Lichen Sclerosus and Vulvar Intraepithelial Neoplasia in Vulvar Cancer

Towards the identification of a true premalignant lesion

Hedwig van de Nieuwenhof

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Towards the identification of a true premalignant lesion

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Chapter 1

General introduction and outline of this thesis

Vulvar squamous cell carcinoma

Vulvar cancer is the fourth most common type of gynaecological cancer (besides cancer of the uterine corpus, ovary and cervix) and comprises five percent of all malignancies of the female genital tract. The majority of vulvar cancers are squamous cell carcinomas (SCC). Vulvar basal cell carcinoma, melanoma, adenocarcinoma, Paget's disease and sarcoma are less common. Vulvar cancer is encountered most frequently in postmenopausal women with a mean age at diagnosis of 65 years.

Aetiology

Vulvar SCC and its precursor lesions originate following two separate pathways.[1,2] (Figure 1) In 2004, the nomenclature of the precursor lesions was revised.[3] The first and most common pathway occurs in older women and leads to mostly differentiated keratinizing SCC, in a background of Lichen Sclerosus (LS) and/or differentiated vulvar intraepithelial neoplasia (dVIN).[2] This type accounts for the majority of all vulvar SCC and is not caused by high-risk HPV. dVIN is seldom diagnosed in its solitary form because it is thought to have a relatively brief intraepithelial phase before progression to invasive carcinoma, and is a difficult clinical and histological diagnosis.[3-5] The oncogenesis of LS to vulvar SCC and its connection with dVIN remains to be elucidated.

The second pathway primarily affects younger women and consists of mainly nonkeratinizing SCCs. In this pathway, an infection with high-risk HPV, predominantly HPV 16 and 18, is involved.[2] Carcinogenesis largely resembles that of cervical SCCs and HPV vaccines will probably prevent most of these HPV 16 and 18 related vulvar SCCs in the future. This type of vulvar SCC is associated with usual VIN (uVIN). The role of the immune system seems to be a central one in the processes associated with both progression to invasive disease and spontaneous regression of uVIN, with almost a fifth of patients having an immunosuppressive condition.[3,6] Tobacco use is very common in patients with an HPV related vulvar (pre) malignancy.[6] Multicentric HPV infections (cervix, vagina and anus) are common in these patients,[6,7] and therefore, a cervical smear should be performed in the work-up of patients with vulvar SCC and uVIN.[7]

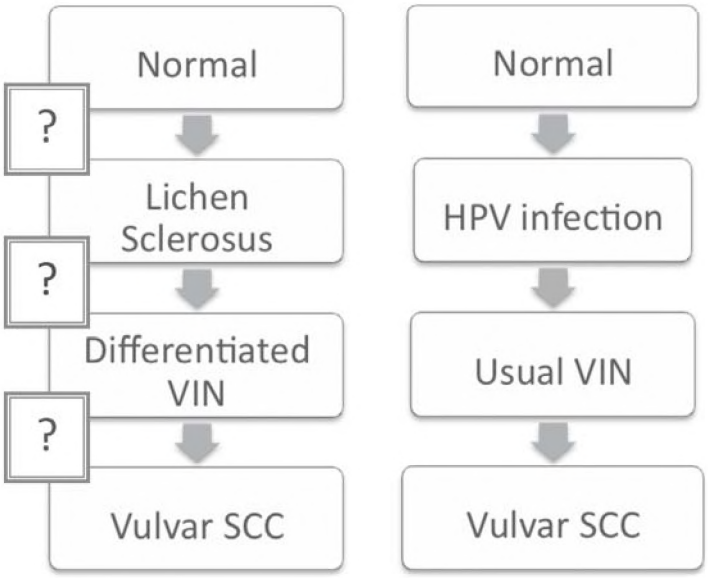

Figure 1 Two pathways leading to vulvar SCC

Diagnostics

The diagnosis of vulvar SCC is often delayed. Self-examination of the vulva is not common, especially among older women. Moreover, patients may feel too embarrassed to visit the family doctor[8] and vulvar SCC may give rise to aspecific symptoms. Owing to the unfamiliarity with vulvar SCC and the similarity of complaints with more common diagnoses such as Candida infections or LS there also may be a doctors' delay.[8]

The diagnosis vulvar SCC is made by an incision- or punch biopsy for histopathological examination. The optimal biopsy is taken from the edge of tumour to normal tissue. An excision biopsy may be an attractive alternative when an earlier biopsy has not led to a diagnosis; however, this may interfere with the sentinel lymph node (SLN) procedure. When there is a multifocal tumour, mapping of the vulva is necessary; multiple biopsies should be taken. Although palpation is unreliable for assessing the node status when small metastases are concerned, it should be performed to diagnose large groin metastases.

Treatment

The cornerstone of treatment of vulvar SCC is surgery, with a limited role for radio- and/or chemotherapy.[9] Surgery provides best chances for local and regional tumour control.[10] In the early 1950s, Stanley Way introduced radical vulvectomy with 'en bloc' bilateral inguinofemoral lymphadenectomy as standard treatment for all operable vulvar SCCs.[11] This strategy resulted in excellent five-year survival rates, however, postoperative wound breakdown and infection after an 'en bloc' procedure occurred with a frequency of up to 61%.[9,12] Psychosexual disturbance and lymph oedema of the legs were major long-term problems.[13] Since then, major modifications have been introduced in the standard surgical treatment to reduce morbidity without compromising the prognosis.[14] The current generally accepted standard treatment is a wide local excision of the tumour with bilateral inguinofemoral lymphadenectomy with separate incisions. In the case of a lateral tumour (>1 cm from the midline), it is sufficient to perform a unilateral lymphadenectomy. The SLN technique has been introduced to further reduce the extent of the surgical treatment.[15]

Prognosis

With appropriate management, the prognosis for vulvar SCC is generally good, with an overall five-year survival rate in operable cases of approximately 70%. Lymph node status is the most important prognostic factor as represented in the International Federation of Gynaecology and Obstetrics (FIGO) staging system (Table 1), which was converted from a clinical to a histopathological staging in 1988. In 2009, a revised FIGO staging for vulvar SCC was introduced;[16,17] major changes consist of the number and morphology of the involved nodes that have been taken into account (*e.g.,* size and whether these are intra- or extra-capsular). The bilaterality of positive nodes has been discarded as an independent prognostic factor.[17] In addition, tumours with adjacent spread to lower urethra, vagina or anus are now classified as FIGO II (formerly FIGO III). Patients with negative inguinofemoral lymph nodes have a five-year survival rate of approximately 90% and this falls to approximately 50% for patients with positive inguinofemoral lymph nodes.[10] The current literature still often uses the FIGO classification as introduced in 1988.

Vulvar SCC	
Stage I	Tumour confined to the vulva
IA	Lesions ≤2 cm in size, confined to the vulva or perineum and with stromal invasion ≤1.0 mm*, no nodal metastasis.
IB	Lesions >2 cm in size or with stromal invasion >1.0 mm*, confined to the vulva or perineum, with negative nodes.
Stage II	Tumour of any size with extension to adjacent perineal structures (⅓ lower urethra ⅓ lower vagina, anus) with negative nodes.
Stage III	Tumour of any size with or without extension to adjacent perineal structures (⅓ lower urethra ⅓ lower vagina, anus) with positive inguinofemoral lymph nodes.
IIIA	– With 1 lymph node metastasis (≥5mm) or, – 1-2 lymph node metastasis (es) (<5mm)
IIIB	– With 2 or more lymph node metastases (≥5mm), or – 3 or more lymph node metastases (<5mm)
IIIC	With positive nodes with extracapsular spread
Stage IV	Tumour invades other regional (⅔ upper urethra, ⅔ upper vagina) or distant structures.
IVA	Tumour invades any of the following: – Upper urethral and/or vaginal mucosa, bladder mucosa, rectal mucosa, or fixed to pelvic bone or – Fixed or ulcerated inguinofemoral lymph nodes
IVB	Any distant metastasis including pelvic lymph nodes.

Table 1 The new FIGO (2009) classification of vulvar SCC[16].

* The depth of invasions is defined as the measurement of the tumour from the epithelial-stromal junction of the adjacent most superficial dermal papilla to the deepest point of invasion.

Recurrence

Recurrence is a frequent event in vulvar SCC. The overall risk of recurrence after primary treatment is 19.4-45.5%.[18-23] Local recurrences are usually diagnosed more than two years after primary treatment[24] and the majority of local recurrences can be cured. At least a subset of local recurrences will be new primary (de novo) tumours due to the residue of a premalignant lesion after surgery. Rouzier *et al.* suggested that when the recurrence was more than 2 cm distal to the primary tumour, it should be regarded as a new primary tumour instead of a local recurrence.[24] The majority of groin and skin bridge recurrences

develop within the first year after treatment. Also pelvic and distant recurrences generally occur shortly after treatment and have a very poor prognosis.[25] With the aim to diagnose local recurrences in an early stage, long term follow up of patients with vulvar SCC seems sensible, and patients should be instructed to perform self examination of the vulva.

Premalignancies of HPV-negative vulvar SCC

The oncogenesis of the HPV-negative vulvar SCC is largely unknown. It is recognized that the majority of patients have LS and/or dVIN in the adjacent tissue. Both entities have been proposed to be premalignant lesions of HPV-negative vulvar SCC.

LS is a chronic inflammatory skin disorder, most patients complain of itch as the most prominent symptom,[26] with a significant impact on their quality of life (Appendix A). There are different reasons to link LS to the development of vulvar SCC.[27] First, in up to 62% of cases of vulvar SCC, LS can be found in adjacent areas.[28,29] Furthermore, as reported in multiple case reports, patients (old and young) with LS may develop vulvar SCC. Third, in series of LS patients that underwent long-time follow-up, 2-6% has been reported to develop vulvar SCC.[29,30] So, only a minority of LS patients will develop a vulvar SCC during her lifetime. Examining retrospective and prospective studies of patients with known LS provides an estimated accumulated frequency of vulvar SCC arising in LS of 4.5% (140 of 3093 reported cases) (summarised in the study of Carlson *et al*[31]). In the prospective cohort of Carli *et al.*, consisting of 211 patients, two patients developed invasive vulvar SCC, resulting in a relative risk of 246.6 compared to a matched control group.[30] In that study, follow up was rather short (median 1 year and 8 months). In another prospective study, a less than 5% risk of progression was cited.[32] The percentage in the review of Carlson was drawn from largely symptomatic series of LS patients.[31] However, a substantial part of LS patients is asymptomatic, and therefore the exact prevalence of LS is unknown. Because this denominator is unknown, the exact frequency of SCC arising in LS cannot be adequately assessed.[31] In practice, a percentage of 4-5% is applied in the literature on LS and in the information for patients with LS.

It is unknown which patients with LS have an increased risk of developing vulvar SCC. The far majority of patients are rather asymptomatic after the initiation of

potent steroid treatment, whereas in other patients the LS remains active, with complaints of itch and pain. Whether this difference has consequences for vulvar SCC development is not known. Remarkably, most women with vulvar SCC present without an antecedent history of clinically and histologically proven LS, but often suffer from LS. The patients were either asymptomatic or the LS was unnoticed by the family doctor.[29,31,33-35] Limited studies have been performed to predict which LS lesion will ultimately progress to vulvar SCC. In addition, these studies often were performed with a small number of lesions. It is suggested that hyperplasia, dysplasia or cellular atypia are precursor features linking progression of vulvar LS to vulvar SCC.[30-32,36,37] Interestingly, these are all characteristics of dVIN,[4,5] but in the days of most of these studies, dVIN was not regarded as a separate entity. In a series of studies by Raspollini *et al*. they could not find a role for the lymphocytic infiltrate in vulvar carcinogenesis. In all specimens (LS unchanged, LS evolved to SCC, and LS adjacent to vulvar SCC) the infiltrates contained CD3+, CD4+, and CD8+ cells, and the amount was not statistically different between the different LS lesions.[38] In addition, they found that LS that later progressed to vulvar SCC (n=8) showed a higher MIB index and p53 expression and increased formation of new blood vessels than LS without progression (n=8).[39,40] We, however, were not able to predict vulvar SCC development on the basis of blood- and lymph vessel parameters.[41]

dVIN is a relatively 'new' lesion; despite its introduction in the 1960's, its existence has long been questioned because it was thought not to represent a clinicopathological entity but a border phenomenon adjacent to vulvar SCC.[42] Liegl and Regauer stated that this atypical epithelial proliferation is common in the proximity of cutaneous carcinomas at other sites and hypothesized that the atypical epithelial proliferation termed dVIN might not be a vulvar specific lesion.[42] Recently, more knowledge about dVIN has been gathered and the new ISSVD nomenclature[3] emphasized the role of dVIN in future studies to the HPV-negative vulvar SCC oncogenesis. Despite this, dVIN is rarely diagnosed as a solitary lesion; therefore, a lot is unknown about its true incidence, malignant potential and biological behaviour. It has been suggested that dVIN is the direct precursor of the HPV-negative vulvar SCC, however strong evidence is lacking and the suggestion of dVIN being a border phenomenon cannot be excluded yet.

To gain more insight in 'Lichen Sclerosus and Vulvar Intraepithelial Neoplasia in vulvar cancer' we have addressed several subquestions in this thesis:

1. What is the exact role of HPV in the two pathways leading to vulvar SCC? (Chapter 3)
2. What is the incidence of both solitary VIN lesions, has this incidence changed in the past years and what is their malignant potential? (Chapter 4)
3. Can we find differences between LS biopsies of patients who later were diagnosed with a vulvar SCC and LS biopsies of patients who did not develop a vulvar SCC? (Chapter 5)
4. Is the malignant progression of LS and dVIN to vulvar SCC accompanied by altered DNA content? (Chapter 6)
5. Can we provide evidence for the fact that dVIN is a likely precursor of vulvar SCC based on the inflammatory response? (Chapter 7)

Chapter 2

Review of squamous premalignant vulvar lesions

H.P. van de Nieuwenhof
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Abstract

Vulvar squamous cell carcinoma (SCC) develops following two different pathways, which have their own premalignant lesions. In the absence of human papillomavirus (HPV), vulvar SCC can develop in a background of lichen sclerosus (LS), differentiated vulvar intraepithelial neoplasia (VIN) or both. The other pathway leading to vulvar SCC is associated with HPV and the HPV-associated premalignancy is usual VIN.

In this review we will discuss the history, epidemiology, aetiology, histology, clinical characteristics, treatment options, malignant potential and prevention strategies of the three squamous premalignant vulvar lesions.

1. Introduction

1.1 Vulvar squamous cell carcinoma and its premalignant lesions

Vulvar squamous cell carcinoma (SCC) accounts for approximately 3-5% of all gynaecological malignancies and 1% of all carcinomas in women, with an incidence rate of 1-2/100.000.[43] In the Netherlands (16 million inhabitants) about 230 new patients with vulvar SCC are diagnosed.[44] Typically, these cancers occur in the seventh decade when comorbidity is common. A rise in absolute numbers of vulvar SCC is expected because of the proportional increase in the average age of the population. Squamous cell carcinomas (SCC) are the most common vulvar carcinomas. The cornerstone of treatment of SCC of the vulva is surgery.[9]

There are two different types of vulvar SCC with their own associated premalignant lesions (Figure 1). The most common type occurs in elderly women and leads to mostly differentiated keratinising SCC, in a background of lichen sclerosis (LS) and often differentiated vulvar intraepithelial neoplasia (dVIN).[2] There is no association with high-risk Human Papillomavirus (HPV) infection. dVIN is underreported, has a relatively brief intraepithelial phase before progression to invasive carcinoma, and is a difficult histological diagnosis.[3,45] The oncogenesis of LS to vulvar carcinoma and its connection with dVIN is not exactly known.

The second type of vulvar SCC consists of mainly non-keratinising carcinomas and primarily affects younger women. This type of vulvar SCC is caused by an infection with high-risk HPV, predominantly HPV 16 and 18.[2,46] This type of carcinoma is associated with warty and/or basaloid VIN. These HPV-associated usual VIN (uVIN) lesions are seen adjacent to approximately 30% of the vulvar SCCs. In the processes associated with both progression to invasive disease and spontaneous regression of uVIN the immune system seems to play an important role.[47] Multicentric HPV infections affecting both cervix, vagina and/or anus have been described, twenty-two percent of uVIN patients has a concurrent cervical intra-epithelial neoplasia (CIN)[48] and up to 71% of uVIN patients has a previous, concomitant or subsequent history of vaginal intraepithelial neoplasia (VAIN), CIN or cervical carcinoma.[49-51] Despite a stable pattern in the incidence of vulvar SCC,[52,53] the incidence of uVIN and vulvar SCC is increasing in women aged 50 years and younger.[52,54-57] This might be due to a higher incidence of HPV infection of the genital tract and/or to an increased awareness of uVIN.

In this review an outline is given on vulvar premalignant lesions. For an overview of vulvar carcinomas we refer to the review of de Hullu *et al.*[9] Clinical and histopathological characteristics, the different treatment modalities and malignant potential of premalignant vulvar lesions are described.

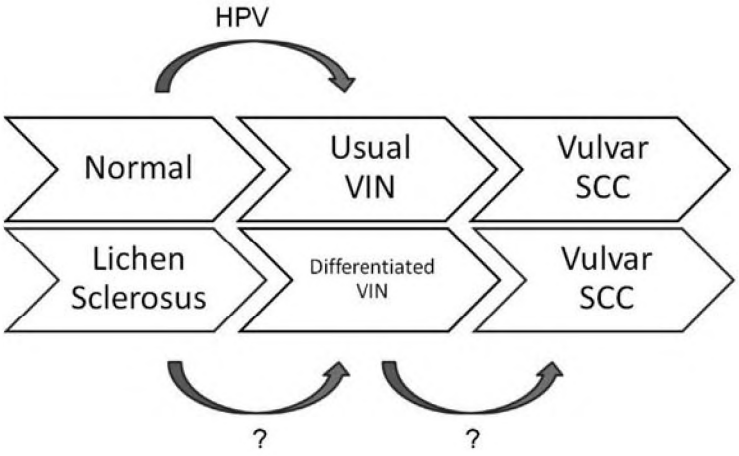

Figure 1 Two pathways leading to vulvar SCC.

1.2 Data sources

Relevant studies were identified by a computer search in the Pubmed database (last date of search February 4th 2008). We searched the medical literature using combinations of the following terms: vulvar intraepithelial neoplasia, simplex, differentiated, usual, classic, VIN, lichen sclerosus, HPV, incidence, vaccines, vaccination, treatment, surgery, steroids, ointments, imiquimod, interferon, photodynamic therapy, 5 fluorouracil, cidofovir, recurrence, progression, oncogenesis and malignant potential. With the button 'related articles' in Pubmed, additional papers were identified. The reference lists from the selected articles were used in order to identify other relevant publications. Also gynaecologic oncology handbooks and relevant theses were used.

1.3 Diagnostics

1.3.1 Biopsy

Vulvar biopsy is the only method to get a histopathological diagnosis when there is a vulvar lesion of uncertain significance, or in the presence of persisting symptoms, such as vulvar irritation or itching. The optimal biopsy is a punch- or small incision biopsy, preferably taken from the edge of the lesion, including a small piece of normal tissue (Figure 2). In case of a suspected malignancy, the biopsy should be taken from the most suspicious part of the lesion. A punch biopsy will produce a core-shaped specimen that is very small, but descends through the full thickness of the epithelium. It can be performed under local, topical anaesthesia. Infiltration anaesthesia, using lidocaine 1% (which may be combined with adrenalin), may be preceded by the application of EMLA® spray or cream, which contains lidocaine and prilocaine as a surface anaesthetic. Disposable punch biopsy devices are available, and a minimal size of 4 mm is advisable for a reliable diagnosis.

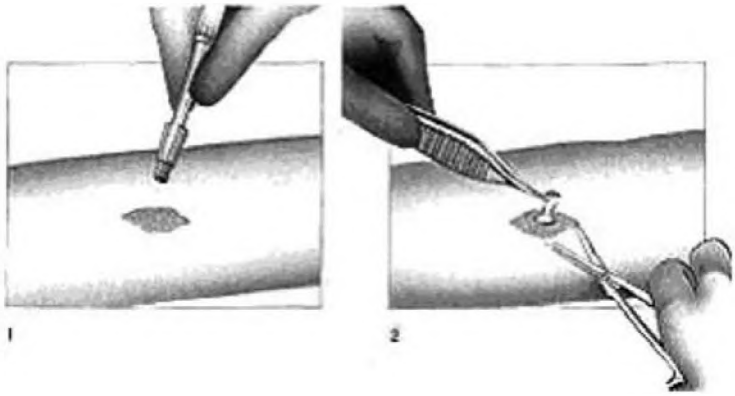

Figure 2 Taking a punch biopsy. 1) The disposable punch biopsy device should be placed on the edge of the lesion with normal tissue. In a gentle, turning motion, the punch should be inserted in the skin. 2) After careful retraction of the device, the skin core can be cut off, carefully holding it in position with a tweezer to prevent crush artefacts.

After taking the biopsy, pressure should be applied to the biopsy site, and when necessary for haemostasis, the wound can be closed by one or two approximating resolvable stitches or by using a silver nitrate stick. When the lesion is multifocal,

the most divergent lesions should be biopsied to obtain the diagnoses of the entire vulvar area. Obtaining the right diagnosis is greatly helped by providing the pathologist with a description of the clinical picture and providing a differential diagnosis. When the results of a punch biopsy are inconclusive, an incision or excision (especially in unifocal lesions that cause symptoms) biopsy can be an attractive alternative. A photograph can be taken for consultation with other medical specialties, to note the localisations of the biopsies that were taken, and to document changes during follow up (Figure 6B).

1.3.2 Vulvoscopy

Vulvoscopy consists of careful observation and the possible use of a 5% acetic acid solution the entire vulvar area to facilitate examination of the vulva by the colposcope. It may result in identification of previously unidentified, subclinical lesions and better define the distribution of clinically evident disease.[58] After application of acetic acid solution clearly demarcated, dense acetowhite areas in the epithelium and abnormal vascular patterns, like mosaicism and punctuation (which reflect the underlying capillary distribution) can be seen.[58] However, acetowhitening has low sensitivity as a predictor of HPV infections,[59,60] although it can be used to determine the extent of the VIN lesion. Another disadvantage is the use of acetic acid, which can be very painful on ulcerative or de-epithelised lesions. In conclusion, vulvoscopy gives limited additional information in the diagnostics of vulvar disease and the painful experience make that there is only a limited role for vulvoscopy. Moreover, performance of a biopsy remains required to establish the diagnosis.

1.4 Psychology and sexology

The effect of vulvar lesions on quality of life is unmistakable. Many LS patients feel embarrassed and have sexual problems.[61,62] Some psychological disturbance has been noted in surveys of women with LS, but this disturbance is thought to be a consequence rather than a cause of the disease.[63,64] Patients may be embarrassed by the disfiguring changes that may occur and avoid sexual intimacy. Limited data that focus on the effect of LS on sexual function are available. A study regarding the impact of LS on women's sexual satisfaction showed that women with LS were less likely to be sexually active (vaginal intercourse, oral intercourse, and masturbation) than controls.[65] The majority of women with LS reported dyspareunia, apareunia and difficulty achieving orgasms and 71% of the patients with a reduced frequency of intercourse experienced an improvement in sexual

functioning after treatment with topical steroids.[66] Introital dyspareunia in patients with LS may be related to three different causes; dryness of the vulvar mucosa, posterior synechiae of the labia minora and rarely introital stenosis. The dryness can be treated with topical steroids and lubricants. The synechiae of the labia minora and the introital stenosis may require limited surgical intervention.[67]

Also the diagnosis VIN can have a great impact on the quality of life. In a review of eight studies on the sexual function for patients treated for VIN, only a small number of women had chronic vulvar pain after vulvar excision, however the majority of women did not return to baseline sexual function.[68] The type of surgery significantly correlates with the magnitude of sexual difficulties, with greater sexual problems among those who underwent radical vulvectomy than among patients treated with wide local excision (WLE).[69-71] However, in the study of Green *et al.* the sexual dysfunction after vulvectomy did not correlate with the extent of surgery or type of vulvectomy.[72] Not all changes in sexual functioning after cancer treatment automatically lead to sexual dysfunctioning. In patients treated for a gynaecological malignancy, sexual dysfunctioning depended on personal and social factors, and the context in which changes in sexual functioning occurred.[73] It is likely that this mechanism can also be applied to patients suffering from a vulvar premalignancy.

Most studies focussed on surgically treated patients; less information is available about the initial effect of pain and pruritus, the two main complaints in patients with LS and VIN lesions. Sargeant and O'Callaghan report that patients with vulvar pain have a significantly worse health-related quality of life in comparison with women without vulvar pain. Moreover, women without vulvar pain were significantly happier in their relationships than those with pain. In addition, women with vulvar pain reported significantly higher levels of distress related to sexual activities. It is important to teach a couple to broaden their sexual repertoire, such that intercourse does not become the major part of sexual activities. Assisting couples dealing with vulvar conditions may lead to an increase in couple happiness and a decrease in sex-related distress.[74]

In conclusion, the number of studies regarding sexuality and LS and/or VIN is very limited. This important aspect of care for patients with LS and/or VIN deserves clinical and scientific attention.

2. Lichen sclerosus

2.1 History

Over the years, a variety of names and descriptions has been used for the disease that is currently named lichen sclerosus (LS). In 1892, Darier was the first to describe the histological features of the disease.[75,76] Lichen sclerosus et atrophicans has been an often used term for a long time. In 1975, the removal of 'et atrophicans' was proposed by the Terminology Committee of the International Society for the Study of Vulvar Disease (ISSVD, later the name was changed to International Society for the Study of Vulvovaginal Disease), because not all LS is histologically atrophic.[77,78]

2.2 Epidemiology

LS occurs at all ages, but not in neonates and is rare in the first year of life. It has a bimodal peak incidence in prepubertal girls and menopausal women.[79] In a study to the prevalence of LS in childhood, a prevalence of 1:900 was found.[80] The majority of patients consists of women aged 50-70 years, while 5-15% of the LS patients are children.[62] Recently, an increase in incidence in childhood LS was noted in the United Kingdom.[80] No definite associations with age at menarche, menopause, first pregnancy or sexual intercourse, number of sexual partners, number of pregnancies, education, smoking habits, Body Mass Index (BMI), hysterectomy, or use of oral contraceptives or hormone-replacement therapy have been found.[76,81] However, the symptoms of LS can be modified by hormonal events in some women.[82] The female to male-ratio is 6:1–10:1,[75,76] and most reports have been on Caucasians. In young girls, the signs and symptoms of LS are sometimes mistaken for sexual abuse.[83] However, childhood LS and sexual abuse are not mutually exclusive diagnoses.[84] In a series of 42 cases of childhood LS, in 12 cases there was evidence of sexual abuse.[85]

The true incidence of LS is unknown and difficult to establish as the care of patients is fragmented: specialties treating LS include dermatology, gynaecology, urology, geriatrics, family medicine and paediatrics, depending on the location of the disease and the patient's age and the responsiveness to therapy. Furthermore, patients can feel embarrassed or frightened and not visit any doctor or have LS that runs an asymptomatic course.[62,86] No recent studies of the incidence of LS in the general population are available. In 1971, Wallace calculated incidences of 1:300 to 1:1000 in new patients, referred to a general hospital.[87] Only a limited number of studies report on the prevalence or incidence of LS in specific groups

of patients. Among women with chronic vulvar symptoms 7-13% suffered from LS.[88,89] The rate of biopsy proven vulvar LS in one general gynaecology private practice was approximately 1.7% (1 in 60 women).[79] One study suggests that 1 in 30 elderly women (nursery home population) has LS.[90]

2.3 Aetiology

Based upon epidemiologic data, a number of mechanisms have been proposed to explain the cause and development of LS. As the highest incidence of LS is observed in patients with low oestrogen physiological states (prepubertal girls and postmenopausal women), hormonal factors have been suggested as a cause for LS. Friedrich and Kalra have shown apparent underlying abnormalities in androgen metabolism (defect in 5 alpha reductase) in women with LS.[63] However, neither oestrogens nor testosterone bring benefit in terms of treatment of symptoms or prophylaxis of the disease.[63,91]

There is a strong association of LS with autoimmune disorders. Between 21.5 and 34% of all patients have an autoimmune disease and up to 74% are found to have auto-antibodies.[92-94] Alopecia areata and vitiligo were the most common associated disorders, but thyroid disease, pernicious anaemia, diabetes mellitus, and cicatrical pemphigoid were also reported.[62,92,93,95] Nevertheless, no statistically significant differences in the natural history of LS were noticed between those patients with autoimmune disorders and those without associated autoimmune disorders.[91] In patients with LS, the family history for autoimmune disease is often positive.[96] There is no evidence that screening for autoimmune disorders in LS patients is beneficial.[97] However, thorough history taking is important and when there are signs and symptoms of concurrent disease, appropriate tests should be ordered. Immunogenetic studies have demonstrated a significant association with HLA class II antigens, particularly DQ7.[62,98] This association was strongest in the patients with early-onset LS.[96] Most HLA class II-related diseases (rheumatoid arthritis, lupus erythematosus) have an immunologic basis. This suggested immunogenetic profile may be one of many presumed factors involved in LS development, extent, and risk of malignancy.[98] The frequency of one of the alleles of the interleukin1 receptor antagonist (IL-1RN) gene is related to increasing disease severity. Thus, IL-1RN may be a candidate gene or severity factor for LS or may possibly be a linked marker to another, as yet undefined, gene.[99] Studies by Carli *et al.* give support to the involvement of the skin immune system in pathogenesis of LS.[100,101]

A genetic influence favouring LS can be derived from familial LS reports.[62,91] LS has been reported in identical and non-identical twins,[93,95] sisters, and mothers and daughters.[96] However, no consistent genetic pattern has been determined and the number of case reports of familial LS is limited.

Several infectious agents have been postulated to induce LS, but such a relationship has not been conclusively demonstrated.[102] LS shares several clinical and histological signs with morphea and acrodermatitis chronica atrophicans (ACA), which both have been associated with Borrelia burgdorferi. Borrelia has been identified in samples of LS by modified silver stain,[103] and using immunoperoxidase methods.[104] However, polymerase chain reaction (PCR) assessment has yielded conflicting results, summed up by Weide *et al.*[105] In Europe and Asia, borrelial DNA was detected in nine of 28 of the cases with LS, whereas in the United States (US) none of 48 patients was positive. Possible explanations are that a subset of LS is caused by a special subspecies of B. burgdorferi that is present in Europe and Asia but does not occur in the US, or that B. burgdorferi is no causal inducer of LS. Also technical differences of the detection methods might contribute to the difference. In some LS lesions however, B. burgdorferi might very well play a role as an observational study by Shelly *et al.* showed positive effects of penicillin and cephalosporin therapy on patients with LS who had responded poorly to treatment with potent topical corticosteroids. All patients (n=13, four men and nine women) showed a significant response, evident within a few weeks.[106] A small study of only four LS patients, suggested an aetiological role of Mycobacterium because of the finding of acid-fast bacteria in all skin biopsies.[107] Furthermore, hepatitis C (HCV) has been suggested because of a case-report of LS associated with chronic hepatitis C and the suspected but non-proven association of HCV with lichen (ruber) planus.[108]

No relationship between HPV infection and LS was found in women[2,91] but has been shown in men.[109-111] In a study of 23 boys (4-14 years) with penile LS, HPV-DNA was detected with PCR in 70%,[112] although it is not clear whether there is a causal relation between LS and HPV. Powell *et al.* compared genital carriage of HPV DNA in prepubertal girls with and without vulvar disease and concluded that HPV appeared to be common in all pre-pubertal girls, but children with LS more often carried high-risk HPV types and their mothers had a high incidence of abnormal cervical smears.[113]

In the itch-scratch-lichen sclerosus hypothesis, LS is postulated to occur as a Köbner phenomenon (the occurrence of LS lesions at sites of injured skin) in women with the susceptible immuno-phenotype who scratch because of genital

irritants.[114,115] Whimster showed that healthy skin grafted onto the vulva became affected by LS and a full-thickness graft from diseased vulva transplanted onto the thigh became normal.[62] This suggests that local dermal factors may facilitate disease expression. This hypothesis is supported by a recent study that showed that the use of moisturizer on LS-affected vulvar skin helps to prevent relapse after use of dermatocorticosteroids.[116] In childhood LS, it has been postulated that sexual abuse could trigger LS as a Köbner effect.[117]

In conclusion, no cause for LS has been found. Careful history taking remains important and further research is needed. When the aetiology is clarified, more specialised and targeted therapies can be developed and prevention might be possible.

2.4 Histology

The classical histological features of LS include a thinned epidermis with loss of normal rete pegs, basal layer vacuolar changes, and paucity of melanocytes.[118] Furthermore, a wide band of homogenised collagen below the dermo-epidermal junction and a band-like lymphocytic infiltrate below the homogenised area are present. The dermis shows variable degrees of oedema.[61,79,91] Oedema is a stage of LS. After oedema, hyalinisation and fibrosis can occur. In severe cases the subepithelial vacuolar change may progress to the formation of bullae.[118] A certain degree of spongiosis and vacuolar degeneration may exist in the cells of the deepest layer. Hyperkeratosis and follicular plugging are highly variable, but may be marked.[118] An example of the typical histology of LS can be found in Figure 3A.

In a recent 'Clinical Opinion' by Jones *et al.*, it was suggested that it should be the role of the pathologist to firstly diagnose LS, then place it into one of the following three categories: atrophic (classic) LS, LS with histological evidence of epidermal thickening, and LS with dVIN.[119]

Recently, Regauer *et al.* have introduced a hypothesis describing early LS as a separate entity. The histological features that they describe as belonging to early LS are quite subtle, and often more prominent in the adnexal structures than in interfollicular skin. Biopsy specimens of early LS rarely display all features that they describe as typical for early LS.[120] So far, no studies following this concept have been described, and up till now there has been little debate about the concept of early LS.[121] Regauer's criteria have not gained any acceptance and have no pathogenetic basis.

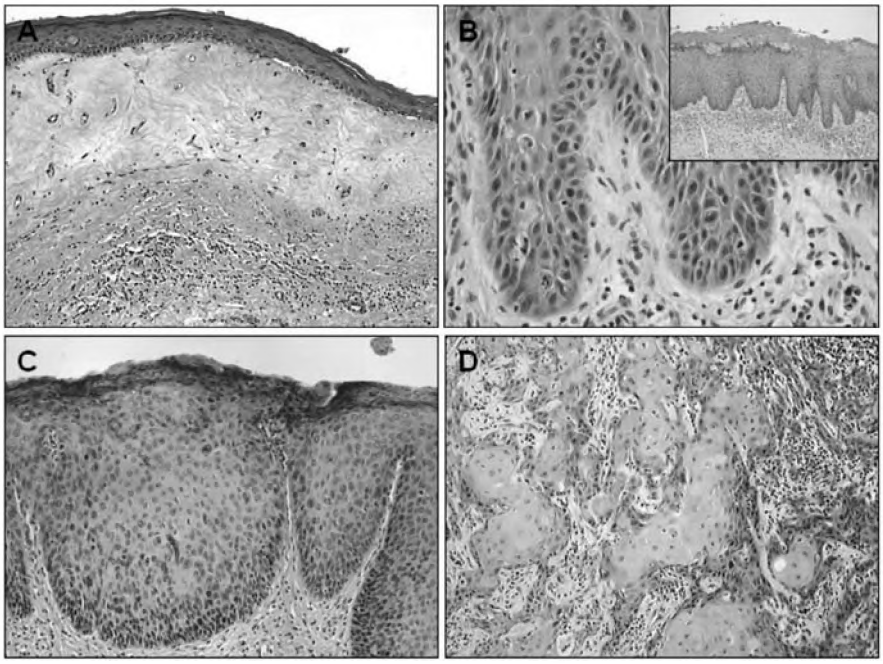

Figure 3 Histological pictures of lichen sclerosus, differentiated VIN, usual VIN and vulvar SCC. A) In lichen sclerosus the epithelium is thinned and without atypia. There is loss of rete ridges, a homogenous, hyalinised zone of oedema beneath the basement membrane and a bandlike infiltrate of lymphocytes B) H&E stained slides of a differentiated VIN lesion (adjacent to SCC). Nuclear atypia and the presence of mitotic figures in differentiated VIN are confined to the basal cell layers. Hyperkeratosis and dyskeratosis are present and the rete ridges are elongated. C) H&E stained slide of a usual VIN lesion without adjacent SCC; atypical nuclei can be found throughout the entire epithelium. D) Invasive nests of vulvar SCC (adjacent to differentiated VIN).

2.5 Clinical characteristics

LS runs a relapsing and remitting course and there is a poor correlation between the extent of the clinical signs and the symptoms.[86] At least one third of patients may be asymptomatic.[79] Complications, most often encountered in severe cases of LS, are secondary infection, adhesion of the clitoral hood with formation of a smegmatic pseudocyst and narrowing of the introitus, which can make intercourse impossible and micturition difficult.[76]

Next to the genital form of LS, in 11-20% of the patients, extra-genital manifestation of the disease is present.[122,123] These can be found on the trunk and neck, upper legs, around the wrists and on the head. Extragenital LS consists of white papules and atrophic maculae. Sporadically, an oral form of LS, without genital or cutaneous manifestations, is described, which consists of asymptomatic white macular lesions.[124,125] Most extra-genital manifestations of LS are asymptomatic, however some rare cases of severe itching of the scalp have been described.[122] Because other dermatoses, such as lichen (ruber) planus, vitiligo, psoriasis inversa and oestrogen deficiency, can mimic LS, the diagnosis in adult patients should preferably be confirmed with a punch biopsy,[61,91] performed as described earlier (see Paragraph 1.3.1).

2.5.1 Signs

Typically, the lesions are white plaques and papules, often with areas of erythema, ecchymosis, hyperkeratosis, pallor, fissuring, telangiectasia, hyperpigmentation, bullae, excoriation, oedema and/or ulceration. The signs can vary from subtle (Figure 4A) to very extensive (Figure 4B). In advanced LS, often there is destruction of vulvar architecture, with scarring of the clitoral prepuce, resorption of the labia minora and narrowing of the introitus.[79] Typically, there is a figure of eight-pattern in the genital area, as can be seen in Figure 4B. The labia minora and majora are most frequently affected, but the entire vulva, perineum and peri-anal area can be affected.[58] In contrast with lichen (ruber) planus, in LS, the vaginal mucosa is usually unaffected, however incidental cases of vaginal LS have been reported.[126] In an extensive study by Cooper *et al.*, pallor and atrophy (wrinkled skin and textural change) were the most frequent findings in both adults and children (in 89% and 62% of patients, respectively). Erythema, atrophy, pallor, hyperkeratosis, and ulceration were all significantly more frequent in women but purpura was more frequent in children (p=0.001).[123] An example of prepubertal LS can be seen in Figure 4C.

2.5.2 Symptoms

Presenting symptoms of LS may include intense pruritus, soreness, pain, burning, dyspareunia, dryness, irritation, urinary complaints, constipation/bowel pain, bleeding and blistering. Painful skin fissures can occur with or after sexual intercourse and defecation. Anogenital pruritus and soreness are the most frequently reported symptoms in both women and children. Prepubertal girls usually present with soreness or itching of the vulva, constipation or dysuria. The

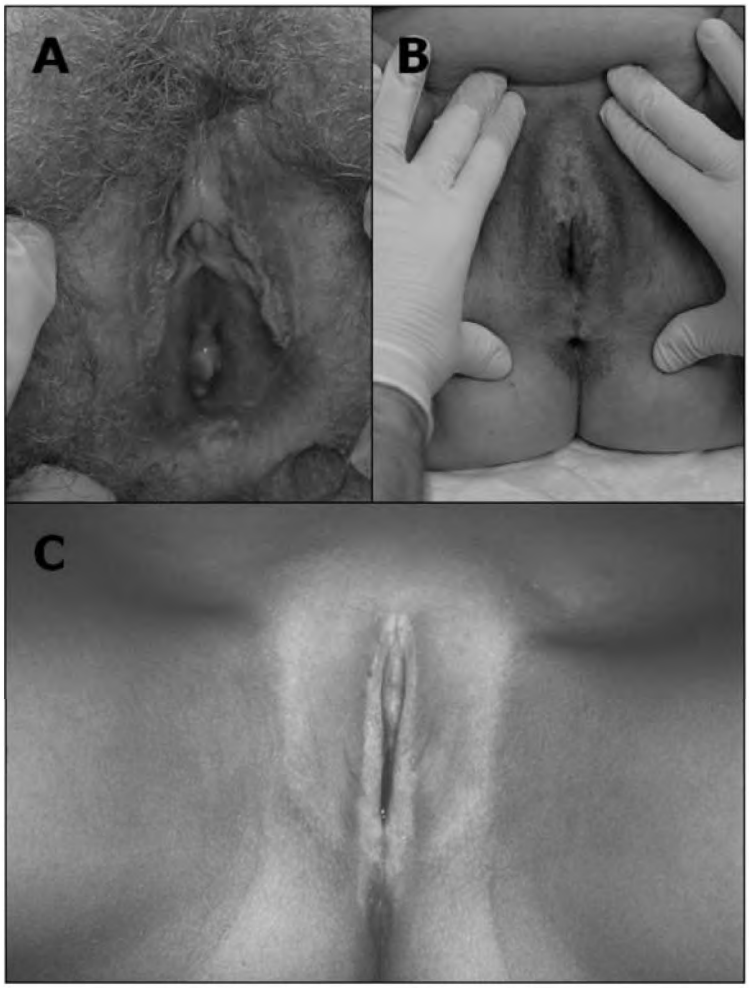

Figure 4 Clinical picture of different patients with lichen sclerosus. A) Lichen sclerosus in a pre-menopausal woman showing subtle loss of architecture and erythema. B) Advanced stage lichen sclerosus in a postmenopausal woman, showing the typical 'figure of eight'. C) Lichen sclerosus in a prepubertal girl.

signs of LS in young girls may be confused with those of sexual abuse, especially because of local bleeding.[76,80,123] As described earlier, LS and sexual abuse may coexist (Paragraph 2.2 Epidemiology).

2.6 Treatment

2.6.1 Introduction

In general, LS has no cure in adults, and lifelong treatment is required. However in some cases, especially prepubertal girls, LS can spontaneously resolve.[61,127] The reasons for treating LS are alleviating and minimising symptoms and the possible prevention of architectural changes.[61,86,120,127] It is not known whether successful control of the disease reduces the long-term risk of malignancy,[128] although a protective effect from malignant evolution is suggested.[127]

2.6.2 Surgery

The role of surgery for the treatment of LS is limited in the absence of dVIN or malignancy.[61,86,129] Surgical options include vulvectomy (with or without a skin graft), laser ablation and cryosurgery. There is a high recurrence rate in patients with LS who have been treated with surgery, even if full thickness skin grafts have been placed.[61,86,129] Next to architectural changes due to surgery, scarring can cause additional anatomical changes. Therefore, surgical treatment of LS should be used as a last resort to release a buried clitoris, to separate fused labia or to widen a narrowed introitus. Perineoplasty can provide good functional results for women with introital stenosis related to LS.[67] Vaginal dilation and corticosteroids may be necessary after operation.

2.6.3 Medical

2.6.3.1 Dermatocorticosteroids

The treatment with the best evidence of efficacy is topical ultrapotent corticosteroid ointment. Corticosteroids have anti-inflammatory, antipruritic, and vasoconstrictor effects. Various studies show good clinical response and histological improvement with the use of these drugs.[86,123,127,128] The use of ultrapotent topical steroids for LS has now become accepted for fist-line management of LS in both children and adults. Recent British Association of Dermatologists (BAD) (www.bad.org.uk) guidelines advocate their use, and give specific guidance on length of initial treatment and on maintenance treatment. There have been no randomised controlled trials on the efficacy of corticosteroids, but evidence for efficacy comes from several small case series.[130-132] Ultrapotent topical steroids can reverse some of the histological changes caused by LS,[120,130,133] however there is no convincing evidence that the treatment of LS influences its prognosis. Cooper *et al.* investigated the reversibility of clinical signs with treatment. They stated that in some patients no signs of LS were present at

follow-up, but there is no information on the recurrence rates after use of ultra-potent steroids was stopped.[123]

There is no universally accepted treatment schedule. In general, short-term use of an ultrapotent corticosteroid is followed by less frequent use of the high-potency steroid (or long-term use of a lower potency topical steroid). Thickened hypertrophic plaques may respond better to intralesional steroids, which can be given in combination with prilocaine.[134] The side effects of topical corticosteroids are burning, irritation, dryness, flaking, maceration, hypopigmentation, and dermal atrophy. Maintenance therapy is often advised, as symptoms can recur in women who terminate therapy.[86,128] Long-term maintenance therapy of vulvar LS with a moisturising cream can maintain the symptom relief induced by topical corticosteroids.[116] However, this study was performed as an open trial without the use of a control group that did not use a moisturizer.

2.6.3.2 Testosterone

Some reports have noted that patients with LS have decreased serum levels of testosterone compared to age-matched controls without the disease.[63] Topical treatment with testosterone has been used and controversy exists about its effectiveness.[135] Topical testosterone can cause virilisation (clitoral enlargement, voice alterations), especially in hyperandrogenic women.[136]

In a prospective, randomised, double-blind evaluation of 58 cases of vulvar LS, 2% testosterone propionate was as effective as petrolatum[137] and in several studies it was less effective than clobestasol.[138,139] Numerous studies emphasise a lack of efficacy and report severe side effects of testosterone; therefore testosterone is considered to be obsolete nowadays.[61,76,86,136]

2.6.3.3 Retinoids

Retinoids are vitamins, because retinol (vitamin A) is not synthesised in the body and must be derived from diet. Retinoids are also considered hormones because retinol is transformed into molecules that bind to nuclear receptors, exhibit their activity, and are subsequently inactivated. Topical retinoids normalise hyperkeratinisation and have demonstrated significant anti-inflammatory effects. Skin diseases like psoriasis and related disorders, congenital disorders of keratinisation, acne, photo-aging and hypovitaminosis A are approved indications of retinoid treatment.[140]

Retinoids appear to reduce connective tissue degeneration in LS. Although orally administered retinoids showed good results, use of these drugs is limited because

of significant and potentially harmful side effects such as cheilitis, xerosis, teratogenicity, elevated liver enzymes, hypertriglyceridemia, abdominal pain, and alopecia. Therefore, retinoids should be saved for severe LS cases that are non-responsive to corticosteroid treatment, and consultation with a dermatologist is recommended before initiating therapy.[61,86]

2.6.3.4 Tacrolimus / pimecrolimus

Tacrolimus and pimecrolimus have an anti-inflammatory and immunomodulating effect and were first introduced as a new second-line treatment for atopic dermatitis. The main side effects are local sensation of heat and burning and therefore it is often discontinued by patients.[91,141] New research has been published on the use of tacrolimus and pimecrolimus for LS and reported to be successful.[141] No long-term effects of tacrolimus and pimecrolimus use in LS patients have been investigated and described yet. Although tacrolimus is not genotoxic and does not interact directly with DNA, it may have a potential to impair local immunosurveillance. Carcinogenicity studies conducted with topical application of tacrolimus in mice demonstrated a dose-dependent development of lymphoma. At the Food and Drug Administration (FDA), 19 cases of tacrolimus (Protopic)-related malignancies have been reported, including nine lymphomas, 10 cutaneous tumours, seven of which occurred at the site of tacrolimus application, as well as cases of SCC, cutaneous sarcoma, malignant melanoma and other tumour types. Therefore the use of tacrolimus should be limited and preferably conducted in a well-designed study.

2.6.3.5 Other

Various other (experimental) treatments for LS have been summed up in recent review articles: antimalarial agents, oral potassium para-aminobenzoate, calcipotriol, oxitomide and photodynamic therapy using aminolevulinic acid. However, the number of cases in these studies is limited and the results are inconclusive.[62,86,91,141]

2.6.4 Supportive care

Correlated complaints of LS should also be treated. Topical oestrogen is recommended for vaginal atrophy. In secondary infections, antibacterial- or antifungal therapy may be needed. Vulvodynia may be treated with xylocaine 5% (or 3%) ointment and antidepressants are a treatment option in unresponsive cases.[61,76] Nocturnal itching can be relieved with antihistamines like hydroxyzine

hydrochloride (10-40 mg A.N.); the sedative effect contributes to its usefulness to relieve pruritus.

In general, and especially in patients with LS, vulvar care and hygiene is important: use lipid-rich neutral creams (*e.g.,* cremor lanette) to soften the skin, no or only neutral soap, cotton underwear, no panty liners and scratching should be avoided. The use of lubricants to facilitate sexual intercourse is recommended.[128] Recently, the results of a prospective non-randomised trial were published, showing that long-term maintenance therapy of vulvar LS with a moisturising cream can maintain the symptom relief induced by topical corticosteroids.[116] Written information and information about support groups should be offered.[128]

2.7 Malignant potential

There are different reasons to link LS to the development of vulvar SCC.[142] First, the majority of vulvar SCCs has LS, squamous cell hyperplasia or dVIN in the adjacent epithelium. In up to 62% of cases of SCC of the vulva, LS can be found in adjacent areas.[28,29] Furthermore, as reported in multiple case reports, patients (old and young) with LS may develop vulvar SCC. Third, in series of LS patients that underwent long-time follow-up, 2-6% has been reported to develop vulvar SCC.[29,30]

A protective effect from symptom control is suspected but has not been proven.[123] Neither the presence nor duration of symptoms nor the loss of vulvar architecture is a useful indicator of malignant potential in LS.[123]

There is an impression that itch and scratch damage (related to the duration of symptoms), is associated with the risk of developing vulvar SCC.[114] Chronic inflammation seems to play an important role, but the exact pathogenesis of progression to malignancy has not yet been established.[91] The immunogenetic profile may also determine (part of) the risk of malignant disease.[76]

Extra-genital LS is not associated with malignancy,[91] and could prove to be an interesting control group in the search for factors that determine the risk of malignant transformation in vulvar LS. Furthermore, the exact mechanism of malignant progression remains to be elucidated. dVIN is the possible precursor lesion of HPV-negative SCC but the exact pathogenesis from LS to dVIN and a subsequent SCC is unknown. Some authors claim a role for squamous cell hyperplasia as a next stage after LS in the development of HPV-negative vulvar SCC but the exact pathway remains to be elucidated.[143] Recently, high p53-expression in LS has been postulated as a marker for increased likelihood to progress to vulvar SCC.[39,144]

2.8 Prevention

Since the cause of LS is unknown, no prevention strategies are available. It has been suggested that an early start with dermatocorticosteroids might prevent signs of advanced LS in patients with early LS.[120] It has been suggested that treatment with steroids also prevents the development of a malignancy. The long-term follow-up of women with LS has been addressed in the guidelines series of the British Association of Dermatologists (BAD); it is suggested that yearly follow-up and examination should be undertaken. Balasubramaniam and Lewis audited the rate of follow-up by general practitioners (GPs) after discharge from secondary care. Twenty-six percent of patients had not been seen for two years after discharge, and 38% had never seen their GP at all. Seventeen percent had seen their GP specifically about their LS but had not been examined. Interestingly, 82% of patients were receiving ongoing therapy for their LS.[145] There is no evidence that follow-up in LS leads to earlier detection or prevention of a vulvar malignancy. However, a prospective study by Oonk *et al.* indicated that routinely scheduled follow-up meetings with patients treated for vulvar carcinoma resulted in the detection of smaller recurrences in a substantial proportion of patients compared with self-reported recurrences.[146] It is recommended that women with vulvar LS are followed in specialised clinics when difficulty exists with symptom control or when there is clinical evidence of localised skin thickening. Follow-up by a specialist (instead of a GP) is also recommended when the pathologist expresses concern and is unable to make a definitive diagnosis of dVIN.[119]

3. Differentiated Vulvar Intraepithelial Neoplasia

3.1 History

dVIN was first described in the 1960s by Abell as a highly differentiated form of vulvar carcinoma in situ (CIS) and designated 'intraepithelial carcinoma of simplex type' to distinguish it from 'intraepithelial carcinoma of Bowen's type' (which nowadays would be called uVIN). In 1977, the term 'differentiated' was introduced to highlight the marked differentiated morphologic features of this entity.[4] In the subsequent ISSVD revisions of the classification that followed, 'VIN 3, severe dysplasia, differentiated type' was replaced by the current term 'VIN, differentiated type'. The nomenclature of the WHO spoke of 'carcinoma in situ (simplex type) (VIN3)'.

3.2 Epidemiology

dVIN accounts for a small proportion (<2-5%) of all VIN lesions compared with the usual type.[51,147] Because of a difficult clinical and histological diagnosis there probably is a considerable underdiagnosis. dVIN characteristically occurs in postmenopausal women (mean age 67 years) and is associated with LS.[4] As dVIN is seldom found in an isolated form, some authors believe it is actually part of the adjacent vulvar SCC.[42] In cases where dVIN is found without any sign of invasion, often the patient has been previously treated for vulvar SCC, and also suffers from LS,[148] however also isolated dVIN lesions can occur in patients without a history of vulvar SCC. Isolated dVIN is believed to have high malignant potential. Unlike uVIN, dVIN is mostly unicentric.[5]

3.3 Aetiology

HPV is very uncommon in dVIN and does not play an important role in its genesis.[5,147,149,150] The aetiology of dVIN remains to be elucidated. Because of its high concurrence with LS, a causative role for LS is likely but also other factors might play a role. Squamous cell hyperplasia has a close relationship to dVIN. However, it is still unclear whether it is a direct precursor of invasive vulvar SCC.[5] Squamous cell hyperplasia with atypia might represent a step in the carcinogenesis.[151] Some have suggested a role for p53-alterations but a study by Regauer *et al.* suggested that p53-expression in LS is a sign of ischemic stress rather than a sign of dVIN.[42]

3.4 Histology

Histologically, dVIN is a more subtle lesion than uVIN and can be mistaken easily for a benign dermatosis or epithelial hyperplasia. The recognition of dVIN is hindered by a high degree of cellular differentiation combined with an absence of widespread architectural disarray, nuclear pleomorphism and diffuse nuclear atypia.[152] The atypia in dVIN lesions is strictly confined to the basal and parabasal layers of the epithelium, where the cells have abundant cytoplasm and form abortive pearls.[4,58] Scattered mitotic figures are common in the basal areas, but can extend into the upper levels of the epidermis.[5] Typically, the epidermis is thickened and has a parakeratotic surface reaction.[4] The nuclei are relatively uniform in size and contain coarse chromatin and prominent nucleoli leading to paradoxical maturation abutting on the epithelial-stromal junction. The superficial layers of the epithelium have normal maturation and do not show koilocytosis. Furthermore, in dVIN the epithelium exhibits elongation of rete pegs.[5,58,147]

Intercellular bridges typically are very prominent, probably as a result of loss of cohesion between cells rather than spongiosis.[4] dVIN is characterised by an increased amount of eosinophilic cytoplasm in dysplastic cells immediately above the basal cell layer. This is indicative of premature differentiation or keratinisation. At low power, the epithelium looks eosinophilic because of the large amount of intracytoplasmic keratin. These abnormal keratinocytes are the hallmark of dVIN. At higher power, nuclear changes of enlargement, pleomorphism, hyperchromatism and mitoses are present.[4,58,148] In the underlying or adjacent papillary dermis, a chronic inflammatory cell infiltrate, consisting of predominantly lymphocytes, plasma cells, and occasionally eosinophils, is present[4] (Figure 3B).

When dVIN invades the dermis, nodules of enlarged eosinophilic keratinocytes appear to fall from the basilar epidermis or from elongated rete ridges. In superficial biopsies, the distinction of dVIN from early invasive vulvar squamous cell carcinoma may be difficult.[4]

The use of MIB1 can be helpful to distinguish between normal vulvar epithelium and dVIN as the basal cell layer in dVIN has a higher proliferation index (percentage of MIB1 positive cells), than normal vulvar epithelium, where the basal cell layer often is negative for MIB1.[153] Immunostaining for p53 may be of value in identifying dVIN in difficult cases. The p53-labeling-index (LI) of the basal cell layer in dVIN is often >90%, and p53-positive cells extend above the basal cell layer (suprabasilar extension) into higher levels of the epidermis.[5]

3.5 Clinical characteristics

3.5.1 Signs

dVIN is infrequently seen as a solitary lesion, and is more commonly identified adjacent to LS and/or invasive SCC. However, also a patient with dVIN that developed vulvar SCC without presence of LS has been described.[154]

It can present as an area of grey-white discoloration with a roughened surface (Figure 5A), an ulcerative red lesion (Figure 5B), an erythematous red lesion (Figure 5C and D), or as an ill-defined raised white plaque. It may be difficult to distinguish from the often-present background of LS.[5] In general, dVIN is believed to produce less bulky lesions than uVIN does. Yang and Hart described a series of 12 patients with lesions varying from 0.5-3.5 cm in greatest dimension.[5]

3.5.2 Symptoms

Patients are, due to the underlying LS, often symptomatic with a long-lasting history of itching and other LS-related symptoms (described in Paragraph 2.5.2).[5]

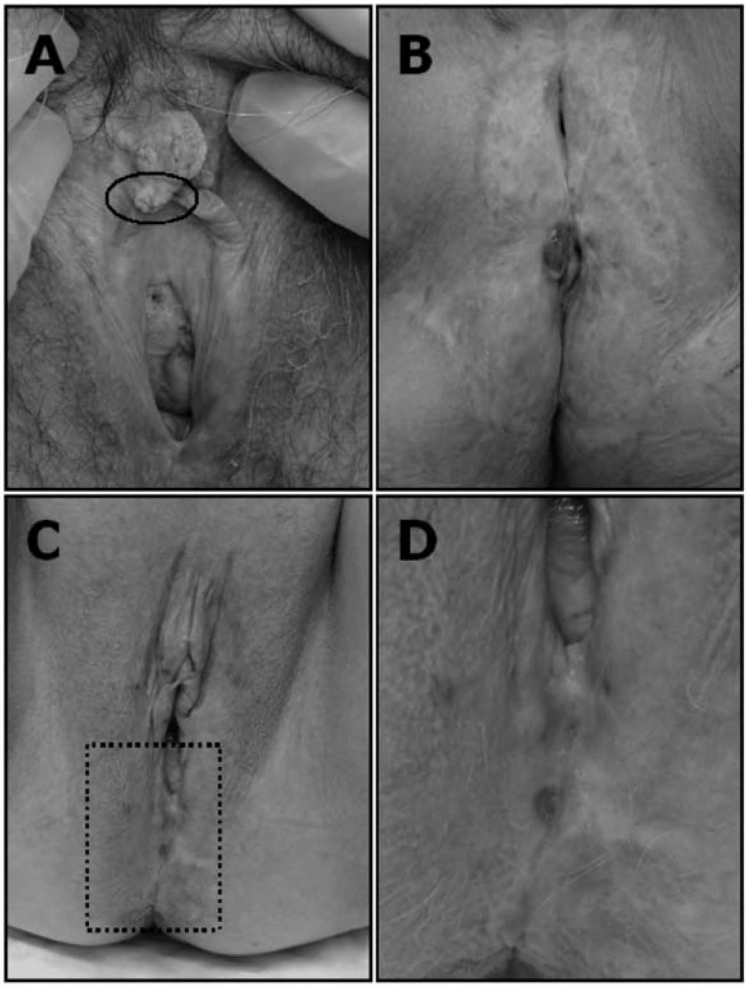

Figure 5 Clinical pictures of differentiated VIN. A) Differentiated VIN arising in a background of lichen sclerosus. An incision biopsy was taken at the site of the ellipse and showed differentiated VIN. Histological examination of the excision of the entire lesion revealed vulvar squamous cell carcinoma, with a depth of invasion of more than 1 mm. B) Differentiated VIN in a patient who was treated by multiple excisions and a vulvectomy with plastic surgery because of therapy resistant lichen sclerosus. C) The red lesion within the dotted line did not improve upon dermatocorticosteroid application and was removed by excisional biopsy. The histology showed differentiated VIN. D) A close-up of the differentiated VIN lesion.

3.6 Treatment

dVIN generally requires a more extensive surgical approach than uVIN, and as dVIN often is unifocal, radical surgical excision is the preferred treatment. dVIN is usually confined to non hair-bearing areas or the external sides of atrophic labia minora, where vulvar reconstruction is feasible. Recurrent lesions are common and so far, there is no place for medical therapy.[147] Follow-up of patients previously treated for dVIN should occur at a specialised vulvar clinic or by a vulvar specialist (a consultant dermatologist or gynaecologist who has had additional and educated training in managing vulvar disease).[119]

3.7 Malignant potential

dVIN is associated with the development of keratinising SCC.[5,155] The exact pathway of carcinogenesis has not yet been established. It is suggested that dVIN is highly proliferative and might be more likely to progress to an invasive SCC than LS and uVIN.[5,154,156] The observation that most SCCs are dVIN-related and most VIN lesions without concurrent invasion are of the usual type, combined with the frequent finding of dVIN adjacent to rapidly growing invasive vulvar SCC, reinforces this presumption.[2,5] Therefore, any suspicious area should be biopsied.[5,147]

4. Usual Vulvar Intraepithelial Neoplasia

4.1 History

Historically, various terms have been used to define vulvar SCC precursors. In 1912, Bowen described 'squamous intraepithelial lesions' (also referred to as Bowen's disease) and since then a myriad of clinical and histopathological terms have been employed to describe what is currently known as uVIN.[147] The first series of Bowen's disease of the vulva was described in 1943 by Knight *et al.* referred to in[157]. In 1965, Kaufman separated non-neoplastic from neoplastic vulvar lesions. He divided the precancerous lesions into three groups: Queyrat's erythroplasia, Bowenoid carcinoma in situ, and carcinoma simplex.[147] In 1976, the ISSVD simplified the terminology: carcinoma in situ and vulvar atypia were replaced by Vulvar Intraepithelial Neoplasia (VIN).[147] According to our Pubmed search, VIN was introduced into medical literature in 1980.

4.2 Grading

For vulvar grading, two systems have been used. The three-grade system of the ISSVD made a subdivision between the lesions according to the amount of epithelium involved by dysplasia, similar to CIN lesions. In VIN 1 the maturation is present in the upper two-thirds of the epithelium, and the superficial cells contain variable but usually mild dysplasia. In VIN 2 the dysplasia involves the lower two-third of the epithelium, and in VIN 3 the dysplasia extends into the upper third of the epithelium, but not involving the full thickness.[58,158] When the entire epithelium is involved, the lesion is classified as carcinoma in situ, which is gathered in VIN 3. The two grade system of Bethesda makes a subdivision between Low grade vulvar intraepithelial lesions (LGVILS) and High grade vulvar intraepithelial lesions (HGVILS).[150] We will discuss both systems of grading.

4.2.1 ISSVD classification

In 2003, the ISSVD decided to abolish the three grade system of VIN because clinicopathological data did not appear to support the concept of a continuum spectrum of VIN lesions leading to vulvar carcinoma, that does exist for CIN and cervical carcinoma.[3,147] The abandonment of VIN 1 and the consolidation of VIN 2 and 3 into one category simply termed (high-grade) VIN, best fitted the studies that have been performed on grading of VIN so far.[3] In Table 1 an overview of 'old' and 'new' nomenclature is given. VIN as a single diagnostic category included two different vulvar premalignancies that have a different malignant potential.[147] In the light of the two different types of vulvar carcinoma the VIN terminology has been modified by the ISSVD, and a 2-tier classification has been suggested: VIN usual type and VIN differentiated type.[3,159] uVIN has been divided into basaloid and warty subtypes. The warty subtype was earlier described as Bowen's disease. Basaloid VIN occurs in an older age group, is more likely to become invasive and less likely to regress. These two subtypes may be seen in a single VIN lesion and cases with mixed features are common.[159] Both subtypes are associated with high-risk HPV types, especially HPV 16, often occur as multicentric genital tract neoplasia and should be considered as premalignant lesions.[147] In clinical practice, no difference is made between the two uVIN lesions. For dVIN, we refer to Chapter 3 of this review.

4.2.2 Bethesda classification

Many gynaecologists and some pathologists have suggested analogous terms of high-and low-grade changes of the vulva as also exist for CIN lesions. In 2005,

Old nomenclature	New nomenclature[3]
VIN 1	No cancer precursor
(Classic) VIN 2/3	Usual VIN (uVIN) - Warty VIN - Basaloid VIN - Mixed (warty-basaloid)
(Well-)differentiated VIN 3 / VIN simplex	Differentiated VIN (dVIN)

Table 1 Overview of the old and new nomenclature of VIN lesions.

Medeiros *et al.* proposed a Bethesda-like grading system of low and high-grade vulvar intraepithelial lesions: low grade vulvar intraepithelial lesions (LGVILS) and high grade vulvar intraepithelial lesions (HGVILS).[150] When a Bethesda like system is applied to vulvar histopathology, by far most frequent lesions fitting into the LGVILS category are condylomata acuminata, which carry a negligible risk for malignant progression and require no follow up. It seems inappropriate and unnecessary that such lesions are placed in a new category of vulvar premalignant lesions.[159]

In our opinion a Bethesda-like grading system is not sufficient because no distinction is made between uVIN and dVIN. Because of the extensive differences in clinicopathological behaviour and malignant potential between uVIN and dVIN lesions, we favour the above-mentioned ISSVD classification.

4.3 Epidemiology

The incidence of uVIN is approximately five per 100.000 women per year and increasing worldwide.[160] In several countries the age-adjusted incidence rates have surpassed the incidence rate of invasive vulvar carcinoma.[52,55,161,162] uVIN has a predilection for relatively young women, often in their 30s and 40s. Cigarette smoking is reported in about 60-80% of the cases. Condylomata, a history of genital herpes, and infection with Human Immunodefiency Virus (HIV) are particularly common in young women with uVIN.[4] Moreover, the use of immuno-suppressants to prevent rejection after transplantation or to treat a chronic autoimmune disease increases the risk of developing uVIN. The increase in incidence of uVIN lesions is in concordance with the increase in HPV prevalence. Despite the increase in uVIN, no increase in the overall incidence of SCC of the

vulva was found.[52,53] In young women an increase in SCC incidence is seen, possibly due to the younger age of first sexual intercourse and the increasing incidence of HPV infections.[52,54-57,162] The increase of uVIN without a clear increase of vulvar carcinoma may have different reasons. The main reason is the more liberal use of vulvar biopsy, which contributed to earlier diagnosis of VIN lesions that might have been missed in the past. This earlier diagnosis and treatment of VIN may prevent the ultimate development of invasive SCC. Moreover, the malignant progression is estimated to be <10%, so only a limited number of uVIN lesions progress to invasive vulvar SCC.[163] For more information about the malignant potential see Paragraph 4.8 of this review.

4.4 Aetiology

In the 1970s, zur Hausen first proposed the hypothesis that HPV, and not herpes simplex as previously thought, was the most likely aetiological agent for the development of cervical carcinoma.[164,165] From laboratory studies as well as epidemiological studies it became clear that HPV plays a major role in the aetiology of not only CIN and cervical carcinoma, but of all squamous anogenital cancers (vulvar, vaginal and anal SCC).[157] In 1982, detection of HPV DNA in VIN was described for the first time.[166] Only high-risk HPV subtypes, mainly HPV 16 and 18, are able to induce uVIN or vulvar SCC.

The mean HPV positivity in VIN lesions is 85% (calculated from nine studies,[1,50,51,167-172] see Table 2), and in the largest study 100% HPV positivity has been shown.[50] It is important to know which type of VIN (uVIN or dVIN) is subject of study, which DNA detection method has been used and which HPV types are determined. The difference in HPV positivity between u- and dVIN is illustrated by Table 2. A possible explanation for the higher percentage of HPV in VIN comparable to SCC of the vulva (about 30%) is that SCC can have developed in both LS and dVIN as well as in uVIN.

The immune system seems to play an important role in clearance and persistence of the HPV infection and development of uVIN. Women infected with HIV are approximately four times more likely to be infected with HPV. The prevalence of uVIN in HIV infected women was reported to range from 0,5 to 37%.[173] Also women who use immunosuppressants to prevent rejection after transplantation, had a 10 to 30 fold risk of cancer of the vulva, mainly caused by HPV 16 and 18, that are found at a higher rate in transplant patients compared with immunocompetent women.[174-176]

Study	Number VIN lesions	Test	Percentage HPV positive
Hoevenaars, 2008[1]	45 uVIN	HrHPV*	84%
	75 dVIN		0%
Van Beurden, 1995[51]	47 x uVIN	HPV	92%**
	1 x dVIN		
Hampl, 2006[50]	183 VIN	HPV	92%
Hillemanns,2006 [167]	30 VIN	HPV	80%
Hørding, 1991[168]	19 VIN	HPV 6,11,16,18,33	79%
Lerma, 1999[169]	12 uVIN 6 dVIN	HPV HPV	42% 0%
Rufforny, 2005[170]	24 VIN	HPV 16	79%
Skapa, 2007[171]	82 uVIN	HPV	99%
	12 dVIN	HPV	8%
Srodon, 2006[172]	34 VIN	HrHPV	100%
Total	186 uVIN 290 VIN*** 93 dVIN		89.9% 89.8% 1.0 %

Table 2 HPV positivity in VIN lesions.
* HrHPV: high risk HPV subtypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 68, 73, and 82
uVIN = usual VIN
dVIN = differentiated VIN
When specified, only VIN 2/3 cases were included
** It is not mentioned whether the dVIN lesion contained HPV. The dVIN lesions were not taken into account in the calculation in the HPV positivity of dVIN lesions. The uVIN lesions were considered to be 92% HPV positive.
*** Subtype of VIN not specified, it is known that >90% of VIN lesion are of the usual type.

4.5 Histology

Histopathologically, uVIN lesions are easy to recognize. An example of the histology of uVIN can be found in Figure 3C. Typically, the epidermis is thickened and is accompanied by a surface reaction of hyperkeratosis and/or parakeratosis

of the most superficial of cell layers.[4,58] The characteristic microscopic features include a wind-blown appearance of the epidermis that produces a disorganisation of keratinocytes, high nuclear-to-cytoplasmic ratio, nuclear hyperchromasia, irregularities of the nuclear membranes, and numerous mitotic figures at all levels of the epidermis. Dyskeratotic cells with dense eosinophilic cytoplasm and pyknotic nuclei probably representing apoptotic cells are common. Cells with a clear or vacuolated cytoplasm that appears to form clear halos around shrunken pyknotic nuclei corresponding to so-called 'koilocytes' may be numerous. Multipolar and abnormal mitotic figures are easily found. uVIN lesions characteristically are aneuploid in upper cell layers of the epithelium.[4] The histological correlates of aneuploidy have been reported to be abnormal mitotic figures and nuclear enlargement with hyperchromatism and pleomorphism, especially in the basal and parabasal zones. Two types of uVIN exist; in the warty pattern the involved epidermis has an undulating or spiked surface creating a warty or condylomatous appearance and there is a striking cellular pleomorphism. Multinucleated cells and koilocytes are common. In the basaloid pattern, the surface is relatively flat and non-papillamatous with a diffuse replacement of the epidermis by a homogenous population of small, undifferentiated keratinocytes with scanty cytoplasm. However, there are overlapping features of these two patterns: mixtures of warty and basaloid patterns are commonly found in the same lesion.[4]

4.6 Clinical characteristics

4.6.1 Signs

uVIN lesions can have a variety of clinical appearances. The key features are raised, well demarcated and asymmetrical lesions. They often produce large whitish (Figure 7) or erythematous plaques (Figure 6A); while some lesions are pigmented (Figure 6C). The most frequently affected sites are the labia majora, the labia minora and posterior fourchette. Other, less affected sites are the clitoris, mons pubis, perineal and perianal areas.[177] Multifocal involvement of the vulva occurs in more than 40% of cases.[4,177] Multicentric intraepithelial or invasive squamous neoplasia (of the cervix, vagina or anus) is also common, occurring in approximately 25-66% of the uVIN patients.[4,48,50,51] The multicentricity of uVIN is age-related; decreasing from 59% in women aged 20-34 to 10% in patients over 50 years of age.[147] Multicentric uVIN are more often HPV positive than the unicentric uVIN lesions.[51,167,168] Because of the often-concurring uVIN and CIN, a cervical smear should always be performed. In Figure 5A, 5C-D, and 6, different

appearances of uVIN are depicted: varying from white and condylomatous-like to brown lesions.

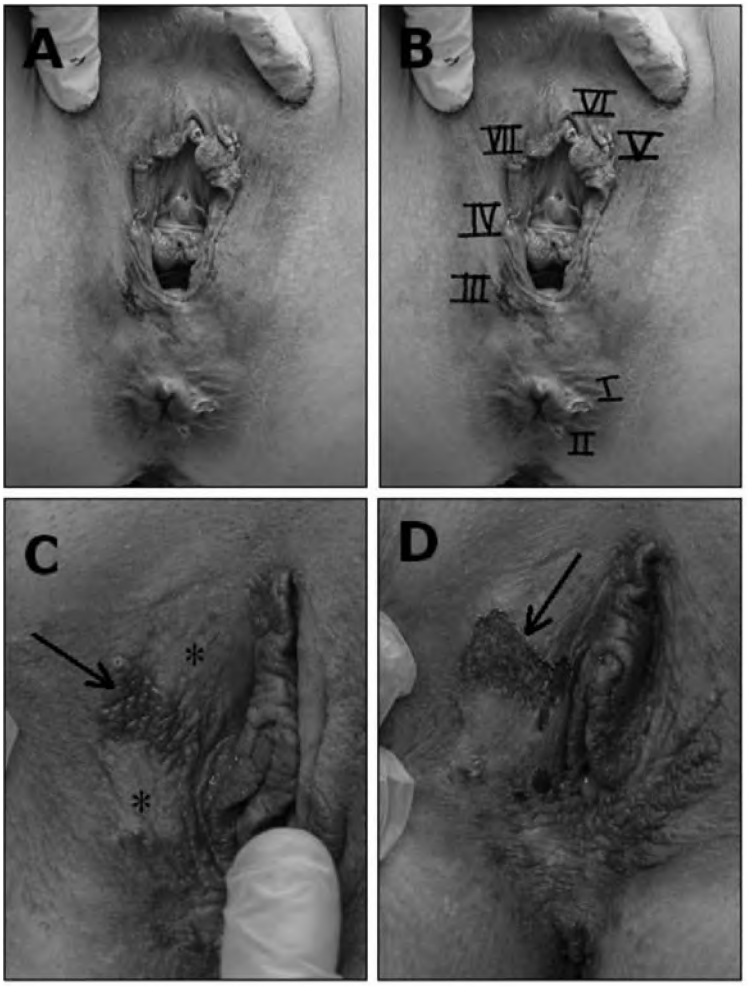

Figure 6 Clinical pictures of usual VIN. A+B) mapping of usual VIN. In order to get information of all suspicious looking lesions (I, IV, V, VI and VII), and the extent of the usual VIN lesions, two normal looking areas were also biopsied (II and III). Six biopsies showed usual VIN and in biopsy III no abnormalities were found. C) The brown area of biopsy-proven usual VIN (indicated by the arrow) was treated by laser vaporisation. The areas indicated by the asterix, were previously treated by laser vaporisation. D) In the area indicated by the arrow, the upper layers of the epithelium were vaporised.

4.6.2 Symptoms

The most common presenting complaint is pruritus, present in about 60% of the patients. Other presenting symptoms can be pain, ulceration and dysuria.[177] A significant proportion of patients have no specific complaints, apart from the finding of an abnormal vulvar area by self examination (22%).[177]

4.7 Treatment

4.7.1 Introduction

There are several options for treatment of uVIN, with a major role for surgery. When a large number of treatment options exist for a particular condition, it is likely that none of those treatments provides an excellent solution to the problem. This seems to be the case considering the treatment of uVIN.[178] The paucity of robust evidence is of critical importance supporting most of the various treatments suggested for uVIN.[178]

Ideally, a treatment of uVIN should address some or all of the following:[178]

- Exclude invasive disease at the outset
- Relief of symptoms
- Eradication of HPV infection
- Minimal disruption of adjacent normal epithelium
- Restoration of normal epithelial architecture
- Reduction in the risk of malignant progression
- Sustained remission

uVIN is considered as an uncommon chronic skin disorder with a high risk of recurrence after treatment and a risk of progression to invasive vulvar SCC. Removal has been standard treatment for all suspicious and/or symptomatic lesions. Due to the recurrent character of uVIN, young women are at risk to require repeated treatment over a prolonged period of follow-up. As a consequence, they may suffer from psychosexual morbidity.[70,72] This leads to the need for alternative treatment options.

4.7.2 Surgery

4.7.2.1 Cold knife surgery

In the past, extensive surgery has been performed for uVIN. During the 1970s, several authors addressed vulvectomy as overtreatment for uVIN. The main reasons to favour more limited surgery were the mutilating treatment for a disease with an increasing incidence in young women, with a recurrent character and the lack of knowledge about the biological behaviour of the disease.[179]

In 1984, Wolcott *et al.* reported an increase in recurrences when wide local excision (WLE) was performed instead of radical vulvectomy, and they found that positive surgical margins were a significant predictor of recurrence.[180] This observation paved again the way towards more extensive surgery for uVIN since that time.[177] Recent reports showed that positive surgical margins rarely predict the development of invasive disease. Therefore WLE, consisting of removal of all visible lesions, is the surgical technique of choice.[179,181]

4.7.2.2 Laser excision, vaporisation, and Loop Electrosurgical Excision Procedure (LEEP).

Vulvar laser surgery was introduced more than a decade ago and seemed an appropriate treatment particularly for uVIN because of the low thermal effect on vulvar tissue.[147,182] Laser excision combines the advantages of both surgical excision (high cure rate and correct diagnosis), and laser vaporisation (cosmetic and functional results).[147] Laser vaporisation is a destructive technique and has the disadvantage of destroying the treated tissue, without the possibility of histological evaluation.[147] In laser excision and vaporisation, at the end of the procedure the treated areas can be washed with 5% acetic acid to ensure that no residual lesion is left behind.[182] Before treatment with laser vaporisation, invasive disease must have been excluded. Both treatments can be performed under local anaesthesia. When a large vulvar area is involved, more treatment sessions may be necessary, as treatment of a large area is painful. Loop electrosurgical excision/ fulguration procedure (LEEP) may be an alternative to laser vaporisation with the advantage of provision of a specimen for pathology; there is a lot of experience with this treatment by the widespread use in patients with CIN.

In several studies, surgical techniques have been compared. In a review about the effect of surgical treatment in relation to recurrence and progression of uVIN, the recurrence rates in 1921 surgically treated patients have been evaluated. The recurrence rates were comparable with an overall recurrence rate of about 20%.[181] In Figure 6 C & D, the effect of laser vaporisation is shown. The areas marked with an asterix were previously treated by laser and had the same appearance as the area indicated by the arrow in between. In Figure 6D the situation directly after the laser vaporisation is shown.

4.7.3 Medical

A lot of medical treatments have been tried to avoid surgery in patients with uVIN lesions. Medical treatment does not provide a specimen for histological evaluation and occult invasion may be missed. Therefore, mapping of the vulva is important, before starting any treatment to prevent missing invasive disease.

4.7.3.1 5-Fluorouracil (5-FU)

5-FU is a topical used chemotherapeutical agent. Topical 5-FU was first used in the 1960s on skin metastases from melanoma and adenocarcinoma of the colon.[183] In 1985, Sillman *et al.* published a review about the use of 5-FU in lower genital intraepithelial neoplasia. Up to then, 68 patients with uVIN 3 were treated with 5-FU. Twenty-three patients achieved remission, five patients improvement, and 40 patients failed to respond. Pain and burning frequently limited the duration of 5-FU treatment.[183] In other studies 5-FU was badly tolerated and yielded limited results.[184-187] In conclusion, topical 5-FU has no prominent role in the current treatment for uVIN because of the severe side effects and the high failure rates.

4.7.3.2 Imiquimod

Imiquimod (Aldara®, 3M Pharmaceuticals, St Paul, MN), an imidazoquinoline amine, is classified as an immune response modifier. It is widely used in the treatment of genital warts with proven efficacy in terms of clearance of the lesions, and a lower recurrence rate compared with conventional surgical treatments.[188-190]

Analogous to high grade CIN, uVIN microenvironment is associated with a decreased expression of the pro-inflammatory Th1 cytokines, Tumour Necrosis Factor α (TNF α) and Interferon γ (IFN γ). Imiquimod acts by activating macrophages and thereby inducing secretion of these pro-inflammatory cytokines. This in turn promotes a Th1 adaptive immune response leading to the secretion of IFNγ and the up-regulation of cell mediated immunity.[191] By inhibiting viral replication, imiquimod focuses on the cause of uVIN and preserves the anatomy and function of the vulva.[192,193]

Several studies evaluated the effect of imiquimod on uVIN lesions; however, all but two studies are small and uncontrolled with various lengths of follow up. In most study settings, a two to three times per week 5% dose regimen was used. After several applications most patients with uVIN experienced side effects, varying from irritation to burning, itching, and/or ulcer development. The side effects made several patients stop applying the imiquimod treatment. Responses were reached after six to 30 weeks of treatment.[193]

Up till now, 110 treated patients are reported in uncontrolled studies, with a complete response rate of 47%, and a partial response rate of 28%. However, in 23% treatment failed and 20% recurrences were found. Two patients showed progression to invasive carcinoma. Two randomised, double blinded, placebo controlled studies (32 and 52 patients) have been described. In the study of Mathiesen *et al.*, 81% of the patients reached complete remission and another 10% a partial remission (from VIN 3 to 1). The patients in the placebo group were offered imiquimod after the study period; another four out of seven had a complete response, two patients had a partial response. All together, 25 out of 31 patients had a complete or partial response.[194] In the study of van Seters *et al.* 81% of patients showed reduction in lesion size, whereas none of the placebo subjects experienced a response. Imiquimod was significantly associated with histological regression and viral clearance. Moreover, relief of itchiness and pain significantly were reduced in comparison with the placebo. Three patients progressed to invasion (<1mm); two of them after placebo, one after imiquimod.[195]

In a study to the T cell responses triggered by imiquimod application, it was demonstrated that these T cell responses were unable to eliminate the virus completely, possibly the reservoir of HPV remaining in the vulvar epithelium is too large, inaccessible or not processed and presented in the appropriate way to the immune system. The magnitude and specificity of the T cell response had no correlation with clinical response to imiquimod.[196]

Follow up of patients treated with imiquimod is still short; long-term effects cannot yet be established. The results of imiquimod are promising but large randomised controlled trials are needed to obtain data on the long term effects. Considering the side effects of imiquimod, its use should be restricted to motivated patients.

4.7.3.3 Interferon

Interferons inhibit replication of viruses, and anti-proliferative effects on non-neoplastic cells have also been described.[197] Interferon is used in rheumatoid arthritis and hepatitis B & C for its antiviral, anti-proliferative and immuno-modulatory activity. In the 1980s, the use of interferon gel in patients with CIN and/or cervical carcinoma was studied. In cervical carcinoma, the tumour regressed to about a third of its original size.[198] In CIN, four out of nine patients treated with interferon vs. seven out of ten patients treated with a placebo showed clinical remissions.[199]

Two studies on the effect of interferon-α in uVIN patients (n=23) have been performed. Nine complete responses, two of which recurred at 12 months, and eight partial responses were noted. There were few side effects associated with treatment in these studies.[197,200] Due to the limited effect of interferon and its high costs, its use in the treatment of uVIN is not widely accepted.

4.7.3.4 Cidofovir

Cidofovir is a deoxycytidine monophosphate analogue, which has potent antiviral activity against a broad range of DNA viruses, including HPV.[201] The drug is approved for treatment of cytomegalovirus retinitis in patients with Acquired Immune-Deficiency Syndrome (AIDS).[201] Its effect in HPV-related disease might result from induction of apoptosis in HPV-infected cells. Cidofovir can reduce E6 and E7 expression and allow accumulation of tumour suppressor proteins p53 and pRb in vitro.[202]

So far, only one study on the use of Cidofovir in uVIN has been reported. Twelve patients with uVIN, ten of which completed follow up, were treated with Cidofovir 1%. Four patients showed complete resolution of all symptoms and visible lesions, and three patients had partial responses. One patient had invasive disease diagnosed in a residual lesion following a partial response to treatment. Local side effects were limited to ulceration of the affected mucosa, with no effect seen on surrounding normal tissue.[203] More studies are needed to investigate whether Cidofovir will have a role in the treatment of uVIN lesions.

4.7.3.5 Therapeutic Vaccination

Recently, two vaccines have been introduced for the prevention of CIN lesions and cervical carcinoma. One vaccine, Gardasil® (Sanofi-Pasteur MSD), acts against HPV 6, 11, 16, and 18 and is approved by the Food and Drugs Administration (FDA). The other one, Cervarix ™ (Glaxo Smith Kline), acts against HPV 16 and 18 and is available from November 1st 2007. Clinical trials have been carried out to find out whether TA/HPV, a recombinant vaccine encoding modified HPV 16 and 18 E6 and E7, could have a role in the treatment of CIN and/or VIN lesions.

Davidson *et al.* compared the immune responses of patients with uVIN lesions to healthy volunteers. In patients with HPV 16 positive uVIN lesions, both antibody- and T-cell responses to the L1 protein were stimulated, but not always sufficient to clear the lesions. E2-specific T-cell responses were rare, and possibly their absence facilitates the persistence of usual VIN lesions.[47] Because of the above-mentioned observations, therapeutic vaccination studies were performed in uVIN

lesions to enhance T-cell mediated immunity in patients with uVIN lesions. Results from those studies show that after various schedules of vaccination, only 1 of 85 patients showed a complete response, 23 of 85 patients had a partial response, and four patients showed progression.[204-206] Viral load diminished post vaccination,[205] but the same HPV subtypes were detected pre- and post-vaccination,[204] and in most patients the grade of uVIN lesion remained unchanged.[204] In a combined analysis of three randomised trials a 49% reduction in lesion size in all high-grade uVIN was seen, irrespective of whether or not HPV DNA was isolated from the lesion.[207] In women positive for HPV DNA, HPV 16/18 vaccination does not accelerate clearance of the virus and should not be used to treat prevalent infection.[208,209] There are currently no therapeutic HPV vaccines that have shown high efficacy in clinical trials.[210]

4.7.3.6 Photodynamic therapy (PDT)

Photodynamic therapy uses the interaction between a tumour-localising photo sensitizer and light of an appropriate wavelength to bring about molecular oxygen-induced cell death.[211] Topical 5-aminolaevulinic acid (ALA) based PDT is particularly attractive because this drug is activated by conversion to protoporphurin IX in rapidly growing cells thus reducing incidental damage to surrounding normal tissues.[212]

The safety and efficacy of photodynamic treatment in uVIN has been evaluated in five studies. A total of 100 patients treated by PDT have been reported so far. A complete response rate of 40% was achieved.[213-217] Patients, who did not reach complete response, did have relief of symptoms.[217] Small unifocal lesions were most responsive to PDT,[215] and multifocal uVIN proved to be difficult to treat.[214,215] uVIN lesions that failed to respond to PDT were more likely to have detectable HPV compared with the responsive uVIN lesions.[213,215] In general, treatment was well tolerated and excellent tissue preservation without scarring was achieved.[217] The optimal light dose has to be determined; better results were achieved when 100J/cm^2 instead of 50J/cm^2 was used.[213,217]

In comparison with surgical techniques, PDT appears to be equally effective to laser vaporisation or local excision.[214] PDT did not induce ulcers or scar formation and the healing time was far below the healing time of lesions treated with laser vaporisation.[214] PDT seems an appropriate treatment for small, unifocal lesions, but these lesions are also easy to remove surgically. The technique is to be promising, but larger studies are needed to confirm its effectiveness in uVIN lesions, to establish the optimal light dose and the effect on HPV.

4.7.3.7 Observation

In case of little or no complaints, some patients may not want to undergo treatment. Considering the relatively low malignant potential, a wait and see policy is an option.[218] Careful observation, at least every three months, is required and greatly helped by photographs. In our clinic, asymptomatic women who deny treatment despite a treatment advice, are seen in our outpatient vulvar clinic every three months. These patients are instructed to contact us when any abnormality occurs and we have a low threshold to perform a biopsy in case of suspected progression.

4.7.3.8 Other

Dinitrochlorobenzene (DNCB) induces a type of delayed hypersensitivity reaction after topical application. DNCB was generally successful in six patients. However, recurrences developed, side effects were serious and histopathology was disturbed by the therapy.[219]

Bleomycin is a topical used chemotherapeutical agent and was used in one study, and yielded a poor response, with five out of twelve patients progressed to invasive carcinoma and only two patients responded.[220]

Indole-3-carbinol is a natural substance derived from the breakdown of glucosinolates, which is present in large concentrations in cruciferous vegetables and has been shown to be anti-carcinogenic and anti-oestrogenic. Its use in the treatment of uVIN has been studied in one uncontrolled randomised phase II trial. It significantly improved symptoms but, of nine of ten patient who agreed to undergo biopsy post treatment, eight biopsies confirmed residual high-grade uVIN.[221]

4.8 Malignant potential

Generally, the malignant potential of uVIN is considered to be low in comparison with dVIN. This opinion is mainly based on the malignant progression in treated patients. An example of vulvar SCC adjacent to untreated uVIN is shown in Figure 7. The rate of invasion after various primary treatments ranges from 1.4% to 20%.[177,180,187,222-228] In the meta-analysis of van Seters *et al.*, 3,3% (108/3322) of patients developed a vulvar SCC during follow up after primary treatment,[181] and in the study of Jones *et al.* 17/405 (4.2%) progression was seen after treatment.[229] The rates of malignant transformation of uVIN are high when compared to the rates found in treated CIN lesions: in four studies, only 31/13969 (0.22%) patients developed invasive cervical SCC after treatment for CIN lesions.[230-233]

The first study on the malignant potential in untreated VIN 3 lesions, showed

malignant progression in 7/8 cases. However, in the above mentioned meta-analysis vulvar SCC developed in 9% (8/88) of untreated uVIN patients.[181] Jones *et al*. described 10/63 (15.8%) women who developed invasive disease before treatment of uVIN.[229] Advanced age, raised lesions, radiotherapy, and an immune compromised state are known risk factors for progression of uVIN.[234] No differences in progression rate could be found between uni- and multifocal lesions.[181]

Two strikingly different patterns of invasive vulvar SCC in women previously treated for uVIN were found. In one group (2.0%), invasion occurred within seven years of treatment (median 2.4 years). Close examination of these cases points to progression of inadequately treated uVIN, as invasion occurred either at the site of previous treatment and/or there was histological evidence of previous positive surgical margins. In the second group (1.8%) invasion occurred many years after treatment of uVIN (median 13.8 years). In all of these cases, the SCC developed at some distance from the previously treated uVIN lesions, thus representing 'de novo' tumours. These late-occurring SCCs appear to represent lesions arising in an HPV induced 'field of risk'.[229]

In contrast to progression to invasive carcinoma, also spontaneous regression of uVIN has been described in 14 women 15-27 years of age. All women had multifocal, pigmented lesions.[235] In the review of van Seters *et al* 1.2% of the patients showed spontaneous regression, all were younger than 35 years. In 17 patients, regression was related to giving birth.[181] An improvement of the immune compromised state, *e.g.*, by refraining from the use of immune suppressants could also lead to spontaneous regression of uVIN. From our own clinical experience, cessation of smoking greatly improves the signs and symptoms of uVIN.

4.9 Recurrence

The relapsing and recurring nature of uVIN is likely to represent an ongoing battle between viral and immune factors in the natural control of this disease.[47] Recurrence rates of uVIN lesions, even after extensive surgical procedures, are common. A number of studies has reported the influence of surgical margins status on recurrence rates. Surgical margins are often positive, irrespective of the type of operation performed.[147] In two large reviews recurrences were significantly lower after free surgical margins than after involved surgical margins.[181,229] Two other studies could not confirm surgical margins as a risk factor for recurrence.[177,236]

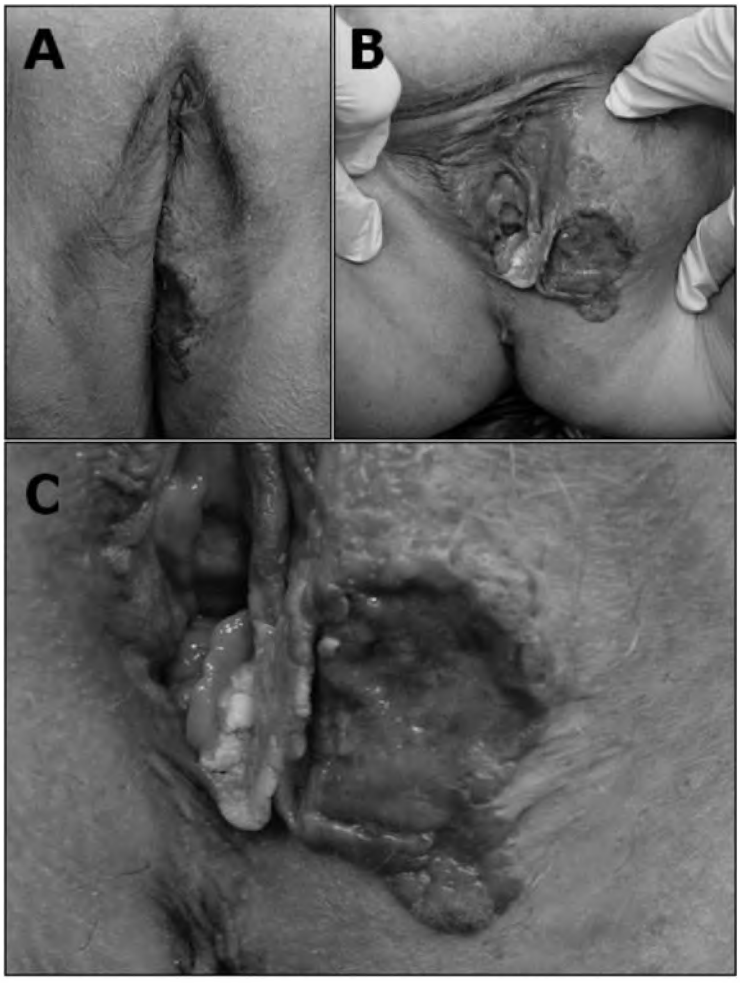

Figure 7 Clinical pictures of usual VIN with adjacent vulvar SCC.
A) The patient had been treated for vulvar itching for months before she visited our outpatient clinic. B) The left labium minus shows white, flat, warty lesions, adjacent to an ulcerative lesion that showed to be vulvar squamous cell carcinoma upon biopsy. C) Because of the size of the tumour (>4cm diameter) the patient was not eligible for the sentinel node study protocol and inguinofemoral lymph node dissection, combined with a wide local excision, was performed.

The presence of positive surgical margins rarely predicts the development of invasive SCC (occurring in 27% of patients with a recurrence and in 24% of patients without recurrence),[226] and therefore it is stated that local excision (instead of vulvectomy) can be performed because of the low risk of developing invasive SCC. Consecutive local resections can better preserve the anatomy and function of the vulva than primary extensive surgery.[179] In the opinion of van Seters *et al.*, the extent of excision should not be enlarged to obtain free surgical margins hoping to diminish the chance of progression to invasive vulvar SCC. Besides, in patients that show progression, mostly the invasion is superficial (<1mm), leaving WLE without inguinofemoral lymph node dissection as a treatment option, with excellent survival rates.[181,218]

Other factors associated with recurrence are HIV positivity,[237] use of immune suppressants and multifocal lesions.[238] p53 gene mutation may play a role in uVIN pathogenesis, independent of high-risk HPV infection and may predict recurrence or progression to vulvar SCC.[222] Irrespective of margin status, the majority of recurrences were observed within the first three years of follow-up, emphasising the fact that careful follow-up of these women is essential.[177]

Concerning symptoms of uVIN, only patients with unifocal uVIN lesions benefited from the extensive removal of affected skin, while symptoms in other extensively operated patients recurred. In contrast, 75% of the patients who underwent restricted surgery benefited from their therapy, even after long-term follow up. The recurrences of symptoms in extensively operated patients were not always due to uVIN or scar forming, but were also caused by the psychological and sexual distress of the surgery.[218]

4.10 Prevention

Prevention would be the best 'treatment' for uVIN lesions. Prophylactic vaccination of healthy individuals against the aetiological agent protects against acquisition of the disease, but confers some risk to otherwise healthy women and requires massive programmes to vaccinate a significant fraction of the population.[210] With the aim to prevent cervical carcinoma, recently two prophylactic vaccines have been introduced to prevent infections with high-risk HPV subtypes (HPV 16 and 18). The main public health goal of the introduction of an HPV vaccine is to reduce the incidence of cervical carcinoma and its precursor lesions. The secondary goal is reduction of the incidence of other HPV-associated cancers and benign and premalignant conditions caused by HPV.[239] The studies on HPV-vaccines have demonstrated that prevention of HPV-infection and CIN is

successful, with persistent effects up to five and a half years,[240] with sustained antibody levels through five and a half years.[241] The question rises whether these vaccines have a role in the prevention of uVIN and vulvar SCC. Preliminary results show a promising efficacy; Paavonen *et al.* state that the quadrivalent HPV vaccine (Gardasil®) prevents HPV 16 and 18- related vaginal intraepithelial neoplasia (VAIN) and uVIN for at least two years post-immunisation.[242] In a combined analysis of three randomised clinical trials with a mean follow up of three years, the vaccine was 97% effective in preventing uVIN associated with HPV16 and 18 in a population that was naive to these viruses at the time of first vaccination, and 100% effective in a population that was naive throughout completion of the vaccination regimen. Vaccine efficacy in the intention-to-treat population, which included women who could already have acquired HPV 16 or 18 infection and those with vulvar HPV-related disease prior to vaccination, was 71%.[207]

Approximately 30% of all vulvar SCCs and up to 94% of uVIN lesions are caused by infection with HPV. The majority of the HPV positive tumours contain HPV 16 and/ or 18. Although the incidence of vulvar SCC in younger women is increasing, it remains a rare neoplasm and uVIN lesions have a low progression rate into invasive carcinoma after treatment.[181] When the promising results for CIN are also true for uVIN, a very large part of the HPV induced VIN lesions and SCCs might be prevented by vaccination against HPV 16 and/or 18. It is important to vaccinate girls before infection with HPV occurs, so before their first sexual intercourse.

In several countries the costs for the vaccines are covered by the health care insurances and in other countries mass vaccination already started, *e.g.*, in Germany and Austria. In the Netherlands the government will soon make a decision whether the vaccination will be incorporated in the vaccination programme or will be covered by the health care insurances.

5. Conclusion

In the absence of HPV, vulvar SCC can develop in a background of LS, dVIN or both. A variety of medical treatments for LS exist, with dermatocorticosteroids as the treatment option of first choice. It is unknown whether the medical treatment of LS decreases the risk of vulvar SCC development. Patients with LS should be followed at least once a year. In case of difficulty with symptom control, clinical evidence of localised skin thickening or uncertainty of the diagnosis (when dVIN

cannot be excluded), follow-up should take place in a specialised clinic. In contrast with the non-surgical approach to LS, the treatment of dVIN, which is often present in a background of LS, should be surgical to prevent progression to invasive vulvar SCC.

uVIN is an entity with a growing interest because of the increasing incidence, recurrent character and the young age of women with this condition. uVIN is associated with HPV infection in about 90%; mainly HPV 16 and 18 are involved. The therapy used to be a radical vulvectomy, but nowadays the extent of the surgical procedure has been reduced to a local excision of the lesions. Moreover, to further limit the role of surgery, several medical, mostly antiviral, agents have been proposed. Some of these agents, such as imiquimod, show promising results, but randomised controlled trials are needed to confirm the often-preliminary findings. The first results of HPV vaccination-studies in HPV naive women are promising. Vaccination can potentially prevent around one third of all vulvar SCC and the majority of uVIN lesions.

Considering the burden of disease, risk of malignancy, and long lasting need for treatment and follow-up, vulvar premalignancies deserve attention in training programmes, clinical studies and patient care.

Chapter 3

The etiologic role of HPV in vulvar squamous cell carcinoma fine tuned

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Abstract

Purpose: High risk (hr) human papillomavirus (HPV) plays a role in the development of a subset of vulvar squamous cell carcinomas (SCC). Uncertainty exists about the true impact of HPV in this tumour type as conflicting reports have been published with diverging prevalence rates. This study was performed to fine tune the role of hrHPV infection in vulvar SCC development in relation to clinical prognosis.

Experimental design: 130 vulvar SCCs of patients with known survival data were analysed for histology of the adjacent lesion (differentiated (d) or HPV-associated usual (u) vulvar intraepithelial neoplasia (VIN)), in relation to $p16^{INK4A}$ expression as marker of HPV activity, and presence and integration of hrHPV-DNA.

Results: uVIN was present adjacent to vulvar SCC in 25/130 cases. uVIN-associated SCCs had high $p16^{INK4A}$ expression and 24/25 SCCs contained integrated hrHPV DNA. dVIN was found adjacent to 105/130 vulvar SCCs. hrHPV was detected in 11 (10.5%) dVIN-associated vulvar SCC, but correlated with high $p16^{INK4A}$ expression in only one case. Integration of viral DNA was never observed in dVIN associated SCCs which suggests that a causal relationship of hrHPV in dVIN-associated tumours is highly unlikely. The Disease Specific Survival (DSS) of the dVIN-associated vulvar SCC patients was significantly worse compared to patients with a uVIN-associated tumour.

Conclusions: hrHPV is causally associated with the development of uVIN-associated SCCs, which comprise 19% of all vulvar SCCs, but not with dVIN-associated vulvar SCCs. dVIN-associated vulvar SCCs have a significantly worse prognosis.

Introduction

The association between Human Papilloma Virus (HPV) and vulvar squamous cell carcinoma (SCC) oncogenesis is less straightforward than for cervical carcinomas, which are causally associated with HPV in nearly 100% of the cases.[243] In previous work, we have demonstrated that vulvar SCCs may develop via two different pathways, with their own specific premalignant lesions.[1,2] The first and most common pathway occurs in elderly women and mainly leads to keratinising SCC, often in a background of lichen sclerosis (LS) and differentiated vulvar intraepithelial neoplasia (dVIN). The second pathway of vulvar SCC occurs in younger women. This type of carcinoma is associated with warty or basaloid VIN, currently referred to as usual VIN (uVIN).

HPV has been found in a subset of vulvar SCCs, suggesting a causal relationship. The exact impact of HPV in this tumour type is uncertain as diverging prevalence rates have been published. Sixteen studies comprising 1181 vulvar SCC patients demonstrated on average 35% HPV positivity, and 25% positivity for HPV 16 and/or 18.[2,35,46,50,169,171,244-253] In the United States, a higher percentage HPV positivity was found (63.2%) than in European countries (34.7%).[254] However, HPV-DNA presence alone does not *per se* indicate viral involvement in the carcinogenic process.[255] Infection with HPV is an early event in the multi-step process of vulvar carcinogenesis and HPV integration into host cell genome seems to be related to the progression of vulvar dysplasia.[167] Viral integration generally disrupts the E2 region, resulting in enhanced expression of E6 and E7. E6 and E7 have the ability to bind and inactivate p53 and pRb, which promotes rapid progression through the cell cycle without a p53-mediated control of DNA integrity. High $p16^{INK4A}$ expression is caused by the functional inactivation of pRb and may be used as a readout for active HPV.[256,257]

With the aim to fine tune the etiologic role of HPV in vulvar SCC oncogenesis we used the histology of the adjacent VIN lesion in relation to $p16^{INK4A}$ expression and HPV integration. As a consequence, the number of vulvar SCCs that may be prevented by the current HPV vaccines can be indicated more accurately and it can be determined whether the two different pathways of vulvar SCC lead to different survival patterns.

Methods

Patients

Between 1988 and 2005, 167 patients were treated with a curative intent for primary vulvar SCC at Radboud University Nijmegen Medical Centre, the Netherlands. Tissue samples of the primary tumours of all patients were available for assessment of the adjacent VIN lesion (i.e,. into uVIN, which is >90% HPV related and dVIN, which is not HPV related[258]), $p16^{INK4A}$ staining as molecular marker for HPV activity,[249] the presence of HPV by short fragment PCR and DNA In Situ Hybridisation (ISH) to demonstrate the physical status of HPV as HPV integration is a key event in carcinogenesis.[259]

In 28 specimens, amplification of the housekeeping β-globin gene by PCR failed, and these were not included in the study. Five tumours did not have sufficient material for immunohistochemical analysis and in four tumours we were not able to classify the adjacent VIN lesion, due to a massive inflammatory response or ulceration. A total number of 130 patients were available for the study. Clinico-histopathological characteristics and follow-up data were obtained from medical charts (Last date of follow-up: August 1st 2008).

Histology of the adjacent VIN lesion

Based on the specific criteria established for the histopathological characteristics, premalignant lesions flanking vulvar SCC were classified as dVIN, with or without the presence of LS, and uVIN.[45,260] All slides were re-evaluated by an expert gynaecopathologist (JB).

$p16^{INK4A}$ expression

Tissue sections (4μm) of the archival paraffin-embedded tissue samples adjacent to that used for H&E slides to assess the adjacent VIN lesion were mounted onto SuperFrost glass slides (Menzel-Gläser, Braunschweich, Germany) and dried overnight at 37°C. The sections were dewaxed in xylene and rehydrated through graded alcohols. Endogenous peroxidase was blocked with 3% hydrogenperoxide in phosphate-buffered saline (PBS; pH 7.4) for 30 minutes. Slides were rinsed three times in PBS for 5 minutes, and antigen retrieval performed with boiling citrate buffer (0.01 M; pH 6.0) for 10 minutes. After cooling down to room temperature (RT), slides were briefly washed in PBS for 10 minutes. Subsequently, the slides were preincubated with 20% normal horse serum and then incubated with the primary antibody $p16^{INK4A}$ (clone JC-8, Immunologic, Duiven, the Netherlands),

diluted 1:200 in PBS with 1% bovine serum albumine (BSA) (overnight). Slides were rinsed in PBS (10 minutes) and postantibody blocking was performed for 30 minutes (horse-anti mouse IgG). Staining was developed with diaminobenzidine/ hydrogenperoxide for 5 minutes, intensified with copper sulphate and counter-stained with Mayer's haematoxylin. The slides were dehydrated through graded alcohols and xylene, and finally mounted with Permount mounting medium (Fisher Chemicals, NJ, USA). Negative controls (buffer only) and HPV-positive CIN 3 lesions served as $p16^{INK4A}$ positive controls in each run.

Interpretation of $p16^{INK4A}$: nuclear and cytoplasmic $p16^{INK4A}$ staining were both considered as a positive reaction. The results were reported in a semi quantitative fashion: *negative* (-) if <5% of the cells had nuclear or cytoplasmic staining, *slightly positive* (1+) if 5 to 25% of the cells were stained, *moderately positive* (2+) if staining was present in 25 to 75% of the cells, and *markedly positive* (3+) if >75% of the cells showed nuclear or cytoplasmic staining.[247,249]

HPV presence and genotyping

DNA was isolated from formalin fixed paraffin-embedded tissue sections (6µm) with the EZ1 robot (with the DNA tissue kit of Qiagen) according to standard procedures[261] and used for PCR analysis. A negative water control was included with each batch of 10 samples. Broad-spectrum HPV DNA amplification was performed using a short PCR fragment (SPF-PCR) assay. The SPF-PCR system amplifies a 65 bp fragment of the L1 open reading frame, allowing the detection of at least 43 HPV types. Subsequent HPV genotyping was performed via a reverse hybridisation line probe assay (LiPA), allowing simultaneous typing of the 25 HPV-genotypes. The combined SPF-PCR-LiPA system for detection and genotyping of HPV has been described in detail elsewhere.[261]

Physical status of HPV by In Situ Hybridisation(ISH)

The ISH procedure for HPV16/18 and HPV31/33 on 3 µm thick paraffin sections was performed as described previously.[262,263] In brief, tissue sections were pretreated with proteolytic reagents, hybridised, and the biotinylated DNA-DNA hybrids were immunohistochemically detected by the peroxidase-labelled avidin-biotin-complex (ABC-PO). For detection of HPV16/18/31/33 DNA sequences, ISH was followed by an immunohistochemical detection method utilizing catalysed reporter deposition (CARD) signal amplification, which enables the detection of low copy numbers. Only a punctate signal in the nuclei was considered to represent viral integration.[264,265]

Statistical methods

Clinical data were entered in a computerised database and analysed using SPSS software (version 16.0.1 for Windows, SPSS). Disease Specific Survival (DSS) was defined as the time from the date of primary treatment to the date of death due to vulvar SCC or last date of follow up. Differences in DSS between the tumours were investigated using Kaplan-Meier statistical method. χ^2 and Student's t test were used to calculate differences in clinicohistopathological characteristics. For all tests a significance level of $p<0,05$ was chosen.

Results

Histopathological review of vulvar SCC slides showed 25 (19.2%) cases of uVIN adjacent to vulvar SCC, and 105 cases (80.8%) of dVIN (of which in combination with LS in 59 cases) (See Figure 1a and 1d for representative H&E slides of uVIN and dVIN). LS as a solitary adjacent lesion was present in three cases and was considered to belong to the dVIN-associated type.[28-30,37]

To investigate whether the tumour tissues displayed HPV activity, we performed $p16^{INK4A}$ staining (Figure 1b-c and 1e-f). Ninety-one tumours (70%) showed a $p16^{INK4A}$ expression pattern in which less than 5% of the tumour cells displayed cytoplasmic, and/or nuclear $p16^{INK4A}$ positivity. In four tumours 5-25% positive cells were observed. 25-75% and more than 75% positive cells were observed in nine and 26 tumours, respectively and were considered a true positive result. All 25 uVIN-associated tumours displayed high $p16^{INK4A}$ expression (more than 25% positive tumour cells), compared to only 10/105 (9.5%) dVIN-associated tumours (Figure 2).

All vulvar SCCs were analysed for presence of HPV DNA. Forty-five of the 130 tumours (34.6%) tested positive for HPV, of which 33 (25.4%) for hrHPV types. Of the hrHPV types, HPV 16 was most common and present in 18 of the 33 tumours (54.5%) as the only HPV type, and in two tumours (6%) a combination with HPV 16 and another HPV type was found. HPV 33 was the second most common type and detected in seven of the 33 HPV-positive tumours (21.2%). HPV 18 was present in two tumours as the solitary subtype, and in combination with HPV 16 and 54 in one additional tumour (Table 1).

In 24 out of 25 (96%) uVIN-associated tumours hrHPV DNA was detected, whereas 11 (10.5%) dVIN-associated tumours were found positive for hrHPV DNA (Figure 2).

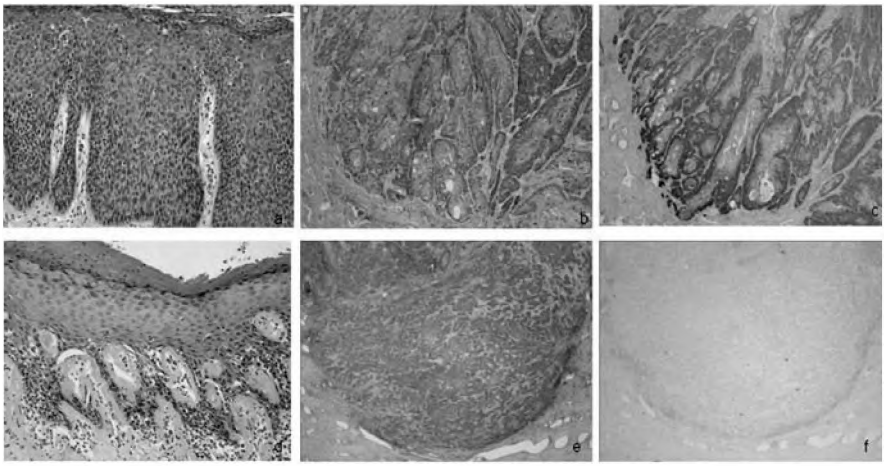

Figure 1 Examples of VIN lesions adjacent to vulvar squamous cell carcinomas (SCC), SCCs associated with both types of VIN and its $p16^{INK4A}$ expression.
Subdivision of vulvar SCCs was among other things based on the histology of the adjacent VIN lesion and $p16^{INK4A}$ staining. uVIN (a) associated tumours (b) showed >25% $p16^{INK4A}$ expression in all cases(c). dVIN (d) associated tumours (e) displayed ≤25% $p16^{INK4A}$ expression (f) in 90.5% of the SCCs.

However, of those 11 dVIN associated hrHPV positive tumours only one single tumour displayed high $p16^{INK4A}$ expression. The remaining 10 dVIN hrHPV positive samples were all $p16^{INK4A}$ negative, suggesting that the HPV found in these dVIN-associated tumours was not active. As $p16^{INK4A}$ expression is linked to HPV activity the physical status of hrHPV DNA by ISH for HPV types 16, 18, and 33 was demonstrated. Twenty-three uVIN-associated SCCs were positive for HPV 16, 18, or 33. One tumour was positive for HPV 58. All 23 HPV 16, 18, and 33 positive uVIN-associated SCCs were analysed, together with all dVIN-associated SCCs that tested positive for HPV DNA and/or displayed high $p16^{INK4A}$ expression. Integration was observed in all 23 HPV 16-, 18-, or 33 positive uVIN-associated SCCs (See Figure 3a for a representative slide of a tumour positive for HPV 31/33 by ISH).

Of these, 17 were positive for HPV 16 and/or 18. However, none of the $p16^{INK4A}$ expressing and/or HPV 16-, 18-, or 33 DNA containing dVIN-associated vulvar SCCs displayed HPV integration (See Figure 2 & 3b for a representative slide of a tumour negative for HPV 16/18 by ISH), suggesting a non significant clinical presence of hrHPV in dVIN-associated SCCs.

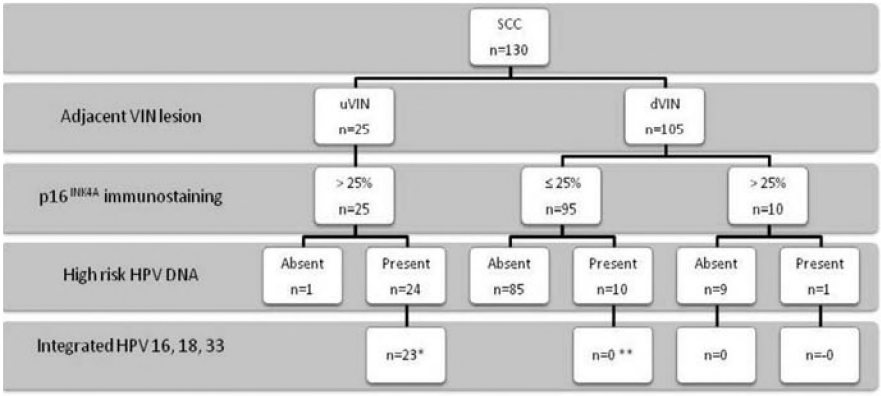

Figure 2 Schematic overview of histology of the adjacent VIN lesion, $p16^{INK4A}$ expression, hrHPV presence and integration.
A causal relation between hrHPV and vulvar SCC is only plausible for uVIN-associated SCCs. In dVIN-associated vulvar SCCs positive for hrHPV or with high $p16^{INK4A}$ expression, this was not accompanied by HPV integration.
* Of the 24 uVIN-associated SCCs, 23 were positive for 16, 18, or 33 and available for integration analysis.
** Of the ten HPV-positive dVIN-associated SCCs with low $p16^{INK4A}$ expression, six were positive for HPV 16, 18, or 33 and available for integration analysis.

	Number	Percentage
HPV negative	85	65.4
Low risk HPV types	12	9.2
54	1	91.7
X	11	8.3
High risk HPV types	33	25.4
16	18	54.4
16, 18, 54	1	3
16, 58	1	3
18	2	6.1
33	7	21.2
52	1	3
52, 66	1	3
53	1	3
58	1	3

Table 1 Overview of HPV types in 130 vulvar SCCs.

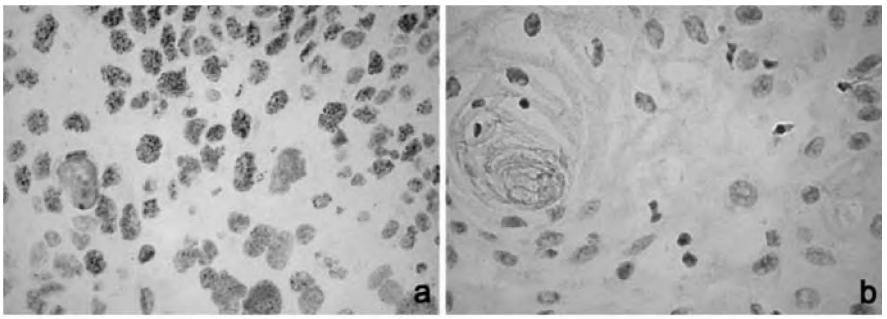

Figure 3 Examples of tumour cells positive or negative for ISH for HPV types 16/18 and 31/33.
Integration analysis was performed to fine tune the etiologic role of HPV in vulvar SCCs. All uVIN-associated SCCs showed HPV in an integrated form (a, positive for HPV 31/33), whereas none of the HPV positive or high $p16^{INK4A}$ expressing dVIN-associated SCCs had HPV integrated (b).

With the aim to find out whether there are differences in disease specific survival (DSS) between the two types of vulvar SCCs, the DSS of all vulvar SCC patients and subdivided between uVIN and dVIN associated vulvar SCCs was determined. In addition, DSS for vulvar SCCs subdivided between hrHPV positive and negative was established. For all vulvar SCC patients, five year DSS rate was 77% (Figure 4a). A subdivision between u- and dVIN showed a five year DSS rate of 70% for dVIN-associated SCC patients. Survival for uVIN-associated SCC patients was significantly better with a five year DSS rate of 100% (log rank p=0.012) (Figure 4b). A subdivision between hrHPV positive or negative did not result in a different DSS (log rank p=0.646) (Figure 4c). Stratification for uVIN, HPV negative dVIN, and HPV positive dVIN revealed an unexpected worse survival of the latter (Figure 4d). Similarly, stratification for HPV positive/$p16^{INK4A}$ positive (24 uVIN and one dVIN), HPV positive/$p16^{INK4A}$ negative (10 dVIN) and HPV negative (94 dVIN and one uVIN) vulvar SCCs demonstrated reduced DSS for the HPV positive/$p16^{INK4A}$ negative subset (Figure 4d insert). As such, the lack of a difference in DSS between HPV positive and HPV negative tumours (Figure 4c) can be explained, but why these HPV positive/$p16^{INK4A}$ negative dVIN-associated SCC carry a poor DSS remains elusive.

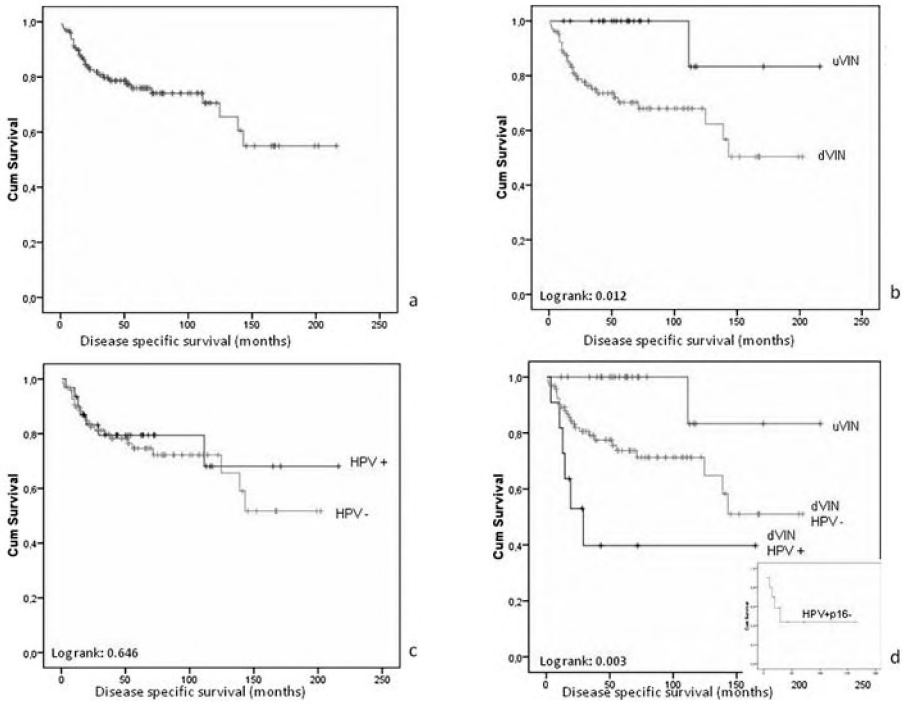

Figure 4 Disease specific survival curves.

Five year Disease Specific Survival (DSS) for all vulvar SCC patients is 77% (a). Subdivision between the vulvar SCCs based on histology of adjacent VIN lesion, demonstrated a better DSS for uVIN-associated tumours than dVIN-associated tumours (p=0.012) (b). Five year DSS for uVIN-associated tumours is 100%, whereas DSS for dVIN-associated tumours is 70%. Based on hrHPV positivity, no significant differences could be demonstrated (p=0.646) (c). This was mainly due to the attribution of hrHPV positive dVIN-associated SCCs (Figure 4d), or HPV positive, $p16^{INK4A}$ negative (insert) with a worse DSS. When all uVIN-associated tumours may be prevented by vaccines in the future, the disease specific survival of vulvar SCC patients will slightly decrease from 77% to 70% (a and b).

Discussion

HPV detection by PCR alone results in an overestimation of the percentage vulvar SCCs that are causally associated with HPV. We demonstrated the presence of hrHPV in 26.9% of the vulvar SCCs, but our more detailed analysis demonstrated a plausible causal relationship between hrHPV and vulvar SCC carcinogenesis only in uVIN-associated vulvar SCCs (19.2%). In dVIN-associated SCCs the detected HPV was not found integrated or activated and thus was not considered to be causally involved. Thirteen percent of the vulvar SCCs were considered to be caused by HPV 16 and/or 18. In addition, the uVIN-associated vulvar SCCs had a better DSS than dVIN-associated vulvar SCCs.

To assess the extent of the aetiological role of HPV in the carcinogenic process, we established histology of adjacent VIN lesion, $p16^{INK4A}$ overexpression, and determination of HPV integration status. Since dVIN is not associated with HPV,[258] it is unlikely that the tumour directly adjacent to this dVIN lesion is caused by HPV. In our study, a subdivision based on adjacent VIN lesion resulted in two clearly perceptible groups of vulvar SCCs (Figure 2). High $p16^{INK4A}$ expression was observed in 9.5% dVIN associated SCCs and suggested active HPV involvement but only in one tumour the high $p16^{INK4A}$ expression was accompanied by hrHPV presence. However, although $p16^{INK4A}$ overexpression is strongly related to active HPV infection as a result of functional inactivation of pRb, it may also occur as an endogenous response towards abnormal cell proliferation due to an eroded checkpoint function.[247,249] $p16^{INK4A}$ thus is an indicator that a carcinogenic cascade has commenced but may also be expressed in HPV-independent tumours where its overexpression may counteract signals that abnormally drive cell proliferation, a checkpoint function that is eroded in premalignant and tumour cells.[266] Furthermore, the observation that integration of HPV was not observed in HPV-positive dVIN-associated tumours supports our suggestion that HPV did not contribute to the development of this tumour type. However, we cannot rule out that that episomal HPV does not have an oncogenic potential. Viral integration is not a prerequisite for oncogenic activity, as a proportion of HPV-16-positive cervical tumours contains episomal HPV DNA either alone or in coexistence with integrated HPV sequences.[267,268] In contrast with dVIN-associated tumours, uVIN-associated tumours were causally associated with hrHPV; all expressed high $p16^{INK4A}$ expression and the HPV types found were integrated in the genome.

The analysis of the presence and integration of hrHPV, expression of $p16^{INK4A}$, and histology of the adjacent lesion indicated that it is very unlikely that hrHPV is

causally related to the development of dVIN-associated vulvar SCCs. When performing a survival analysis for dVIN and uVIN-associated SCC, we found a significantly worse DSS for dVIN (Figure 4b). Unexpectedly, we found reduced DSS for HPV positive dVIN compared to HPV-dVIN-associated vulvar SCCs (Figure 4d). We cannot provide an explanation why presence of HPV, that most likely did not induce the dVIN-associated vulvar SCC, is associated with poor DSS. The low sample number of HPV positive dVIN (n=11) compared to HPV negative dVIN (n=94) may have influenced this finding. Further retrospective studies using samples from other centres are needed to determine whether the poor DSS of HPV positive dVIN is actually a real finding or a chance occurrence. Stratification based on $p16^{INK4A}$ expression did not result in a different allocation of samples in the three subsets because the sample set contains only one HPV positive/$p16^{INK4A}$ positive dVIN and one HPV negative uVIN-associated vulvar SCC and therefore DSS curves were highly comparable to uVIN, HPV positive dVIN and HPV negative dVIN.

In this study we demonstrate that with HPV DNA detection alone the number of vulvar SCCs with a clinically relevant infection is overestimated. This has implications for the protective role of HPV vaccines for vulvar SCCs. There is increasing evidence that the recently introduced vaccines against HPV 16 & 18 will significantly reduce the incidence of cervical carcinoma and its premalignancies.[242,269] This has led to the implication of HPV vaccination in nationwide vaccine programmes in most European countries. Although most studies have focused on the impact these vaccines will have on cervical cancer, limited research has been performed on how the vaccines may affect vulvar SCC incidence. Still, in September 2008, the Food and Drug Administration (FDA) approved one vaccine (Gardasil®) to be used to prevent some cancers of the vulva and vagina.[270] In our detailed analysis it appears that only uVIN associated (25/130) vulvar SCCs are causally associated with hrHPV. Of those uVIN associated vulvar SCCs, 17 (13% of all vulvar SCCs) were HPV 16/18 positive and will possibly be prevented by the current HPV vaccines. We demonstrate that the uVIN-associated vulvar SCC have a more favourable prognosis compared to dVIN-associated vulvar SCCs. Vaccination may therefore prevent a small subset of vulvar SCC with relatively good prognosis, without broadly affecting vulvar SCC incidence and survival rates. Currently, HPV vaccines covering more hrHPV types are under development, and most, if not all, uVIN-associated carcinomas may be prevented by these vaccines in the future. As this strategy eliminates the tumours with a relatively good prognosis, five year survival for vulvar SCC patients could slightly decrease from 77% in the pre-

vaccination era to 70% after the possible prevention of all uVIN-associated vulvar SCCs (Figure 4 a-b).

In conclusion, hrHPV is causally associated with the development of uVIN-associated vulvar SCCs. Although hrHPV was found in 10.5% of dVIN-associated vulvar SCCs, it does not seem to be causally associated with tumour development. Integration of HPV 16 or 18 was observed in 17 uVIN-associated vulvar SCCs (13%), which may be prevented by HPV vaccines in the future. This is lower than previously supposed and leaves the majority of the vulvar SCCs with worst DSS unsolved.

Chapter 4

Vulvar squamous cell carcinoma development after diagnosis of VIN increases with age

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Abstract

Objective: The purpose of the present study is to investigate the trends in incidence of both usual (u) and differentiated (d) vulvar intraepithelial neoplasia (VIN) separately, their malignant potential and the relation with other HPV related anogenital lesions in the Netherlands during a 14 year period.

Methods: The incidences of both types of VIN and vulvar SCC were retrieved from the Nationwide Netherlands Database of Histo- and Cytopathology. Population data were retrieved from the Database of Statistics Netherlands.

Results and Conclusions: In the study period the incidence of uVIN and dVIN increased, while the incidence of vulvar SCC remained stable. The overall percentage of uVIN patients that were later diagnosed with vulvar SCC was 5.7%, which was significantly lower than the percentage for dVIN patients (32.8%). In addition to this 5.6 fold increased conversion rate, the time of progression from dVIN to SCC development was significantly shorter than that of uVIN (p=0.005). Percentage of uVIN patients that were later diagnosed with SCC significantly increased with age (p=0.005) whereas the time to SCC significantly shortened with age (p=0.05). Forty-one percent of uVIN patients had a past, concomitant or future HPV associated lesion of the lower genital tract, which is in contrast to the 3% for dVIN patients. In short, an increase in diagnoses of both uVIN and dVIN has not led to an increase in vulvar SCC incidence. The malignant potential of dVIN is higher than for uVIN. For uVIN the malignant potential increases with age.

Introduction

Vulvar squamous cell carcinoma (SCC) is the fourth most common gynaecological type of cancer with an annual incidence of 2-3 per 100.000 women.[44,271] Vulvar SCC usually arises from premalignant vulvar intraepithelial neoplasia (VIN). In general, there are two different types of VIN, *i.e.,* differentiated VIN (dVIN) and usual VIN (uVIN) that both can progress towards vulvar SCC.[2,3] Whereas uVIN is causally related with human papillomavirus (HPV) infection, dVIN is HPV unrelated.[2]

dVIN is a recently recognised, but difficult to diagnose entity for clinicians as well as pathologists.[3,45] dVIN is found adjacent to SCC in 70-80% of vulvar SCCs, but solitary dVIN lesions are rare. This may be caused by a relatively brief intraepithelial phase before progression to invasive carcinoma,[5,154,156] and suggests a high malignant potential of dVIN. dVIN is often seen in a background of lichen sclerosuis (LS) and occurs in elderly women (mean age 65).[272] The aetiology of SCC via LS and dVIN is largely unknown. The HPV-associated (mainly HPV 16 and 18) uVIN mainly leads to non-keratinising vulvar SCC and it primarily affects younger women around the age of 45.[51] uVIN lesions are seen adjacent to approximately 20-30% of the vulvar SCCs. Multicentric intraepithelial or invasive squamous neoplasia (of cervix, anus or vagina) occur in approximately 53-66% of uVIN patients.[50,51] Most of these lesions are cervical (intraepithelial) neoplasias, as in most countries, as well as in the Netherlands, well-organised mass screening programmes exist for the prevention of cervical carcinoma. In contrast to the presumed high malignant potential of dVIN, SCC percentages of 3- 5% were found in patients treated for uVIN.[51,161,181,229]

The incidence of uVIN is increasing worldwide,[52,55,161,162,181,271,273] which is in concordance with the increase in HPV prevalence. Despite the increase in uVIN, no increase in the overall incidence of SCC of the vulva has been seen.[52,53,271] In some publications, an increase in SCC incidence in young women was reported, possibly due to the younger age of first sexual intercourse and the increasing incidence of HPV infections.[54-57] Since the introduction of the new nomenclature, the incidence of uVIN and dVIN lesions has not been studied separately. The aim of the present study is to investigate the trends in incidence of both types of VIN, their malignant potential and the relation with other HPV related anogenital lesions in the Netherlands during a 14 year period.

Materials and Methods

Data concerning the incidence of VIN and vulvar SCC were collected via PALGA, the Nationwide Netherlands Database of Histo- and Cytopathology,[274] which has national coverage from 1991 onwards. Analogous with other studies concerning the incidence of cancer and using cancer registry data,[275-277] we calculated the incidence of VIN and vulvar SCC.

VIN

All patients with a primary VIN lesion diagnosed between January 1st, 1992 and December 31st, 2005 in the Netherlands were selected and a total number of 2935 patients were retrieved. We excluded patients with a first diagnosis of VIN diagnosed before 1992 with a recurrent lesion between 1992 and 2005. For all patients, the first report that mentioned VIN or vulvar SCC was included. For every pathology-report, all VIN lesions were subdivided into u- or dVIN. We based this subdivision on the conclusion drawn by the pathologist and additional indications mentioned in the reports (Figure 1). In the analyses we adopted the new International Society for Study of Vulvovaginal Disease (ISSVD) classification[3] (we did not include VIN 1 lesions, and grouped VIN 2 and 3 together as uVIN). We only included solitary VIN lesions, (*i.e.,* not adjacent to vulvar SCC). VIN lesions that progressed to vulvar SCC within three months were excluded, as these were considered 'missed invasion'. A total number of 1893 patients was available for analyses, including 1826 uVIN patients and 67 dVIN patients.

For each patient with VIN, prior and subsequent pathology reports of vulvar biopsies or excisions were available and when a vulvar SCC occurred more than three months after the first histological diagnosis of VIN, it was scored as malignant progression. In addition, the pathology reports contained all histological reports of biopsies or excisions of the female lower anogenital tract.

Vulvar SCC

The PALGA database revealed 4648 patients with vulvar cancer between January 1st, 1992 and December 31st, 2005 in the Netherlands. We excluded patients with a non-squamous vulvar carcinoma and patients with a first diagnosis of vulvar SCC diagnosed before 1992 with a recurrent lesion between 1992 and 2005. For all patients, the first report that mentioned vulvar SCC was included. The total number of primary vulvar SCC patients was 2701.

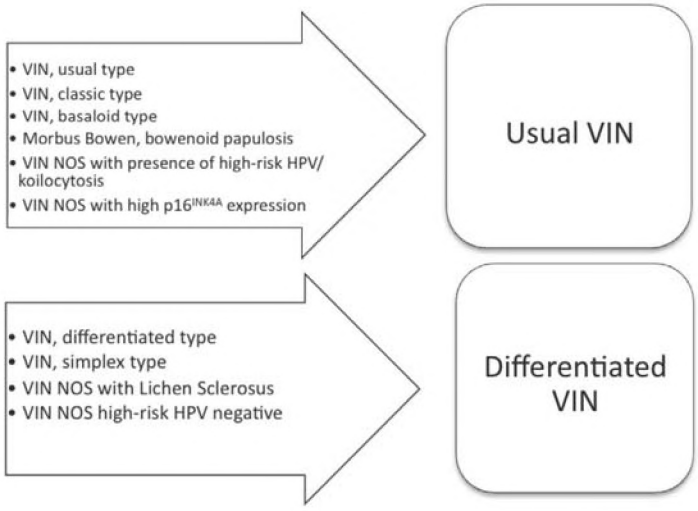

Figure 1 Histological diagnoses that support the diagnosis of usual or differentiated VIN. VIN NOS: VIN not otherwise specified.

Population data

Population data in order to calculate European standardised rates of the Netherlands were obtained from the Database of Statistics Netherlands (http://www.cbs.nl/).

Statistical analysis

Incidences of both types of VIN and vulvar SCC were calculated per 100.000 person years (European Standardised Rate, ESR). Age was categorised into 15-year strata (15-29, 30-44, 45-59, 60-74, over 75 years of age). Differences between the incidences over time were calculated using the univariate general linear model. The Student's t test was used to compare the mean ages and times to SCC development. A significance level of $p<0.05$ was chosen.

Results

In the study period, 2701 patients were identified with vulvar SCC, 1826 patients with solitary uVIN and 67 patients with solitary dVIN. The median age of patients with uVIN (47.8 years) and dVIN (67.0 years) differed significantly ($p<0.001$). The median age at diagnosis of vulvar SCC patients was 70.4 years, which is significantly higher compared to uVIN, but comparable with dVIN.
In order to determine trends in incidence of vulvar SCC, uVIN and dVIN, their incidences over the 14 year study period were calculated (Figure 2). The incidence of uVIN almost doubled from 1.2/100.000 patients in 1992 to 2.1/100.000 patients in 2005 ($p<0.001$). The incidence of dVIN increased nine-fold from 0.013/100.000 patients to 0.121/100.000 patients ($p=0.019$). In contrast, the incidence of vulvar SCC remained unchanged from 2.6/100.000 patients in 1992 to 2.5/100.000 patients in 2005 ($p=0.670$). To determine the age with highest incidence rate of vulvar SCC, uVIN and dVIN, the age specific incidences were calculated (Figure 3). A gradual and strong increase in vulvar SCC incidence was observed starting at the age of 60. uVIN showed a bimodal peak incidence at the ages of 40-44 and at 75-79. dVIN has the highest prevalence at the age of 75-79. Of all premalignant lesions, uVIN was diagnosed in 98.9% of all VIN lesions in 1992, which decreased to 94.5% in 2005, despite the overall increase in uVIN incidence (Figure 2). Conversely, the diagnosis of dVIN increased in this time period to 5.5% of all VIN lesions in 2005, with a significant increase in especially the last three years of the study period.

With the objective to study possible differences in the incidence of malignant progression from VIN to vulvar SCC the incidence of SCC more than three months after the initial diagnosis of VIN was determined. The overall percentage of uVIN patients subsequently diagnosed with vulvar SCC was 5.7% (104 patients). The risk of a subsequent diagnosis of SCC significantly increased with the age of diagnosis of uVIN, beginning with 2.7% for the age group 15-29 and increasing to 8.5% for the group >75 years of age ($p=0.005$) (Figure 3). In addition, the time between diagnosis of uVIN and SCC significantly shortened with increasing age; 50 months for the 15-29 age group and 25 months for the >75 age group (Figure 4).

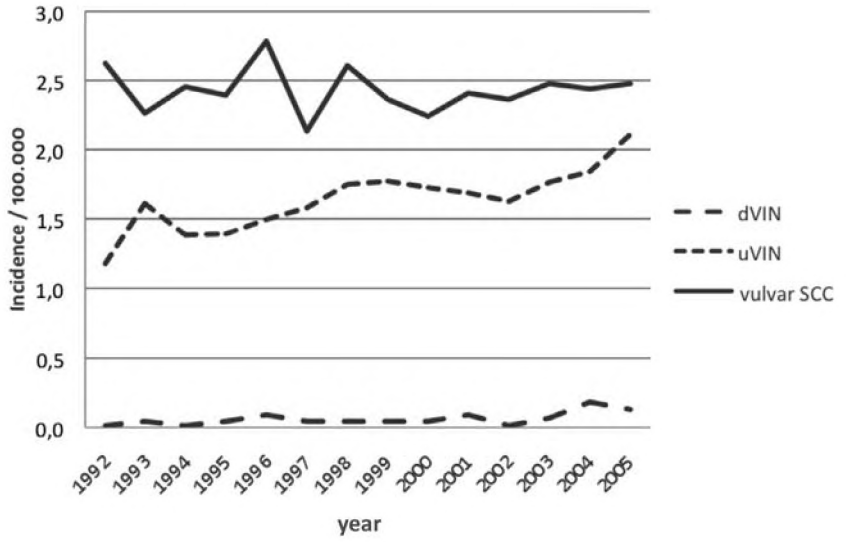

Figure 2 Overview of incidence vulvar SCC, uVIN and dVIN for all patients.
The incidence of vulvar SCC remained stable whereas the incidences of both types of VIN increased.

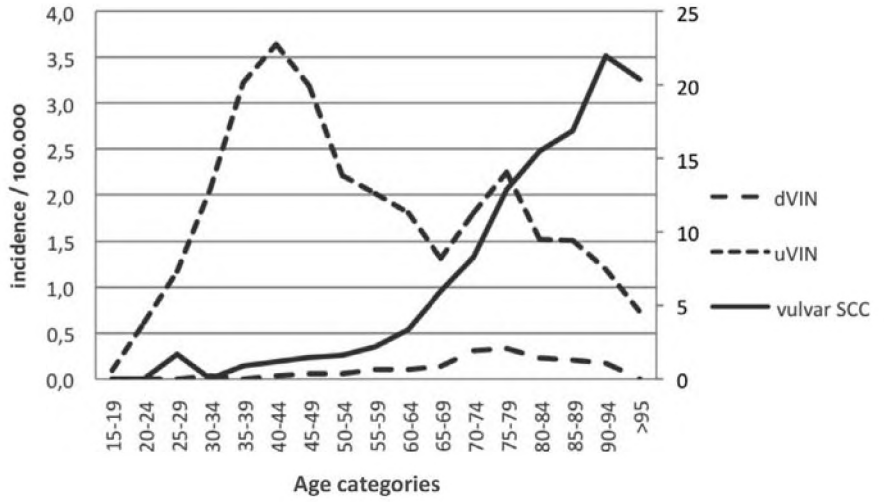

Figure 3 Age specific incidence for vulvar SCC, u- and dVIN from 1992-2005.
A gradual and strong increase in vulvar SCC was found. uVIN shows a bimodal peak incidence at the ages of 40-44 and 75-79. dVIN has the highest prevalence at the age of 75-79. Left y-axis: uVIN and dVIN incidence; right y-axis: vulvar SCC incidence.

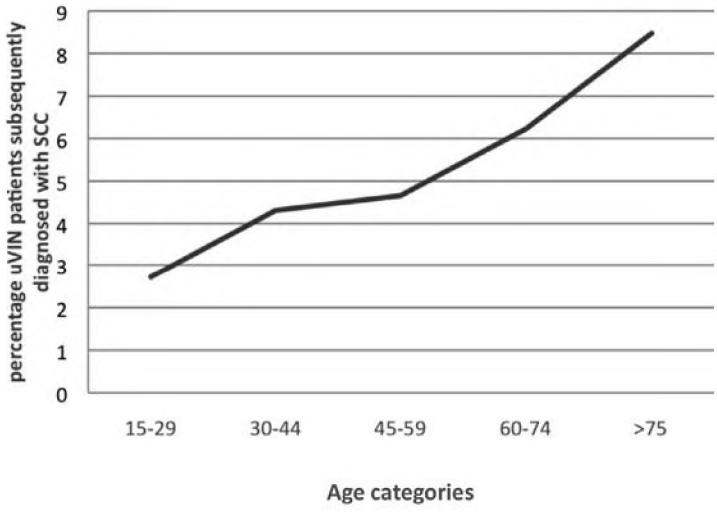

Figure 4 Percentage of patients with uVIN that subsequently diagnosed with vulvar SCC.

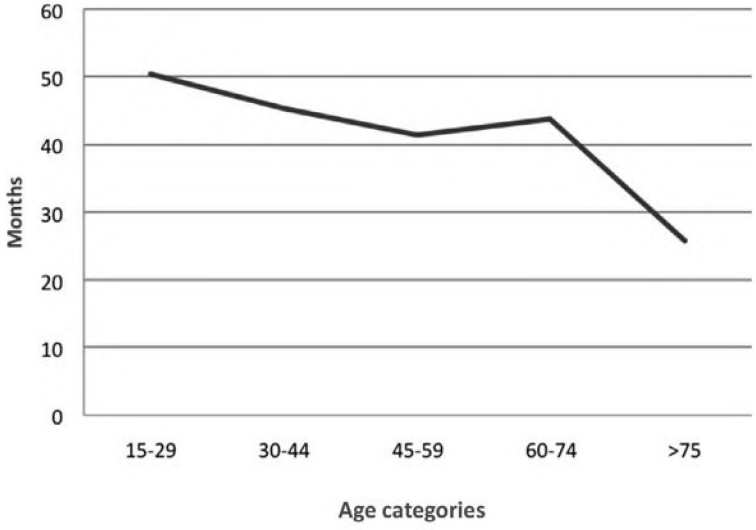

Figure 5 Time from usual VIN to vulvar SCC development.
The time from uVIN to vulvar SCC significantly decreases with increasing age.

The number of dVIN patients that were subsequently diagnosed with SCC was too small to give a trend over a 14 years study period (22 patients) but the overall percentage of dVIN lesions with subsequent diagnosis of SCC was 32.8%. The median time from uVIN towards SCC was 41.4 months (range 3 - 156 months), whereas the median time from dVIN to SCC was significantly shorter: 22.8 months (range 3 - 8.4 months) (p=0.005). Thirty percent of all SCCs after diagnosis of uVIN developed within one year of follow up after the diagnosis of uVIN.

The database contained additional information about previous, concomitant or subsequent intraepithelial neoplasia of the cervix, vagina and/or anus (CIN, VAIN and/or AIN, respectively). Forty-one percent of patients with uVIN had an associated HPV induced lesion of the lower female anogenital tract, comprising cervical, anal, and vaginal (pre) neoplasia or combinations of those (Table 1). This percentage remained stable from 1992-2005. We found that the most common type was a CIN lesion or cervical carcinoma (33.5% of all uVIN patients). In contrast, epithelial abnormalities of the genital tract were observed in only two of the 67 patients diagnosed with dVIN.

	VAIN	AIN	AIN, VAIN	CIN	CIN, AIN	CIN, AIN, VAIN	CIN, VAIN	No multicentric HPV associated lesion
1826 uVIN	45 (2.5%)	89 (4.9%)	7 (0.7%)	471 (25.8%)	93 (5.1%)	10 (0.6%)	37 (2.0%)	1074
67 dVIN	2 (2.9%)	0	0	0	0	0	0	65

Table 1 Overview of prevalence of previous, concomitant or subsequent multicentric HPV associated intraepithelial neoplasias or invasive carcinomas.

Discussion

During the 14 year study period the incidence of uVIN and dVIN increased significantly, while the incidence of vulvar SCC remained stable. In the recent years vulvar premalignant lesions have gained more public awareness, and a more liberal use of biopsies may have led to the observed increase in incidence worldwide.[52,55,161,162,271,273] Increased awareness not only contributes to the doubling in uVIN diagnoses, but also it may result in removal of these lesions at an early

stage of disease before these become invasive. In addition, the malignant potential of uVIN lesions is considered to be low: about 3% to 5% of all uVIN patients progress towards SCC,[161,181,229] and is in line with our data (5.7%).

Other studies reported impressive increases in uVIN incidence, up to 400% increase.[52,55,161,162,271] Some of those also reported an increase in vulvar SCC in young patients,[52,54-57] but in this study incidence of vulvar SCC under the age of 44 remained stable. There are several differences between these previous publications and this study. First, we excluded recurrent VIN lesions; in addition, we excluded patients with a VIN lesion that developed into a vulvar SCC within three months, because it is most likely that the diagnosis of (micro) invasive SCC was missed. Furthermore, we used a different statistical method; the above mentioned studies mostly studied two cohorts and compared them with a χ^2 test. Our data give an overview of the trends over a 14 year period, and we have calculated whether there was an increase or decrease over those years. Finally, the incidence of HPV related lesions of the lower female genital tract may not show an increase as found in several other countries, possibly due to a limited increase in HPV prevalence. For example, the incidence of adenocarcinoma in situ lesions of the cervix has not increased in the Netherlands, which is in contrast with the incidence in the United States of America.[277]

The overall percentage of dVIN patients subsequently diagnosed with vulvar SCC was 32,8%, which was significantly higher compared to uVIN patients (5.7%). Furthermore, the time period between diagnosis of VIN and SCC was significantly shorter for dVIN than for uVIN patients (22.8 compared to 42 months). dVIN is a recently recognised, and difficult to diagnose lesion due to the high degree of cellular differentiation, absence of widespread architectural disarray, nuclear pleomorphism and diffuse atypia.[4] Default from the assumption that about 75% of the vulvar SCC is HPV unrelated (2075 SCCs in this study) and have dVIN as premalignant lesion (67 solitary dVIN lesions in this study), the incidence of solitary dVIN may be extremely underreported. This may be explained by the fact that dVIN as a solitary lesion, or even adjacent to SCC, easily may be mistaken for benign dermatosis or epithelial hyperplasia.[45] It is thought that dVIN has a short intraepithelial period and a rapid progression towards SCC and therefore is less likely to be found without the presence of a carcinoma.[5,154,156] The percentage of 32.8% would probably even be higher and the time to SCC shorter when invasion within the three months after dVIN were included. It is unlikely that dVIN is a new lesion that somehow developed in the past few years. We presume that of all lesions previously diagnosed as benign dermatosis or epithelial hyperplasia, a

large proportion would currently be diagnosed as dVIN. As such, trends in increased incidence of dVIN in the past years are merely a reflection of increased diagnosis of dVIN as a newly described entity rather than a truly increased incidence. Currently, well-defined histopathological features and immunohistological stainings exist to recognise dVIN more easily.[1,4,5,45] This is reflected in the increased diagnosis of dVIN as a solitary lesion in the last three years of the study. A large study on the incidence of dVIN either as a solitary lesion or adjacent to SCC is needed to elucidate this point.

A bimodal peak incidence of uVIN incidence was observed at 40-44 and 75-79 years of age (Figure 3). As the incidence of SCC strongly increases with age, the time from uVIN to vulvar SCC is much longer for the patients aged 40-44 than for patients aged 75-59. For uVIN patients we found that the percentage of uVIN patients that are subsequently diagnosed with vulvar SCC increased with the age of uVIN diagnosis. In addition, the time from uVIN towards the diagnosis of SCC was significantly shorter for patients in the older age group. This has not been described before but is in line with two reports on recurrence of CIN after treatment.[278,279] This finding may possibly be explained by altered immunity for elderly women compared to younger women. In young women uVIN may reflect the immaturity of the immune system and a hindered elimination of HPV. In older women uVIN may reflect a failure of the immune system to suppress HPV resulting in recurrent uVIN lesions and possibly vulvar SCC. In their review, van Seters *et al.* found that the patients with uVIN who were subsequently diagnosed with SCC often had immunosuppressant treatment,[181] which is supportive for a role for the immune system. Another explanation may be that older women are less likely to perform self examination of the vulvar region than younger women.

Forty-one percent of uVIN patients had a past, concomitant or future HPV-associated lesion of the female lower anogenital tract, whereas dVIN patients hardly presented these types of lesions. In two other reports, percentages of 53%[50] and 67% (concerning a smaller number of patients)[51] were found, which are higher than reported in this study. In contrast to the study of Hampl *et al.*[50] we did not include vulvar condylomas as an associated HPV induced vulvar lesion. Vulvar condylomas are merely caused by low-risk HPV 6 and 11, which is in contrast to the high-risk HPV types that are associated with uVIN, CIN, VAIN and AIN. As a result, the malignant potential of vulvar condylomas is thought to be negligible. CIN lesions were the most frequently found associated lesions, which is not unexpected as in the Netherlands women aged 30 – 60 years of age are screened for cervical abnormalities every five years. The majority of HPV associated lesions

of the female lower genital tract may be prevented by the current HPV 16-18 vaccines that have been introduced recently and implemented in national vaccination programmes, as the majority of these HPV lesions are caused by these HPV types. The multicentricity of the HPV induced lesions argues to examine the entire lower female genital tract for possible HPV induced lesions, once a lesion is found.

In conclusion, this study has demonstrated that an increase in both types of VIN has not led to an increase in the incidence of vulvar SCC. In addition, dVIN lesions are rarely seen as a solitary lesion, but are diagnosed more often in the last years of the study, most likely as a result of recently established histopathological criteria. The malignant potential of dVIN is higher than for uVIN. For uVIN, the malignant potential increases with age.

Acknowledgements

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Chapter 5

Differentiated vulvar intraepithelial neoplasia is often found in lesions, previously diagnosed as lichen sclerosus, that have progressed to vulvar squamous cell carcinoma

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Abstract

Background: Vulvar lichen sclerosus (LS) is thought to be the precursor lesion of HPV unrelated vulvar squamous cell carcinoma (SCC). It remains unclear why only 2-5% of all LS lesions progress to SCC. It has been proposed that differentiated vulvar intraepithelial neoplasia (dVIN) is the direct precursor lesion. However, dVIN is only recently recognized as a separate entity and is a difficult diagnosis which may easily be missed or mistaken for a benign dermatosis. The aim of this study was to test the hypothesis that of all lesions that have been diagnosed as LS in the past, a part might currently be diagnosed as dVIN and to identify histopathological differences between LS lesions with and without progression to vulvar SCC.

Methods: All LS slides were revised by two expert gynaecopathologists, who were unaware of the course of the patient. The presence of hyperkeratosis, parakeratosis, dyskeratosis, hyperplasia, basal cellular atypia of keratinocytes, (atypical) mitoses, oedema, hyalinisation and subepithelial inflammation were documented. Furthermore, the presentation of the rete ridges and basal cell layer were examined and the epithelial thickness counted.

Results: After revision of LS biopsies without progression (n=61), 58 were reclassified as LS. In contrast, revision of LS biopsies with progression yielded concordant diagnoses in 18/60 cases (30%). Importantly, 25/60 (41.7%) lesions were reclassified as dVIN. Median time from dVIN to vulvar SCC was significantly shorter (27.5 months) than from LS to vulvar SCC (83.8 months) ($p<0.001$). LS that did progress to SCC, but that did not meet the criteria for dVIN, more often showed parakeratosis ($p=0.004$), dyskeratosis ($p<0.001$), hyperplasia ($p=0.048$) and basal cellular atypia ($p=0.009$) compared to LS without progression.

Conclusion: dVIN diagnosis has been frequently missed and is associated with rapid progression to SCC. Patients with LS with dys- and parakeratosis, hyperplasia and/or basal cellular atypia should be kept under close surveillance as these lesions also tend to progress to SCC.

Introduction

Vulvar squamous cell carcinoma (SCC) is the fourth most common type of gynaecological cancer and affects the external female genitalia. It accounts for approximately 3-5% of all gynaecological malignancies, with an incidence rate of 1-2/100,000.[10] Vulvar SCC is a disease that occurs in the 6th-7th decade of life, and an increase in incidence is to be expected due to the ageing of women. Vulvar SCC treatment has a great impact on the quality of life because of the extensive surgery that is often required.[9] Improvements for the patients are established by individualization of treatment (*e.g.*, the Sentinel Lymph Node technique[15]) and prevention of the disease by more insight in and treatment of the associated premalignancies.

There are two different pathways leading to vulvar SCC with their own premalignant lesions; the most common pathway, that accounts for approximately 80% of all SCCs, is HPV-independent,[280] but its aetiology is still unknown. These, mostly differentiated keratinizing, SCCs often arise in Lichen Sclerosus (LS) and it has therefore been suggested that LS is the precusor lesion of SCC.[28,29,281] LS is a chronic inflammatory skin disease and mainly affects the female anogenital area.[76,258] The classical histological findings of LS are a thinned epidermis and/or loss of rete ridges, hyperkeratosis, oedema and/or hyalinization, and a chronic band like inflammatory cell infiltrate of the dermis[58] (Figure 1), but there are a lot of variations to these classical histological characteristics, leading to a myriad of lesions that may be classified as LS. Although LS is considered a premalignant condition, only 2-5% of the patients with LS ultimately develop a vulvar SCC.[29,30] Currently, no molecular markers have been proven to identify LS lesions that are at risk to develop vulvar SCC. It has been hypothesized more recently, that differentiated Vulvar Intraepithelial Neoplasia (dVIN) is the direct precursor of vulvar SCC. dVIN (Figure 2A) is often found directly adjacent to SCC and is characterized by a thickened epithelium that is typically associated with elongation and anastomosis of rete ridges (Figure 2B). Dys- and parakeratosis are usually present (Figure 2 C & D), associated with prominent intercellular bridges (Figure 2B). The basal keratinocytes are large and pleomorphic with a relatively large amount of eosinophilic cytoplasm. Keratin pearl formation within the rete ridge may be seen. The nuclear chromatin is vesicular rather than coarse, and the nuclei have prominent nucleoli (Figure 2E), usually most prominently in the basal and parabasal keratinocytes.[58] Although dVIN is seen adjacent to approximately 80% of vulvar SCCs,[280] it is seldom diagnosed as a solitary lesion.[282] This discrepancy

may be explained by underdiagnosis; dVIN may easily be missed or mistaken for a benign dermatosis, such as lichen simplex chronicus and squamous hyperplasia.[4,5,45] In addition, it has been proposed that dVIN has a relatively short intraepithelial phase before progression towards vulvar SCC[282] which makes it more difficult to diagnose isolated dVIN. It has been hypothesized that dVIN may develop from LS and carries a higher malignant potential than LS does.[5,28,36,148,283,284] We hypothesize that of all lesions that have been diagnosed as LS in the past, a part would currently be diagnosed as dVIN.

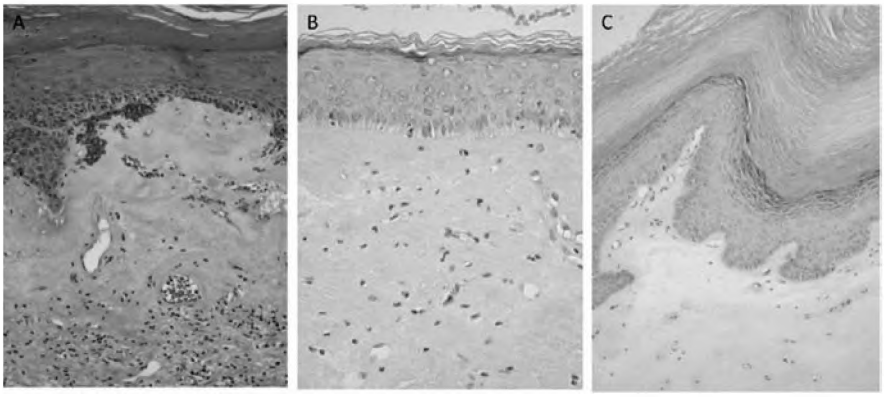

Figure 1 Classical cases of lichen sclerosus (LS). A: Typical example of LS with a thinned epidermis and loss of rete ridges, hyperkeratosis, hyalinization, and a chronic band like inflammatory cell infiltrate of the dermis
B: Lichen sclerosus with atypia, C: Lichen sclerosus with hyperplasia. Original magnification. A-C: 100x.

The terminology and criteria for the diagnosis LS have been subject to quite some revisions in the past 40 years and this unevitably has led to diagnoses of LS that would currently be classified differently, including dVIN. Before the 1975 ISSVD classification[285] of vulvar dystrophies, confusing terms such as 'kraurosis', 'leukoplakic vulvitis' and 'atrophic vulvitis' were used. The term 'vulvar dystrophy' replaced all of these terms. In 1987, the term 'non-neoplastic epithelial disorders of the vulva' was introduced to replace 'dystrophy', listing LS, squamous hyperplasia and other dermatoses.[286] In 2005, Regauer proposed the term 'early' LS, to be set apart from 'atrophic' LS[120], suggesting there clearly are two types of LS, each with its own histopathological criteria, but this subdivision has not gained

worldwide acceptance.

It is currently unknown which LS lesion carries risk of malignant progression. This is in part due to the wide variety of histopathological entities that have been collectively diagnosed as LS in the past. The first aim of this study was to test the hypothesis that of all lesions that have been diagnosed as LS in the past, a part would currently be diagnosed as dVIN. The second aim was to determine whether, in retrospect, there are histopathological differences between LS lesions that did and did not progress to vulvar SCC. Recognition of the LS at risk for SCC development would greatly help to select patients who need close follow-up.

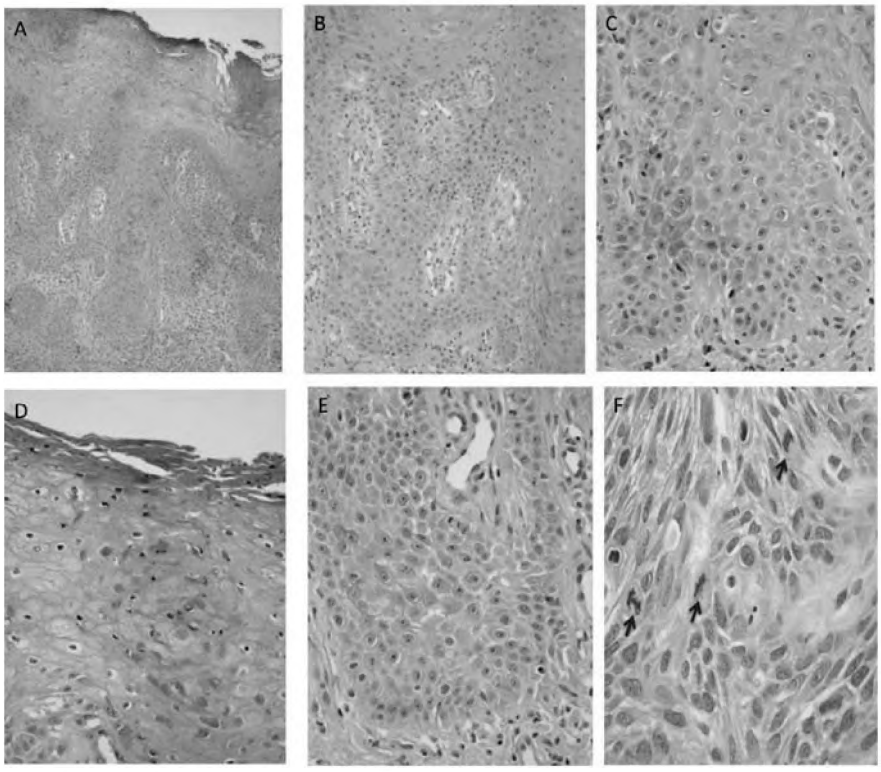

Figure 2 Differentiated VIN.

Low power overview of dVIN (A) with the five most important histopathological characteristics that can be recognized in a H&E stain: elongated rete ridges with anastomosis (B), disorderly basal cell layer and dyskeratosis(C), parakeratosis (D), prominent nucleoli (E), and atypical mitoses, indicated by arrows (F).

Original magnifications: 50x (A), 100x (B,D), 200x (C,E) and 400x (F).

Patients and Methods

Patient selection

Lichen sclerosus without progression

Patients with LS who were not diagnosed with a subsequent vulvar SCC were selected from the outpatient clinic at the department of Obstetrics and Gynaecology of the Radboud University Nijmegen Medical Centre (RUNMC), the Netherlands. We used the nationwide Netherlands database of Cyto- and Histopathology (PALGA)[287] to confirm that these patients did not develop a vulvar SCC with a minimum follow up of ten years.

Lichen sclerosus with progression

Patients with vulvar SCC who were treated at the departments of Obstetrics and Gynaecology of the RUNMC or the University Medical Centre Groningen between January 1st 1988 and December 31th 2008 were selected for this study. With the use of PALGA, all prior vulvar biopsies with the diagnoses 'Lichen sclerosus (et atrophicus)' and 'vulvar dystrophy' were retrieved. When multiple prior biopsies were available from different moments, the most recent biopsy was included for this study (with a minimum interval between the biopsy and vulvar SCC of three months). In case multiple biopsies were taken at the same time, the lesion with the most severe dysplastic characteristics was selected.

The haematoxylin and eosin (H&E)-stained slides were retrieved and reviewed by two expert gynaecopathologists (JB and HH) independently and unaware of the course of the patient. Discrepancies were resolved in a consensus meeting with these two gynaecopathologists. In the analyses, we categorised the diagnoses vulvar dystrophy and LS together, because in earlier days the term vulvar dystrophy was used for the same entity as LS. Concordance between the original and revised diagnosis was calculated.

Collected data

The patients' age, time of diagnosis of SCC and time to progression were obtained from medical charts and electronic patient files. All slides were scored on the presence of hyperkeratosis, parakeratosis (Figure 1C and 2D), dyskeratosis (Figure 2C), hyperplasia (Figure 1C), basal cellular atypia (Figure 1 B), presence of mitotic figures (Figure 2F), oedema, hyalinization (Figure 1A) and presence of subepithelial inflammation (Figure 1A). In addition, we examined the presentation of the rete ridges (Figure2B), the basal cell layer (Figure 2C) and the epithelial thickness.

Statistical analysis

Calculations were performed using Statistical Package for Social Sciences 16.0 (SPSS, Chicago, IL).

Descriptive statistics were used to reproduce study results as percentages, means, medians and standard deviations. Chi-Square tests were used to identify significant differences between LS with and without progression. The Independent-Samples t-Test was used to compare the age for two groups. P-values less than 0.05 were considered to be statistically significant.

Results

Lichen sclerosus without progression

Sixty-one patients with LS without progression and a minimal follow up of ten years were retrieved from the pathology archives of the RUNMC. Median age at the time of the diagnosis was 59.5 (range 16-83 years) Three biopsies did not fulfil criteria for LS and were excluded. In 58 of the 61 cases (95%), the diagnosis was not changed.

Lichen sclerosus with progression

During the study period 1988-2008, 273 patients with vulvar SCC were treated in Nijmegen and 548 patients were treated in Groningen. In Nijmegen, 33 cases that sufficed the criteria of a prior biopsy with 'Lichen sclerosus(et atrophicus)' or 'vulvar dystrophy' were identified and 72 in Groningen. Of these 105 cases, a total number of 60 biopsies were received from the pathology archives of the RUNMC/ University Medical Centre Groningen and the referring hospitals in the surrounding areas. We did not receive 35 biopsies we requested from the hospitals where the biopsies were taken and ten biopsies were excluded from analysis for different reasons (poor quality H&E staining, or slides with too little tissue to review). The study population consisted of 60 LS patients with progression to vulvar SCC. Median age at the time of diagnosis of LS was 64.6 (range 30-90) years. These patients were older compared to LS patients without progression ($p<0.001$).

All biopsies were revised and scored. In total, diagnoses of 42 biopsies were changed by the two expert gynaecopathologists (70%). Twenty-five of the 60 (41.7%) lesions previously diagnosed as LS, but that did progress to SCC, were reclassified as dVIN (Figure 2). The biopsies that were changed into dVIN mainly

came from the original diagnosis categories of LS with an associated lesion such as an HPV induced lesion (VIN 1, 3, Morbus Bowen, Buschke Löwenstein), or from LS with hyperplasia, atypia or both. The highest percentage of concordance between the original and changed diagnoses was achieved for 'simple' LS without an associated lesion (42.9%). The original and revised diagnoses are given in Table 1. Of all biopsies, six were not classified as LS/dVIN. Twenty-nine biopsies were diagnosed as LS, of which one was classified as early LS (categorized as LS),[120] and three were classified as LS with hyperplasia (Figure 1C). Five LS biopsies had basal cellular atypia (Figure 1B), and two biopsies were classified as LS with hyperplasia and basal cellular atypia. These seven biopsies did not comply with other criteria for dVIN.

Differences in the time to progression into vulvar SCC between LS and dVIN biopsies were calculated. For this analysis, the six biopsies without LS or dVIN were excluded. The time to progression for dVIN (27.5 months) was shorter than for LS (83.8 months) (p=0.001) (Table 2). The number of lesions in the subcategories of LS were too small to calculate differences in times until progression.

Histopathological differences between lichen sclerosus with and without progression

For this comparison, only biopsies were included that were indeed classified as LS after revision (29 with progression, 58 without progression). LS biopsies with progression showed more often dyskeratosis (31% vs. 0% p<0.001) and parakeratosis (48.3% vs. 19% p=0.004) than LS biopsies without progression. In addition, LS biopsies with progression more often had hyperplasia (24.1% vs. 8.6% p=0.048) and basal cellular atypia (20.7% vs. 3.4% p=0.009). The other histopathological characteristics did not differ between both groups (Table 3).

		Diagnosis after revision						
Original diagnosis	Total	LS	LS with hyperplasia	LS with atypia	LS with hyperplasia and atypia	dVIN	Other	Concordance n (%)
Lichen sclerosus	42	18	4	3	1	10	6	18/42 (42.9)
LS with HPV related lesion	5				1	4		0/5
LS with atypia	4					4		0/4
LS with hyperplasia	9	1		1		7		0/9
Total	60	19	4	6	2	25	6	18/60 (30)

Table 1 Overview of original and diagnoses after revision.

Diagnosis after revision		Median age (range)	Median time to vulvar SCC (months) (range)
Lichen sclerosus	29	64.6 (30-90)	83.8 (9-230)
– Lichen sclerosus	19	67.6 (52-79)	61 (9-200)
– Lichen sclerosus with hyperplasia	3	51.8 (30-54)	98.6 (13-135)
– Lichen sclerosus with atypia – Lichen sclerosus with hyperplasia and atypia	5 2	59.1 (53-64) 69.7 (65-73)	115 (91-231) 30 (26-35)
Differentiated VIN	25	64.4 (42-90)	27.5 (5-144)
No Lichen sclerosus, no differentiated VIN	6	64 (54-72)	31.4 (4-63)

Table 2 LS with progression; diagnoses after revision by two expert gynaecopathologists.

Histological features	LS without progression (N= 58)		LS with progression (N = 29)		P value
	Number	%	Number	%	
Hyperkeratosis					.875
No	21	(36.2)	11	(37.9)	
Yes	37	(63.8)	18	(62.1)	
Parakeratosis					.004
No	47	(81)	15	(51.7)	
Yes	11	(19)	14	(48.3)	
Dyskeratosis					<0.001
No	58	(100)	20	(69)	
Yes	0	(0)	9	(31)	
Rete ridges					.235
Normal	2	(3.4)	5	(6.9)	
Reduced	51	(87.9)	21	(82.8)	
Widened	4	(6.9)	0		
Merged	1	(1.7)	1	(3.4)	
Elongated and merged	0		1	(3.4)	
Widened and merged	0		1	(3.4)	
Epithelial thickness					.332
(cells)	1	(1.7)	1	(3.4)	
<5	27	(46.6)	10	(34.5)	
5-9	24	(41.4)	11	(37.9)	
10-14	5	(8.6)	4	(13.8)	
15-19	1	(1.7)	3	(10.3)	
≥20					
Hyperplasia					.048
No	53	(91.4)	22	(75.9)	
Yes	5	(8.6)	7	(24.1)	
Dysplasia					.213
No	57	(98.3)	27	(93.1)	
Yes	1	(1.7)	2	(6.9)	
Basal cellular atypia					.009
No	56	(96.6)	23	(79.3)	
Yes	2	(3.4)	6	(20.7)	
Mitotic figures					.510
No	49	(84.5)	26	(89.7)	
Yes	9	(15.5)	3	(10.3)	
Basal cell layer					.600
Normal	52	(89.7)	27	(93.1)	
Disorderly	6	(10.3)	2	(6.9)	
Oedema					1.000
No	22	(37.9)	11	(37.9)	
Yes	36	(62.1)	18	(62.1)	
Hyalinisation					.099
No	12	(20.7)	2	(6.9)	
Yes	46	(79.3)	27	(93.1)	

Inflammation at epithelial-dermal junction					.658
Absent	22	(37.9)	9	(31)	
Focal	12	(20.7)	5	(17.2)	
Diffuse	24	(41.4)	15	(51.7)	
Inflammation subepithelial					.647
Bandlike	25	(43.1)	14	(48.3)	
Not bandlike	33	(56.9)	15	(51.7)	
Intraepithelial presence inflammation cells					.759
No	24	(41.4)	13	(44.8)	
Yes	34	(58.6)	16	(55.2)	

Table 3 Overview of original and diagnoses after revision.

Discussion

In this study we found that 41.7% of LS biopsies, taken from patients who showed progression into vulvar SCC, showed the histological characteristics of dVIN. In the majority of cases dVIN was found in biopsies of which the original diagnosis was LS with an HPV induced lesion or with hyperplasia and/or atypia. In addition, we found that LS biopsies with dys- and parakeratosis, hyperplasia and atypia may be regarded as LS with an elevated risk of vulvar SCC development.

By revising numerous biopsies which were earlier classified as LS, we show that a substantial part nowadays would be diagnosed as dVIN. Remarkably, most revisions were made when the original report mentioned LS with an associated lesion (an HPV induced lesion, hyperplasia and/or atypia) (Table 1). Most concordance was achieved for 'simple' LS lesions without an associated lesion. The histological diagnosis of dVIN is difficult; the recognition of dVIN is hindered by a high degree of cellular differentiation combined with an absence of widespread architectural disarray, nuclear pleomorphism and diffuse nuclear atypia.[4] The atypia in dVIN lesions is strictly confined to the basal and parabasal layers of the epithelium.[4,58] In addition, despite dVIN was first described in the 1960s by Abell as a highly differentiated form of vulvar carcinoma in situ (CIS),[288] until more recently, the entity has not gained wide attention because its existence as a clinicopathological entity has long been questioned.[42] Since more insight is gained that vulvar SCCs originate from two different pathways, each with its own

premalignant lesions,[1,2] dVIN has been designated the direct precursor of the HPV independent pathway. Earlier, all VIN lesions were classified as being HPV related, which explains the high incidence of LS with HPV induced lesions, which turned out to be dVIN instead of the nowadays so-called usual VIN (uVIN) lesions.[3] In addition to the difficult histopathological diagnosis, it is thought that the intraepithelial period of dVIN is short, all being reasons why dVIN has been seldom diagnosed as a solitary lesion. In this study, we show that dVIN is not as rare as a solitary lesion as previously thought, but there is significant underdiagnosis. The recognition of dVIN is of utmost importance because the malignant potential of dVIN is thought to be high,[282] and therefore treatment differs between LS (topical superpotent corticosteroid ointment[127]) and dVIN (surgical excision[258]).
After exclusion of the lesions that were changed into dVIN, we found that LS biopsies with progression more often showed dys- and parakeratosis, hyperplasia and basal cellular atypia in comparison with LS biopsies without progression. It has been suggested that hyperplasia, dysplasia or cellular atypia are precursor features linking progression of vulvar LS to vulvar SCC[30,31,36,37] and that squamous cell hyperplasia with atypia might represent a step in the carcinogenesis towards vulvar SCC.[151] Although the numbers of LS lesions with atypia or hyperplasia were small in our study, it may be recommended that these patients require a more careful follow up because they have a higher risk to develop vulvar SCC.[37] The characteristics of dys- and parakeratosis have not been associated with progression towards vulvar SCC before, but were not included in the analyses of the earlier studies. In our study, dysplasia was not a characteristic of LS biopsies with progression. We hypothesize that in the studies that have defined features linking progression of LS to SCC, also dVIN lesions may have been included, and therefore dysplasia was found to be associated with progression. Patients with progression were significantly older at the time of the biopsy, which has also been associated with malignant progression in other studies,[31,37] but the difficulty is that it is unknown for how long the patients have been suffering from LS at the time of the biopsy.

From this study, we cannot draw strong conclusions regarding the malignant potential of dVIN because we have not included dVIN lesions without progression. In the LS group without progression, no dVIN lesions were found. In an earlier study, we saw that 32.8% of solitary dVIN lesions progressed to vulvar SCC.[282] In that study, we only used the reports in the PALGA database; we did not revise the VIN lesions. Now that we demonstrate that dVIN is more often present in its

solitary form than previously thought, its high malignant potential should be appreciated. The median time between dVIN biopsies and vulvar SCC development in this study was 27.5 months, which is comparable with the 22.8 months in our other study.[282] In total, we have identified 92 (67[282] and 25 (this study) solitary dVIN lesions with and without progression, which is still rather a small number to draw strong conclusions from.

This study has several limitations; the first is the possible different localization of the biopsies and the subsequent SCC. We do not have access to these data of patients as this is poorly recorded. The concept of field cancerization is plausible, because in most patients the greatest part of the vulva is affected by LS. But it is unknown whether different types of LS were present in one patient at the same time. In addition, there is a large time range between LS lesions and vulvar SCC development, the longest being 19 years.

In total, 821 patients were treated for a vulvar SCC during the study period, and of only 105 patients a biopsy of LS or vulvar dystrophy was identified. We used PALGA,[274] which has national coverage from 1992 onwards, so it may be that more biopsies were taken before 1992 that we did not retrieve. However, the majority of vulvar SCC patients do not have a recorded and histopathologically proven history of LS, but suffer from LS, either asymptomatic or unnoticed.[29,31,33-35] This may result in delayed diagnosis by both patients and doctors. In our vulvar clinic, the policy is to biopsy every women with a suspicion of LS, because this diagnosis has the consequence of a lifelong follow up (at least yearly).

In conclusion, we found that in patients with progression, the original diagnosis LS often was changed into dVIN. When adhering to strict criteria (elongated rete ridges with anastomosis, a disorderly basal cell layer, dys- and parakeratosis, prominent nucleoli and atypical mitosis) the diagnosis of dVIN should be more easy to make, especially when the patient is suffering from LS as well. We conclude that dVIN suffers from underdiagnosis rather than from rarity. When the diagnosis LS with an HPV associated lesion, atypia or hyperplasia is made by a pathologist, the diagnosis dVIN should at least be considered by both the pathologist and the gynaecologist/dermatologist. Furthermore, when the diagnosis dVIN cannot be established, but when LS with dys- and/or parakeratosis, hyperplasia and basal cellular atypia is diagnosed, close follow up of the patient is warranted as these LS lesions are at increased risk for malignant progression. A prospective study and translational research are needed to confirm our hypothesis that patients with LS lesions with dys- and/or parakeratosis, hyperplasia and basal cellular atypia are at risk to develop vulvar SCC.

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Chapter 6

High levels of p53 expression correlate with DNA aneuploidy in (pre-) malignancies of the vulva

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Abstract

Aims: The molecular pathogenesis of HPV-unrelated vulvar squamous cell carcinoma (SCC) is not well known. Whether malignant progression of lichen sclerosus (LS) and differentiated vulvar intraepithelial neoplasia (dVIN) to vulvar SCC could be accompanied by altered DNA content has not been studied extensively.

Methods & results: DNA-content in isolated nuclei of microdissected normal vulvar epithelium (n=2), LS (n=9), dVIN (n=13), solitary dVIN (n=6) and SCC (n=17) from 26 patients, was measured via DNA image cytometry. p53 expression was determined by immunohistochemistry on consecutive tissue sections. Thirty-eight percent of dVIN lesions (5/13) and 65% of SCCs (11/17) were DNA aneuploid or tetraploid. In lesions that contained dVIN and adjacent SCC, the ploidy status of dVIN did not exceed that of SCC. We observed a strong correlation between high p53 expression and DNA aneuploidy. This relation was also present at the level of single nuclei, measured by sequential image cytometry of p53-immunohisto-chemistry followed by DNA image cytometry on formalin-fixed tissue sections. Similarly, we found p53-positive non-proliferating cells with increased DNA content in the superficial compartment of 6 solitary dVIN that were not associated with SCC, indicating ascending aneuploid cells from the basal compartment.

Conclusions: DNA ploidy measurements suggest that dVIN has a higher malignant potential than LS, and thus is a more likely precursor of SCC. Furthermore, high p53 expression correlates with increased DNA content and aneuploidy, but it requires further research to unveil a possible causal relation.

Introduction

Vulvar cancer is the fourth most common gynaecologic cancer and accounts for approximately five percent of the malignancies of the female genital tract.[289] Based on etiologic characteristics, two separate pathways that lead to vulvar squamous cell carcinoma (SCC) can be distinguished.[2] About a third of all vulvar SCCs develops through a high-risk human papillomavirus (HPV)-dependent pathway in which premalignant stages of vulvar SCC are usual vulvar intraepithelial neoplasia (uVIN)-lesions that mainly affect younger women. However, the majority of vulvar SCCs arise in the absence of HPV, predominantly in older women with lichen sclerosus (LS). LS is a chronic inflammatory disease of the vulvar skin and mucosa characterised by markedly thinned epithelium with loss of rete ridges, culminating in architectural changes of the vulva and can give rise to severe itching. Differentiated VIN (dVIN) is thought to play a role in this HPV-independent pathway as a premalignant lesion with a high malignant potential although the molecular pathogenesis remains elusive.[2] Women with LS have a two to six percent lifetime risk to develop vulvar SCC.[258] dVIN is seen relatively infrequently in its pure form and is commonly identified as an ulcerative or verrucous lesion, adjacent to invasive SCC, often in a background of LS. dVIN can be a difficult histological diagnosis as the atypia is confined to the basal cell layers whereas the rest of the epithelium shows normal maturation.[58] The possible malignant progression of LS via dVIN and subsequently rapidly to vulvar SCC, may be caused by mitotic defects and consequently chromosome missegregation and instability.

DNA aneuploidy, a frequently identified genomic abnormality, can occur in the earliest stages of malignant transformation, although it is unknown whether DNA aneuploidy is a cause or consequence of malignant progression.[290] Impaired functioning of cell cycle checkpoint proteins contributes to proliferation of cells with impaired genomic integrity. To prevent survival of aneuploid cells, cell cycle checkpoint proteins block cell proliferation after abnormal division.[291] In normal skin, only a small percentage of cells divides and has a tetraploid status. Due to increased proliferation, the percentage of tetraploid cells increases and may result in the development of aneuploid cells when loss of function of the cell cycle regulator proteins occurs.[291] The tumour suppressor protein p53 is a key regulator of maintaining normal diploid status[292] and halts cell division and/or eliminates cells that have acquired irreparable DNA damage.[293] A relationship between high p53 expression, morphological variability of nuclei and DNA aneuploidy in breast

carcinomas was demonstrated by Haroske and co-workers[294] more than a decade ago, but has not been studied in vulvar carcinomas and their precursor stages before.

In vulvar SCCs, ploidy has been studied mostly in relation to prognosis. It has been reported that aneuploidy in vulvar SCC varies from 13% to 83%, but no relation with prognosis has been described so far.[169,295-300] Aneuploidy in vulvar LS varies from 0% to 33% of the cases.[31,169,300,301] The relation between the expression of cell cycle proteins and ploidy status of vulvar lesions has not been extensively studied. Lerma *et al.* concluded that p53 expression is a late event in malignant progression as 72% of the mainly HPV-negative SCCs were aneuploid and only 56% of the SCCs showed p53-overexpression.[169] Recently, high p53 expression in the basal cell layers of LS has been postulated as a marker for increased likelihood to progress to vulvar SCC.[39]

The aim of this study was to analyse ploidy in different groups of HPV-negative vulvar lesions to test the hypothesis that in LS, dVIN, and SCC, increased DNA content abnormality is associated with an increased malignant potential of the histological entity. In addition, we evaluated the correlation between aneuploidy and the expression of p53 with the intention to shed light on the potential malignant conversion of LS and dVIN towards SCC.

Methods

Sample Selection

Formalin-fixed and paraffin-embedded tissue sections of 28 patients who underwent a local excision or (partial) vulvectomy because of a (pre-) malignancy of the vulva, were randomly selected from the archives of the Department of Pathology, Radboud University Nijmegen Medical Centre. All tissue sections were collected between 1996 and 2009. All original H&E-stained slides were re-examined by an expert gynaecopathologist (JB), and all lesions were classified according to WHO criteria.[58] Tissues were obtained according to local ethical guidelines and approved by the local regulatory committee.

The median age of all patients was 71.5 years (range 37-90 years). From most patients, more than one type of lesion was present in the excised tissue and the total number of lesions available for analysis was 47: two samples of normal epithelium, nine LS lesions, 13 dVIN lesions, and 17 samples with SCC. In addition, 6 solitary dVIN not associated with micro- or macro-invasive SCC were included in

this study. See Table 1 for an overview of the lesions and tissue composition. In the tissue sections with LS and dVIN, and 1 dVIN lesion, micro-invasive vulvar SCC was present in the tissue, but was too small for ploidy analysis (Table 1). All patients with SCC had LS in the excised material; when it was present in the same tissue block, both entities were analysed.

Lesions were tested for high risk HPV DNA using a broad-spectrum HPV detection/genotyping assay (SPF10-LiPA) and were all HPV-negative. The combined SPF-PCR-LiPA system for detection and genotyping of HPV has been described in detail elsewhere.[261,302]

Lesion	Number of	
	Patients	Samples
LS only	1	1
dVIN (adjacent to microinvasive SCC)	1	1
dVIN (without adjacent (micro) invasive SCC	6	6
SCC only	5	5
LS+dVIN (all adjacent to (micro)invasive SCC)	3	6
LS+SCC	2	4
LS+dVIN+SCC	3	9
dVIN+SCC	5	10
N+SCC	1	2
N+dVIN+SCC	1	3
Total	28	47

Table 1 Number of patients and samples
LS = lichen sclerosus (n=9)
SCC = squamous cell carcinoma (n=17)
dVIN = differentiated VIN (n=19)
N = normal epithelium (n=2)

Tissue dissection and isolation of nuclei for Feulgen staining

From each paraffin-embedded tissue block, a 50 µm thick section was cut and dewaxed through xylene and rehydrated through graded alcohol. The tissue sections were washed three times in demineralised water and phosphate-buffered saline (PBS), before microdissection of the different epithelial lesions. Excised tissue was digested with pepsine (Sigma Aldrich, 2 ml 0.5%(w/v), pH1,5) for 60 minutes at 37˚C. After complete tissue dissolution, 2 ml of PBS was added

to stop the reaction. The suspension was passed through a 50 µm Celltrics filter, and spinned down for 10 min at 150g. The pellet of nuclei was collected and counted using a Coulter Counter. Subsequently, the suspension was centrifuged again and the pellet was re-suspended in PBS to a dilution of 200.000 nuclei per ml. A cytospin of 20.000 nuclei was prepared (100 µl,10 min, 100g), followed by fixation in Böhm fixative (60 min at room temperature (RT)), rinsed with methanol and air-dried. For ploidy measurement, Schiff-Feulgen staining was performed as described elsewhere.[303]

Ploidy examination with Q-path DNA software

Image cytometry with Q-path DNA software (Leica Imaging Systems Ltd. Cambridge, UK) was performed to determine DNA ploidy of isolated nuclei. For each analysis DNA content of 4000 nuclei was calculated automatically. Images of overlapping nuclei were manually excluded from the analysis. Ploidy status was determined according to the consensus criteria of the European Society for Analytical Cellular Pathology (ESACP).[304-306] In each sample, lymphocytes were identified by their size and roundness and included as an internal control for true diploid status.

p53 and MIB1 immunohistochemistry

p53

Four-micrometer-thick sections of formalin-fixed paraffin-embedded tissue blocks were mounted onto SuperFrost slides and dried overnight at 37°C. The sections were dewaxed in xylene and rehydrated through graded alcohols. Following quenching of endogenous peroxidases, slides were rinsed three times in PBS (pH 7.4) for 5 minutes, and antigen retrieval performed with boiling sodiumcitrate (0.01 M; pH 6.0, 10 minutes). After cooling down to RT, tissue sections were rinsed in PBS for 10 minutes and subsequently incubated with a primary monoclonal antibody towards p53 (clone DO-7 (Neomarkers), 1:1000 in PBS containing 1% (w/v) bovine serum albumine (BSA)) for 1h at RT. Subsequently the slides were rinsed in PBS (10 minutes) and post antibody blocking was performed for 15 minutes (Powervision Plus, Dako SA, Glostrup, Denmark). This was followed by incubation with polymeric-horse-radish peroxidase conjugated goat-anti-rabbit IgG, (30 minutes, RT). Staining was developed with diaminobenzidine (DAB) in the presence of H_2O_2, counterstained with Mayer's haematoxylin, dehydrated in ethanol and xylene, and finally mounted with Permount (Fisher Chemicals, NJ, USA). Negative controls (buffer only) and positive controls were included in each analysis.

Because the atypical cells in dVIN are located in the basal parts of the epithelium, nuclear p53-positivity in the lower one-third of the epithelium was estimated. In the SCCs and LS lesions, the percentage of p53-positive cells in the entire lesion was estimated.

MIB1

MIB1 immunohistochemistry was performed as has been described in detail elsewhere.[153]

Combined p53 immunohistochemistry and DNA image cytometry

Tissue sections (7 µm) mounted on Superfrost slides were stained for p53 as described above, but developed with the water soluble 3 amino-9-ethyl-carbazol (AEC) instead of DAB, and mounted with Imsol without coverslips. Images of representative areas were digitised and stored in 24 bit RGB using a 3CCD camera (Sony 950P, Sony, Japan) attached to a Zeiss AxioPhot microscope (Carl Zeiss, Germany) with 20x objective (Plan Neofluar, NA=0.5; specimen level pixel size 0.64x0.64 µm2). Subsequently, p53 stained sections were washed in demineralised water (37°C, 1 hour), followed by an overnight wash-step in demineralised water to remove all traces of Imsol. After a brief third wash with demineralised water, tissue sections were submerged in Böhm fixative (60 min, room temperature), rinsed with methanol, air-dried and Schiff-Feulgen staining was performed. This procedure removes all p53 staining as well as the haematoxylin counter staining. Contours of nuclei in stored p53 images were extracted and shown as an overlay in the live camera image, to facilitate acquisition of exactly the same locations in the Feulgen stained sections. Images of the Feulgen stained sections were acquired using the same camera and microscope setup, using a band pass filter (565.5+/-20nm). Recognition of nuclei in images from Feulgen stained specimens was initially performed by applying a region growing algorithm on manually indicated seed points. If required, results of region growing were interactively corrected. Integrated optical density (IOD) of both Feulgen and p53 staining were calculated for 500 to 1000 nuclei in each specimen. To obtain an internal reference for diploid Feulgen IOD and for p53 negativity, approximately 200 nuclei in the tissue stroma were measured in each specimen. When comparing the p53-negative basal regions with the superficial p53-positive regions, the operator manually identified the basal and superficial regions.

Statistical analyses

To determine the correlation between DNA ploidy and semi-quantitative scoring of p53 expression, Chi-square tests were performed. To compare ploidy status of p53-positive versus p53-negative cells, the IOD_{p53} and $IOD_{Feulgen}$ distributions for these groups were compared using the two sample Kolmogorov-Smirnov test. Statistics were performed using SPSS software (SPSS Inc, Chicago, Il, USA).

Results

Ploidy Analysis

DNA content of various entities of vulvar (pre-) malignancies was performed by DNA image cytometry on isolated nuclei from microdissected tissues (Figure 1A-D). The ploidy status of all samples is summarised in Table 2. Both normal epithelium samples were DNA diploid. DNA hypoploidy was observed in one out of nine LS lesions (11%), whereas the other eight LS samples were DNA diploid. Five of the 13 analysed dVIN lesions (38%) were DNA aneuploid. From the 17 carcinomas, nine were DNA aneuploid tumours (53%), and two tumour samples (12%) were DNA tetraploid. The remaining six tumour samples (35%) were DNA diploid. For lesions in which both dVIN and SCC were present, the ploidy status of dVIN never exceeded the ploidy status of the associated SCC.

	Hypoploid	Diploid	Aneuploid	Tetraploid	Total
LS	1	8	-	-	9
dVIN	-	8	5	-	13
SCC	-	6	9	2	17
N	-	2	-	-	2
Total	1	24	14	2	41

Table 2 Ploidy-status versus type of lesion.
N: normal epithelium
LS: lichen sclerosus
dVIN: differentiated VIN
SCC: squamous cell carcinoma

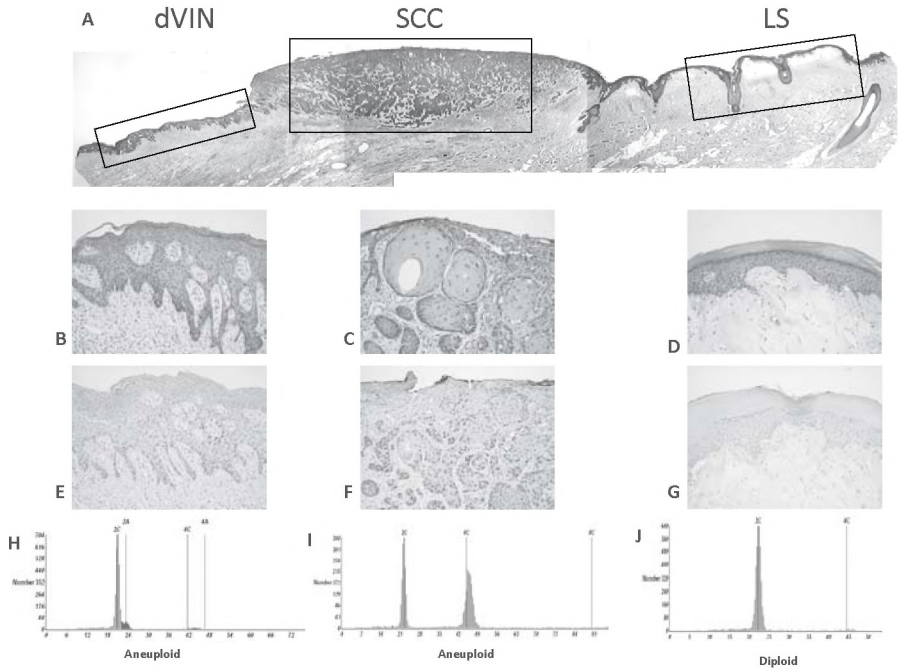

Figure 1 Analysis of DNA ploidy and p53 expression in tissue containing LS, dVIN and SCC. A) H&E staining of the composite lesion (constructed of three images, as shown by the dashed dividing lines, original magnification 1.25x). The black rectangles represent the excised areas for ploidy analysis.

B) H&E stained detail of dVIN lesion with nuclear atypia and the presence of mitotic figures in the basal cell layers. Hyperkeratosis and dyskeratosis are present and rete ridges elongated. C) H&E stained detail of SCC. D) In the H&E stained section of LS, flattened and mildly hyperkeratotic epithelium is present. Loss of rete ridges is clearly visible. There is little cellular or nuclear atypia. A zone of hyalinised dermis and subepithelial oedema of variable thickness is present. The characteristic band-like infiltrate of lymphocytic cells is located beneath this zone.

E) Immunohistochemistry revealed nuclear p53-posititivity in dVIN, most prominent in the basal cell layers with suprabasal extension. In this section, p53-postivity of the lower one third of the epithelium is estimated at 80%. F) Approximately 75% of the cells of the invasive nests of the SCC are positive for p53. G) p53-negative LS lesion.

Original magnification B-G: 50x

Each histogram (H-J) displays the ploidy condition of the vulvar lesion beneath it. The x-axis in the ploidy histogram represents the integrated optical density (IOD) of the nuclei population, whereas the y-axis identifies the number of nuclei in the specific population. dVIN (H) was aneuploid with a 2A-peak (aneuploid peak) shown next to a 2C-peak (diploid peak). Tetraploidy in an SCC lesion (I), identified with a 4C-peak (tetraploid peak) higher than the 2C-peak. Diploid LS lesion (J).

p53 expression

Subsequently, consecutive tissue slides of the samples described above were subjected to p53 expression analysis by immunohistochemistry (Figure 1E-G). Results are summarised in Table 3. In all 13 dVIN present in composite lesions p53-positivity was confined to the lower one-third of the epithelium (Figure 1E), sometimes with suprabasal extension. In the SCCs, p53 expression (Figure 1F) varied from 5% to 95% (median 75%) positive cells (Table 3). In LS, the percentage of p53-positive (Figure 1G) did not exceed 30%. In the composite lesions that contained dVIN and SCC, the percentage of p53-expressing cells in dVIN was less or comparable to p53 expression by the corresponding SCC in all but one case (*i.e.,* patient XVII in Table 3).

Patient	Diagnosis	Ploidy-status#	p53-expression*
I	LS dVIN	Diploid Diploid	10% 10%
II	LS	Diploid	2%
	dVIN	Diploid	1%
III	dVIN	Diploid	10%
	SCC	Diploid	5%
IV	LS	Diploid	0%
	dVIN	Aneuploid	NA
V	LS	Diploid	10%
	SCC	Aneuploid	75%
VI	SCC	Diploid	40%
VII	dVIN	Aneuploid	75%
	SCC	Aneuploid	80%
VIII	dVIN	Diploid	5%
IX	SCC	Diploid	0%
X	LS	Hypoploid	30%
XI	SCC	Diploid	30%
XII	LS	Diploid	2%
	dVIN	Aneuploid	80%
	SCC	Tetraploid	75%
XIII	dVIN	Diploid	25%
	SCC	Diploid	50%
XIV	SCC	Tetraploid	20%

XV	LS	Diploid	5%
	dVIN	Diploid	50%
	SCC	Aneuploid	80%
XVI	N	Diploid	0%
	dVIN	Diploid	80%
	SCC	Aneuploid	90%
XVII	dVIN	Aneuploid	100%
	SCC	Aneuploid	70%
XVIII	LS	Diploid	30%
	dVIN	Aneuploid	75%
	SCC	Aneuploid	85%
XIX	dVIN	Diploid	90%
	SCC	Aneuploid	95%
XX	LS	Diploid	20%
	SCC	Diploid	65%
XXI	N	Diploid	0%
	SCC	Aneuploid	95%
XXII	SCC	Aneuploid	95%

Table 3 Analysis of DNA ploidy and p53 expression in composite vulvar lesions.

p53 expression and DNA ploidy

In order to determine a possible correlation between the percentage of p53-positive cells and DNA content in a tissue entity, both results were combined. This revealed that the high percentage of p53-expressing cells in the lower one-third of the epithelium (dVIN) or the entire lesion (SCC or LS or normal tissue) significantly correlated with aneuploidy determined by Feulgen staining on isolated cells (Table 4, chi-square=27.6, $p<0.001$). Ninety-three percent (14/15) of all non-diploid lesions displayed more than 70% of p53-positive cells. In contrast, in 92% of the DNA diploid cases (22/24), p53 expression was observed in less than 70%. As such, we subjectively set the cut-off value for high p53 expression at 70%. Despite high p53 expression in two dVIN lesions adjacent to an aneuploid SCC (case XVI and XIX) DNA content was determined to be diploid, whereas the H&E staining displayed the typical features of dVIN.[58] Case XIX is shown in Figure 2. Collectively, these data suggest a positive correlation between high percentage of p53-positve cells and altered DNA content in tissue specimens.

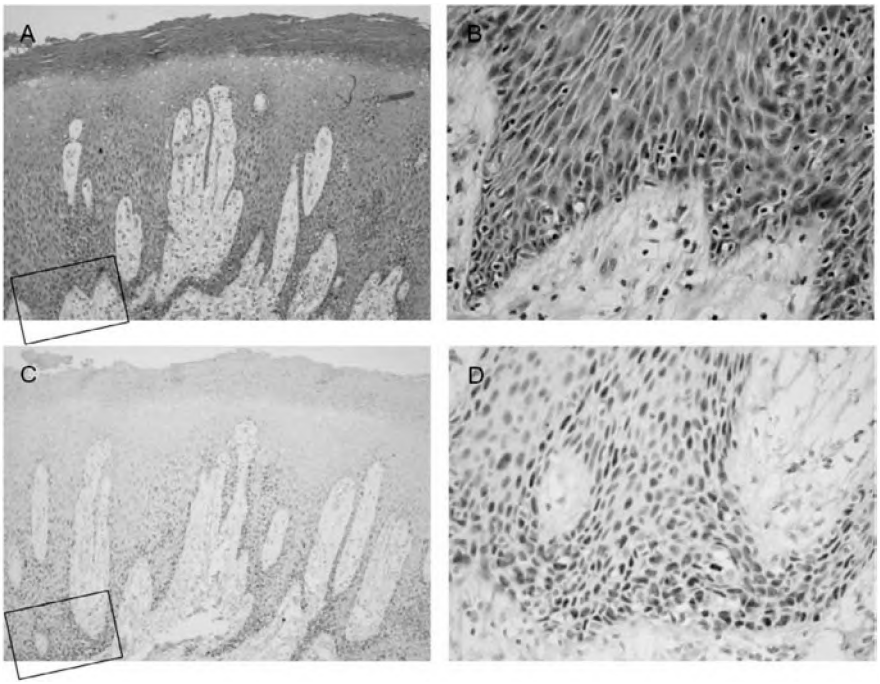

Figure 2 dVIN of case XIX.
A: H&E staining of a diploid dVIN lesion adjacent to an aneuploid carcinoma with elongated and bridging rete ridges. B) Detail of the boxed area in A showing atypia in the basal layer. C) A consecutive tissue section stained for p53 expression demonstrates more than 70% of the cells within the lower one-third component positive for p53. D) Detail of the boxed area in C. Original magnifications: A,C:50x, B,D:200x.

	p53 <70%	p53 ≥70%	Total
Diploid	22	2[a]	24
Non-diploid	1[b]	14	15[*]
Total	23	16	39

Table 4 Ploidy-status versus high p53-expression
[a] both dVIN lesions, b SCC, * p<0.001 (Chi-square test)

Combined ploidy analysis and immunohistochemistry

The above described correlation was derived from two different approaches, which made it impossible to determine the relation between DNA-content

aberration and p53 expression in individual cells. To overcome this problem, we subjected tissue sections to sequential p53 immunohistochemistry and Feulgen DNA staining (Figure 3AB), and measured the staining intensity in between these two staining methods. The integrated optical density (IOD) of p53 in p53-negative stromal nuclei reached a maximum value of 1.6. Visual inspection of p53 staining confirmed that higher values corresponded to p53-positive nuclei. Therefore, p53-positivity was defined as IOD IOD_{p53}>1.6. Mean values of $IOD_{Feulgen}$ of p53-negative nuclei in each tumour specimen is presented as a dotted line (Figure 3C, top panels), which allows easy visualisation of the shift in the distribution of $IOD_{Feulgen}$ towards increased intensity in p53 expressing cells (Figure 3C, middle panels). Comparison of the Feulgen IOD distribution of p53-negative versus p53-positive epithelial nuclei showed a significant difference for all patients assessed ($p<0.001$). When all IOD values for p53 and Feulgen are presented as a scatter plot (Figure 3C, bottom panels), a positive trend between both IODs can be observed in all samples that display high levels of p53 expression (i, ii, and iii). Thus, compared to p53-negative nuclei, p53-positive nuclei displayed an increased DNA content.

DNA ploidy, p53 expression and cell proliferation in solitary dVIN

Expression of p53 was not observed in the superficial layers of dVIN and could imply that aneuploidy is confined to the lower layers. We have therefore analysed the relationship between DNA ploidy and p53 expression in single cells of 6 solitary dVIN samples in more detail and summarised the results in Table 5. The frequency plot of sample 2 is shown in Figure 4. Compared to the DNA content of normal reference cells within the stroma (DNA Index (DI)=1), the lower one-third component of dVIN (basal epithelium) contains nuclei with increased DNA content (DI>1: 27.5%; Figure 4 lower left panel). Because of the effect of truncation of nuclei within (relatively thin) tissue sections, ploidy patterns show asymmetric (right skewed) distribution. In contrast, the upper one-third component (superficial epithelium) only contains 4.7% nuclei with a DI>1. This indicates that the superficial component indeed contains a low percentage of cells with an abnormal DNA content.

In all samples, the basal layers contained significantly more p53+ cells (Table 5A). Similarly, DNA content was significantly higher in the basal component compared to the superficial component for all samples (Table 5A). Strongly increased DNA content in the superficial layer was also observed in two of the six samples analysed (*i.e,.* sample 1 and 5). When comparing DNA content between p53

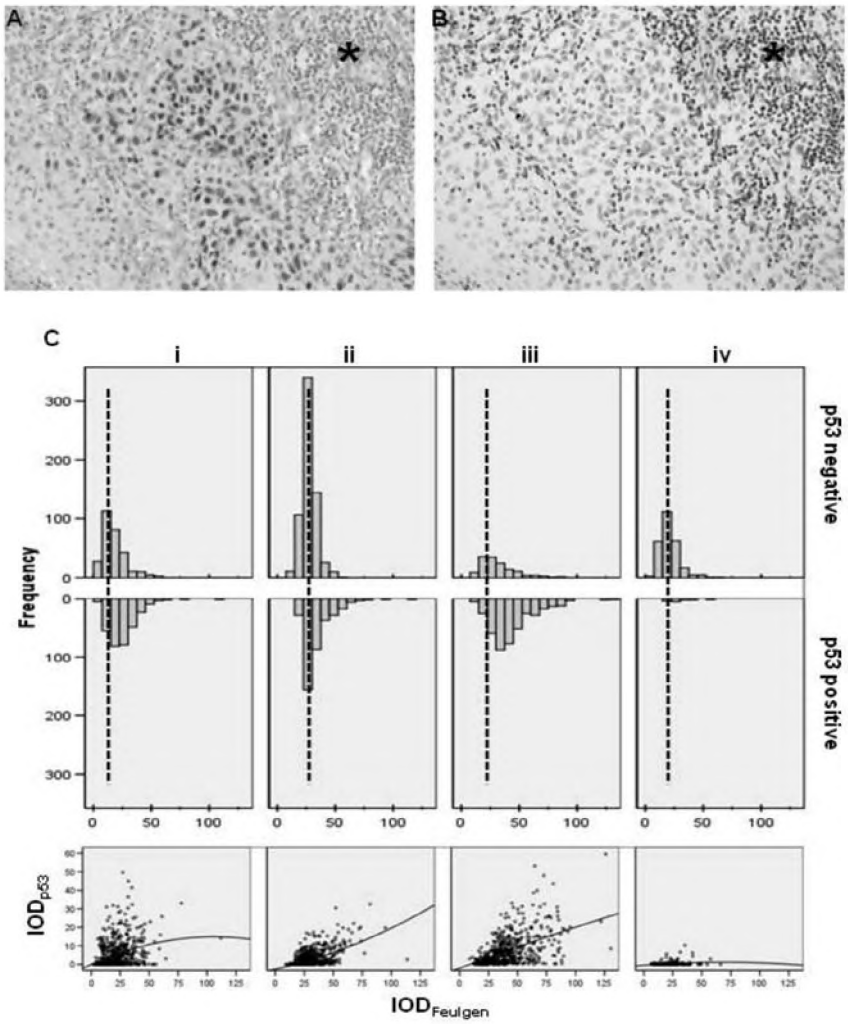

Figure 3 p53 staining intensity correlates with DNA content.
Sequential staining for p53 (A) and DNA (B) was performed on tumour tissue and the respective IOD values determined on selected cells. Yellow asterisks indicate the region of inflammatory cells from which cells were selected to serve as a diploid and p53 negative internal control. The frequency distribution (C) of $IOD_{Feulgen}$ values of p53 negative (IOD_{p53}<1.6, top panels) and p53 positive (IOD_{p53}>1.6, middle panels) nuclei from four different patients (i-iv) are presented as histograms and demonstrates a shift towards increased DNA content when p53 expression is increased. Dotted line: mode $IOD_{Feulgen}$ of p53-negative nuclei. Scatterplots of $IOD_{Feulgen}$ and IOD_{p53} (IOD_{all}>0, bottom panels) reveals a similar positive correlation between p53 staining intensity and DNA content in individual cells. Original magnification A,B:50x

negative and p53 positive cells in the basal compartment, for all cases a strongly significant increase was observed for the number of cells with DI>1 (Table 5B). With the exception of sample 3, such a relationship was not found for cells in the superficial component due to the low number of p53-postive cells.

We have previously shown that the lower compartment of dVIN is highly proliferative[153] and which also contributes to increased DNA content in this study. Therefore, we have analysed MIB-1 expression in the 6 solitary dVIN lesions and found a mean percentage of positive cells of 43.1% (95% CI 18.1% - 68.1%) in the basal compartment compared to 2.1% (95% CI 1.1-4.8%) in the superficial compartment. The low abundance of proliferative cells in relation to the percentage of cells with DI>1% in the superficial component, indicates that DNA content is in part due to aneuploidy.

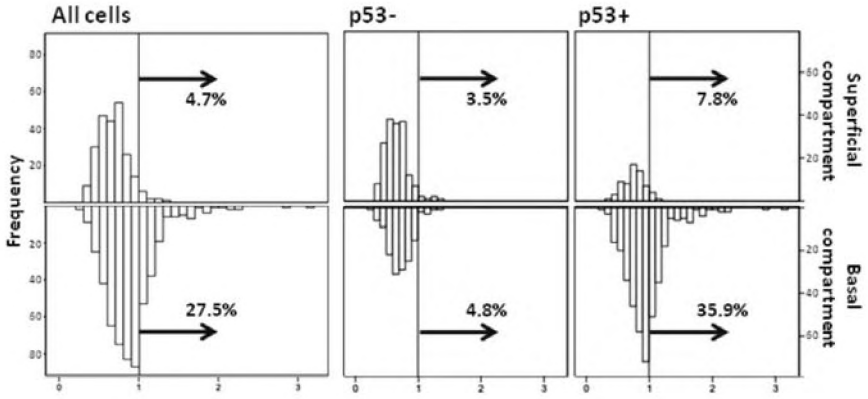

Figure 4 DNA ploidy histograms for one example case (case 2). Upper panels show data from the superficial compartment; lower panels for the basal compartment. Panels on the left show data for all cells, middle and right panels distinguish between p53 negative and p53 positive cells, respectively. The percentages shown inside individual graphs indicate the percentages of cells with DI>1 (indicative for aneuploidy).

Sol dVIN	p53 positive cells (%)			Cells with DI >1* (%)		
	Basal**	Superficial ***	p	Basal	Superficial	p
1	20.0	9.8	0.003	47.4	21.7	<0.001
2	72.9	27.2	<0.001	27.5	4.7	<0.001
3	41.4	9.4	<0.001	3.9	5.2	<0.001
4	30.9	3.3	<0.001	16.6	7.8	0.003
5	4.1	0.6	<0.001	20.7	10.6	<0.001
6	34.9	12.9	<0.001	26.4	8.7	<0.001

Table 5A p53 positivity and DNA content in solitary dVIN.

Sol dVIN	Basal compartment** Percentage cells with DI >1			Superficial compartment*** Percentage cells with DI >1		
1	43.9	61.0	<0.001	20.2	35.7	NS
2	4.8	35.9	<0.001	3.5	7.8	NS
3	1.6	7.1	<0.001	3.5	21.6	<0.001
4	8.4	34.9	<0001	6.9	33.3	NS
5	19.7	44.0	<0.001	10.7	0	NS
6	21.8	35.0	<0.001	9.3	5.0	NS

Table 5B DNA content in relation to p53 expression and localisation within dVIN.
* DI (DNA index)=1: DNA content of normal reference cells within the stroma.
** Basal compartment: the lower one-third component of dVIN.
*** Superficial compartment: the upper one-third component of dVIN.

Discussion

In this study we demonstrate that DNA aneuploidy in vulvar lesions correlates with high expression of p53 in tissue at the cellular level, which has not been described before. Based on a higher percentage of DNA aneuploid cases among dVIN than in LS, we believe that at least a subset of dVIN lesions is a premalignancy with a higher malignant potential compared to LS.

Our finding of a lack of DNA aneuploidy in LS is coherent with the results published by Scurry *et al.*[300] They performed cytomorphometric analysis on 20 LS samples and found that 50% of those samples were diploid, and that the other 50% were

hypoploid. The authors concluded that the occurrence of hypoploidy in LS reflects reduced mitotic activity that relates to a loss of DNA.[300] We only found one out of nine LS lesions to be DNA hypoploid and have described previously that the proliferative activity in LS is comparable to that in other vulvar premalignancies.[153] However, the absence of DNA aneuploidy in all but one hypoploid LS in our study suggests that LS has less malignant potential than dVIN. Recently, Raspollini *et al.* showed that high MIB1 and p53 labelling indices in LS might identify those vulvar LS cases with a high likelihood of evolving into SCC.[39] However, these high-risk LS lesions show histological features that resemble the criteria for dVIN.[36] The results of our study suggest that these so called 'atypical LS' might be non-diploid dVIN lesions.

The atypia in a dVIN lesion is confined to the lower one-third of the epithelium (Figure 1B).[45] However, the entire epithelium of the dVIN lesion was microdissected and used for DNA image cytometry. Therefore, samples were 'contaminated' with p53-negative cells and could have resulted in an underestimation of DNA aneuploidy in dVIN. Furthermore, in situ quantification of DNA demonstrated that increased DNA content in the superficial layers was observed which cannot be explained by mitotic activity as MIB1 positivity in these layers was low. However, it is unlikely that all dVIN lesions would have been DNA aneuploid, as DNA diploid SCCs were found, and as there were no patients with a DNA aneuploid dVIN lesion next to a DNA diploid SCC. From these observations we conclude that aneuploid cells predominantly reside within the basal compartment of dVIN, but that migration towards the superficial compartment can occur.

It remains unclear how p53 could contributes to the development of vulvar SCC from dVIN. Lerma *et al.* concluded that p53 expression is a late event in malignant progression as 72% of their SCCs were DNA aneuploid and only 56% of the SCCs showed p53-overexpression (which was defined as >10% of the tumour cells)[169], but they do not provide simultaneous results on ploidy status and p53 expression in individual lesions. In vulvar SCCs, ploidy has been studied mostly in relation to prognosis and no results on ploidy status of VIN lesions exist. Despite the limited number of cases in our study, the percentage of DNA aneuploidy in vulvar SCC (9/17, 53%) is in line with the percentages found in previous reports of 13% to 83%.[169,295-300] The causal relationship between high p53 expression in vulvar epithelium and DNA aneuploidy remains elusive. Possibly, destabilisation of chromosomes due to centrosome amplification contributes to the cancer susceptibility phenotype associated with mutation (resulting in over-accumulation of p53) or stabilisation of p53.[291] In this study, immunohistochemical detection of

p53 expression could not be correlated with p53 mutation because no antibody can discriminate between either forms.

Rolfe *et al.* found that p53 mutations develop in LS and squamous hyperplasia, and are intrinsic to the clonal evolution that leads to vulvar SCC.[307] Our immunohistochemical data supports this hypothesis, although we were unable to directly correlate immunohistochemical detection of p53 with p53 mutational status due to insufficient high quality DNA in the microdissected tissue. Further correlative studies of p53 expression with mutational status in vulvar dVIN and SCC are warranted.

It has been suggested that loss of function of cell cycle regulator genes influences chromosome segregation, leading to DNA aneuploidy.[291,293,308] The absence of $p16^{INK4A}$ in cells generates supernumerary centrosomes, which can eventually lead to the production of aneuploid daughter cells as a result of unequal segregation of the genomic material during mitosis.[309] In our study, we were unable to correlate the role of $p16^{INK4A}$ and $p14^{ARF}$ to DNA aneuploidy since the staining of $p16^{INK4A}$-expression was not different in DNA diploid and DNA aneuploid cases (data not shown).

In conclusion, a high percentage of p53 expressing cells in vulvar lesions appears to be a surrogate marker for DNA aneuploidy. DNA aneuploidy was observed in vulvar lesions in which more than 70% of the epithelial cells expressed p53. Furthermore, high levels of p53 expression correlate with increased DNA content in individual cells. For dVIN, both adjacent to vulvar SCC and solitary, increased p53 expression and DNA ploidy are found in the basal cells compared to the superficial compartment, although ascending aneuploid cells can also be detected. The observation that the degree of aneuploidy in dVIN is higher compared to LS, but less or equal compared to SCC, suggests that dVIN has a higher malignant potential than LS. The mechanism of oncogenesis and the progression of LS to dVIN remains elusive.

Chapter 7

Specific intraepithelial localisation of mast cells in differentiated vulvar intraepithelial neoplasia and their possible contribution to vulvar squamous cell carcinoma development

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Abstract

Aim: The aetiology of vulvar squamous cell carcinomas (SCC) that are not causally associated with high risk human papillomavirus remains largely elusive. The aim of this study was to analyse the inflammatory response in its presumed precursor lesions, lichen sclerosus (LS) and differentiated vulvar intraepithelial neoplasia (dVIN), and provide evidence that dVIN is a likely precursor of vulvar SCC.

Methods and results: Immunohistochemical analyses for CD4, CD8, CD20, CD68, S100, and tryptase positive immune cells was performed and quantified in LS (n=7), dVIN (n=19), SCC (n=11), and normal vulvar tissue (n=8). The subepithelial inflammatory response in dVIN and SCC was comparable, but absent in LS. Abundant intraepithelial mast cells were observed in dVIN only, and was confirmed by electron microscopy, toluidine blue staining and cKIT expression. Adjacent keratinocytes displayed increased proliferation as determined by MIB-1 positivity. Electron microscopy revealed intraepithelial mast cell degranulation. Intraepithelial mast cells were not or infrequently observed in vulvar hyperplasia (n=13), condylomata acuminata (n=5), keratinocytic intraepidermal neoplasia of sun-exposed skin (KIN, n=15), epidermal hyperplasia of head and neck (n=12), and psoriasis (n=3).

Conclusions: These data indicate that dVIN can be recognized by intraepithelial mast cells and that this might promote the progression of dVIN to SCC.

Introduction

Vulvar squamous cell carcinoma (SCC) accounts for approximately 3-5% of all gynaecological malignancies and 1-2% of all carcinomas in women, with an incidence rate of 1-2/100.000.[10,258] Vulvar SCC can develop via two different oncogenic pathways.[1,2] Approximately 20% of all vulvar SCCs are associated with usual vulvar intraepidermal neoplasia (uVIN) and are caused by an infection with oncogenic Human Papillomavirus (HPV).[280] The aetiology of HPV-negative vulvar SCCs is unknown. These tumours commonly arise in a background of lichen sclerosus (LS).[2] Differentiated vulvar intraepidermal neoplasia (dVIN) is often found adjacent to this type of tumour. Cellular atypia and architectural disarray in dVIN is confined to the basal and suprabasal layers.[1-3,45] Intriguingly, the normal keratinocyte differentiation programme appears intact as the development of a terminally differentiating epidermis is not impaired and is thus a difficult to recognise entity by non-expert pathologists. Solitary dVIN lesions are rare,[282] which might be caused by under diagnosis due to its histological resemblance to epidermal hyperplasia and/or by a relatively brief intraepithelial phase prior to invasive carcinoma.[5,154,156] Alike SCC, but in contrast to LS, an often strong inflammatory response can be observed directly at the epidermal-dermal junction of dVIN. These observations contributed to our hypothesis that the development of HPV-negative SCC follows a gradual progression from LS to dVIN to carcinoma.

Progression of a premalignant lesion towards an invasive tumour concurs with a persistent stromal response. Recruitment of immune cells can be observed in or around developing neoplasms that regulate different aspects of tumour development (*e.g.,* fibroblast activation and the early angiogenic response) by providing amongst others soluble growth and matrix remodelling enzymes.[310] This stromal response creates a microenvironment that can support neoplastic progression.[310-312] Recruitment of B-cells and deposition of immunoglobulins appear key events in the initial stromal response towards a developing neoplasm.[312] Similar to a wound healing response, mast cells are among the first cells of the innate immune response at the damaged site. Under normal conditions mast cells are pre-stationed in tissues and continuously monitor their microenvironment for signs of distress. When tissue homeostasis is disturbed, mast cells instantly release soluble mediators that induce mobilisation and infiltration of immune cells (*e.g.,* macrophages and T-cells) into damaged tissue as well as activate vascular and fibroblast responses in order to orchestrate elimination of invading

organisms and initiate local tissue repair.[313] On the other hand, mast cells accumulate at the periphery of premalignancies, where they initiate the early events of neoplastic progression, *i.e.,* fibroblast activation, extracellular matrix remodelling and activation of angiogenesis.[314,315]

In order to find similarities and differences in the composition of the inflammatory response in vulvar premalignant stages (LS and dVIN) and vulvar SCC that can support our hypothesis, we have quantified a subset of inflammatory cells, *i.e.,* T-cells (CD4 and CD8), B-cells, dendritic cells, macrophages and mast cells. These analyses revealed a similarity between the composition of the inflammatory response in dVIN and SCC. Furthermore, we demonstrate intraepithelial localisation of mast cells in dVIN, which was not observed in other benign or premalignant epidermal hyperplasia nor in vulvar SCC. This suggests that mast cells might be involved in malignant conversion of dVIN to SCC.

Material and Methods

Human tissues

Formaldehyde-fixed and paraffin-embedded tissue containing normal vulvar tissue (n=8), LS (n=7), dVIN (n=19), HPV-negative vulvar SCC (n=11), uVIN (n=8), vulvar hyperplasia (n=13), condylomata acuminata (n=5), keratinocytic intraepidermal neoplasia of sun-exposed skin (KIN, n=15), epidermal hyperplasia of head and neck (n=12), and psoriasis (n=3) were obtained from the pathology archives of the Radboud University Nijmegen Medical Centre. In this set of vulvar samples, all SCCs arose in a background of LS and contained dVIN at its border. Three out of 19 dVIN were solitary and not associated with a SCC. For electron microscopy, fresh 4 mm biopsies of clinical suspect dVIN lesions were obtained from vulvectomies and split in two. One half was processed for H&E staining and the other fixed in 2.5% glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer (pH 7.4). All specimens were re-evaluated by expert pathologists (JB and KMH). Tissues were obtained according to local ethical guidelines and approved by the local regulatory committee.

Immunohistochemistry

Tissue sections (4 µm) on Superfrost slides (Menzel-Gläser, Braunschweig, Germany) were deparaffinised in xylene, rehydrated through graded alcohols and rinsed three times in phosphate-buffered saline (PBS; pH 7.4) for 10 minutes.

Following a sodium-citrate antigen retrieval step (0.01 M, pH 6.0, 95°C, 10 minutes), tissue sections were pre-incubated with 20% normal goat serum in PBS and subsequently incubated overnight at 4°C with antibodies towards CD8 (mouse monoclonal, clone C8/144B, 1/80, Dako, Glostrup Denmark), CD20 (mouse monoclonal, clone L26, 1/300, Dako), CD68 (mouse monoclonal, clone KP1, 1/4000, Dako), S100 (rabbit polyclonal, 1/2000, Dako), or tryptase (mouse monoclonal clone AA1, 1/400, Dako). For detection of CD4 (mouse monoclonal, clone BC/1F6, 1/10, Biocare Medical, Concord CA, USA), antigen retrieval was performed by incubating tissues in TRIS-buffered EDTA (1mM, pH 9.0) at 95°C for 10 minutes. All antibodies were diluted in PBS containing 1% bovine serum albumine (BSA). Slides were rinsed in PBS for 10 minutes and incubated with biotinylated anti-rabbit IgG or anti-mouse IgG (Vector, Burlingame, CA) for 30 minutes at room temperature (RT). A biotin-avidin peroxidase or alkaline phosphatase complex was generated according to standard procedures (Vector). Peroxidase was visualised with 3-amino-9-ethylcarbazole (AEC, ScyTek, Logan, UT), or 3,3'-diaminobenzidine (DAB, Sigma-Aldrich) and briefly counterstained with Mayer's haematoxylin. Slides were dried and mounted with Permount (Fisher Scientifc, Fairlawn, NJ, USA). Negative controls (buffer only) were included in each analysis.

For Ki67/Tryptase double staining, the sodium-citrate antigen retrieval step and incubation with normal serum was followed by incubation with monoclonal mouse-anti Ki67 (clone MIB-1, 1:100, Dako) overnight at 4°C. After washing the slides with PBS for 10 minutes, tissue sections were incubated with biotinylated anti-mouse IgG (Vector), an avidin-peroxidase complex (Vector) generated and visualised with DAB. Subsequently, the tryptase staining protocol (AEC) was performed.

Immunofluorescence

After deparaffinisation and rehydration, tissues were left in PBS overnight at 4°C. Following sodium-citrate antigen retrieval, tissues were incubated with the mouse monoclonal antibody towards tryptase (1/400) and rabbit polyclonal antibody towards CD117 (1/200, Dako) overnight at 4°C. After extensive wash steps with PBS, tissues were incubated with Alexa488-conjugated goat-anti-rabbit (Invitrogen, 1/200) for 30 minutes TRYP at room temperature in the dark. Following extensive wash steps with PBS, tissues were incubated with Alexa594-conjugated rabbit-anti-mouse CD117 (Invitrogen, 1/200, 30 minutes room temperature), washed extensively with PBS, incubated with 4,6'-diamino-2-phenylindole (DAPI, 80 ng/ml in PBS, 30 seconds), and mounted with Vectashield (Vector).

Toluidine blue

The toluidine blue staining protocol for mast cells was performed according to standard procedures. Briefly, following deparaffinisation and rehydration, tissues were incubated 0.1% Toluidine blue (Sigma-Aldrich) for 2-3 minutes, rinsed in running tap water for 2 minutes, quickly dehydrated through graded alcohols and xylene, and mounted with Permount.

Cell quantification

Specifically stained cells within the epithelium and adjacent stoma were counted manually by two observers independently (HvdN, LvK) in 4-8 non-overlapping fields at 40x magnification and expressed as a percentage of all inflammatory cells in the respective tissue component. The sub-epithelial component (*i.e.,* stroma) was defined as the dermal component within 100-150 μm from the epidermal-dermal interface in which a direct interaction of inflammatory cells with the epidermal component is likely. For LS, this implies that the characteristic band-like infiltrate under the broad hyalinised zone of superficial dermis was not included in the analysis.

In order to determine the amount of tryptase-positive cells in various epithelia, cells were counted manually in 4-8 non-overlapping fields of which the epidermal surface area was measured using KS400 image analysis software (Karl Zeiss, Weesp, The Netherlands).[153,316] Subsequently, epidermal mast cells were expressed as number of cells per mm^2.

Electron microscopy

Processing of tissue samples for electron microscopy was performed as described previously.[317] After presence of dVIN was confirmed in H&E, the glutaraldehyde-fixed tissue fragments were postfixed in cacodylate-buffered 1% OsO_4 for 2 hours, dehydrated, and embedded in Epon 812 (Merck, Darmstadt, Germany). Ultrathin sections were cut on an ultratome (Leica, Reichert Ultracuts, Wien, Austria), and contrasted with 4% uranyl acetate for 45 min and subsequently with lead citrate for 4 min at room temperature. Sections were examined in a Jeol 1200 EX2 electron microscope (JEOL, Tokyo, Japan).

Statistical analysis

Data were analysed using SPSS software (version 16.0.1 for Windows, SPSS) and box plots generated. Because the percentages of cells in stroma and epithelium, and mast cells per mm^2 epithelium were not normally distributed, nonparametric

Mann-Whitney tests were performed to compare means between groups. A probability value of <5% was considered statistically significant.

Results

Profiling of the inflammatory response

Immunohistochemical analyses were performed to determine the localisation and amount of T-helper cells (CD4), cytotoxic T-cells (CD8), B-cells (CD20), macrophages (CD68), dendritic cells (S100), and mast cells (tryptase) (Figure 1) in LS (n=6), dVIN (n=7) and HPV-negative vulvar SCC (n=5)

Whereas the mean amount of all inflammatory cells in the sub-epidermal component of LS is low (11 ± 3 cells per high power field (HPF, average of 4-8 non-overlapping fields at 40x), it is highly increased in both dVIN and SCC (88 ± 31 and 84 ± 32 cells per HPF, respectively). To gain insight in similarities and differences in the composition of these infiltrates, specific cell types were expressed as mean percentage of the total amount of cells (Figure 2A). Within the stromal component, the percentage of CD4-positive cells in SCC was significantly increased compared to LS and dVIN (p=0.04, Mann-Whitney, 2-tailed), and indicated an altered CD4:CD8 ratio in SCC compared to LS and dVIN.

In LS, CD20-positive B-cells were localised to the patchy infiltrate in the mid dermal region, whereas these were also diffusely dispersed within the subepidermal dermis in dVIN and SCC. In dVIN, the percentage of B-cells in sub-epidermal infiltrate of dVIN was 2-fold increased (p=0.04) compared to that of SCC. Furthermore, an increased number of macrophages was observed in LS compared to dVIN and SCC (p=0.01).

Within the epithelium of LS, a higher percentage of CD8-positive cells was observed compared to dVIN (p=0.2) and SCC (p=0.03). Furthermore, the LS epithelium contained significantly more dendritic cells than dVIN (p=0.04). Intriguingly, a high percentage of mast cells were detected within the epithelium of dVIN, not in LS (p=0.005) and infrequently in SCC (p=0.01). Intra-epithelial macrophages were observed in dVIN and SCC, but not in LS (p=0.01).

Vulvar hyperplasia without cellular or nuclear atypia does not confer a risk for neoplastic progression. We therefore compared the inflammatory response in vulvar hyperplasia with dVIN (Figure 2B).

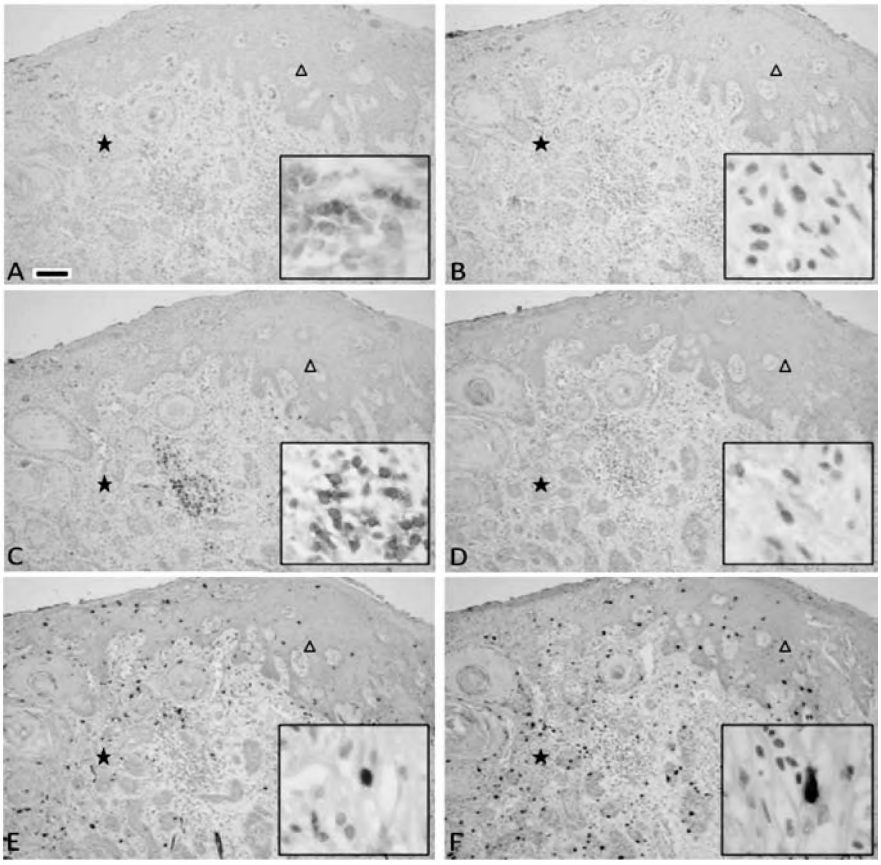

Figure 1 Detection of immune cells in vulvar lesions.
A diffuse distribution of CD4-positive T-helper cell (A) and CD8-positive cytotoxic T-cells (B) within the stromal component SCC (*) and adjacent dVIN (D) was observed. CD20-positive B-cells (C) predominantly localised in clusters. D68-positve macrophages (D) were also found in these clusters and throughout the stromal component. S100-positive dendritic cells (E) and tryptase-positive mast cells (F) were detected within the stroma and epithelium.
Inserts in A-F: high power magnification of specific staining. Scale bar A: 100 nm.

No significant differences were observed in the composition of the stromal inflammatory response, but the ratio CD4/CD8 was smaller in vulvar hyperplasia compared to dVIN, albeit not significantly different (0.5±0.2 and 0.9±0.5, respectively). The epithelial infiltrate in hyperplasia consisted predominantly out

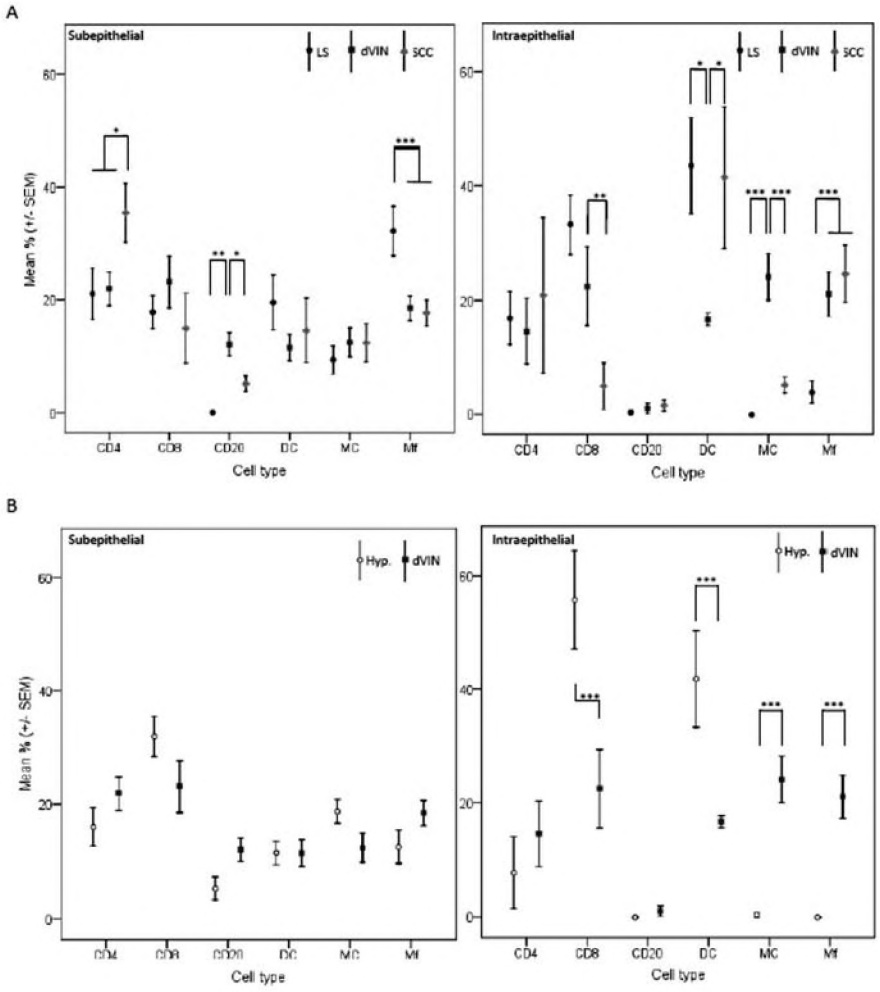

Figure 2

A. Error bar plot of the percentage (mean ± SEM) of T-helper cells (CD4), cytotoxic T-cells (CD8), B-cells (CD20), dendritic cells (DC), mast cells (MC), and macrophages (Mf) within the sub-epidermal and intraepithelial inflammatory infiltrate of LS (n=7), dVIN (n=19) and SCC (n=11). * p≤0.05 (Mann-Whitney); ** p≤0.03; *** p≤0.01.

B. Similar error bar plot, now comparing the inflammatory response in benign vulvar hyperplasia (n=13) and premalignant dVIN (n=19).

of CD8 positive cells (55%±9), whereas these cells contributed to 22%±9 of stromal epithelial cells in dVIN. However, no differences were observed in absolute number of CD8 cells per high power field. Strikingly, cells of the innate immune response (mast cells, and macrophages) were detected in dVIN, but not in hyperplasia.

To validate that the tryptase-positive cells were indeed mast cells, additional stainings were performed. An immunofluorescent analysis for tryptase and the mast cell marker cKIT (CD117) revealed that all tryptase-positive cells within the stroma and epithelium of dVIN also expressed cKIT (Figure 3A-B). Using toluidine

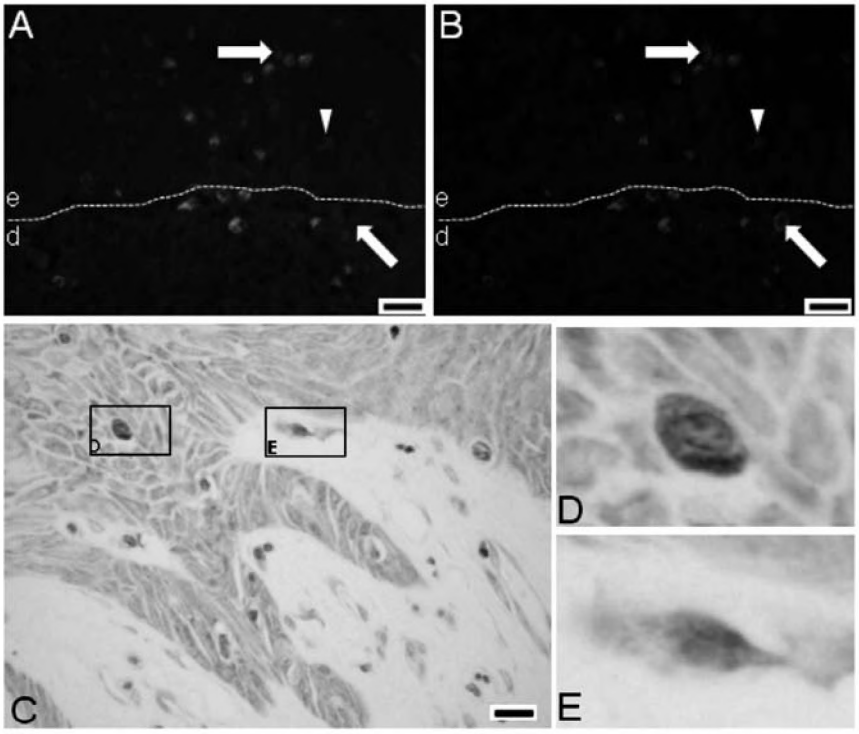

Figure 3 Tryptase-positive cells within the epithelium are mast cells.
Double immunofluorescence for tryptase (A, green) and the mast cells marker CD117 (B, red) demonstrated that all tryptase-positive cells express CD117. Conversely, CD117-positive but tryptase-negative cells (arrows) can be observed. Toluidine blue staining of a dVIN (C) demonstrated the typical reddish granual appearance of intraepithelial (D) and stromal (E) mast cells. Dotted line in D and E indicates the basement membrane. Arrowhead indicates a mitosis. e: epithelium, d: dermis. Scale bar A: 12.5 nm; D, E: 25nm.

blue, intraepithelial mast cells were readily identified by their typical granular reddish appearance (Figure 3C-D). The amount of toluidine blue cells was approximately 50% less compared to tryptase immunohistochemistry (data not shown) and suggested mast cell degranulation. Collectively, these data demonstrated intraepithelial localisation of mast cells in dVIN.

High infiltration of mast cells in dVIN, but not in other cutaneous epidermal abnormalities

The identification of mast cells within the epithelium of dVIN suggested that this could be a marker for this premalignancy. Therefore, we have analysed the amount of intraepithelial mast cells per mm^2 in different types of epidermal abnormalities (Figure 4).

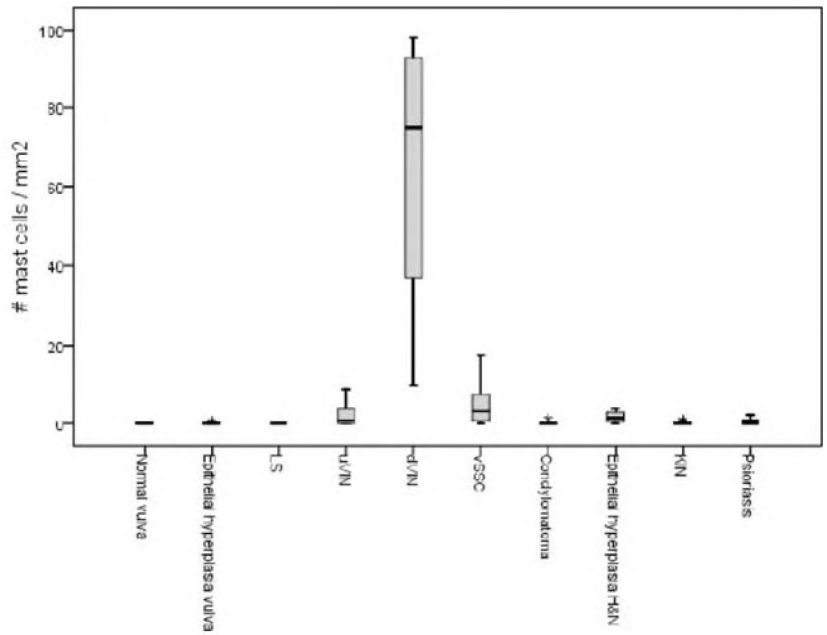

Figure 4 Quantification of intraepithelial mast cells in cutaneous epithelia.
Box plot of the amount of mast cells per mm2 epithelium in vulvar epithelium (normal, n=8; hyperplasia, n=13; LS, n=7; usual VIN (uVIN), n=8; dVIN, n=19; vulvar SCC, n=11; condylomata acuminata, n=5), hyperplasia of head and neck (H&N) epithelium (n=12), keratinocytic intraepidermal hyperplasia of skin (KIN, n=15), and psoriasis (n=3). *** $p<0.001$, Mann-Whitney compared to any other entity.

Intraepithelial mast cells were not detected in normal vulvar epithelium (n=8) and LS (n=7). Mast cells were detected in two of 13 epidermal hyperplasias, but the density in these two cases was very low (0.05±0.02/mm^2). Mast cells were not always detected in HPV-associated uVIN (n=8, 2.5±3.2/mm^2), vulvar SCC (n=11, 5.7±6.3/mm^2) and condylomata acuminata (n=5, 0.3±0.7/mm^2). In contrast, a high number of intraepithelial mast cells was observed in all dVIN, albeit at different densities (n=19, 64.1±30.1/mm^2; $p<0.001$, Mann-Whitney test compared to any other entity). In dVIN, 80% of the intraepithelial mast cells were confined to the first three layers of basal epithelium (Figure 5). In non-vulvar epidermal abnormalities, a limited number of intraepithelial mast cells was infrequently detected in psoriasis (n=3, 0.8±1.7/mm^2), epidermal hyperplasia of the head and

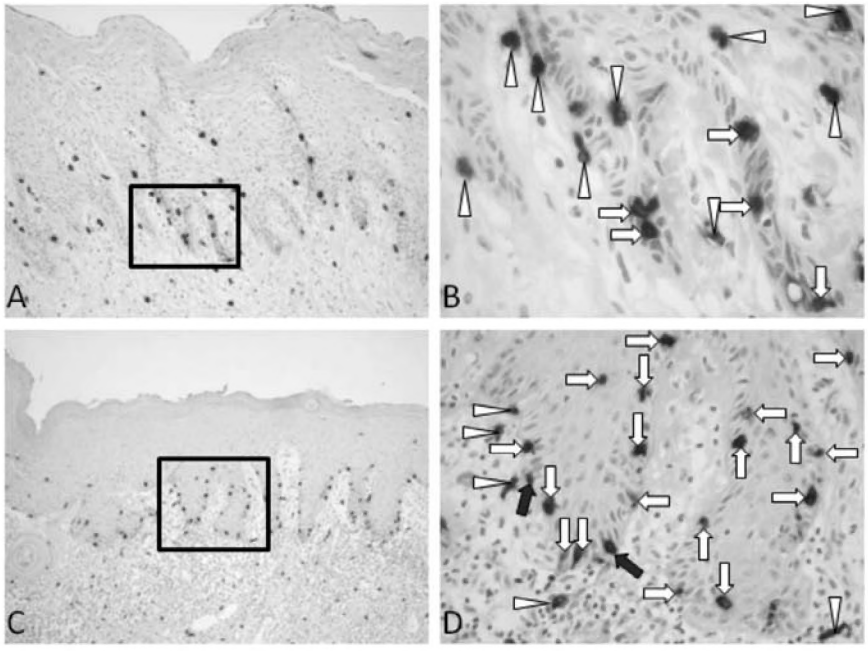

Figure 5 Intraepithelial mast cell in dVIN predominantly localise to the basal compartment. Representative images of two different dVIN lesions (A, C) containing a high amount of tryptase-positive intraepithelial mast cells (brown) that predominantly localise to the basal component. B and D are high power magnifications of the areas indicated in A and C, respectively. Intraepithelial (arrows) and stromal (arrowheads) mast cells are indicated. Scale bar A, C: 100 nm; B, D: 25 nm.

neck (n=12, 1.9±1.6/mm^2), and cutaneous keratinocytic intraepidermal neoplasia (KIN, n=15, 0.2±0.4/mm^2).

Intraepithelial mast cells and keratinocyte proliferation

Degranulation of mast cells can stimulate cell proliferation. Intriguingly, intraepithelial mast cell staining was accompanied by a focal and diffuse tryptase staining of keratinocytes suggesting release of proliferation–inducing tryptase. Occasionally, we observed keratinocyte mitosis adjacent to intraepithelial mast cells (Figure 6A), but could not demonstrate a significant correlation between the abundance of mitotic figures and presence of mast cells (data not shown). To investigate this in more detail, a double immunohistochemical staining for tryptase and the proliferation marker Ki67 was performed using the monoclonal antibody MIB-1. The latter identifies all cells that are not in the G0 phase of the cell cycle, and thus identifies all proliferative cells in addition to those in M-phase. These analyses indicated MIB1-positive keratinocytes in close proximity of intraepithelial mast cells (Figure 6B & C) and supports previous observations that mast cells can increase keratinocyte proliferation.[318-320] However, MIB-1 positive keratinocytes also were observed in the absence of mast cells (Figure 6D), indicating that intraepithelial localisation of mast cells is not a prerequisite for keratinocyte proliferation.

Intraepithelial mast cells are predominantly degranulated

The observations that the intensity of tryptase staining of intraepithelial mast cells was reduced compared to that of stromal mast cells (Figure 6C) and that a local diffuse tryptase staining of the epidermis could be observed (Figure 6A) suggested mast cell degranulation. To confirm that the content of the granules of intraepithelial mast cells could be released, electron microscopy was performed on 5 dVIN biopsies to study the stromal and intraepithelial mast cells in dVIN biopsies in detail. Dermal mast cells were readily identified by their characteristic granules with different electron densities. (Figure 7A). In contrast, the electron density of intraepithelial mast cell granules (Figure 7B-E) was strongly reduced and suggested a release of their contents into the epidermal environment. The electron micrographs further indicated that the basement membrane of the epithelium infiltrated with mast cells remained intact (Figure 7E) and is in line with the observation that migration of inflammatory cells across basement membranes is proteolysis independent.[321]

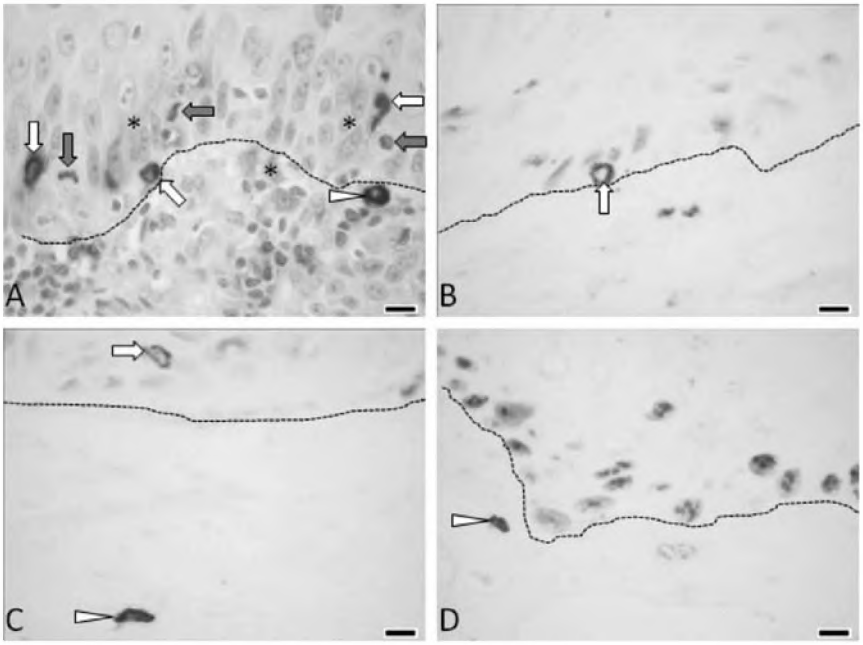

Figure 6 Increased keratinocyte proliferation adjacent to intraepithelial mast cells. dVIN (A) in which mitotic figures (blue arrows) can be observed adjacent to DAB-stained tryptase-positive intraepithelial mast cells (white arrows). A dermal mast cells is indicated by an arrow head. Diffuse tryptase staining in close proximity of mast cells can be detected (asterisks). Sequential staining for Ki67 (brown) and tryptase (red) (B-D) revealed proliferating (brown) keratinocytes adjacent to mast cells (B,C), but intraepithelial localisation of mast cells is not a prerequisite for high keratinocyte proliferation (D). In general, the intensity of tryptase staining of dermal mast cells (arrowheads) is stronger compared to that of intraepithelial mast cells (arrows). Counterstaining was not performed. Dotted line in B-D indicates basement membrane. Scale bar A-D: 12.5 nm.

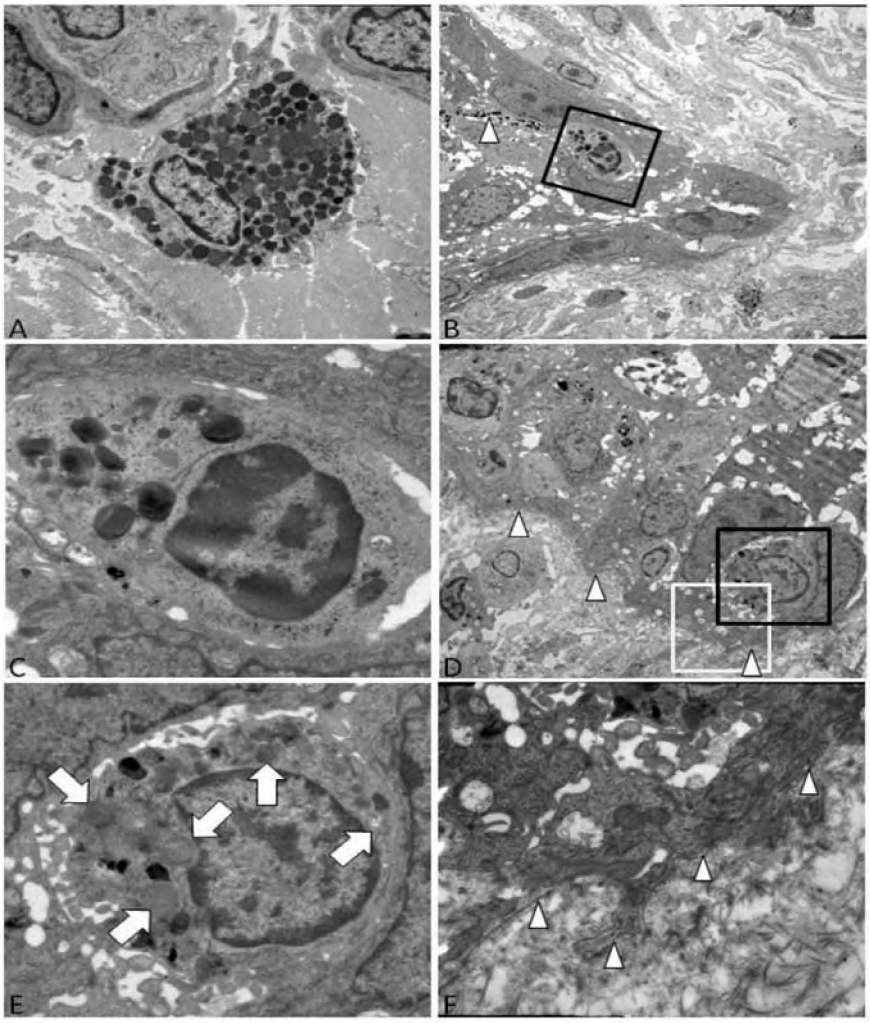

Figure 7 The number of electron-dense granules of intraepithelial mast cells is reduced. Electron micrographs of a dermal (A) and intraepithelial (B-E) mast cell. The characteristic scala of granules with varying electron density can be observed in a dermal mast cell (A). In contrast, the granular content of an intraepithelial mast cell (boxed area in B) is strikingly different (C, enlargement of indicated area in B). Arrow head in B indicates melanin deposits. Similarly, the intraepithelial mast cell in panel D (black box), does contain granules (E) albeit not electron dense (arrows). The basement membrane (arrow heads) in close proximity of the intraepithelial mast cell (F, white box in D) appears intact (arrow heads). Original magnifications A: 6000x; B, D: 2000x, C, E: 6000x; F:10.000x.

Discussion

The aetiology of HPV-independent vulvar SCC is unknown. The suggested brief intraepithelial phase of dVIN before progression to invasive carcinoma[5,154,156] urges for the identification of cellular and/or molecular markers and better understanding of its biology. By profiling the inflammatory response in LS, dVIN and SCC we have started to provide support for the gradual progression model that states that SCC evolves from dVIN. Furthermore, we demonstrate that intraepithelial localisation of mast cells could be marker for dVIN, and that this could promote keratinocyte proliferation.

Mast cells within the epithelium of dVIN are readily observed by immunohistochemical staining for mast cell tryptase. These cells also proved positive for the mast cell marker cKIT/CD117. However, less intraepithelial mast cells were observed when dVIN lesions were stained with toluidin blue. Since this staining highlights the content of mast cell granules, and electron microscopic analysis of intraepithelial mast cells revealed a reduced electron density of these granules, it allowed to conclude that mast cell degranulation occurs within the epidermis of dVIN. Our findings that mast cells hardly infiltrate the proliferative epithelium of vulvar, cutaneous and mucosal hyperplasia, condylomata acuminata, uVIN and SCC indicates that the epidermal localisation of mast cells in dVIN is not merely a marker of epithelial proliferation.

Degranulation of mast cells can have a wide variety of effects in skin. The contribution of dermal mast cells to cutaneous malignancies has been well described.[322] Their infiltration into the epithelium and subsequent degranulation potentially alters keratinocyte homeostasis. Mast cell tryptase is a mitogen for keratinocytes. It can activate protease-activated receptor (PAR)-2 on keratinocytes and increase their proliferation *in vitro*.[319,320] In atopic dermatitis, mast cells likewise infiltrate the intraepithelial component and correlate with increased keratinocyte proliferation.[318] Furthermore, mast cell chymase can activate pro-IL18 that is produced by keratinocytes.[323,324] In transgenic mice, high levels of IL18 in the epithelium contributes to the development of atopic dermatitis-like inflammatory skin lesions.[325] Once activated, keratinocytes release inflammatory, chemotactic and growth promoting cytokines, and cytokines regulating humoral and cellular immunity.[326] The subsequent strong inflammatory response alters tissue homeostasis and can promote malignant progression of initiated cells.[311]

It remains elusive why mast cells migrate into the epithelium of dVIN, but not as prominent in other vulvar epidermal abnormalities. We did not find a correlation

between the amount of stromal and intraepithelial mast cells, which suggests that mast cells within dVIN is not a spill-over of an increased concentration of stromal mast cells. Furthermore, the amount of stromal mast cells in SCC is comparable to that of dVIN but migration into the epidermal component of the tumour is limited. This indicates a preferential migration of mast cells into dVIN. Mast cells have also been observed in the epidermis of atopic dermatitis,[318] bronchial epithelium in asthma,[327] and mucosa of chronic and stress-induces inflammatory bowel disease.[328] In all of these examples, recruitment and activation of mast cells depends on the presence of an irritating agent, from which could be concluded that also for dVIN a noxe could exist. However, there is no evidence of the presence of, *e.g.*, a microorganism or irritating agent that could be involved in the aetiology of dVIN.

dVIN and its associated SCCs commonly arise in the background of LS. Also for LS, the cause remains elusive.[258] Although LS can be asymptomatic, most patients experience itching and burning sensations. The latter provokes scratching and subsequent damaging of the already atrophic skin. Clinically, these 'unstable' LS often present as erythematic lesions. Histological examination of these lesions frequently shows solitary dVIN or micro-invasive SCC,[258] although exact numbers are unknown. This raises the possibility that scratching and damaged skin might induce mast cell recruitment and activation that leads to the induction of a chronic inflammatory response that can support neoplastic progression of initiated cells.

In summary, we have demonstrated that mast cells specifically infiltrate the epidermal component of dVIN and mainly localise to the basal and suprabasal layers in which atypia is found. Our data suggest that identification of intraepithelial mast cells by tryptase immunohistochemistry aids in diagnosis dVIN and requires further studies. The majority of intraepithelial mast cells have released the content of their granules. This appears to correlate with increased proliferation of the adjacent keratinocytes and suggest that mast cells could promote the neoplastic progression of dVIN towards SCC. The mechanism by which mast cells are triggered to infiltrate the epidermal component of dVIN and why they are not found in the adjacent SCC remains elusive.

Acknowledgements

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Chapter 8

General discussion

Is dVIN a border phenomenon or a precursor lesion?

Because dVIN is rarely seen in its solitary form, is has been argued that dVIN is a border phenomenon of HPV-negative vulvar SCC, instead of being a precursor lesion.[42] In Chapter 4 we have shown that although dVIN was seldom diagnosed in its solitary form, it does occur. Solitary dVIN was more often diagnosed in the last three years of the study, reflecting more knowledge of pathologists and clinicians to recognize this lesion. Moreover, in Chapter 5 we describe that, after revision, solitary dVIN is more often found in biopsies previously diagnosed as LS in patients that later developed a vulvar SCC. Based on these studies, we have concluded that dVIN is not solely a border phenomenon of the HPV-negative vulvar SCC, but at least also a precursor lesion.

Does dVIN suffer from underdiagnosis or from rarity?

dVIN is a difficult diagnosis, both clinically and histopathologically. Clinically, it appears mostly as subtle erosive lesions in a background of LS. In LS, areas of erythema often occur, which are difficult to discern from dVIN. Histopathologically, the recognition of dVIN is hindered by a high degree of cellular differentiation combined with an absence of widespread architectural disarray, nuclear polymorphism and diffuse nuclear atypia.[4] The atypia in dVIN is strictly confined to the basal and suprabasal layers of the epithelium.[4,58] In Chapter 4 we describe that of all VIN lesions diagnosed in the Netherlands between 1992 and 2005, only a minority were dVIN, in comparison to the HPV induced usual VIN. Its incidence had increased in the more recent years, and because it is unlikely that dVIN is a new lesion that somehow developed in the past few years, this is merely a reflection of more knowledge about dVIN of both gynaecologists and pathologists. In Chapter 3, we demonstrate that dVIN is found adjacent to 80% of 130 vulvar SCCs, which strongly reflects the gap between the incidence of the solitary diagnosis and the incidence of dVIN adjacent to a vulvar SCC. We presume that of all lesions previously diagnosed as benign dermatosis (such as LS) or epithelial hyperplasia, a large proportion would currently be diagnosed as dVIN. Indeed, in Chapter 5 we have revised LS slides of patients who were later diagnosed with vulvar SCC, and it appeared that dVIN was present in 42% of these patients, previously diagnosed as having LS. From this study, we have hypothesized that dVIN will be more often diagnosed as a solitary lesion with the current well-defined histopathological features and acquaintance of the lesion by both gynaecologists and pathologists.

Still, there is a large gap between the number of solitary dVIN lesions and dVIN lesions found adjacent to vulvar SCC. Besides the difficult diagnosis, this could be attributed to the short intraepithelial phase of dVIN. In Chapter 4 we found that the time between the diagnosis of dVIN and subsequent vulvar SCC was 22.8 months. This was the first time that this assumed 'short intraepithelial phase' was quantified. In Chapter 5, the time from dVIN to SCC was comparable with that study, being 27.5 months. In the upcoming years, the gap between solitary dVIN and dVIN adjacent to vulvar SCC will probably diminish. This may be achieved by paying more attention to dVIN by both clinicians and pathologists and this may eventually lead to more insight in the true biological behaviour of dVIN and an earlier detection of dVIN, which, after proper treatment (surgical excision), may lead to a decrease of HPV-negative vulvar SCCs. This early detection will be of great importance in the future because the incidence of HPV-negative vulvar SCC is thought to increase in the upcoming years due to the ageing of society. Most women with vulvar SCC present without a history of LS, however, these patients often suffer from either asymptomatic or unnoticed LS,[29,31,33-35] leading to a delay in diagnosis by both patients and doctors. These patients never have been treated, which may suggest, although evidence is lacking, that treatment with topical corticosteroids may have some preventive effect in vulvar SCC development.[123] The anti-inflammatory effectiveness of these topical corticosteroids may be the explanation for this possible effect.

In conclusion, dVIN is seldom diagnosed as a solitary lesion, but this is merely attributable to underdiagnosis rather than to rarity.

Is LS or dVIN the direct precursor of HPV-negative vulvar SCC?

Because LS as well as dVIN are seen adjacent to HPV-negative vulvar SCC, both are considered premalignant lesions. In what way vulvar SCC arises from LS and dVIN still has to be clarified. Review of the published literature on LS and SCC as performed in Chapter 2 illustrates conspicuous associations between these two entities. Other itchy conditions (*e.g.,* eczema, psoriasis) do not show such a relation with vulvar carcinogenesis. Other malignancies than SCCs (*e.g.,* basal cell carcinoma) are rarely associated with LS. Remarkably, LS has only a link with cancer when present on the genital area (vulva, penis[329]); only two case reports have been published about skin cancer arising in extragenital LS.[330,331] Carlson found a compiled frequency of vulvar SCC arising in LS of 4.5%, based on case reports and retrospective studies.[31] However, this percentage of 4.5% was

hindered by the poorly defined terminology which had been used for LS. In addition, mainly symptomatic women were included in those studies. As the incidence of asymptomatic LS is unknown, a better estimate of the real malignant potential of LS cannot be given. All together, the coexistence of LS and vulvar SCC has been shown, but without illustrative evidence of vulvar SCCs arising from LS. The topographical association with most vulvar SCCs arising within fields of LS has been the most compelling evidence linking these two conditions.

As described in Chapter 3, we found that dVIN was more often found (102 cases) adjacent to a vulvar SCC than LS (62 cases). The percentage of 47.7% (62/130) of LS being present adjacent to vulvar SCC may be biased by the fact that we only searched for adjacent lesions in the same slide that contained the tumour. LS may be more often present when also slides are revised which are more distal from the tumour. However, in most if not all cases, dVIN was present adjacent to the vulvar carcinoma and in between LS and the tumour, suggesting that dVIN but not LS is the direct precursor lesion.

In Chapter 5 we have studied LS lesions with and without subsequent progression to vulvar SCC. In LS biopsies with progression, in 42% dVIN was found after revision. The median time between LS biopsy and vulvar SCC development was 83.8 months, whereas this was significantly shorter for dVIN (27.5 months), indicating a higher malignant potential for dVIN than for LS, also suggesting that dVIN is the direct precursor lesion.

In Chapter 6 and 7 we have studied LS in the carcinogenic cascade leading to vulvar SCC in more detail. In Chapter 6 we studied DNA aneuploidy, a frequently identified genomic abnormality, which can occur in the earliest stages of malignant transformation. DNA aneuploidy was not found in LS, making it unlikely to be a premalignant lesion. In contrast, DNA aneuploidy was found in 38% of dVIN lesions and in 53% of tumour samples, indicating that dVIN has a higher malignant potential than LS. The percentage of p53 expression cells in LS turned out to be 30% or lower. On the other hand, this p53 expression was higher than in normal tissue (although limited in number in our study). p53 is a key regulator of maintaining a normal diploid status and an increased expression may indicate that the skin has difficulties with maintaining this diploid status in LS. The amount of p53 expressing cells increased with increasing ploidy status. These increasing percentages of aneuploidy and p53 expression suggest an increasing malignant potential from LS to dVIN and vulvar SCC.

In Chapter 7, a similarity between the composition of the inflammatory response in dVIN and SCC was shown, which was different from that in LS. The mean

amount of inflammatory cells in the subepithelial component of LS was low. In dVIN the total amount of inflammatory cells was significantly higher, and comparable with the amount found in vulvar SCC. As progression of a premalignant lesion is often accompanied by a persistent inflammatory stromal response, this challenges the role of LS as the direct premalignant lesion.
With these studies, we have more evidence than based on incidence studies alone that dVIN and not LS is the direct precursor lesion of HPV-negative vulvar SCC. We have not found any strong evidence that LS is a true premalignant condition.

The microenvironment in LS and dVIN probably contributes to vulvar SCC development

In Chapter 7 we have studied the inflammatory response in LS, dVIN and SCC, with the aim to find similarities and differences in the composition of it. In general, solid tumours consist of both malignant cells and a number of stromal cell types.[332] Cells in the stromal compartment are a dynamic, flexible asset to tumour development, reorganizing and evolving in response to cues from malignant cells as the tumour advances.[333] The stromal compartment consists of a subset of inflammatory cells, subdivided between the innate and adaptive immune cells. The innate immune system, also referred to as the first line of immune defence against infection, consists of granulocytes (neutrophils, basophils, and eosinophils), dendritic cells, macrophages, natural killer cells and mast cells). When tissue homeostasis is disrupted by an invading microbe or virus, macrophages and mast cells release chemokines and cytokines, which attracts leukocytes. This acute activation of the innate immune response activates the adaptive immune response, which comprises B lymphocytes, CD4 T helper cells and CD8 cytotoxic T cells. In chronically inflamed tissue, this may lead to excessive tissue remodelling, loss of tissue architecture, protein and DNA changes by oxidative stress and an increased risk of cancer development. This increased risk is caused by the functions of the inflammatory cells, which influence proliferation and survival, angio- & lymphangiogenesis, invasion & metastasis and immune evasion & suppression. We found that the mean amount of inflammatory cells in LS in the sub-epidermal component of LS is low, which was in contrast to the amount found in dVIN and SCC. Intriguingly, a high percentage of mast cells was detected within the epithelium of dVIN, which was absent in LS and scanty in SCC. In addition, we showed that mast cells were not found in other intraepithelial lesions. With the aim to unravel their possible function in the dVIN epithelium,

we found that the mast cells may play a role in keratinocyte proliferation. In addition, we found that mast cells in the epithelium were predominantly degranulated, suggesting a release of their contents into the epidermal environment.

Mast cells may play a crucial role in angiogenesis. They have been known to accumulate around blood vessels during homeostasis[334-336] and produce pro-angiogenic compounds.[337,338] Recent studies have suggested that mast cells play an important role in the 'angiogenic switch' during tumour growth.[339] At early stages in tumour development, mast cells direct angiogenesis in the developing tumour, while at later stages tumour cells take control of growth and angiogenesis and growth becomes mast cell-independent.[340] The latter may be an explanation why few mast cells were present in vulvar SCC, when compared to dVIN.

Based on these data, one could hypothesize that the so-called early LS, as suggested by Regauer,[120] would carry a higher risk of malignant progression than advanced, atrophic LS. We propose a hypothetical new model for HPV-negative vulvar carcinogenesis (Figure 1). In 'early' LS, the lymphocytic infiltrate is tagged along the basement membrane and it is known that premalignant progression is accompanied by a stromal response of mainly inflammatory cells. These inflammatory cells (mainly cytotoxic T cells) may induce tissue remodelling, loss of tissue architecture, protein and DNA changes by oxidative stress, thereby increasing the risk of dVIN and probably vulvar SCC development. 'Early' LS may also 'regress' into 'advanced, atrophic' LS. In 'advanced' LS there is often an oedematous zone beneath the basement membrane, under which and a bandlike lymphocytic infiltrate is present. The inflammatory cells have descended and no longer are supposed to interact with the keratinocytes of the epithelium of LS. Vulvar SCC, however, is more often seen in the advanced stages of LS. It may be argued that the preneoplastic changes already occur in the earliest stages, when early LS is replaced by dVIN and therefore, atrophic LS is seen adjacent to dVIN. Because LS is often present on the whole vulvar area, there is a field cancerization effect. It is possible that several subtypes of LS are present within the same patient, with a different malignant potential. This may explain why in some areas a vulvar SCC develops, whereas in other areas the LS remains quiet.

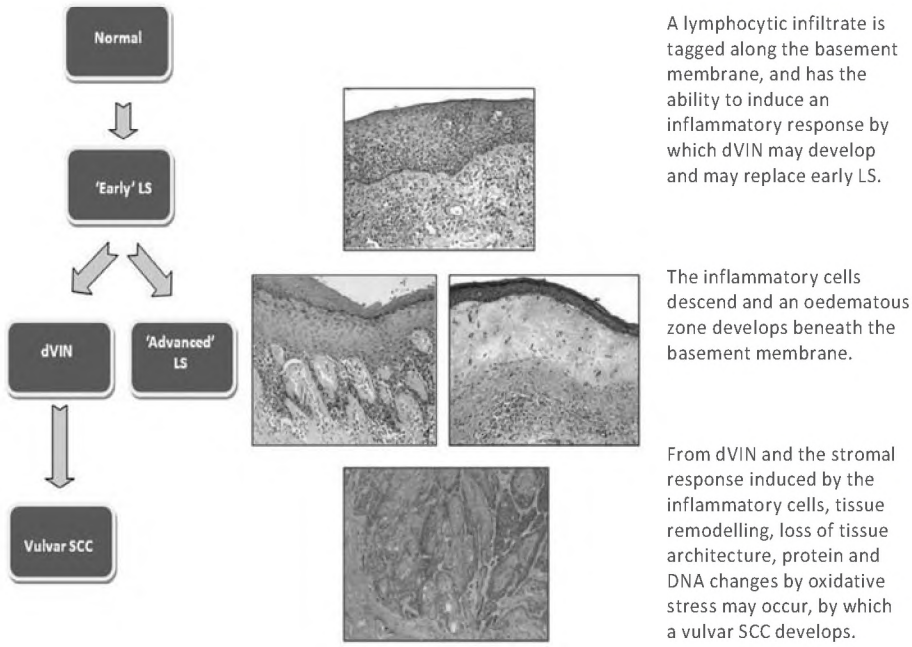

Figure 1 Hypothesis of a new model for HPV-negative vulvar SCC oncogenesis.

What will be the role of HPV vaccines in preventing vulvar SCCs?

The introduction of HPV vaccines has led to a lot of media attention. The main goal of the vaccines is to prevent cervical (pre) malignancies. Because a subset of vulvar SCCs is also positive for HPV, the preventive effect is thought not to be applicable for cervical (pre) malignancies only. In a short period, several reviews have been published regarding HPV infections in vulvar SCCs and address the possible impact of the HPV vaccines on the incidence of vulvar SCC,[254,341-344] with HPV16/18 percentages between 32%[342] and 57.5%.[343] However, as mentioned by Gillison *et al.* HPV detection by itself should not be considered as sufficient evidence for a causal relation.[342] In Chapter 3 the role of HPV is fine tuned; we have demonstrated that HPV detection alone is not sufficient to prove a causal

relation with vulvar carcinogenesis. We have shown that there are vulvar SCCs with dVIN adjacent to it, which are positive for HPV DNA. With in situ hybridization we have shown that this HPV was not integrated into the genome, and with $p16^{INK4A}$ immunohistochemistry it was demonstrated that the HPV was not active in most cases. All together, there are HPV positive vulvar SCCs that are unlikely to be caused by that HPV infection. This study has implications for the amount of vulvar SCCs that may be prevented in the future, which is less than suggested in the reviews mentioned, based on HPV positivity alone. HPV vaccines will have a role in the prevention of uVIN related vulvar SCCs, but not in the dVIN related vulvar SCCs.

Future perspectives

We have gained more insight in the oncogenesis of HPV-negative vulvar SCC, with special focus on the role of dVIN in this cascade, but the mechanisms behind this oncogenesis remain to be unravelled. To gain evidence for the proposed hypothesis as outlined above, the concept of early LS may be subject of future research. Especially, the composition of the inflammatory infiltrate of early LS may be the focus of study. It would be of great interest to know why an early LS lesion either progresses towards dVIN or regresses to advanced LS. Unfortunately, mouse models of vulvar SCC are not used on a large scale.[345] With the use of these models, the carcinogenic cascade of early LS, advanced LS, dVIN towards vulvar SCC might be monitored.

In addition, it would be of great interest to know the genetic mutations that occur from LS to dVIN and vulvar SCC. It has been demonstrated that p53 mutations are shared in both dVIN and vulvar SCC, which supports a genetic relationship between the two.[346] The main problem with this kind of research is that DNA has to be isolated from paraffin embedded formalin fixed tissues. LS only has a very thin epithelium and dVIN lesions are often very tiny adjacent to vulvar SCC with the inflammatory infiltrate directly underneath it. We have tried to isolate enough DNA from these lesions, but unfortunately, we did not succeed. Fresh frozen tissue would be more suitable for DNA isolation and this kind of research.
Furthermore, it would be very helpful when a subgroup of LS patients could be distinguished with an increased risk of malignant progression. We have access to a group of LS patients with and without towards vulvar SCC. In Chapter 5 we have focused on the histopathological characteristics of these lesions. Future research

may be focused on the inflammatory infiltrate as studied in Chapter 7 to find out whether there are differences in the inflammatory infiltrate between them. We have demonstrated that mast cells are prominently present in dVIN (Chapter 7), but the question remains by which mechanisms mast cells enter the epithelium, e.*g.,* which cell in LS is responsible for the attraction of mast cells? Furthermore, we have demonstrated that mast cells are likely to play a role in keratinocyte proliferation, but do they also exert other functions? Also other cells may be of interest; we have focussed on several inflammatory cells, but also other inflammatory cells (*e.g.,* regulatory T cells, interleukins, natural killer cells) may be studied. The regulation of inflammatory reactions in cancer is not fully understood, but recent research has focused on the role of the IL-17/IL23 axis. IL-17 and IL-23 signalling could promote tumour growth and metastasis and directly suppress antitumour immune responses.[347] However, there is also evidence to suggest that IL-17, Il-23 and Th17 cells could assist in the generation of antitumor immune responses. Well-established tumours produce high levels of TGFß and IL-6, as both of these cytokines can also act as differentiation factors for Th17 cells. Tumour-derived TGFß may help the differentiation of FoxP3+ regulatory T cells, thus resulting in immunosuppression of antitumour responses.[348] These inflammatory cells may be studied in vulvar SCC and its precursors as well to establish the role in vulvar carcinogenesis.

In addition, attention is needed to educate pathologists and gynaecologists to better recognize dVIN and to adhere to a strict nomenclature for vulvar premalignant lesions. Both clinically and histopathologically dVIN is hard to recognize. Gynaecologists need to biopsy every suspicious lesion in a background of LS and pathologists need to be trained in the recognition of dVIN. The recognition of a premalignancy in general may lead to its treatment and possibly the prevention of malignant progression. The incidence of the HPV-negative vulvar SCC may increase due to the ageing of society, so it is of utmost importance that the oncogenic cascades are better understood and that the current knowledge is incorporated in daily practice.

Chapter 9

Summary & Samenvatting

Summary

Chapter 1

Vulvar SCC arises following two different pathways. The minority is caused by an infection with high-risk HPV, with usual VIN as premalignant lesion. The majority is HPV independent and arise in a background of LS. dVIN is thought to be the direct precursor of this vulvar SCC. How LS develops into dVIN and eventually vulvar SCC is currently unknown.

To be able to answer the question: 'what is the role of dVIN in the oncogenesis of vulvar SCC?' we have several sub questions that are addressed in this thesis:

1. What is the exact role of HPV in the two pathways leading to vulvar SCC? (Chapter 3)
2. What is the incidence of solitary dVIN, has this incidence changed in the past years and what is its malignant potential? (Chapter 4)
3. Can we find differences between LS biopsies of patients who later are diagnosed with a vulvar SCC and LS biopsies of patients who did not develop a vulvar SCC? (Chapter 5)
4. Is the malignant progression of LS and dVIN to vulvar SCC accompanied by altered DNA content? (Chapter 6)
5. Can we provide evidence for the fact that dVIN is a likely precursor of vulvar SCC based on the inflammatory response? (Chapter 7)

Chapter 2

Chapter 2 is a review about the vulvar premalignant lesions, LS, dVIN and uVIN. The signs and symptoms, malignant potential, and treatment options of these lesions are described.

Chapter 3

With the aim to find out what the exact role of HPV is in the two pathways leading to vulvar SCC, we have performed a study on 130 vulvar SCC cases for the presence of the adjacent VIN lesion, its $p16^{INK4A}$ expression, and HPV DNA presence and integration in the genome. With the results of this study we also wanted to estimate the prophylactic effect of HPV 16/18 vaccines for vulvar SCC. We showed that HPV presence not automatically means a causal relationship with the tumour development. Based on HPV presence alone the preventive effect of HPV vaccines is overestimated. Moreover, we showed that the dVIN-associated vulvar SCCs had a worse disease specific survival (DSS) than the uVIN-associated vulvar SCCs,

while a comparison between HPV positive and HPV negative vulvar SCCs did not result in different DSS curves. This was explained by a worse DSS for dVIN-associated vulvar SCCs that were HPV positive. Summarised, HPV found in dVIN-associated vulvar SCCs is not likely to be causally associated with the tumour development. uVIN-associated vulvar SCCs may be prevented by vaccines in the future, leaving the problem of dVIN-associated vulvar SCCs, with worst DSS unsolved.

Chapter 4

It has been thought that dVIN is underdiagnosed as a solitary lesion and that its malignant potential is high, but this has not been studied in detail before. In Chapter 4 we have performed a study to the incidence of both VIN lesions and vulvar SCC in the Netherlands from 1992 – 2005. Of all VIN lesions, the vast majority were usual VIN lesions, of which the incidence significantly increased in the 14 years of study. Only a minority of all VIN lesions were of the differentiated type, but we found an increase in incidence, especially from 2002 onwards. Remarkably, despite an increase in both premalignant lesions, the incidence of vulvar SCC remained stable. In contrast to the 5.7% malignant potential of uVIN, a percentage of 32.8% was found for dVIN. The time from VIN diagnosis to vulvar SCC development was shorter for dVIN (22.8 months) compared to uVIN (41.4 months). We also demonstrated that this malignant potential for uVIN increased with age, probably reflecting an altered immunity for elderly women, reflecting a failure of the immune system to suppress HPV resulting in recurrent uVIN lesions and possibly vulvar SCC. Forty-one percent of patients with uVIN have a previous, concomitant or subsequent intraepithelial neoplasia of the cervix, vagina and/or anus. From this study we have concluded that an increase of both uVIN and dVIN has not led to an increase in vulvar SCC incidence, and that dVIN carries a higher malignant potential than uVIN.

Chapter 5

Because only a minority of patients with LS will ultimately develop vulvar SCC, we compared the LS biopsies of patients who were later diagnosed with vulvar SCC (both in Nijmegen and Groningen) to LS biopsies of patients who never developed vulvar SCC. All biopsies were revised by two expert gynaecopathologists and histopathological characteristiscs were scored. We found that of the 60 LS biopsies with later progression, in 42% dVIN was found. After we excluded those dVIN lesions, a comparison was made with the LS biopsies of patients without

progression. Para- and dyskeratosis, atypia and hyperplasia were the histopathological features linking LS to vulvar SCC development. In addition, patients with later progression were significantly older than patients without progression at the time the biopsy was taken. The median time to vulvar SCC development was significantly longer for LS than for dVIN lesions. In this study we have shown that dVIN is not as rare as previously thought but this it was often overlooked in the past.

Chapter 6

In Chapter 6 we have studied the p53 expression and DNA ploidy status in LS, dVIN and HPV negative vulvar SCCs. The aim of this study was to find out whether progression of LS and dVIN to vulvar SCC is accompanied by altered DNA content. In addition, we have taken the p53 expression of these lesions into account to study the relation between the expression of this cell cycle protein and ploidy status. We have found that the majority of LS lesions were diploid, reflecting a normal DNA content in these lesions. Lesions in which both dVIN and vulvar SCC were present, the ploidy status of dVIN never exceeded the ploidy status of the carcinoma. In all dVIN lesions, the p53 expression was confined to the basal and suprabasal layers, sometimes with suprabasal extension. We showed a positive correlation between high percentage of p53 positive cells and altered DNA content in the tissues studied. We also have examined this correlation in individual cells, demonstrating a difference in Feulgen Integrated Optical Density between p53 negative and p53 positive epithelial nuclei. In solitary dVIN lesions the lower one-third component of dVIN contains nuclei with increased DNA content, in contrast to the upper one-third component, which contains a low percentage of cells with an abnormal DNA content. In addition, the basal compartment was more proliferative than the superficial compartment. All together, in this study we have demonstrated that based on ploidy data and p53 expression, dVIN has a higher malignant potential than LS. The mechanism of oncogenesis and the progression of LS to dVIN still cannot be unravelled with these data.

Chapter 7

Because progression of a premalignant lesion towards an invasive tumour concurs with a persistent stromal response, the purpose of the study in Chapter 7 was to analyse the composition of the inflammatory response in LS, dVIN and vulvar carcinoma. We studied the localisation and amount of T helper cells, cytotoxic T cells, B cells, macrophages, dendritic cells, and mast cells. The mean amount of all

inflammatory cells in the sub-epithelial component was low in LS, and highly increased in dVIN and vulvar SCC. Remarkable was the high percentage of mast cells within the epithelium of dVIN, which were not found in LS and only infrequently in SCC. Significantly more mast cells were found in the epithelium of dVIN compared to other intraepithelial or hyperplastic lesions. MIB-1 positive keratinocytes were observed in close proximity of intraepithelial mast cells, but we also found MIB-1 positive keratinocytes in the absence of mast cells, suggesting that intraepithelial localisation of mast cells is not a prerequisite for keratinocyte proliferation. The electron density of intraepithelial mast cells was strongly reduced, suggesting that mast cells have released their contents into the epidermal environment. With this study we have shown that intraepithelial localisation of mast cells could be used as a marker for dVIN, which may facilitate its recognition. We have provided support for the gradual progression from dVIN towards vulvar SCC. Moreover, intraepithelial mast cells can promote proliferation. From this study it remains elusive why mast cells migrate to the epithelium of dVIN and whether they exert other functions, apart from induction of proliferation.

Samenvatting

Hoofdstuk 1

Het vulvacarcinoom kan op twee manieren ontstaan. Een klein deel wordt door een infectie met hoog risico HPV veroorzaakt, met usual VIN als premaligniteit. De meerderheid wordt veroorzaakt op een HPV onafhankelijke manier, in een achtergrond van LS. dVIN wordt verondersteld het directe voorstadium te zijn van dit type vulvacarcinoom. Hoe LS zich ontwikkelt tot dVIN en verder tot vulva carcinoom, is op dit moment nog onbekend.

Dit proefschrift heeft als doel de vraag te beantwoorden wat de rol is van dVIN in de oncogenese van het vulvacarcinoom. Hiertoe hebben we verschillende subvragen geformuleerd welke besproken worden in dit proefschrift.

1. Wat is de precieze rol van HPV in het ontstaan van de twee typen vulva-carcinoom? (Hoofdstuk 3)
2. Wat is de incidentie van solitaire dVIN, is deze incidentie veranderd over de afgelopen jaren en wat is de maligne potentie van dVIN? (Hoofdstuk 4)
3. Zijn er verschillen tussen LS biopten van patiënten die later wel of niet zijn gediagnosticeerd met een vulvacarcinoom? (Hoofdstuk 5)
4. Gaat de maligne progressie van LS en dVIN tot vulvacarcinoom gepaard met een veranderde DNA inhoud? (Hoofdstuk 6)
5. Kunnen we bewijs leveren dat dVIN het voorstadium is van het HPV negatieve vulvacarcinoom op basis van de ontstekingsrespons? (Hoofdstuk 7)

Hoofdstuk 2

Hoofdstuk 2 is een review over vulvaire premaligniteiten, te weten LS, dVIN en uVIN. Van deze laesies worden de symptomen, klinische kenmerken, maligne potentie en behandelopties beschreven.

Hoofdstuk 3

In hoofdstuk 3 beschrijven we wat de exacte rol van HPV is bij de twee typen vulvacarcinoom. We hebben naast 130 vulvacarcinomen gekeken of er sprake was van LS, dVIN of uVIN, de p16 expressie, HPV DNA aanwezigheid en of dit gevonden HPV geïntegreerd in het genoom aanwezig was. Met deze resultaten willen we tevens kijken wat het preventieve effect kan zijn van de HPV vaccinaties. Het bleek dat HPV aanwezigheid niet automatisch betekende dat dit een causale relatie had met het ontstaan van de tumor. Wanneer het preventieve effect alleen gebaseerd wordt op het aan- of afwezig zijn van HPV, leidt dit tot een overschatting.

Ook bleek dat de overleving van patiënten met een dVIN geassocieerd vulvacarcinoom slechter was in vergelijking met patiënten met een uVIN geassocieerd vulvacarcinoom, terwijl een vergelijking gebaseerd op HPV aanwezigheid dit verschil niet toonde. De overleving van HPV positieve, dVIN geassocieerde tumoren was erg slecht in onze populatie. De conclusie van dit hoofdstuk is dat als er HPV wordt gevonden in een dVIN geassocieerd carcinoom, dit HPV niet de oorzaak is van de tumor. Alleen uVIN geassocieerde tumoren kunnen voorkomen worden door de HPV vaccinaties. Echter, de tumoren met de slechtste overleving blijven na HPV vaccinatie bestaan.

Hoofdstuk 4

Over dVIN wordt vaak geschreven dat deze onder gediagnosticeerd wordt in zijn solitaire vorm en dat de maligne potentie ervan hoog is, maar dit werd nooit eerder aangetoond. In hoofdstuk 5 hebben we de incidentie van de beide VIN soorten en het vulvacarcinoom in Nederland in de periode 1992-2005 bestudeerd. Van alle VIN laesies bestond het overgrote deel uit uVIN, waarvan de incidentie significant is toegenomen gedurende de studie periode. Slechts een klein deel bestond uit solitaire dVIN laesies, maar een toename in de incidentie werd ook voor dVIN gezien, in het bijzonder vanaf 2002. Ondanks deze stijging van beide VIN laesies, is de incidentie van het vulvacarcinoom gelijk gebleven. De maligne potentie van uVIN was 5.7% in onze studie, wat aanmerkelijk lager is dan de 32.8% die we vonden voor dVIN. De tijdsduur van VIN laesie tot het ontwikkelen van het vulvacarcinoom verschilde tussen uVIN (41.4 maanden) en dVIN (22.8 maanden). Tevens bleek dat de maligne potentie van uVIN toenam met een toenemende leeftijd waarop uVIN werd vastgesteld, wat verklaard zou kunnen worden door de veranderde immuniteit op hogere leeftijd, waardoor de HPV infectie niet onderdrukt kan worden. Dit kan mogelijk eerder leiden tot recidiverende uVIN laesies en mogelijk een uVIN gerelateerd vulvacarcinoom. Tevens had 41% van de patiënten met uVIN een voorafgaande, gelijktijdige, of toekomstige HPV geïnduceerde laesie van cervix, vagina en/of anus.

Hoofdstuk 5

Slechts een kleine minderheid van alle patiënten met LS ontwikkelt op latere leeftijd een vulvacarcinoom, maar het is nog onbekend welke patiënten hierop een hoger risico hebben. We hebben LS biopten vergeleken van patiënten die later wel en die geen vulvacarcinoom hebben gekregen. Alle biopten werden gereviseerd door twee pathologen met expertise op gynaecologische pathologie.

Histopathologische kenmerken werden bekeken en gescoord. In de biopten van patiënten die later een vulvacarcinoom hebben ontwikkeld, vonden we in 42% dVIN. Deze hebben we uit de vergelijking tussen de LS biopten met en zonder later vulvacarcinoom gelaten. Deze vergelijking liet zien dat LS biopten met latere progressie vaker dys- of parakeratose, atypie of hyperplasie hadden. Ook waren de patiënten die later een vulvacarcinoom hebben ontwikkeld ten tijde van het biopt ouder dan de patiënten die geen vulvacarcinoom hebben ontwikkeld. De tijd tussen LS en vulvacarcinoom ontwikkeling was langer dan de tijd tussen dVIN en het vulvacarcinoom. Deze studie laat zien dat solitaire dVIN niet zo zeldzaam is, maar dat deze laesie vaak over hoofd werd gezien in het verleden.

Hoofdstuk 6

In hoofdstuk 6 hebben we ons gericht op de vraag wat de p53 expressie en ploidie status is in LS, dVIN en het HPV negatieve vulvacarcinoom. Het doel van deze studie was om te kijken of de progressie van LS naar dVIN en het vulvacarcinoom gepaard gaat met een veranderde DNA inhoud. Ook hebben we gekeken naar de p53 expressie van deze laesies om de relatie tussen p53 expressie en DNA inhoud te bestuderen. Bijna alle LS laesies waren diploid, wat een normale DNA inhoud impliceert. In laesies waar zowel dVIN als vulvacarcinoom voorkwamen, was de DNA inhoud van dVIN nooit méér dan in het naastgelegen vulvacarcinoom. In de dVIN laesies, was de p53 expressie alleen in de basale en suprabasale lagen aanwezig, soms met suprabasale extensie. Er was een positieve correlatie tussen de p53 expressie en de DNA inhoud in alle laesies. Ook hebben we gekeken naar DNA op cel niveau, wat een verschil in Feulgen Integrated Optical Density toonde tussen p53 positieve of negatieve cellen. In solitaire dVIN laesies bevatten de cellen in het onderste 1/3 gedeelte een verhoogde DNA inhoud, wat verschilde van cellen in de bovenste 1/3 laag van het epitheel. Ook bleek dat cellen in het basale deel proliferatiever waren dan in de bovenste lagen. In deze studie hebben we laten zien dat, gebaseerd op ploidie gegevens en p53 expressie, dVIN een hogere maligne potentie heeft dan LS. Het oncogenetische mechanisme hierachter kunnen we hiermee niet achterhalen.

Hoofdstuk 7

Progressie van een premaligniteit naar een invasief carcinoom gaat vaak gepaard met een reactie in het onderliggende stroma. In hoofdstuk zeven hebben we naar de samenstelling van het ontstekings infiltraat gekeken in LS, dVIN en het vulvacarcinoom. We hebben de lokalisatie en hoeveelheid van T helper cellen,

cytotoxische T cellen, B cellen, macrofagen, dendritische cellen en mestcellen bestudeerd. LS laesies bevatten weinig cellen in het sub-epitheliale compartiment, en het aantal ontstekingscellen was significant verhoogd in dVIN en vulva-carcinoom. Wat erg opviel was de aanwezigheid van mestcellen in het epitheel van dVIN, welke niet aanwezig waren in LS en slechts weinig in het vulvacarcinoom. In een vergelijking met andere intraepitheliale of hyperplastische laesies bleek dit karakteristiek te zijn voor dVIN. In de omgeving van mestcellen vonden we een verhoogde proliferatie van keratinocyten, maar ook vonden we proliferatie verder weg van mest cellen wat suggereert dat mestcellen geen voorwaarde zijn voor cel proliferatie. De electronen dichtheid van intraepitheliale mestcellen was verlaagd ten opzichte van stromale mest cellen wat degranulatie suggereert. In deze studie hebben we laten zien dat intraepitheliale mestcellen als marker voor dVIN kunnen dienen, wat de herkenning hiervan makkelijker maakt. Op basis van het ontstekings infiltraat hebben we aanwijzingen dat er een graduele progressie is van LS via dVIN naar het vulvacarcinoom. Ook bleek dat mescellen cel proliferatie kunnen bevorderen. Waarom mestcellen in het epitheel van dVIN voorkomen en wat, naast cel proliferatie, hun functie is, blijft nog onduidelijk.

Appendix A

The effect of vulvar lichen sclerosus on quality of life and sexual functioning

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Abstract

Objective: Lichen sclerosus (LS) is a chronic skin disorder mostly seen on the female anogenital skin. The aim of this study was to evaluate the QoL and sexuality in female LS patients and to compare their scores with healthy controls. Additionally, we wanted to find factors associated with impaired sexual functioning in LS patients.

Methods: Members of the Dutch LS foundation and support group were asked to fill in three questionnaires: the Dermatology Quality of Life Index (DLQI), Female Sexual Distress Scale (FSDS) and the Female Sexual Function Index (FSFI). 215 out of 368 patients returned their questionnaire (58.4%). Their scores were compared to a control group which consisted of 61 women of similar age ($p=0.472$) without a skin disorder.

Results and Conclusions: Of all domains of QoL, LS interfered most with sexual functioning. Patients significantly scored lower on all subscales of the FSFI (desire ($p=0.016$), arousal ($p<0.001$), lubrication ($p<0.001$), orgasm ($p<0.001$), satisfaction ($p<0.001$) and pain ($p<0.001$), indicating worse sexual functioning. These problems with sexual functioning brought about significant sexual distress ($p<0.001$). Patients who experienced more influence on their QoL had more sexual difficulties, leading to more sexual distress independent of their age.

Introduction

Lichen sclerosus (LS) is a chronic skin disorder that affects patients of all age groups, particularly women. It is most commonly seen on the female anogenital skin, but it may occur on extragenital areas as well. LS is a tissue destructive disease that may lead to anatomical changes; resorption of the labia minora, hooding and burial of the clitoris, labial fusion and introital stenosis may occur (Figure 1). The origin of LS is unknown and in LS patients there is an increased incidence of vulvar squamous cell carcinomas with a life-time risk of about 5%, which is a relative risk of 300 compared to women in the general population.[29,30] Most patients complain of itching, but a burning sensation, dyspareunia, dysuria and painful defecation are also reported.[258] The current standard treatment of LS is the use of superpotent corticosteroid ointment.[130-132] Although this treatment is effective in the majority of LS patients, most experience exacerbations at times.

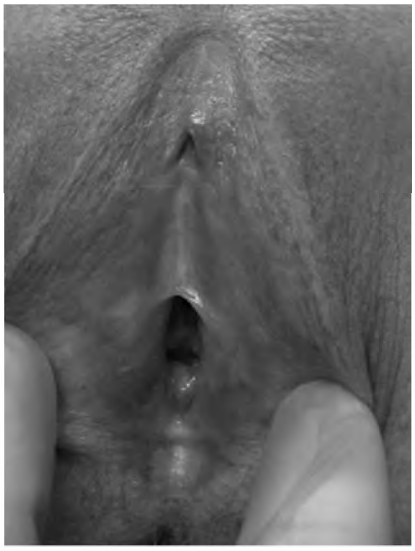

Figure 1 LS with narrowing of the introitus by anterior labial fusion.

Limited data that focus on the effect of LS on quality of life (QoL) and sexual functioning are available. Probably many LS patients feel embarrassed and do have sexual problems.[61,62] Patients may be uncomfortable by the disfiguring changes that may occur and may therefore avoid sexual intimacy. In a study on the QoL in 43 chronic LS patients, it was found that patients who are seeking care

for LS have considerable QoL impairment: the majority of patients reported both an impact on sexual functioning and frustration and a significant impact on general happiness. Their self esteem and confidence were also affected. Data in that study showed that all major QoL domains were affected except for work/ school functioning.[349] Dalziel reported that LS had a detrimental effect on sexual functioning, but only a small number of patients (45) were questioned. Furthermore, she did not use a validated questionnaire and the results were not compared with a control group.[66]

QoL and sexuality may be assessed in various ways; in our study on QoL and sexual functioning in female LS patients we choose to use the Dermatology Life Quality Index (DLQI), the Female Sexual Distress Scale (FSDS) and the Female Sexual Function Index (FSFI).
Few studies have addressed QoL and sexuality in LS patients, and these studies included a limited number of patients and/or lack a control group. In addition, they did not use validated questionnaires. Therefore, it is still largely unknown what the exact effect of LS on the QoL and sexuality is. The aim of this study was to evaluate the QoL and sexuality with validated questionnaires in detail in a large group of female LS patients and to compare their scores with healthy controls. Additionally, we wanted to find factors associated with impaired sexual functioning in LS patients.

Methods

Patients

The Dutch LS foundation and support group was initiated in 2006. At the beginning of our study, 430 LS patients (32 male, 398 female) were member of this foundation. Patients are only allowed to become a member of the foundation after they declare that they have a diagnosis of LS, preferably by taking a biopsy. Since LS is a chronic skin disease, all patients are under follow up by a gynaecologist, dermatologist or family doctor. Unfortunately, we did not know whether they all used some form of treatment at the time of the study. All female members were invited to participate in our study by an email message. Thirty email messages could not be delivered. As a result, a total number of 368 female patients with LS were asked to participate in the study. In the email message a link to a website was included where the questionnaires could be filled in electronically and

anonymously. In addition, patients were asked for several sociodemographic patient characteristics (age and marital status) and medical data (duration of LS).

Control group

Within the Radboud University Nijmegen Medical Centre, there is collaboration between the departments of Obstetrics & Gynaecology and the department of Dermatology. These two departments designed a QoL study for psoriasis patients using the same questionnaires as used in this study on LS patients. Both LS and psoriasis patients were asked to invite female friends of about the same age to fill in the questionnaires to form a control group for both studies. They could participate in the study when they did not have psoriasis, LS or another dermatological condition.

Questionnaires

The DLQI is the first dermatology-specific QoL instrument and to date it is the most commonly used.[350,351] It consists of 10 questions concerning patients' perception of the impact of their skin disease on different aspects of their QoL and has been validated for dermatology patients.[349] (Some examples of questions: "Over the last week, how much has your skin influenced the clothes you wear?" and "Over the last week, how much has your skin made it difficult for you to do any sport?") The questionnaire is structured with each question having four alternative responses: 'not at all', 'a little', 'a lot', or 'very much', with corresponding scores of 0, 1, 2, 3 respectively. The answer 'not relevant' is scored as '0'. The DLQI is calculated by summing the score of each question, resulting in a minimum of 0 and a maximum of 30. The higher the score, the greater the impairment of QoL.[351]

The FSFI is a 19 item questionnaire that has been developed as a brief, multidimensional self-report instrument for assessing the key dimensions of sexual functioning in women. The FSFI has six subscales: desire, arousal, lubrication, orgasm, satisfaction and pain. The questionnaire was designed and validated for the assessment of female sexual functioning and QoL in clinical trials and epidemiological studies.[352] Individual domain scores are obtained by adding the scores of the individual items that comprise the domain and subsequently multiplying the sum by the domain factor. The full scale score is obtained by adding the six domain scores.[352] Higher scores indicate better sexual functioning. A FSFI total score of 26.55 or less is the cut-off point for differentiating women with and without sexual dysfunction.[353]

The FSDS is a 12 item self-rating instrument for assessing sexually related personal distress and has been extensively used. The scale showed a high degree of discriminative ability in distinguishing between sexually dysfunctional and sexually functional women.[354,355] Every item requires an answer that is rated as: never (0 points), rarely (1 point), occasionally (2 points), frequently (3 points), or always (4 points). The total score, ranging from 0 to 48, provides a measure of sexual distress, in which the higher the score, the higher the level of sexual distress[355] A total score of ≥15 on the FSDS is the recommended cut-off score to establish the presence of sexually related personal distress.[355] Both the FSFI and FSDS have been validated for a Dutch population.[356]

Statistics

Data were entered in a computerised database and analyzed using SPSS software (version 16.0.1 for Windows, SPSS). Differences between patients and controls were calculated using the Mann-Whitney U test for independent, nonparametric distributed data. Correlations were calculated using Pearson bivariate correlations and a linear regression model was used to calculate which factor was associated with sexual dysfunctioning. P values <0.05 were considered statistically significant.

Results

Patients of the Dutch LS foundation and support group returned 215/368 (58.4%) questionnaires. The median age was 50 years (range 20-76 years). The majority of the patients (80%) were married or living together. Only 82 patients filled in for how long they had suffered from LS. The median duration of complaints was 5.0 years (mean 8.0, range 1-45 years). Sixty-one healthy controls filled in the questionnaires. The median age of the control group was 50 years (range 24-69), which did not differ significantly with the median age of the LS patients (p=0.472).

DLQI

To find out whether LS has influence on the QoL in patients with LS, they were asked to fill in the DLQI. As this is a questionnaire about the influence of dermatology disorders on QoL, only LS patients were asked to complete this questionnaire, because the control group consisted of patients without a dermatological condition. The mean total score of LS patients was 11.92 points

(SD 6.18), which indicates a very large effect on the QoL.[350] LS patients had the highest mean score on the item regarding sexual difficulties. LS did not interfere much with working or studying (Figure 2).

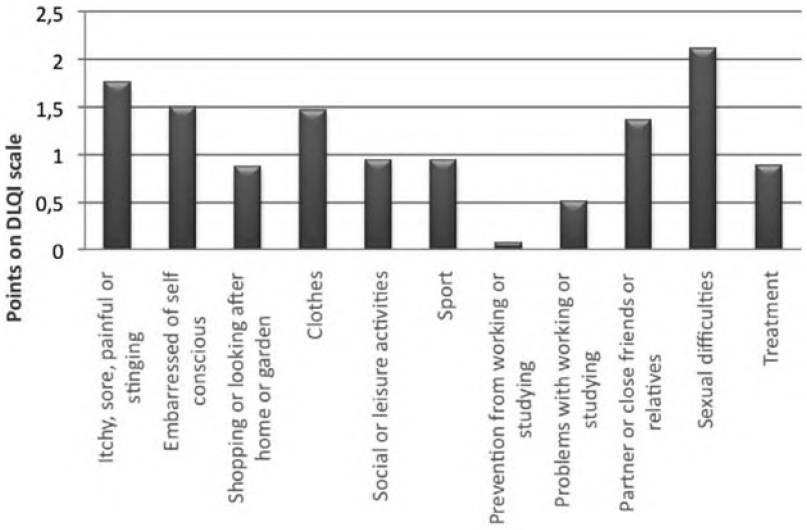

Figure 2 Mean DLQI scores.
In total, 212 patients filled in all questions of the DLQI questionnaire.

FSFI

LS patients had a mean total score of 18.79 (SD 7.95), that of the control group was 27.43 (SD 4.03) ($p<0.001$). With a cut off value of 26.55,[353] this indicates sexual dysfunctioning in LS patients, not in controls. Patients (n=187) scored significantly lower on all the subscales for the FSFI compared to the control group (n=61) (Mann Whitney U test: $p<0.001$ for subscales arousal, lubrication, orgasm satisfaction and pain, $p=0.016$ for desire). The smallest difference was on the subset 'desire' (Figure 3).

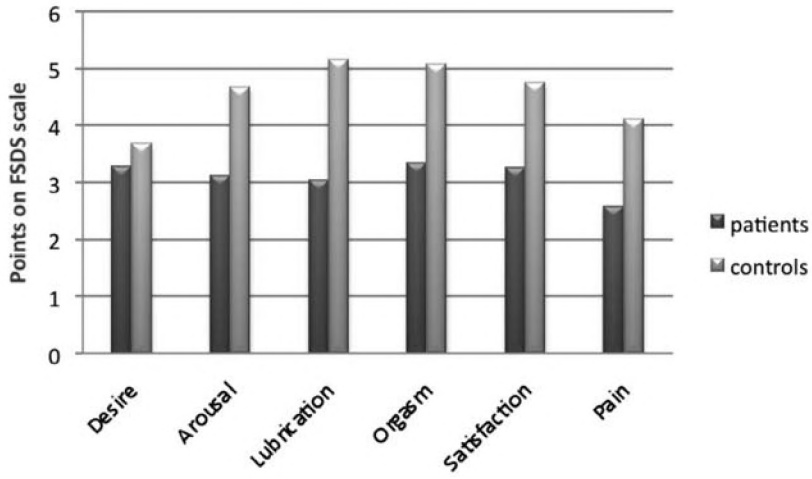

Figure 3 Mean FSFI scores.
In total, 187 patients filled in all questions of the FSFI questionnaire. These scores were significantly different from controls on all domains (Mann-Whitney U test).

FSDS

Mean total score for the FSDS was 26.08 (SD 11.81) for patients and 9.97 (SD 8.26) for controls ($p<0.001$). Using a cut off score of ≥15,[355] this indicates sexual distress in LS patients, not in controls. LS patients (n=206) had significantly higher mean scores on all 12 items of the FSDS in comparison with healthy controls (n=61) (Mann Whitney U test $p<0.001$ for all items) (Figure 4).

With the aim to find out what factors are associated with sexual dysfunctioning, a regression analysis was performed. Of the factors age, marital status, duration of LS (years) and the total DLQI scores, the total DLQI score predicted sexual dysfunctioning significantly ($p=0.003$ B=-0.363), indicating that patients who experience that their LS has a large effect on their QoL, have more sexual problems.

The amount of itch and/or pain (DLQI question 1) correlated significantly with the mean scores of the FSDS ($r=0.442$; $p<0.001$). Patients who experienced more itch and/or pain, had more sexual distress. Moreover, they had more problems regarding arousal ($r=-239$; $p<0.001$), lubrication ($r=-0.221$; $p=0.002$), orgasm

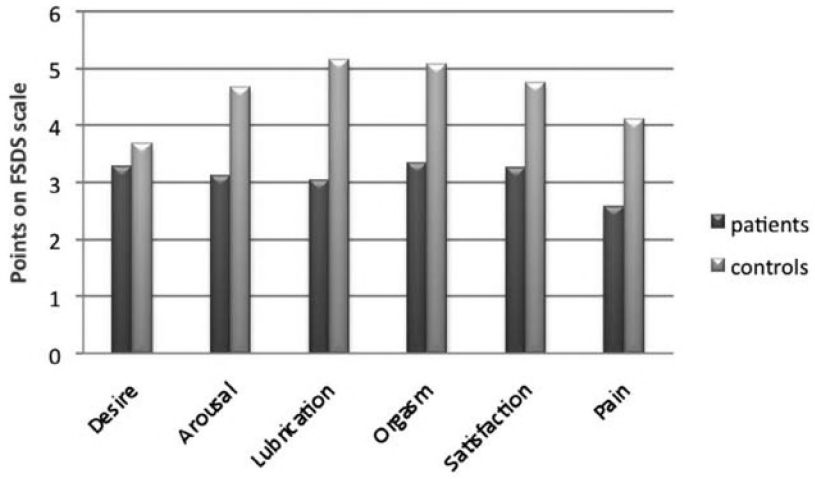

Figure 4 Mean FSDS scores.
In total, 206 patients filled in all questions of the FSDS questionnaire. These scores were significantly different from controls on all domains (Mann-Whitney U test).

(r=-0.172; p=0.001), satisfaction (r=0.239; p=0.001) and pain (r=-0.151; p=0.032). Older patients had lower mean scores on the subscales desire (r=-0.151; p=0.034), arousal (r=-0.241; p=0.001), lubrication (r=-0.251; p<0.001), pain (r=-0.317; p<0.001) and total score (r=-0.246; p<0.001) of the FSFI questionnaire, indicating worse sexual functioning. The age of patients did not correlate with the FSDS scores (r=-0.50; p=0.485).
There was no correlation between the amount of itch/pain and desire (r=-0.111; p=0.117). Patients that reported to have sexual problems (DLQI question 9), had more sexual distress (total score FSDS) (r=0.624; p<0.001). In patients with LS, the marital status was not associated with sexual functioning (Chi square p=0.596).

Discussion

In this study we show that LS has a considerable influence on the QoL and sexual functioning. This influence on sexual functioning causes significant sexual distress. Patients who experience a great impact on their QoL, have worse sexual functioning.

Sexual difficulties in LS patients may be attributed to three main causes for dyspareunia. The skin is sensitive and delicate which easily tears and may cause superficial dyspareunia. The fear for pain lowers arousal, decreases lubrication, and causes the pelvic muscles to contract, which indeed results in dyspareunia. In LS, anatomical changes may also occur, like hooding and burial of the clitoris, labial fusion and introital stenosis, which may make intercourse painful or give rise to problems with achieving orgasms.

In our study, the mean DLQI score was 11.92. Basra *et al.* performed a review of validation data about the DLQI, which included a myriad of dermatological conditions.[350] These data show that our mean score is comparable to the scores of atopic dermatitis, hyperhidrosis, psoriasis and dermatomyositis sine myositis.

There are several limitations about our study. We had no access to socio-demographic information which may have influenced the sexual functioning of patients. It may be hypothesized that patients suffering from LS may be more anxious or depressive than women in the control group, which may have influenced the results. In addition, only a minority of patients filled in the question: for how long have you had LS? This may be because the LS was set up gradually or that patients did not remember this anymore. Moreover, we did not have a fully matched control group. Patients of LS and psoriasis may be embarrassed to tell their friends they suffer of LS/psoriasis or ask them to fill in the questionnaires about sexual dysfunctioning. However, with these 61 control patients, we found highly significant differences between LS patients and controls. Furthermore, we had a response rate of 58.4% and not all patients completely filled in the questionnaires; 28 patients did not fill in the FSFI, which might be due to the very intimate and personal questions about sexual functioning in this questionnaire. This may introduce a responder bias, because those most bothered by their symptoms are more likely to respond.
This study highlights the need for attention for sexual functioning when treating patients with LS. Treatment (topical corticosteroids) may make a difference in the ability to have intercourse.[66] Fusion of the labia may require simple perineotomy under local anaesthesia. However, in the case of introital stenosis, perineoplasty may be performed with the aim to ameliorate sexual intercourse.[67] No data are available about the use of lubricants, the role of the sexologist or other treatment options. Despite this lack, we often advice our patients to use lubricants to diminish friction with intercourse. When patients are motivated, we also refer them to a sexologist.

In conclusion, we have shown that patients with LS experience an effect on the QoL, especially with sexual functioning. In addition, we have found that sexual functioning is impaired on all the subscales. These difficulties in sexual functioning have led to significant sexual distress. In general, patients who experienced more influence on their QoL had more sexual difficulties, leading to more sexual distress.

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Joep, lief vriendje. Ben ik toch nog eerder klaar dan jij!!! We hebben vele discussies gehad over mijn zeer dunne proefschrift (waarschijnlijk net zo dik als jouw eerste hoofdstuk) en het nut van jouw onderzoek. We hebben erom kunnen lachen en ik weet zeker dat je trots bent vandaag. Ik hoop dat jij je proefschrift ook snel en goed kunt afronden. Jij bent mijn beste vriendje!!

Curriculum Vitae

Hedwig van de Nieuwenhof werd geboren op 21 november 1980 in Veghel waar zij ook opgroeide. Met een duidelijk plan om geneeskunde te gaan studeren worstelde zij zich door een β pakket aan het Gymnasium van het Zwijsen college te Veghel. Door een uitloting volgde er eerst een jaar psychologie aan de universiteit van Maastricht, maar in 2000 kon aan de studie geneeskunde worden begonnen. Haar studententijd begon in een klein, koud kamertje in de Waterstraat waar toch nog 8 andere dames bijpaste voor het wekelijkste jaarclub eten. Al snel ontmoette zij Joep van Gennip en zij gingen snel op zoek naar een geschiktere kamer aan de Heijendaalseweg. Voor de aanvang van de coschappen heeft zij 2 maanden rondgereisd in Ecuador, Peru en Bolivia waarna er een IFMSA stage gynaecologie volgde in Marilia, Brazilië. Na de regulier coschappen volgde er een keuze coschap obstetrie in het Jeroen Bosch Ziekenhuis (lokatie Groot Ziekengasthuis) en een afsluitend coschap gynaecologische oncologie in het UMC St Radboud. Na een wetenschappelijke stage op diezelfde afdeling, behaalde zij het artsexamen in november 2006. Enthousiast gemaakt voor wetenschappelijk onderzoek door die laatste stage, startte zij in 2007 met een promotieonderzoek bij de pijler gynaecologische oncologie van het UMC St Radboud (promotor prof. dr. Leon Massuger en copromotores dr. Joanne de Hullu en dr. Léon van Kempen). Een AGIKO aanvraag werd gehonoreerd, en in maart 2010, bij 38 weken zwangerschap, werd het onderzoek afgerond. In april 2010 is Floris geboren. In augustus 2010 begon zij als ANIOS te werken op de afdeling Verloskunde en Gynaecologie van het Jeroen Bosch Ziekenhuis (lokatie Groot Ziekengasthuis) en zal daar in januari 2011 starten met de opleiding tot gynaecoloog (opleider dr. H.P. Oosterbaan).