Molecular and clinical aspects of vulvar squamous premalignancies

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Molecular and clinical aspects of vulvar squamous premalignancies

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Contents

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Chapter 1

General Introduction and Outline of the Thesis

General Introduction

Vulvar Squamous cell carcinoma and its premalignant lesions

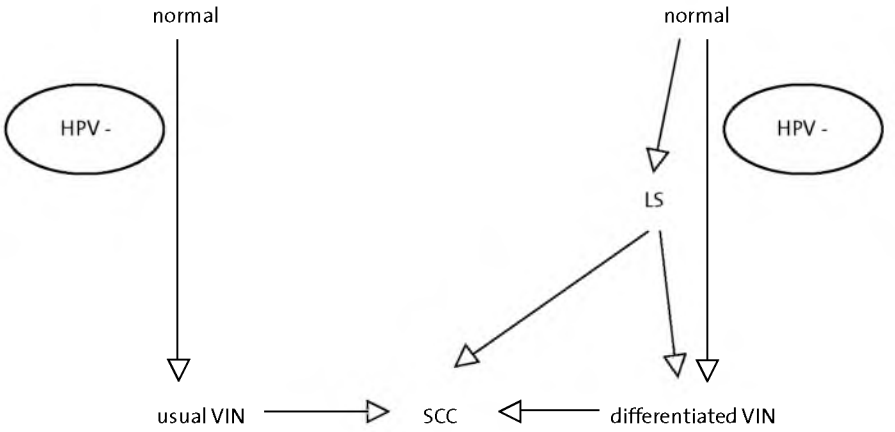
Vulvar squamous cell carcinoma (SCC) accounts for approximately 3-5% of all gynaecological malignancies and 1% of all carcinomas in women, with an incidence rate of 1-2/100.000.[1] In the Netherlands (16 million inhabitants) about 230 new patients with vulvar SCC are diagnosed annually.[2] Typically, 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. Vulvar SCC may spread by three routes. Initial spread occurs usually to the inguinofemoral lymph nodes. The number of inguinofemoral lymph node metastases is the most important prognostic factor. Hematogenous spread is rare in early stage disease, while spread by direct extension also is infrequent. Surgery is first choice in the treatment of patients with SCC of the vulva.[3] In the second half of the last century overall survival figures rose from 20% to over 60% after the introduction of radical vulvectomy with "en bloc" bilateral inguinofemoral and pelvic lymphadenectomy instead of simple local excision.[4;5] The current surgical standard of care is the triple incision approach with radical wide local excision (WLE) or complete radical vulvectomy with uni- or bilateral inguinofemoral lymphadenectomy. The use of the triple incision technique has lowered treatment related morbidity. The sentinel lymph node (SLN) procedure is a technique for determining the status of the regional lymph nodes with even less treatment-related morbidity. Recently, a large multicenter observational study provided level 3 evidence indicating that it appears safe to omit inguinofemoral lymphadenectomy in case of a negative SLN.[6]

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 sclerosus (LS) and often differentiated vulvar intraepithelial neoplasia (dVIN).[7] 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.[8;9] The oncogenesis of LS to vulvar SCC and its connection with dVIN is not exactly known.

The second type of vulvar SCC consists of mainly non-keratinising carcinomas, primarily affecting younger women. This type of vulvar SCC is caused by an infection with high-risk HPV, predominantly HPV 16 and 18.[10] 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.[11] Multicentric HPV infections affecting cervix,

vagina and/or anus have been described; twenty-two percent of uVIN patients has a concurrent cervical intra epithelial neoplasia (CIN)[12] and up to 71% of uVIN patients has a previous, concomitant or subsequent history of vaginal intraepithelial neoplasia (VAIN), CIN or cervical carcinoma.[13-15] Despite a stable pattern in the incidence of vulvar SCC,[16;17] the incidence of uVIN and vulvar SCC is increasing in women aged 50 years and younger.[16;18-21] This might be due to a higher incidence of HPV infection of the genital tract and/or to an increased awareness of uVIN.

Figure 1: Two pathways leading to vulvar squamous cell carcinoma



Diagnostics

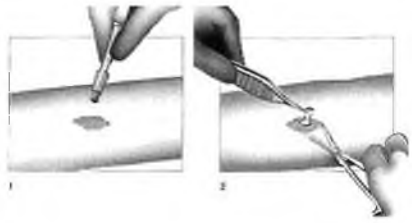
Biopsy

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.

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 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 forceps to prevent crush artefacts.

Vulvoscopy

Vulvoscopy consists of careful observation, and the possible use of a 5% acetic acid solution on the 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.[22] 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.[22] However, acetowhitening has low sensitivity as a predictor of HPV infections,[23,24] although it can be used to determine the extent of the VIN lesion. The use of acetic acid can be very painful. In conclusion, vulvoscopy provides 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.

Psychology and sexology

The effect of vulvar lesions on quality of life is unmistakable. Many LS patients feel embarrassed and have sexual problems.[25;26] 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.[27;28] 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.[29] 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.[30] 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.[31]

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.[32] 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).[33–35] 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.[36] 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.[37] 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.[38]

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.

Lichen Sclerosus

History

Over the years, a variety of names and descriptions have 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.[39;40] 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 its name was changed to International Society for the Study of Vulvovaginal Disease), because not all LS is histologically atrophic.[41;42]

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.[43] In a study on the prevalence of LS in childhood, a prevalence of 1:900 was found.[44] The majority of patients consists of women aged 50-70 years, while 5-15% of the LS patients are children.[26] Recently, an increase in incidence in childhood LS was noted in the United Kingdom.[44] 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.[40;45] However, the symptoms of LS can be modified by hormonal events in some women.[46] The female to male-ratio is 6:1–10:1,[39;40] and most reports have been on Caucasians. In young girls, the signs and symptoms of LS are sometimes mistaken for sexual abuse.[47] However, childhood LS and sexual abuse are not mutually exclusive diagnoses.[48] In a series of 42 cases of childhood LS, in 12 cases there was evidence of sexual abuse.[49]

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.[26;50] 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.[51] 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.[52;53] The rate of biopsy proven vulvar LS in one general gynaecology private practice was approximately 1.7% (1 in 60 women).[43] One study suggests that 1 in 30 elderly women (nursery home population) have LS.[54]

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.[27] However, neither oestrogens nor testosterone bring benefit in terms of treatment of symptoms or prophylaxis of the disease.[27;55]

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.[56-58] Alopecia areata and vitiligo were the most common associated disorders, but thyroid disease, pernicious anaemia, diabetes mellitus, and cicatrical pemphigoid were also reported.[26,56,57;59] 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.[55] In patients with LS, the family history for autoimmune disease is often positive.[60] There is no evidence that screening for autoimmune disorders in LS patients is beneficial.[61] 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.[26;62] This association was strongest in the patients with early-onset LS.[60] 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.[62] 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.[63] Studies by Carli et al. give support to the involvement of the skin immune system in pathogenesis of LS.[64;65]
A genetic influence favouring LS can be derived from familial LS reports.[26;55] LS has been reported in identical and non-identical twins,[57;59] sisters, and mothers and daughters.[60] 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.[66] 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,[67] and using immunoperoxidase methods.[68] However, polymerase chain reaction (PCR) assessment has yielded conflicting results, summed up by Weide et al.[69] 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.[70] 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.[71] 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.[72]
No relationship between HPV infection and LS was found in women[55] but has been shown in men.[73-75] In a study of 23 boys (4-14 years) with penile LS, HPV-DNA was detected with PCR in 70%,[76] 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.[77]
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.[78,79] 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.[26] 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.[80] In childhood LS, it has been postulated that sexual abuse could trigger LS as a Köbner effect.[81]

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.

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.[82] 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.[25,43,55] 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.[82] 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.[82] 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.[83]

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.[84] 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.[85] Regauer's criteria have not gained any acceptance and have no pathogenetic basis.

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.[50] At least one third of patients may be asymptomatic.[43] 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.[40]

Next to the genital form of LS, in 11-20% of the patients, extra-genital manifestation of the disease is present.[86,87] 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.[88,89] Most extra-genital manifestations of LS are asymptomatic, however some rare cases of severe itching of the scalp have been described.[86]

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,[25;55] performed as described earlier.

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.[43] 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.[22] In contrast with lichen (ruber) planus, in LS, the vaginal mucosa is usually unaffected, however incidental cases of vaginal LS have been reported.[90] 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 ($p=0.001$)[87]. An example of prepubertal LS can be seen in Figure 4C.

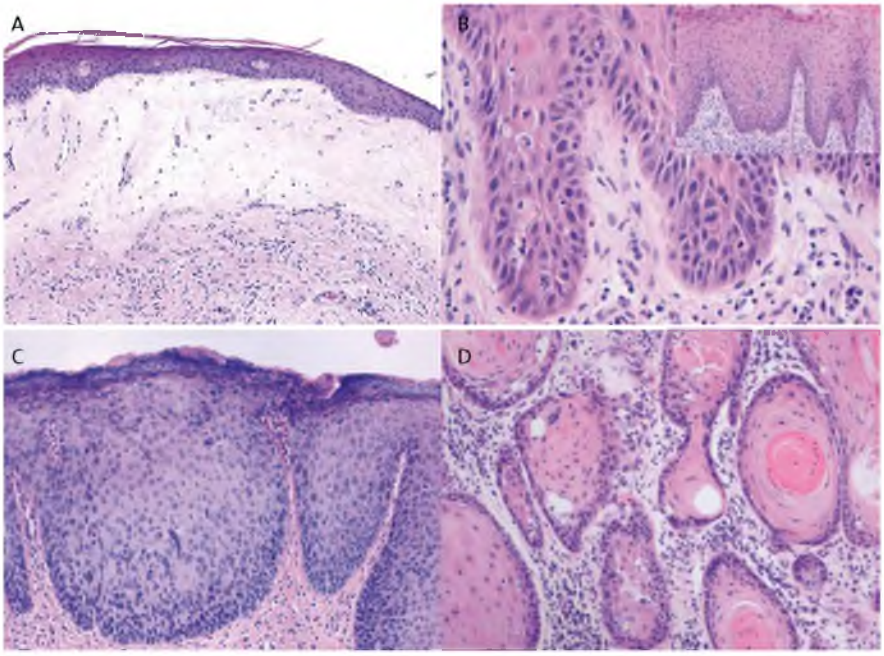
Symptoms

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 signs of LS in young girls may be confused with those of sexual abuse, especially because of local bleeding.[40;44;87] As described earlier, LS and sexual abuse may coexist.

Treatment

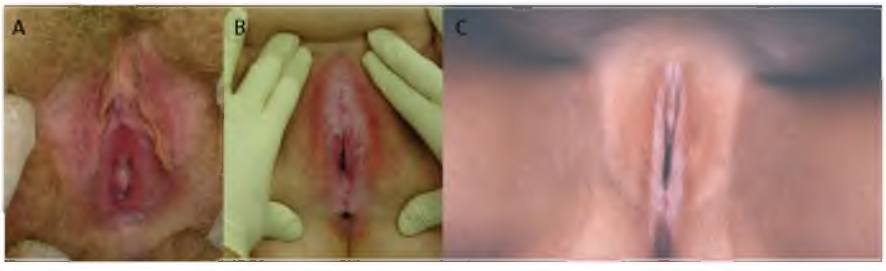
In general, LS has no cure in adults, and lifelong treatment is required. However in some cases, especially prepubertal girls, LS can spontaneously resolve.[25;91] The reasons for treating LS are alleviating and minimising symptoms and the possible prevention of architectural changes.[25;50;84;91] It is not known whether successful control of the disease reduces the long-term risk of malignancy,[92] although a protective effect from malignant evolution is suggested.[91]

Figure 3: Histological pictures of lichen sclerosus, differentiated VIN, usual VIN and vulvar squamous cell carcinoma.



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 squamous cell carcinoma). 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 squamous cell carcinoma; atypical nuclei can be found throughout the entire epithelium.
D. Invasive nests of vulvar squamous cell carcinoma (adjacent to differentiated VIN).

Figure 4: Clinical picture of different patients with lichen sclerosus.



A. LS in a pre-menopausal woman showing subtle loss of architecture and erythema.
B. Advanced stage LS in a postmenopausal woman, showing the typical 'figure of eight'.
C. Lichen sclerosus in a prepubertal girl.

Surgery

The role of surgery for the treatment of LS is limited in the absence of dVIN or malignancy.[25;50;93] 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.[25;50;93] 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.[31] Vaginal dilation and corticosteroids may be necessary after operation.

Medical treatment

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.[50;87;91;92] 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.[94-96] Ultrapotent topical steroids can reverse some of the histological changes caused by LS,[84;94;97] 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.[87]

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.[98] 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.[50;92] Long-term maintenance therapy of vulvar LS with a moisturising cream can maintain the symptom relief induced by topical corticosteroids.[80] However, this study was performed as an open trial without the use of a control group that did not use a moisturizer.

Testosterone

Some reports have noted that patients with LS have decreased serum levels of testosterone compared to age-matched controls without the disease.[27] Topical treatment with testosterone has been used and controversy exists about its effectiveness.[99] Topical testosterone can cause virilisation (clitoral enlargement, voice alterations), especially in hyperandrogenic women.[100] In a prospective, randomised, double-blind evaluation of 58 cases of vulvar LS, 2% testosterone propionate was as effective as petrolatum[101] and in several studies it was less effective than clobestasol.[102;103] Numerous studies emphasise a lack of efficacy and report severe side effects of testosterone; therefore testosterone is considered to be obsolete nowadays.[25;40;50;100]

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.[104]

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.[25;50]

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.[55;105] New research has been published on the use of tacrolimus and pimecrolimus for LS and reported to be successful.[105] 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.

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.[26;50;55;105]

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.[25;40] 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.[92] 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.[80] Written information and information about support groups should be offered.[92]

Malignant potential

There are different reasons to link LS to the development of vulvar SCC.[106] 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.[107,108] 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.[108;109] A protective effect from symptom control is suspected but has not been proven.[87] Neither the presence nor duration of symptoms nor the loss of vulvar architecture is a useful indicator of malignant potential in LS.[87]

There is an impression that itch and scratch damage (related to the duration of symptoms), is associated with the risk of developing vulvar SCC.[78] Chronic inflammation seems to play an important role, but the exact pathogenesis of progression to malignancy has not yet been established.[55] The immunogenetic profile may also determine (part of) the risk of malignant disease.[40]

Extra-genital LS is not associated with malignancy,[55] 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.[110] Recently, high p53-expression in LS has been postulated as a marker for increased likelihood to progress to vulvar SCC.[111,112]

Differentiated Vulvar Intraepithelial Neoplasia

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.[7] In the subsequent ISSVD revisions of the classification that followed, 'VIN III, 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)'.

Epidemiology

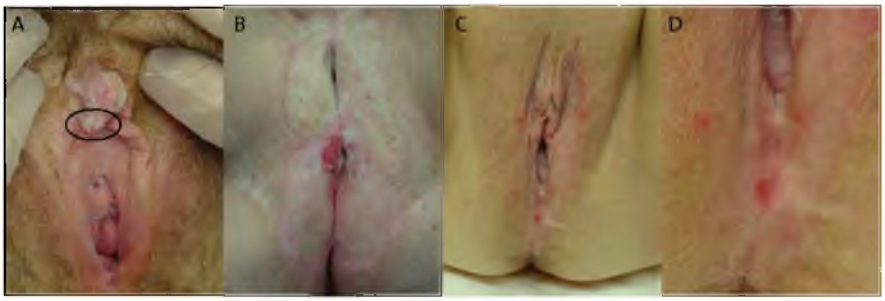
dVIN accounts for a small proportion (<2-5%) of all VIN lesions compared with the usual type.[15,113] Because of a difficult clinical and histological diagnosis there probably is a considerable underdiagnosis. dVIN characteristically occurs in postmenopausal women (mean age 67 years) and is associated with LS.[7] As dVIN is seldom found in an isolated form, some authors believe it is actually part of the adjacent vulvar SCC.[114] 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,[115] 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.[116]

Aetiology

HPV is very uncommon in dVIN and does not play an important role in its genesis.[113,116-118] 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.[116] Squamous cell hyperplasia with atypia might represent a step in the carcinogenesis.[119] 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.[114]

Figure 5: Clinical pictures of differentiated VIN.



A. Differentiated VIN arising in a background of LS. 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.

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.[7] 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.[7;22] Scattered mitotic figures are common in the basal areas, but can extend into the upper levels of the epidermis.[116] Typically, the epidermis is thickened and has a parakeratotic surface reaction.[7] 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.[22,113;116] Intercellular bridges typically are very prominent, probably as a result of loss of cohesion between cells rather than spongiosis.[7] 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, and hyperchromatism and mitoses are present.[7,22,115] In the underlying or adjacent papillary dermis, a chronic inflammatory cell infiltrate, consisting of predominantly lymphocytes, plasma cells, and occasionally eosinophils, is present[7] (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.[7] 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.[116]

Clinical characteristics

Symptoms

Patients are, due to the underlying LS, often symptomatic with a long-lasting history of itching and other LS-related symptoms.[116]

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.[120]
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.[116] 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.[116]

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.[113] 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).[83]

Malignant potential

dVIN is associated with the development of keratinising SCC.[113;116] 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.[116,120;121] 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.[116] Therefore, any suspicious area should be biopsied.[113;116]

Usual Vulvar Intraepithelial Neoplasia

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.[113] The first series of Bowen's disease of the vulva was described in 1943 by Knight et al.[122] 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.[113] In 1976, the ISSVD simplified the terminology: carcinoma in situ and vulvar atypia were replaced by Vulvar Intraepithelial Neoplasia (VIN).[113] The term VIN first appeared in literature in 1980.

ISSVD classification

The old 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.[22,123] When the entire epithelium is involved, the lesion is classified as carcinoma in situ, which is gathered in VIN 3. 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.[9;113] 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.[9] 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.[113] In the light of the two different types of vulvar carcinoma the VIN terminology has been further modified by the ISSVD, and a 2-tier classification has been suggested: VIN usual type and VIN differentiated type.[9,124] 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.[124] 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.[113] In clinical practice, no difference is made between the two uVIN lesions.

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

Old nomenclature	New nomenclature[9]
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)

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, 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).[118] 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.[124] 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.

Epidemiology

The incidence of uVIN is approximately five per 100.000 women per year and increasing worldwide.[125] In several countries the age-adjusted incidence rates have surpassed the incidence rate of invasive vulvar carcinoma.[16,19,126,127] 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.[7] Moreover, the use of immunosuppressants 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.[16,17] 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.[16,18-21,127] 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.[128]

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.[129,130] 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).[122] In 1982, detection of HPV DNA in VIN was described for the first time.[131] Only high-risk HPV (hr-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,[14,15,132-138] see Table 2), and in the largest study 100% HPV positivity has been shown.[14] 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%.[139] 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.[140-142]

Table 2: HPV positivity in VIN lesions

Study	Number VIN lesions	Test	Percentage HPV positive
Hoevenaars, 2008[133]	45 uVIN	Hr-HPV*	84%
	75 dVIN		0%
Van Beurden, 1995[15]	47 x uVIN	HPV	92%**
	1 x dVIN		
Hampl, 2006[14]	183 VIN	HPV	92%
Hillemanns, 2006[132]	30 VIN	HPV	80%
Hørding, 1991[134]	19 VIN	HPV 6,11,16,18,33	79%
Lerma, 1999[135]	12 uVIN	HPV	42%
	6 dVIN	HPV	0%
Rufforny, 2005[136]	24 VIN	HPV 16	79%
Skapa, 2007[137]	82 uVIN	HPV	99%
	12 dVIN	HPV	8%
Srodon, 2006[138]	34 VIN	Hr HPV	100%
Total	186 uVIN		89.9%
	290 VIN***		89.8%
	93 dVIN		1.0 %

* Hr-HPV: 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.

Histology

Histopathologically, uVIN lesions are easy to recognise. 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.[7,22] 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.[7] 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.[7]

Clinical characteristics

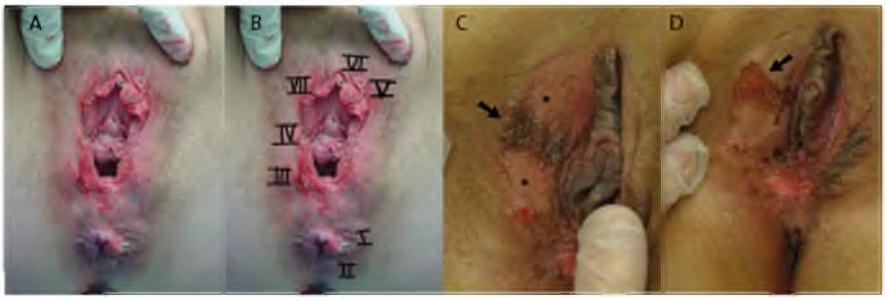
Signs

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.[143] Multifocal involvement of the vulva occurs in more than 40% of cases.[7,143] Multicentric intraepithelial or invasive squamous neoplasia (of the cervix, vagina or anus) is also common, occurring in approximately 25-66% of the uVIN patients.[7,12,14,15] The multicentricity of uVIN is age-related; decreasing from 59% in women aged 20-34 to 10% in patients over 50 years of age.[113] Multicentric uVIN are more often HPV positive than the unicentric uVIN lesions.[15,132,134] Because of the often-concurring uVIN and CIN, a cervical smear should always be performed. In Figure 6 and 7, different appearances of uVIN are depicted: varying from white and condylomatous-like to brown lesions.

Symptoms

The most common presenting complaint is pruritus, present in about 60% of the patients. Other presenting symptoms can be pain, ulceration and dysuria.[143] A significant proportion of patients have no specific complaints, apart from the finding of an abnormal vulvar area by self examination. (22%).[143]

Figure 6: Clinical pictures of usual VIN.

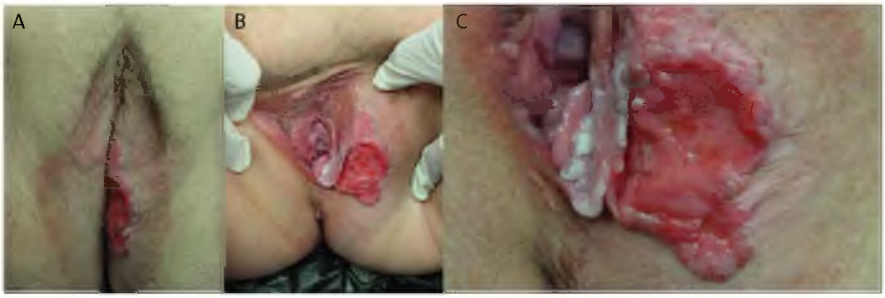


A+B. mapping of usual VIN. In order to get information of all suspicious looking lesions (I, IV, V, VI and VII), and the extent of the usual VIN lesions, two normal looking areas were also biopsied (II and III). Six biopsies showed usual VIN and in biopsy III no abnormalities were found.

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.

Figure 7: Clinical pictures of usual VIN with adjacent vulvar squamous cell carcinoma.



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.

Treatment

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.[144] The paucity of robust evidence is of critical importance supporting most of the various treatments suggested for uVIN.[144] Ideally, a treatment of uVIN should address some or all of the following:[144]

- 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.[34;36] This leads to the need for alternative treatment options.

Surgery as treatment for uVIN

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.[145] 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.[146] This observation paved again the way towards more extensive surgery for uVIN since that time.[143] 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.[145;147]

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.[113;148] Laser excision combines the advantages of both surgical excision (high cure rate and correct diagnosis), and laser vaporisation (cosmetic and functional results).[113] Laser vaporisation is a destructive technique and has the disadvantage of destroying the treated tissue, without the possibility of histological evaluation.[113] 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.[148] 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. 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 because of its 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%.[147] 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.

Medical treatment of uVIN

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.
Other medical treatment options, like 5-Fluorouracil (5-FU)[149], Interferon[150;151], photo dynamic therapy (PDT)[152] and Cidofovir[153] have been described, but are not discussed here in detail because of limited experience and results.

Imiquimod

Imiquimod (Aldara®), 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.[154-156]
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 (IFNγ). 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.[157] By inhibiting viral replication, imiquimod focuses on the cause of uVIN and preserves the anatomy and function of the vulva.[158,159]
Several studies evaluated the effect of imiquimod on uVIN lesions; 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.[159]
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.[160] 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.[161]
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.[162]

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.

Therapeutic vaccination
Two commercial vaccines against HPV (Gardasil™ and Cervarix™) are currently available in many countries worldwide. Both are produced with recombinant technologies, consist of self-assembled virus-like particles (VLPs), and have shown high immunogenicity. They have been found to be highly efficient in preventing persistent infections and lesions not only from the uterine cervix, but also from the anus, vagina, and vulva. Both vaccines have been shown to be generally safe in the phase II and phase III randomized controlled trials. Gardasil™ is a quadrivalent recombinant vaccine that protects against HPV 6, HPV11, HPV16 and HPV18.[163]. Cervarix™ is a bivalent vaccine that protects against HPV16 and HPV18. It has been chosen by the Dutch government for the Dutch vaccination programme. Cross protection for HPV31, HPV33 and HPV45 for cervical lesions has been described, but has not been evaluated for vulvar lesions.[164]

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 uVIN lesions.[11] 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.[165-167] Viral load diminished post vaccination,[166] but the same HPV subtypes were detected pre- and post-vaccination,[165] and in most patients the grade of uVIN lesion remained unchanged.[165] 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.[168] 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.[169,170] Currently the effect of other new therapeutic HPV vaccines is tested.[171]

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.[172] 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.

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%.[143,146,173-180] 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,[147] and in the study of Jones et al. 17/405 (4.2%) progression was seen after treatment.[181] 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.[182-185]

The first study on the malignant potential in untreated VIN III 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.[147] Jones et al. described 10/63 (15.8%) women who developed invasive disease before treatment of uVIN.[181] Advanced age, raised lesions, radiotherapy, and an immune compromised state are known risk factors for progression of uVIN[186]. No differences in progression rate could be found between uni- and multifocality.[147]

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'.[181]

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.[187] 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.[147] 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.

Outline of the thesis

Despite all surgical advances, the morbidity of the treatment of vulvar SCC is still impressive. A rise in absolute numbers of vulvar SCC is expected because of the proportional increase in the average age of the population. Ultimately, around 20 percent of all vulvar SCC patients will develop a recurrence, making repeated, mutilating surgery with or without radiotherapy necessary.

Improving the care for patients with vulvar (pre-)malignancies requires further understanding of the oncogenesis of vulvar SCC. In 1990, Neill et al. suggested two different kinds of vulvar carcinoma exist: one arising in vulvar dystrophy (currently LS) and another that develops after an infection with HPV, followed by VIN.[188] Additional research led to the belief that vulvar SCC indeed develops following at least two different pathways.

In chapter 2 and 3, the two pathways leading to vulvar SCC are characterised by the differences in the presence of hr-HPV DNA and the immunohistochemical expression of $p16^{INK4A}$, $p14^{ARF}$ and MIB1. In chapter 4 we investigated MIB1-expression in vulvar tissue, in order to find an explanation for the differences in malignant potential.

The malignant potential of LS is believed to be low; 3-5% of the patients develop vulvar SCC. Therefore, most guidelines recommend regular follow-up. In chapter 5 we describe the follow-up of LS patients at the departments of Gynaecology and Dermatology in the Radboud University Nijmegen Medical Centre. Follow-up in LS patients could be improved when one is able to differentiate between LS lesions that will progress into dVIN and/or vulvar SCC and those that remain solitary LS. In chapter 6 we investigated whether microvessel parameters in LS with associated SCC differ from the microvessel parameters in solitary LS. In chapter 7 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. In the final chapter 8, we propose future studies and hypothesis based on the results of the abovementioned studies, to gain further insight in the development of HPV-negative vulvar SCC and to improve the care for patients with vulvar disease.

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Chapter 2

Vulvar squamous cell carcinoma is a multifactorial disease following two separate and independent pathways

Abstract

Two separate pathways leading to vulvar carcinoma have been suggested. First, a human papillomavirus (HPV)-dependent pathway, where pre-malignant stages of vulvar cancer are the classic vulvar intraepithelial neoplasia (VIN) lesions. Second, an HPV-independent pathway, associated with differentiated VIN III lesions and/or lichen sclerosus. To obtain insight into the mechanisms underlying these pathways, we determined the relationship between HPV DNA and the expression of $p14^{ARF}$ and $p16^{INK4A}$ in non- and (pre)malignant vulvar lesions. Seventy-three archival samples of non- and (pre)neoplastic vulvar lesions were selected and tested for hr-HPV DNA using a broad-spectrum HPV detection/genotyping assay (SPF_{10}-LiPA) and the expression of $p14^{ARF}$ and $p16^{INK4A}$. The prevalence of HPV increased with the severity of the classic VIN lesions; in VIN I no hr-HPV was detected, in VIN II 43% and in VIN III 71% of the samples were hr-HPV positive. Roughly the same was true for the expression of $p14^{ARF}$ and $p16^{INK4A}$. The simultaneous expression of $p14^{ARF}$ and $p16^{INK4A}$ was highly associated with the presence of hr-HPV DNA. Hr-HPV was detected in only a single case of the differentiated VIN III lesions, whereas no expression of $p14^{ARF}$ was found and 16^{INK4A} was present in only 2 cases. All 16 samples of vulvar cancer were hr-HPV DNA negative although in respectively 63% and 25%, $p14^{ARF}$ and $p16^{INK4A}$ was expressed. No relation was found between hr-HPV and the expression of $p14^{ARF}$ and $p16^{INK4A}$ in the 20 non-neoplastic vulvar lesions.

Our results provide further evidence that vulvar squamous cell carcinoma is a multifactorial disease that develops from two different pathways. First, an HPV-dependent pathway with a remarkable resemblance to CIN lesions and cervical carcinoma and second, an HPV-independent pathway in which differentiated VIN III lesions, which are hr-HPV-negative, may be precursors.

Introduction

Vulvar cancer is the fourth most common gynecologic cancer. In the United States, in 2004, 3970 new cases of vulvar cancer (4.8% of malignancies of the female genital tract) were diagnosed (Cancer Facts and Figures 2004, American Cancer Society). Cigarette smoking, vulvar dystrophy, vulvar intraepithelial neoplasia (VIN), human papillomavirus (HPV) infection, immunodeficiency syndromes, a previous history of cervical cancer, and northern European ancestry are risk factors for the development of vulvar carcinoma.[1]

The majority of vulvar malignancies are squamous cell carcinomas. It has been suggested that vulvar squamous cell carcinoma and its precursor lesions may develop following two separate pathways, based on etiological and histopathologic characteristics, thus suggesting a heterogeneous etiology.[2-5]

The first pathway leads mainly to nonkeratinizing (basaloid and/or warty) carcinomas and primarily affects younger women. In this pathway infection with high-risk human papillomavirus (hr-HPV), predominantly HPV 16 and 18, seems to be involved.[2;3;5-7] This type of carcinoma is associated with warty and/or basaloid VIN, which is often referred to as "classic" or Bowenoid VIN.[8-11] In a recently proposed new nomenclature, the HPV-positive VIN lesions are called VIN, usual type.[12;13] The HPV-positive vulvar squamous cell carcinomas are supposed to have a significantly better prognosis than HPV-negative cancer. The second pathway is rarely associated with high-risk HPV, occurs in older women and leads to mostly differentiated keratinizing squamous cell carcinoma, in a background of non-neoplastic epithelial disorders (e.g., lichen sclerosus). Its possible precursor form is referred to as "simplex" or (well-) differentiated VIN (III) and is often difficult to distinguish from benign vulvar lesions like lichen sclerosus, chronic inflammation or normal vulvar skin.[8;11-14] The atypia in differentiated VIN III lesions is confined to the basal and parabasal layers of the epithelium, in which the cells have abundant cytoplasm and form abortive pearls and 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 layer of the epithelium has a normal maturation and does not contain koilocytosis.

It is well established that high-risk HPV infection plays an essential role in the carcinogenesis of tumors of the anogenital tract. Cancer development is the result of a complex mechanism in which, among others, HPV oncogenic proteins E6 and E7 bind host regulatory proteins, especially tumor gene products p53 and phosphorylated retinoblastoma protein (pRb). These changes may respectively lead to the degradation of p53 by the E6 oncoprotein, and to the functional inactivation of pRb through binding to the E7 gene product.[15-17] Because of the loss of tumor suppressor function the release of the transcriptional factor E2F-1 from the E2F–pRb complex may occur, allowing for the activation of $p16^{INK4A}$.[17-19] E2F also regulates $p14^{ARF}$, creating a connection between the

pRb and p53 tumor suppressor pathways.[20-22] The INK4A/ARF gene encodes the proteins $p16^{INK4A}$ and $p14^{ARF}$. $P16^{INK4A}$ is a cyclin-dependent kinase (CDK) inhibitor that regulates the activity of CDK-4 and –6. $P14^{ARF}$ mediates p53 activation by inhibiting MDM2-mediated degradation of p53 and is induced by hyperproliferative signals such as E2F, c-myc and ras. Over-expression of $p16^{INK4A}$ in cervical intraepithelial neoplasia (CIN) and cervical carcinomas with high-risk HPV infection has recently been extensively reported.[16,18,23-26] Little is known about the expression of $p14^{ARF}$ in CIN with only a few reports so far.[16,27] The relationship between the presence of HPV DNA and the expression of both $p14^{ARF}$ and $p16^{INK4A}$ in vulvar lesions has not been studied until now, which is of interest because both proteins are encoded by the same transcription factor and their pathways are activated through HPV oncogenic proteins as well as by several other factors (i.e. p53, c-myc, ras).

This study was designed to evaluate whether the presence or absence of high-risk HPV combined with the expression of the cell cycle related biomarkers $p14^{ARF}$ and $p16^{INK4A}$ in different groups of vulvar lesions may provide more insight into the pathways leading to vulvar carcinoma.

Materials and Methods

Sample selection

Seventy-three samples of different non- and (pre)neoplastic vulvar lesions were randomly selected from the archives of the Department of Pathology, Radboud University Nijmegen Medical Centre (Nijmegen, The Netherlands). All tissue specimens were collected between 1992 and 2000 and routinely fixed in 4% buffered formalin and paraffin embedded. The hematocylin and eosin-stained slides were re-examined blindly and independently by two consultant pathologists specialized in gynecological pathology (JG and JB) and diagnosed based on published histomorphologic criteria (classic VIN I, classic VIN II, classic VIN III, differentiated VIN III, squamous cell carcinoma, lichen sclerosus or chronic inflammation).[28] Subsequently, divergently scored lesions were presented to a third gynecopathologist, who formulated a final diagnosis (which in all cases was identical to the consensus diagnosus reached by the 2 pathologists). In 66 of the 73 cases (90%) the independent diagnoses of the 2 pathologists were identical. Altogether, the kappa value of was 0.94, indicating an excellent rate of interobserver reproducibility.[29] All samples of squamous cell carcinoma were classified as being well, moderately or poorly differentiated (grade 1-3, WHO classification).[28]

Thus, the final cases included in this study consisted of 10 samples of normal vulvar tissue with chronic inflammation, 10 samples of lichen sclerosus, 5 classic VIN I lesions, 7 classic VIN II lesions, 17 classic VIN III lesions, 8 differentiated VIN III lesions and 16 samples of squamous cell carcinoma (14 keratinizing and 2 non-keratinizing).

The median age was 57 years (range, 8-91). Details of the different diagnostic groups are presented in Table 1.

Table 1. Numbers of samples in each diagnostic group and median age per group

Diagnostic group	Final diagnosis		Initial diagnosis by pathologist		Median age (yr) (min;max)
			JB	JG	
Classic VIN I	5		4	5	48 (36;68)
Classic VIN II	7		9	9	54 (37;74)
Classic VIN III	17		15	14	48 (29;82)
Differentiated VIN III	8		8	9	66 (62;88)
Squamous cell carcinoma	16	(2)	15	16	71 (37;91)
Well-differentiated	9		-	-	62 (37;91)
Moderately differentiated	5	(1)	-	-	75 (44;87)
Poorly differentiated	2	(1)	-	-	87.5 (87;88)
Lichen sclerosus	10		10	9	57.5 (8;68)
Inflammation	10		10	10	32.5 (21;72)
Not conclusive	0		2	1	-
Total	73		73	73	57 (8;91)

Numbers in parenthesis in consensus diagnosis column = number of nonkeratinizing squamous cell carcinoma samples.

Immunohistochemistry

Four-micrometer-thick sections of the archival paraffin-embedded tissue samples were mounted onto polylysine-coated slides and dried overnight at 58°C. The sections were dewaxed in xylene and endogenous peroxidase was blocked using H_2O_2 in methanol for 15 minutes and the slides were rinsed three times in phosphate-buffered saline (PBS; pH 7.4) for 5 minutes. The slides were placed in a citrate buffer (0.01 M; pH 6.0), heated in a household microwave oven (3 minutes at 850 W until boiling; followed by 10 minutes at 180 W). The sections were allowed to cool down to room temperature (RT) and briefly washed in PBS (10 minutes). Subsequently the slides were pre-incubated with 20% normal goat serum and then incubated with primary antibodies $p16^{INK4A}$ (clone 16P04, Neomarkers, Fermont, CA) diluted 1:500 in PBS with 1 % bovine serum albumine (BSA) (60 minutes, RT) and $p14^{ARF}$ (clone 14P02, Neomarkers) diluted 1:200 in PBS with 1% BSA (60 minutes, RT). Subsequently the slides were rinsed in PBS (10 minutes) and postantibody blocking was done for 15 minutes (Powervision Plus, Dako SA, Glostrup, Denmark). This was followed by

incubation with polymeric-horse-radish peroxidase-goat anti-mouse/rabbit/rat IgG (30 min, RT). Staining was developed with diaminobenzidine (mixed with H_2O_2), counterstained with Mayer's hematoxylin, dehydrated in ethanol and xylene and finally mounted. Negative controls (buffer only) and HPV-positive classic VIN III and CIN3 lesions served as $p16^{INK4A}$ and $p14^{ARF}$ positive controls in each run.

Interpretation of $p16^{INK4A}$ and $p14^{ARF}$

For $p16^{INK4A}$, nuclear and cytoplasmic staining was 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.[14;18;30;31]

For $p14^{ARF}$, only nuclear or nucleolar staining was considered to be a positive reaction (no cytoplasmic staining was seen in any case). Because of a less extended expression pattern of $p14^{ARF}$, a different semi-quantitative scoring system was used: negative (-) if <1% of cells were stained, slightly positive (1+) if the percentage was in the range of 1 to 5% and moderately positive (2+) if >5% of the cells were stained.[16;21]

All stains were analyzed by one pathologist (HS). In case of doubt a second pathologist (JB) was consulted.

HPV detection and genotyping

Four-micrometer-thick tissue sections of each archival sample were put into a reaction tube and incubated overnight at 56 °C in 200 µl of 10 mM tris–HCL with 1 mM EDTA, 0.2% Tween-20, and proteinase K (0.3 mg/ml). Proteinase K was inactivated by 10 min incubation at 100 °C. The sample was centrifuged for 10 min at 11.000 rpm and 10 µl was directly used for PCR analysis. A water blank control was processed with each batch of ten 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 for the detection of at least 43 HPV types. Subsequent HPV genotyping was performed via a reverse hybridization line probe assay (LiPA), allowing for simultaneous typing of the following 25 HPV-genotypes: HPV 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70 and 74. The combined SPF-PCR-LiPA system for detection and genotyping of HPV has been described in detail elsewhere.[32;33]

Statistical analysis

The interobserver variability was established by determining the kappa value. A kappa (κ) value of 1 indicates 100% interrater reliability. Generally, $\kappa > 0.80$ represents excellent agreement, $0.80 \geq \kappa > 0.60$ represents substantial agreement, $0.60 \geq \kappa > 0.40$ represents moderate agreement; $0.40 \geq \kappa > 0.20$ represents fair agreement, and κ ffi 0.20 represents slight or poor agreement.[29]

Spearman's rank correlation (Spearman's ρ) was used to investigate the correlation between the expression of $p14^{ARF}$ and $p16^{INK4A}$ and the grade of squamous cell carcinoma and the grade of dysplasia in classic VIN. To test whether the expression of $p14^{ARF}$ and $p16^{INK4A}$ differed between cases infected and not infected with high-risk HPV, the non-parametric Mann-Whitney U test for unpaired observations was used.

To facilitate the statistical analysis of the combined expression of $p14^{ARF}$ and $p16^{INK4A}$, the variables were converted to binary variables. For $p16^{INK4A}$, no staining (-) and slightly positive (+) immunohistochemical stainings were considered negative; moderately positive (++) and markedly positive (+++) immunohistochemical stainings were considered positive. Because of the less extended expression pattern and the absence of staining in the inflammed and lichen sclerosus samples, for $p14^{ARF}$, no staining (-) was considered negative and slightly (+) and moderately (++) stained samples were considered positive. The association between the presence of $p14^{ARF}$ and $p16^{INK4A}$ expression was assessed by 2 x 2 contingency tables and using the Fisher exact test.[34] All analyzes were performed by using SPSS® version 12.0.1 software (SPSS Inc., Chicago, IL). A significance level of $p<0.05$ was considered to be significant.

Results

The immunohistochemical staining and HPV detection and typing results of all 73 samples are presented in Table 2.

HPV detection and typing

No high-risk HPV DNA was detected in the VIN I lesions. High-risk HPV was detected in 3 of the 7 classic VIN II lesions (43%), and in 12 of the 17 classic VIN III lesions (71%). Only one of eight differentiated VIN III lesions contained high-risk HPV DNA (13%). In the 10 lesions with chronic inflammation, also 1 sample was found to be positive for high-risk HPV DNA (10%) and in none of the samples of squamous cell carcinoma and lichen sclerosus lesions high-risk HPV DNA was detected. Low-risk HPV was detected in two classic VIN I lesions and in two classic VIN II lesions (all HPV6).

Tablel 2. Analysis of HPV, $p16^{INK4A}$ and $p14^{ARF}$ in 73 vulvar lesions

Case	HPV	$p14^{ARF}$	$p16^{INK4A}$
1 classic VIN I	–	–	+++
2 classic VIN I	–	–	–
3 classic VIN I	–	–	–
4 classic VIN I	6	–	+
5 classic VIN I	6	–	+
6 classic VIN II	–	–	–
7 classic VIN II	–	–	–
8 classic VIN II	16	+	+++
9 classic VIN II	16	++	+++
10 classic VIN II	31	+	+++
11 classic VIN II	6	–	++
12 classic VIN II	6	+	+++
13 classic VIN III	–	++	+++
14 classic VIN III	–	++	–
15 classic VIN III	–	++	–
16 classic VIN III	–	++	+++
17 classic VIN III	–	–	+++
18 classic VIN III	16	++	+++
19 classic VIN III	16	++	+++
20 classic VIN III	16	++	+++
21 classic VIN III	16	+	+++
22 classic VIN III	16	+	+++
23 classic VIN III	16	–	+++
24 classic VIN III	16	++	+++
25 classic VIN III	16	–	+++
26 classic VIN III	16	+	+++
27 classic VIN III	33	++	+++
28 classic VIN III	16	++	+++
29 classic VIN III	31	++	+++
30 differentiated VIN III	–	–	–
31 differentiated VIN III	–	–	–
32 differentiated VIN III	–	–	–
33 differentiated VIN III	–	–	+
34 differentiated VIN III	–	–	+
35 differentiated VIN III	–	–	–
36 differentiated VIN III	–	–	–
37 differentiated VIN III	16	–	–
38 SCC well differentiated	–	++	+++

39 SCC well differentiated	–	-	-
40 SCC well differentiated	–	++	+
41 SCC well differentiated	–	++	++
42 SCC well differentiated	–	-	-
43 SCC well differentiated	–	-	-
44 SCC well differentiated	–	+	-
45 SCC well differentiated	–	-	-
46 SCC well differentiated	–	-	-
47 SCC moderately differentiated	–	-	-
48 SCC moderately differentiated	–	+	-
49 SCC moderately differentiated	–	+	-
50 SCC moderately differentiated	–	++	-
51 SCC moderately differentiated (NK)	–	++	+++
52 SCC poorly differentiated	–	++	-
53 SCC poorly differentiated (NK)	–	++	-
54 Lichen Sclerosus	–	–	–
55 Lichen Sclerosus	–	–	–
56 Lichen Sclerosus	–	–	+
57 Lichen Sclerosus	–	–	+
58 Lichen Sclerosus	–	–	+
59 Lichen Sclerosus	–	–	–
60 Lichen Sclerosus	–	–	–
61 Lichen Sclerosus	–	–	–
62 Lichen Sclerosus	–	–	–
63 Lichen Sclerosus	–	–	+
64 Inflammation	–	–	–
65 Inflammation	–	–	–
66 Inflammation	–	–	–
67 Inflammation	–	–	–
68 Inflammation	–	–	–
69 Inflammation	–	–	–
70 Inflammation	–	–	–
71 Inflammation	–	–	–
72 Inflammation	–	–	–
73 Inflammation	16	–	–

(-) negative; (+) slightly positive; (++) moderately positive; (+++) markedly positive;
(SCC) squamous cell carcinoma; (NK) nonkeratinizing.

p14^{ARF} expression in vulvar lesions

Examples of the nuclear and nucleolar staining with p14^{ARF} in classic VIN III and vulvar squamous cell carcinoma is shown in the figures 1A and B, respectively. No cytoplasmic staining was observed. The level of p14^{ARF} expression increased with the degree of dysplasia in the classic VIN lesions (Spearman's ρ=0.63; p<0.001): no expression in classic VIN I lesions, 57% (4/7) was slightly to moderately positive in the classic VIN II lesions and the majority of the classic VIN III lesions were slightly (18%) or moderately (65%) positive (3/17 and 11/17 respectively). In the diagnostic groups of differentiated VIN III, lichen sclerosus and chronic inflammation no p14^{ARF} expression was found. Expression of p14^{ARF} in vulvar carcinoma samples was heterogeneous: 38% (6/16) of the samples showed no expression; 19 % (3/16) of the samples were slightly positive and the remaining 44% (7/16) were moderately positive. There was no statistically significant correlation between the expression of p14^{ARF} and the grade of squamous cell carcinoma (Spearman's ρ=0.4; p=0.1).

p16^{INK4A} expression in vulvar lesions

In the classic VIN lesions the level of expression of p16^{INK4A} increased with the degree of the VIN (Spearman's ρ=0.5; p=0.005): in the classic VIN I lesions 60% (3/5) of the samples were slightly to markedly positive for p16^{INK4A}; in the classic VIN II lesions there was moderate to marked expression in 71% (5/7) of the samples and 88% (15/17) of the classic VIN III lesion samples were markedly positive for p16^{INK4A}. Figure 1C is an example of nuclear and cytoplasmic staining for p16^{INK4A} in a classic VIN III lesion. In classic VIN I-III lesions, the positivity for p16^{INK4A} parallels the presence of atypical cells at different levels within the epithelium (Fig. 1C and D).

Seventy-five percent (12/16) of the vulvar carcinoma samples showed no expression of p16^{INK4A}; in one sample (6%) there was slight (5-25%) staining and 19% (3/16) of the samples were moderately to markedly positive for p16^{INK4A}. No statistically significant correlation between the expression of p16^{INK4A} and the grade of differentiation of the squamous cell carcinoma was found (Spearman's ρ=-0.21; p=0.43).

None of the inflammatory lesions showed any expression of p16^{INK4A}. Six lichen sclerosus samples were negative and the other four were slightly positive (nuclear or cytoplasmic staining in 5-25% of the cells). Two of eight differentiated VIN III lesions were slightly positive for p16^{INK4A}.

Combined results of HPV detection and p14^{ARF} / p16^{INK4A} expression

Sixty-eight percent (19/28) of the p14^{ARF} positive vulvar lesions simultaneously expressed p16^{INK4A} and from the p16^{INK4A} positive lesions, 79% (19/24) had a concordant expression of p14^{ARF}. In the classic VIN lesions the expression of p14^{ARF} and p16^{INK4A} was significantly associated (Fisher exact test, p=0.028); for the squamous cell carcinomas there was no significant association (Fisher exact test, p=0.25). All high-risk HPV positive classic VIN lesions were accompanied by the expression of p16^{INK4A} and 13 of

15 had a concordant expression of $p14^{ARF}$ (87%). The two other high-risk HPV positive lesions (a differentiated VIN III lesion and a sample of inflammation) were negative for $p16^{INK4A}$ and $p14^{ARF}$. The expression of $p14^{ARF}$ and $p16^{INK4A}$ in classic VIN lesions was more frequently observed in the presence of high-risk HPV (Mann-Whitney U test=57.5; p=0.037 and Mann-Whitney U test=37.5; p=0.002 respectively). The simultaneous expression of $p14^{ARF}$ and $p16^{INK4A}$ in squamous cell carcinoma lesions was 19% (3/16), of which all were negative for high-risk HPV. The differentiated VIN III lesions showed no simultaneous expression for $p14^{ARF}$ and $p16^{INK4A}$.

Discussion

This article provides further evidence that vulvar squamous cell carcinoma and the preneoplastic precursor lesions may develop following two different pathways: HPV-dependent and HPV-independent pathway. The mechanistic aspects leading to these different pathways will be discussed.

Development of vulvar squamous cell carcinoma: HPV-dependent pathway

The presence of high-risk HPV DNA in classic VIN lesions has been described to vary from 0 to 90%, depending on the stage of dysplasia (higher VIN lesions are more often positive for HPV DNA), type of HPV, and sensitivity of the method of HPV detection.[5;9;14;35;36] The HPV genotypes in this study correspond to the types that have been described in the literature: generally HPV 16 is considered to be the most common genotype in vulvar lesions although HPV type 18, type 31, type 33 and type 45 also have been reported. Low-risk HPV types can also be found, being predominantly HPV type 6 and type 11.

In classic VIN lesions, the percentages of both $p14^{ARF}$- and $p16^{INK4A}$-expressing cells increased with the degree of dysplasia and the simultaneous expression was associated with the presence of hr-HPV DNA. Eighty-seven percent (13/15) of the hr-HPV positive classic VIN lesions expressed both $p14^{ARF}$ and $p16^{INK4A}$. There is a remarkable resemblance with CIN lesions, where the expression of $p14^{ARF}$ and $p16^{INK4A}$ has also been reported to be associated, with a close relation to the presence of high-risk HPV.[16;27] $p16^{INK4A}$ expression in vulvar lesions has also been described by Chan et al., who found a comparable increase of $p16^{INK4A}$ expression with the increase in the grade of VIN lesions.[30] The HPV status, however, was not assessed. The expression of $p14^{ARF}$ in vulvar lesions has not been published.

The nonkeratinizing carcinoma is considered to be the endpoint of the HPV-dependent pathway leading to vulvar carcinoma. The nonkeratinizing vulvar carcinomas in this study were negative for HPV DNA, whereas others reported a presence of hr-HPV in these lesions from 69 to 100%.[14] A possible explanation for the negative results might

be the subset of HPV types that was tested for was not extensive enough; we only tested for 25 types of HPV. In cervical cancer studies the HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 68, 73 and 82 have been classified as high-risk and the HPV types 26, 53, and 66 as probable high-risk types of HPV. The HPV types 6, 11, 34, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP6108 have been classified as low-risk types.[17;33;37] The detected HPV types were classified accordingly for the vulvar lesions in this study. It might be possible that there are unidentified high-risk vulvar HPV types. One of the nonkeratinizing HPV-negative squamous cell carcinomas and two HPV DNA negative classic VIN III lesions were positive for $p16^{INK4A}$ and $p14^{ARF}$, supporting this possibility, because the simultaneous expression of $p16^{INK4A}$ and $p14^{ARF}$ was highly associated with the presence of hr-HPV DNA.

Unfortunately, there were only two nonkeratinizing vulvar carcinomas in this study, which makes it difficult to compare the results with other studies. The small amount of nonkeratinizing squamous cell carcinomas in the randomly selected group of samples in this study, might be explained by the hypothesis that the easier to recognize classic VIN lesions, which proceed to nonkeratinizing squamous cell carcinomas, have a long transition period before they develop into squamous cell carcinoma and thus HPV-related VIN lesions are surgically removed in an early stage. The more difficult to diagnose and identify differentiated VIN III lesions (probably the precursor of the keratinizing squamous cell carcinoma) is assumed to have a short existence period with rapid progression into carcinoma.

Development of vulvar squamous cell carcinoma: HPV-independent pathway

Our results support the hypothesis that, next to an HPV-dependent pathway, a second HPV-independent pathway leading to vulvar squamous cell carcinoma exists. All keratinizing squamous cell carcinomas in this study were hr-HPV negative which is in accordance with the results of others.[3;4;11;38] Furthermore, seven of eight (88%) differentiated VIN III lesions also were hr-HPV negative. The median age of the women with differentiated VIN III lesions and squamous cell carcinomas is comparable and remarkably higher than the median age in the group of HPV-dependent classic VIN lesions. Also, only 14% (3/22) of the presumed HPV-independent lesions (differentiated VIN III and keratinizing squamous cell carcinomas) show simultaneous expression of $p14^{ARF}$ and $p16^{INK4A}$. The absence of hr-HPV, the low presence of combined expression of $p14^{ARF}$ and $p16^{INK4A}$, and the high median age in both groups of differentiated VIN III and keratinizing squamous cell carcinoma, make it likely that differentiated VIN III precedes vulvar keratinizing squamous cell carcinoma. The heterogeneous expression patterns for either $p14^{ARF}$ or $p16^{INK4A}$ can not be explained by the presence or absence of hr-HPV. Perhaps $p14^{ARF}$ and $p16^{INK4A}$ expression in vulvar squamous neoplasms are more complicated than the cervical counterparts.

The absence of $p14^{ARF}$-expression in lichen sclerosus, inflammation and differentiated VIN III lesions and the presence of $p14^{ARF}$-expression in 57% of the squamous cell

carcinomas (8/14) suggests that (over)expression of $p14^{ARF}$ is associated with tumor progression. However, the role of $p14^{ARF}$ in vulvar lesions in general cannot be entirely explained in this way, as there was considerable $p14^{ARF}$ expression in the classic VIN lesions, which are by definition non-invasive. Possibly, $p14^{ARF}$ is activated differently in both pathways; through E6 and/or E7 in the HPV-dependent pathway and through other factors in the HPV-independent pathway (e.g., c-myc or ras). It has been suggested that an alteration in the p53 gene is important in the genesis of differentiated VIN III and the development of vulvar carcinoma in the absence of HPV.[11;39;40]

The International Society for the Study of Vulvovaginal Disease (ISSVD), which recently proposed a modified terminology for vulvar lesions, emphasizes that there is no evidence that the VIN 1 to 3 morphologic spectrum reflects a biologic continuum. They also state that VIN I is no cancer precursor; the fact that we only found lr-HPV and limited expression of $p16^{INK4A}$ might support this. Further research on both keratinizing and nonkeratinizing vulvar squamous cell carcinomas with other p53/mdm2/$p14^{ARF}$-pathway- and pRb/cyclinD1/$p16^{INK4A}$-pathway-related biomarkers is needed to gain further insight in mechanisms taking place in the pathways that lead to invasive vulvar squamous cell carcinoma and to investigate the parallels with CIN that have been drawn.

Lichen sclerosus and inflammation

Women with lichen sclerosus are reported to have an increased risk of developing squamous cell carcinoma, although still the vast majority of women with lichen sclerosus do not develop vulvar cancer.[8,11;41] In this study 40% of the lichen sclerosus lesions were slightly positive for $p16^{INK4A}$ and consistently negative for $p14^{ARF}$, which is comparable to the expression in differentiated VIN III lesions. These results are comparable to other studies on lichen sclerosus and $p16^{INK4A}$ expression: Riethdorf et al. [14] found lichen sclerosus to contain focal and heterogeneous $p16^{INK4A}$ expression in 42%, with little to no expression in other benign vulvar lesions and normal skin. From the seven benign lesions studied by Chan et al., six were positive for $p16^{INK4A}$, with <25% of the cells showing nuclear staining.[30] There was no expression for $p14^{ARF}$ or $p16^{INK4A}$ in the group of inflammation. Neither $p14^{ARF}$ nor $p16^{INK4A}$ can be helpful in distinguishing inflammation or normal vulvar epithelium from differentiated VIN III lesions.

In conclusion, the results of this study are in agreement with the existence of two distinct pathways leading to vulvar cancer. The HPV-dependent pathway resembles the pathway leading to the development of cervical cancer and seems to result in predominantly nonkeratinizing vulvar cancer. The mechanism(s) involved in the HPV-independent pathway presumably leading to keratinizing vulvar carcinomas, as yet, remains unclear and further investigations are needed to determine the position of differentiated VIN III lesions and lichen sclerosus in this pathway.

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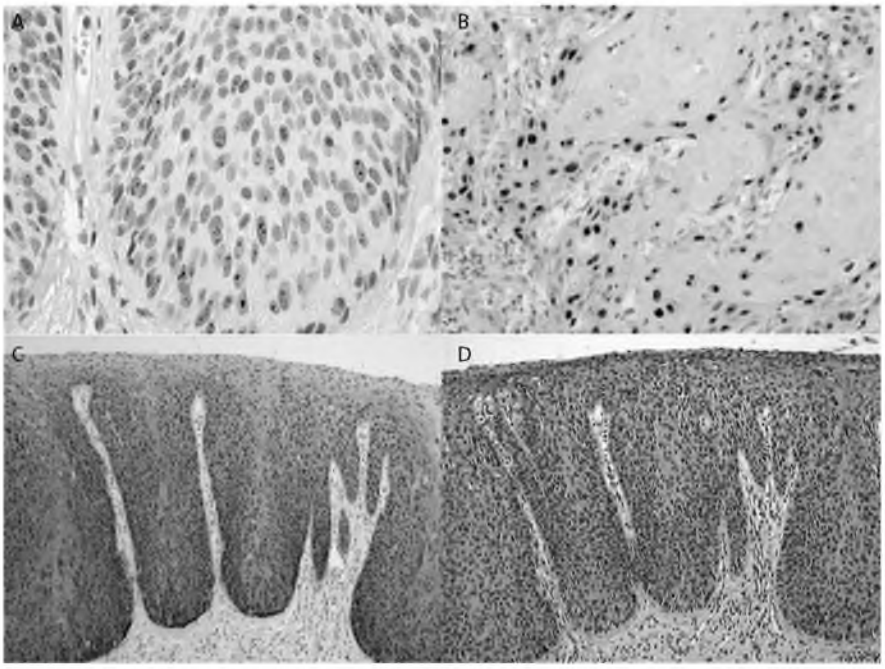
Figure 1



Hematoxylin and eosin and indirect immunohistochemical staining of 4-µm paraffin sections of a classic VIN III lesion and a squamous cell carcinoma. The peroxidase-labeled immunohistochemical complexes were visualized with diaminobenzodine (DAB) (brown-black).

A. $p14^{ARF}$-stained slide, showing a basal part of the epithelium of a classic VIN III lesion with speckled positive nuclei and/or nucleoli.

B. Squamous cell carcinoma stained with $p14^{ARF}$: positive nuclei (speckled) can be seen especially at the invasive border of the lesion.

C. In a classic VIN III lesion $p16^{INK4A}$-positive cells (nuclei and cytoplasm) can be seen in all layers of the epithelium.

D. Hematocylin and eosin-stained section of the classic VIN III lesion, showing atypical cells throughout the whole thickness of the epithelium.

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Chapter 3

A panel of $p16^{INK4A}$, MIB1 and p53 proteins can distinguish between the two pathways leading to vulvar squamous cell carcinoma

Abstract

Two pathways leading to vulvar squamous cell carcinoma (SCC) exist. The expression of proliferation- and cell-cycle-related biomarkers and the presence of high-risk (hr) HPV might be helpful to distinguish the premalignancies in both pathways. Seventy-five differentiated VIN-lesions with adjacent SCC and 45 usual VIN-lesions (32 solitary and 13 with adjacent SCC) were selected, and tested for hr-HPV DNA, using a broad-spectrum HPV detection/genotyping assay (SPF_{10}-LiPA), and the immunohistochemical expression of MIB1, $p16^{INK4A}$ and p53. All differentiated VIN-lesions were hr-HPV- and p16-negative and in 96% MIB1-expression was confined to the parabasal layers. Eighty-four percent exhibited high p53 labeling indices, sometimes with parabasal extension. Eighty percent of all usual VIN-lesions were hr-HPV-positive, p16-positive, MIB1-positive and p53-negative. Five (of seven) HPV-negative usual VIN lesions, had an expression pattern like the other HPV-positive usual VIN lesions. In conclusion, both pathways leading to vulvar SCC have their own immunohistochemical profile, which can be used to distinguish the two types of VIN, but cannot explain differences in malignant potential.

Introduction

Vulvar squamous cell carcinoma (SCC) accounts for 3-4% of all female genital cancers. There are two types of vulvar SCC that have different clinical and pathological features.[1,2] Both types of vulvar cancer are preceded by their own type of vulvar intra-epithelial neoplasia (VIN). Based on histopathological characteristics, VIN lesions can be divided into usual VIN (also known as Bowenoid or classic VIN, basaloid or warty subtype) and differentiated VIN (formerly named simplex VIN or well-differentiated VIN).[3] Recently, the International Society for Vulvovaginal Disease (ISSVD) has proposed a revised nomenclature for vulvar lesions.[2,3]

The majority of vulvar SCCs occur in elderly patients with lichen sclerosus and develop following an human papillomavirus (HPV)-negative pathway.[4,5] Its premalignancy, differentiated VIN, can be difficult to distinguish from a benign vulvar lesion (e.g. chronic inflammation) or normal epithelium.[5,6] It is assumed that differentiated VIN is highly proliferative and might rapidly progress into an invasive neoplasm, because it is seldom found without (micro-invasive) vulvar carcinoma and often adjacent to HPV-negative vulvar SCC.[2,5,6] Since differentiated VIN is often unifocal and the amount of skin involved is limited, surgical treatment by means of a wide local excision probably reduces the risk of progression to invasive carcinoma.[7]

Usual VIN is often multifocal, occurs in younger women and is associated with smoking and HPV, predominantly HPV16 and -18, and can lead to HPV-positive vulvar SCC.[8] One third of all vulvar SCCs is associated with HPV.[9] The risk of malignant transformation of usual VIN to an invasive carcinoma appears to be 3-4%. The viral gene products E6 and E7 interfere with two pathways of cell cycle regulation. HPV E6 can interact with p53, leading to p53 dysfunction, which allows for an absence of cell cycle arrest.[6,10] HPV E7 can inactivate pRb which can result in an over-expression of p16^{INK4A} and hyperproliferation.[11,12]

Proliferative activity in tissues can be visualized using MIB1, a proliferation marker which is a monoclonal antibody against the Ki-67 nuclear antigen, present in human proliferating cells in all stages of the cell cycle besides the G_0 phase.[13] In several (pre-) malignant lesions, MIB1-expression can be used for grading, estimating prognosis, and prediction of biological behaviour.[14-18] The tumor suppressor p53 detects genetic alterations in cells in G_1-phase, resulting in cell cycle arrest or apoptosis. It frequently is mutated in HPV-negative vulvar SCC.[19] Immunohistochemically, p53 is detected frequently in vulvar SCC and differentiated VIN, most likely because of cellular accumulation of the mutated abnormal protein.[6]

The lack of knowledge about the oncogenesis of vulvar SCC and the malignant potential of VIN lesions result in the absence of an evidence based protocol for the optimal treatment and follow-up for patients with VIN. The aim of the present study

was to investigate the patterns of MIB1, $p16^{INK4A}$ and p53-expression and the presence of HPV in VIN lesions and adjacent SCCs to gain insight in the oncogenesis of vulvar squamous cell carcinoma, and test whether these parameters can be helpful to distinguish the two types of VIN lesions.

Materials and methods

Patients and histopathology

All patients with a histological diagnosis of VIN with or without concurrent primary vulvar carcinoma between 1990 and 2002, with available microscopic slides and paraffin blocks, were selected from the database of the Department of Pathology of the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands (n=162). No recurrent vulvar carcinomas were selected. When a patient had a VIN lesion preceding or after the diagnosis of vulvar carcinoma, only the carcinoma and the adjacent VIN lesion were used for analysis. This led to a reduction with 25 cases. Another seventeen patients were excluded because of the absence of VIN according to current criteria, in which VIN1 is no longer considered to be a premalignancy.[3]

All original hematoxylin-and-eosin-stained slides were reviewed by one pathologist with special expertise in gynecopathology [JB]. The histological diagnosis of the vulvar lesion was based on Kurman et al., Sideri et al. and Wilkinson et al..[3,20,21] The differentiation grade of vulvar SCCs was determined according to WHO criteria. In figure 2A, 2E-inset, 3A, and 3A-inset, H&E stained sections of respectively solitary usual VIN, SCC associated with usual VIN, and differentiated VIN with adjacent SCC are shown.

After revision, a total number of 120 patients with a VIN lesion were eligible for analyses. Eighty-eight patients had an associated primary vulvar carcinoma and 32 patients did not have nor developed a vulvar carcinoma (last date of follow-up December 2006). No patients with differentiated type VIN without a previous or subsequent vulvar SCC were diagnosed and therefore this entity was not present in this study. Of the 88 patients with an associated vulvar carcinoma, 13 had a concurrent usual VIN lesion and in 75 patients the carcinoma was adjacent to a differentiated VIN lesion. Representative sections for each case were selected for immunohistochemical analysis. A minimum distance of 0.5 cm between differentiated VIN and SCC in the same slide was required. When normal vulvar epithelium was available in the tissue sample, a site most distant from the (pre-) malignant vulvar lesion was selected for analysis of one or more immunohistochemical parameters (n=62; 40 patients with a differentiated VIN lesion with associated SCC, nine patients with usual VIN with associated SCC and 13 patients with a solitary usual VIN lesion).

Material of 32 patients was also used in previous studies by the same group; mostly providing lichen sclerosus and normal vulvar epithelium (not in investigation in this paper).[9;22] When the use of VIN and/or SCC was duplicated, new H&E staining as well as immunohistochemical- and HPV-analysis was performed.

Recently, a patient with a solitary dVIN lesion was treated at our hospital. She had lichen sclerosus and five years ago she underwent a hemivulvectomy with bilateral inguinofemoral lymph node dissection because of a multifocal, macro-invasive squamous cell carcinoma of the vulva. Afterwards she received radiotherapy because of two positive lymphnodes in the left groin.

HPV DNA detection

Four micrometer thick tissue sections of each archival sample were put into a reaction tube and incubated overnight at 56°C in 200 µl of 10 mM tris–HCL with 1 mM EDTA, 0.2% Tween-20, and proteinase K (0.3 mg/ml). If the VIN lesion and vulvar carcinoma were not available in the same archival tissue sample, two tissue sections (placed in one reaction tube) were used for HPV analysis. Proteinase K was inactivated by 10 min incubation at 100°C. The sample was centrifuged for 10 min at 11.000 rpm and 10 µl was directly used for PCR analysis. A water blank control was processed with each batch of ten 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 for the detection of at least 43 HPV genotypes. Subsequent HPV genotyping was performed via a reverse hybridization line probe assay (HPV SPF_{10} Line BLOT 25, LABO Bio-Medical products B.V., Rijswijk, The Netherlands), allowing for simultaneous typing of the following 25 HPV-genotypes: HPV 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70 and 74. The combined SPF-PCR-LiPA system for detection and genotyping of HPV has been described in detail elsewhere and is considered highly sensitive.[23;24] In cervical cancer studies the HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 have been classified as high-risk and the HPV types 26, 53, and 66 as probable high-risk types of HPV.[12;25] The HPV types detected in this study were classified accordingly.

Immunohistochemistry

Serial tissue sections (4-µm thick) of formalin-fixed and paraffin-embedded blocks were cut with the first and the last sections hematoxylin and eosin-stained for control. After deparaffinizing and hydration, endogene peroxidase was blocked by an incubation in 1,5% H_2O_2 in phospate-buffered saline (PBS) for 15 minutes. Antigen retrieval was performed by microwave heat induction. The slides were pre-incubated with 20% normal goat serum (10 min) and then incubated with primary antibodies p53 (clone DO7, Dakocytomation, Denmark) 1:400, p16^{INK4A} (clone 16PO4, Neomarkers, Fermont, CA, USA) 1:500, and MIB1 (clone MIB1, Dakocytomation, Denmark) 1:200, all suspended in 1 % bovine serum albumine (BSA)/PBS (60 min, RT). Subsequently, post-antibody blocking was done for 15 min (powervision plus). This was followed by

incubation with polymeric-horse-radish peroxidase-goat anti-mouse/rabbit/rat IgG (30 min, RT). The slides were developed with diaminobenzidine (mixed with H_2O_2) and the p53 and p16^{INK4A} slides were rinsed in $CuSO_4$ for amplification; all slides were counterstained with Mayer's hematoxylin (30 sec), dehydrated and finally mounted. All incubation steps were followed by three washes in PBS. Titration experiments were performed to determine the aforementioned optimal dilutions for the primary antibodies and in each series a positive control was included (CIN3 lesion).

Quantification of immunohistochemical results

The immunoreactivity of p16^{INK4A} in the VIN lesion (and, when present, in their adjacent vulvar carcinoma and normal tissue) was scored based on the localization and extent of the p16^{INK4A}-immunoreactivity within the epithelium. Three categories were discerned: (1) no p16^{INK4A}-positivity, (2) focal p16^{INK4A}-positivity, and (3) diffuse, transepidermal positive p16^{INK4A}-staining.[26-28] For statistical purposes, focal p16^{INK4A}-staining was considered 'negative'.

For MIB1, the localization of the immunoreactivity within the epithelium was assessed, and four categories were discerned: (1) basal or parabasal staining, (2) positivity confined to cells in the lower one third of the epithelium, (3) staining of cells in the lower two thirds of the epithelium, or (4) diffuse, transepithelial positive staining.[9] For statistical purposes, MIB1-staining in the (para)basal layers or in the lower one-third of the epithelium was considered 'low' and MIB1-staining in the lower two-thirds or the entire epithelium was considered 'high'.

For p53, cells were considered to be positive in case of nuclear staining. The extent of p53-positivity was evaluated by determining the percentage of p53-positivity in basal layer cells after counting 200 consecutive cells (labeling index (LI)). The pattern of p53-staining was assessed by recording the location of the positive cells in the levels of the epithelium. The term "suprabasal extension" was used when p53-positive cells were found in both the basal layer and in higher layers of the epithelium.[5] In carcinomas, the percentage of p53-positive cells was estimated after evaluation of the entire lesion present in the slide.

When the carcinoma was micro-invasive (n=6, four adjacent to usual VIN, two adjacent to differentiated VIN) no immunohistochemical staining results could be scored.

Statistics

On the basis of the histological diagnosis, patients were divided in three groups (usual VIN with SCC, usual VIN without SCC and differentiated VIN with SCC). The difference in age was tested using the non-parametric Kruskal-Wallis test. Differences in presence of HPV, p16^{INK4A}-expression and MIB1-localisation were tested using the chi-square test. The differences in p53-LI in VIN lesions and p53-positivity in SCCs between groups were tested using the non-parametric Mann-Whiney-U test. For all analyses a p-value of <0.05 was considered to be statistically significant.

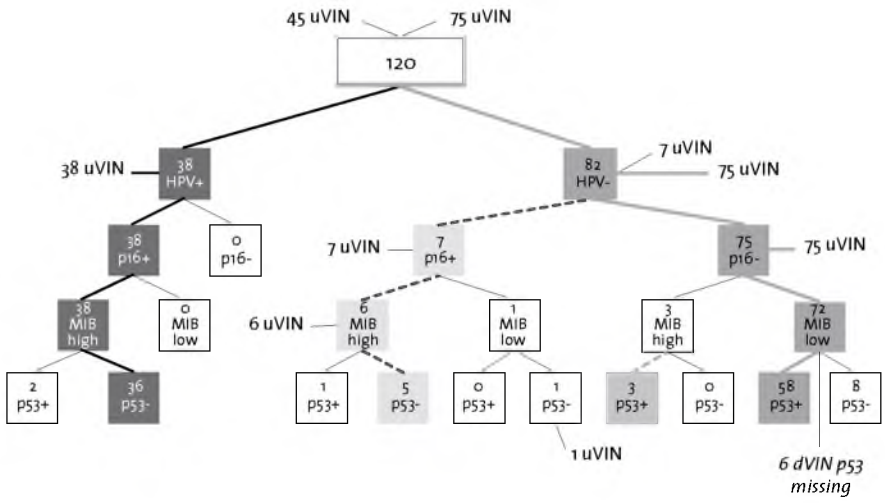
Results

Patients with a vulvar carcinoma adjacent to usual type VIN had a lower median age (52 years, SD 13.4 years) compared to patients with differentiated VIN with associated carcinoma (74 years, SD 12.5 years). Patients with usual VIN without an associated vulvar carcinoma had a median age of 36 years (SD 10.8 years) at the time of diagnosis. The differences in age at the time of diagnosis were highly statistically significant (Kruskal-Wallis Test, p<0.001). In sixty percent of the patients with differentiated VIN a (clinical and/or histological) diagnosis of LS was noted in the patient chart; in this study no examination for LS on the histology specimen was performed.

The FIGO and TNM stages of the vulvar carcinomas in both groups were comparable: 64% of the dVIN associated carcinomas were FIGO stage I or II vs. 75% of the usual VIN related carcinomas. Ninety-three percent of the dVIN-associated carcinomas were T1/T2 and 63% were No vs. 83% and 75% of the usual VIN related carcinomas.

In Figure 1, the combined results of the presence of HPV and the expression of p16^{INK4A}, MIB1 and p53 are summarized in a flow-chart.

Figure 1: Flow diagram showing simultaneous HPV-positivity, p16^{INK4A}-expression and p53-LI in the 120 lesions.



uVIN = usual VIN
dVIN = differentiated VIN
A p53-LI > 0.5 was considered p53-positive (p53+)
MIB low : MIB1-positive cells in the (para)basal layers or the lower one third of the epithelium
MIB high: MIB1-positive cells in the lower two thirds of the epithelium or the entire epithelium

HPV

Usual VIN was significantly more often hr-HPV positive than differentiated VIN; 38 of 45 cases of usual VIN were hr-HPV positive (84%); all cases of differentiated VIN were hr-HPV-negative (chi-square, p<0.001). Usual VIN without associated carcinoma showed comparable percentages of positivity for hr-HPV with usual VIN with associated carcinoma; 26 of 32 cases (81%) and 12 of 13 cases (92%) respectively (data not shown, chi-square, p>0.05). One usual VIN lesion without associated SCC was positive for low-risk HPV (HPV 6). All hr-HPV-positive usual VIN lesions were diffusely positive for $p16^{INK4A}$ and had MIB1-expression up to high in the epithelium. Ninety-five percent (36/38) had a p53 LI of ffi0.5.

$p16^{INK4A}$

The staining pattern of $p16^{INK4A}$ in usual VIN was cytoplasmic and nuclear, with more nuclear than cytoplasmic staining (see Figure 2B). Irrespective of the type of adjacent lesion, normal tissue showed no or minimal immunostaining for $p16^{INK4A}$.

The $p16^{INK4A}$ immunoreactivity in vulvar carcinomas showed similarities with the $p16^{INK4A}$ immunoreactivity in its associated VIN lesion: all usual VIN lesions were positive for $p16^{INK4A}$, whereas in differentiated VIN, all 75 lesions were negative for $p16^{INK4A}$. All usual VIN lesions without SCC were positive for $p16^{INK4A}$. In the group of differentiated VIN lesions, only one case was positive for $p16^{INK4A}$. The difference in $p16^{INK4A}$-positivity in the carcinomas adjacent to differentiated VIN (3/73: 4%) and usual VIN (8/9: 89%) was significant (data not shown, chi-square, p<0.001).

MIB1

A uniformly nuclear and mostly very strong MIB1-staining was seen in all types of vulvar lesions, with no cytoplasmic staining (see Figures 2C, 2E and 3B). MIB1 immunoreactivity in normal epithelium (irrespective of the type of adjacent VIN lesion) was parabasal with a negative basal cell layer in all cases.

In SCC adjacent to differentiated VIN the median estimated positivity for MIB1 was 70% (range 10-100%) whereas in SCC adjacent to usual VIN the median estimated MIB1-positivity was 80% (range 50-100) (Mann-Whitney-U, p=0.06). There was a significant difference in the localization of MIB1-staining between the two types of VIN lesions (chi-square, p<0.001); usual VIN lesions showed MIB1 staining up to high in the epidermis in 44 of 45 of cases (98%), in contrast to the MIB1-staining confined to the lower layers of the epithelium in differentiated VIN in 72 of 75 cases (96%).

p53

Analyzing the p53-expression patterns in VIN lesions, two distinct patterns became apparent. In differentiated VIN lesions, the basal cell layer often was positive for p53, and in most lesions there was "suprabasal extension" as can be seen in Figure 3C. In usual VIN lesions less cells of the basal layer were positive. The suprabasal positivity

in usual VIN, occasionally showed a distinct clustered pattern, in which central parts of the epithelial rete ridges were positive for p53 whereas the rest of the epithelium was negative for p53 as can be seen in Figure 2D. In normal vulvar epithelium no p53-expression was found. The expression of p53 in the two types of carcinoma can be found in Figure 1F (carcinoma adjacent to usual VIN) and Figure 3D (carcinoma adjacent to differentiated VIN).

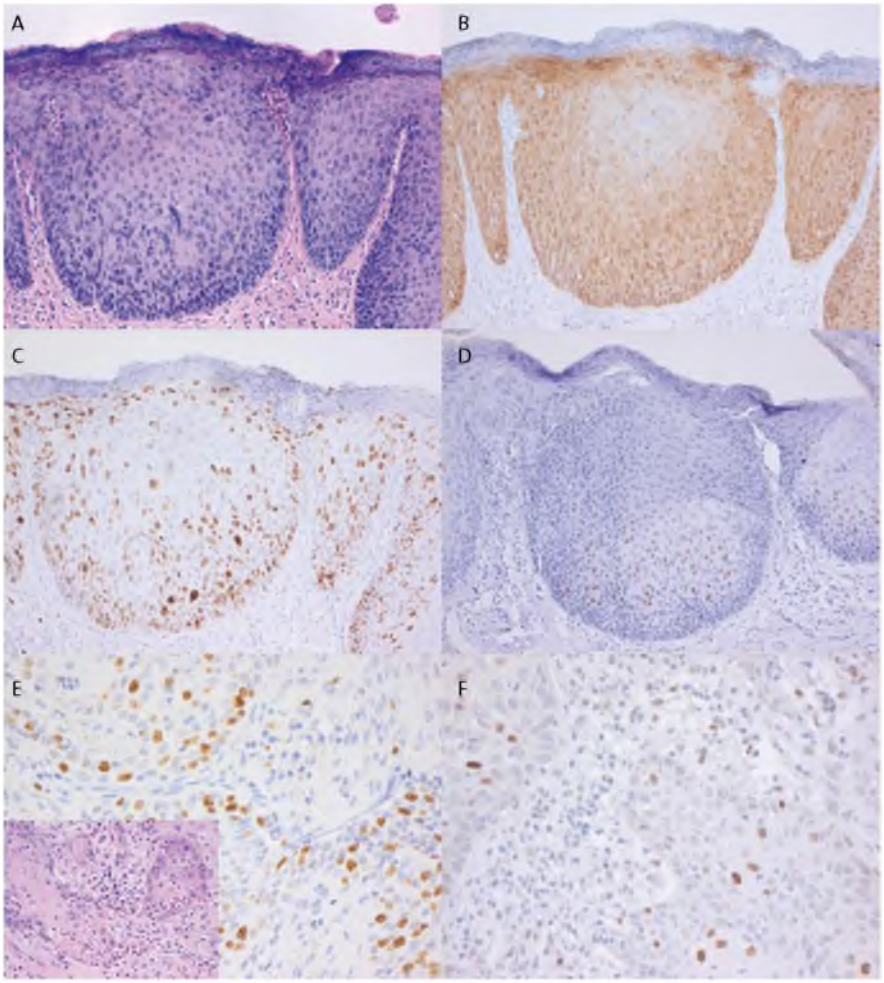
The median LIs and the percentages of p53-positivity in the carcinomas are shown in Table 1. Analyzing the p53 LIs revealed that the p53 LI in differentiated VIN adjacent to VC was significantly higher than the p53 LI in usual VIN adjacent to VC (Mann-Whitney-U, $p<0.001$). The difference in p53 LIs between usual VIN with and without VC was not significant (Mann-Whitney-U, $p<0.08$). The median percentages of p53-positivity in vulvar carcinoma adjacent to differentiated VIN were significantly higher than in vulvar carcinoma adjacent to usual VIN (Mann-Whitney-U, $p=0.008$).

Discussion

Two separate pathways lead to the development of vulvar SCC, which have their own precursor lesions, with a unique immunohistochemical profile that corresponds with the profile in the adjacent carcinoma. We believe that the use of this immunohistochemical profile can be of particular help in the correct and timely diagnosis of VIN.
In VIN lesions as well as the adjacent carcinomas, the expression of p16^{INK4A} was highly associated with the presence of HPV. This close relation has already been demonstrated in the vulva[9;29;30], the cervix[31], the head and neck region[32;33], the skin[34], and the anorectal region.[35] In the oral cavity, immunohistochemical p16^{INK4A} detection has proven to be fully equivalent to HPV detection.[36] Others have shown that clinically meaningful viral HPV infections can be reliably measured with an algorithm of p16^{INK4A} immunostaining followed by PCR on p16^{INK4A}-positive cases.[33] In this study, the use of p16^{INK4A} alone was sufficient to identify all usual VIN lesions, and the immunohistochemical profiles of five of the seven HPV-negative usual VIN lesions (see Figure 1) suggest that even though we used a highly sensitive and accurate HPV detection method[24], the results in at least five usual VIN lesions were false-negative.

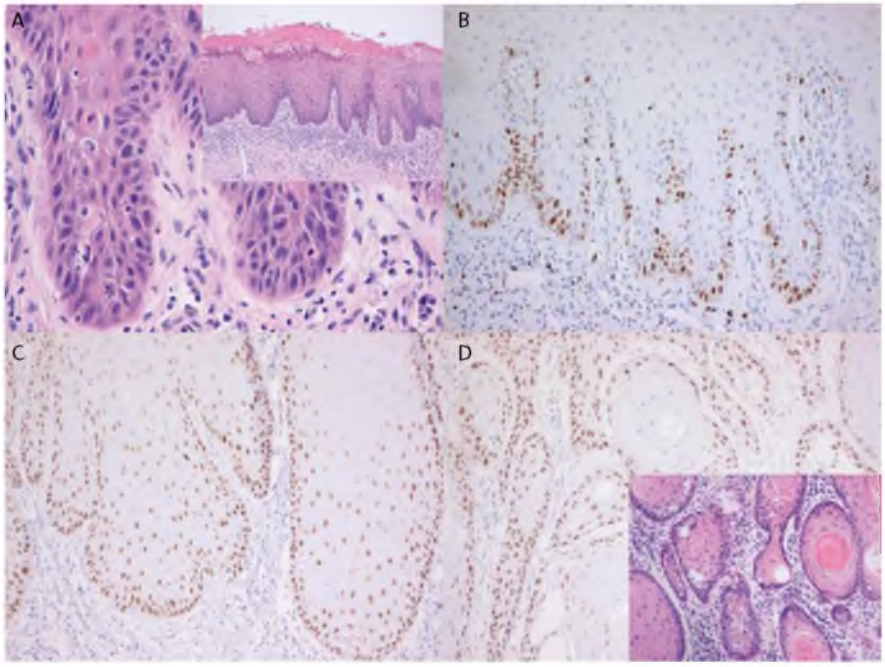
High-HPV DNA was found in only 12/88 (14%) of all the vulvar SCCs in this study, all adjacent to a usual VIN lesion and HPV16 was, as in earlier publications on HPV in the genital area, most prevalent.[8;37] Previous studies on vulvar carcinomas have reported hr-HPV infection in 0-57% of the cases, depending on the HPV detection method and the types of SCC that were analyzed.[9;19;38-40]

Figure 2: solitary usual VIN lesion (A-D) and squamous cell carcinoma adjacent to a usual VIN lesion (E-F)



A. H&E stained slide of a usual VIN lesion without adjacent squamous cell carcinoma; atypical nuclei can be found throughout the entire epithelium.
B. the entire epithelium is positive for $p16^{INK4A}$.
C. MIB1-positive cells can be found in at least the lower 2/3 of the epithelium in usual VIN.
D. Clusters of p53-positive cells can be found in the epithelium of a usual VIN lesion.
E. MIB1-postive nests in vulvar squamous cell carcinoma. On the H&E photo in the inlay, mitotic figures and atypia can be seen.
F. in the carcinoma adjacent to usual VIN, around 25% of the cells are positive for p53.

Figure 3: differentiated VIN lesion with adjacent squamous cell carcinoma



A. H&E stained slides of a differentiated VIN lesion (adjacent to squamous cell carcinoma). Nuclear atypia and the presence of mitotic figures in the differentiated VIN lesion is confined to the basal cell layers. Hyperkeratosis and dyskeratosis are present and the rete ridges are elongated.
B. In differentiated VIN, MIB1-positivity is confined to the basal and parabasal layers of the epithelium.
C. In differentiated VIN, p53-posititivity is most prominent in the basal cell layers with suprabasal extension.
D. Around 90% of the cells of the invasive nests of the squamous cell carcinoma adjacent to differentiated VIN are positive for p53.

Similar to the previously published series of Yang and Hart, the differentiated VIN-lesions adjacent to SCC showed a high p53 LI and a comparable expression-pattern with suprabasal extension.[5] Usual hr-HPV-positive VIN lesions, however showed a much lower p53 LI and the lower percentage in the HPV-positive carcinomas in our study, has also been described by others.[41-43] As previously described by Santos et al., in HPV-positive SCCs, $p16^{INK4A}$ and p53 tended to be mutually exclusive.[30] Nogueira et al. described comparable results for VIN in women younger vs. older than 55 years of age, without testing for HPV.[44] It is likely that the group of women younger that 55 years consisted of mainly high grade, HPV-positive, usual VIN lesions whereas the group of women over 55 years probably mainly consisted of HPV-negative, differentiated VIN lesions. The clustered positivity in the epithelium of usual VIN has never been described.

In normal vulvar tissue, irrespective of the adjacent type of VIN, no expression of p53 and $p16^{INK4A}$ was found and MIB1-expression in normal vulvar tissue was confined to the lower one-third of the epithelium. As was shown in a recent publication, MIB1 can be used to distinguish normal vulvar epithelium from differentiated VIN and other premalignancies because of a MIB1-negative basal cell layer in normal vulvar epithelium.[22] This feature combined with the absence of expression of the cell cycle related proteins investigated in this study, can improve the timely recognition of differentiated VIN as it is often overlooked or mistaken for a benign dermatose such as pseudoepitheliomatous hyperplasia and lichen simplex chronicus.[45;46] The differences in age of the two groups of VIN lesions were highly statistically significant. This fits the epidemiological data known from literature; usual VIN occurs in younger women and LS associated differentiated VIN and keratinizing vulvar SCC occurs at a higher age.

The fact that no isolated, solitary differentiated VIN lesions have been found in this study supports the idea that differentiated VIN is a lesion with a short intra-epithelial phase that rapidly progresses to vulvar SCC.[6,7] This is supported by the fact that in this study all differentiated VIN lesions presented adjacent to vulvar SCC, and mostly had a size of more than 1 cm. We strongly believe in the high malignant potential of differentiated VIN. Incidental cases of differentiated VIN occurred in our hospital, but all after a patient had been treated for vulvar SCC. We also found some cases of differentiated VIN on biopsy, and invasive carcinoma in the subsequent vulvectomy or excision (performed within two weeks of diagnosis). Nevertheless, there is controversy regarding the actual role of differentiated VIN in the development of vulvar SCC. It has also been described as the in-situ carcinoma component adjacent to the invasive carcinoma[47], and our results cannot confirm nor reject this hypothesis. Furthermore, differentiated VIN can be difficult to diagnose, both clinically and histopathologically.[42;45;46;48] When differentiated VIN is found in the surgical margins of an excision, this might have consequences for the further treatment and follow-up of the patient. Better recognition and uniform use of nomenclature will facilitate future research and the comparison of published results. The changes in nomenclature of squamous vulvar lesions proposed by the ISSVD are not yet uniformly used.[3]

In conclusion, both pathways leading to vulvar SCC have their own molecular background. Future studies should focus on the exact role of p53 in the development of HPV-negative vulvar squamous cell carcinoma and the malignant potential of differentiated VIN. Using a robust immunohistochemical panel with the proteins p16^{INK4A}, p53, and MIB1, the two types of VIN lesions can be accurately distinguished and recognized. Timely diagnosis and thus early recognition of differentiated VIN lesions should lead to a more extensive treatment strategy for this kind of VIN lesion.

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Table 1: p53 LIs and range in vulvar carcinoma and VIN

	median	range
Vulvar carcinoma (74#)	**%**	**<min-max>**
adjacent to differentiated VIN (66)	67,5 %	<0-95>
adjacent to high-grade VIN(8)	30,0 %	<15-60>
VIN (114*)	**LI**	
differentiated type (69)	0.85	<0.16-1.00>
high-grade VIN, adjacent to VC (13)	0.025	<0.00-0.12>
high-grade VIN, without VC (32)	0.058	<0.00-0.55>

When the carcinoma was micro-invasive (n=6) p53 positivity could not be estimated and in 8 cases there was no carcinoma left in the p53 slide (but was present in H&E), therefore the total number of carcinomas in this table is less than 88.
* In 6 cases there was no VIN lesion left in the p53 slide (but was present in H&E), therefore the total number of VIN lesions is less than 120.
VC= vulvar carcinoma

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Chapter 4

MIB1-expression in basal cell-layer: a diagnostic tool to identify premalignancies of the vulva

Abstract

Lichen sclerosus, high-grade usual vulvar intraepithelial neoplasia (VIN) and differentiated VIN have a different malignant potential. The objective of this study was to quantify the proliferative activity in the basal region of the epithelium of vulvar premalignancies. Furthermore, we investigated whether MIB1 expression in the basal region of vulvar epithelium can be helpful in diagnosing differentiated VIN, which may be hard to discern from normal epithelium. MIB1 was used to immunohistochemically visualise proliferating cells within formalin-fixed, paraffin-embedded, archival tissue sections of different vulvar premalignancies (N=48) and normal vulvar epithelium (N=16). Automatic digital image analysis software was developed to quantify the proliferating fraction in different parts of the epithelium (MIB1 positivity index). MIB1 expression differed among the various vulvar premalignancies; a MIB1-negative basal cell layer was a distinct feature of normal vulvar epithelium. No MIB1-negative basal cell layer was noted in differentiated VIN or other vulvar premalignancies. Owing to this negative cell layer, the MIB1 proliferation index in normal vulvar epithelium was significantly lower than in vulvar premalignancies. In conclusion, MIB1 expression can be a helpful tool in diagnosing a premalignancy and has additional value especially to distinguish differentiated VIN neoplasia from normal vulvar epithelium, but cannot explain the differences in malignant potential.

Introduction

Vulvar cancer is the fourth most common gynaecologic cancer and comprises 5 percent of all malignancies of the female genital tract. Based on clinical and pathological features, vulvar squamous cell carcinoma can be subdivided into two different types, which seem to develop from their own associated premalignancies.

The most frequent type of vulvar carcinoma occurs mainly as a unifocal lesion in elderly women and is related to lichen sclerosus and/or differentiated vulvar intraepithelial neoplasia (VIN). This type of tumour is probably not associated with human papillomavirus (HPV) infection.[1-4] The less common type, accounting for about one-third of all vulvar squamous cell carcinomas, is associated with HPV, predominantly HPV-types 16 and 18. This type of tumour often occurs as a multifocal lesion in relatively young women and is usually preceded by high-grade usual VIN lesions (also referred to as classic VIN lesions).[3;5;6] Recently, the International Society for the Study of Vulvovaginal Disease (ISSVD) has proposed a new nomenclature for premalignant vulvar lesions (see Table 1).[7]

In usual VIN, the epithelium is thickened and accompanied by hyperkeratosis and/or parakeratosis. Although a spectrum of architectural abnormalities may be seen, the lesion is readily recognizable as an intraepithelial neoplasm by the pathologist due to cytological abnormalities of the epithelial cells throughout the whole thickness of the epithelium.[8] Differentiated VIN is clinically and pathologically more difficult to recognize. There is little or no atypia above the basal or parabasal layers and it has a high degree of cellular differentiation, which combined with an absence of widespread architectural disarray hinders recognition.[2;3;9] Differentiated VIN is relatively infrequently diagnosed in its pure form and is often seen adjacent to lichen sclerosus and/or rapidly growing invasive vulvar squamous cell carcinoma.[3;8;10] Besides the difficulties in clinical and histological recognition of differentiated VIN, it is suggested that this form of VIN is highly proliferative and might be more likely to progress to an invasive neoplasm than lichen sclerosus and HPV-related VIN lesions.[2;3;11] Currently, no biomarker or diagnostic tool to predict possible invasive behaviour of premalignant vulvar lesions is available. Because of the risk of malignant progression it is current practice that all patients with vulvar premalignancies undergo regular check-ups. However, there is no evidence that this follow-up prevents the development of cancer or results in earlier detection of a malignancy.[12]

Proliferative activity in tissues can be visualized using a proliferation marker like MIB1, which is a monoclonal antibody against the Ki-67 antigen, a nuclear antigen present in human proliferating cells in all stages of the cell cycle, but not in the G_0 phase.[13,14] In many (pre)malignant lesions qualitative MIB1-expression is used for grading and estimating prognosis.[10;15-18] Bulten et al. used a method in which epithelial MIB1-expression patterns in cervical lesions were digitally quantified. The method proved to be an

objective, reproducible and reliable method of classification for dysplastic changes in cervical epithelium.[15] Systematic quantitative evaluation of MIB1-expression in the basal region of premalignant vulvar lesions has not yet been performed.[10,19-23]

The primary objective of this study was to quantify the proliferative activity in the epithelial cell layers in differentiated VIN and other vulvar premalignancies with the aim to find an explanation for the differences in malignant progression. Furthermore, we investigated whether MIB1-expression in the basal regions of the vulvar epithelium may be helpful in diagnosing differentiated VIN.

Materials and Methods

Specimens

Sixty-four specimens from vulvar biopsies, vulvar excisions or vulvectomies, diagnosed as non- or premalignant vulvar lesions between 1992 and 2002, were retrospectively obtained from the archives of the Department of Pathology at the Radboud University Nijmegen Medical Centre (Nijmegen, the Netherlands). All specimens were routinely fixed (4% buffered formalin) and paraffin embedded. Standard 4 µm thick haematoxylin and eosin-stained sections were used for the classification of the lesions. Two expert-pathologists [JB and JMMG], specialised in gynaecological pathology, blindly and independently re-examined the slides and classified them according to current WHO criteria and the recent modification of the ISSVD.[7,24] They agreed on all diagnoses.

The 64 tissue specimens in this study consisted of: 22 usual type VIN lesions, 14 differentiated VIN lesions, 12 lesions with lichen sclerosus and 16 normal epithelium samples (10 patients with vulvodynia who were treated with a partial resection of the vulvar vestibule and six normal epithelium samples from free tumour margins in vulvectomy specimens of patients treated for vulvar cancer).

Immunohistochemistry

Four-micrometer thick paraffin sections were mounted onto polylysine-coated slides and dried overnight at 58°C. The sections were dewaxed in xylene and endogenous peroxidase was blocked using H_2O_2 in methanol for 15 minutes and the slides were rinsed three times in phosphate-buffered saline (PBS; pH 7,4) for 5 minutes. The slides were placed in a citrate buffer (0,01 M; pH 6,0) and heated in a household microwave oven (3 min at 850 W until boiling; followed by 10 min at 180 W). The sections were allowed to cool down to room temperature (RT) and were briefly washed in PBS (10 min). Subsequently, the slides were pre-incubated with 20% normal goat serum and incubated with the primary antibody Ki-67 (clone MIB1, Dakocytomation, Denmark) 1:100 in PBS with 1% BSA (60 min, RT). Subsequently, the slides were rinsed in PBS (10 min) and post-antibody blocking was done for 15 min (powervision plus). This was

followed by incubation with polymeric-horse-radish peroxidase-goat anti-mouse/rabbit/rat IgG (30 min, RT). The slides were developed with diaminobenzidine (mixed with H_2O_2), counterstained with Mayer's haematoxylin, dehydrated in ethanol and xylene and finally mounted. In each run a buffer only and a vulvar squamous cell carcinoma served as negative and positive controls.

Quantification of immunohistochemical staining / Image analysis

Quantitative analysis of MIB1 staining was achieved using digital image analysis of microscopic images. Image acquisition was performed using a 3CCD colour video camera (Sony DXC-950P, Sony Corp, Japan) mounted on a conventional light microscope (Axioskop 2 plus, Carl Zeiss AG, Germany) and attached to a personal computer with frame grabber card (Matrox Meteor-II Multichannel, Matrox Imaging, Dorval, Canada). Images were acquired using a 20x objective (Plan Neofluar, NA=0.5, resulting specimen level pixel size 0.39 μm^2). Prior to analysis of the immunohistochemical staining, an image of an empty microscopic field was acquired, which was used for correction for unequal illumination. In each tissue section we aimed to measure 8 microscopic fields, representative of the lesion. Image acquisition and analysis were performed using a custom written macro in KS400 image analysis software (Carl Zeiss AG, Germany). For each digitised red green blue (RGB) image, the following procedure was performed. The operator interactively defined the location of the basement membrane by drawing a line with a mouse cursor. From this line, 20 strata of each 5 µm thickness were automatically determined, covering the basal region of the epithelium. Within these areas, pixels with a ratio between the red and green RGB component of more than 1.03 and a red intensity under 180 were labelled as belonging to MIB1 positive nuclei. In the same way, pixels with a ratio between blue and green intensity over 1.07 and red camera channel under 210 were labelled as belonging to haematoxylin stained, MIB1 negative nuclei. See figure 1A and 1B for the different steps in the process. Thresholds were determined from a set of training slides and were found adequate for almost all slides analysed in this study. When the initial thresholds led to unrealistic patterns, adjustment was performed by the operator (data not shown).

For each measured field, the total area of positive nuclei per stratum of 5 µm and the total area of all nuclei per stratum was automatically recorded. For each stratum, the ratio between the MIB1 positive nuclear area and total nuclear area was calculated (single layer). This ratio, x100%, was used as a measure of MIB1 positivity: the MIB1 positivity index. In addition, the cumulative MIB1 positivity indices were calculated by dividing the sum of the MIB1 positive nuclear area in multiple strata by the sum of the total nuclear area in the same strata (x100%).

In normal vulvar epithelium, the presence or absence of inflammatory cells directly underlying the basement membrane was recorded for each measured field.

Statistical analysis

All measured fields were averaged to calculate mean values per patient. These values were used to calculate mean values per type of lesion.

All analyses were performed using SPSS 12.0.1 software (SPSS Inc, Chicago, Illinois, USA). To test whether the positivity index in the lowest 5 µm of normal vulvar epithelium differed between the fields with and without inflammatory cells underneath the basal membrane, the non-parametric Mann-Whitney-U-test for unpaired observations was used. The Kruskal-Wallis one-way analysis of variance by ranks was used to test whether the different positivity indices were different for at least one of the four diagnostic groups. When a significant difference was found, distribution-free all-treatments multiple comparisons based on pairwise rankings with correction for tied observations were used to disclose which of the diagnostic groups differed significantly.[25]

Results

Pathology

In figure 1C, 1E and 1G, H&E stained sections of differentiated VIN, usual VIN and lichen sclerosus are shown.

The epithelial cells of *usual VIN* (figure 1C) have a high nuclear:cytoplasmic ratio and lack cytoplasmic maturation above the basal and parabasal layers. Mitotic activity is present above the basal layer with mitotic figures that are often abnormal in appearance and reach the upper cell layers. Multinucleation and dyskeratosis, including formation of intraepithelial squamous pearls, may be seen. Nuclear pleomorphism and hyperchromasia are present; however, nucleoli are uncommon.

The atypia in *differentiated VIN* lesions (figure 1E) is strictly confined to the basal and parabasal layers of the epithelium, where the cells have abundant cytoplasm and form abortive pearls. 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 a normal maturation, exhibit hyperkeratosis and do not show koilocytosis.[24] Furthermore, in differentiated VIN the epithelium exhibits elongation of rete pegs.

In *lichen sclerosus* (figure 1G) the loss of rete ridges is clearly visible. There is little or no cellular or nuclear atypia. A zone of homogeneous collagenised subepithelial oedema of variable thickness is present as is a band of lymphocytic infiltration beneath this zone. The epithelium is flattened and/or thinned, and mild hyperkeratosis is present.

MIB1 expression pattern

In all lesions, MIB1 staining was present in the basal and/or parabasal regions of the epithelium and the basal membrane was always clearly identifiable. As can be seen in figure 1 (D, F and G), MIB1 expression varied in the different vulvar premalignan-

cies. In usual VIN all cell-layers were positive for MIB1 (see figure 1D). In differentiated VIN (figure 1F) the majority of the epithelium was MIB1-negative with a thin layer of MIB1 positive cells parabasally. MIB1 positivity was restricted to the lower 1/3 of the epithelium in lichen sclerosus (figure 1H). Furthermore, normal epithelium had a distinct MIB1 expression pattern. Like in lichen sclerosus, the lower 1/3 of the normal vulvar epithelium was MIB1 positive, but a MIB1 negative cell layer directly above the basement membrane was visible (figure 1A-B). A simplified schematic representation of the different expression patterns is depicted in figure 2.

MIB1 positivity index

In figure 3, an overview of the single (figure 3A) and cumulative (figure 3B) MIB1 positivity indices in different areas of the epithelium are shown. The thick line represents the median positivity index, boxes represent quartiles and the lines indicate extreme values. The MIB1 positivity indices per patient were based on a median number of 6 (range 4-10) measurements. In normal epithelium, there was no statistically significant difference in MIB1 positivity index in the lowest 5 µm of the epithelium between the measured fields *with* or *without* inflammatory cells underneath the basal membrane (data not shown, Mann-Whitney-U-test, p>0.05). The MIB1 positivity indices in normal epithelium from vulvar vestibulitis patients and from patients with a carcinoma did not differ from one other (data not shown, Mann-Whitney-U-test, all p-values>0.05)

In table 2, an overview of p-values (distribution-free all-treatments multiple comparisons based on pairwise rankings with correction for tied observations) can be found, comparing cumulative and single layer MIB1 positivity indices between the four diagnostic groups.

In normal vulvar epithelium, the lowest 10 µm of the epithelium was almost negative for MIB1. The cumulative MIB1 positivity indices at 5 and 10 µm in normal epithelium were significantly lower than the corresponding areas in any of the other lesion types (all p-values <0.001). In the higher strata, single layer MIB1 positivity indices in normal vulvar epithelium were comparable to the single layer MIB1 positivity indices in differentiated VIN. Due to the big difference in the lowest two layers, the difference between the cumulative MIB1 positivity indices remained statistically significant. Comparing the MIB1 positivity indices between lichen sclerosus and normal vulvar tissue revealed differences in only the layers up to 15 µm.

In usual VIN lesions, a MIB1 positivity index of almost 40% or more was observed in all layers of the epithelium, causing the cumulative MIB1 positivity indices to be around the same values. In none of the other epithelia, single layer MIB1 positivity indices (at 55 µm or higher) were as high as those in usual VIN (all p-values < 0.001). Comparing the cumulative MIB1 positivity indices of usual VIN with the other lesions gave the same results (all p-values < 0.05). The differences with normal epithelium were most striking, as all cumulative and single layer MIB1 positivity indices were significantly higher in usual VIN.

The cumulative MIB1 positivity indices up to 10 µm in lichen sclerosus were significantly lower than these strata in differentiated VIN. The single and cumulative MIB1 positivity indices higher in the epithelium were not significantly different and the pattern was comparable (see figure 2 and figure 3).

Discussion

In general, it is suggested that differentiated VIN has a higher malignant potential than other vulvar premalignancies.[8;26] This is not explained by our quantitative analysis of proliferative activity. The MIB1 positivity indices in the epithelium of differentiated VIN were not statistically different from the MIB1 positivity indices in well established other precursors of vulvar carcinoma, i.e. usual VIN. The proliferative activity in the lower strata was comparable whereas the MIB1 positivity indices covering a higher part of the epithelium were significantly higher in usual VIN, which is thought to be of lesser malignant potential. Several other investigators calculated a MIB1 positivity or labelling index, measuring the entire thickness of the epithelium or only the basal cell layer, but none of the groups used digital quantitative image analysis and mostly no distinction was made between differentiated and usual VIN, or only usual VIN was analysed.[27] Only scoring the MIB1-pattern or using a semi-quantitative scoring method is less accurate. Due to deep rete pegs the measurement of the entire epithelium, as previously published by Bulten et al for cervical lesions, is technically not possible in differentiated VIN lesions.[15] We calculated the MIB1 positivity index by dividing the measured MIB1 positive nuclear area by the total nuclear area. In this manner, the thickness of the epithelium that can be highly variable amongst different vulvar premalignancies and amongst different patients was no confounder in the measurements. When it would be possible to measure the entire epithelium, the differences in cumulative MIB1 positivity indices between differentiated VIN and usual VIN would probably be more prominent.

We noticed a remarkable difference in the MIB1 staining pattern of vulvar premalignancies versus normal vulvar epithelium. In normal vulvar epithelium, a MIB1-negative cell layer above the basal membrane was observed, which was absent in differentiated VIN. There was no MIB1-negative cell layer in other vulvar premalignancies and therefore the presence of a negative MIB1 layer seems to be a characteristic of normal vulvar epithelium and the absence of such a layer above the basal membrane, indicates the presence of a premalignancy. Scurry et al. describe similar results in their qualitative analysis of Ki-67 of lichen sclerosus and normal epithelium.[21] Although they did not digitally quantify their measurements and did not specifically mention a MIB1-negative basal cell layer, they describe that the MIB1 staining in normal epithelium was localized more parabasally and in lichen sclerosus more basally. The lack of effect of inflammatory cells beneath normal vulvar epithelium on the presence of a MIB1-

negative basal cell layer and the absence of a MIB1-negative basal cell layer in vulvar premalignancies can be seen as disturbance of cell growth in vulvar premalignancies.

Lichen sclerosus and usual VIN lesions are readily recognizable as they have distinct characteristics at histopathological examination. Differentiated VIN is often very difficult to recognize and sometimes hard to discern from normal epithelium.[2,3,9] Using MIB1-expression to detect a proliferating basal cell layer in combination with the characteristic MIB1 staining pattern with only positive cells in the rete pegs and little positivity in the upper layers of the epithelium, might improve the early recognition of differentiated VIN. A simplified schematic representation of the different expression patterns is depicted in figure 2. Further investigations have to provide evidence whether this feature of MIB1 is applicable in daily practice.

Despite its atrophic clinical aspect, lichen sclerosus has a relatively high proliferative activity, which was significantly higher than in normal vulvar tissue, which might be responsible for its malignant potential. This is in contrast with Tan et al., who found a lower MIB1 positivity index in vulvar lichen sclerosus compared to normal controls. However, they did not use digital imaging techniques to quantify MIB1-positivity and only counted the basal and suprabasal cell layers.[29] Lichen sclerosus and differentiated VIN showed comparable patterns of MIB1 positivity indices, both single and cumulative. This attributes to the assumption that lichen sclerosus and differentiated VIN belong to the same entity that might proceed to HPV-negative vulvar carcinoma.[1-3,30] Differentiated VIN might develop from lichen sclerosus and/or squamous hyperplasia, through loss of apoptosis regulation, like the development of squamous cell carcinoma in oral lichen planus lesions.[31] The role of the regulator of apoptosis, p53, and its mutations, in differentiated VIN and HPV-negative vulvar carcinoma remains to be elucidated.[8,32-34]

In conclusion, the use of MIB1 in the diagnosis of premalignant vulvar lesions might prove helpful, especially to discern differentiated VIN from normal vulvar epithelium. In all vulvar premalignancies the relationship between basal and parabasal cell-layers seems to be disturbed. However, the MIB1 positivity index was not higher in differentiated VIN lesions, which are presumed to be more likely to become invasive, than in usual VIN lesions.

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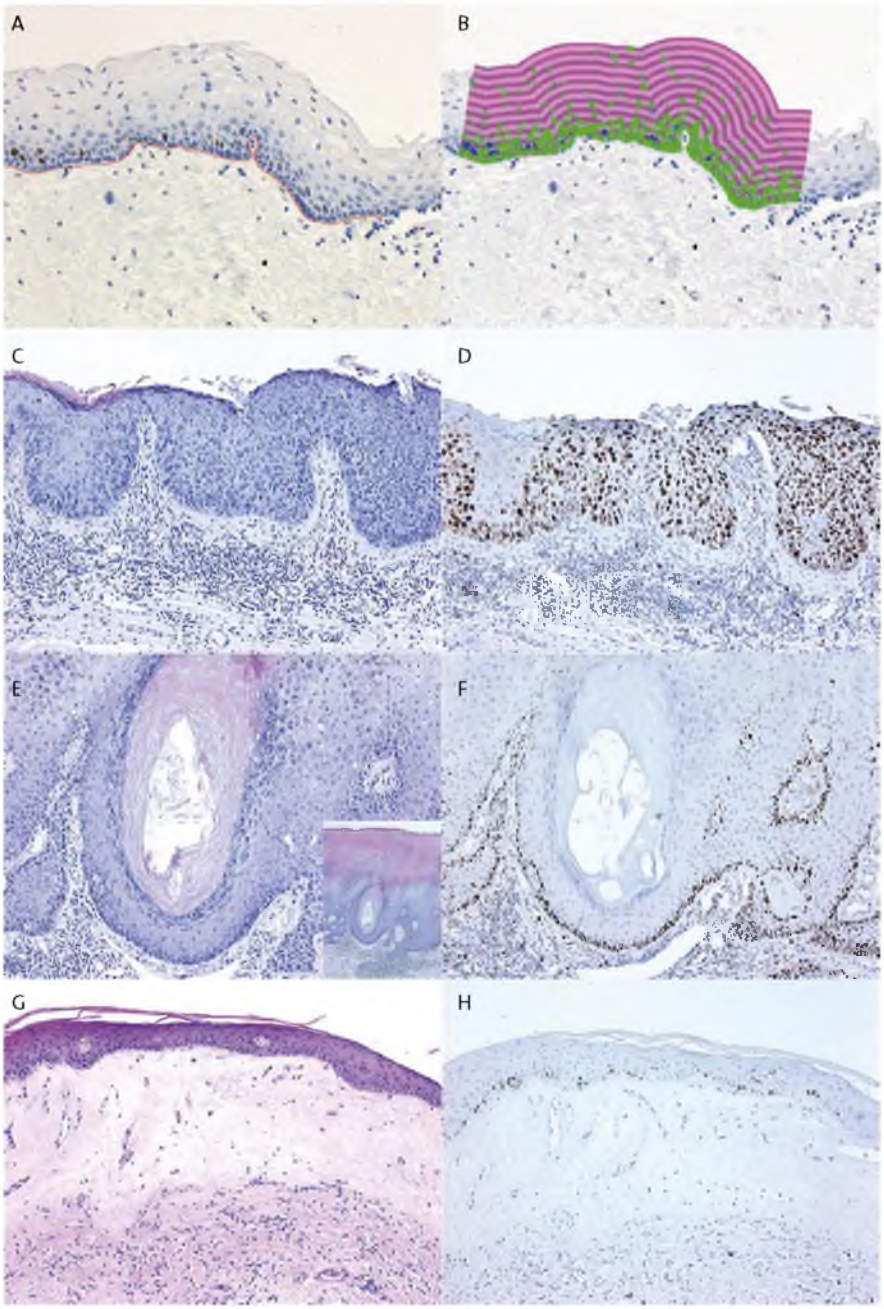
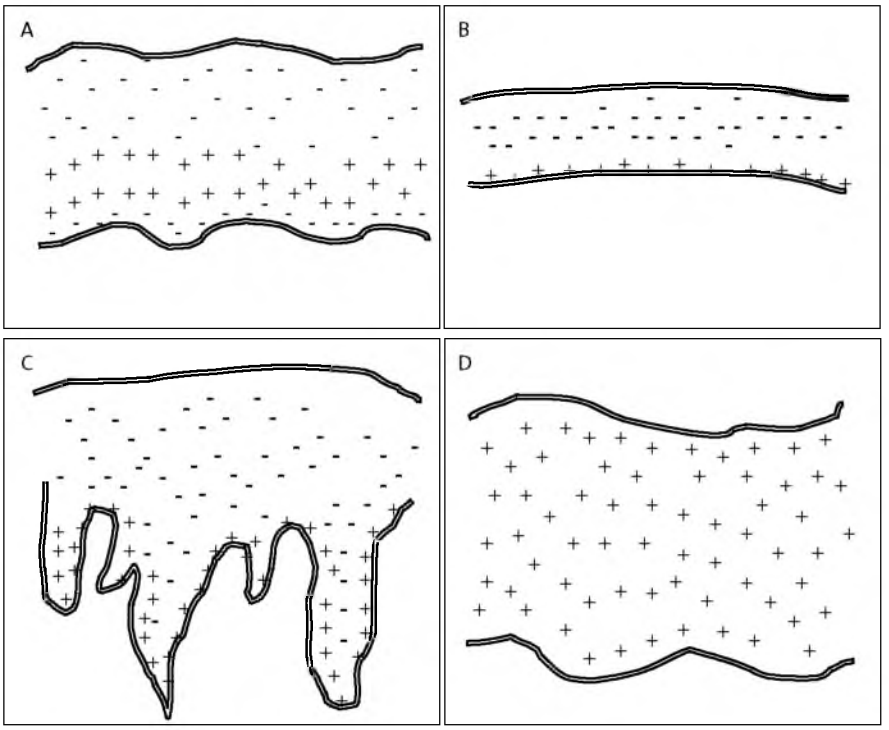
Figure 1



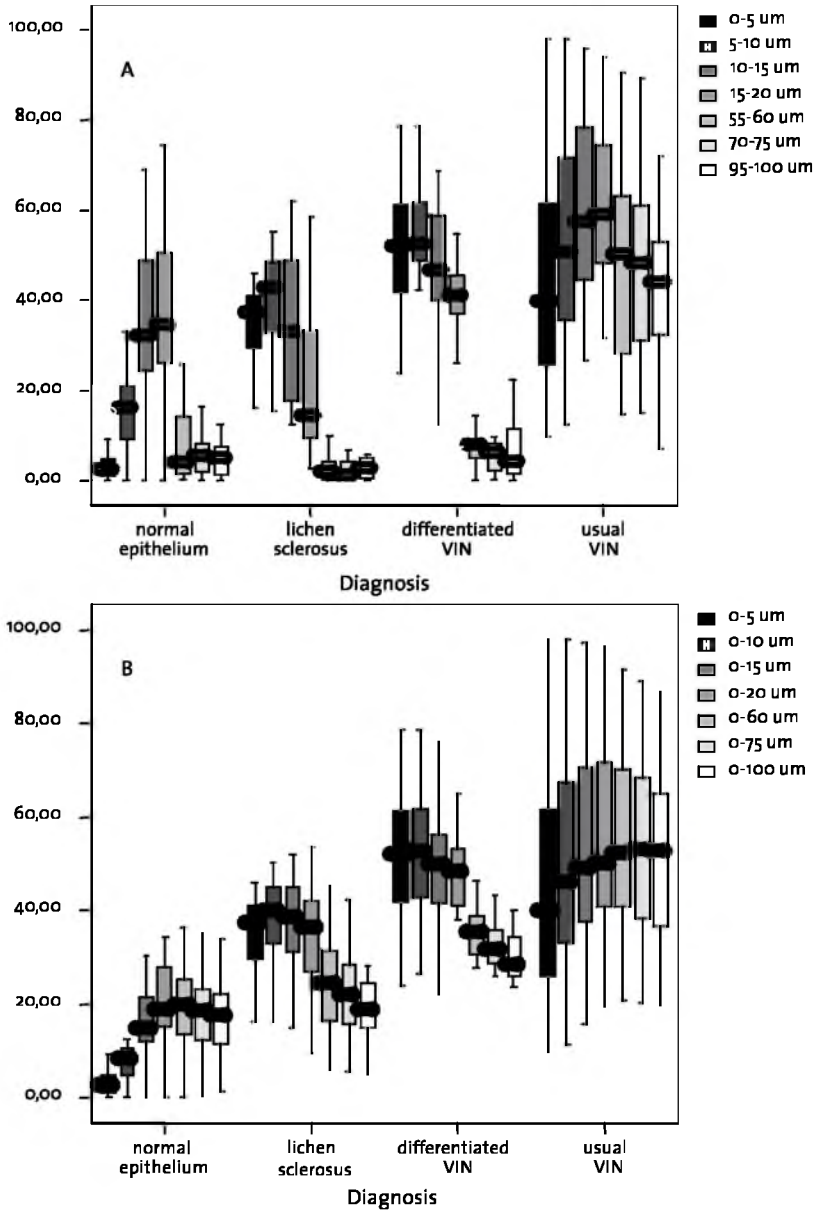
A-B. Photos of MIB1-staining and the digital image analysis process in normal vulvar epithelium.
A. A red line under the basement membrane, drawn by the operator.
B. An 100µm wide area covering part of the epithelium was identified. 20 strata of each 5µm width, in which MIB1-negative (green) and MIB1-positive (blue) nuclei are shown. Note the negative MIB1 cell layer above the basement membrane.
C-H. H&E and MIB1-staining of 4 µm paraffin sections of a usual VIN lesion, differentiated VIN and lichen sclerosus. The peroxidase-labelled immunohistochemical complexes were visualized with diaminobenzodine (DAB) (brown-black).
C. H&E-stained section of a usual VIN lesion, showing atypical cells throughout the whole thickness of the epithelium.
D. In usual VIN MIB1-positive cells can be seen in all layers of the epithelium.
E-1. An abortive pearl that can be found in differentiated VIN.
E-inset The atypia in differentiated VIN is confined to the basal layers of the epithelium.
F. In differentiated VIN the MIB1-positive cells are confined to the basal layers.
G. In lichen sclerosus little atypia can be seen.
H. In lichen sclerosus the MIB1-positive cells are confined to the basal layers.
Original magnifications: (A, B) x20; (C, D, E-1, F, G, H) x10; (E-inset) x5

Figure 2
Simplified schematic representation of the MIB1-expression in



A. normal vulvar epithelium,
B. lichen sclerosus,
C. differentiated VIN and
D. usual VIN.

Figure 3



Boxplots of MIB1 positivity indices. The thick line represents the median positivity index, boxes represent quartiles and the lines indicate extreme values.
A. single layer MIB1 positivity indices,
B. cumulative MIB1 positivity indices.

Table 1. Old and new nomenclature for vulvar lesions[7]

Old nomenclature	New nomenclature
VIN1	No cancer precursor
(Classic) VIN2/3	High-grade VIN, usual type / usual VIN
(Well-)Differentiated VIN(3) / VIN simplex	Differentiated VIN

Table 2. Comparisons of single layer and cumulative MIB1 positivity indices

Lesion types compared	p-value single layer MIB1 positivity indices		p-value cumulative MIB1 positivity indices	
normal vs lichen sclerosus	0-5µm	P < 0.001	5µm	P < 0.001
	5-10µm	P < 0.01	10mu	P < 0.001
	10-15µm	NS	15µm	P < 0.01
	15-20µm	NS	20µm	NS
	55-60µm	NS	60µm	NS
	70-75µm	NS	75µm	NS
	95-100µm	NS	100µm	NS
normal vs differentiated VIN	0-5µm	P < 0.001	5µm	P < 0.001
	5-10µm	NS	10mu	P < 0.001
	10-15µm	NS	15µm	P < 0.001
	15-20µm	NS	20µm	P < 0.001
	55-60µm	NS	60µm	P < 0.01
	70-75µm	NS	75µm	P < 0.01
	95-100µm	NS	100µm	P < 0.05
normal vs usual VIN	0-5µm	P < 0.001	5µm	P < 0.001
	5-10µm	P < 0.001	10mu	P < 0.001
	10-15µm	P < 0.05	15µm	P < 0.001
	15-20µm	P < 0.01	20µm	P < 0.001
	55-60µm	P < 0.001	60µm	P < 0.001
	70-75µm	P < 0.001	75µm	P < 0.001
	95-100µm	P < 0.001	100µm	P < 0.001
lichen sclerosus vs differentiated VIN	0-5µm	P < 0.05	5µm	P < 0.05
	5-10µm	NS	10mu	P < 0.05
	10-15µm	NS	15µm	NS
	15-20µm	NS	20µm	NS
	55-60µm	NS	60µm	NS
	70-75µm	NS	75µm	NS
	95-100µm	NS	100µm	NS
lichen sclerosus vs usual VIN	0-5µm	NS	5µm	NS
	5-10µm	NS	10mu	NS
	10-15µm	P < 0.05	15µm	NS
	15-20µm	P < 0.001	20µm	NS
	55-60µm	P < 0.001	60µm	P < 0.001
	70-75µm	P < 0.001	75µm	P < 0.001
	95-100µm	P < 0.001	100µm	P < 0.001
differentiated VIN vs usual VIN	0-5µm	NS	5µm	NS
	5-10µm	NS	10mu	NS
	10-15µm	NS	15µm	NS
	15-20µm	P < 0.05	20µm	NS
	55-60µm	P < 0.001	60µm	P < 0.05
	70-75µm	P < 0.001	75µm	P < 0.05
	95-100µm	P < 0.001	100µm	P < 0.01

NS = not significant (p>0.05)

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Chapter 5

Lichen sclerosus: treatment and follow-up at the departments of Gynaecology and Dermatology

Abstract

Objective: To compare the treatment and followup of patients with lichen sclerosus (LS) at the departments of Gynaecology and Dermatology at the Radboud University Nijmegen Medical Centre (RUNMC), Nijmegen, the Netherlands, to evaluate the need for a multidisciplinary vulvar clinic.

Materials and Methods: Treatment and follow-up data of all women with histologically proven (between January 1995 and January 2001) anogenital LS visiting the outpatient clinics of the departments of Obstetrics & Gynaecology and Dermatology were collected (last date of follow-up: January 2008).

Results: Eighty-four patients with LS were included in this study; ten patients (12%) were treated by both specialties. At the Gynaecology-department, LS patients more often received surgical treatment, topical estrogens and lidocaine ointment, whereas at the Dermatology-department local class II/III corticosteroids were more often prescribed. Follow-up frequencies were similar in both specialties and took place at 3-4 visits in the first year, and at least once a year afterwards. One patient developed vulvar squamous cell carcinoma (SCC). This patient had withdrawn from follow-up and was diagnosed with carcinoma 74 months after the LS had been diagnosed.

Conclusions: Although no hospital guidelines existed, management of patients with LS agreed with current recommendations in the literature, although differences in secondary and supportive therapy existed, due to differences in expertise. The relatively high percentage of patients treated by both specialties with a high frequency of visits emphasizes the need for a multidisciplinary clinic for vulvar disease.

Introduction

Lichen sclerosus (LS) is an idiopathic chronic inflammatory dermatose with an incidence between 1:300 and 1:1000.[1] Most cases occur in postmenopausal women and approximately 7-15% appear in prepubertal girls. Hormonal factors, autoimmune disorders, association with HLA class II antigens, infectious causes, as well as a genetic influence have been associated with LS.[1]

In a series of LS patients that underwent long-time follow-up, 2-6% developed vulvar SCC.[2] Because of the risk of malignant progression, lifelong surveillance of all women with LS is considered essential.[3] No evidence exists that regular check-ups reduce the risk of malignant progression. Malignant progression has never been evaluated in relation to treated versus non-treated disease, or the length of time the disease has been present.[4]

Pruritus is the most common complaint among women with LS. Others may present with pain, a burning sensation, an altered appearance of the vulva, irritation, soreness, dyspareunia or dysuria. A considerable number of patients (33%) are asymptomatic, but do have clinical signs of LS upon physical examination.[5] There is often a considerable delay in diagnosis, which may be due to patient's embarrassment and reluctance of the physician to fully evaluate the symptoms as well as unfamiliarity with this disease. A wide variety of healthcare providers, including gynaecologists, dermatologists and general practitioners may treat the disease. The heterogeneity of medical specialties involved in the management of LS probably reflects the lack of a uniform approach to this disease. In LS, the cornerstone of treatment is the use of high-potent dermatocorticosteroid creams.[6] Surgery is considered to be a last resort to treat complications secondary to the LS.[7]

At the departments of Gynaecology and Dermatology of the Radboud University Nijmegen Medical Centre (RUNMC), Nijmegen, the Netherlands, no guidelines concerning the treatment and follow-up of LS existed. This study was designed to compare the treatment and follow-up of LS at the departments of Gynaecology and Dermatology, and to evaluate the need for a multidisciplinary vulvar clinic.

Materials and Methods

Study population

All consecutive patients who were histologically diagnosed with anogenital LS between January 1995 and January 2001 at the outpatient clinics of the departments of Gynaecology and Dermatology by means of the PALGA-registry (**P**athologisch-**A**natomisch **L**andelijk **G**eautomatiseerd **A**rchief: the nationwide network and registry of

histo- and cytopathology in the Netherlands) of the department of Pathology (RUNMC). After exclusion of all women with a previous vulvar SCC or vulvar SCC at the first histopathology specimen, 84 patients were eligible for analysis.

Patient characteristics, lesion characteristics, methods of treatment and follow-up data were obtained from the gynaecological and dermatological records and anonymously stored in an SPSS database. Medical treatment was divided into topical treatment and systemic therapy and the type of treatment was recorded. Surgical treatment was subdivided in local excision, extensive excision/vulvectomy and laser vaporisation. Information was retrieved up to the last follow-up in the RUNMC or to date (until 1 January 2008).

The study was carried out in accordance with the applicable rules concerning the review of research ethics committees and informed consent.

Statistics

All analyses were performed using SPSS® version 15.0.1 Software (SPSS Inc, Chicago, IL, USA). Differences in the use of medical treatment modalities between the 2 departments were tested for significance using a Fisher exact probability test. The Chi-square test was used to calculate differences between the follow-up frequencies of the different departments. A p-value <0,05 was considered statistically significant.

Results

Patient characteristics

Of the 84 women that were diagnosed with histologically proven LS between 1995 and 2001, 42 women (50%) had been referred to the department of Gynaecology and 42 women (50%) to the department of Dermatology. Forty-eight patients (57%) had been referred by their GP, 18 (21 %) by a general gynaecologist, 13 (15%) by a dermatologist, one by a urologist, one by a specialist in internal medicine, and three patients (4%) visited the gynaecologist in the RUNMC for a different benign gynaecological problem (see Table 1). Most patients at the department of Gynaecology were referred by their GP. Both departments received approximately a quarter of their patients from a referring department in their own specialty.

At the first visit to our hospital the mean duration of symptoms was 3.3 months (range 0.2-21 months). The symptoms at the first consult were pruritus in 63 women (75%), dyspareunia in 40 women (48%), pain in 26 women (31%), dysuria in 11 women (13%), architectural changes in seven women (8%), and problems with defecation in four women (5%). One woman (1%) experienced no symptoms. At the first visit, the clinical examination of the vulva was suspicious for LS in 69 women (82%).

Treatment

Of all 84 women referred to the RUNMC, 38 (42%) were treated at the department of Gynaecology, 36 (46%) at the department of Dermatology and 10 (12%) by both specialties (referred to one department with internal consultation at the other). The different treatment modalities used by gynaecologists and/or dermatologists and the statistical significance of the differences are shown in Table 2.
The patients treated with class III and IV corticosteroids had an average of 2 exacerbations per year; patients treated with other medication, had more exacerbations: 2.5/year. The mean time to exacerbation of LS after vulvectomy was 15 months. Ranges can be found in Table 3.

Follow-up

Of all women with LS, 70 (83%) were followed for at least one year; 29 patients (32.2%) at the department of Gynaecology, 32 patients (40%) at the department of Dermatology and 9 patients (10%) by both specialties. The median time of follow-up of all patients was 58 months (range 0 - 137 months).
The mean numbers of visits per year during the first five years after the first consult are shown in Figure 1. The follow-up frequency was equal at the two departments. Patients visiting both departments had a significantly higher frequency of follow-up (Chi square, $p<0.05$).
Four patients (5%) did not show up for follow-up and were considered lost to follow-up. Thirty-five patients (42%) are still followed at the RUNMC to date. For unclear reasons, in at least 32 patients (38%) it was decided to stop further follow-up (after a mean follow-up time of 36 months). The remaining 13 patients (15%) were referred to their general practitioner (GP) or referring specialist for further follow-up.

Malignant progression

One patient (1.2%) of the cohort developed vulvar SCC. This healthy, premenopausal woman was originally referred to the department of Gynaecology at the RUNMC because of menorragia at the age of 54. During clinical examination LS was diagnosed and confirmed by a biopsy. After 89 months, with no follow-up, she visited the RUNMC again because of a suspicious vulvar lesion. This lesion was locally excised with adequate margins (≥8mm), and histopathological examination showed SCC with an invasion of 2-3 mm. Regular follow-up, 2-4 times a year, takes place at the department of Gynaecology (RUNMC). In the years after her primary tumor, seven recurrences of SCC occurred at 15, 24, 41, 50, 58, 63 and 67 months after the primary tumor. At the moment she is in good clinical condition.

Discussion

This retrospective study evaluates the treatment and follow-up of LS in two specialties (Gynaecology and Dermatology), which to our knowledge has never been described in detail so far.[8] The data of our study show that in the majority of patients with LS a regular long-term follow-up takes place. There was no difference in frequency of follow-up between the departments of Gynaecology and Dermatology. A variable number of follow-up visits took place in the year after diagnosis and at least once yearly in the following years. Reasons for the relatively high number of visits in the first year of follow-up are the taking of another biopsy and the start and evaluation of (new) therapy.

Currently, the guideline of the British Association of Dermatologists (BAD) regarding LS advocates life long follow up for LS that continues to be poorly controlled and for patients that have persistent disease with history of a previous vulvar SCC. Difficult cases with complications may be best managed in a vulval clinic with a multidisciplinary team, including a dermatologist and a gynaecologist.[9] In a consensus document prepared at a workshop at the ISSVD World Conference (2006), follow-up in specialist clinics is also recommended when the pathologist expresses concern and is unable to make a definitive diagnosis of differentiated vulvar intra-epithelial neoplasia (dVIN), when difficulty exists with symptom control or when there is clinical evidence of localized skin thickening.[4] The American College of Obstetricians and Gynaecologists (ACOG) suggests annual examinations for patients whose LS is well controlled, and more frequent visits for those with poorly controlled disease.[10] In general, the BAD as well as the ACOG advise at least 2 follow up visits after the initial consultation: (i) at 3 months to assess response to treatment and (ii) 6 months later to ensure that the patient is confident in treating their problem, before discharge to the care of the primary physician (GP). If patients continue steroids, the BAD suggests yearly visits to a primary care physician.

All three published reports on the management of LS, indicate moderate to strong topical steroids as the treatment option of choice.[4,9,10] The BAD guideline recommends clobetasol propionate initially once a night for 4 weeks, then on alternate nights for 4 weeks and for the final third month, twice weekly.[9] Topical steroid therapy is not without complications, including the possibility of contact sensitization, skin changes, and secondary infection. In the RUNMC, all patients with clinically active LS, even when asymptomatic, are treated to possibly prevent anatomical changes and exacerbations of LS symptoms. In both specialties, class IV corticosteroids were the most frequently used treatment for patients with LS. Whether the long-term topical treatment with a potent steroid cream can reduce the risk of malignant evolution remains unclear.[10]

In the Netherlands no guidelines directed by the Dermatology or Gynaecology Society regarding the treatment of LS exist. Considering the treatment of patients with LS, we noted a remarkable difference between the departments of Gynaecology and Derma-

tology. Surgical treatments were exclusively performed by gynaecologists, especially in the past, and topical estrogen and lidocaine ointments were significantly more often prescribed by gynaecologist, whereas dermatologists prescribed class II and class III corticosteroids, antimalarials and retinoids significantly more often. These differences, however, are inherent to the different specialties: in The Netherlands, dermatologists are not trained to perform vulvar surgery and have experience with a much greater scale of medical therapies for dermatoses than gynaecologists. This emphasizes the importance of a multidisciplinary approach.

It is remarkable that in a substantial part of the patients with LS, for unclear reasons, no further follow-up took place in the RUNMC. However, we did not have information on the follow-up at general practioners. We believe that, because progression to SCC may occur at any time after the diagnosis of LS,11 lifelong follow-up is essential. Uncomplicated cases of LS could very well be followed by family physicians with enough experience in vulvar disease. Patients should be well educated about alarm signs and symptoms, and be instructed to visit their GP in case of persistent ulceration or new growth. During follow-up, biopsy taking of all suspicious lesions (mapping) plays an essential role in the early diagnosis of SCC.

There is an underreporting of young patients in this study as we have only included biopsy proven LS in our study and usually biopsy is not performed in pre-pubertal girls. The patients presenting with defecation problems were 8,50, 50 and 73 years old at time of diagnosis; in pre-pubertal girls this symptom is usually reported more often.

Twelve percent of the patients with LS were treated by both departments. In this group much more treatment modalities were used and the frequency of follow-up was significantly higher than in the patients treated by specialists from a single department. Possibly this high frequency of follow-up is caused by more and more severe complaints and/or non-responsiveness to some treatment options. For these patients, referral to a multidisciplinary clinic for vulvar disease is advisable, because of the advantages for early diagnosis, the best possible treatment and interdisciplinary teaching.[12] A multidisciplinary clinic for vulvar disease should at least have specialists for the departments of Dermatology and Gynaecology. In case of sexual dysfunction or psychological problems, referral to a (specialized) sexologist or psychologist should be offered.

At least 1 patient that had withdrawn from follow-up, developed SCC. Because of the low number of patients and the retrospective character of this study, no firm conclusions regarding the malignant potential of LS and the effect of follow-up can be drawn. 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.[13] A prospective study by Oonk *et al.* indicated that routinely scheduled follow-up meetings with patients with vulvar carcinoma resulted in the detection of smaller recurrences in a substantial proportion of patients compared with self-reported recurrences.[14] Further research is necessary to show if this also applies for LS; a multidisciplinary vulvar clinic would be an excellent setting for this type of research.

In our hospital, a large number of patients with LS was treated with extensive excisions or even vulvectomy. There was a relatively short time to relapse of LS symptoms. These surgeries have all taken place in the beginning of the investigated period (late 1990s). Because of the mutilating effect, high rate of recurrence and possible initiations of new LS lesions, which are reported in literature,[7,9] vulvectomies or large excisions are no longer considered a suitable treatment for LS. Surgical intervention in LS should not be aimed at removing the disease, but at resolving complications of the disease: to release a buried clitoris, to separate fused labia or to widen a narrowed introitus in case of complaints about clitoral symptoms (pain or sexual clitoral dysfunction).

The treatment of LS patients varies, due to the differences in the specialties. To optimise the management of patients with LS strict protocols are advisable. So far, biomarkers that can predict which lesions are at risk to become malignant are lacking. Future research should be aimed at exploring different follow-up schemes. A multidisciplinary approach at a vulvar clinic provides an excellent setting to conduct this kind of research.

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Table 1: Overview of referring specialists

Referring specialist	Gynaecology	Dermatology	Total
General Practitioner	30	18	48
Gynaecology	11*	10	21
Dermatology	1	12*	13
Urology	-	1	1
Internal Medicine	-	1	1
Total	**42**	**42**	**84**

* Patients sent from gynaecology to gynaecology or dermatology to dermatology were referrals for a second opinion.

Table 2: Overview of treatment of LS patients per specialty

Treatment	Gynaecology (n=38)	Dermatology (n=36)	Both specialties (N=10)
Local excision	5*	0*	0
Extensive excision / vulvectomy	14*	0*	5
Laservaporisation	2	0	2
Corticosteroids class I	0	2	0
Corticosteroids class II	0*	10*	1
Corticosteroids class III	3*	17*	6
Corticosteroids class IV	30	24	10
Intralesional corticosteroids	0	0	1
Estrogen local	10*	3*	5
Estrogen systemic	3	1	1
Testosterone local	8	4	4
Retinoids oral	0*	5*	3
Coal tar	0	1	1
Tacrolimus	0	1	3
Imiquimod	0	1	0
Antimalarials	0*	5*	2
Lidocaine	3*	0*	3
Antidepressants	1	1	1

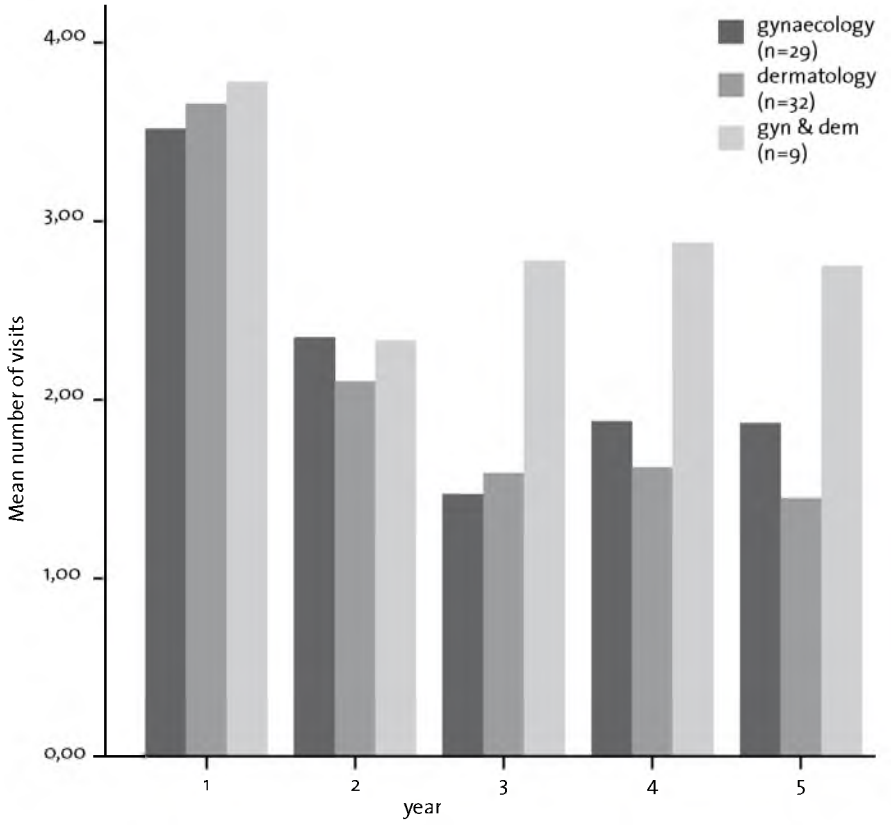
* Statistically significant difference(Fisher exact probability test; P<0.05)

Table 3: Exacerbations of LS after / with treatment

	Exacerbation (% patients)	Mean time to exacerbation	Exacerbation rate (mean)
Vulvectomy (n=19)	60%	19 months (range 2-150)	-
Class III-IV Corticosteroids (n=31)	49%	-	2/year (range 0.3-5)
Multiple treatments* (n=28)	69%	-	2.5/year (range 1-10)

Derived from patients that had a follow-up of at least 1 year
* medical treatments other than corticosteroids III-IV, laservaporisation and/or local excision

Figure 1: Bar chart of mean number of visits for LS per year



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Chapter 6

The prognostic value of blood and lymph vessel parameters in lichen sclerosus for vulvar squamous cell carcinoma development: an immunohistochemical and electron microscopy study

Abstract

Objective: The objective of the study was to quantify vessel type and -density in lichen sclerosus (LS) to find a marker for its malignant potential.

Study design: Quantitative analysis was performed on paraffin-embedded tissue samples of 28 patients with LS (7 adjacent to vulvar squamous cell carcinoma, 21 solitary), and immunohistochemical staining for CD34 (vascular and lymphangiogenic lymph endothelial cells), D2-40 (lymphatic-specific marker) and α-SMA (pericyte marker). Electron microscopy was performed on fresh tissue.

Results: No significant differences in vessel density or other vessel parameters could be demonstrated between the 2 groups. In hyalinized lesions, vessel diameter and α-SMA positivity was reduced compared with nonhyalinized lesions. Electron microscopy revealed detachment of pericytes from vascular endothelial cells and increased thickening of basement membrane, while endothelial cell function did not appear strongly impaired.

Conclusion: Malignant potential of LS cannot be predicted by vessel characteristics. Hyalinization in LS is associated with pericyte detachment from the basal lamina of vascular endothelial cells.

Introduction

Lichen sclerosus (LS) is a chronic inflammatory skin disease that may occur in any cutaneous surface, but has a distinct preference for the anogenital area. It is more commonly found in women than in men, and extragenital LS can be found in up to 20% of all women suffering from vulvar LS.[1] The etiology of LS remains elusive.

Multiple associations with autoimmune disorders, sexual hormones, infection or repeated trauma (itch and scratch hypothesis/Koebner phenomenon)[2] have been reported and genetic as well as immunologic factors are thought to play a role.[3] The classic histological features of lichen sclerosus are epidermal thinning, decreased rete ridge length, band-like dermal inflammation of varying intensity, with or without edema and/or hyalinization.[4] LS is diagnosed in all age groups, including infancy, but is most prevalent in postmenopausal women. An incidence of 1:300-1000 in gynecological and dermatological female patients is estimated but the exact numbers are unknown.[5]

Women suffering from genital LS have a 4-6% risk of developing vulvar squamous cell carcinoma (SCC).[5-7] Furthermore, in 20-60% of the cases of vulvar SCC, LS can be found in adjacent areas.[8-10] Because of the risk of malignant progression, it is current practice that all patients with vulvar LS undergo regular check-ups, although there is no evidence that this follow-up prevents the development of vulvar SCC or results in earlier detection of a malignancy.

Until now, there is no biomarker available that can identify vulvar LS lesions in which SCCs are more likely to develop. Microvessel density (MVD) has been suggested to be of predictive value for tumor development and progression in skin tumors[11] and multiple types of gynecological tumors.[12,13] The induction of the angiogenic response is considered a key step in the transition from a premalignancy toward an invasive neoplasm[14-16] and high MVD is related to poor survival in vulvar cancer patients.[17] Furthermore, Raspollini *et al.* suggested that MVD could identify those cases of LS that have the potential to evolve to vulvar SCC.[18] In addition to vascular endothelial cells, pericyte number and function could have a prognostic value. Pericytes envelop microvascular endothelial blood vessels and are key regulators of vessel homeostasis.[19,20] In bladder and colorectal tumors, loss of pericyte function is associated with poor prognosis.[21,22] Pericytes have not been studied in vulvar lesions.

Edema and hyalinization may be considered surrogate markers for the (dys)functioning of microvasculature: edema and deposition of proteins (hyalinization) may be the results of leaky microvessels and/or inadequate drainage. Although edema and hyalinization are both manifestations of vascular malfunction, hyalinization is thought to occur after the edematous phase in LS.[23,24]

The aim of this study was to analyze MVD and pericyte characteristics in vulvar LS associated with vulvar SCC and solitary vulvar LS in order to establish a possible diagnostic significance in the identification of malignant potential.

Materials and methods

Sample Selection

Paraffin-embedded tissue of 28 LS patients were selected from the archives of the Department of Pathology, Radboud University Nijmegen Medical Centre (Nijmegen, The Netherlands). Two distinct groups of patients were distinguished: solitary LS (n=21) and LS directly adjacent to vulvar squamous cell carcinoma (n=7). LS was considered to be solitary when the patient had no history of differentiated vulvar intraepithelial neoplasia (VIN) and/or vulvar SCC prior to or after the biopsy. All Hematoxylin and Eosin (H&E)–stained slides were re-examined by an expert gynecopathologist (JB). In each lesion, the presence of edema and hyalinization was scored.
Because the stored tissue samples were anonymously studied, this part of the study was exempt from institutional review board approval.
For electron microscopy, fresh 4 mm biopsies of LS lesions were obtained and split in 2. 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). Tissues were obtained according to local ethical guidelines and approved by the local regulatory committee.

Immunohistochemistry of CD34, D2-40 and α-SMA

Paraffin sections (4µm) were mounted onto Superfrost slides (Menzel-Gläser, Braunschweig, Germany), and dried overnight at 37°C. Tissues were dewaxed in xylene, rehydrated through graded alcohol baths and rinsed three times in phosphate-buffered saline (PBS; pH 7.4) for 10 minutes. Following an antigen retrieval step (Sodium citrate (0.01 M; pH 6.0), 95°C, 10 minutes), tissue sections were preincubated with 20% normal goat serum in PBS and subsequently incubated with the primary antibodies for CD34 (Dako, Glostrup, Denmark, 1:750), D2-40 (Dako, 1:100) or α-SMA (NeoMarkers, Fremont, CA, USA, 1:15000), for 60 minutes at RT. All antibodies were diluted in PBS containing 1% bovine serum albumin.

Slides were rinsed in PBS for 10 minutes and incubated with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature (RT). A biotin-avidin alkaline phosphatase complex was generated according to standard procedures (Vector Laboratories). Alkaline phosphatase was visualized with Fast blue (20 ml Tris HCl pH 8.2, 4 mg Naphtol AS-MX, 4.8 mg Levamisole and 20 mg Fast Blue BB salt) and counterstained with Nuclear fast red (Vector Laboratories). Slides were mounted with Imsol (Klinipath, Duiven, The Netherlands) and subsequently with Permount (Fisher Scientific, Fairlawn, NJ, USA). Negative controls (buffer only) and positive controls (normal skin) were applied in each run.

Quantification of CD34, D2-40 and α-SMA staining

Image acquisition was performed using a 3CCD color video camera (Sony DXC-950P, Sony Corp, Japan) mounted on a conventional light microscope (Axioskop 2 plus, Carl Zeiss AG, Germany) and attached to a personal computer with frame grabber card (Matrox Meteor-II Multichannel, Matrox Imaging, Dorval, Canada). Images were acquired using a ×20 objective (Plan Neofluar, NA=0.5, resulting in specimen level pixel size of 0.39 μm^2). Prior to analysis of the immunohistochemical staining, an image of an empty microscopic field was acquired, which was used for correction for unequal illumination. Image acquisition and analysis were performed using a custom written macro in KS400 image analysis software (Carl Zeiss).[25]

Thresholds were determined from a set of training slides and were found adequate for almost all slides analyzed in this study. When the initial thresholds led to unrealistic patterns, adjustment was performed by the operator (data not shown). For each patient, MVD, mean vessel area, and mean vessel perimeter in the lichenoid area between the band-like inflammatory infiltrate and epidermis was calculated per surface area in multiple non-overlapping images (range 3-13, mean 6.4).

Electron microscopy

Processing of tissue samples for electron microscopy was performed as described previously.[26] After presence of LS was confirmed in H&E, the glutaraldehyde-fixed tissue fragments were postfixed in cacodylate-buffered 1% osmiumtetroxide containing 1% potassiumhexacyanoferrate(II) for 1 h, 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 1200EX-II electron microscope (JEOL, Tokyo, Japan).

Statistical Analysis

Calculation of vessel parameters per patient was performed using Statistical Package for the Social Sciences (version 15.0.1; SPSS, Chicago, IL). Because the vessel variables were not normally distributed, the non-parametric Mann-Whitney U test was used to analyze differences between the groups. The relationship between the presence of edema or hyalinization in the two groups of LS was calculated according to the Fisher's exact test. $P<.05$ was considered to be statistically significant.

To test our hypothesis that no differences exists for MVD between SCC-associated and solitary lesions, and also between non-hyalinized and hyalinized lesions, equivalence testing was used[27] next to the commonly performed testing for differences between groups. The null hypothesis for equivalence testing is that the difference between the means of two groups exceeds a certain threshold ε, with alternative hypothesis that no such difference exists. The critical t-value for rejecting the null hypothesis may be computed from the inverse non-central F-distribution, with non-centrality parameter $\lambda=n_1 n_2 \varepsilon^2/(n_1+n_2)$.[27] In the present study, the constant ε was

fixed at a value of 20% of the average MVD for the respective experiment, which we consider an appropriate threshold for a difference of physiological significance.

Results

Clinicopathological features

The histopathological analysis of all cases of LS showed hyperkeratosis, a thin epidermis and a variable chronic inflammatory cell infiltrate. The presence of edema and hyalinization was variable and recorded for each patient. We found no cases of edema without hyalinization and half of the cases showed hyalinization without edema, confirming that hyalinization occurs after the edema (Table 1). The presence of hyalinization was not related to the presence of SCC adjacent to the LS (Table 2; P=.673).

Immunohistochemistry

LS lesions were immunohistochemically stained for CD34, D2-40 and α-SMA (Figure 1) on consecutive sections. In addition to vascular endothelial cells, CD34 also stains lymphangiogenic but not quiescent lymph vessels.[28] We did not observe CD34-positive vessels that were also positive for the specific lymph vessel marker D2-40 (Figure 1, B and C), which indicates that lymph vessel growth is not evident in these LS samples. Subsequently, the respective vessels number, area and perimeter were quantified. No significant differences in the total number of CD34, D2-40 or α-SMA-positive vessels could be demonstrated between solitary LS and SCC-associated LS (Figure 2). In addition, no differences in CD34, α-SMA or D2-40-positive vessel area and vessel perimeter were observed in both LS groups (data not shown).

We found a significantly reduced number of α-SMA-positive vessels in hyalinized LS, whereas the number of CD34-positive and D2-40-positive vessels was not reduced in hyalinized LS (Figure 3). However, all vessels were significantly smaller in hyalinized LS (Figure 4). A trend towards smaller vessels and less SMA positivity in edematous lesions was observed, but these differences were not statistically significant (data not shown).

Electron microscopy

Loss of α-SMA expression suggested impaired pericyte function or even lack thereof. To further analyze pericyte characteristics in LS, transmission electron microscopy was performed on fresh LS specimens with and without hyalinization. Microvessels in LS are enveloped by pericytes in nonhyalinized LS (Figure 5, A and B), but are detached from the basal lamina of microvascular endothelial cells in hyalinized regions of LS (Figure 5, C and D). At higher magnification (Figure 5, E), thickening of the basement membrane is evident. Furthermore, inter epithelial junctions (IEJs) are closed and vesiculovacuolar organelles (VVOs) can be seen, which suggests that endothelial cell function is not strongly impaired.

Comment

Our study shows that vessel parameters in LS are not associated with development of vulvar SCC. Neither did we find a correlation between edema or hyalinization and the presence of SCC adjacent to LS. However, vessels were significantly smaller in LS with hyalinization, and reduced α-SMA-positivity was observed. Electron microscopy revealed that pericytes are detached from the basal lamina, and thickening of the basement membrane in hyalinized regions of LS. Hyalinization was observed in both types of LS and we did not find a significant difference between hyalinization and tumor development (Table 2). Therefore, although pericyte detachment occurs in hyalinized LS, we propose that this does not increase risk for tumor development.

The role of angiogenesis in vulvar oncogenesis is still unclear and studies focusing on the different vulvar premalignancies give conflicting results. Raspollini *et al.* described that expression of vascular endothelial growth factor (VEGF) was highest among the group of LS that evolved to vulvar SCC.[18] However, Maclean *et al.* showed that VEGF was not expressed in normal vulva, solitary LS or in LS adjacent to SCC.[29] Likewise, Bamberger *et al.* could not demonstrate a predictive value of VEGF expression.[30] Furthermore, it has been suggested that increased density of sub-epithelial CD34-positive micro-vessels could identify those LS that are at greater risk for SCC development.[18]

Our study does not corroborate these findings. We did not find differences in vessel density, area or perimeter, nor did we detect lymphangiogenesis as we have not observed D2-40 positive vessels that also expressed CD34.[28] Our data confirm a previous report that micro vessel density in LS does not predict its malignant potential.[30]

Unexpectedly, we found a correlation between pericyte coverage of vascular endothelial cells and the presence of hyalinization: reduced α-SMA positivity and detachment of mural cells was observed in hyalinized lichenoid areas in both types of LS. Pericytes are generally considered to be contractile cells that stabilize vessel walls and participate in the regulation of blood flow in the microcirculation.[31] Pericytes may also influence endothelial permeability, proliferation, survival, migration and maturation.[31] Regulation of blood vessel homeostasis and response to angiogenesis or vascular permeability-inducing stimuli involves a complex spatio-temporal regulation through several molecules.[32] This provides a balance between the activation of endothelial cells to expand the vasculature and the interaction of endothelial cells with pericytes to ensure a stable, functional vasculature. This interaction is in part under control of the vascular endothelial growth factor (VEGF)-VEGF receptor system.[33] Currently, we do not know whether VEGF receptor activity is altered in vessels with detached pericytes, which is subject of our future investigations. However, despite detachment of pericytes and loss of their α-SMA expression in hyalinized LS, these vascular endothelial tubes do not display widening of the inter-endothelial junction

(IEJ). Vesicular-vacuolar organelles (VVOs) that are involved in transcellular transport from vessel lumen to the interstitium can be readily detected in these vessels. This suggests that vascular function might be still intact and that the thickening of the basement membrane prevents the vessels from leaking.

The probability that LS is a vulvar SCC precursor lesion is debatable. The number and dilation of vessels in solitary LS is identical to that in LS directly adjacent to SCC. In tumors, however, MVD is strongly increased (shown by Bancher-Todesca *et al.*[34] and our unpublished results). Our data demonstrate the lack of an angiogenic switch in LS juxtaposed to SCC. Since the induction of an angiogenic program is considered to be required for progression of a premalignant lesion towards an invasive tumor, we argue that LS is not a vulvar SCC precursor lesion. However, it has to be noted that the number of patients included in this study may have been too small for this statement to be unequivocal. An *a priori* power analysis using vessel density values (n, average, SD, α, ß) as input data indicates that at least 1400 samples need to be analyzed in order to detect a significant difference. However, the opposite statistical approach (i.e. testing that absolute difference in blood vessel density in the two groups is less than 20%) demonstrated no differences in the density of CD34 and aSMA-positive vessels in solitary and SCC-associated LS ($P<0.05$). However, due to the low number of D2-40 positive vessels, we cannot exclude a difference in lymph vessel density.

It is tempting to speculate that HPV-negative differentiated vulvar intraepithelial neoplasia (dVIN), which can be found in the edges of the tumor is the true precursor. MVD has been reported to be of value for determining the potential malignant progression of HPV-associated usual VIN to SCC.[34;35] Whether this also holds true for dVIN remains elusive and is subject of further studies.

Table 1. Hyalinization vs edema

	Edema	No edema	Total
Hyalinized	7	13	20
Non-hyalinized	0	8	8
Total	7	21	28

Fisher's exact test, $P = .065$. In lichen sclerosus, edema was found only in the hyalinized cases

Table 2. Hyalinization vs association with carcinoma

	Solitary LS	SCC associated LS	Total
Hyalinized	15	5	20
Non-hyalinized	6	2	8
Total	21	7	28

Fisher's exact test, P= .673.

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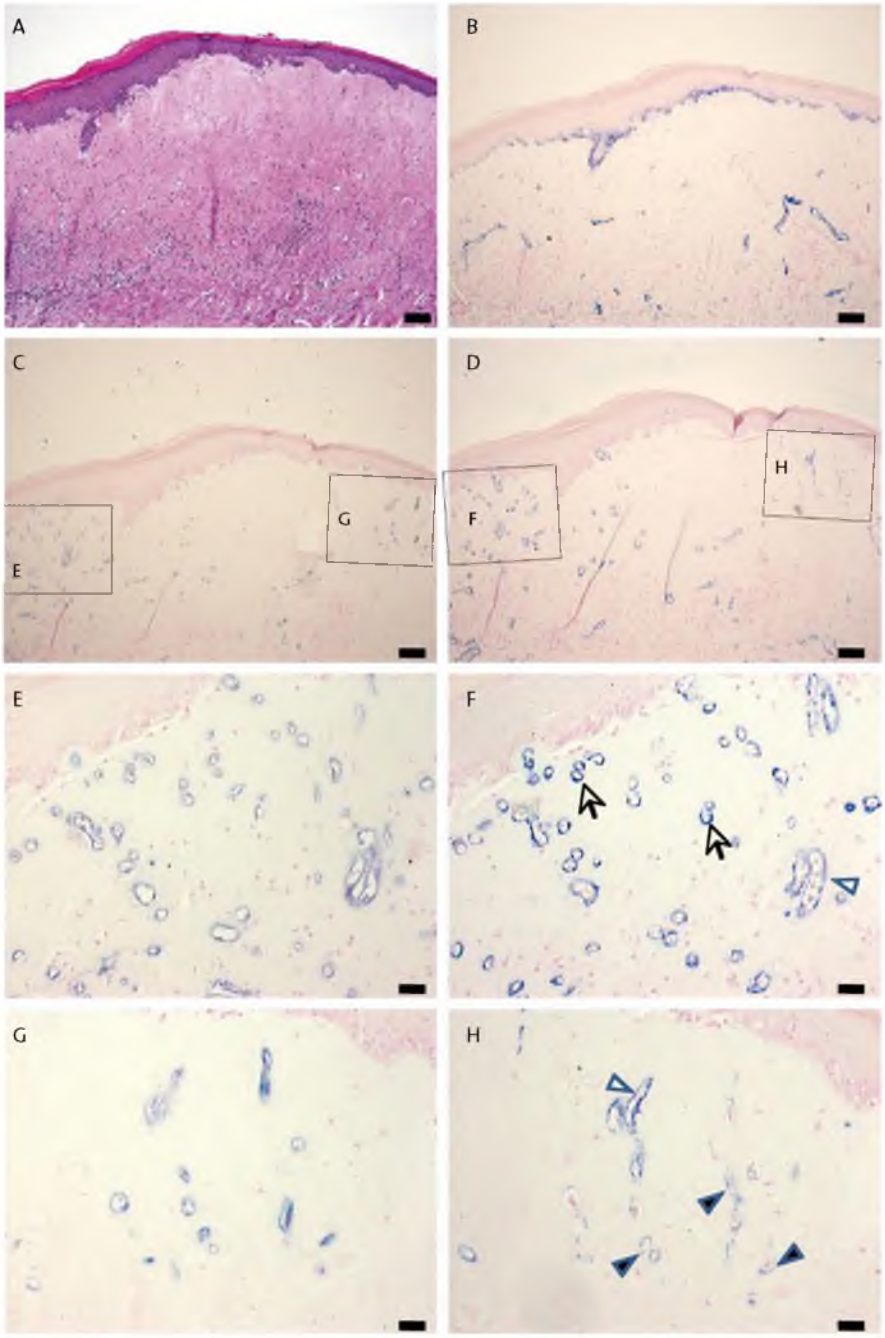
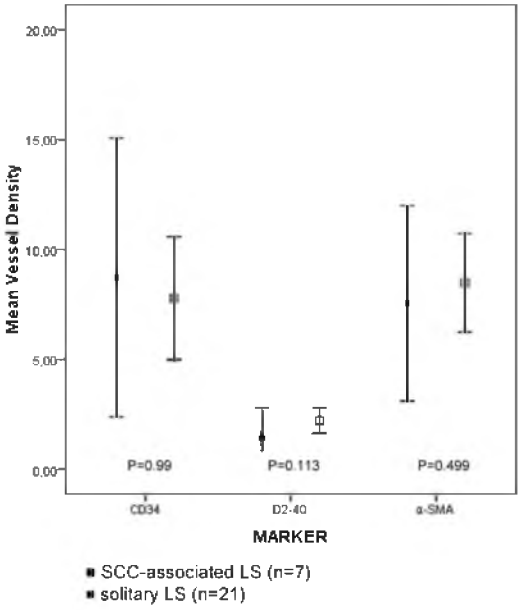
Figure 1. Immuohistochemical analysis of blood and lymph vessels and pericytes in LS.



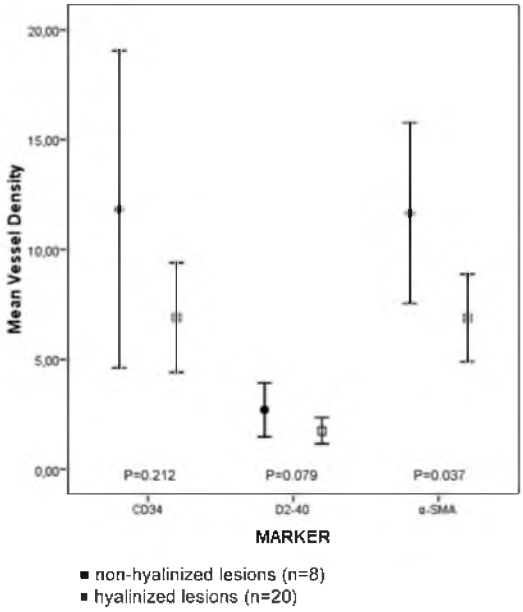
A. H&E staining of LS with the typical features of LS: thinned epithelium, loss of rete ridges and a band-like inflammatory infiltrate beneath a zone of variable degrees of edema and hyalinization.
B. D2-40 is expressed by the basal cell layer and lymphatic vessels
C. CD34 stains blood vessels and lymphangiogenic endothelium. We did not find overlap with
B. D2-40 staining indicating that lymphangiogenesis did not occur. The CD34 staining intensity was comparable in
E. nonhyalinized as well as (G) hyalinized regions. In contrast,
D. α-SMA expression was strong (*arrow*) to moderate (*white arrowhead*) in
F. non-hyalinized regions of LS, but moderate (*white arrowhead*) or very weak (*black arrowhead*) in
H. hyalinized regions.
Scale bars: A-D, 100μm; E and F: 30μm.

Figure 2. No correlation between MVD and LS type



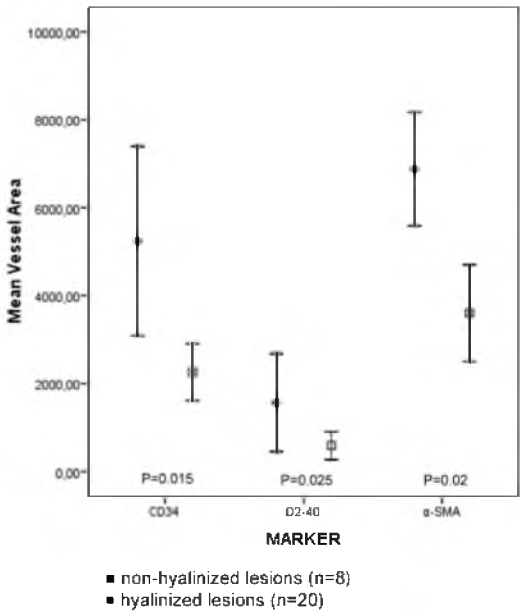
Mean of MVD (± 2SEM) for CD34, D2-40 and α-SMA-positive vessels in solitary LS (n=21) and LS associated with SCC (n=7).

Figure 3. α-SMA-positive MVD is reduced in hyalinized LS



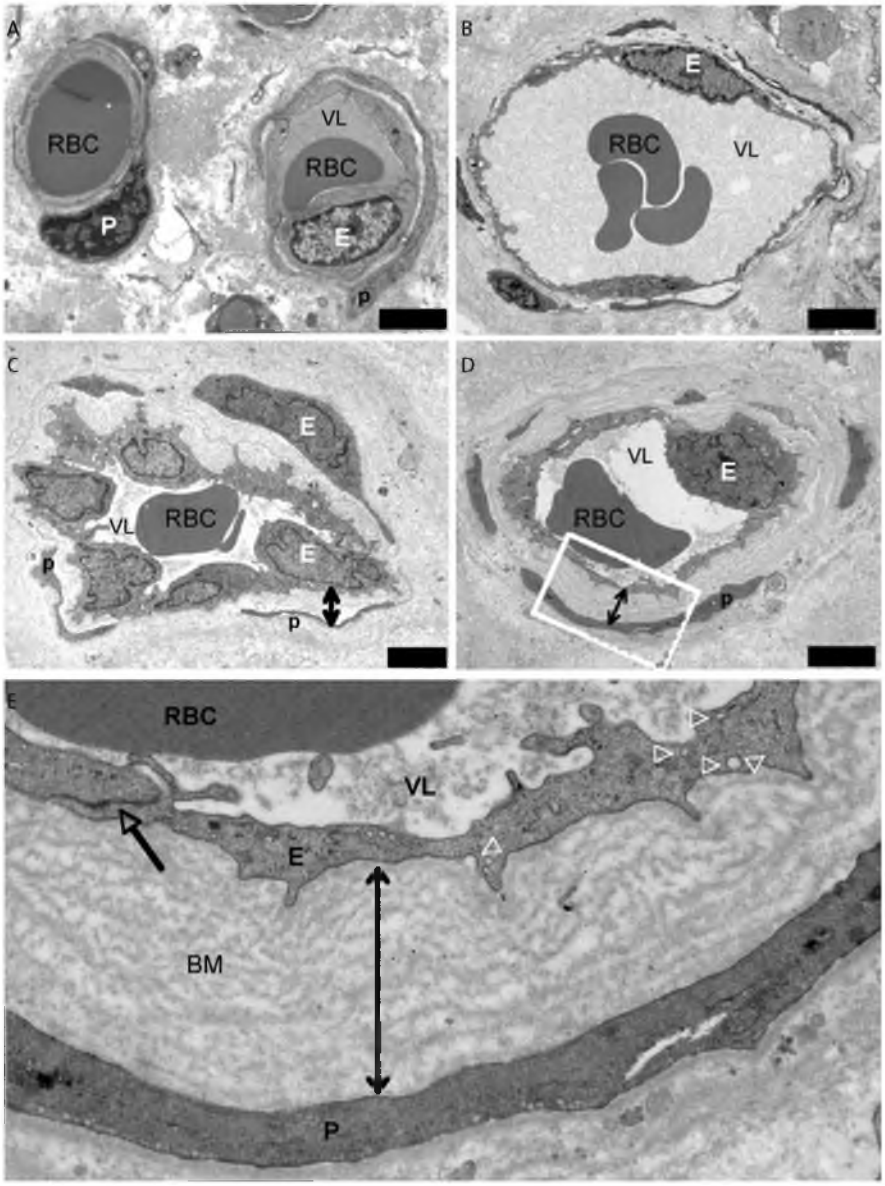
Mean of MVD (± 2SEM) for CD34, D2-40 and α-SMA-positive vessels in nonhyalinized LS (n=8) and hyalinized LS (n=20).

Figure 4. Reduced mean vessel area in hyalinized LS



Mean of vessel area for CD34, D2-40 and SMA in non-hyalinized (n=8) and hyalinized LS (n=20)

Figure 5. Representative electron-micrographs of micro vessels in lichen sclerosus



A and B. The microvascular endothelium is closely covered by pericytes in nonhyalinized lichen sclerosus

C and D. Microvessels in hyalinized zones of lichen sclerosus display pericyte detachment (*double-headed arrows*) and thickening of the basement membrane. (E) At higher magnification, closed endothelial fenestrae (*black arrow*) and vesiculovacuolar organelles (*white arrowheads*) are visible.

BM, basement membrane ; *E*, endothelial cell; *RBC*, red blood cell; *P*, pericyte; *VL*, vascular lumen. Scale bars: 5µm.

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Chapter 7

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

Abstract

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. DNA-content in isolated nuclei of microdissected normal vulvar epithelium (n=2), lichen sclerosus (n=9), differentiated vulvar intraepithelial neoplasia (n=13), and squamous cell carcinoma (n=17) from 22 patients was measured via DNA image cytometry. For additional analysis, 6 differentiated vulvar intraepithelial neoplasia lesions were selected, bringing the number of patients to 28. p53 expression was determined by immunohistochemistry on consecutive tissue sections. Thirty-eight percent (5/13) of differentiated vulvar intraepithelial neoplasia lesions and 65% (11/17) of squamous cell carcinomas were DNA aneuploid or tetraploid. In lesions that contained differentiated vulvar intraepithelial neoplasia and adjacent squamous cell carcinoma, the ploidy status of differentiated vulvar intraepithelial neoplasia did not exceed that of squamous cell carcinoma. 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 immunohistochemistry followed by DNA image cytometry on formalin-fixed tissue sections. Similarly, we found p53-positive nonproliferating cells with increased DNA content in the superficial compartment of 6 additional solitary differentiated vulvar intraepithelial neoplasia lesions that were not associated with squamous cell carcinoma, indicating ascending aneuploid cells from the basal compartment. DNA ploidy measurements suggest that differentiated vulvar intraepithelial neoplasia has a higher malignant potential than lichen sclerosus, and thus is a more likely precursor of squamous cell carcioma. 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 gynecologic cancer and accounts for approximately five percent of the malignancies of the female genital tract.[1] Based on etiological characteristics, 2 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 papilloma virus (HPV)-dependent pathway in which premalignant stages of vulvar cancer are usual vulvar intraepithelial neoplasia (VIN)-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 characterized 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 2% to 6% lifetime risk to develop vulvar SCC.[3] 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.[4] 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.[5] 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[6]. 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.[6] The tumor suppressor protein p53 is a key regulator of maintaining normal diploid status[7] and halts cell division and/or eliminates cells that have acquired irreparable DNA damage.[8] A relationship between high p53 expression, morphological variability of nuclei and DNA aneuploidy in breast carcinomas was demonstrated by Haroske and co-workers[9] more than a decade ago, but has not been studied in vulvar carcinomas and their precursor stages before.

In vulvar carcinomas, ploidy has been studied mostly in relation to prognosis. It has been reported that aneuploidy in vulvar carcinomas varies from 13% to 83%, but no relation with prognosis has been described so far.[10-16] Aneuploidy in vulvar LS varies from 0% to 33% of the cases.[14,16-18] 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.[14] 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.[19]

The aim of this study was to analyze 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 toward 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 Center. All tissue sections were collected between 1996 and 2009. All original hematoxylin and eosin (H&E)-stained slides were re-examined by an expert gynecopathologist (J.B.), and all lesions were classified according to World Health Organization criteria.[4] 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: 2 samples of normal epithelium, 9 LS lesions, 13 dVIN lesions, and 17 samples with SCC. In addition, 6 solitary dVINs 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 analyzed.

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.[20;21]

Table 1. Number of patients and samples

Lesion	No. of patients	No. of 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**

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 demineralized water and phosphate-buffered saline (PBS) before microdissection of the different epithelial lesions. Excised tissue was digested with pepsine (Sigma Aldrich, Zwijndrecht, The Netherlands, 2 mL 0.5%[wt/vol], 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 (Partec GmbH, Münster, Germany) and spinned down for 10 min at 150g. The pellet of nuclei was collected and counted using a Coulter Counter (Beckman Coulter, Woerden, The Netherlands). Subsequently, the suspension was centrifuged again; and the pellet was re-suspended in PBS to a dilution of 200 000 nuclei per milliliter. A cytospin of 20 000 nuclei was prepared (100 µL, 10 minutes, 100g), followed by fixation in Böhm fixative (60 min at room temperature [RT]), rinsing with methanol and air-dryin. For ploidy measurement, Schiff-Feulgen staining was performed as described elsewhere.[22]

Ploidy examination with Q-path DNA software

Image cytometry with Q-path DNA software (Leica Imaging Systems Ltd, Cambridge, United Kingdom) 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).[23-25] In each sample, lymphocytes were identified by the the 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 (Menzel Gläser, Braunschweig, Germany) 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 3 times in PBS (pH 7.4) for 5 minutes; and antigen retrieval was performed with boiling sodiumcitrate (0.01 mol/L, 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 toward p53 (clone DO-7 [Neomarkers, Labvision, Fremont, CA, USA], 1:1000 in PBS containing 1% [wt/vol] bovine serum albumin) for 1 hour 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 horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (30 minutes, RT). Staining was developed with diaminobenzidine (DAB) in the presence of H_2O_2, counterstained with Mayer's hematoxylin, dehydrated in ethanol and xylene, and finally mounted with Permount (Fisher Scientific, Fair Lawn, 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.[26]

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-carbazole instead of diaminobenzidine, and mounted with Imsol without coverslips (Klinipath, Duiven, The Netherlands). Images of representative areas were digitized and stored in 24 bit RGB using a 3CCD camera (Sony 950P, Sony, Tokyo, Japan) attached to a Zeiss AxioPhot microscope (Carl Zeiss, Jena, Germany) with 20x objective (Plan Neofluar, NA=0.5; specimen level pixel size 0.64x0.64 µm2). Subsequently, p53-stained sections were washed in demineralized water (37°C, 1 hour), followed by an overnight wash step in demineralized water to remove all traces of Imsol. After a brief third wash with demineralized water, tissue sections were submerged in Böhm fixative (60 minutes, RT), rinsed with methanol, air-dried; and Schiff-Feulgen staining was performed.

This procedure removes all p53 staining as well as the hematoxylin 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 ± 20 nm). 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 semiquantitative scoring of p53 expression, χ^2 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 2-sample Kolmogorov-Smirnov test. Statistics were performed using SPSS software (SPSS Inc, Chicago, IL).

Table 2. Ploidy-status *vs* type of lesion

	Hypoploid	Diploid	Aneuploid	Tetraploid	Total (100%)
LS	1 (11%)	8 (89%)	-	-	9
dVIN	-	8 (62%)	5 (38%)	-	13
SCC	-	6 (35%)	9 (53%)	2 (12%)	17
N	-	2 (100%)	-	-	2
Total	**1 (2%)**	**24 (59%)**	**14 (34%)**	**2(5%)**	**41**

N: normal epithelium
LS: lichen sclerosus
dVIN: differentiated VIN
SCC: squamous cell carcinoma

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 summarized in Table 2. Both normal epithelium samples were DNA diploid. DNA hypoploidy was observed in 1 (11%) of 9 LS lesions, whereas the other 8 LS samples were DNA diploid. Five (38%) of the 13 analyzed dVIN lesions were DNA aneuploid. From the 17 carcinomas, 9 (53%) were DNA aneuploid tumors; and 2 (12%) tumor samples were DNA tetraploid. The remaining 6 (35%) tumor samples 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.

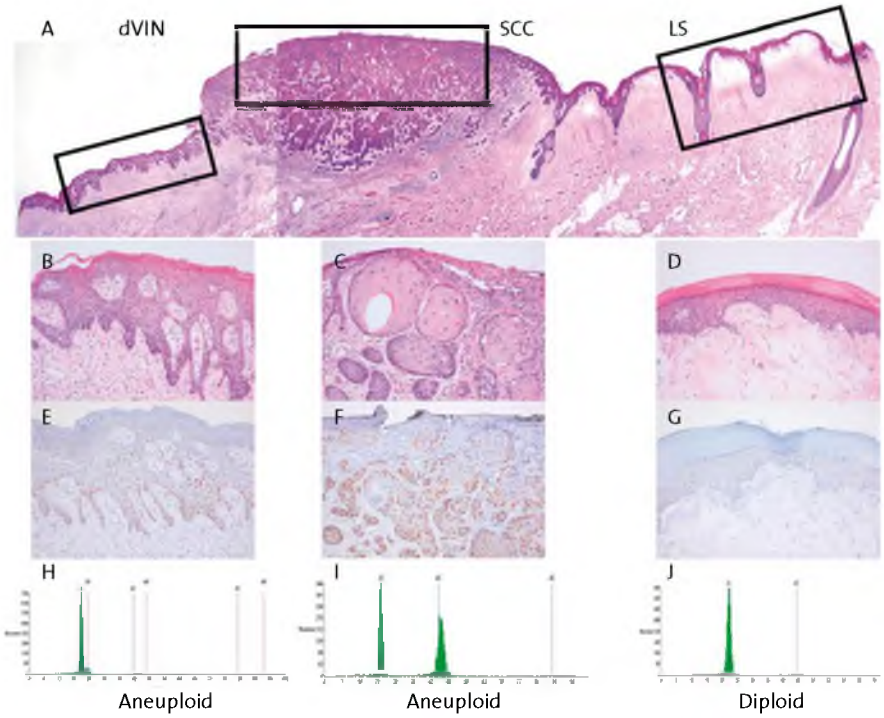
p53 expression

Subsequently, consecutive tissue slides of the samples described above were subjected to p53 expression analysis by immunohistochemistry (Figure 1E-G). Results are summarized in Table 3. In all 13 dVINs 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 (ie patient XVII in Table 3).

p53 expression and DNA ploidy

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, LS or normal tissue) significantly correlated with aneuploidy determined by Feulgen staining on isolated cells (Table 4, $\chi^2 = 27.6$, $P < .001$). Ninety-three percent (14/15) of all non-diploid lesions displayed more than 70% of p53-positive cells. In contrast, in 92% (22/24) of the DNA diploid cases, 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.[4] 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 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 12.5x). 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 hyalinized dermis and subepithelial edema of variable thickness is present. The characteristic band-like infiltrate of lymphocytic cells is located beneath this zone.
E. Immunohistochemistry revealed nuclear p53-positivity 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).

Combined ploidy analysis and immunohistochemistry

The above-described correlation was derived from 2 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 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_{p53} greater than 1.6. The mean value of $IOD_{Feulgen}$ of p53-negative nuclei in each tumor specimen is presented as a dotted line (Figure 3C, top panels), which allows easy visualization 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 ($P < .001$). 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 with 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, which could imply that aneuploidy is confined to the lower layers. We have therefore analyzed the relationship between DNA ploidy and p53 expression in single cells of 6 solitary dVIN samples in more detail and summarized the results in Table 5. The frequency plot of sample 2 is shown in Figure 4. Compared with 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 greater than 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-positive cells (Table 5A). Similarly, DNA content was significantly higher in the basal component compared with 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 analyzed (ie samples 1 and 5). When comparing DNA content between p53-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 greater than 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[26], which also contributes to increased DNA content in this study. Therefore, we have analyzed MIB-1 expression in the 6 solitary dVIN lesions and found a mean percentage of positive cells of 43.1% (95% confidence intervfal [CI], 18.1% - 68.1%) in the basal compartment compared with 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 greater than 1% in the superficial component indicates that DNA content is in part due to aneuploidy.

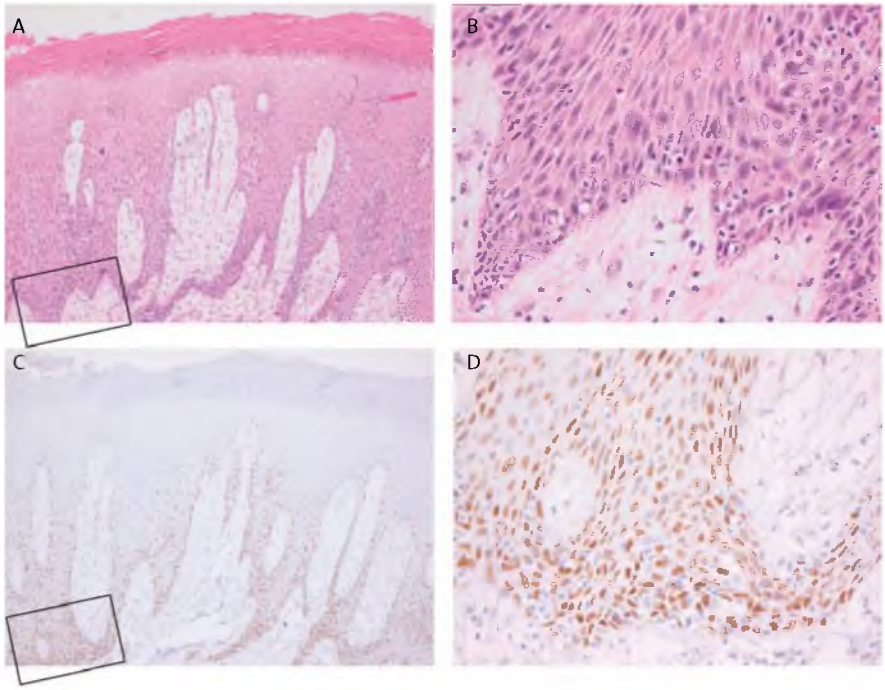
Discussion

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 in dVIN than in LS, we believe that at least a subset of dVIN lesions is a premalignancy with a higher malignant potential compared with LS.

Our finding of a lack of DNA aneuploidy in LS is coherent with the results published by Scurry *et al.*[16] 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.[16] We only found 1 of 9 LS lesions to be DNA hypoploid and have described previously that the proliferative activity in LS is comparable to that in other vulvar premalignancies.[26] However, the absence of DNA aneuploidy in all but 1 hypoploid LS in our study suggests that LS has less malignant potential than dVIN. Recently, Raspollini *et al.* showed that high MIB1 and p53 labeling indices in LS might identify those vulvar LS cases with a high likelihood of evolving into SCC.[19] However, these high-risk LS lesions show histological features that resemble the criteria for dVIN.[27] The results of our study suggest that these so called 'atypical LS' might be non-diploid dVIN lesions.

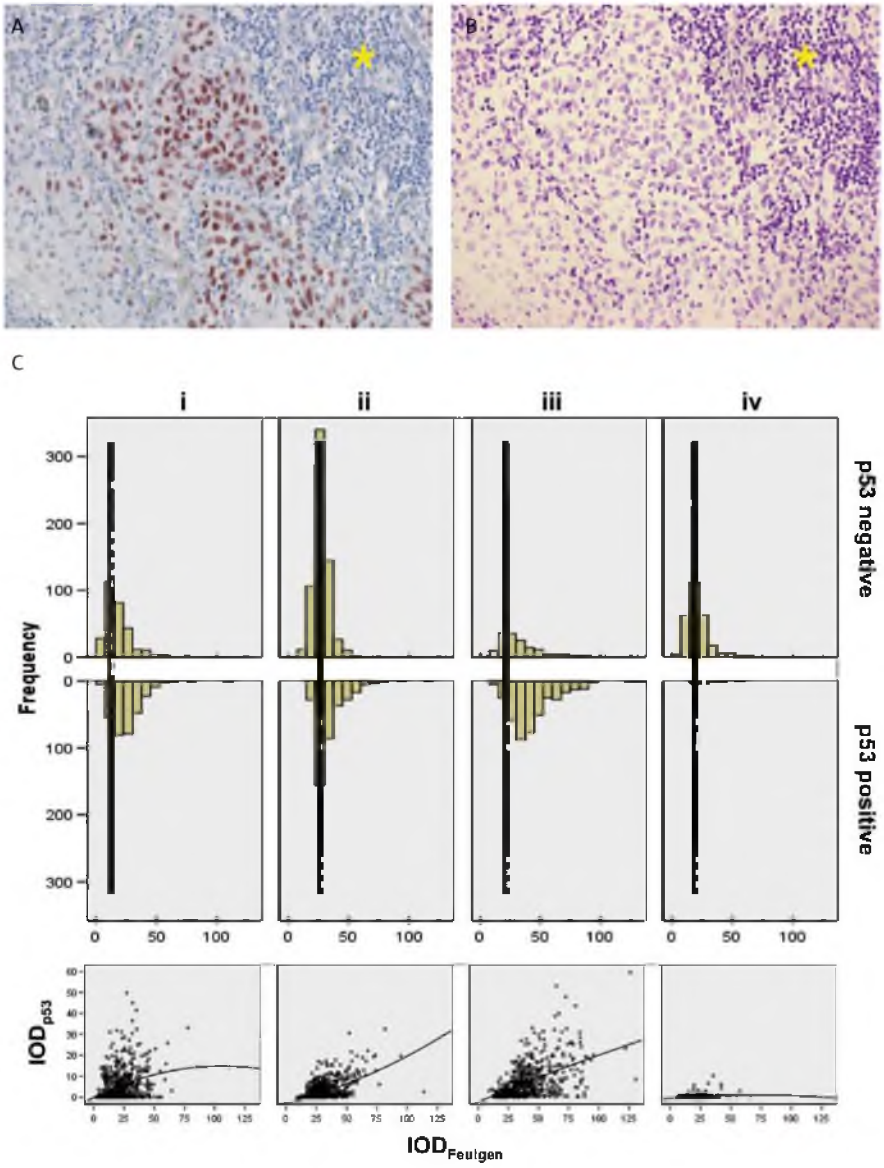
The atypia in a dVIN lesion is confined to the lower one-third of the epithelium (Figure 1B).[28] However, the entire epithelium of the dVIN lesion was microdissected and used for DNA image cytometry. Therefore, samples were "contaminated" with p53-negative cells and could have resulted in an underestimation 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 toward the superficial compartment can occur.

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 and C:50x, B and D:200x.

Figure 3: p53 staining intensity correlates with DNA content.



Sequential staining for p53 (A) and DNA (B) was performed on tumor 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 4 different patients (i-iv) is 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_{al}>0, bottom panels) reveal a similar positive correlation between p53 staining intensity and DNA content in individual cells. Original magnification A and B:50x.

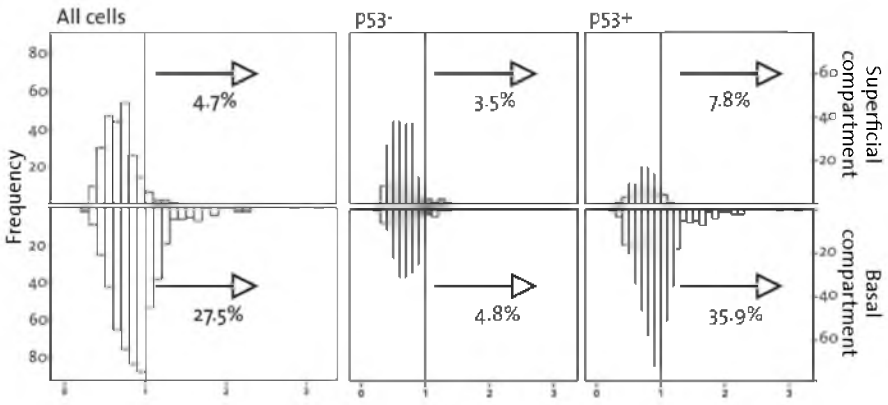
It remains unclear how p53 could contribute to the development of vulvar SCC from dVIN.
Lerma *et al*. concluded that p53 expression is a late event in malignant progression as 72% of their SCCs were DNA aneuploid and only 56% of the SCCs showed p53 overexpression (which was defined as >10% of the tumor cells)[14]; but they do not provide simultaneous results on ploidy status and p53 expression in individual lesions. In vulvar carcinomas, 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%.[10-16] The causal relationship between high p53 expression in vulvar epithelium and DNA aneuploidy remains elusive. Possibly, destabilization of chromosomes due to centrosome amplification contributes to the cancer susceptibility phenotype associated with mutation (resulting in over-accumulation of p53) or stabilization of p53.[6] 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.[29] Our immunohistochemical data support 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.[6,8,30] 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.[31] 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 with the superficial compartment, although ascending aneuploid cells can also be detected. The observation that the degree of aneuploidy in dVIN is higher compared with LS, but less or equal compared with SCC, suggests that dVIN has a higher malignant potential than LS. The mechanism of oncogenesis and the progression of LS to dVIN remains elusive.

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 greater than 1 (indicative for aneuploidy).

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

Patient	Diagnosis	Ploidy-status#	p53-expression*
I	LS	Diploid	10%
	dVIN	Diploid	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%

*p53-expression: in dVIN: percentage in lower one-third of the epithelium; in LS and SCC the entire lesion was assessed
NA: not available

Table 4 Ploidy-status vs high p53-expression

	p53<70%	p53≥70%	Total
Diploid (DNA index 0.9-1.1)	22	2[a]	24
Non-diploid (aneuploid or tetraploid)	1[b]	14	15*
Total	23	16	39

[a] both dVIN lesions, [b] SCC, * P<.001 (χ^2test)

Table 5 p53 positivity and DNA content in solitary dVIN (A) and DNA content in relation to p53 expression and localization within dVIN (B)

A

Solitary dVIN	p53-positive cells (%)			Cells with DI > 1[a] (%)		
	Basal[b]	Superficial[c]	*P*	Basal	Superficial	*P*
1	20.0	9.8	.003	47.4	21.7	<.001
2	72.9	27.2	<.001	27.5	4.7	<.001
3	41.4	9.4	<.001	3.9	5.2	<.001
4	30.9	3.3	<.001	16.6	7.8	.003
5	4.1	0.6	<.001	20.7	10.6	<.001
6	34.9	12.9	<.001	26.4	8.7	<.001

B

Solitary dVIN	Basal compartment			Superficial compartment		
	Percentage cells with DI > 1			Percentage cells with DI > 1		
	p53 -	p53 +	*P*	p53 -	p53 +	*P*
1	43.9	61.0	<.001	20.2	35.7	NS
2	4.8	35.9	<.001	3.5	7.8	NS
3	1.6	7.1	<.001	3.5	21.6	<.001
4	8.4	34.9	<.001	6.9	33.3	NS
5	19.7	44.0	<.001	10.7	0	NS
6	21.8	35.0	<.001	9.3	5.0	NS

[a] DI = 1: DNA content of normal reference cells within the stroma
[b] Basal compartment: the lower one-third component of dVIN
[c] Superficial compartment: the upper one-third component of dVIN

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Chapter 8

General discussion

General discussion

In this thesis, clear evidence is presented that there are two separate pathways leading to vulvar squamous cell carcinoma (SCC).[1,2] Both pathways have their own premalignancies and patient characteristics. In the HPV-associated pathway, oncogenesis resembles the development of cervical carcinoma. In the HPV-negative pathway, the mechanism of cancer development is unclear. So far, differentiated VIN (dVIN) is the most likely candidate in the search for the true precursor lesion.

HPV-positive pathway

The development of vulvar SCC through the HPV-related pathway resembles the development of cervical cancer. As shown in the meta analysis by Smith *et al.* including 44 studies, HPV16 is the most prevalent HPV type in both vulvar SCC (29.3%) and uVIN (71.2%). The next most common HPV type was HPV18 in vulvar SCC (5.6%) and HPV33 in uVIN (7.7%). When HPV prevalence was calculated as the percentage of only HPV–positive invasive vulvar cancer cases, 75.5% of HPV positivity was attributable to HPV16, followed by HPV18 (14.3%) and HPV56 (1.7%).[3]

A decrease in incidence of HPV related vulvar squamous lesions is to be expected with the introduction of the prophylactic HPV vaccine. Two commercial vaccines against HPV (Gardasil™ and Cervarix™) are currently available in many countries worldwide. They have been found to be highly efficient in preventing persistent infections and lesions not only from the uterine cervix, but also from the anus, vagina, and vulva. Both vaccines have been shown to be generally safe in phase II and phase III randomized controlled trials. Gardasil™ is a quadrivalent recombinant vaccine that protects against HPV 6, HPV11, HPV16 and HPV18.[4] Cervarix™ is a bivalent vaccine that protects against HPV16 and HPV18, and has been chosen by the Dutch government for the Dutch vaccination programme. Cross protection for HPV31, HPV33 and HPV45 for cervical lesions has been described, but has not been evaluated for vulvar lesions.[5]

With the introduction and effectuation of HPV vaccination, a decrease in the incidence of uVIN and associated vulvar SCCs is to be expected. Novel therapies and treatments might decrease the morbidity and possibly mortality of HPV-related vulvar squamous (pre-)malignancies. Several therapeutic vaccination strategies have been tested (mainly for HPV16) and were found to be effective on immunological, serological or clinical parameters.[6-9]. Recently, the immunogenicity and efficacy of a synthetic long-peptide therapeutic vaccine was tested in women suffering from HPV16 positive uVIN.[10] Clinical response (12 months post vaccination) was achieved in 79%, and complete response appeared to be correlated with induction of HPV16-specific immunity.

Imiquimod, the immunomodulator that has been proven to be effective in the treatment of uVIN[11], seems to fail in patients that lack HPV16-specific type 1 T-cell immunity. This indicates that a combination therapy, in which the HPV16-specific

T-cell response is induced or boosted by vaccination and the affected skin is treated with imiquimod, may increase the number of patients that benefit from treatment.[12] Currently the combined effect of Imiquimod and therapeutic vaccination is investigated.[13] It has to be noted that data on Imiquimod is still limited, side-effects can be severe, and long-term follow-up is unknown.

Altogether, HPV-positive vulvar SCCs account for less than 20% of the total amount of vulvar cancers.[14] Preventive HPV-vaccination has only just started, and therapeutic vaccines are currently not common practice and only carried out in trials. When these strategies prove to be effective, the percentage is HPV-related vulvar lesions is likely to decrease. Given the often multicentricity of HPV, cervical smears should be taken in all patients with HPV-related vulvar lesions.[15] Special attention should be paid to immune-compromised individuals (e.g. transplant recipients, auto-immune disease patients treated with immunosuppressants, and HIV infected women) who are at increased risk of both anogenital HPV infections and their associated (pre) malignancies, and recurrence after treatment of uVIN.[16,17]

Development of HPV-negative vulvar squamous cell carcinoma

The biggest challenge in the management for vulvar cancer patients lays in unravelling the pathway leading to HPV-negative vulvar SCC. This type of cancer occurs mainly in elderly patients and has a worse prognosis than its HPV-related counterpart.[14] In our aging population, the incidence is likely to increase. In the HPV-related pathway, the well-investigated cervical cancer development equivalent has led to understanding the HPV-related vulvar SCC oncogenesis.

Recently, for the development of penile SCC, a bimodal classification with two distinct premalignancies has been described.[18,19] Next to the HPV-related precursor of penile SCC, called bowenoid penile intraepithelial neoplasia (PIN) (the penile counterpart of uVIN), non HPV-related differentiated PIN has been described. This lesion has similar characteristics as dVIN: subtle morphologic features and an association with LS. Similar to what we have described for HPV-negative vulvar SCC, in the HPV-unrelated penile SCC development, mutations of the p53-gene have been suggested.[20]

In SCCs of the head and neck (HNSCC), HPV-positive cancers, seem to differ from HPV-negative HNSCC. Patients with HPV-positive HNSCC tend to be younger and have a lower intake of tobacco and alcohol. In patients with oropharyngeal cancer, tumor HPV status is a strong and independent prognostic factor for survival.[21] The concept of "field cancerization" is well accepted for patients frequently exposed to carcinogens (like alcohol and tobacco), suggesting that the entire oral mucosa is exposed to carcinogens and multiple foci of transformed tissue clones are expected in these patients at high risk for malignancy[22;23]. In the HPV-negative HNSCCs no lesion similar to dVIN has been described.

The question remains why only the minority of patients with LS develops SCC. It might be a coincidence (due to age factors) but that cannot explain the fact that LS is present in such a high percentage of vulvar SCC specimens. Differentiated VIN is most often observed in association with SCC in a background of LS, but solitary dVIN in a background of LS also occurs. Interestingly, most dVIN tissue samples also contain atypical LS that is characterised by a strong inflammatory response directly underneath the epidermal-dermal junction. It has been proposed by Regauer and co-workers[24] that these LS lesions are in fact early LS with the capacity to revert to normal epithelium, but which can also progress towards irreversible, advanced LS with the typical deep dermal band-like infiltrate and hyalinated and edemic dermal zone. Possibly, the inflammatory response and activated vasculature in early LS might also trigger the development of (pre)malignancies just like chronic inflammation is associated with carcinogenesis (e.g. Crohn's disease and colorectal carcinoma, gastric ulcers and gastric adenocarcinomas).[25] Since inflammation has been suggested as a critical component in tumor progression,[26] further analysing the inflammatory response, might help us understand vulvar cancer oncogenesis. The analysis of inflammatory infiltrate of LS and dVIN shows a promising role of mast cells.[27] When the features of LS that are related to an increased risk of dVIN and/or SCC development are known, care for LS patients may be improved. When it is possible to distinguish patients with a high risk of malignancy from low-risk patients, studies should be carried out to investigate the effect of different screening intervals. Possibly, low-risk patients can be followed less frequently and preferably by their GP, whereas high-risk patients are better followed in a hospital setting at higher frequency.

Currently, most research is carried out on histological specimens, using immunohistochemistry. Further improving the care for LS patients, could be aimed at non-invasive methods of diagnosing (an increased risk) of malignant transformation, possibly by new advances in vulvar cytology.

Differentiated VIN is a not yet universally accepted diagnosis. The histological features are well described, but its cellular differentiation and absence of widespread architectural disarray makes this type of VIN difficult to recognize. Expert gynaecopathologists, mostly working in university hospitals, often do recognize dVIN lesions, as most patients with vulvar cancer are treated and followed in a university hospital in the Netherlands. Not all pathologists agree to the same features as part of the diagnosis, and some believe the lesion as such does not exist and it represents an *in-situ* carcinoma component adjacent to an invasive SCC[28]. Several lesions have been described that could be interpreted as dVIN: *squamous hyperplasia with atypia*, *atypical lichen sclerosus, hypertrophic lichen sclerosus with acanthosis*, all mostly related to LS. On the recent meeting of the ISSVD (Edinburgh, UK, 2009) a focus group was formed, working on the criteria of dVIN in order to gain widespread acceptance of the nomenclature and diagnosis. Interobserver studies, comparing the histological analysis of different pathologists and their final diagnoses and nomenclature will provide insight in the reproducibility of features of dVIN.

The low number of published solitary dVIN lesions in studies, might be explained by the hypothesis that dVIN has a short intra-epithelial phase that rapidly progresses into vulvar SCC. The lack of a universally accepted histological definition and the variety of descriptions and "names" possibly referring to the same entity, are also contributors to the underestimated incidence of dVIN. Investigating biopsies of patients who developed a vulvar SCC after the diagnosis of LS will be helpful in unravelling the biological behaviour. It is likely that revision of the available pre-cancer biopsies will reveal additional cases of solitary dVIN among cases previously labelled as LS.[29] Assessment of biopsy intervals will also provide information on the length of the intra-epithelial phase, which in the HPV-negative pathway is expected to be shorter than in uVIN.[30] Another factor in the relative underreporting of dVIN might be the fact that, whatever is causing dVIN, might not result in a clinical lesion that is likely to be biopsied.[31] We believe that the reddish or ulcerative lesions in LS patients are potential dVIN lesions (see figure 5CD, Chapter 1), and should be biopsied. However, mapping biopsies in individual patients, show dVIN at clinically "quiet" appearing LS changed vulvar skin (personal experience). Therefore, studies should be initiated correlating clinical lesions to the morphological and histological features in dVIN, like has been done in lichen sclerosus.[32]

In the past few years, in the Netherlands, the care for patients with vulvar complaints has become more centralized in special vulvar clinics. Mostly, this is a collaboration between the departments of gynaecology and dermatology. Often, additional specialists (e.g. sexologist, physiotherapist) are available for quick referral. Multidisciplinary vulvar clinics can provide specialized medical and supportive care for patients with extensive vulvar disease and/or complaints. There is no evidence that routine follow-up of patients with vulvar premalignancies will improve the prognosis of patients or change the risk of malignant progression nor the prognosis in patients who develop vulvar SCC.

Besides improvement of care for a subgroup of patients with difficult to treat vulvar complaints, these specialized vulvar clinics provide an excellent population for observational and follow-up studies. Our retrospective chart study on vulvar lichen sclerosus patients showed a subgroup of patients with a lot of symptoms that will especially benefit from the care in a vulvar clinic.[33] Currently, the vulvar clinic in Nijmegen is subject of investigation regarding the quality of care for vulvar patients. So far, no studies on the preferences of and quality of care for vulvar patients have been published.

As long as no uniform nomenclature and definitions are used, publications will be difficult to compare. Still, articles not taking the new accepted nomenclature of the ISSVD into account are being published.[34] To facilitate future studies and to further unravel the pathway leading to HPV-negative vulvar SCC, all studies should ideally use the same nomenclature and include pathological review. Uniform nomenclature, as introduced by the ISSVD,[35] is therefore essential and should be adopted worldwide.

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Chapter 9

Summary, Samenvatting, Bibliography, Dankwoord, Curriculum Vitae

Summary

Chapter 1
Vulvar squamous cell carcinoma (SCC) develops following two different pathways, which have their own premalignant lesions. In the absence of human papilloma virus (HPV), vulvar SCC can develop in a background of lichen sclerosus (LS), differentiated vulvar intraepithelial neoplasia (dVIN) or both. The other pathway leading to vulvar SCC is associated with HPV and the HPV-associated premalignancy is usual VIN (uVIN). Improving the care for patients with vulvar (pre-)malignancies requires further understanding of the oncogenesis of vulvar SCC. The aim of this thesis is to gain insight in the oncogenesis of vulvar squamous cell carcinoma. Both pathways leading to vulvar squamous cell carcinoma were investigated.
This chapter reviews the history, epidemiology, aetiology, histology, clinical characteristics, treatment options, malignant potential and prevention strategies of LS, uVIN and dVIN as an introduction to the experimental work in the rest of the thesis.

Chapter 2
To obtain more insight into the mechanisms underlying the two pathways leading to vulvar SCC, we determined the relationship between HPV DNA and the expression of cell cycle related biomarkers $p14^{ARF}$ and $p16^{INK4A}$ in non- and (pre)malignant vulvar lesions. The prevalence of HPV increased with the severity of the classic VIN lesions and so did the expression of $p14^{ARF}$ and $p16^{INK4A}$. The simultaneous expression of $p14^{ARF}$ and $p16^{INK4A}$ was highly associated with the presence of hr-HPV DNA. Hr-HPV was detected in only a single case of dVIN, whereas no expression of $p14^{ARF}$ was found and 16^{INK4A} was present in only 2 cases. All samples of vulvar SCC were hr-HPV DNA negative although in a substantial part $p14^{ARF}$ and/or $p16^{INK4A}$ was expressed. No relation was found between hr-HPV and the expression of $p14^{ARF}$ and $p16^{INK4A}$ in the non-neoplastic vulvar lesions. These results provide further evidence that vulvar squamous cell carcinoma is a multifactorial disease that develops from two different pathways.

Chapter 3
It is important to distinguish the two types of VIN because the both have their own type of treatment and a different malignant potential. Therefor three groups of VIN lesions were investigated; seventy-five dVIN lesions with adjacent SCC and 45 usual VIN lesions: 32 solitary and 13 with adjacent SCC. All were tested for hr-HPV DNA, using a broad-spectrum HPV detection/genotyping assay (SPF_{10}-LiPA), and the immunohistochemical expression of MIB1, $p16^{INK4A}$ and p53. All dVIN-lesions were hr-HPV-negative and $p16^{INK4A}$-negative and in nearly all, MIB1-expression was confined to the parabasal layers. Eighty-four percent exhibited high p53 labeling indices, sometimes with parabasal extension. Eighty percent of all usual VIN-lesions were hr-HPV-positive, $p16^{INK4A}$-positive, MIB1-positive and p53-negative. Both pathways leading to vulvar SCC have their own immunohistochemical profile, which can be used to distinguish the two types of VIN, but cannot explain differences in malignant potential.

Chapter 4
To further investigate the differences in malignant potential in vulvar premalignancies, the proliferative activity in the epithelial cell layers in different vulvar premalignancies and normal skin was quantified. In chapter 3, a distinct MIB1-expression pattern in dVIN lesions was found. In this study, we tested whether the expression of MIB1 in the basal regions of the vulvar epithelium may be helpful in diagnosing dVIN and can explain the differences in malignant potential. Automatic digital image analysis software was developed to quantify the proliferating fraction in different parts of the epithelium (MIB1 positivity index). MIB1 expression differed among the various vulvar premalignancies; a MIB1-negative basal cell layer was a distinct feature of normal vulvar epithelium. No MIB1-negative basal cell layer was noted in dVIN or other vulvar premalignancies. Owing to this negative cell layer, the MIB1 proliferation index in normal vulvar epithelium was significantly lower than in vulvar premalignancies. MIB1 expression can be a helpful tool in diagnosing a premalignancy and has additional value especially to distinguish dVIN from normal vulvar epithelium, but cannot explain the differences in malignant potential.

Chapter 5
The malignant potential of LS is believed to be low; 3-5% of the patients develop vulvar SCC. Therefore, most guidelines recommend regular, low-frequency follow-up. In this chapter, we describe the follow-up of LS patients at the departments of Gynaecology and Dermatology in the Radboud University Nijmegen Medical Centre, where no guidelines concerning the treatment and follow-up of LS existed. By doing so, the need for a multidisciplinary vulvar clinic was evaluated. Treatment and follow-up data of all women with histologically proven (between January 1995 and January 2001) anogenital LS visiting the outpatient clinics of the departments of Obstetrics & Gynaecology and Dermatology were collected (last date of follow-up: January 2008).
Eighty-four patients with LS were included in this study; ten patients (12%) were treated by both specialties. At the department of Obstetrics & Gynaecology, LS patients more often received surgical treatment, topical estrogens and lidocaine ointment, whereas at the department of Dermatology local class II/III corticosteroids were more often prescribed. Follow-up frequencies were similar in both specialties and took place at 3-4 visits in the first year, and at least once a year afterwards. One patient developed vulvar squamous cell carcinoma (SCC). This patient had withdrawn from follow-up. Management of patients with LS, agreed with current recommendations in the literature. However, differences in secondary and supportive therapy existed, due to differences in expertise. The relatively high percentage of patients treated by both specialties with a high frequency of visits emphasizes the need for a multidisciplinary clinic for vulvar disease.

Chapter 6
Follow-up in LS patients could be improved when one is able to differentiate between LS lesions that will progress into dVIN and/or vulvar SCC and those that remain solitary LS. Vessel type and vessel density might be predictors of malignant potential.

Therefore vessel parameters and pericyte characteristics in vulvar LS associated with vulvar SCC and solitary vulvar LS lesions were compared.
Quantitative analysis of immunohistochemical staining for CD34 (vascular and lymphangiogenic lymph endothelial cells), D2-40 (lymphatic-specific marker) and α-SMA (pericyte marker) was performed on paraffin-embedded tissue samples of 28 patients with LS (7 adjacent to vulvar SCC, 21 solitary). Electron microscopy was performed on fresh tissue. No significant differences in vessel density or other vessel parameters could be demonstrated between the 2 groups. In hyalinized lesions, vessel diameter and α-SMA positivity was reduced compared with nonhyalinized lesions. Electron microscopy revealed detachment of pericytes from vascular endothelial cells and increased thickening of basement membrane, while endothelial cell function did not appear strongly impaired. Malignant potential of LS cannot be predicted by vessel characteristics. Hyalinization in LS is associated with pericyte detachment from the basal lamina of vascular endothelial cells.

Chapter 7
Chapter 7 describes the expression of p53 and DNA ploidy status in LS, dVIN and HPV-negative vulvar SCC. The goal of this study was to investigate whether the progression of LS towards dVIN and vulvar SCC is accompanied by an altered DNA content. Also, the expression of p53 was assessed to investigate the relationship between p53-expression and DNA ploidy.
Almost all LS lesions were diploid, implicating normal DNA content. In lesions that contained both dVIN and vulvar SCC, the ploidy status of the dVIN never exceeded the ploidy status of the adjacent vulvar 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 immunohistochemistry followed by DNA image cytometry on formalin-fixed tissue sections. Similarly, we found p53-positive nonproliferating cells with increased DNA content in the superficial compartment of solitary dVIN lesions that were not associated with SCC, indicating ascending aneuploid cells from the basal compartment. 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.

Chapter 8
In the final chapter, future studies and hypothesis based on the results of the abovementioned studies are proposed and discussed, in order to gain further insight in the development of HPV-negative vulvar SCC and to improve the care for patients with vulvar disease.

Samenvatting

Hoofdstuk 1

Vulvair plaveiselcelcarcinoom (PCC) kan ontstaan via 2 verschillende routes, elk met een eigen voorstadium (premaligniteit). Zonder de aanwezigheid van humaan papillomavirus (HPV) kan een vulvair PCC ontstaan in een gebied met lichen sclerosus (LS), gedifferentieerde vulvaire intraepitheliale neoplasie (dVIN) of beide. De andere route, die leidt tot een vulvair PCC is wel geassocieerd met HPV. De HPV-geassocieerde premaligniteit is de zogenaamde usual VIN (uVIN). Het verbeteren van de zorg voor patiënten met een vulvaire (pre-)maligniteit kan alleen, wanneer het onstaan van kanker (oncogenese) verder wordt doorgrond. Het doel van dit proefschrift is om inzicht te krijgen in de ontstaanswijze van het vulvaire PCC. Beide routes die leiden tot het vulvair PCC werden onderzocht.

Het eerste hoofdstuk geeft een overzicht van de geschiedenis, epidemiologie, etiologie, histologie, klinische verschijnselen, behandelingsopties, maligne potentie en preventie-strategie van LS, uVIN en dVIN. Het dient als introductie op het experimentele werk, dat wordt beschreven in de overige hoofdstukken van het proefschrift.

Hoofdstuk 2

Om meer inzicht te krijgen in de mechanismen, die ten grondslag liggen aan de 2 routes, die leiden tot vulvair PCC, bepaalden we de relatie tussen de aanwezigheid van HPV DNA en de aanwezigheid van eiwitten die gerelateerd zijn aan de cel-cyclus (expressie van de de biomarkers $p14^{ARF}$ en $p16^{INK4A}$) in niet- en (pre-)maligne vulvaire afwijkingen. Het voorkomen van hoog-risico HPV (hr-HPV) nam toe met de ernst van de uVIN afwijking; deze relatie was ook aanwezig voor de expressie van $p14^{ARF}$ en $p16^{INK4A}$. De gelijktijdige expressie van $p14^{ARF}$ en $p16^{INK4A}$ had een sterke relatie met de aanwezigheid van hr-HPV DNA. Hr-HPV werd slechts in 1 dVIN afwijking aangetroffen, terwijl er in deze groep geen expressie van $p14^{ARF}$ kon worden aangetoond en $p16^{INK4A}$-expressie slechts aanwezig was bij 2 patiënten. Alle vulvaire PCCs waren hr-HPV DNA negatief, alhoewel in een deel $p14^{ARF}$ en/of $p16^{INK4A}$ wel tot expressie kwam. Er kon geen relatie worden aangetoond tussen hr-HPV en de expressie van $p14^{ARF}$ en $p16^{INK4A}$ in de niet-premaligne vulvaire afwijkingen. Deze resultaten bevestigen, dat vulvair PCC een multifactoriële ziekte is, die kan ontstaan via twee verschillende routes: met en zonder de aanwezigheid van hr-HPV.

Hoofdstuk 3

Het is van belang om onderscheid te maken tussen de twee types VIN vanwege verschil in enerzijds de behandeling en anderzijds de kans op maligne ontaarding. In de zoektocht naar een betere herkenning van de twee types VIN en een verklaring voor het verschil in maligne potentie zijn er drie groepen VIN afwijkingen onderzocht: vijfenzeventig dVIN afwijkingen met naastgelegen PCC en 45 uVIN afwijkingen (32 solitair en 13 met naastgelegen PCC). Er werd gekeken naar de aanwezigheid van hr-HPV DNA met behulp van een breedspectrum HPV detectie- en genotypering-assay (SPF_{10}-

LiPA). Daarnaast werd ook gekeken naar de expressie van MIB1, $p16^{INK4A}$ en p53; dit zijn eiwitten die gerelateerd zijn aan de cel-cyclus en proliferatie. Alle dVIN afwijkingen waren hr-HPV-negatief en $p16^{INK4A}$-negatief en in vrijwel alle gevallen was de expressie van MIB1 alleen aanwezig in de lagen net boven de basaalmembraan (parabasaal). Vierentachtig procent had een hoge p53 labelings index, soms met een uitbreiding richting het oppervlak. Tachtig procent van alle uVIN afwijkingen was hr-HPV-positief, $p16^{INK4A}$-positief, MIB1-positief en p53-negatief. De twee routes, die leiden naar vulvair PCC hebben hun eigen profiel van HPV en immunohistochemische expressie van eiwitten, dat gebruikt kan worden om de twee VIN types te onderscheiden, maar dat profiel geeft geen verklaring voor het verschil in maligne potentie.

Hoofdstuk 4

Om de verschillen in maligne potentie van vulvaire voorstadia verder te onderzoeken, werd de mogelijkheid tot groei (proliferatie) in de epitheliale cellagen in getal uitgedrukt voor verschillende vulvaire premaligniteiten en normale vulvaire huid. In hoofdstuk 3 werd een opvallend expressie-patroon van MIB1 bij dVIN gevonden. In de studie beschreven in hoofdstuk 4 werd onderzocht of de expressie van MIB1 in de basale cellagen van vulva-epitheel behulpzaam kan zijn bij het stellen van de diagnose dVIN en of het de verschillen in maligne potentie kan verklaren.

Speciaal voor de studie ontwikkelde, digitale beeldanalyse software werd gebruikt om de groeifractie in de verschillende delen van het epitheel te berekenen (MIB1 positiviteits-index). Het patroon van MIB1-expressie was verschillend in de diverse vulvaire weefsels: normaal vulva-epitheel had een MIB1-negatieve basale cellaag als uniek kenmerk. In dVIN of de andere premaligniteiten was deze MIB1-negatieve basale cellaag niet aanwezig. Dankzij deze negatieve cellaag was de MIB1-positiviteits-index in normaal vulvair epitheel significant lager dan in de vulvaire premaligniteiten. MIB1-expressie kan behulpzaam zijn bij het stellen van de diagnose van een vulvaire premaligniteit en heeft toegevoegde waarde in het maken van onderscheid tussen dVIN en normaal vulva-epitheel, maar kan het verschil in maligne potentie niet verklaren.

Hoofdstuk 5

De maligne potentie van LS wordt in de literatuur als laag ingeschat: 3-5% van de vrouwelijke LS patiënten ontwikkelt een vulvair PCC. De meeste richtlijnen adviseren regelmatige controles in een lage frequentie. In dit hoofdstuk wordt de follow-up van LS-patiënten bij de afdelingen Verloskunde & Gynaecologie en Dermatologie in het UMC St Radboud beschreven. Daarnaast vond ook evaluatie plaats van de behoefte aan een multidisciplinaire vulvapoli waar gynaecologen en dermatologen samen patiënten zien met name met moeilijk te behandelen klachten.

Van alle vrouwen met een histologisch bewezen anogenitale LS (tussen januari 1995 en januari 2001), die de polikliniek van de afdeling Verloskunde & Gynaecologie en/of Dermatologie bezochten, werden gegevens over behandeling en follow-up verzameld (laatste follow-up: januari 2008).

Het beloop van 84 patiënten werd geanalyseerd; tien patiënten werden behandeld door beide specialismen. Op de afdeling Verloskunde & Gynaecologie werden LS patiënten vaker behandeld door middel van chirurgie, lokale oestrogeen-crèmes en lidocaïnezalf, terwijl op de afdeling Dermatologie vaker klasse II/III corticosteroïden werden voorgeschreven. De follow-up frequentie was gelijk op beide afdelingen: 3 tot 4 bezoeken in het eerste jaar, en daarna ten minste eenmaal per jaar. Eén patiënte ontwikkelde een vulvair PCC. Zij had zich onttrokken aan follow-up. De behandeling van patiënten met LS was in overeenstemming met de thans geldende richtlijnen in de literatuur. Er zijn wel verschillen ten aanzien van de therapie van tweede keus en ondersteunende therapie, door verschil in expertise. Het relatief hoge percentage patiënten, dat door beide specialisten wordt behandeld met een hoge frequentie van polibezoeken, onderschrijft de behoefte aan een multidisciplinaire vulvapoli.

Hoofdstuk 6

De follow-up van patiënten met LS kan verbeterd worden, wanneer het mogelijk is onderscheid te maken tussen LS afwijkingen, die zullen ontaarden in dVIN en/of PCC, en LS afwijkingen, die dat niet zullen doen. Vaattype en vaatdichtheid zouden voorspellers van maligne potentie kunnen zijn. Daarom werden vaat-parameters en eigenschappen van de cellen rondom bloedvaten (pericyten) in solitaire LS en LS geassocieerd met vulvair PCC vergeleken.

Kwantitatieve analyse van immunohistochemische kleuringen voor CD34 (aankleuring van vasculaire en lymfatische endotheel cellen), D2-40 (lymfvat-specifieke merker) en α-SMA (pericyt merker) werd verricht op LS afwijkingen naast een vulvair PCC en op solitaire LS afwijkingen. Tussen de 2 groepen konden geen significante verschillen in vaatdichtheid of andere vaat-parameters worden aangetoond. Electronen microscopie van vers LS weefsel liet het loslaten van pericyten van het vasculaire endotheel zien, naast een toegenomen dikte van de basaalmembraan, terwijl de endotheliale celfunctie niet leek te zijn aangedaan. De maligne potentie van LS kan derhalve niet worden voorspeld met behulp van vaat-eigenschappen.

Hoofdstuk 7

In hoofdstuk 7 wordt beschreven wat de p53-expressie en ploïdie status is in LS, dVIN en het HPV negatieve vulvacarcinoom. Het doel van deze studie was om te onderzoeken of de progressie van LS naar dVIN en het vulvacarcinoom gepaard gaat met een andere DNA inhoud. Ook is onderzoek gedaan naar de p53-expressie van deze afwijkingen om de relatie tussen p53-expressie en DNA inhoud te bestuderen. Bijna alle LS afwijkingen waren diploïd, wat een normale DNA inhoud impliceert. In afwijkingen waarin zowel dVIN als vulvacarcinoom voorkwam, was de DNA inhoud van dVIN nooit méér dan in het naastgelegen PCC. In de dVIN afwijkingen was de p53-expressie alleen in de basale en suprabasale lagen aanwezig, soms met uitbereiding naar de hogere lagen. Er was een positieve correlatie tussen hoge expressie van p53 en de aanwezigheid van DNA aneuploïdie. Deze relatie was zowel aanwezig op weefsel niveau als op het niveau van individuele cellen. In de oppervlakkige lagen

van de solitaire dVIN afwijkingen vonden we p53-positieve, niet-prolifererende cellen met een toegenomen DNA inhoud. Dit lijkt erop te wijzen, dat aneuploïde cellen kunnen opstijgen vanaf het basale compartiment. DNA ploïdie metingen suggereren, dat dVIN een hogere maligne potentie heeft dan LS, en dus een meer waarschijnlijke voorloper van PCC is. Er dient nog meer onderzoek te worden gedaan om de mogelijk causale relatie tussen hoge p53-expressie en toegenomen DNA inhoud en aneuploïdie te onderzoeken.

Hoofdstuk 8
In het laatste hoofdstuk worden toekomstige studies en hypotheses, gebaseerd op de resultaten beschreven in dit proefschrift, voorgesteld en bediscussieerd. Genoemde studies zijn noodzakelijk om meer inzicht te krijgen in de ontwikkeling van HPV-negatief vulvair PCC en om de zorg voor patiënten met vulvaire afwijkingen te verbeteren.

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Oral Presentations

Oncogenesis of vulvar squamous cell carcinoma follows two separate and immunohistochemically different pathways.
Awarded: "Best Translational Research Presentation"
15th International meeting of the European Society of Gynaecological Oncology (ESGO)
October 2007, Berlin, Germany

MIB1-expression in basal cell-layer: a diagnostic tool to identify premalignancies of the vulva.
15th International meeting of the European Society of Gynaecological Oncology (ESGO)
October 2007, Berlin, Germany

Aneuploidy in vulvar (pre)malignancies is associated with high p53-expression
Awarded: "Best presentation – moderated poster session"
Gynaecongres, NVOG (Dutch Society for Obstetrics and Gynaecology)
May 2008, Haarlem, the Netherlands.

High levels of p53-expression correlate with DNA aneuploidy in (pre-)malignant lesions of the vulva.
Biennal Meeting International Society for Vulvovaginal Disease (ISSVD)
September 2009, Edinburgh, Scotland.

Bloedvat en lymfevat parameters in lichen sclerose kunnen het ontstaan van vulvair plaveiselcelcarcinoom niet voorspellen: een immunohistochemische en electronen-microscopische studie.
2e Symposium Experimenteel Onderzoek Heelkundige Specialismen (SEOHS)
November 2009, Nijmegen, The Netherlands

Differentiated vulvar intraepithelial neoplasia is often found in lesions, previously diagnosed as lichen sclerosus, that have progressed to vulvar squamous cell carcinoma
Biennal Conference, European College for the Study of Vulval Disease (ECSVD)
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Lieve Ellen en Yvette, we kennen elkaar vanaf de eerste Nijmeegse studieweken; toen al waren onze levens verschillend en dat zijn ze gebleven... Gelukkig is daarin altijd plaats voor een dagje sauna, avondjes uit (eten) en natuurlijk Sinterklaas. We hebben veel leed maar ook veel lief samen gedeeld de afgelopen jaren. Ik ben trots op onze vriendschap en blij met jullie steun en begrip!

Lieve Irene, Marieke, Sefke, Iris, Helga, Laura, Wendy, Susana, Maud, Marijke en alle andere Circe-dames: mogen jullie je alweer voor mij in't net hijsen..... Dit is (voorlopig) de laatste keer hoor!

Joop en Henny, wat heb ik het met jullie getroffen. Weekendjes met of bij jullie zijn een heerlijk rustpunt. Esther en Saskia, jullie zijn de leukste schoonzussen die je je maar kan wensen, thuis en op vakantie.

Lieve zusjes, Sylvia & Charlotte. “Nee, we zijn geen drieling...” en “Ja, dat is mijn zus...” antwoorden we alle drie regelmatig. Gelukkig hebben we behalve onze looks, ook een hele hoop andere dingen gemeen, daarom is het zo fijn met jullie! Ik denk dat we met z’n drieën voorlopig wel genoeg scripties hebben geschreven, zullen we papa nu maar aan het werk zetten?

Lieve papa en mama, er staat altijd een bed, glas wijn of gedekte tafel klaar, om (zelfs met een heel dispuut of 10 collega’s) te genieten van een goed gesprek, heerlijk eten en ‘gewoon thuis zijn’. De van jullie geërfde liefde voor koken en mijn hotelschool ambities hebben het moeten afleggen tegen Geneeskunde; het tot 2 keer toe worden uitgeloot, heeft heel wat tranen en bekeuringen gekost, maar het is toch gelukt. Dank voor jullie liefde en de ruimte om eigen keuzes te maken en het warme nest om af en toe in terug te keren.
Mam, je bent er altijd! Je inbindkunsten worden nu ook al beroemd buiten Nijmegen en ik denk dat de verzoeken blijven binnenstromen.
Papa, al voordat ik begon aan het onderzoek wist ik dat als ik ooit ging promoveren, jij mijn paranimf zou worden. Kan ik eindelijk weer eens zeggen: “boekje voorlezen papa”. Na een lange zoektocht tijdens mijn co-schappen naar het vak dat het beste bij mij past, kwam ik toch steeds uit bij de gynaecologie. Ik weet dat je trots om me was geweest, welk vak ik ook had gekozen, maar ik vind het erg bijzonder om mijn liefde voor ons vak met je te kunnen delen.

Lieve oma, u bent als geen ander in staat om groots te genieten van kleine dingen. Ik ben er trots op dat u op de eerste rij zit 2 december!

Liefste Boudewijn, het boekje van je meisje is af. Al meer dan 10 jaar probeer je de medische wereld waarin ik me begeef te begrijpen: het is heerlijk om met jou te kunnen relativeren. Zonder jou was het ECHT niet gelukt.

Curriculum Vitae

Irene van der Avoort werd op 14 februari 1978 in Eindhoven op de wereld gezet door haar moeder met hulp van haar vader. Ze groeide op in Hardenberg en verhuisde op haar 17e jaar naar Helmond. Na haar eindexamen aan het Dr. Knippenbergcollege in Helmond, was het tijd om te gaan studeren.
Toen ze was uitgeloot voor Geneeskunde, werd ze nageplaatst voor Biomedische Gezondheidswetenschappen (BGW) in Nijmegen. Tijdens haar studententijd was ze als lid actief bij Carolus Magnus en Dispuut Circe. Daarnaast was ze lid van de Opleidingscommissie en tijdens haar co-schappen secretaris van de KoRaad. Een stage bij professor Barker (Medical Research Council, Southampton, UK) was al gepland, toen ze bij de derde keer meeloten toch werd ingeloot voor Geneeskunde. Ze besloot om de studie BGW eerst af te maken, voordat ze begon aan haar doctoraal Geneeskunde. Tijdens de afstudeerstage werkte ze mee aan het promotieonderzoek van Ron van Golde naar mannelijke subfertiliteit. Met de nodige vrijstellingen haalde ze in 2002 haar doctoraal geneeskunde en kon ze beginnen met haar co-schappen, die in augustus 2004 werden afgesloten met het artsexamen.
Direct daarna kon ze voor 4 maanden aan de slag als zaalarts op de afdeling Gynaecologie in het UMC St Radboud te Nijmegen. Daarna startte ze als arts-onderzoeker en werd samen met Leon Massuger, Joanne de Hullu en Hans Bulten een interne AGIKO-subsidie verkregen, zodat het onderzoeksproject, dat heeft geleid tot dit proefschrift, kon worden voortgezet. Ze was betrokken bij de opzet van de vulvapoli in het UMC St Radboud en daar van 2006 tot 2008 ook werkzaam.
Van april 2008 tot en met september 2009 volgde ze het eerste perifere deel van de opleiding tot gynaecoloog in het Catharina Ziekenhuis in Eindhoven (opleider dr. T.H.T. Hasaart). Vanaf 1 oktober 2009 volgt ze in het UMC St Radboud het academische deel van de opleiding (opleider prof.dr. D.D.M. Braat).
Irene woont samen met Boudewijn de Boe in Nijmegen.