic T-cell stimulation assay. In agreement with this, in uVIN patients treated with imiquimod, no interference of frequent NSAID-use with clinical outcome was observed. However, we did notice that high $CD1a^+$ and $CD207^+$ cell counts in frequent NSAID-users before treatment seemed to predict a favorable response to imiquimod treatment.

Our data indicate that NSAID-use does not seem to interfere with moDC and moLC function and does not interfere with immunomodulatory properties of imiquimod in uVIN patients. Therefore, NSAIDs can safely be used to reduce imiquimod side effects in uVIN patients during treatment.

INTRODUCTION

Usual type Vulvar Intraepithelial Neoplasia (uVIN) is a premalignant skin disorder, which is caused by a persistent infection with high-risk human papillomavirus (hrHPV).[1,2] In the etiology of uVIN, inhibition of the host immune response seems to be an important determinant. Earlier studies demonstrated that immunodeficiency and smoking (which reduces local immunity) are important risk factors for the development of HPV-related premalignant and malignant lesions.[3-7]

The concept that the immune response is insufficient in patients with uVIN has lead to treatment with the immune response modifier imiquimod. Imiquimod binds to toll-like receptor (TLR) 7 and 8 at the cell surface of dendritic cells (DCs). Binding induces consecutive activation of nuclear factor-kappa B (NF-κB). This activation results in secretion of multiple cytokines, such as TNFα, type-1 IFN and IL-12, and activation of DCs, thus inducing a strong cellular immune response.[8,9] Upon treatment of uVIN with imiquimod, 35% of patients had a complete response (100% reduction in lesion size),[10] which lasted for >5 years in all but one case.[11] In addition, another 46% of patients showed a partial reduction in lesion size of at least 25%. Only in patients where the numbers of immunocompetent cells were normalized, the clinical response was ≥75%.[10] Based on these results, imiquimod is now considered first-line treatment for uVIN in the Netherlands.

Treatment with imiquimod, however, is not always tolerated well. Patients using imiquimod frequently complain of side effects such as severe local inflammation, itching or burning, flulike symptoms, weariness and headache.[10,12] We observed that patients treated with imiquimod commonly use nonsteroidal anti-inflammatory drugs (NSAIDs) or paracetamol in order to reduce these side effects. NSAIDs have been described to inhibit the enzymatic activity of cyclooxygenase (COX) 1 and 2, thereby impairing the conversion of arachidonic acid to prostaglandins (PGs), which results in an anti-inflammatory effect.[13] Additionally, several studies demonstrated that NSAIDs also suppress NF-κB activation[14-18] and in this way can inhibit DC differentiation.[14,19-20]

The effects of concomitant use of NSAIDs during imiquimod treatment are unknown. However, since the function of imiquimod is to enhance the patients' cell-mediated immune response against HPV and thus cure uVIN, the aim of this study was to investigate whether NSAIDs have an inhibitory effect on the cell-mediated immune response and thereby can interfere with imiquimods immunomodifying activities. Because it is not possible to isolate enough immune cells from VIN biopsies, we cultured DCs and Langerhans cells (LCs, a specialized dendritic cell that populates the epidermis[21]) from peripheral blood mononuclear cells (PBMCs) to elucidate the effect of NSAIDs on differentiation and function of antigen presenting cells. Furthermore, in patients who participated in an earlier study,[10] we analyzed whether frequent use of NSAIDs had an effect on imiquimod response and on immunocompetent cell counts before and after imiquimod treatment.

MATERIAL AND METHODS

Isolation of CD14+ cells and culture of moDCs and moLCs

PBMCs were isolated from buffy coats of healthy donors by Ficoll-paque (Ficoll Paque Plus, GE Healthcare Biosciences, Uppsala, Sweden) centrifugation. After isolation, PBMCs were washed and CD14 microbeads (MACS Microbeads, Miltenyi Biotec, Bergisch Gladbach, Germany) were added to isolate $CD14^+$ cells. The $CD14^+$ fraction was counted and the monocytes were placed in 24 well plates at a density of 1×10^6 cells/ml/well. Monocytes were cultured in RPMI 1640 containing Glutamax (Gibco Invitrogen, Carlsbad, USA), supplemented with 10% FCS and gentamycin, at 37˚C in a humidified atmosphere containing 5% carbon dioxide. Growth factors used for the generation of moDCs were 1,000 IU/ml GM-CSF (Immunotools, Friesoythe, Germany) and 200 IU/ml IL-4 (R&D systems, Abingdon, UK). TGF-β1 (10 ng/ml, PeproTech, Rocky Hill, USA) was added to moDC cultures to generate moLCs.[21,22] On day two of culture, 0.5–2.5 mM aspirin (Sigma, Zwijndrecht, the Netherlands), 100–500μM ibuprofen (Sigma) or ethanol (Sigma) was added to the wells to measure the effect of aspirin or ibuprofen on moDC and moLC maturation. On day 6, cells were stimulated with 2 ng/ml CPG 1668 (Gibco) to mimic viral infection.

Fluorescence-activated cell sorting (FACS) analysis

On day 7, cells were harvested and the expression of membrane markers was assessed by flowcytometry using the following fluorescent labeled antibodies: CD11c APC, HLA-DR APC-Cy7 for moDC culture and HLA-DR PE-Cy7 for moLC culture (all BD Biosciences, Franklin Lakes, USA), CD1a PE-Cy5, CD14 APC, CD80 PE (all BD Pharmingen, San Diego, USA), CD207 PE (Immunotech, Marseille, France), CD40 APC, CD86 PE (both eBioscience, Hatfield, UK), CCR7 FITC and CCR6 FITC (both R&D systems). Cells were labeled according to the manufacturers' instructions and appropriate isotype-matched antibodies served as negative controls. After staining with the viability marker 4',6-diamidino-2-phenylindole (DAPI, Invitrogen Molecular Probes, Carlsbad, USA), cells and data were analyzed using the LSR II (BD Biosciences) and FlowJo software (Treestar, Ashland, USA).

Isolation and fluorescent labeling of naive CD4+ T cells

T cells were purified from human buffy coat. Naive T cells were obtained by negative selection using naive $CD4^+$ T-cell isolation kit (Miltenyi) according to manufacturers' instructions. All steps were performed with MACS buffer (PBS supplemented with 5 mM EDTA and 1% BSA) at 4°C. In brief: all cells, except $CD4^+$ naive T cells, were indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies (CD8, CD14, CD16, CD19, CD36, CD45RO, CD123, TCRγ/δ and Glycophorin A) followed by incubation with antibiotin microbeads. Isolation of pure naive T cells was achieved by depletion of the magnetically labeled non-$CD4^+$ naive T cells. For fluorescent cell labeling, cells were washed twice with serum-free medium and labeled in a final concentration of 5 μM carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen,

Molecular Probes) for 10 min in serum-free medium at 37°C. Adding excess ice-cold culture medium stopped the reaction.

Allogeneic Mixed Lymphocyte Reaction (MLR)

CFSE labeled and CFSE nonlabeled allogeneic peripheral blood naive T cells were co-cultured in duplicate with moDCs or moLCs with and without NSAIDs with a DC:T-cell ratio of 1:10 in culture medium in 96 well round bottom plates for 5 days at 37°C. At day 5, T cells were harvested and stained with the antibodies CD4 APC (eBioscience) and CD3 PE (BD Pharmingen) to discriminate between T cells and moDCs or moLCs. T-cell proliferation was measured by flowcytometry using a LSR II (BD Biosciences) and FlowJo software (Treestar).

Measurement of prostaglandin E_2 and cytokine production

Levels of prostaglandin E_2 (PGE_2) were measured in day 7 supernatant of moDC and moLC cultures by using a competitive enzyme immunoassay (R&D systems) according to the manufacturer's instructions.

Cytokine levels were measured in MLR culture supernatants (moLCs cultured with T cells) using ELISA kits according to the manufacturer's instructions. Il-4, IFNγ, type 1 IFN, TNFα (all eBioscience) and IFN-inducible protein-10 (Ip-10, BD Biosciences), were measured in day 5 supernatant of allogeneic MLR.

Study population and immunohistochemical staining of biopsies

Twenty-five women with multifocal uVIN who participated in our double-blinded, placebo-controlled randomized clinical trial (RCT)[10] were treated with imiquimod twice a week for 16 weeks. Biopsy samples of these patients taken before and 4 weeks after treatment were analyzed for the presence of $CD1a^+$ LCs, $CD207^+$ DCs expressing Langerin, $CD208^+$ DCs, $CD123^+/CD11c^-$ plasmacytoid DCs, $CD94^+$ natural killer (NK) cells, $CD14^+$ monocytes, $CD4^+$ T-helper cells, $CD8^+$ cytotoxic T cells, $CD25^+/HLA\text{-}DR^+$ regulatory T cells, and $CD68^+$ macrophages, as described previously.[23]

All patients kept a daily record chart to report use of their study medication as well as any concomitant medication. Drugs most commonly used in this study were over-the-counter NSAIDs. All patients who were treated with imiquimod were divided into two groups according to the following criteria for the frequency of NSAID-use: 1) Low users: never or sporadically, *i.e.* no more than three times during the whole study period for one or two days, and 2) frequent users: daily or regularly, *i.e.* more than three times during the whole study period, for 1 up to 5 days in a row.

Statistical evaluation

Data analysis was performed with the use of the SPSS 15.0 software package for Windows. For the *in-vitro* experiments, differences in cytokine levels and in percentage of proliferated T cells between groups were compared using the Kruskal-Wallis test.

We used the Fisher Exact test to evaluate differences in NSAID-use between patients with ≥75% clinical response, defined as a clinical reduction in lesion size of at least 75%, versus patients with <75% clinical response, defined as a clinical reduction in

lesion size of less than 75%. We used the Mann Whitney U test to evaluate differences in immunocompetent cell counts between the different subgroups (frequent- or low NSAID-users with ≥75% response versus <75% response). A two-tailed *P*-value of $P<0.05$ was chosen to represent statistical significance.

RESULTS

In vitro effect of aspirin and ibuprofen on moDC maturation and on moDC induced T-cell proliferation

To investigate the effect of aspirin and ibuprofen on moDC maturation, $CD14^+$ monocytes (+ GM-CSF and IL-4) were cultured from day 2 till day 7 in the presence of 0.5–2.5mM aspirin or 100–500μM ibuprofen as described in *Materials and Methods*. Since uVIN patients have a permanent HPV-infection, we added CPG on day 6 to mimic the stimulatory effect of viral DNA and detect the effect on moDC maturation and function. We performed a competitive immunoassay on day 7 culture supernatants to demonstrate that NSAIDs reduced PGE_2 levels. PGE_2 production was reduced by more than 20% after stimulation with ibuprofen and by more than 70% after stimulation with aspirin (data not shown).

After FACS analysis on day 7, living (DAPI-negative) moDCs ($CD11c^+$, $HLA\text{-}DR^+$ cells) were gated (Fig. 1a). From these viable cells, we analyzed the expression of the maturation markers CD80, CD86, CD40, and HLA-DR and of the chemokine-receptors CCR6 (highly expressed in immature DCs[24]) and CCR7 (highly expressed in mature DCs[24]). As shown in Figure 1b, a dose-dependent increase in expression of HLA-DR and CD86 was observed upon stimulation with aspirin or ibuprofen, while CD80, CD40 and CCR6 expression was somewhat decreased upon stimulation with the highest dose of aspirin or ibuprofen. No difference in expression was observed for CCR7. Similar results were observed in cell cultures without CPG stimulant (data not shown).

In order to investigate whether interaction of NSAID-treated moDCs with $CD4^+$ T cells results in T-cell activation, we co-cultured moDCs with allogeneic T cells and subsequently analyzed T-cell proliferation. First, living $CD4^+CD3^+$ T cells were gated and the percentage of proliferated cells was analyzed by gating the CFSE-negative T-cell population (Fig. 1c). Figure 1d shows the percentage of proliferated T cells in co-cultures with moDCs stimulated with CPG, CPG + 2.5 mM aspirin or with CPG + 500 μM ibuprofen. MoDCs stimulated with CPG in the presence or absence of ibuprofen or aspirin did not differ significantly in their ability to activate T cells.

Effect of aspirin and ibuprofen on moLCs

Our next step was to investigate the effect of NSAIDs on moLCs, since LCs play a major role in initiation of the cellular immune response in the skin.[25] Previously, we observed that LC counts are reduced in the epidermis of VIN patients.[23]

In order to investigate the effect of NSAIDs on moLCs, we cultured PBMCs with GM-CSF, IL-4 and TGF-β to generate a high percentage of Langerhans-like moDCs. As

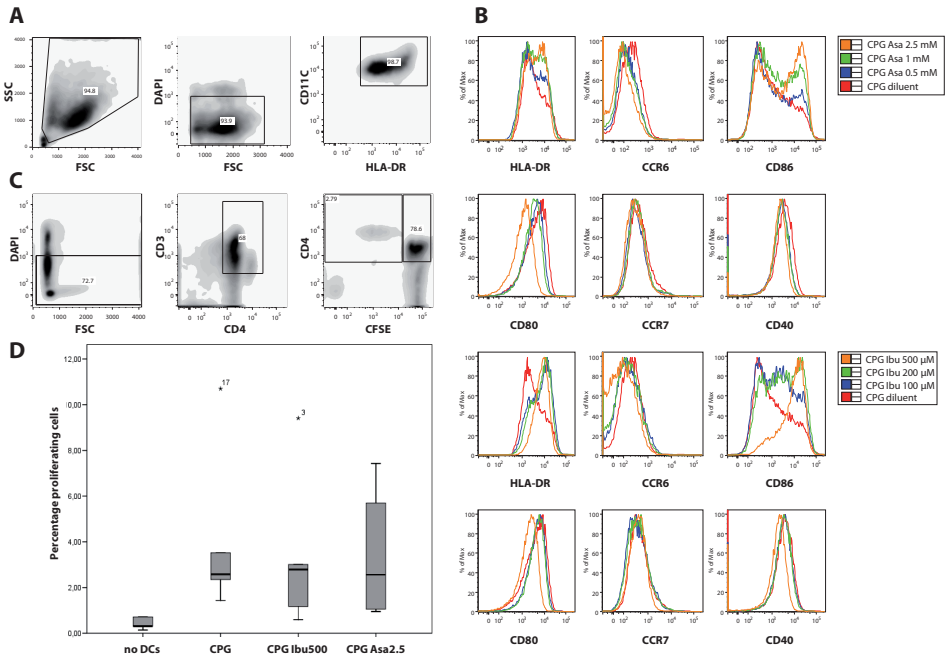

Figure 1. A. Gating of monocyte-derived dendritic cells (moDCs). DAPI-negative living cells are gated and from this population, CD11c^{+} HLA-DR^{+} cells are selected as moDCs. **B.** Expression of HLA-DR, CCR6, CD86, CD80, CCR7 and CD40 in moDCs ± aspirin (Asa) and expression in moDCs ± ibuprofen (Ibu). **C.** Gating of proliferated T cells. CD4^{+}CD3^{+} cells are selected as T cells. Proliferated T cells are CFSE^{low}. **D.** Percentage of proliferated T cells in co-cultures with moDCs stimulated with CPG, with CPG + ibuprofen 500 µM (Ibu500) and with CPG + aspirin 2.5 mM (Asa2.5). No significant differences were observed between CPG, CPG + ibuprofen and CPG + aspirin (Kruskal-Wallis test). FSC, forward scatter; SSC, sideward scatter; CFSE, carboxyfluorescein succinimidyl ester; Asa, aspirin; Ibu, ibuprofen.

is shown in Figure 2a, CD1a – a cell-specific marker for epidermal LCs[25] – is expressed on the surface of cells cultured with TGF-β. However, upon stimulation with aspirin or ibuprofen, more cells became CD1a negative suggesting an impaired differentiation. The strongest effect was observed after stimulation with ibuprofen. The expression of CD207 – Langerin, which is a LC specific C-type lectin[25] – decreased upon aspirin stimulation but increased upon ibuprofen stimulation. When we measured the expression of the maturation markers CD80, CD86 and HLA-DR, an increase in CD86 expression was found upon stimulation with aspirin or ibuprofen (Fig. 2a), as was observed in conventional moDCs. However, HLA-DR expression increased upon stimulation with ibuprofen, but decreased upon stimulation with aspirin. CCR6 increased upon ibuprofen stimulation and no differences were found for the other markers (Fig. 2a).

To assess the effect of NSAID-stimulated moLCs on T-cell proliferation, we performed again an allogeneic MLR. However, moLCs cultured with or without ibuprofen or aspirin did not differ significantly in their ability to activate T cells (Fig. 2b).

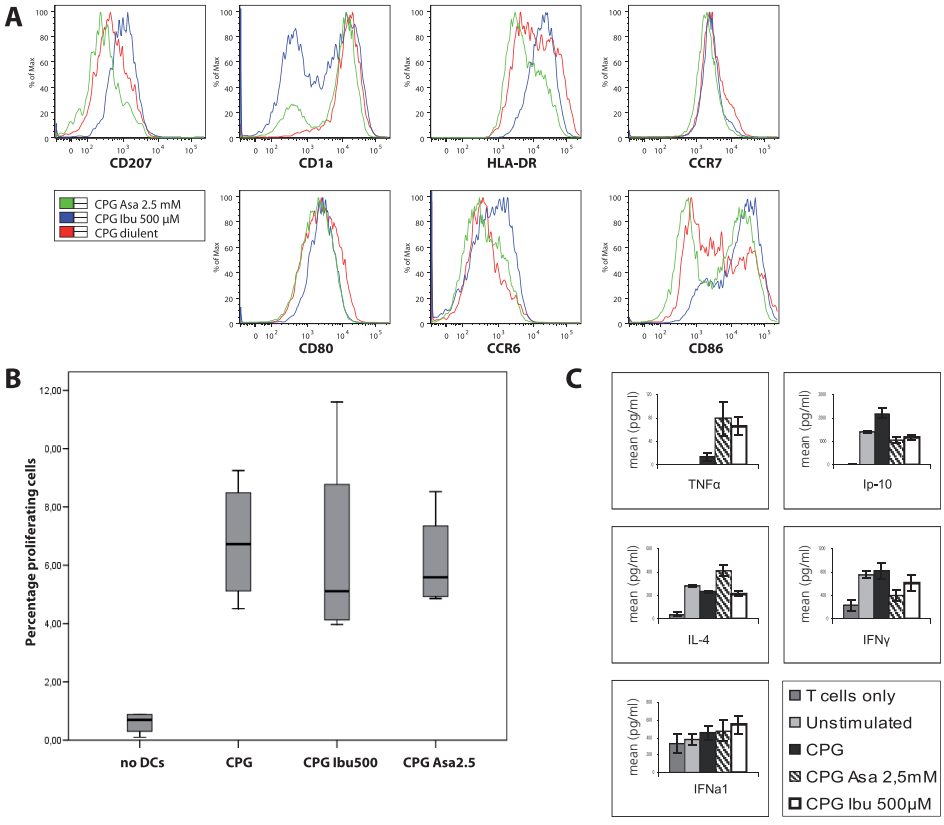

Figure 2. A. Expression of CD207, CD1a, HLA-DR, CCR7, CD80, CCR6 and CD86 in monocyte-derived Langerhans cells (moLCs) ± aspirin (Asa) and expression in moLCs ± ibuprofen (Ibu). **B.** Percentage of proliferated T cells in co-cultures with moLCs with CPG, CPG + ibuprofen 500 µM (Ibu500) and with CPG + aspirin 2.5 mM (Asa2.5). No significant differences were observed between CPG, CPG + ibuprofen and CPG + aspirin (Kruskal-Wallis test). **C.** Cytokine production measured in allogeneic MLR supernatants with moLCs and T cells. Mean ± SEM, data from four buffy coats. No differences between CPG, CPG + aspirin 2.5 mM and CPG + ibuprofen 500 µM (Kruskal-wallis test). Asa, aspirin; Ibu, ibuprofen.

The levels of different inflammatory cytokines were measured in supernatant of MLR cultures to assess the effect of NSAIDs on cytokine production. Although, Ip-10 and Il-4 were significantly higher in MLR supernatants than in T cells cultured without moLCs, no effect of aspirin or ibuprofen was observed on cytokine production (Fig. 2c).

In vivo effect of NSAID-use on immunocompetent cell counts before and after imiquimod treatment, and response rate after treatment

Results from our *in-vitro* experiments demonstrated no effect of NSAIDs on phenotype and function of moDCs and moLCs. Because we were interested in a possible interference of NSAIDs with imiquimod treatment, we next investigated the effect of NSAID-use on imiquimod response in VIN patients who were treated with imiquimod

in a previous double-blinded, placebo-controlled RCT.[10] According to our definition, 12 of 25 patients were frequent NSAID-users; 5 of the 12 had a clinical response to imiquimod of ≥75% and seven patients had a response of <75%. Thirteen patients were low NSAID-users; eight of them had a clinical response of ≥75% response and five patients had a response of <75%. No significant difference was measured in NSAID-use between patients with ≥75% response and patients with <75% response. This indicates that NSAID-use does not influence the success of imiquimod treatment. Interestingly, when we compared immunocompetent cell counts between different subgroups, we did observe that frequent NSAID-users with ≥75% response had significantly higher CD1a$^+$ and CD207$^+$ cell counts in the epidermis before imiquimod treatment than frequent NSAID-users with <75% response (P=0.007 and P=0.004 respectively, Fig. 3a and 3b) or than any of the low NSAID-users. Cell counts for the different immunocompetent cells are shown in supplementary Table 1 (http://www.erasmusmc.nl/47393/1584119/1603959/Terlou).

In conclusion, elevated CD1a$^+$ and CD207$^+$ cell counts in frequent NSAID-users seem to predict a good clinical response to imiquimod treatment, but NSAID-use, as such, does not negatively affect the success of imiquimod treatment.

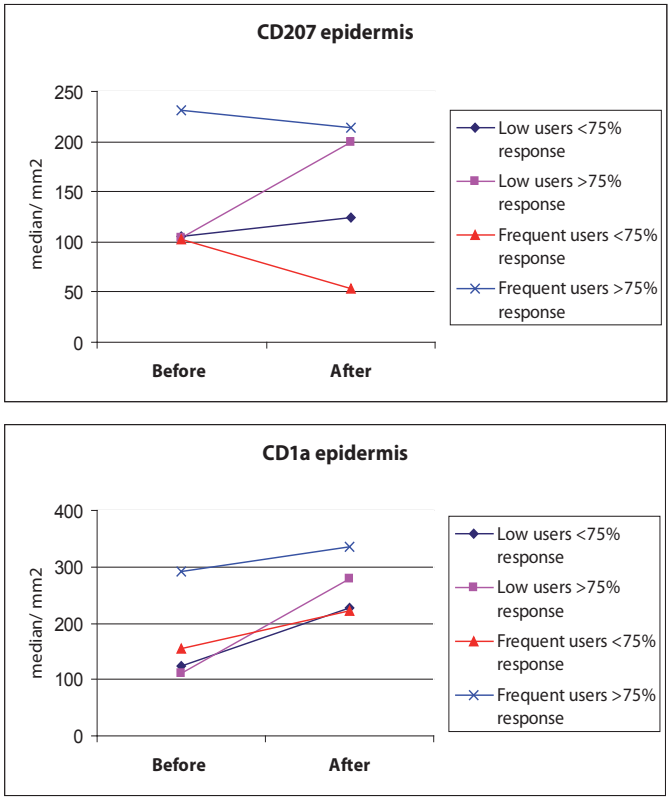

Figure 3. Number of CD1a$^+$ and CD207$^+$ cells before and after imiquimod treatment in frequent NSAID-users and low NSAID-users with ≥75% and <75% clinical response. Frequent NSAID-users with ≥75% response have significantly higher CD1a$^+$ and CD207$^+$ cell counts in the epidermis before imiquimod treatment than frequent NSAID-users with <75% response (P=0.007 and P=0.004, Mann-Whitney U Test).

DISCUSSION

In our study, we investigated whether NSAIDs have an inhibitory effect on the cell-mediated immune response and thereby can interfere with imiquimods immunomodifying activities. For this purpose, moDCs and moLCs were cultured *in vitro* in the presence of aspirin and ibuprofen and expression of relevant surface markers, stimulatory function and production of proinflammatory cytokines were measured. Although an effect was observed on the expression of moDC and moLC maturation markers (HLA-DR, CD86, CD80, CD40, and CCR6), NSAIDs did not affect the ability of moDCs and moLCs to stimulate T cells, neither could we observe an effect on the production of cytokines. Additionally, we investigated whether frequent use of NSAIDs had an effect on imiquimod response in patients with uVIN. We found that frequent NSAID-users with high $CD1a^+$ and $CD207^+$ cell counts before imiquimod treatment had a favorable response to imiquimod treatment. In other words, in frequent NSAID-users high $CD1a^+$ and $CD207^+$ cell counts seem to predict the imiquimod response. However, in accordance with our *in vitro* findings, we did not observe a reduced imiquimod response in VIN-lesions of frequent NSAID-users. In summary, our results indicate that NSAIDs do not inhibit moDC and moLC stimulatory function and do not seem to interfere with imiquimods immunomodulatory properties in uVIN patients.

NSAIDs have been used for more than 100 years to reduce pain and inflammation, in particular in patients with various rheumatologic and inflammatory disease states for adequate control of their condition. The anti-inflammatory and analgesic efficacy of NSAIDs is mainly due to the inhibition of COX-1 and COX-2, which convert arachidonic acid to PGs.[13] PGs play an important role in the function of immune cells by modulating production of cytokines, chemokines and their receptors.[26] Additionally, PGs such as PGE_2 are mandatory for DC migration[27,28] and enhance T-cell proliferation and differentiation.[29]

Another way in which NSAIDs affect the immune response is by inhibiting NF-κB activation.[14] NF-κB has been regarded as a key element in the response of cells to inflammatory stimuli and targets proinflammatory cytokines, chemokines and cell adhesion molecules.[16] Inhibition of NF-κB also affects maturation of DCs.[30] Several studies have investigated the effect of NSAIDs on DCs.[19,20, 31-35] Hackstein *et al.*[19] reported an inhibition of maturation and function of murine DCs after stimulation with aspirin, while Matasic *et al.*[20] also demonstrated an impairment of immunostimulatory function in human DCs after aspirin stimulation. This effect was due to inhibition of NF-κB, since no effect was observed upon stimulation with selective COX-1 or COX-2 inhibitors.[20] In contrast, other studies demonstrated that NSAIDs can stimulate DC function by inhibition of PGE_2 production. PGE_2 enhances Il-10, which down-regulates Il12-p70 production and thereby down-regulates DC stimulatory capacity.[34,35] These sometimes contradictory reports are difficult to compare, since different cell types, protocols or drugs were used. Moreover, various pathways involved in DC maturation can be affected upon stimulation with NSAIDs.

In our study, no effect on moDC function was measured after stimulation with aspirin or ibuprofen, despite the fact that the concentrations of aspirin and ibuprofen used in our experiments were within the range to inhibit COX-1, COX-2 and NF-κB activation.[17,18] In addition, we also failed to observe an effect of NSAID-use during imiquimod treatment of VIN. A possible explanation for this is that a significant percentage of NSAIDs bind to plasma albumin, and *in vivo* NSAIDs are partly converted into more or less biologically active metabolites,[16,36] which could result in plasma concentrations of NSAIDs in patients too low to affect DC function. It is also possible that the effect of NSAIDs in these patients is overruled by the strong stimulatory effect of imiquimod on NF-κB. After binding on TLR 7 or 8 on the surface of the DC, imiquimod induces activation of NF-κB, which results in a profound cellular immune response.[8,9]

In conclusion, our data indicate that NSAIDs do not interfere with imiquimod immunomodifying activities and therefore can safely be used to reduce the symptoms of imiquimod side effects in uVIN patients during treatment. Furthermore, elevated $CD1a^+$ and $CD207^+$ cell counts in frequent NSAID-users seem to predict a good clinical response to imiquimod treatment.

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TREATMENT OF VAGINAL INTRAEPITHELIAL NEOPLASIA WITH IMIQUIMOD: RESULTS OF A PILOT STUDY

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In revision

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AN AUTOIMMUNE PHENOTYPE IN VULVAR LICHEN SCLEROSUS AND LICHEN PLANUS: A TH1 RESPONSE AND HIGH LEVELS OF MICRORNA-155

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ABSTRACT

Vulvar lichen sclerosus and lichen planus are T-cell mediated chronic skin disorders. Although autoimmunity has been suggested, the exact pathogenesis of these disorders is still unknown.

Therefore, the aim of the current study was to investigate the molecular and immunological mechanisms critical in the pathogenesis of vulvar lichen sclerosus and lichen planus. By using gene expression profiling and real-time RT-PCR experiments, we demonstrated a significantly increased expression of the pro-inflammatory cytokines (*IFNγ*, *CXCR3*, *CXCL9*, *CXCL10*, *CXCL11, CCR5, CCL4, CCL5*) specific for a Th1 IFNγ induced immune response. In addition, *BIC*/microRNA-155 (miR-155) – a microRNA involved in regulation of the immune response – was significantly upregulated in lichen sclerosus and lichen planus (9.5- and 17.7- fold change respectively). Immunohistochemistry showed a significant T cell response, with pronounced dermal infiltrates of $CD4^+$, $CD8^+$ and $FOXP3^+$ cells. In conclusion, these data demonstrate an autoimmune phenotype in vulvar lichen sclerosus and lichen planus, characterized by increased levels of Th1 specific cytokines, a dense T cell infiltrate and enhanced *BIC*/miR-155 expression.

INTRODUCTION

Vulvar lichen sclerosus (LS) and lichen planus (LP) are both chronic inflammatory skin disorders that have a negative impact on quality of life and may proceed to malignant disease.[1]

LS can affect any part of the skin, but is most frequently seen in the anogenital area. Although it can occur at all ages, even among children, LS is most common in the fifth to sixth decade.[2,3] Women with vulvar LS often present with severe pruritus and soreness of the vulvar and perianal area.[2-4] Typically, lesions appear as a figure-of-eight pattern around the vulva and anus, with white atrophic lesions or hyperkeratosis. In advanced stages, there is destruction of the vulvar anatomy, characterized by resorption of the labia minora, narrowing of the introitus and a buried clitoris.[3]

LP also generally develops in the fifth to sixth decade.[5,6] In contrast to LS, the mucosa can be involved in LP, resulting in vaginal and oral lesions.[7,8] Patients usually present with vulvar soreness, pruritus, burning, dyspareunia, and/or vaginal discharge. Erosive lesions, with denuded epithelium associated with typical white Wickham's striae, can arise at the vulvar or vaginal mucosa, and alterations of normal vulvar architecture may occur. In severe cases, adhesions and stenosis can lead to complete obliteration of the vagina.[5-7]

Histologically, both disorders are characterized by a band-like lymphocytic infiltrate and a thinned epidermis with vacuolar changes in the basal layer. In long standing, classic LS the lymphocytic infiltrate is located under a band of homogenized collagen below the dermoepidermal junction, while in LP this infiltrate is situated directly beneath the epidermis.[1] However, histological evaluation of LS and LP can be difficult, because especially the first phase of LS can show histological overlap with LP.[9]

Local application of ultrapotent corticosteroids is still the treatment of choice for both disorders. If corticosteroids are not helpful in patients with LP, the use of topical calcineurin inhibitors (tacrolimus or pimecrolimus) may be considered.[10] However, all these treatment options only reduce symptoms and generally does not stop progression.[1] This illustrates the importance of new treatment strategies. One example of such a new treatment option is photodynamic therapy (PDT). The possible antisclerotic and anti-inflammatory effect of PDT might be induced by the reduction of TGF-β1 and/or the increase of IL-10 after PDT.[11] Since progression to vulvar squamous cell carcinoma has been reported for LS as well as LP, life-long follow-up of these patients is indicated.[1]

The etiology of these disorders has not yet been elucidated. Various studies have suggested different etiological factors. Cases of familial LS and LP have been reported and an association with HLA class II antigens has been found, suggesting a genetic background.[12-14] Other studies have suggested a role for local factors and infectious agents, such as an infection with Borrelia burgdorferi, human papillomavirus (HPV) or hepatitis C virus. However, the results of these studies are inconclusive and none of these agents has been proven to be pathogenetic.[15-17] Current opinion suggest

an autoimmune pathogenesis for both disorders, since patients with LS and LP are more frequently diagnosed with autoimmune disorders such as vitiligo and thyroid disease, and the presence of autoantibodies, for example to extracellular matrix protein 1 (ECM1) in patients with LS, has been observed.[18,19]

In summary, since it is not clear what causes LS or LP, the aim of this study was to elucidate the molecular mechanisms involved in the pathogenesis of vulvar LS and LP.

MATERIALS & METHODS

Patient samples

Fresh frozen and formalin-fixed tissues of patients with a clinically and histologically confirmed diagnosis of LS and LP were selected from the anonymous pathology archive of the Erasmus University Medical Center. Control samples were obtained from healthy women who underwent elective vulvar surgery for cosmetic reasons.

Fresh tissues were directly frozen in liquid nitrogen after collection and were stored at -80ºC until further analysis. Cryosections of 10 µm were obtained and the first and tenth section in a series of 10 was stained to assess tissue morphology. The remaining sections were used to isolate total RNA using Trizol (Invitrogen, Life Technologies, Philadelphia, PA, USA) and quality and quantity was assessed on the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA).

RNA was isolated from 14 control samples, 12 LS and 11 LP samples. From these samples, we selected 14 control, 8 LS and 5 LP samples for the microarray experiment. To confirm microarray data, we performed RT-PCR experiments using RNA of 4 LS and 2 LP of the microarray samples, combined with RNA of other samples (4 LS and 6 LP).

For immunohistochemistry we selected cryo- and paraffin sections of LS (n=16), LP (n=9) and control samples (n=10). Once more, to confirm microarray data, these samples were others than used for the microarray experiments.

The medical ethical committee of the Erasmus University Medical Center approved our study design and the protocol was compliant with the Helsinki Guidelines.

Microarray and microarray data analysis

Affymetrix U133plus2 GeneChips were used to obtain gene expression profiles of 8 LS (median age 58 years, range 22–76), 5 LP (median age 59 years, range 51–78) and 14 control samples (median age 40 years, range 17–54). In addition, nine samples of patients with usual type vulvar intraepithelial neoplasia (uVIN) (median age 39, range 33–48), a vulvar disease with a strong lymphocytic dermal infiltrate, were used for the analysis of *BIC*/miR-155 gene expression. Isolation of RNA, staining, washing and scanning procedures were performed as described by the manufacturer (Affymetrix, Santa Clara, CA, USA).[61] Raw (.CEL files) and normalized microarray data have been deposited in the GEO repository at NCBI under accession number GSE5563.

Differentially expressed genes were identified by using statistical analysis of microarrays (SAM). A False Discovery Rate (FDR) less than 1% was considered

statistically significant. Using OmniViz software hierarchical clustering of differentially expressed genes was performed.

Ontological analysis, using Ingenuity Pathway software (Ingenuity® Systems, www.ingenuity.com), was employed to assess the functional relevance of the observed differences in gene expression profiles.

Quantitative real-time RT-PCR

IFNγ, IL17A, IL17F, IL22RA1 and *CXCL10*. Validation of the expression of these genes was performed in 8 LS (of whom 4 were also used for microarray), 8 LP (of whom 2 were used for microarray) and 8 control samples in duplicate using real-time quantitative RT-PCR (38 cycles, 15 sec at 95ºC, 30 sec at 59-62ºC and 1 min at 72ºC) using the Opticon I (Applied Biosystems, Foster City, CA, USA) and SYBR Green ITM (Applied Biosystems). cDNA samples (10 ng each) were amplified with gene specific primer pairs (0.5 μM) in a total volume of 25 μl including 12.5 μl SYBR Green PCR master mix (Applied Biosystems). The housekeeping gene β-actin was used for normalization and all used PCR primers were intron spanning. The used primer sequences were as follows.

β-*actin*: 5′-TCCCTGGAGAAGAGCTACGA-3′(forward);
5′-AGGAAGGAAGGCTGGAAGAG-3′ (reverse).
IFNγ: 5′-TGACCAGAGCATCCAAAAGA-3′(forward);
5′-CATGTATTGCTTTGCGTTGG-3′ (reverse).
IL17A: 5′-ACCAATCCCAAAAGGTCCTC-3′(forward);
5′-GGGGACAGAGTTCATGTGGT-3′(reverse).
IL17F: 5′CCTCCCCCTGGAATTACACT-3′(forward);
5′-TTCCTTGAGCATTGATGCAG-3′(reverse).
IL22RA1: 5′ACCCCAGACACGGTCTACAG-3′(forward);
5′-GTAGAGCTCCGTGAGGTTGC-3′(reverse).
CXCL10: 5′-CCACGTGTTGAGATCATTGC-3′(forward);
5′-TTCTTGATGGCCTTCGATTC-3′(reverse).

The relative fold increase of real-time RT-PCR results was determined by the $2^{-\Delta\Delta Ct}$ method[62] using the average expression of the housekeeping gene (β-actin) as a control.

MicroRNA-155 (miR-155). Validation of the expression of miR-155 was performed using a two-step TaqMan RT-PCR assay (Applied Biosystems) according to manufacturers' protocols in 5 LS, 5 LP and 5 control samples. The TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) was used for the preparation of cDNA; 10 ng of total RNA was used per 15 μl RT reaction. Reverse transcription was performed in a PTC-200 Peltier Thermal Cycler (MJ Research, Biozym, Landgraaf, Belgium) for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then held at 4°C. The RT products were subsequently amplified with sequence-specific human miRNA primers using the Applied Biosystems 7900HT Real-Time PCR system. The reactions were incubated in duplicate in a 96-well plate at 95°C for 10 min followed by 40 cycles at 95°C for 15

sec and at 60°C for 1 min. Amplification signals were computed with the SDS v.2.3 software (Applied Biosystems). The relative fold increase of miR-155 to control was determined by the $2^{-\Delta\Delta Ct}$ method[62] using the average expression of two endogenous controls, RNU44 and RNU48.

Immunohistochemistry

Immunohistochemical staining was performed on 6 µm thick frozen tissue sections. The following markers were used: CD4, marker for T helper cells (MT.310; Dako, Glostrup, Denmark); CD8, marker for cytotoxic T cells (DK25; Dako); FOXP3, marker for regulatory T cells (Treg) (EBioscience Ltd., Hatfield, UK) and CD19, marker for B cells (Beckmann Coulter, Brea, USA), in dilutions of respectively 1:50, 1:50, 1:50 and 1:25. Staining was performed as described previously.[63]

For identification of immature dendritic cells (DCs)/ Langerhans cells, staining for CD1a (clone O10, Immunotech, Marseille, France) in a dilution of 1:10 was performed on paraffin-embedded tissue sections as described earlier.[55]

Stained cells were counted throughout the entire epidermal thickness and 100 µm deep into the dermis in a blinded session. Because the cell infiltrate in LS tissue is localized in the mid-dermis below a subepidermal sclerotic zone, LS tissues were counted 100 µm deep into the mid-dermal infiltrate. After measuring the total area of 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

Data analysis was performed with the use of the SPSS 17.0 software package for Windows. Immunohistochemistry and PCR data were analyzed using the non-parametric Kruskal-Wallis test and the Mann-Whitney test for independent samples. Correlation studies were performed with the non-parametric Spearman's correlation coefficient. A two-tailed *P*-value of $P<0.05$ was chosen to represent statistical significance.

RESULTS

Identification of differentially expressed genes in LS and LP

To study signaling pathways involved in both disorders, we performed gene expression arrays. Using SAM analysis we found 6643 differentially expressed probesets between LS and controls, 7354 between LP and controls and 623 between LS and LP (Fig. 1). The number of differentially expressed genes between LS and LP was only 10% of the number of genes differentially expressed between LP and controls, or between LS and controls (the complete list of differentially expressed genes can be obtained from supplementary Table 1, http://www.erasmusmc.nl/47393/1584119/1603959/Terlou).

By using Ingenuity Pathway Analysis we identified biological functions that were significantly regulated in LS and LP as compared to control samples. The following biological functions were found ($P<0.001$): 1) Antigen presentation, 2) Cell-mediated

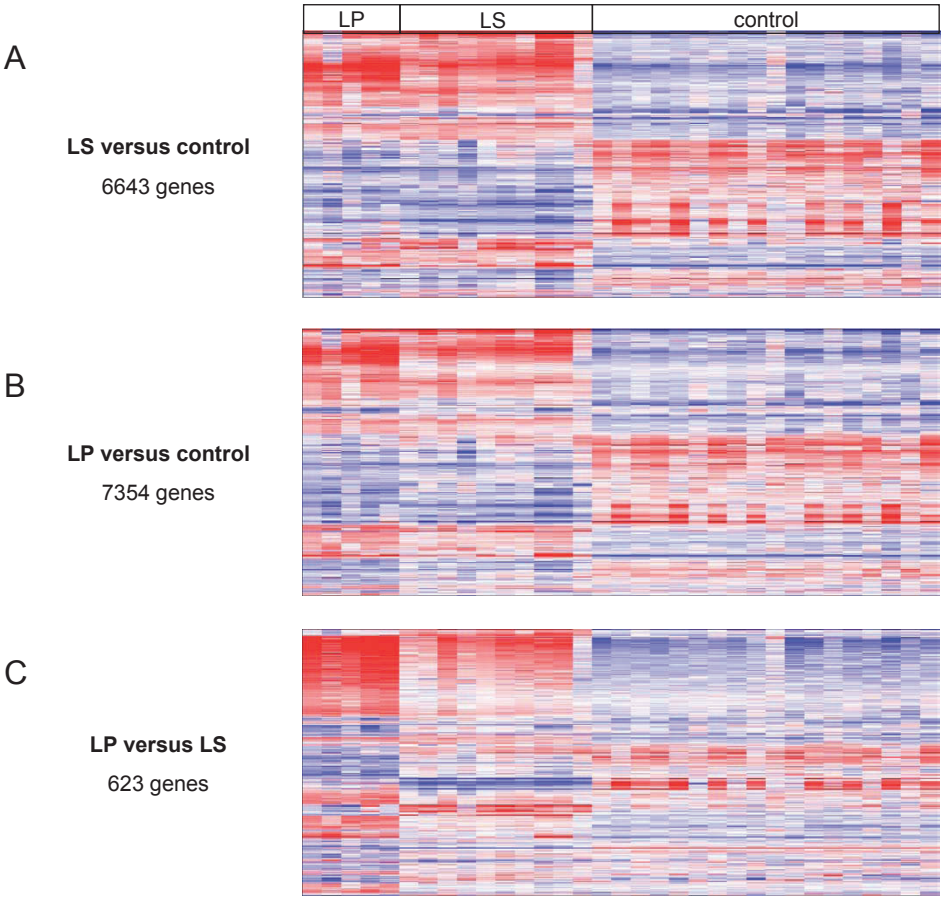

Figure 1. Gene expression profiles of lichen sclerosus (LS) and lichen planus (LP). Unsupervised clustering of expression profiles from 18 control, 5 LP and 10 LS samples. **A.** 6643 genes were differentially regulated in LS versus control. **B.** 7354 genes were differentially regulated in LP versus control. **C.** 623 genes were differentially regulated in LP versus LS. Each row represents one gene (red: upregulated, blue: downregulated), each column one expression profile from one tissue sample.

immune response, 3) Humoral immune response and 4) Inflammatory response (supplementary Fig. 1, http://www.erasmusmc.nl/47393/1584119/1603959/Terlou). High regulation of these biological functions is caused by the fact that most differentially expressed genes between LS/LP and control tissues are involved in pathways related to the immune response. This result was also found by Edmonds et al., who studied gene expression profiling in male genital lichen sclerosus.[20]

In order to investigate the immune response in more detail, we used the KEGG cytokine-cytokine receptor pathway (www.genome.jp/kegg/) as a background for our own immune signaling data. Many cytokines were upregulated in LS and LP (Fig. 2). For most genes this upregulation was more pronounced in LP than in LS. By

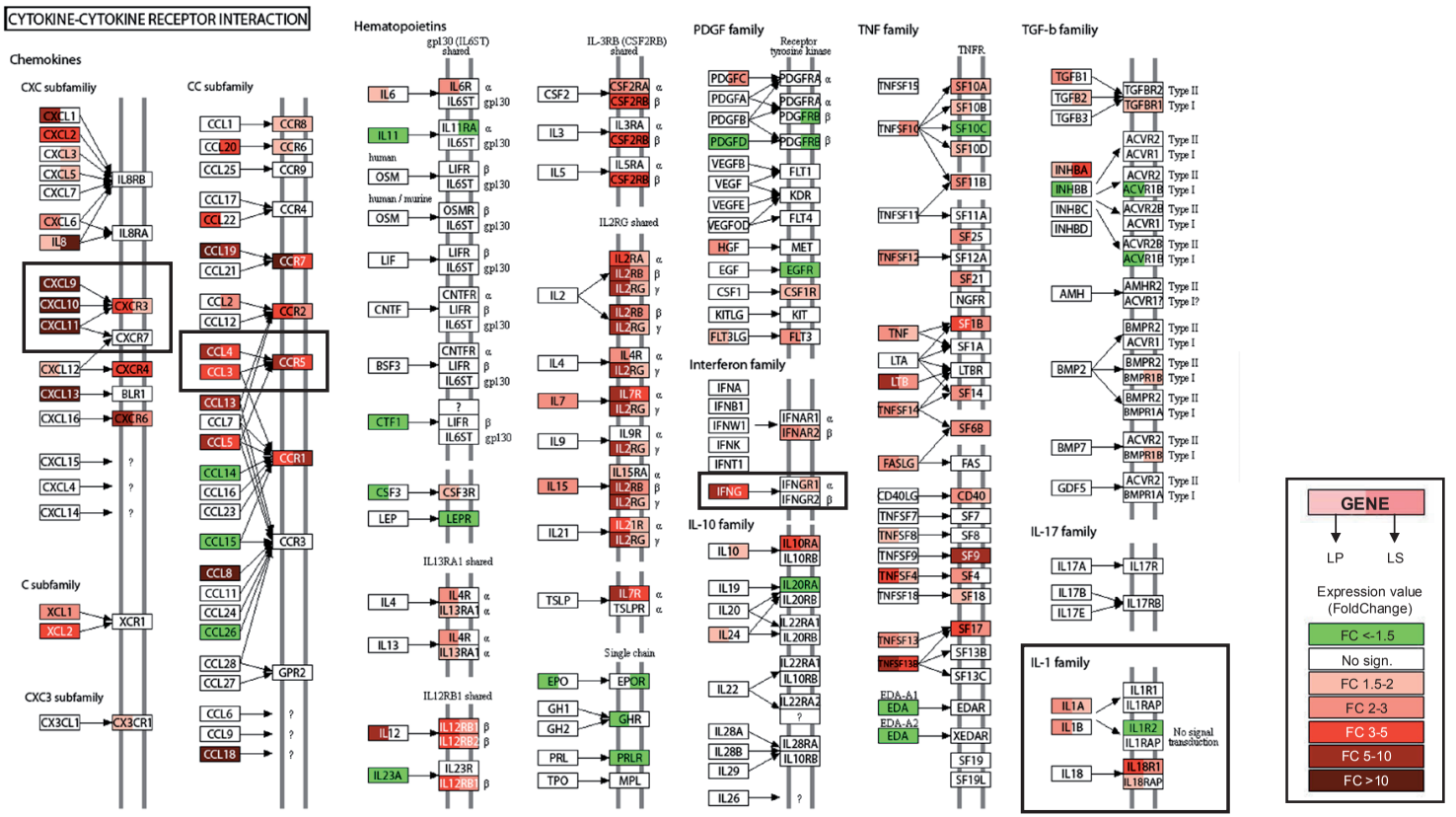

Figure 2. Cytokine - cytokine receptor pathway in lichen sclerosus (LS) and lichen planus (LP). The Cytokine-cytokine receptor scheme form the KEGG database (www.genome.jp/kegg/) was used to overlay gene expression values in LS and LP compared to controls. Each box represents one gene: the color represents the fold change expression value in LP (left side) and LS (right side). Black boxes mark 1) CXCR3 and CCR5 and their ligands, 2) IFNγ and 3) the IL-1 family.

studying those cytokines in more detail, we focused on well-known pro-inflammatory and anti-inflammatory cytokines.[21] Of the pro-inflammatory cytokines we found upregulation of *IL1*, *IL6* (only in LP), *IL7*, *IL15*, *IFN*γ and *TNF*α. For most of the anti-inflammatory cytokines no modulation was found (for example for *IL4* and *IL13*), and only slight upregulation of *TGF*β*1* (only in LP) and *TGF*β*2* (only in LS). Interestingly *IL11*, also known as an anti-inflammatory cytokine, was downregulated in LS and LP. In summary, significantly more pro-inflammatory cytokines were highly expressed in LS and LP, which is regarded as an indication for a cellular T helper type 1 (Th1) response.[22]

IFNγ is one of the key players in the Th1 response and its mRNA was strongly upregulated in LS and LP (3.5- and 7.8-fold, respectively, Fig. 2). It is produced by T helper cells ($CD4^+$ T cells), which are characterized by chemokine-receptors *CXCR3* and *CCR5*.[23] As shown later (Fig. 4), in the dermis significantly higher levels of $CD4^+$ T cells were seen in LS and LP, and as expected both *CXCR3* and *CCR5* were highly expressed in both disorders. Besides this strong expression of *CXCR3* and *CCR5*, their activating ligands *CXCL9*, *CXCL10*, *CXCL11* and *CCL3, CCL4,* respectively, were also strongly upregulated. These results indicate that the infiltrate of $CD4^+$ T cells seems to be involved in the production of significant amounts of IFNγ, *CXCL9*, *CXCL10*, *CXCL11, CCL3* and *CCL4,* thus further stimulating a cellular Th1 response in LS and LP. Other stimulatory factors for production of Th1 cytokines and consequently for the differentiation of T cells to the Th1 phenotype, are cytokines of the IL-1 family, namely interleukin-1 alpha (IL1α), interleukin-1 beta (IL1β) and interleukin-18 (IL18) (Fig. 2, IL-1 family is indicated with a black box). We found upregulation of IL1α and the IL18 receptor in LS and LP and upregulation of IL1β in LP. To activate these inflammatory cytokines, caspase 1 is necessary. As expected, caspase 1 (CASP1) was significantly upregulated in LS (1.8-fold) as well as in LP (2.0-fold).

All the observations described above suggest a strong Th1 response. However, to investigate the potential role of Th2 and Th17 next to Th1, we performed real-time RT-PCR on important regulators of these responses. A clear and pronounced upregulation of *IFN*γ and *CXCL10* was confirmed, indicating a Th1 response, while we found no significant regulation of key players in the Th2 (*CCR3*, *IL4*) and Th17 (*IL17A*, *IL17F*, *IL22R*) response (Fig.3A).

One other important finding concerning the immune response was the strong upregulation of *BIC*, a gene which encodes for microRNA-155 (miR-155). MiR-155 plays an important role in regulating homeostasis of the immune system. It regulates production of cytokines, chemokines and transcription factors, but has also been shown to promote T cell-dependent autoimmune inflammation.[24,25] We found strong upregulation of *BIC*/miR-155 in LS (9.5-fold) and LP (17.7-fold) compared to controls (Fig. 3B). This upregulation was confirmed by TaqMan RT-PCR (LS: 8,5-fold and LP:17.5-fold, Fig. 3A). In contrast, no differential gene expression of *BIC*/miR-155 was observed between controls and uVIN samples, a vulvar disease with a strong lymphocytic dermal infiltrate (Fig. 3B).

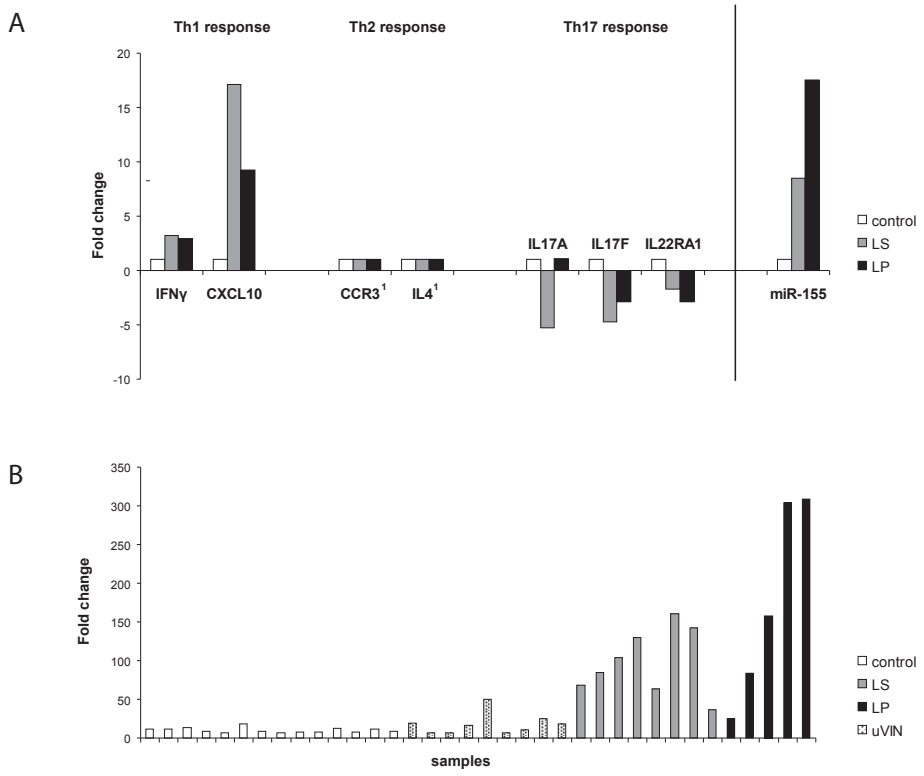

Figure 3. Real-time RT-PCR results. **A.** Differences in gene expression are visualized for key players in the Th1, Th2 and Th17 response and validation of miR-155 expression by TaqMan RT-PCR (control n=5, lichen sclerosus (LS) n=5 and lichen planus (LP) n=5). [1] No significant difference on microarray data and therefore no real-time RT-PCR was performed. **B.** Gene expression of *BIC*/miR-155 in controls (n=14), LS (n=8), LP (n=5) and usual type vulvar intraepithelial neoplasia (uVIN) (n=9).

Immune cell counts in LS and LP

In order to document the composition of the cellular infiltrate, presence of $CD4^+$ T cells, $CD8^+$ T cells, $FOXP3^+$ Treg cells, $CD1a^+$ DCs/Langerhans cells and $CD19^+$ B cells was assessed. In LS no differences in immune cell counts were observed in the epidermis compared to controls. However, in the dermis, cell counts for $CD8^+$ T cells and $FOXP3^+$ Treg cells were significantly higher than in controls ($P<0.001$ and $P=0.013$ respectively, Fig. 4 and supplementary Table 2, http://www.erasmusmc.nl/47393/1584119/1603959/Terlou). In contrast, $CD1a^+$ cells were significantly decreased in the dermis ($P=0.008$, supplementary Table 2).

In the epidermis of LP, $FOXP3^+$ Treg cells were significantly increased compared to controls ($P=0.024$), and no differences were observed for the other immune cells. In the dermis, however, a significant increase in $CD4^+$ T cells, $CD8^+$ T cells, $FOXP3^+$ Treg cells and $CD19^+$ B cells was observed in LP in comparison with controls (Fig. 4 and supplementary Table 2). Similar to differences in gene expression levels, differences in immune cell counts were more pronounced in LP than in LS.

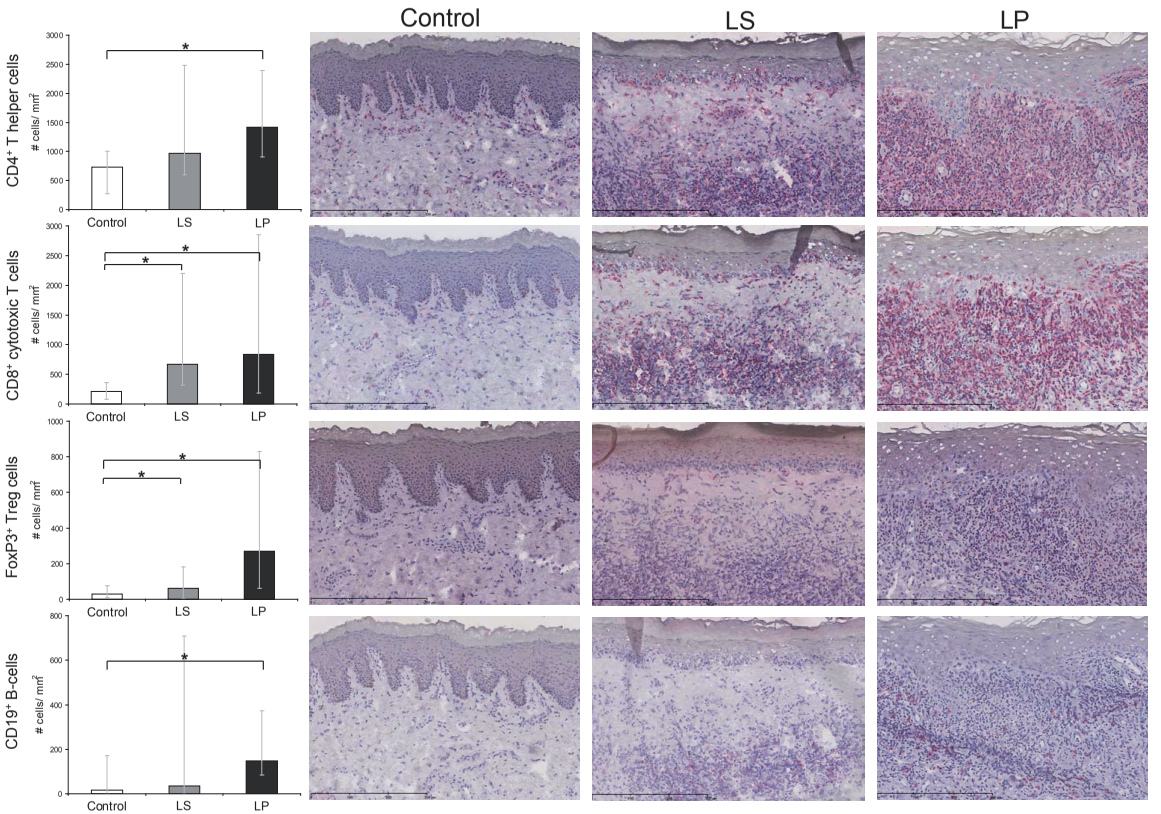

Figure 4. Immune cell counts in lichen sclerosus (LS) and lichen planus (LP). Numbers of immune cells in controls (n=10), LS (n=16) and LP (n=9) and immunohistochemical staining for CD4, CD8, FOXP3 and CD19 (20x magnification). Median ± range. *, *P*<0.05 (Mann-Whitney test). Bar represents 300µm.

DISCUSSION

Recent publications indicate that LS and LP may be autoimmune disorders. Firstly, like autoimmune diseases, genital LS and LP are more common in women than in men.[3,26] Secondly, patients with LS and LP are more frequently diagnosed with other autoimmune disorders such as vitiligo and thyroid disease, and patients with LP show more frequently circulating autoantibodies.[18] Also, the presence of autoreactive T cells against basement membrane zone components has been observed in LS and LP.[19,27-29] Lastly, an association of LS with HLA class II antigen DQ7 and of LP with HLA DRB1*0201 has been demonstrated.[12-14]

From the above, it is hypothesized that aberrant immune signaling plays a role in the pathogenesis of LS and LP, although the exact molecular mechanisms which lead to these immunological changes have not yet been clarified. Here we will discuss our main findings which further corroborate that autoinflammatory mechanisms are the basis of both LS and LP.

LS and LP are characterized by a strong Th1 response

In both disorders, the lymphocytic infiltrate contained primarily T cells. In LS patients, a significant increase in $CD8^+$ T cells and Treg cells was observed, while $CD4^+$ T cell counts in LS were not significantly increased (P=0.07). For LP, a more pronounced increase for all immune cell counts was observed, with significantly increased T- and B cell counts. These data are in agreement with published investigations describing dense T cell infiltrates in LS and LP.[30-33]

To investigate whether these T cells are involved in a Th1 or Th2 response, we studied the different chemokine receptors. Th1 cells are characterized by the expression of CXCR3 and CCR5, while Th2 cells express CCR3 and CCR4.[23] We found high levels of *CXCR3* and *CCR5* in LS and LP and no expression of *CCR3* and *CCR4*, indicating an infiltrate of Th1 cells. Although the role of Th1 response in autoimmune disorders is not completely understood, it seems to play an important role in the induction, maintenance and exacerbation of chronic inflammation. This inflammation is controlled by the presence of Th1 cells, which produce IFNγ. High levels of IFNγ will attract and stimulate different immune and epithelial cells. In reaction to this, these cells will produce ligands for the CXCR3 and CCR5 receptors, namely cytokines CXCL9, CXCL10, CXCL11, CCL3 and CCL4 (Fig. 2), thereby attracting more Th1 cells to the site of inflammation. As a result the Th1 response will be maintained and intensified, resulting in chronic inflammation.[34] The important role for the Th1 phenotype in LS and LP was further illustrated by the upregulation of IL1α and CASP1. The binding of CASP1 to cytokines of the IL-1 family results in activation of the inflammatory processes and suggest the involvement of inflammasomes.

The role of the above described cytokines in relation to autoimmunity has previously been investigated and reviewed.[34-36] In various autoinflammatory and autoimmune disorders, like multiple sclerosis,[37-39] rheumatoid arthritis,[40] systemic lupus erythematosus (SLE),[41] Graves' disease,[42,43] type 1 diabetes mellitus[44] and

Table 1. Expression of cytokines and miR-155 in different autoinflammatory/autoimmune disorders according to literature.

Disorder	Cytokine profile
Multiple Sclerosis[37-39,58]	CXCR3↑, CXCL9↑, CXCL10↑, IFNγ↑, miR-155↑
Rheumatoid arthritis[40,56,59]	CXCR3↑, CXCL9↑, CXCL10↑, IFNγ↑, miR-155↑
Systemic Lupus Erythematosus[41,57]	CXCR3↑, CXCL9↑, CXCL10↑, IFNγ↑, miR-155↑
Graves' disease[42,43]	CXCR3↑, CXCL9↑, CXCL10↑, IFNγ↑
Type 1 diabetes mellitus[44]	CXCR3↑, CXCL9↑, CXCL10↑, IFNγ↑
Vitiligo[45]	CXCR3↑, IFNγ↑

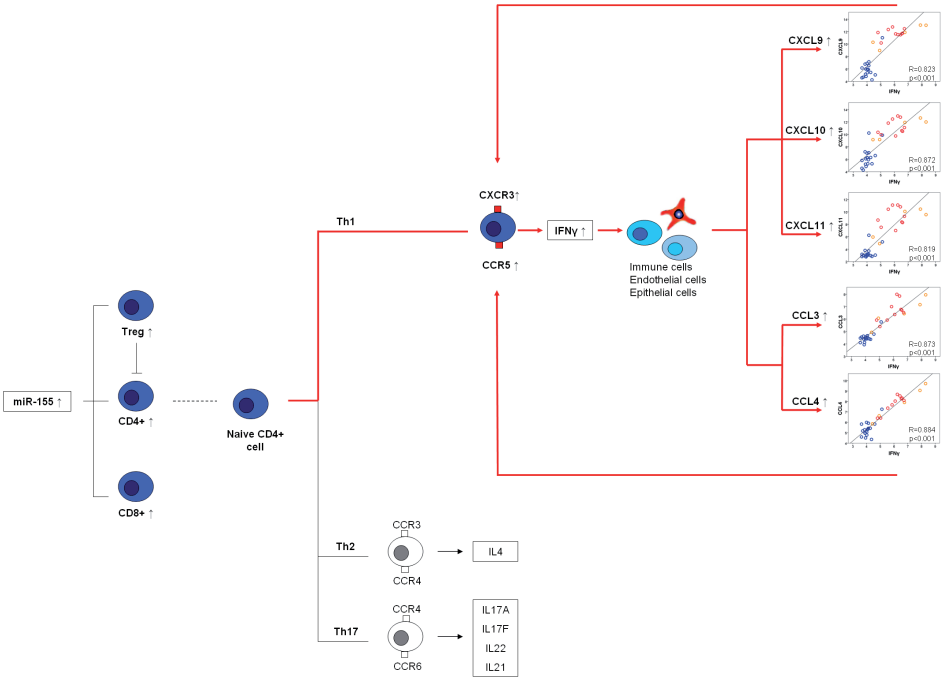

Figure 5. Hypothesized mechanism of pathogenesis of lichen sclerosus (LS) and lichen planus (LP). MiR-155 is strongly upregulated in LS and LP, and possibly affects Treg cell mediated suppression of $CD4^+$ T cells by affecting the suppressive function of Treg cells and by reducing the susceptibility of $CD4^+$ T cells for Treg cell mediated suppression. The T cell response is skewed to Th1. Upregulation of CXCR3 and CCR5 (Th1 receptors) results in increased production of IFNγ, which attracts different dendritic, epithelial and endothelial cells. In response, these cells will produce high amounts of CXCL9, CXCL10, CXCL11, and CCL3, CCL4 which in turn intensify and maintain the Th 1 response. Correlations between IFNγ and these ligands are shown on the right (Spearman's correlation coefficient). Blue: controls (n=18); red: LS (n=10); orange: LP (n=5).

vitiligo,[45] high levels of CXCR3 and its ligands are described (Table 1). In Figure 5 a schematic representation of this feed-forward autoinflammatory process is shown, which, according to our data, plays a major role in the autoinflammatory pathogenesis of LS and LP. Furthermore, the upregulation of CASP1 and IL1α, also suggests a role for inflammasome activity in both disorders, as previously has been described for various autoimmune diseases.[46] Future investigations should focus on the role of inflammasomes in LS and LP.

Next to increased immune cell counts and expression of pro-inflammatory cytokines, we also observed an increased expression of miR-155. As indicated in Figure 5, elevated expression of miR-155 may be a primary molecular event that initiates the activation of immune cells. This concept will be discussed in the next section.

MiR-155 is profoundly upregulated in LS and LP

MicroRNAs are small endogenous noncoding RNAs that post-transcriptionally regulate gene expression by base-pairing to imperfect complementary target sites on the RNA, or by partially blocking translation.[47,48] It is known that miR-155 plays a critical role in regulating homeostasis of the immune system. It regulates production of cytokines, chemokines and transcription factors, and is induced by endotoxins via toll-like receptor signaling.[24,49-51] Furthermore, miR-155 is expressed in several types of activated immune cells, such as macrophages, DCs, B and T cells, and promotes T cell differentiation towards Th1.[24,49,52-54]

Based on this, it can be hypothesized that increased expression of miR-155 in LS and LP originates from the dense T cell infiltrate in both disorders. However, we did not observe overexpression of *BIC*/miR-155 in uVIN, a vulvar disease with also a strong dermal lymphocytic infiltrate (Fig. 3B). A possible explanation for the difference in expression of miR-155 in LS/LP and in uVIN could be that the lymphocytic infiltrate in LS and LP primarily consists of *activated* T cells, while in uVIN the T cell response seems to be ineffective since these T cells are unable to clear HPV, the underlying cause of uVIN.[55]

A role for miR-155 in autoimmune diseases has been demonstrated recently, and increased expression was observed in different autoinflammatory and autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis (Table 1).[25,56-59] One important mechanism inducing autoimmunity is loss of immune tolerance. Normally, unwanted immune responses are controlled by the suppressive function of Treg cells, which are known to play a critical role in maintenance of immune tolerance. In a mouse model studying SLE, it was observed that artificially enhanced miR-155 expression resulted in an altered Treg cell phenotype and thereby in a reduced suppressive function of Treg cells (despite an increase in Treg cell counts).[57] In addition, another study showed that increased miR-155 levels in $CD4^+$ T cells resulted in reduced Treg cell mediated suppression.[60] For LS and LP a similar mechanism may be at work: because of enhanced miR-155 levels the observed increased numbers of Treg cells may not fully affect $CD4^+$ T cells thus resulting in impaired immune tolerance towards self-antigens causing autoimmunity.

LS and LP have profound characteristics of an autoimmune disorder

Summarizing our data, we demonstrate high levels of *BIC*/miR-155 and increased gene expression of pro-inflammatory cytokines involved in autoimmunity, with an important role for CXCR3 and CCR5 and their ligands resulting in a dense infiltration of T cells. Overall, these observations were more pronounced in LP than in LS. In addition, Cooper et al. found recently significant higher levels of auto-antibodies in 52/126 (41%) women with LP and 39/190 (21%) women with LS compared to controls.[18] We also found autoantibodies in a high percentage of patients with LP (32%) and LS (14%) (data not shown). Taken together with previous data about associations with HLA-DR, other autoimmune diseases and demonstration of autoreactive T cells, an autoimmunological basis for LS and LP seems likely. Future studies should focus on the role of miR-155 and the immune response in these disorders, which may be a focus for more targeted therapies.

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GENERAL DISCUSSION

GENERAL DISCUSSION

Vulvar and vaginal premalignant epithelial disorders are a burden for the patient, with severe and long-lasting symptoms and a clear psychosexual impact. Although the awareness for these disorders seems to be increased over the last years, reflected by more specialized vulvar clinics and a search for new treatment modalities, unresolved questions about the exact pathogenesis of these diseases remain and better treatment options are still very much needed.

Treatment of usual type VIN with imiquimod

Vulvar intraepithelial neoplasia (VIN) is one of the vulvar disorders with a premalignant potential.[1,2] The incidence of usual type VIN (uVIN), which is caused by a persistent infection with high-risk type human papillomavirus (HPV), has increased especially in younger women.[3,4] Because treatment options like surgery and laser vaporization are mutilating, a trend towards less invasive treatments that preserve anatomy and sexual function has been observed in recent years. Topical treatment is attractive because it can be applied directly by the patient and is easily monitored for efficacy. Furthermore, new treatment modalities for uVIN aiming to enhance patients' immune response and thereby clear the persistent high risk type HPV infection, are of interest. The rationale behind such treatment modalities is that the immune response in uVIN lesions seems to be ineffective, since uVIN lesions are characterized by an immunosuppressive state in the epidermis, while an insufficient immune response to HPV occurs in the dermis of uVIN.[5-8] In addition, it has been demonstrated that patients with persistent disease show no detectable anti-HPV T-cell responses.[9]

Several studies demonstrated that the immune response modifier imiquimod, which is applied as imiquimod 5% cream, is a promising therapy for uVIN.[10,11] Our group performed a double-blinded, placebo-controlled randomized clinical trial (RCT) and demonstrated a complete response after 16 weeks of treatment in 35% of patients and a reduction in lesion size of at least 25% in an additional 46% of patients. More importantly, HPV was cleared in 58% of patients, suggesting an adequate clearance of the underlying cause of uVIN.[11] From these results, it was concluded that imiquimod should be the first-line treatment option for uVIN. However, until now long-term follow-up data on imiquimod as a treatment for uVIN were not available. In this thesis it was demonstrated that the recurrence rate in complete responders after imiquimod treatment is relatively low compared to other treatments such as local excision, laser vaporization or photodynamic therapy (PDT), which have recurrence rates of respectively 15–50%, 23–40% and 48%.[2,12-14] In our study, 8 of 9 complete responders (89%) were still disease-free after imiquimod treatment with a median follow-up of 7.3 years. Two additional patients with an initial partial response became complete responder after a prolonged treatment with imiquimod and were also still disease-free at long-term (>5 years) follow-up.[15] From these data we conclude that in case of a complete response, imiquimod is an effective treatment in the long-term. However, these recurrence rates are based on a small number of

patients, and the study described in this thesis is the only one reporting on long-term clinical response after imiquimod treatment so far.

Other therapeutic approaches for usual type VIN

Although imiquimod is an effective treatment in a number of uVIN patients, there is still a significant group of patients that do not respond or have only a partial response upon imiquimod treatment. In case of a partial response, imiquimod is effective to reduce lesion size and could therefore be used preceding surgery, thereby limiting the mutilating effects of surgery. However, in these partial responders high recurrence rates are still a problem, since we observed that most partial and non-responders who were secondary treated with surgery for their residual lesions suffered from recurrences.[15] Therefore, treating these patients is a challenge and accordingly other approaches are needed to improve the response rates in these patients.

Longer treatment. A straightforward approach is to prolong the treatment period of imiquimod. We observed that small lesions are more likely to respond to imiquimod treatment, and two patients with a partial response during the initial RCT became complete responder after a prolonged treatment with imiquimod.[15] In case of side effects as local inflammation, burning or flu-like symptoms, patients can use nonsteroidal anti-inflammatory drugs (NSAIDs) or paracetamol to reduce these side effects without interfering with imiquimods immunomodifying activities, as we demonstrated in chapter 5.[16] This might increase patients' compliance to treatment, in particular when longer treatment periods are needed. For these reasons we consider prolonged treatment with imiquimod a feasible addition to the current 16-week period of treatment.

Therapeutic vaccination. Another way to modulate the local immune response in favor of clearance of a persistent HPV infection is boosting the local immune response by therapeutic vaccination. A recent study[17] reported on 20 women with HPV-16–positive uVIN that were vaccinated three or four times with a mix of long peptides from the HPV 16 viral oncoproteins E6 and E7. Three months after vaccination, 5 of 20 patients had a complete response, increasing to 9 of 19 (47%) at 12 months after last vaccination. A partial response was observed in seven patients after 3 months and in six after 12 months follow-up. Complete response rate was maintained at 24 months of follow-up. Interestingly, the authors also found that complete response was associated with lesion size. Patients with smaller lesions had a significantly stronger HPV-specific T-cell response with higher IFNγ than patients with large lesions, indicating that the vaccine-induced HPV16-specific T-cell response is regulated differently in patients with larger lesions.[18] Summarizing these data, therapeutic vaccination can lead to a complete response in uVIN patients by inducing HPV-16 specific immunity. However, similar to treatment with imiquimod, still a number of patients do not or only partially respond.

Combination therapy. Since it has been demonstrated that HPV-16–positive VIN patients with a pre-existing HPV-specific type 1 T-cell response are more likely to have a strong response to imiquimod treatment,[19] another treatment strategy could

be to combine imiquimod with HPV vaccination, which has been investigated in one study.[20] In this study, 19 VIN patients were treated with imiquimod on the vulva for 8 weeks followed by three doses of therapeutic HPV vaccination, and complete histologic regression was observed in 32% of women at week 10, increasing to 58% at week 20 and 63% at week 52.[20] These results are promising, but data on long-term follow-up are still needed. Moreover, starting with HPV vaccination followed by imiquimod treatment could improve response to treatment. With such a treatment protocol, an HPV-specific type 1 T-cell response is induced before start of imiquimod treatment. Because a preexisting HPV-specific type 1 T-cell response is associated with a favorable clinical outcome upon imiquimod treatment,[19] this might result in a complete response in more patients.

Lifestyle change. Finally, it is of interest to investigate the impact of smoking on response to immunomodifying therapies in uVIN patients. It is known that smoking is an important risk factor for uVIN,[21] which is reflected by the significant number of smokers in the above mentioned studies (*i.e.* 23/26 of imiquimod treated patients smoked in the RCT performed by our group[11] and 15/19 patients smoked in the study from Daayana *et al.*[20]). Smoking increases nitric oxide levels in tissues and it is known that high nitric oxide levels in cells infected with high-risk type HPV result in decreased levels of the tumor suppressor genes p53 and pRb, thus negatively affecting DNA integrity and bypassing successful immunoclearance of the disease.[22] In our pilot study investigating the efficacy of imiquimod as a treatment for vaginal intraepithelial neoplasia (VAIN), a negative correlation between smoking and histologic response to imiquimod treatment was observed (chapter 6). This diminished response to imiquimod might be due to an already impaired local immune response in smokers. Hence, smokers should be encouraged to stop smoking in particular before start of immunomodifying treatments.

Future perspectives on treatment of HPV-related vulvar and vaginal disorders

Recently, prophylactic HPV vaccination was implemented in the general population in most European countries for the prevention of HPV-related premalignant and malignant diseases.[23] For that reason, the incidence and as a consequence the impact of HPV-related diseases will probably diminish in the future. However, since prophylactic vaccination is only directed to high-risk type HPV 16 and 18, premalignant diseases caused by other high-risk HPV types still need treatment. Moreover, it will take several decades until prophylactic vaccination reduces the incidence of HPV-related diseases in the general population.[24] In the mean time, effective treatment is needed. Combination of different treatment strategies as imiquimod and therapeutic vaccination might increase response rate in patients with uVIN.

In addition, immunomodifying treatments could also be useful for other HPV-related disorders of the anogenital tract, such as VAIN and cervical intraepithelial neoplasia (CIN). Although VAIN is relatively rare, its treatment is difficult with high recurrence rates and considerable morbidity. Given that treatments as vaginectomy

can impair sexual function, application of imiquimod could be a promising alternative treatment. We demonstrated a complete histologic response after imiquimod treatment in 3/9 (33%) of VAIN patients and a partial response was observed in three patients. Although the sample size is small, these results are promising and need further investigation in larger studies. Furthermore, response rates should be compared with other treatment options as laser vaporization.

Finally, immunotherapy as a treatment for cancer has been tested extensively in the past few years. Immunotherapies such as cytokines or dendritic cell vaccines induce a Th1 immune response and activate cytotoxic T-lymphocytes to eliminate the tumor.[25,26] In case VIN has already progressed to invasive cancer, there might also be a role for such immunotherapies in the treatment of vulvar squamous cell carcinoma.

Non-HPV related vulvar disorders: immunity in lichen sclerosus and lichen planus

Like HPV-related vulvar disorders, lichen sclerosus (LS) and lichen planus (LP) seem to be diagnosed more frequently, probably due to an increased awareness by patients and specialists. Although evidence exist that both disorders are chronic autoimmune mediated diseases, the exact pathogenesis of LS and LP is still unclear.[27,28] Furthermore, both disorders are difficult to treat. Topical ultrapotent corticosteroids are currently the treatment of choice for LS and LP.[27,29] However, treatment only reduces symptoms and side effects as dermal atrophy make long-term treatment often complicated. To develop more curative therapies, more knowledge is needed about the exact pathogenesis of LS and LP. For that reason, the molecular and immunological mechanisms critical in the pathogenesis of LS and LP were investigated in this thesis (chapter 7). We observed an autoimmune phenotype in both diseases, with a strong Th1 response illustrated by a dense T-cell infiltrate and high levels of Th1 specific cytokines.

Another important finding was the overexpression of microRNA-155 (miR-155). The role of microRNAs (miRNAs) – which act as post-transcriptional gene regulators – was unknown until recently. Over the last decade, more and more studies have found that miRNA regulatory pathways are important in the pathogenesis of various diseases and altered expression of miRNAs has been demonstrated in cancers, autoimmune diseases, but also inflammatory skin conditions.[30-32] MiR-155 is a miRNA that plays a role in regulating homeostasis of the immune system, as it regulates production of cytokines, chemokines and transcription factors and is induced by endotoxins via toll-like receptor signaling.[33,34] In addition, miR-155 shows increased expression in activated immune cells and has proven to be involved in autoimmunity.[35-37] It plays an important role in driving chronic inflammation that is inappropriately directed at tissue-specific antigens,[35] and high levels of miR-155 have been observed in different autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis.[38,39] Although we demonstrate increased expression of miR-155 in LS and LP, which could be an important step in pathogenesis, the potential role of miR-155 in these disorders is still unclear. To explore the role of miR-155, it is important to identify the cells that

express miR-155 and investigate which genes are targeted by miR-155 in LS and LP. Also, miRNA profiling studies are necessary to further identify and characterize other disease-specific miRNAs.

Future perspectives on treatment of vulvar LS and LP

We demonstrated an autoimmune phenotype in vulvar LS and LP, which is also supported by the finding of autoantibodies in a high percentage of patients, and by previous data about associations with HLA-DR, other autoimmune diseases and demonstration of autoreactive T cells.[40,41] In the future, approaches targeting the immune response should be considered to find curative treatments. An interesting perspective for more targeted therapies could be the suppression of miR-155 by topical application of antimiRNA oligonucleotides (antagomirs). However, development of antimiRNA oligonucleotides that can bind strongly to the miRNA and are stable enough in physiological conditions remains a challenge.[42] Moreover, the exact pathogenesis and role of miR-155 first need further clarification.

Final remark

Treatment of vulvar and vaginal premalignant epithelial disorders is challenging. Although promising less invasive treatment options have been investigated over the past few years, no "gold-standard" treatments exist for these diseases. In addition, very long-term follow-up is required, since disease recurrence is common. Therefore, an individual treatment approach is essential.

Finally, since vulvar and vaginal epithelial disorders can have a negative impact on quality of life and on sexual functioning,[43,44] it is important that clinicians pay attention to these psychosexual sequelae. The founding of patient support groups and the development of specialized vulvar clinics in which dermatologists, gynecologists and sexologists work together, are of great significance to improve individual patient care.

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9

SUMMARY
SAMENVATTING

SUMMARY

Vulvar and vaginal premalignant disorders are a burden for the patient and have a high psychosexual impact. Women with vulvar premalignant lesions such as vulvar intraepithelial neoplasia (VIN), often have severe and long-lasting symptoms and treatment is complicated. In the past, surgery was the treatment of choice, but recurrence rates are high. In addition, surgical treatments can mutilate the vulva, causing psychosexual distress. As a consequence, several studies investigating less invasive treatment options that preserve anatomy were initiated over the last years. These new treatment modalities aim to modify patients' immune response and thereby clear the initial cause of the disease: a persistent infection with human papillomavirus (HPV). Furthermore, topical treatments are attractive because they can be applied directly by the patient and are easily monitored for efficacy.

The aim of this thesis was to investigate immune regulation of premalignant vulvar and vaginal disorders, and we focused in particular on the clinical and immunological effects of treatment with the immune response modifier imiquimod.

In **Chapter 1** a general introduction is given about the vulva, the immune response and the premalignant vulvar disorders *VIN*, *lichen sclerosus* and *lichen planus*.

An extensive overview of *VIN*, *vulvar Paget's disease* and *melanoma in situ*, is given in **Chapter 2**. Of the premalignant vulvar disorders, VIN is diagnosed more and more frequently. In contrast, vulvar Paget's disease and melanoma in situ are two rare diseases. Nevertheless, because of their malignant potential, a vulvar specialist should also be aware of these diagnoses and be familiar with the best way to manage these diseases.

Usual type VIN (uVIN) is caused by a persistent infection with HPV, and the lack of an appropriate immune response is of critical importance in the etiology of uVIN. Imiquimod is an immune response modifier that activates the cellular immune response. For that reason, it was previously tested as a treatment for uVIN. In a recent placebo-controlled, double-blinded randomized clinical trial (RCT) by our group (van Seters *et al*.), it was observed that 35% of patients had a complete response after treatment with imiquimod and 46% a partial response. All complete responders remained disease-free at one year follow-up. However, long-term follow-up results after imiquimod treatment were still lacking. Therefore, as is described in **Chapter 3,** we performed a study in which we assessed the long-term (>5 years) efficacy of imiquimod as a treatment for uVIN. Twenty-four of 26 patients who were treated with imiquimod during the initial RCT, were seen for follow-up. Median follow-up period was 7.2 years. uVIN recurred in one of nine complete responders. Of the initial partial responders, two became disease-free after additional imiquimod treatment. In the other partial responders, uVIN recurred at least once after the initial RCT. In long-term complete responders, lesion size at study entry was smaller and these patients had a significantly better global quality of life at follow-up than patients with residual disease and/or recurrence after imiquimod treatment. Our data indicate that

treatment with imiquimod is an effective long-term therapy for uVIN. Small lesions are more likely to respond completely to imiquimod treatment; therefore treatment period in the initial RCT might have been too short to be effective for larger lesions. Consequently we advise prolonging the treatment period until regression has stopped.

Chapter 4 describes differences in immune cell levels and in the expression of $p16^{INK4a}$, which is a specific marker for high-risk type HPV, in uVIN tissue before and after imiquimod treatment in relation to HPV clearance and clinical response. Immune cell counts and $p16^{INK4a}$ expression before and after imiquimod treatment were investigated in patients who cleared HPV and patients who did not clear HPV after treatment, and in healthy controls. Before imiquimod treatment, both HPV cleared and HPV non-cleared patients showed significantly upregulated immune cell counts compared to healthy controls. However, in patients that cleared HPV and showed histologic regression after imiquimod treatment, immune cell counts and $p16^{INK4a}$ expression were normalized to control levels. These data indicate that imiquimod-induced clearance of HPV results in normalization of counts for certain immune cells and is strongly correlated with histologic regression of uVIN.

Based on the previously shown efficacy of imiquimod as a treatment for uVIN, imiquimod is now considered first-line treatment for uVIN in the Netherlands. Treatment with imiquimod, however, is not always tolerated well. Patients using imiquimod frequently complain of side effects such as severe local inflammation, itching or burning, flulike symptoms, weariness and headache. We observed that patients treated with imiquimod commonly use nonsteroidal anti-inflammatory drugs (NSAIDs) or paracetamol in order to reduce these side effects. Since the effect of NSAIDs in patients using imiquimod is unknown, we describe in **Chapter 5** whether NSAID-use, which has been documented to inhibit the cell-mediated immune response, interferes with the outcome of imiquimod treatment and thereby can interfere with imiquimods immunomodifying activities. We demonstrate in an *in vitro* study about the effect of NSAIDs on dendritic cells (DCs) and Langerhans cells (LCs) that NSAIDs neither affect the ability of DCs and LCs to stimulate T-cell proliferation nor the production of cytokines (although an effect was observed on the expression of DC and LC maturation markers). In agreement with this, in uVIN patients treated with imiquimod, no interference of frequent NSAID-use with clinical outcome was observed. Our data indicate that NSAID-use does not seem to interfere with DC and LC function and does not interfere with immunomodulatory properties of imiquimod in uVIN patients. Therefore, NSAIDs can safely be used to reduce imiquimod side effects in uVIN patients during treatment.

Chapter 6 describes the efficacy and tolerability of imiquimod as a treatment for vaginal intraepithelial neoplasia (VAIN), which is also caused by a persistent HPV infection. In this pilot study, fourteen patients with histological confirmed VAIN were treated with imiquimod twice weekly for 16 weeks. Twelve of 14 patients completed treatment, while two patients stopped due to severe side effects. Three of nine patients (33%) from whom biopsies were available after treatment had

a complete histologic response, and three patients (33%) showed partial regression. In addition, one of three patients from whom only cytology was available, had a negative PAP-smear after treatment, and one showed a partial response. Furthermore, smoking was negatively correlated with histologic response to imiquimod. These preliminary data indicate that self-administration of imiquimod, which can be done easily by the patient at home and conserves anatomy and sexual function, can be an effective alternative treatment for VAIN in particular in patients with multifocal VAIN or contraindications for surgery. However, due to possible side effects, patients should be carefully instructed beforehand and followed during treatment.

In **Chapter 7**, the molecular and immunological mechanisms involved in vulvar lichen sclerosus and lichen planus were investigated in order to further clarify the pathogenesis of both diseases. By performing gene expression profiling and RT-PCR experiments, we demonstrated a significantly increased expression of pro-inflammatory cytokines specific for a Th1 IFNγ induced immune response. Immunohistochemical staining also showed a significant T-cell response, with pronounced infiltrates of $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells and $FOXP3^+$ regulatory T cells. In addition, we found that *BIC*/microRNA-155 (miR-155) – a microRNA involved in regulation of the immune response – was significantly upregulated in lichen sclerosus and lichen planus. Furthermore, 34/106 (32%) of the women with lichen planus and 6/42 (14%) of the women with lichen sclerosus had a positive titer for serum autoantibodies. Taken together, these data demonstrate an autoimmune phenotype in vulvar lichen sclerosus and lichen planus, characterized by increased levels of proinflammatory cytokines and *BIC*/miR-155.

In **Chapter 8**, the main findings of this thesis are discussed and related to current and future research perspectives about treatment of premalignant vulvar and vaginal disorders.

SAMENVATTING

Vulvaire en vaginale premaligne afwijkingen hebben een grote impact op het psychoseksueel functioneren. Vrouwen met vulvaire premaligne afwijkingen, zoals vulvaire intraepitheliale neoplasie (VIN), hebben vaak ernstige en langdurige klachten, en daarnaast is de behandeling gecompliceerd. In het verleden had operatieve behandeling de voorkeur, maar de recidiefkans is groot. Daarnaast kan een operatie de vulva mutileren, hetgeen gepaard gaat met seksueel disfunctioneren en psychosomatische stress. Ook operatieve behandeling van premaligne vaginale aandoeningen kan leiden tot seksueel disfunctioneren.

In de afgelopen jaren zijn er verscheidene studies geïnitieerd waarin alternatieve behandelingen werden onderzocht, zoals lokale behandeling in de vorm van een crème die door de patiënt zelf kan worden aangebracht. Deze nieuwe behandelingen hebben als doel om de afweer van de patiënt te beïnvloeden en daarmee het onderliggende proces van de premaligne afwijkingen – namelijk een infectie met het humaan papillomavirus (HPV) – te bestrijden. Een ander groot voordeel van deze behandelingen is dat ze de vulva niet mutileren.

Het doel van dit proefschrift was om de immuunregulatie in premaligne vulvaire en vaginale aandoeningen te onderzoeken, waarbij wij ons in het bijzonder hebben geconcentreerd op de klinische en immunologische effecten van behandeling met de immunomodulator imiquimod.

Hoofdstuk 1 geeft een algemene inleiding over de vulva, het afweersysteem en de premaligne vulvaire afwijkingen *VIN*, *lichen sclerosus* en *lichen planus*.

In **Hoofdstuk 2** wordt een uitgebreid overzicht gegeven van *VIN*, de *vulvaire ziekte van Paget* en *melanoma in situ*. VIN wordt steeds vaker en op jongere leeftijd gediagnosticeerd. De vulvaire ziekte van Paget en melanoma in situ zijn beiden relatief zeldzame aandoeningen. Toch is het – vanwege hun kans op maligne ontaarding – belangrijk dat vulvaspecialisten bekend zijn met deze diagnoses en de optimale behandeling van deze ziekten.

Usual type VIN (uVIN) wordt veroorzaakt door een persisterende infectie met het humaan papillomavirus (HPV). Een onvoldoende effectieve afweer tegen de HPV infectie speelt een belangrijke rol in de etiologie van uVIN. Imiquimod is een immunomodulator die de cellulaire afweer activeert. Derhalve is onderzocht of imiquimod een effectieve behandeling is voor uVIN. Uit een recent placebogecontroleerd, dubbelblind, gerandomiseerd onderzoek (RCT) van onze onderzoeksgroep (van Seters *et al*.), bleek dat 35% van de patiënten een complete respons had na imiquimod behandeling en 46% een partiële respons. Alle 'complete responders' waren na 1 jaar nog steeds ziektevrij. Het effect op lange termijn was tot nu toe echter nog niet bekend. Daarom hebben wij het lange termijn effect van imiquimod als behandeling voor uVIN onderzocht, zoals beschreven in **Hoofdstuk 3**. Vierentwintig van de 26 patiënten die behandeld werden met imiquimod tijdens de eerdere RCT, kwamen voor een follow-up bezoek. De mediane follow-up periode was

7.2 jaar. Een recidief trad op in 1 van de 9 complete responders. Daarnaast hadden twee van de partiële responders een complete remissie van de restafwijkingen na een additionele imiquimod behandeling. Alle andere partiële responders hadden tenminste één recidief na de eerdere RCT.

Tevens bleek dat in complete responders de grootte van de uVIN laesie voor start van de RCT kleiner was en dat deze patiënten een betere score op kwaliteit van leven hadden tijdens de follow-up, dan de patiënten die nog een restafwijking en/ of recidief hadden. Onze data laten dan ook zien dat behandeling van uVIN met imiquimod effectief is op de lange termijn. Omdat kleine laesies eerder volledig lijken te reageren op imiquimod dan grotere laesies, is de behandelperiode mogelijk te kort geweest om effectief te zijn voor de grotere laesies. Daarom adviseren wij te behandelen totdat de afwijkingen niet meer verder in regressie gaan.

Hoofdstuk 4 beschrijft verschillen in het aantal immuuncellen en in de expressie van $p16^{INK4a}$ (een marker specifiek voor de aanwezigheid van hoog-risico type HPV) in uVIN weefsel voor en na imiquimod behandeling in relatie tot HPV klaring en klinische respons. We hebben gekeken naar het aantal immuuncellen en naar $p16^{INK4a}$ expressie in de huid van gezonde controles, in de huid van uVIN patiënten die HPV klaarden en in de huid van uVIN patiënten die HPV niet klaarden na imiquimod behandeling. Vóór de behandeling was er sprake van een verhoogd aantal immuuncellen in zowel patiënten die HPV klaarden als patiënten die HPV niet konden klaren in vergelijking met gezonde controles. Echter, na de behandeling normaliseerde het aantal immuuncellen en $p16^{INK4a}$ expressie in patiënten die HPV hadden geklaard en histologische regressie toonden, maar niet in patiënten bij wie HPV nog steeds aanwezig was. Hieruit kunnen we concluderen dat HPV klaring geïnduceerd door imiquimod resulteert in normalisatie van het aantal immuuncellen, en sterk is gecorreleerd met histologische regressie van uVIN.

Sinds is aangetoond dat imiquimod effectief is als behandeling voor uVIN, is imiquimod de eerstelijns behandeling voor uVIN in Nederland. Behandeling met imiquimod wordt echter niet altijd goed verdragen vanwege de vaak voorkomende bijwerkingen als lokale ontsteking, jeuk of branderigheid, koortsachtige symptomen, vermoeidheid en hoofdpijn. Wij bemerkten dan ook dat patiënten vaak niet-steroïde ontstekingsremmende pijnstillers (NSAID's) of paracetamol gebruiken tijdens imiquimod behandeling om deze bijwerkingen te verminderen. Het effect van NSAID-gebruik tijdens imiquimod behandeling was echter tot nu toe onbekend. Daarom beschrijven wij in **Hoofdstuk 5** of gebruik van NSAID's, waarvan bekend is dat het de cellulaire afweer remt, de uitkomst van imiquimod behandeling beïnvloedt en dus ingrijpt op de immuunmodulerende werking van imiquimod. Met een *in vitro* studie naar het effect van NSAID's op dendritische cellen (DCs) en Langerhans cellen (LCs), laten we zien dat NSAID's geen effect hebben op het vermogen van DCs en LCs om T-cellen te stimuleren of op de productie van cytokines, ondanks dat er wel effecten van NSAID's werden gezien op de expressie van zogeheten DC- en LC- *maturation markers*. In overeenstemming hiermee had frequent NSAID-gebruik ook geen effect op de klinische uitkomst na imiquimod behandeling. Deze resultaten impliceren dat

NSAID-gebruik geen effect heeft op de functie van DCs en LCs en evenmin ingrijpt op de immuunmodulerende eigenschappen van imiquimod in patiënten met uVIN. Daarom kunnen NSAID's veilig gebruikt worden tijdens imiquimod behandeling ter vermindering van bijwerkingen.

Hoofdstuk 6 beschrijft de effectiviteit van imiquimod als behandeling voor vaginale intraepitheliale neoplasie (VAIN), een ziekte die ook wordt veroorzaakt door een persisterende HPV infectie. In een pilot studie hebben wij veertien patiënten met VAIN tweemaal per week behandeld met imiquimod gedurende 16 weken. Twaalf van de 14 patiënten volbrachten de gehele behandeling, twee patiënten stopten vanwege hevige bijwerkingen. Drie van de 9 patiënten (33%) waarvan weefsel beschikbaar was na behandeling, bleken een complete histologische respons te hebben, en drie patiënten (33%) hadden een partiële regressie van de VAIN laesies. Tevens had één van de drie patiënten van wie alleen cytologie beschikbaar was na behandeling, normale cytologie (PAP1) en toonde een andere patiënt een partiële cytologische respons. Daarnaast had roken een negatief effect op de respons na imiquimod behandeling. Deze pilot studie laat zien dat imiquimod ook een effectieve behandeling kan zijn voor patiënten met VAIN, met name voor patiënten met multifocale afwijkingen of waarbij chirurgie niet het middel van eerste keus is. Vanwege mogelijke bijwerkingen is het wel van belang dat patiënten voor de behandeling goed worden geïnstrueerd, en intensief worden begeleid tijdens de behandeling.

In **Hoofdstuk 7** zijn de moleculaire en immunologische mechanismen in vulvaire lichen sclerosus en lichen planus onderzocht om zo de pathogenese van beide ziekten verder te kunnen ophelderen. Met behulp van genexpressie profielen en RT-PCR experimenten zagen wij een verhoogde expressie van pro-inflammatoire cytokines specifiek voor een Th1 INFγ geïnduceerde afweer. Immunohistochemische kleuringen toonden ook een sterke T-celgemedieerde afweer, met een omvangrijk infiltraat van $CD4^+$ T helper cellen, $CD8^+$ cytotoxische T-cellen en $FOXP3^+$ regulatoire T-cellen. Tevens vonden we in lichen sclerosus en lichen planus een significant verhoogde expressie van *BIC*/microRNA-155 (miR-155) – een microRNA dat betrokken is bij de regulatie van de afweer. Daarnaast hadden 34/106 (32%) van de vrouwen met lichen planus en 6/42 (14%) van de vrouwen met lichen sclerosus een positieve titer voor autoantistoffen. Samenvattend vormen deze data sterke aanwijzingen dat er sprake is van een auto-immuun fenotype in vulvaire lichen sclerosus en lichen planus.

In **Hoofdstuk 8** worden de belangrijkste bevindingen van dit proefschrift besproken en gerelateerd aan de huidige en toekomstige onderzoeksmogelijkheden ter behandeling van premaligne vulvaire en vaginale aandoeningen.

LIST OF ABBREVIATIONS

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ALA	aminolevulinic acid
ANA	antinuclear antibody
Anti-Tg	antithyroglobulin antibody
Anti-TPO	antithyroid peroxidase antibody
APC	antigen presenting cell
Asa	aspirin
ASCUS	atypical squamous cells of undetermined significance
BMZ	basement membrane zone
BSA	bovine serum albumin
CFSE	carboxyfluorescein succinimidyl ester
CIN	cervical intraepithelial neoplasia
COX	cyclooxygenase
CR	complete response
DAB	diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
mDC	myeloid dendritic cell
moDC	monocyte-derived dendritic cell
pDC	plasmacytoid dendritic cell
DES	diethylstilbestrol
EGWs	external genital warts
EMPD	extramammary Paget's disease
EORTC	European Organization for Research and Treatment of Cancer
FACS	fluorescence-activated cell sorting
FDR	false discovery rate
FSC	forward scatter
HIV	human immunodeficiency virus
HPV	human papillomavirus
hrHPV	high-risk human papillomavirus
lrHPV	low-risk human papillomavirus
HRP	horse radish peroxidase
HSIL	high-grade squamous intraepithelial lesion
Ibu	ibuprofen
IFN	interferon
IL	interleukin
ISSVD	International Society for the Study of Vulvovaginal Disease
LC	langerhans cell
moLC	monocyte-derived langerhans cell
LLETZ	large loop excision of the transformation zone
LP	lichen planus
LS	lichen sclerosus

LSIL	low-grade squamous intraepithelial lesion
MCP-1	monocyte-chemoattractant protein-1
MHC	major histocompatibility complex
miR	microRNA
MIS	melanoma in situ
MLR	mixed lymphocyte reaction
NF-κB	nuclear factor-kappa B
NK	natural killer
NSAIDs	nonsteroidal anti-inflammatory drugs
NR	no response
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PDT	photodynamic therapy
PGs	prostaglandins
PGE_2	prostaglandin E_2
PR	partial response
QoL	quality of life
RCT	randomized controlled trial
SAM	significant analysis of microarrays
SCC	squamous cell carcinoma
SLE	systemic lupus erythematosus
SSC	sideward scatter
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	T-regulatory
VAIN	vaginal intraepithelial neoplasia
VIN	vulvar intraepithelial neoplasia
dVIN	*differentiated type* vulvar intraepithelial neoplasia
uVIN	*usual type* vulvar intraepithelial neoplasia.

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Santegoets LAM, van Baars R, **Terlou A**, Heijmans-Antonissen C, Swagemakers SM, van der Spek PJ, Ewing PC, van Beurden M, van der Meijden WI, Helmerhorst TJ, Blok LJ. Different DNA damage and cell cycle checkpoint control in low- and high-risk human papillomavirus infections of the vulva. Int J Cancer 2011 Aug 3 *[epub ahead of print]*

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PORTFOLIO

PHD PORTFOLIO

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PHD TRAINING

	Year	ECTS
General courses		
Biomedical English Writing and Communication, EUR	2009	4
Introduction to Data-analysis , NIHES	2009	0.9
Adobe Photoshop and Illustrator CS5 Workshop, Molmed	2011	0.3
In-depth courses		
Basic and Translational Oncology, Molmed	2008	1.7
Colposcopy course, stichting OOG	2008	1
Vulvar Pathology, stichting OOG	2009	0.3
Advanced Immunology, Dept. Immunology EUR	2009	3
Erasmus FACS flow day, Molmed	2009	0.3
National and International conferences/presentations		
International Society for the Study of Vulvovaginal Disease (ISSVD), Edinborough Scotland (oral), *Award for best candidate presentation*	2009	2
Wladimiroff Research day, Erasmus MC, Rotterdam	2009	0.3
Society for Gynecologic Investigation (SGI), Orlando, USA (poster)	2010	1
Wladimiroff Research day, Erasmus MC, Rotterdam (oral)	2010	1
European Academy of Dermatology and Venereology (EADV), Cavtat, Croatia (oral)	2010	2
European College for the Study of Vulval Disease (ECSVD), Munich, Germany (oral)	2010	2
Wladimiroff Research day, Erasmus MC, Rotterdam (oral)	2011	1
National and International seminars and workshops		
Symposium Nederlandse Vereniging voor Vulvapathologie (NVvVP)	2008	0.3
Symposium NVvVP	2009	0.3
Erasmus MC PhD Day	2009	0.3
Postgraduate (ECSVD)	2010	0.3
Symposium "Vulvaire (pre-) maligniteiten: goed bekeken!", UMC St Radboud, Nijmegen	2010	0.3
Attending and presenting in monthly SGGO meetings	2008-2010	1
Attending and presenting in weekly research meetings	2008-2011	2
Grant applications, reviewing papers		
Reviewing paper for Current HIV research	2011	0.3
Reviewing paper for The Australian and New Zealand Journal of Obstetrics and Gynaecology	2010	0.3
Travelgrant: Erasmus Trustfonds	2010	0.3
Teaching activities		
Teaching students and residents in outpatient vulvar clinic	2009-2010	2
Other		
Board member 'Nederlandse Vereniging voor Vulvapathologie'	2009-onwards	10

DANKWOORD

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ABOUT THE AUTHOR

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Annelinde Terlou (18 november 1982) attended secondary school at the Christelijk Gymnasium Utrecht, combining regular schooling with an early career as violinist at the Young Talent Class of the Utrecht Conservatory (1993-2001). After graduation she studied Medicine at the Rijksuniversiteit Groningen. Highlights during her study were internships at Hospital Sao João, Porto, Portugal and at the Antoni van Leeuwenhoek Hospital in Amsterdam under supervision of Dr. M. van Beurden; under supervision of Prof. dr. P.C. Rowe and Prof. dr. C.L. Schwartz she carried out a research project at the Department of Pediatric Oncology at the Johns Hopkins University School of Medicine in Baltimore. This project resulted in her first international publication.

After graduation (cum laude) in 2007, Annelinde worked during 6 months as a resident at the Department of Obstetrics and Gynecology at the St. Antonius Hospital in Nieuwegein, followed by a PhD project on premalignant vulvar and vaginal disorders at the department of Obstetrics and Gynecology at the Erasmus University Medical Center in Rotterdam under supervision of Prof. dr. Th. J. M. Helmerhorst. During her PhD project she presented her work at different national and international conferences, and was awarded with the 'best candidate presentation' at the 20th conference of the International Society for the Study of Vulvar Disease (ISSVD). She combined her PhD project with work as a clinician at the Vulvar Outpatient Clinic of the Erasmus University Medical Center, and became a board member of the 'Nederlandse Vereniging voor Vulvapathologie' (NVvVP).

During her clinical work at the Vulvar Outpatient clinic, she became interested in Dermatology. Therefore, she has started in September 2011 her residency in Dermatology at the VU medical center in Amsterdam under supervision of Prof. dr. Th.M. Starink.